Washington University in St. Louis
Washington University Open Scholarship

Engineering and Applied Science Theses & Dissertations | McKelvey School of Engineering

Summer 8-15-2021

Nanoparticle T cell Engagers as a Modular Platform for Cancer Immunotherapy

Kinan Alhallak
Washington University in St. Louis

Follow this and additional works at: https://openscholarship.wustl.edu/eng_etds

 Part of the Oncology Commons, and the Pharmacy and Pharmaceutical Sciences Commons

Recommended Citation

Alhallak, Kinan, "Nanoparticle T cell Engagers as a Modular Platform for Cancer Immunotherapy" (2021). *Engineering and Applied Science Theses & Dissertations*. 641.
https://openscholarship.wustl.edu/eng_etds/641

This Dissertation is brought to you for free and open access by the McKelvey School of Engineering at Washington University Open Scholarship. It has been accepted for inclusion in Engineering and Applied Science Theses & Dissertations by an authorized administrator of Washington University Open Scholarship. For more information, please contact digital@wumail.wustl.edu.

www.manaraa.com

WASHINGTON UNIVERSITY IN ST. LOUIS

McKelvey School of Engineering
Department of Biomedical Engineering

Dissertation Examination Committee:
Abdel Kareem Azab, Chair
Samuel Achilefu
Hong Chen
Michael Kinch
Laura Sun

Nanoparticle T cell Engagers as a Modular Platform for Cancer Immunotherapy

by
Kinan Alhallak

A dissertation presented to
The Graduate School
of Washington University in
partial fulfillment of the
requirements for the degree
of Doctor of Philosophy

August 2021
St. Louis, Missouri

www.manaraa.com

© 2021, Kinan Alhallak

www.manaraa.com

Table of Contents

www.manaraa.com

List of Figures

www.manaraa.com

www.manaraa.com

List of Tables

www.manaraa.com

Acknowledgments

I would like to sincerely acknowledge my wonderful mentor, Abdel Kareem Azab, for his mentorship and for teaching me that there's a balance between work and having fun. I want to also acknowledge my family and friends for always being there when I ever needed them. Special thanks to Jennifer Sun, Basia Muz, and the others in the lab who helped me in my development as a researcher, scientist, and as a well-rounded person in general.

Kinan Alhallak

Washington University in St. Louis

August 2021

www.manaraa.com

ABSTRACT OF THE DISSERTATION
Nanoparticle T cell Engagers as a Modular Platform for Cancer Immunotherapy

by

Kinan Alhallak

Doctor of Philosophy in Biomedical Engineering

Washington University in St. Louis, 2021

Research Advisor: Abdel Kareem Azab, PhD

Immunotherapy is a class of treatment that stimulates a person's own immune system to recognize, target, and eliminate cancer cells. In recent years, immunotherapy has taken center stage in a variety of malignancies and holds great promise in becoming the "cure" for cancer. T cell-base immunotherapies such as chimeric antigen receptor (CAR)-T cells and T cell engagers (TCEs) have shown promising pre-clinical and clinical results. As the field progresses with novel strategies, the ability to manipulate the immune system with high efficiency, persistency, and robustness, along with limited toxicities has become a major hurdle for clinical translation. To this end, we created a new class of nanoparticle-based T cell engager (nanoTCE) platform by combining biomaterials into immunotherapies to achieve cell-specific immunomodulation, overcome immunosuppression, and address tumor microenvironment heterogeneity.

CAR-T cells are autologous T cells that have been virally transfected outside the body to express an engineered CAR construct, containing a synthesized fragment that targets a desired surface antigen on cancer cells. While this therapy produces favorable results, it is challenged by a long list of limitations, including toxicity, high cost, complex production process, the need for frequent quality testing, and safety concerns with the viral vector. On the other hand, TCEs are a non-cell therapy consists of two single chain variable fragments connected by a protein linker. One fragment recognizes a tumor-associated surface antigen, while the other recognizes the CD3

www.manaraa.com

receptor on a T cell. TCEs demonstrate high potency and efficacy against tumor cells and exploit the use of endogenous T cells, circumventing the limitation of genetically engineering extracted patient T cells to express CARs. The disadvantages of TCEs, however, include toxicity, laborious and tedious production, the need for continuous infusion due to short pharmacokinetics (PK), and the inability to induce persistent T cell activation. Moreover, both CAR-T and TCE therapies target one single antigen, which confer the development of antigen-less clones, tumor escape, and relapse, especially in multi-clonal diseases such as Waldenstrom Macroglobulinemia (WM) and Multiple Myeloma (MM).

To address some of the limitations faced by CAR-Ts and TCEs, we developed nanoparticle-based bispecific T cell engagers (nanoBTCEs), which are liposomes decorated with monoclonal antibodies targeting anti-CD3 on T cells and one cancer antigen on tumor cells. We show that nanoBTCEs 1) have a long half-life of about 60 hours, which enables once-a-week administration instead of continuous infusion; 2) induce T cell activation in the presence of WM and MM cancer cells; and 3) induce T cell-mediated cancer cell lysis of WM and MM cells. Due to the nanoparticulate nature of nanoBTCEs, we improved pharmacokinetics profile compared to regular TCEs, enabled simple and affordable production, and created an off-the-shelf platform for cancer immunotherapy.

Furthermore, for multi-clonal diseases such as MM, we also developed nanoparticle-based multispecific T cell engagers (nanoMuTEs), which are liposomes decorated with anti-CD3 monoclonal antibodies targeting T cells, and monoclonal antibodies targeting more than one cancer antigen. NanoMuTEs targeting multiple cancer antigens showed greater efficacy in MM cells *in vitro* and *in vivo*, compared to nanoBTCEs targeting only one cancer antigen. Unlike nanoBTCEs, treatment with nanoMuTEs did not cause downregulation of a single antigen and

www.manaraa.com

prevented the development of antigen-less tumor escape. To this point, our nanoparticle-based immuno-engaging technology provides a solution for the major limitations of current immunotherapy technologies, such as cost, PK, and tumor escape.

Another major disadvantage TCEs face is weaker T cell activation and persistence compared to CAR-T cells, which is why CAR-T cells have a greater anti-tumor response compared to TCEs. The small molecule phytohemagglutinin (PHA) is a commonly used to activate T cells *ex vivo*. However, it hasn't been used for immunotherapy *in vivo* due to its biological instability and toxicity. We report the encapsulation of PHA in a liposome increased its stability and reduced its toxicity *in vivo*, activated T cells *in vitro* and *in vivo*, and induced killing of tumor cells *in vitro* and *in vivo*. The liposomal PHA is a new form of pan-cancer immunotherapy which acts regardless of tumor antigens and thus does not induce antigen-less tumor escape, while also circumventing current obstacles of T cell exhaustion.

In conclusion, our nanoTCE platform uses nanoparticles to create a relatively simple, reproducible, and off-the-shelf solution to overcome the major limitations of current immunotherapy techniques such as TCEs and CAR-T cells. The nanoTCE targets each antigen with high specificity, creating a more robust immunotherapy technology to induce the immune system for an effective response. More importantly, nanoTCE can be customized to target any combination of desired cancer or immune cell antigen. This simple, customizable, specific, translational, and efficacious nanoTCE platform provides the flexibility to engage any immune cell for the treatment of the cancer of interest and can be used for personalized medicine based on the cancer antigens presented by the patient's tumor.

www.manaraa.com

Chapter 1: Biomaterials for Cancer Immunotherapy

1.1 Non-Cellular Immunotherapies

1.1.1 Delivery of Antibodies

Delivery of antibodies is one of the most pursued immunotherapy strategies. Many of these antibodies bind to cancer-specific antigens to induce immune-mediated cancer killing. Some are designed to target T cells or antigen-presenting cells (APCs) to modulate and refine their responses, while others bridge both mechanisms to create a direct link between immune cells and cancer cells [1, 2]. These have all received great clinical success and have led to approvals by the U.S. Food and Drug Administration (FDA). However, the disadvantages of antibodies include: (i) their short half-lives; (ii) poor tumor penetration; and (iii) the use of targets not unique to cancer cells leading to off-target effects. Here, we examine some of the ways biomaterials are used to assist and improve antibody-based immunotherapies.

1.1.1.1 Tumor-Targeting Antibodies

Antibodies targeting tumor antigens are among the earliest-studied cancer therapies. A great number of cancer-targeting monoclonal antibodies (mAbs) have been approved by the FDA for a variety of malignancies [2]. Therapeutic antibodies function via multiple anti-tumor mechanisms, including antibody-dependent cellular cytotoxicity (ADCC), complement-dependent cytotoxicity (CDC), and induction of T cell immunity through cross-presentation [3]. Examples for mAb-based immunotherapy include (i) alemtuzumab (Campath®), an anti-CD52 antibody that binds and kills leukemia cells for the treatment of B cell chronic lymphocytic leukemia (CLL) via ADCC [4]; (ii) retuximab (Retuxan®), an antibody that binds to CD20 on B cells and eliminates tumors via ADCC [5]; and iii) trastuzumab (Herceptin®), an anti-HER2 antibody for the

www.manaraa.com

treatment of HER2-overexpressing metastatic breast cancer that function via inhibition of tumor proliferation and recruitment of effector cells [6].

Many biomaterials are developed to help achieve specific and controlled delivery of antibodies to increase the potency of the treatment. Guziewicz et al. reported the use of a lyophilized silk fibroin hydrogel as a novel biomaterial for the stabilization and sustained delivery of antibody therapeutics, in which the silk-antibody hydrophobic/hydrophilic interactions prolonged the release of encapsulated antibodies for over 38 days [7]. This enabled the antibody to better accumulate at the tumor site due to longer circulation. Additionally, biocompatible and biodegradable mesoporous silica (SiO_2) films have been explored to achieve sustained release of anti-vascular endothelial growth factor (VEGF) mAb bevacizumab (Avastin®) [8]. This system was able to release functionally-active antibodies and release 98% of the drug over a period of a month. Another study developed an artificial organoid implant comprised of a PEG-heparin cryogel scaffold to customize the release of bispecific antibodies for immunotherapy against acute myeloid leukemia (AML) [9]. This novel cryogel scaffold encapsulated human mesenchymal stromal cells (MSCs) which were genetically modified to secrete anti-CD33/anti-CD3 bispecific antibodies for the activation of T cells. This device effectively supported MSC proliferation and continuously released the bispecific antibodies which overcame limitations of free bispecific antibodies such as their short half-lives and systemic toxicity.

1.1.1.2 Immunostimulatory Antibodies

Another class of antibodies have been developed for the stimulation of immune cells such as T cells and APCs. These include mAbs that function as agonistic ligands for co-stimulatory receptors, enhance activation and/or maturation of APCs, inhibit immunosuppressive

www.manaraa.com

mechanisms caused by cells such as regulatory T cells (Tregs), and inhibit lymphocyte inhibitory receptors [10]. CD40 is among the most studied co-stimulatory receptors found on APCs such as dendritic cells (DCs), B cells, and macrophages; mAbs agonistic to CD40 are known to promote anti-tumor immunity by inducing cytotoxic T cell responses [11, 12]. However, the maximum tolerated dose for CD40 mAb is limited due to the occurrence of inflammatory response in off-target organs and serious systemic toxicities such as cytokine release syndrome (CRS) and T cell depletion [13, 14]. One example of biomaterial-assisted presentation of co-stimulatory signals to APCs was reported by Gu et al. to improve the potency of CD40 mAbs [15]. Luminescent porous silicon nanoparticles conjugated with CD40 mAbs were able to achieve a 30-40-fold increase of B cell activation compared to the non-conjugated nanoparticles. Another study coupled agonistic anti-CD40 antibody with adjuvant CpG oligonucleotides onto the surface of PEGylated liposomes for intratumoral delivery to APCs. The two agents have been tested to demonstrate synergistic anti-tumoral effects, but the potency was concurrent with systemic toxicity. Anchoring the two potent and synergistic agents on liposomes resulted in a high level of retention in the tumor and surrounding tumor microenvironment (TME) [16].

4-1BB (also known as CD137) is a co-stimulatory receptor for T cell activation, and agonistic antibodies for CD137 could generate anti-tumor immunity. However, the systemic administration of CD137 antibody elicited disordered T cell infiltration and inflammation in the liver [17]. To circumvent this lethal immunotoxicity, anti-CD137 and anti-IL-2 antibodies were anchored onto the surface of liposomes, which resulted in rapid accumulation of therapeutics in tumors while lowering systemic exposure [18]. Overall, nanoparticle-assisted delivery achieved anti-tumor activity similar to free agents alone but without life-threatening systemic toxicities.

www.manaraa.com

1.1.1.3 Immune Checkpoint Inhibitors

Immune checkpoint inhibitors are antibodies that compromise the tumor cell's ability to evade immune system [19]. The first FDA-approved immune checkpoint inhibitor was the anti-cytotoxic T-lymphocyte antigen 4 (CTLA-4) antibody, which releases T cells from repressive signals and activate their responses toward cancer [20]. Additionally, checkpoint inhibitors that disrupt the "don't kill me signal" have also taken center stage in the clinic. Inhibition of the interaction between programmed cell death protein 1 (PD-1) on T cells and ligand (PD-L1) on tumor cells has proven to enhance T cell response and induce antitumor activity in patients [21]. While immune checkpoint inhibitors demonstrated remarkable efficacy in a variety of cancers, the inconsistent response rates, repeated dosing, and high toxicity profile remain to be problematic [22].

Many approaches were reported to avoid unwanted side effects while retaining the anti-tumoral effects of checkpoint inhibitors. A microparticle delivery system composed of poly(lactic-co-hydroxymethyl glycolic acid) was formulated to co-deliver CTLA-4 checkpoint inhibitor and anti-CD40 DC-stimulating antibody [23]. The microparticles showed an initial burst release followed by a sustained release for 30 days and showed no adverse effects.

Hydrogel-based platforms were also employed for improving kinetics for antibody release. Li et al. reported a subcutaneously injected alginate hydrogel for dual delivery of anti-PD-1 checkpoint inhibitor and a COX-2 inhibitor [24]. The hydrogel-mediated system resulted in higher anti-PD-1 mAb accumulation in the tumor and comparable serum concentration compared to intraperitoneal injection of free mAb. In addition, the treatment enhanced the presence of $CD4^+$ and $CD8^+$ T cells while reducing Tregs in the tumor and immune system.

www.manaraa.com

Another innovative strategy involved a biodegradable microneedle patch for sustained local release of anti-PD-1 antibody [25]. The microneedle patch composed of hyaluronic acid integrated with pH-sensitive dextran nanoparticles carrying anti-PD-1 antibody and glucose oxidase, which converts blood glucose to gluconic acid. The acidic environment generated by glucose oxidase enabled the release of anti-PD-1 from the nanoparticles and induced robust immune response in mice melanoma models. Similar strategies have also been used to deliver combinations of immune checkpoint inhibitors and immunosuppressive agents [25].

1.1.2 Delivery of Immunomodulators

The TME is composed of cellular components such as tumor, endothelial, epithelial, stromal, and immune cells, as well as the non-cellular components such as the extracellular matrix. Cancer cells alter the TME into an immunosuppressive environment and also depend on the TME for growth, invasion, and metastasis [26]. Modification of the TME with immunomodulators such as small molecule inhibitors, cytokines, and agonists for pattern recognition receptors (PRRs) can be an effective strategy to enhance anti-cancer immunity [27].

1.1.2.1 Small Molecule Inhibitors

The transforming growth factor β (TGFβ) is upregulated in a number of cancer types and play a substantial role in regulating almost every cell component in the TME [28]. Specifically, TGFβ acts as antagonist that interferes with host immunity and is considered one of the most potent mediators of immunosuppression in tumorogenesis [29]. Hence, TGFβ inhibitors were developed to activate T cells and improve current chemotherapeutics. However, systemic administration of TGFβ inhibitors can be extremely toxic owing to the central role of this signaling pathway.

One study aimed to augment T cell function inhibiting TGFβ in particular immune cell subsets [30]. PLGA/PEG nanoparticles carrying TGFβR1 inhibitors were targeted to $CD8^{+}$/PD-

www.manaraa.com

1^+ T cells and resulted in delayed tumor growth and prolonged survival in mice models, whereas free TGFβR1 inhibitors had no effect. Nano-scale liposomal polymeric gels were also developed for co-delivery of hydrophobic TGFβ inhibitors and hydrophilic IL-2 cytokines for activation of melanoma-specific T cell responses [31].

The STAT3 transcription factor is a key immune suppressor that inhibits DC maturation and macrophage function by suppressing antigen presentation and costimulatory molecules, as well as promote proliferation of Tregs that inhibit cytotoxic $CD8^+$ T cell responses [32]. Targeting STAT3 signaling has been proven to be successful in restoring cancer immunity. Novel tumor-targeting liposomes loaded with a STAT3 inhibitor was developed by Liao et al [33]. It was demonstrated that systemic administration of these targeted liposomes resulted in priming of the immune system for an antitumor response, demonstrated by an increase in activated T cells, M1-like macrophages, and DCs in the surrounding TME. This strategy also primed the immune system for a better response against a HER2 DNA vaccine [33].

1.1.2.2 Cytokines

Cytokines are proteins that act as mediators for intracellular signaling to regulate homeostasis of the immune system [34]. Cancer immunotherapy using cytokines is highly desirable for engaging immune response against cancer, and the three main types are interferons (IFNs), interleukins, and granulocyte macrophage colony-stimulating factor (GM-CSF). IFNs are known to elicit immune responses by inducing the maturation of macrophages, natural killer (NK) cells, T cells, and DCs [35]. IFNα-2a, IFNα-2b are the first FDA-approved cytokine-immunotherapy for the treatment of hairy cell leukemia [36]. IFN-γ has been shown to recruit macrophages to the TME and inhibit macrophage polarization towards the M2 tumor-associated phenotype [37]. Interleukins are known to strongly stimulate T cell proliferation and differentiation. IL-2 is

www.manaraa.com

produced mainly by $CD4^+$ T cells and can promote the cytolytic effects and proliferation of T and NK cells, and was the first effective immunotherapy for cancer [38]. IL-12 is known to induce the production of IFN-γ, promote Th1 immune response, and stimulate both the innate and adaptive immune systems [39]. Finally, GM-CSF stimulate immune responses by promoting T cell survival and DC differentiation and antigen presentation [40].

Cytokines administered *in vivo* have poor half-lives and generate severe systemic toxicities, thus limiting their use in the clinic. Therefore, biomaterials have been employed to circumvent this problem. One simple biomaterial application to cytokine delivery is PEGylation for prolonged circulation. Conjugation of PEG to cytokines such as granulocyte colony-stimulating factor (G-CSF), IFN□-2a, IFN□-2b are FDA-approved [41]. Other than PEGylation, several polymers have been conjugated to cytokines for protection and enhanced delivery. Polyoxazolines of various molecular weights have been reported for conjugation to G-CSF for increased stability and safety [42].

A novel delivery platform was reported for slow and sustained release of IL-12 from cholesterol-bearing pullulan-based hydrogel nanoparticle, which lead to prolonged IL-12 concentration in the serum without causing serious toxic events [43]. Additionally, Wang et al. developed a system for targeted delivery of IL-12 to the TME via pH-responsive polymeric nanoparticles, which resulted in a release of IL-12 in the acidic tumor site and subsequent shifting of macrophages from M2 to M1 phenotype, with negligible cytotoxicity [44]. Another study developed an injectable polymeric system composed of gelatin and chondroitin-6-sulfate for localized and sustained delivery of IL-2 to the brain tumor [45]. Active IL-2 was released for 2 weeks *in vitro* and 21 days *in vivo*. The intratumoral treatment induced immunologic memory

www.manaraa.com

and protected 42% of the animals from tumor rechallenge, suggesting that the novel release system is able to confer lasting antitumor immunity.

Sun et al. developed a TME-responsive nanocarrier for cell membrane-targeted delivery of a particular cytokine, TRAIL, to maximize delivery to the membrane bound receptor and minimize the internalization of the carrier [46]. The TRAIL-loaded DNA nanostructures transformed into nanofibers after liposome shell degradation, and the micro-scaled nanofibers efficiently presented the loaded TRAIL to death receptors on the cancer cell membrane and amplified the apoptotic signaling with reduced TRAIL internalization.

1.1.2.3 Agonists of Pattern Recognition Receptors

PRRs are known to be critical costimulatory receptors on innate immune cells that play an important role in initiating inflammatory response in myeloid cells such as macrophages and DCs [27]. They mediate the initial sensing of infection through recognition of pathogen-associated molecular patterns (PAMPs) and endogenous damage-associated molecular patterns (DAMPs) [47]. One important family of PRRs is the Toll-like receptors (TLRs), which is responsible for sensing invading pathogens at the cell surface or within intracellular endosomes and lysosomes [48]. TLR ligands can induce anti-tumor efficacy through activation of phagocytosis and antigen presentation by myeloid cells in the TME, which could serve as a promising cancer therapy strategy. However, systemic administration of TLR ligands may induce nonspecific stimulation of the immune system and the inflammatory toxicities limit the clinical application of such agents [49]. Biomaterials-based delivery strategies could protect TLR ligands from nuclease degradation and minimize toxicities.

An agonist for TLR9 has been identified to exhibit therapeutic potential in cancer treatment, by producing pro-inflammatory cytokines and inducing T cell responses; biomaterial-

www.manaraa.com

based strategies have been used for the delivery of this agonist [50]. Cationized gelatin-based nanoparticles loaded with a TLR9 agonist were targeted to the lymph nodes, where they selectively bound to APCs, delivered the TLR9 agonist, and mediated local immune stimulation [51]. In another study, novel self-assembled DNA immune-nanoflowers were used to deliver a TLR9 agonist to macrophages [52]. These nanoagents demonstrated high potency in triggering activation and proliferation of immune cells and secreted immunostimulatory cytokines such as TNFα and IL-6. The TLR7/8 agonist imidazoquinoline (IMDQ) is another molecule that directs potent cytotoxic T cell activity and induces high levels of type I IFN and IL-12. Nuhn et al. reported a pH-degradable IMDQ-ligated hydrogel nanoparticle for delivery of IMDQ specifically to the lymph nodes, thus dramatically reducing systemic toxicities [53].

1.1.3 Delivery of Other Molecules

1.1.3.1 Engineered Protein Scaffolds

Therapeutic antibodies are challenged by low tissue penetration especially in solid tumors due to their large molecular weights (~150 kDa), which limit their potential for effective immunotherapy. A wide variety of engineered protein scaffolds have been developed recently to overcome drawbacks in mAbs. These non-immunoglobulin family protein structures are equipped with antibody binding sites but are much smaller in size (2-20kDa) and more stable at high temperatures [54].

One example of engineered protein scaffolds include designed ankyrin repeat proteins (DARPins), a 12-19kDa molecule that is flexible in target design and is inexpensive to produce [55]. Several DARPin drugs are in clinical trials, one of which is in a phase 2 trial for multiple myeloma (MM) and works to reverse drug resistance toward front-line chemotherapy drugs (NCT03136653). The smallest of the protein scaffolds are the bicyclic peptides called bicycles,

www.manaraa.com

which are only 2 kDa, and are designed for quick and efficient delivery of tumor-killing toxins. The short half-life of bicycles could be advantageous or problematic depending on the desired goal. Pollaro et al. recently developed a bicycle peptide conjugated to an albumin binding tag, which resulted in a long plasma half-life and deep tissue penetration [56].

1.1.3.2 Bispecific T cell Engagers

Bispecific T cell engagers (BTCEs) represent a new class of cancer immunotherapeutic. They are tandem single chain variable (scFv) fragments connected by flexible linkers, with one scFv specific to a T cell-specific molecule such as CD3, while the other is specific to a tumor-associated antigen (TAA), allowing BTCEs to directly link the T cell to a tumor cell, leading to T cell activation and tumor killing, without the need for antigen presentation or stimulatory signals [57]. Additionally, BTCEs can be used as an "off-the-shelf" product since they are not restricted to major histocompatibility complex (MHC) molecules and are easily scalable.

The most established BTCE is blinatumomab, which simultaneously targets CD3 and the TAA CD19 [58]. Early blinatumomab clinical trials for patients with B-lineage acute lymphoblastic leukemia [59] and non-Hodgkin's lymphoma [60] evidenced favorable results. Following the use of blinatumomab, it resulted in a significantly higher median survival compared to traditional chemotherapy [61]. Goebeler et al. concluded that a continuous injection for 4-8 weeks, due to its short half-life, led to a more effective treatment [60]; however, continually injecting treatment for long periods of time leads to patient discomfort and infections which oftentimes lead to death [62]. The continual injection of BTCEs in the clinic is one of its major clinical disadvantages. Additionally, a reoccurring theme was the neurological adverse effects associated with blinatumomab, which was seen in a majority of clinical studies [59, 60].

www.manaraa.com

1.2 Artificial Cellular Immunotherapies

The pursuit of T lymphocytes for immunotherapy is due to their roles in tumor infiltration and effector cell retargeting [63]. To enhance the activity of the immune system, new strategies are explored for efficient stimulation of antigen-specific immune cells. While recent advances in adoptive T cell therapy (extraction, modification, and infusion of autologous T cells) has led to many successes in the clinic, the cost and efficiency of such therapy are major hurdles and limits its potential [64, 65]. These shortcomings animated the effort to create artificial cells for more effective cancer treatment to better understand of immune behavior [66]. Artificial immune cells can be developed into an "off-the-shelf" product with much shorter production timeline, while allowing better control for antigen presentation and immune activity.

1.2.1 Artificial APCs

In vivo, endogenous APCs are oftentimes restricted in antigen presentation due to immunosuppression from the TME [67]. Artificial APCs (aAPCs) mimic the functions of APCs to rapidly activate and expand T cells *ex vivo* or *in vivo* for cancer therapy. Cellular aAPCs have been created from human leukemia cell lines, *Drosophila* cells, and mouse fibroblasts [66, 68]. While these are physiological in nature, they all require genetic modifications and potentially carry negative regulatory molecules.

Synthetic aAPCs are emerging as an attractive tool for T cell stimulation. These engineered particles are often comprised of lipids, polymers, or inorganic materials, and include three signaling components required for T cell activation, including (i) pMHC–antigen multimers, (ii) antibodies binding to stimulatory receptors (such as anti-CD28 antibodies), and (iii) stimulatory cytokines (such as IL-2) [69]. Development of aAPCs have focused on the

www.manaraa.com

induction of $CD8^+$ cytotoxic T cells (CTLs) via MHC class I stimulation, since these cells are capable of antigen-specific cancer cell destruction and prolonged memory.

1.2.1.1 Spherical aAPCs

Several biomaterial-based, cell-sized, spherical aAPCs have been created to mimic and study the interaction between APC and CTLs. Many studies have been done to determine the optimal size and the fewest components necessary on these synthetic particles without compromising function. One study explored the use of aAPCs made from latex microbeads (5-6 μm) conjugated with H-2Kb-Ig/TRP2 peptide complexes, anti-CD28 antibody, 4-1BB, and CD83 ligands for the rapid expansion of melanoma-specific CTLs [70]. These aAPCs successfully retained CTLs' antigen-specificity toward TRP2-expressing melanoma and mediated an effective anti-cancer response. Similar latex-based aAPCs coated with human leukocyte antigen (HLA)-A2/pIL-13Rα2 complexes, anti-CD28 antibody, and CD83 molecules were used to induce CTLs reactive to HLA^+/IL-13R$\alpha 2^+$ glioma cells [71].

Polystyrene-based aAPCs are fairly common as well. aAPCs coated with HLA/HA-1 peptide complexes and costimulatory anti-CD28 antibodies on 5.6 μm-diameter polystyrene beads were designed to target the expansion of HA-1 specific $CD8^+$ effector memory T cells for the treatment of relapsed leukemia[72]. The ease of preparation and stability of such aAPCs can allow for several rounds of CTL expansion; a similar study using HLA-coated aAPCs were able to maintain primary CTLs for more than 2.5 months in culture [73]. Systematic nano-engineering approaches have also been applied to the design of aAPCs [74]. Hickey et al. reported superparamagnetic iron oxide nanoparticles-based aAPCs conjugated with pMHC- and anti-CD28 antibody to model the interaction between APCs and T cells [75]. It was reported that the size of the aAPCs and stimulatory molecule are as important as ligand availability in achieving T

www.manaraa.com

cell activation, and that particles larger than 300 nm and greater TCR-MHC clustering resulted in higher T cell activation.

Although bead-based aAPCs are homogeneous and easy to manufacture, the rigid and solid structure does not allow for the dynamic remodeling that establish the immune synapse between natural APCs and T cells, neither does it incorporate the release of cytokines [69]. Certain biomaterials were pursued to overcome this limitation. Liposomal formulations have been explored due to their desirable properties for elevated T cell activation such as membrane fluidity and surface nanoclusters reorganization [76-78]. However, lipid-based particles suffered from relative instability comparing to bead-based aAPCs. Mechanically soft elastomer poly(dimethyl siloxane) (PDMS) was also used to fabricate APCs [79]. Softer microbeads conjugated with activating ligands were found to induce higher T cell activation compared to rigid polystyrene beads. Degradable polymers such as poly(lactic-co-glycolic acid) (PLGA) was also utilized to create antigen-presenting microparticles, which included recognition and co-stimulatory ligands conjugated on the surface and IL-2 encapsulated in the biodegradable core [80]. This platform allowed for control over a wide range of particle sizes, sustained release of cytokine factors, and enhanced antigen presentation of modular signals on the aAPC surface.

1.2.1.2 Nonspherical aAPCs

In addition to ligand density and co-stimulatory signals, the area of contact between the T cell and artificial surface is also a crucial parameter for the design of synthetic aAPCs [74]. Although nanoscale aAPCs may have advantageous properties for *in vivo* applications such as improved draining to lymph nodes and reduced accumulation in the reticuloendothelial system, studies have found that larger particle sizes (4-5 μm) provide optimal APC function [13, 75]. Another consideration for area of contact is the shape of aAPCs. Most aAPC systems use spherical

www.manaraa.com

particles to stimulate T cells, while natural APCs are not spherical. With activation, APCs (such as DCs) undergo major changes in cell morphology, leading to significant increases in their overall cell surface area, facilitating interaction with T cells to direct T cell fate [73]. Therefore, the use of nonspherical microscale particles for the design of aAPCs have generated great interest, due to improved mimicry of endogenous interaction with T cells compared to the spherical alternatives [74].

Ellipsoid PLGA-based aAPCs have been explored and found to lead to significant difference in T cell stimulation compared to spherical formulations; specifically, T cells were observed to migrate to and interact preferentially with the long axis of the ellipsoidal aAPCs, suggesting the altered shape of aAPCs elicited higher binding efficiencies with T cells [81]. Additionally, Fadel et al. have developed single-walled carbon nanotubes (SWNT) for facilitation of greater surface area and modifications [82, 83]. Anti-CD3 coated SWNT activated T cells, released IL-2, and was proven to be more efficient comparing to antibody coated polystyrene aAPCs or CD3 mAbs alone. Moreover, the functionalized SWNT preferentially clustered to form 5-6 micron large-scale aggregates, resulting in even higher surface area for T cell interaction and activation.

1.2.2 Artificial T cells

T cells are formidable components of the immune system that are responsible for the killing of tumor cells. However, the TME suppresses T cell activity and many studies have shown that $CD8^+$ cytotoxic T cells reflect “tumor ignorance” and are unable to perform effector functions even when presented with increasing tumor antigen [84]. Efforts are being made to produce T cells with directivity toward target cancer cells. While engineering T cells is a promising strategy, considerable toxicities can be generated due to CRS, macrophage activating syndrome,

www.manaraa.com

and neurotoxicity [85-87]. To circumvent these challenges, other strategies have been explored to create artificial T cells of various constructs.

A team in UCLA reported a novel class of artificial T cells capable of boosting a host's immune system by actively interacting with immune cells through direct contact [88]. These artificial cells are composed of super-soft alginate microparticles that mimic the mechanobiological features of natural T cells. The particles are also adjusted for elasticity with calcium ions, and to mimic the biologic properties, the artificial cells are coated with phospholipids, conjugated with CD4 signalers, and loaded with IL-2 and IFNγ. Another strategy for engineering T cells involve attaching drug-loaded carriers to the membrane of T cells, enabling the direct delivery of cytokines. One publication reported the conjugation of cytokine-loaded liposomal nanocarriers onto the surface of transplanted T cells, which enabled the direct delivery of cytokines to the T cells [89]. Compared with mice treated with free cytokines, this strategy led to rapid T cell expansion at the tumor site and enhanced survival of mice melanoma model. Engineering non-immune cells to have the machinery of T cells is another strategy for artificial T cells. A study by Kojima et al. reported modification of human adipose stem cells with three signaling components to mimic the complex signaling within T cells [90].

1.3 Adoptive Cell Therapy

Aside from using protein- and polymer-based materials, cell-based materials have also been used to reach new limits for cancer immunotherapy. Adoptive cell therapy (ACT), an emerging technique for expanding, engineering, and/or activating autologous or allogeneic immune cells *ex vivo*, has received high praise for its ability to confer curative responses to patients with advanced, refractory, or relapsed tumors. Herein, we review and discuss the ACT techniques

www.manaraa.com

used for each immune cell type (T cells, NK cells, macrophages, and DCs and the advances of each).

1.3.1 T Cells

1.3.1.1 Tumor-Infiltrating Lymphocytes

The idea of manipulating T cells as one of the first ACT strategies and biomaterials for cancer immunotherapy sprouted following the discovery of the T cell's ability to often exterminate hematological malignancies and harm recipient tissue via the graft-versus-leukemia (GvL) and graft-versus-host disease (GvHD) effects, respectively [91]. Massive strides in T cell biology, reprogramming, and engineering have permitted the extensive use of T cells for therapeutic purposes. The very first adoptive transfer of T cells included the extraction of tumor-infiltrating lymphocytes (TILs) from the suppressive TME of melanoma patients [92]. The TME is commonly filled with tumor-specific T cells that are exhausted and insufficiently activated; hence extracting, expanding, stimulating, and infusing these TILs back into patients creates a living biomaterial for the use in ACT. ACT using TILs is achieved by growing them in culture in the presence of tumor cells; once the TILs eliminate the melanoma cells, indicating the fact that the T cells present are tumor-specific, IL-2-containing medium is then used to activate and expand the TILs for infusion back into the patient [93]. This technique has been used for decades [94] and has greatly succeeded in the clinical setting. About 50% of melanoma patients were observed to have favorable response rates following the adoptive transfer of autologous TILs [95, 96]. Current efforts are being put towards the optimization of the TIL therapy, such as the use of lymphodepleting chemotherapy options to enhance the cytotoxic activity of the TILs and shortening the T cells' time in culture to prevent the induction of senescence [97].

www.manaraa.com

1.3.1.2 T Cell Receptor-Engineered T Cells

The ongoing problem, however, associated with the use of TILs is the inability to expand the T cells found in the TME to a sufficient number of cells for the use in ACT. Alternatively, T cells can be isolated from the peripheral blood and transduced with T cell receptor (TCR) genes that are reactive to TAAs, enabling the conversion from naïve to antigen-specific T cells [98]. Generally, the TCR identifies the tumor antigen by binding to the antigenic peptide and MHC present on the tumor cell surface. In this case, the newly-endowed specificity of the TCR confers the ability of the T cell to induce cytotoxicity specifically to the cells with the targeted protein via MHC complex I. Various TAAs have been targeted by TCR-engineered T cells such as MART-1, gp100, CEA, p53, MAGE-A3, and NY-ESO-1 [98-103]. A problem with the initial approach of these TCR-engineered T cells is that the targets on the cancer cell were predominantly also present on normal tissue which, of course, lead to significant toxicities. For instance, when Johnson et al. used TCR-engineered T cells to target MART-1/gp100, the treated patients experienced significant hearing loss and uveitis [100]. In addition to severe toxicities, a weakness of TCR-engineered T cells is its restriction to only targeting MHC molecules when it is evidently known that cancer cells downregulate these MHC components [104].

1.3.1.3 Chimeric Antigen Receptor T Cells

In light of this, chimeric antigen receptor T (CAR-T) cells were created to bind to the cancer antigen independent of the MHC. CARs are recombinant fusion proteins that are derived from the domains of 1) the targeted antigen, 2) T cell activation (CD3), and 3) costimulatory signals (e.g. CD28, 4-1BB, OX40, and ICOS) [105]. The very first CAR only contained CD3 and the targeted antigen to allow for T cell activation and specificity to tumor cells, respectively. However, the lack of persistence and expansion of T cells during therapy lead to the addition of costimulatory domains into the CAR [106]. Additive effects were seen when multiple

www.manaraa.com

costimulatory molecules were put onto the CAR vector [107]. These CAR vectors are derived from viruses to take advantage of its transduction machinery. To create CAR-T cells, blood is first collected from the patient and purified for T cells; these T cells are then activated and expanded with beads covered with CD3 and CD28 antibodies [108]. While these T cells are being activated and expanded, the viral vector with the desired CAR proteins are incubated with the cells and removed after multiple days of incubation. Due to the ability of the vector to present the CAR in the form of RNA [109], this enables the cargo to be entirely integrated within the phenotype of the T cell. When these cells are injected back into the human body, they continually proliferate and lyse their targets, hence creating these "living drugs" for the treatment of cancer (~$500,000 per treatment) [110].

Huge success has been seen with CAR-T therapy for the treatment of hematological malignancies. The first type of CAR-T therapy was used to target CD19 on neoplastic B cells [111]. Other CAR-T targets used for non-Hodgkin lymphoma (NHL), HL, AML, and MM include CD20, CD30, CD33, and BCMA, respectively [112-115]. With the use of this ACT, however, a major complication that arises is CRS, which is an adverse event resulting from a bulk release of cytokines largely orchestrated by macrophages [116]. Complications typically seen for CRS include hypotension, fever, nausea, tachycardia, respiratory insufficiency, among others [117].

Another prohibitively complex aspect of creating CAR-T cells is the ability to only target and kill the cancer without killing each other especially for the treatment of T cell malignancies. To circumvent this issue, a group from the Washington University School of Medicine has created an "off-the-shelf" CAR-T for the treatment of T cell malignancies [118]. Cooper et al. deleted CD7, which is present on both healthy and malignant T cells, from the T cells that were

www.manaraa.com

equipped with the CD7-targeting CAR vector to prevent the CAR-T cells from killing each other. Another study successfully deleted the endogenous TCR and HLA class I from the CAR-T cells for the "off-the-shelf" use in cancer therapy [119].

Compared to hematological disorders, solid tumors have not seen much success with CAR-T cell therapy due to the overwhelming variety of immunosuppressive cells existing in the TME such as tumor-associated macrophages, Tregs, and myeloid-derived suppressor cells. A creative strategy that potentiates the effect of CAR-T therapy is the use of polymer implants to locally deliver the CAR-T cells to the tumor [120, 121]. However, more strategies are warranted for the enhancement of CAR-T cell therapy for solid tumors.

1.3.2 Natural Killer Cells

NK cells have also been used as a living drug and biomaterial for ACT. NK cells are very similar to T cells in the sense that both secrete very similar effector molecules such as perforin, granzymes, and IFNγ. One advantage of using NK cells compared to T cells for ACT is the ability to avoid GvHD with the use of allogeneic NK cells. NK cells do not attack normal cells due to the MHC class I molecules expressed on the normal cells; these molecules inhibit the cytotoxic effects of NK cells by binding to the inhibitory killer immunoglobulin-like receptor (KIR) on the NK cells [122]. On the other hand, for the cells that do not express sufficient levels of MHC class I molecules, the NK cell triggers its activating signals which in turn eliminates the target cell. For example, clinicians have taken advantage of this scenario by taking these alloreactive NK cells and infusing them into patients with refractory AML [123]. In this study, the NK cells protected the patients from relapse due to the lower expressions of MHC class I on the AML blasts. Patients with other cancer subtypes such as melanoma, renal cell carcinoma, and

www.manaraa.com

HL were investigated and showed favorable results with NK cell therapy as well [123]. Despite the absence of MHC class I molecules on tumor cells, NK cells oftentimes do not recognize the tumor.

To aid the NK cells with specificity towards TAAs, NK cells have also been manipulated with the CAR approach. CAR-NK targets used for MM, NHL, and melanoma include CS1, CD20, and GPA7, respectively [124-126]. In contrast to CAR-T cells, CAR-NK cells are able to be "off-the-shelf" for use in cancer treatment. NK-92, an immortal NK cell line, has shown clinical efficacy for ACT [127] and has recently concluded a pre-clinical study using NK-92 cells transduced with a CAR vector for triple-negative breast cancer [128]. Scientists using ACT with NK cells have also pursued functionally-activating these cells with interleukins (e.g. IL-12, IL-15, and IL-18) prior to re-infusion *in vivo* [129] and have instigated a clinical trial for the treatment of AML (NCT01898793) based on the ability of the pre-clinical study to confer memory-like NK cells.

1.3.3 Macrophages

To this day, the treatment of solid tumors remains to be a major obstacle for the field of cancer immunotherapy. The most abundant immune cells found in the TME are myeloid cells. Macrophages, a specific lineage of myeloid cells, are APCs that phagocyte pathogens and infectious agents and present the acquired antigens to activate naïve T cells. Tissue homeostasis is mediated by macrophages and these cells have also been found to have an ambiguous role in the homeostasis of the tumor [130]. In light of this, extensive investigation has been pursued on macrophages for the use in ACT. In 1985, Stevenson et al. were the first to grow and activate monocytes, the precursor to macrophages, *ex vivo* with IFN prior to re-infusion for the

www.manaraa.com

treatment of colon cancer [131]. More recently, a clinical protocol has been published for the use of autologous monocytes for the treatment of ovarian cancer [132].

Another recent approach to increase the specificity of the macrophages to tumor cells is using CAR-Macrophages (CAR-MA); this technique potentiates the effect of CAR therapy for the treatment of solid tumors due to the high quantities of macrophages infiltrating the TME and the ease of acquiring them. The first group that used CAR-MA only demonstrated the ability of the cells to phagocytose CD19- or CD20-coated silica beads when transduced with the CAR vector *in vitro* [133]. In this study, the investigators used the cytosolic domains of Megf10 to trigger phagocytosis. Dr. June and colleagues, on the other hand, have also created CAR-MA by utilizing the HER2-targeting CAR vector in the CD3 receptor of the macrophage [134]. Like the CAR-NK cell therapy, CAR-MA also act as "off-the-shelf" products for cancer treatment.

1.3.4 Dendritic Cells

A major function of DCs is the cross-presentation of exogenous antigens and presenting these antigens to CD4 and CD8 T cells via MHC class II and I molecules, respectively [135]. Stimulating T cells via cross-presentation allows the DCs to be the most potent and efficient APCs in the human body [136]. Taking advantage of the putative role of DCs, multiple research groups have pursued this cell type for ACT. Porgador et al. were the first to show the proof of concept of "educating" the DCs prior to re-infusion *in vivo* [137]. The autologous DCs were incubated with the desired tumor antigen peptide to enable engulfment of the antigens and facilitate T cell immunity when injected back *in vivo*. Cytokines have also been combined with tumor peptides during *ex vivo* DC activation. GM-CSF, IL-2, and IL-4 have all been combined individually with peptides to aid in DC stimulation in clinical trials [138-140].

www.manaraa.com

Another successful approach demonstrated by others utilizes the transduction of DCs with viral vectors *ex vivo*. The primary rationale of transducing DCs with viruses outside rather than inside the body is due to the humoral immune system to secrete neutralizing antibodies following the treatment of adenoviruses [141]. Furthermore, DCs transduced *ex vivo* have shown greater than 95 percent efficiency following viral transfection [142] and have been used in clinical trials for patients with cancers such as renal cell and prostate cancer [143]. Squadrito et al. have recently shown the introduction of chimeric receptors in the form of a viral vector to enhance the uptake of tumor microvesicles of DCs and induce high tumor efficacy for the treatment of breast cancer [144]. *Ex vivo* stimulation of DCs with these techniques have been used in cancer patients for more than a decade and are currently rendering favorable responses and complete remissions [145].

1.4 Gene-Based Immunotherapies

1.4.1 Small Interfering RNA

Regulating gene expression using small interfering RNA (siRNA) is a highly used technique in the field of cancer immunotherapy. siRNA is specifically used to knockdown a gene for a short period of time. These siRNAs undesirably activate the immune system when injected into the human body in its naked form [146] and are recognized by immune cells via the TLRs [147]. Many have taken advantage of the double-edged sword tactic of immune stimulation and utilized it for cancer immunotherapy. For instance, one study silenced a drug resistant-associated gene with a siRNA in melanoma, upregulated IFN□, and induced the stimulation of DCs via TLR7 for cancer treatment [148]. In addition, a siRNA was used against human papillomavirus (HPV)-driven tumors and seen to concomitantly induce an innate immune response via TLR7 and TLR8 [149]. These studies have proven to treat malignancies without actually targeting the immune

www.manaraa.com

system, which provides strong evidence of the multifaceted approach of siRNAs for cancer immunotherapy. However, many challenges exist following the systemic administration of naked siRNA such as its tendency to upregulate the immune system and not alter tumor fate, non-specificity, and low stability [150]. Therefore, current and ongoing siRNA modalities are pursuing established methods for the safe delivery of siRNA including its encapsulation in viral vectors or nanoparticles.

Additionally for gene-silencing treatment to function correctly, the siRNA must enter the cytoplasm of the cell and avoid exocytosis [151]. In fact, around 70% of lipid nanoparticles encapsulated with siRNA have been shown to be excreted by the cell, requiring a more creative method of enclosing siRNA to increase efficiency [152]. Polyethylenimine nanocapsules encapsulated with PD-L1 siRNA have been used to successfully reprogram tumor-associated DCs to induce tumor efficacy [153]. The siRNA polymeric particles were intentionally used for the engulfment by both the DCs and the cancer to enable a change in phenotype and an increase in immunogenicity, respectively. In another case, siRNA particles were used to silence the chemokine receptor CCR2 on monocytes, decreasing the number of tumor-associated macrophages in the TME [154]. siRNA molecules have also been conjugated to metal nanoparticles for the use in biomaterials for cancer immunotherapy. These metal particles were targeted to silence VEGF in tumor-associated macrophages and lung cancer cells *in vivo* [155]. The incorporation of siRNA is not just limited to nanoparticles; one study entrapped an siRNA against IL-4 and mammalian target of rapamycin (mTOR) in a polyethylene glycol (PEG)-based hydrogel for the potential localized treatment of cancer and induce, for instance, macrophage polarization [156].

www.manaraa.com

An interesting approach also pursued was the *ex vivo* silencing of a gene in tumor cells and subsequently injecting these tumor cells back into the patient in a Phase I clinical trial [157, 158]. This siRNA was put into a viral vector and transfected into the patient's tumor cells to prevent the production of an immunomodulatory cytokine, TGF□. Following transfection, the tumor cells were then irradiated to ensure the growth cycle arrest of these cells. The treated patients lived significantly longer than the non-treated patients [158], hence suggesting further investigation for this type of treatment option. However, a couple of disadvantages exist for the use of siRNA knockdown for cancer immunotherapy. siRNA therapy only knocks down the target gene for 24-72 hours following transfection and the efficiency of this knockdown varies tremendously per gene. Therefore, a greater number of therapeutics reprogramming tumor-associated immune cells and restoring tumorigenic cytotoxicity for cancer treatment is highly warranted.

1.4.2 Messenger RNA

1.4.2.1 mRNA Use in DCs

Another way of modulating gene expression in cells is the introduction of messenger RNA (mRNA) to the cell with the gene of interest. Specifically, mRNA augments the desired concentration of protein in the cell, whereas siRNA induces the opposite and silences the gene for the attenuation in protein expression [159]. APCs have been targeted to induce an immune response with mRNA therapy. As mentioned with siRNA, naked mRNA is also degraded very quickly when injected in the human system and needs to reach the cell cytoplasm for protein translation [160]. Mounting evidence is accumulating for the use of mRNAs in cancer immunotherapy. One instance demonstrates the use of mRNA-conjugated protamine for the induction of TLR7 to induce anti-tumor activity *in vitro* and *in vivo* [161]. Another study exhibits

www.manaraa.com

the use of a mRNA-cationic peptide complex for the stimulation of DCs, in particular, via TLR7 [162]. Conjugating mRNA to a cationic peptide deters the premature degradation of the mRNA complex.

The *ex vivo* stimulation of DCs has been pursued with the use of mRNAs and allows the precise manipulation of the antigens desired on DCs. In particular, Langerhans-like DCs were transfected *ex vivo* with mRNAs encoding the desired antigens onto these cells for both MM and melanoma in patients [163]. However, these cells are not available in large quantities and expanding these cells requires extensive protocols and expensive facility equipment for the treatment of these patients. Different techniques have been used for the delivery of mRNA to DCs such as nucleofection, electroporation and sonoporation [164-166] due to the amenability of the DCs. These DCs have been further improved by inducing immunostimulatory cytokines such as IL-12 and IL-18 using mRNAs [167, 168]. This would allow NK cell activation, CD4 differentiation, and induce effector molecule secretions by T cells. In addition, mRNAs coding for PD-1, PD-L1, anti-CTLA-4, or anti-GITR have been investigated in DCs [169-171]. In this case, rather than using mAbs to inhibit immune checkpoints, the authors transfected DCs to express these ligands to block their respective receptors. The success of this approach led to the initiation of a clinical trial utilizing mRNAs coding for anti-CTLA-4 [163]. The process includes the extraction of DCs, incubation with the desired mRNA, and injection intranodal to the patients. A pre-clinical study used a combination of mRNAs encoding CD70, CD40 ligand, and TLR4 for the enhanced stimulation of DCs [172]. Specifically, the DCs, once transfected, generated cytotoxic T cells and converted regulatory to helper T cells. Following this study, a clinical trial was pursued with stage III and IV melanoma tumors and showed to induce an objective response rate of 27% [173].

www.manaraa.com

1.4.2.2 mRNA Use in T Cells

With regards to CAR-T cells, the creation and regulation of viral vectors are complex and expensive. Many groups are trying to make the CAR-T development process more efficient and have begun electroporating T cells with mRNA to encode CAR proteins to circumvent the issues of viral vectors. In this case however, mRNA transfection is only transient and is unable to integrate into the genome of the T cell [174]. Therefore, CAR mRNAs render the T cells as a short-term treatment and are currently under investigation in clinical trials [174]. Creating short-lived CAR-T cells alleviates the problem of the off-tumor toxicity that is typically seen with CAR-T cell therapy; adverse events include tumor lysis syndrome and anaphylaxis. mRNA-transfected CAR-T cells alleviated the symptoms associated with anaphylaxis in a clinical study [175]. As mentioned in the study, CAR therapy with mRNA transfection lack persistence and potency; Foster et al. have shown that purification and modification of the mRNA renders robust T cell responses [176]. The T cells electroporated with the modified mRNA constructs enabled a 100-fold decrease in tumor burden compared to the T cells with unmodified mRNA.

Other methods of producing CAR-T cells includes the incubation of extracted T cells with mRNA-encapsulated nanoparticles [177]. Previous methods of producing CAR-T cells require electroporation in order for the CAR-encoding mRNA to reach the cytoplasm. The mRNA particles, on the other hand, are conjugated with anti-CD3 and anti-CD8 to target the T cells and stimulate endocytosis. One study has successfully demonstrated the delivery of immunotoxins using T cells to the tumor via mRNA transfection [178]. Due to the inability of drugs to home to the tumor cells, hence T cells were transfected to secrete the particular immunotoxin that inhibits cancer cell growth and used to home to the tumor. Neoantigens, newly formed antigens only present on the surface of cancer cells, are very precise and specific targets

www.manaraa.com

for the use in mRNA vaccines. Cationic lipids complexed with mRNA was injected intravenously and was seen to primarily induce CD4 T cell responses [179].

1.5 Conclusions

In summary, immunotherapy has emerged and established itself as a major pillar for curative and palliative care for cancer treatment. However, these therapies have many limitations and are in need of improvement. A range of protein-, polymer-, and cell-based materials have been implemented in the clinical setting as novel strategies to better target and treat multiple cancer subtypes while also minimizing the side effects associated with treatment. With the ability to customize biomaterials based on charge, size, surface functionalization, targeting, and cell-type, there are ample opportunities to further advance treatment for translation into the clinic. Further advancements in the field of biomaterials for cancer immunotherapy will depend on expertise across several disciplines such as immunology, nanomedicine, and material science.

www.manaraa.com

Chapter 2: Bispecific T cell Engagers for the Treatment of Multiple Myeloma: Achievements and Challenges.

2.1 Introduction

MM is a neoplastic plasma cell dyscrasia that primarily arises in the bone marrow, the second most common hematological malignancy, and represents approximately 20% of deaths from hematopoietic cancers [180]. Mainstay therapies for MM, such as corticosteroids, proteasome inhibitors, and immunomodulatory drugs, have shown significant clinical success and improved patient survival [181]. With the never-ending improvements of standard-of-care practices in MM, the current median survival has recently surpassed six years [182, 183]. However, MM is notoriously incurable and patients who fall victim to this disease eventually relapse. Therefore, novel therapeutic strategies used as a monotherapy or in combination with standard-of-care treatment regimens are highly warranted to improve the therapeutic landscape in MM.

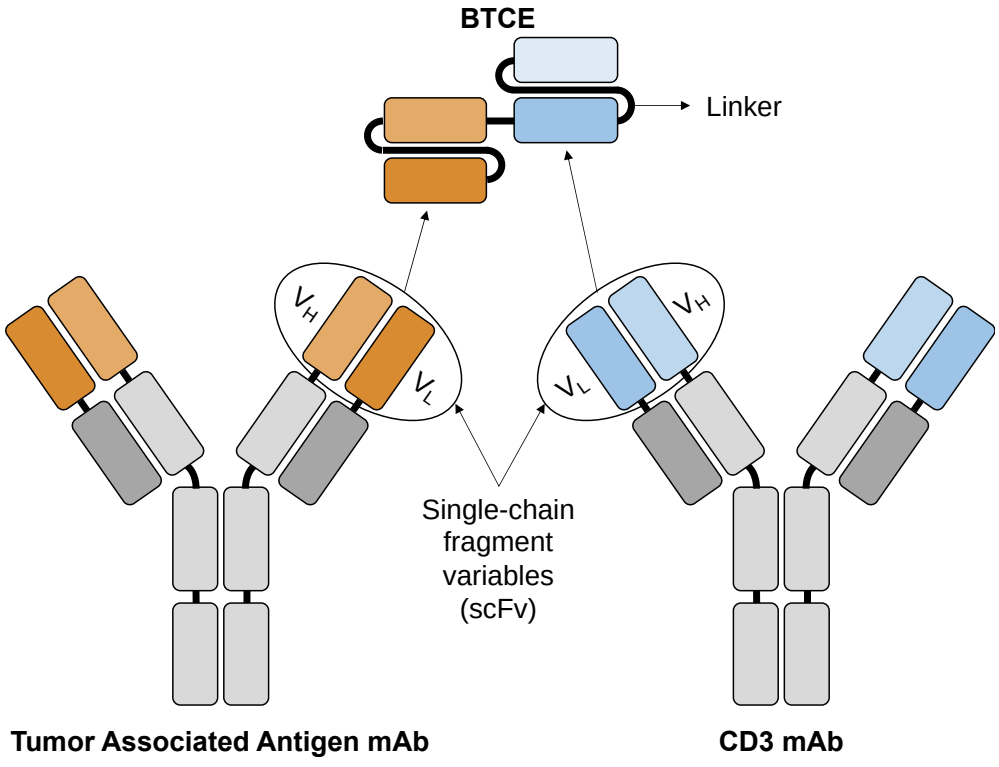
T cell-based immunotherapy is solidifying itself as a major pillar for the treatment of MM. The concept of targeting T cells during the early stages of immunotherapy development was conceived following the observation of the T cell's ability to eliminate blood cancers and harm normal tissue via graft-versus-leukemia and graft-versus-host disease, respectively [184]. This has led to extensive research in immunotherapies focused exclusively on T cells and ways to hone T cell-directed cytotoxicity on cancer cells while mitigating potential deleterious effects. Examples of T cell-based immunotherapy used for MM include immune checkpoint inhibitors, CAR-T cells, and BTCEs [185-187]. In this review, we provide a brief overview of BTCEs being investigated in the clinic currently for the treatment of MM and address the general achievements and challenges of this emerging immunotherapy option.

www.manaraa.com

2.2 Bispecific T cell Engagers

2.2.1 Mechanism of Action

All BTCEs are a class of bispecific antibodies that are made up of two scFvs which are connected by a protein linker [188] as shown in **Figure 1**. These scFvs bind to MM and T cells by targeting the desired MM antigen and the CD3 subunit of the TCR, respectively. The first bispecific antibody that was produced and published on was in 1972, and strategies for improving BTCE antibody manufacture are always ongoing [189-191]. Once the BTCE is bound to the target antigen and CD3, this subsequently leads to formation of a cytolytic synapse, upregulation of T cell activation and granule expression, and polyclonal expansion of the T cells [192-194]. BTCE-induced T cell activation is 1) extremely potent; 2) highly specific; 3) independent of TCR specificity; 4) does not need co-stimulation of CD28 and/or other co-stimulatory molecules; and 5) does not require peptide antigen presentation for target cell lysis.



www.manaraa.com

Figure 1. Creation of the Bispecific T cell Engager. The anti-TAA scFv specifically recognizes the desired TAA on the tumor cell while the anti-CD3 scFv recognizes the CD3 molecule on the T cell. This enables a highly specific and bivalent system for T cell-based immunotherapy.

The activation of T cells is only triggered upon concomitant binding of the TCR and target cell to the BTCE. BTCEs do not activate T cells by solely binding to the TCR due to their low affinity [195]. The general basis of how a BTCE activates a T cell is explained by the kinetic-segregation model [196] (**Figure 2**). CD45 is a transmembrane protein, constituted of a large extracellular domain and an intracellular phosphatase, the phosphatase domain of CD45 interacts with the TCR and dephosphorylates it, and hence prevent its activation [197, 198]. In a resting T cell, the net phosphorylation of the TCR is kept at a minimum due to dephosphorylation by CD45 [196], as shown in **Figure 2A**. Physiologically, when the T cell interacts with an APC, the TCR binds to the MHC with the antigen it is presenting, forming a close-contact zone immune-synapse. The close proximity of the T cells to the APC push away the extracellular domain of CD45, due to its large size (~30-50 nm) [199], which prevents the CD45 from interacting an dephosphorylating the TCR, and allows the activation of the T cell [200] (**Figure 2B**). BTCE-directed lytic synapses formed between T cells and target cells closely mimic those formed naturally through the TCR and MHC class peptide antigen interactions [201]; this is done by initiating an interaction between the T cell and target cell directly through cell specific antigens induced by the BTCE, as shown in **Figure 2C**. Once the BTCE is bound to the target antigen on the cancer cell and CD3 on T cells, the BTCE-induces formation of a close-contact zone immune-synapse that pushes the extracellular domain of CD45 away from the TCR, preventing its dephosphorylation and subsequently allowing T cell activation. The distance between the T cell and the other cells in the close-contact zones in the immune-synapse can be up to 300 nm for sufficient TCR stimulation [202]; nonetheless, smaller contact zones and size of target antigen leads to better activation and efficacy of BTCE [203].

www.manaraa.com

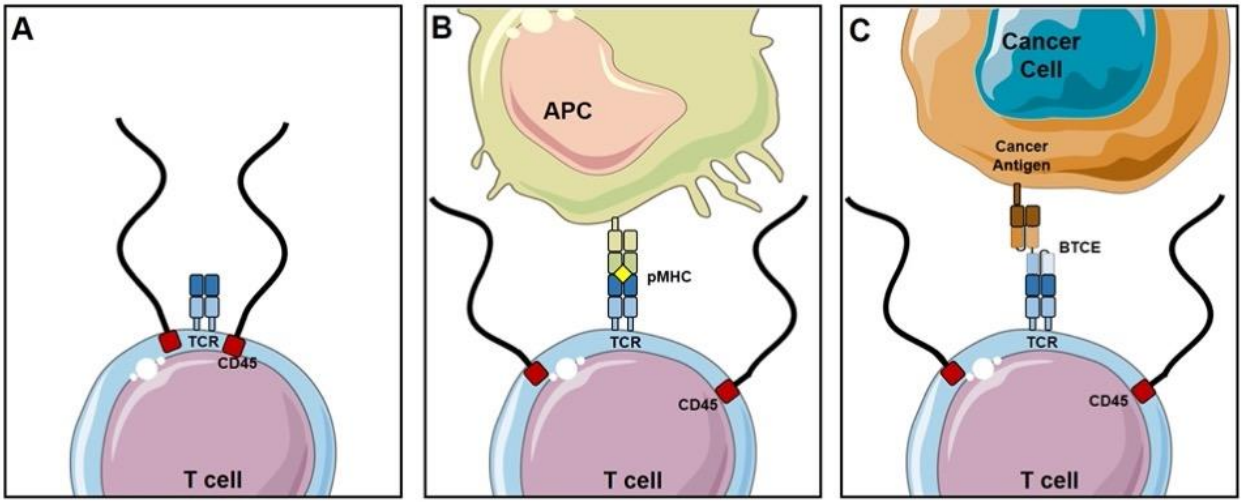


Figure 2. Molecular mechanism of BTCE-induced T cell activation. A) The kinetic-segregation model proposes that the exclusion of CD45 is a prerequisite for T cell activation. **B)** As the APC gets in proximity of the T cell, CD45 is subsequently excluded, and the peptide major histocompatibility complex (pMHC) interacts with the TCR and enables activation. **C)** For BTCE-induced T cell activation, the BTCE brings the tumor cell in proximity of the T cell to exclude CD45 from the close-contact zone and enable subsequent T cell activation.

2.2.2 Advantages

2.2.2.1 High Potency and Efficacy

BTCEs prove highly promising as a therapy due to their high potency and efficacy. The high potency of BTCEs is reflected by the low concentrations (picomolar range or lower) and low effector: target ratios required to demonstrate significant, specific lysis of target cells [192, 193, 201, 204]. In the presence of BTCEs, serial lysis of tumor cells by T cells has been demonstrated, allowing for a robust response, and increasing the efficacy and potency of BTCEs [205]. BTCEs are able to stimulate the production of lytic synapses without the normal TCR/MHC antigen recognition mechanism [194, 206]. The small size of the BTCEs (approximately 55 kDa and 11 nm in length) brings the T cell and target cell into close proximity necessary to form a synapse [207]. This mechanism explains the high efficacy of BTCEs, as they

www.manaraa.com

are able to overcome tumor immunosuppressive mechanisms to evade the immune system, such as downregulation of MHC antigen presentation and molecules for co-stimulation [194, 207].

2.2.2.2 Safety

In addition to their efficacy, BTCEs demonstrate suitable safety. BTCEs have demonstrated high selectivity for target antigens, with no signs of T cell activation in the absence of a target antigen [208]. Unlike CAR-T cells which are already activated ex vivo, with BTCEs, T cells only become activated when a target cell is also present and bound to the BTCE, minimizing potentially harmful cytokine secretion in the absence of the target tumor cell [188, 209]. In a phase I clinical trial of the Amgen's BCMA/CD3 BTCE (NCT03836053) in relapsed and/or refractory MM patients, AMG 420 demonstrated rates of cytokine release syndrome lower than those found for CAR-T cells that are directed to the same target [187].

2.2.2.3 Availability off-the-shelf

As a therapeutic, BTCEs are available in an "off-the-shelf" manner, ready for immediate treatment use [206, 210]. The act through the activation of endogenous T cells, and unlike CAR-T cells, no ex vivo manipulation of patient immune cells is necessary in order to achieve a direct interaction between T cells and target cells [205]. This decreases the need to determine patient tumor-specific antigens for manipulation of T cells ex vivo, which is particularly beneficial as some tumors may not have distinctive antigens for targeting [204, 211].

2.2.2.4 Lower Cost

Currently, a typical drug treatment regimen such as a combination of bortezomib and dexamethasone costs around $125,000 per MM patient [212]. The FDA-approved BTCE for B-ALL, blinatumomab, sells for around $89,000 per course of therapy [213]; whereas, CAR-T cells carry a higher financial burden for MM patients with a cost of around $500,000 per treatment

www.manaraa.com

[214]. The low cost of producing BTCEs stems from the advanced technologies that are currently available for the production of antibodies. This might lead to further developments in perfecting the state-of-the-art techniques used for the assembly of BTCEs and hence decreasing the overall price of using BTCEs for MM treatment.

2.2.3 Challenges

2.2.3.1 Poor Pharmacokinetic Profile

The small size of the traditional BTCE (approximately 55 kDa and 11 nm in length) confers its poor absorption, distribution, metabolism, and excretion properties [188]. Similar to other small proteins, the traditional BTCE is also systemically eliminated via nonspecific catabolism; whereas, monoclonal antibodies (~150 kDa) have prolonged distribution in the blood due to neonatal Fc receptor (FcRn)-regulated protection of the Fc receptor [215, 216]. Blinatumomab and other BTCEs of the same format have a typical half-life of around 2 hours; due to the very short half-life, BTCEs have to be continuously administered intravenously for a cycle of 28 days [217]. Circumventing the poor pharmacokinetic profile of the traditional BTCE is one of the main reasons that more efforts are transitioning to investigating BTCEs that contain an Fc receptor.

Methods to circumvent the poor pharmacokinetic profile of BTCEs include supplementing an Fc region onto the BTCE structure. AMG 701 is an example of this; Amgen included an Fc region onto the scFvs to be able to take advantage of the FcRn-regulated protection of the BTCE [215, 216]. Another example of prolonging the pharmacokinetic profile of the BTCE is including a single chain domain antibody that binds to albumin. This also takes advantage of the FcRn-mediated serum half-life extension. Harpoon Therapeutics use the anti-

www.manaraa.com

albumin technology to extend the half-life of their BTCE by non-covalently binding to albumin which avoids low affinity Fc receptor binding [218].

2.2.3.2 Laborious and Cumbersome to Produce

Generally, creating a particular monoclonal or bispecific antibody takes about six months [219]; this is due to the long and laborious process that is required to successfully create the BTCE of interest. The standard operating procedure for making a BTCE is first started by creating the desired DNA constructs using gene synthesis [191]. Phage display is used to develop the sequences of human variable fragments [220]. Once the preferred gene is isolated, assembled, and sequenced, restriction enzymes are introduced at both ends of the scFv gene to induce ligation of the gene and plasmid for subsequent cloning and plasmid construction [221]. The above process is repeated once more for the creation of the second scFv. Both scFvs are linked together using a short peptide that contain glycine and serine which are most commonly used for linkers [222]; this method is done by polymerase chain reaction. The resulting product is expressed in a bacterial or mammalian system such as Escherichia coli or Chinese hamster ovary cells, respectively, to achieve larger quantities of the BTCE [223, 224]. Following propagation, the BTCE is reduced and refolded to create active molecules. Then the final product is achieved by purification via ion-exchange chromatography. Protein concentration and purity is finally assessed using Bradford assay and sodium dodecyl sulfate–polyacrylamide gel electrophoresis, respectively [221, 224]. Techniques entailing the process of fusing Fc regions to BTCEs adds another dimension of complexity and is described in more detail elsewhere [220].

2.2.3.3 Inability to Target Multiple Antigens

Cancer is a multi-clonal disease; each clone can express different patterns of tumor antigens. Within the same patients the existence of several clones that may express different levels (or no

www.manaraa.com

levels) of tumor antigens expressed on the dominant clone was observed [225], which may significantly limit the efficacy of BTCEs targets one tumor antigen only. To further explain this phenomenon, we demonstrated the concept schematically in **Figure 3**. Assuming a multi-clonal tumor with three different each has high expression of different surface antigens A, B or C, with the clone expressing antigen A as the dominant clone. The estimative approach to treat this tumor would be an anti-antigen A BTCE, which may indeed eradicate the clone with high expression of antigen A but leaving behind the other two clones B and C, which are antigen-less of A, to escape the treatment, proliferate and induce relapse of the disease. In addition, antigen loss or downregulation of specific surface antigens is a common mechanism observed in cancer cells treated with targeted therapy against the specific antigen [226]. For instance, patient treated with BCMA-targeted CAR-T cells, BCMA expression on MM cells was decreased significantly [227], which raises the need to create BTCEs with the ability to target multiple tumor antigens simultaniously to circumvent antigen-less tumor escape and patient relapse.

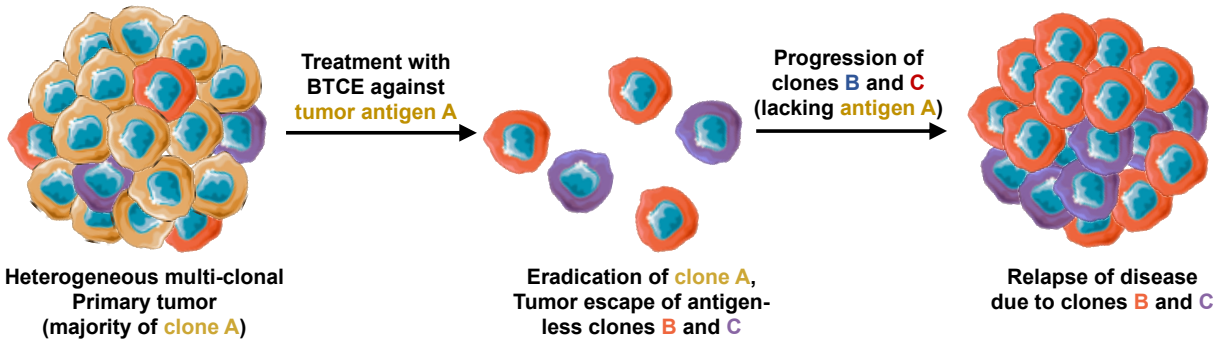


Figure 3. Schematic of the mechanism of tumor escape after treatment. Immunotherapies targeting one antigen due to development of antigen-less tumor clones which cause relapse of the disease.

In addition to the incapability of targeting more than one surface antigen on the cancer cell, there is also a need to target additional antigens on the T cell, other than CD3. Targeting CD28, in addition to CD3, as co-stimulatory receptor was shown to significantly induce more

www.manaraa.com

profound and sustained activation and proliferation of T cells [228]. In CAR-T cells, persistence and antitumor lysis was significantly augmented when co-stimulatory molecules such as ICOS and 4-1BB were incorporated in the CAR [229]; therefore, BTCEs also are in need of the ability to target co-stimulatory receptors to prevent exhaustion and increase the antitumor effects of T cells.

The development of TCEs that target multiple antigens is challenging due to the highly sophisticated nature of producing TCEs. Nabel and colleagues recently created a trispecific TCEs targeting CD38, CD3, and CD28 for the treatment of MM [230]. They investigated the levels of cytokine secretions, T cell activation, and T cell-redirected MM lysis in vitro and in vivo induced by the trispecific TCE. However, several concerns remain following the production of the first trispecific TCE for MM such as safety and feasibility to be able to progress towards the potential of creating a more multivalent TCE for MM.

In addition, Harpoon Therapeutics are currently investigating a trispecific TCE targeting T cells, MM, and albumin using anti-CD3, anti-BCMA, and anti-albumin, respectively. As mentioned before, the rationale for including anti-albumin is to substantially increase half-life. This creates a trispecific TCE that is only ~50 kDa which is a third of the size of a monoclonal antibody [218].

2.3 BTCEs for the Treatment of MM

The central tenet of making an efficacious BTCE is to be able to target the malignant cancer cells without harming normal tissue to reduce off-target toxicities. The optimal antigen target would have high and universal expression on the cancer cells but not on other normal cells [225]. MM tumors are multi-clonal, highly heterogeneous, and genetically unstable [231-233]. Due to the high mutational burden of MM and the multi-clonal nature of the tumors, selecting a single most

www.manaraa.com

preferred target is oftentimes challenging. We have listed below a list of the most pursued antigen targets for the treatment of MM using BTCEs.

2.3.1 BCMA

All BCMA, B cell maturation antigen also known as CD269 and TNFRS17, mediates the survival and growth of B cells and plays a critical role in the maturation and differentiation of B cells to plasma cells [234]. Persistence and long-term survival of plasma cells are hindered when BCMA expression is knocked out of plasma cells [235]. Most importantly, malignant plasma cells express significantly higher levels of BCMA compared to their normal counterparts which validates BCMA as a selective immunotherapeutic target for MM. There is a direct relationship of the overexpression and activation of BCMA as MM progresses [236]. BCMA is used a biomarker for MM due to its significant high expression. In addition, BCMA is universally and preferentially expressed on plasma cells with little to no expression in other hematologic cells. The only exception is plasmacytoid dendritic cells which have been shown to help survival of MM in the bone marrow [237].

Amgen created a BCMA/CD3 BTCE called BI 836909 and investigated the effect of their product in the preclinical setting [208]. BI 836909 is a BTCE with a BCMA and CD3 scFv connected by a protein linker. The hallmarks of T cell activation and cytolytic activity in MM cell lines and primary patient samples were observed. Xenograft tumors and plasma cells in vivo and in cynomolgus monkeys, respectively, were eradicated by BI 836909. BI 836909 was later named AMG 420, and Amgen have an active trial to assess the maximum tolerated dose (MTD) of AMG 420 in patients with relapsed and/or refractory MM (NCT03836053; **Table 1**). The maximum dose tested was 800 μg/day of continuous intravenous administration for four weeks which led to grade 3 adverse events; 400 μg/day was found to be the MTD for this study [238].

www.manaraa.com

Serious adverse events were seen in half of the patient cohort which consisted of peripheral neuropathy and infections. Secondary outcomes included a response rate of 70% at the MTD and an overall response rate of 31%.

Table 1. BTCE Clinical Trials in MM

Clinical Trial Number	Phase	Status	BiTE name	BiTE Target	Completion Date
NCT03173430	I	Terminated	Blinatumomab	CD19	2019
NCT03445663	I/II	Recruiting	AMG 424	CD38	2022
NCT03309111	I/II	Recruiting	GBR 1342	CD38	2021
NCT03275103	I	Recruiting	BFCR4350A	FcRH5	2021
NCT02514239/ NCT03836053	I	Active/ Recruiting	AMG 420	BCMA	2020/ 2025
NCT03287908	I/II	Recruiting	AMG 701	BCMA	2025
NCT03761108	I/II	Recruiting	REGN5458	BCMA	2022
NCT03933735	I	Recruiting	TNB-383B	BCMA	2021

Another Amgen BTCE that is targeted to BCMA is AMG 701. The difference between Amgen's two BTCEs is that AMG 701 has an extra Fc region to extend half-life. AMG 701 has been demonstrated to induce potent and specific MM cell lysis in vitro and in vivo [239]. In Amgen's study, they found that the elimination half-life of AMG 701 is around 112 hours in cynomolgus monkeys. This study has prompted initiation of phase I/II clinical study to investigate the pharmacokinetic, pharmacodynamics, and efficacy of AMG 701 (NCT03287908).

An additional BCMA/CD3 BTCE, REGN5458, is currently being investigated by Regeneron Pharmaceuticals [240]. Structurally, REGN5458 contains an Fc region with BCMA Fab and CD3 Fab domains. Preclinical data conclude that REGN5458 induced T cell-mediated lysis of MM cell lines and primary plasma cells in vitro. Additionally, xenograft tumors were eliminated

www.manaraa.com

when dosed at 4 mg/kg intravenously for twice a week. A phase I/II clinical trial was subsequently initiated to investigate the dose-limiting toxicities of REGN5458 (NCT03761108; **Table 1**). All patients included in the clinical study exhibited MM progression after undergoing three or more prior lines of treatment. Two (50%) patients were minimal residual disease negative following a weekly administration of 6 mg of REGN5458, and five (71%) patients had treatment-emergent adverse events related to the study (NCT03761108).

TeneoBio has also created a BCMA BTCE called TNB-383B that eliminates MM cells in vitro and in mice with minimal toxicity [210]. TeneoBio have shown that the TNB-383B has significantly lower cytokine release with sufficient anti-tumor efficacy compared to other BTCEs targeting BCMA. An advantage of TNB-383B is the use of fully human scFvs in the BTCE structure to avoid any unwanted immune response that can come from using mouse scFvs. Teneobio has teamed up with AbbVie to conduct a clinical trial using TNB-383B to investigate the MTD and pharmacokinetic profile of the BTCE in patients with relapsed or refractory MM (NCT03933735; **Table 1**).

Harpoon Therapeutics have created a novel trispecific TCE (HPN217) that targets T cells, MM, and albumin using anti-CD3, anti-BCMA, and anti-albumin, respectively. HPN217 has been shown to induce cytotoxicity in vitro and has demonstrated greater potent killing of MM cells with higher number of BCMA receptors per cell [241]. Harpoon has also shown CD69 and CD25 upregulation and cytokine secretion which are all hallmark markers of T cell activation. MM cell lysis and pharmacokinetic profiles were shown in mice and cynomolgus monkeys, respectively. The extended half-life of HPN217 was around 85 hours, whereas the normal BTCE half-life is around 2 hours [217].

www.manaraa.com

2.3.2 CD38

The CD38 receptor is a transmembrane glycoprotein that acts as an adhesion molecule and mediator for cell growth and calcium signaling for MM [242]. CD38 is highly expressed on the vast majority of MM cells, however it is also expressed (to lower extent) on various hematopoietic cells, including monocytes, B cells, T cells, and natural killer cells [243, 244]. CD38 has served as a target for the treatment of MM for multiple treatment regimens and have shown promising results in the clinic for monoclonal antibodies, such as daratumumab, isatuximab, and MOR202 [245, 246], and CAR-T cells (NCT03464916); thus, validating CD38 as a therapeutic target for MM.

Amgen's CD38/CD3 BTCE has been investigated in the preclinical setting (AMG 424) [247], and Amgen is currently recruiting patients to begin a phase I/II clinical trial (NCT03445663; **Table 1**). AMG 424 deviates from the traditional BTCE structure that consists of only scFv fragments. An Fc region supports the base of AMG 424 with a CD38 fragment antigen binding (Fab) domain on one side and CD3 scFv on the other. AMG 424 induced MM cell killing in vitro and in vivo and depleted the targeted B cells in cynomolgus monkeys; B cells were the primary outcome in this study due to the technically challenging nature of tracking plasma cells in cynomolgus monkeys. However due to the ubiquitous expression of CD38 on normal tissue, the potential toxicities of AMG 424 were also assessed in the mentioned study. The authors concluded that the depletion of monocytes and T cells only occurred at significantly large doses (EC_{50} of 42 and 325 pmol/L, respectively) compared to the depletion of B cells which only had an EC_{50} of 8 pmol/L in peripheral blood mononuclear cells of cynomolgus monkeys [247]. This preclinical study has led to the initiation of a phase I/II clinical trial for the treatment of patients with relapsed and/or refractory MM (NCT03287908).

www.manaraa.com

Another CD38/CD3 BTCE has been pushed to a phase I/II clinical trial by Ichnos Sciences (NCT03309111; GBR 1342; **Table 1**). According to Ichnos Sciences, the investigators delineate the structure of GBR 1342 to be very similar to that of AMG 424 [248]. The structure of GBR 1342 includes a Fc region with a CD38 scFv and CD3 Fab domain, whereas Amgen created AMG 424 with a CD38 Fab domain and CD3 scFv. GBR 1342 was shown to induce antitumor activity in vitro. The authors also monitored the depletion of T cells and monocytes in cynomolgus monkeys. They found that GBR 1342 depleted T cells and CD38-positive monocytes and observed a rebound of both cell types after approximately 48 hours [249].

There is also a trispecific TCE targeting MM, co-stimulatory molecule of the T cell, and the TCR by using anti-CD38, anti-CD28, and anti-CD3. The rationale for targeting CD28 is to enable enhanced and persistent T cell activation. The trispecific TCE enables cytolysis of MM and activation of T cells in vitro and in vivo. Additionally, Nabel's group investigated the TCE in primates and found that the MTD varied based on administration. Intravenous administration showed an MTD of 30-75 ug/kg whereas for subcutaneous, MTD was greater than 100 ug/kg; this is most likely due to the greater serum antibody levels in the blood following intravenous injection.

2.3.3 FcRH5

FcRH5, also known as CD307, FcRL5, and IRTA2, is an immunoregulatory cell surface molecule that is expressed only on B cells and remains on their surface as they mature to plasma cells, unlike major B-cell markers such as CD19, CD20, and CD22, which are lost in plasma cells [250]. As an immunotherapeutic target, FcRH5 is highly attractive due to its consistent expression on different developmental stages of B cells and the ability to utilize FcRH5 as a general target for other B cell malignancies [251, 252]. FcRH5 are always expressed on plasma

www.manaraa.com

cells, whereas other specific mature B cell markers are downregulated [250]. FcRH5 mRNA is additionally overexpressed in MM compared to other hematopoietic cells. FcRH5 is a universal and novel target and is being pursued for treatment regimens such as CAR-T cells [253].

Genentech created a BTCE with two Fab domains (one targeting FcRH5 and the other targeting CD3) and an Fc portion at the base of the bispecific IgG [254]. The proof-of-concept of Genentech's FcRH5/CD3 BTCE has been extensively investigated in vitro, in vivo, and in cynomolgus monkeys [203, 254]. Preclinically, the FcRH5/CD3 BTCE induced T cell activation in vitro concurring with the kinetic-segregation model, and the authors investigated the ability of the BTCE to induce T cell activation and killing as the targeted epitope location is distal, central, or proximal to the cell membrane [203]; they found that the membrane-proximal epitope produced a more efficient T cell synapse and enhanced killing of MM. Li et al. also exhibited the ability of the FcRH5/CD3 BTCE to redirect T cells to lyse MM patient samples, a MM cell line, and plasma cells in vitro, in vivo, and in cynomolgus monkeys respectively [203]. In addition, Genentech recently optimized their FcRH5/CD3 BTCE to enable negligible antibody-dependent cell-mediated cytoxicity and investigated whether or not this would impair its ability to induce T cell activation and T cell-redirected MM cell lysis [254]. This BTCE will be translated to a phase I clinical trial to primarily determine the adverse events that occur during and after administration of the FcRH5/CD3 BTCE in MM patients (NCT03275103; **Table 1**).

2.3.4 CD19

CD19 is a cell surface marker that acts as a coreceptor in antigen receptor-mediated activation of B cells and enhances intracellular signaling [255]. Normal plasma cells express CD19, whereas generally CD19 is not present on the surface of MM [256]. CD19 has been only shown to be

www.manaraa.com

expressed on MM in rare occasions [257]. However, a certain population of MM expresses very low levels of CD19 and is known to have an aggressive stem-like phenotype [256, 258].

Blinatumomab, a CD19/CD3 BTCE, has been approved for the treatment of B-cell acute lymphoblastic leukemia (B-ALL) [259, 260]. Blinatumomab has been proposed to target this aggressive subset of MM. Yet, there are currently no published studies that investigate blinatumomab for MM preclinically, and the only clinical trial that is studying the feasibility and safety of blinatumomab for the treatment of MM has been terminated recently (NCT03173430; **Table 1**).

2.3.5 CD138

CD138 or syndecan-1 is a canonical cell marker that is highly expressed and very abundant on MM and plasma cells. CD138 has been shown to increase tumor progression and survival and induces angiogenesis, cytoskeletal formation, adhesion, and signaling [261]. It has also been shown to interact with cytokines, chemokines, and growth factors to exert molecular roles in tumorigenesis. The gold standard marker to detect MM is the use of CD138 due to the very high presence of the marker on MM [232]; however, CD138 can be shed which can regulate function and stability [262]. CD138 is universally expressed on MM cells; however, different perturbations to MM cells can decrease expression such as hypoxia which could be the reason for failure of many CD138-targeted therapies [263].

A CD138 BTCE has been made to combat MM cells with the targeted surface marker. This specific BTCE actually includes an Fc portion to engage natural killer cells as well as T cells[264]. This particular aspect of including an Fc region enables increased half-life (which was not shown in this study [264]) and engagement of natural killer cells to induce an even

www.manaraa.com

greater immune response against MM. They found that the BTCE bound to natural killer, T cells, and MM cells to form a complex that induced MM cell killing. The CD138 BTCE was able to upregulate CD69 and CD25 expression and activate CD4 and CD8 T cells. T cell-mediated MM cell lysis was observed using fluorescent microscopy and was able to induce anti-tumor efficacy in vitro and in vivo.

2.3.6 Novel TCE Strategies

CD138 Recently, our group has shown that nanoparticles, particularly liposomes, can be used as a surrogate to bispecific antibodies for the engagement of T cells [265]. We have shown previously that our nanoparticle T cell engager (nanoTCE) was able to circumvent the disadvantages of the traditional BTCE mentioned in this review including poor pharmacokinetic profile, laborious and cumbersome to produce, and inability to target multiple antigens. Our nanoTCE is able to reach 60 hours in blood serum in vivo; can be made simply, reproducibly, and quickly; and customized to target any desired antigen of interest inside or outside the realm of MM [265].

The nanoTCE concept was proven using CD20 as a target for lymphoma. The CD20 nanoTCE was able to induce cancer cell lysis and T cell activation by upregulating CD69 expression in vitro and in vivo [265]. As mentioned previously, MM is highly heterogeneous and targeting one marker creates antigen-less tumors and creates relapse for the MM patient (**Figure 3**). Therefore, we sought to create a nanoparticle multispecific T cell engager (nanoMuTE) to target more than one MM surface marker and reduce any potential creation of antigen-less clones. We targeted three very abundant markers on MM: BCMA, CS1, and CD38 [266]. Each of these markers are individually present on MM cells; however, expression differs from patient

www.manaraa.com

to patient [265]. This creates a sound rationale for the targeting of multiple cancer antigens which is currently impossible for traditional TCEs.

We have shown that each nanoTCE targeting CD3 and BCMA, CS1, or CD38 induced T cell activation (upregulation of CD69) and cytokine secretions (IL-2, IL-6, IL-10, TNF-α, and IFN-γ), while nanoMuTEs, which target all three MM markers and CD3, enabled even greater T cell activation compared to each individual nanoTCE [265]. The same trend was also seen with T cell-mediated MM cell lysis in vitro and in vivo, where nanoMuTEs induced greater MM cytolysis than each nanoTCE. Furthermore, we portray two different models of antigen-less tumor escape. We demonstrated that using single-targeted nanoTCEs induced antigen-less tumor escape due to the elimination of MM cells only expressing one single marker; whereas, nanoMuTEs eliminated all MM cells with BCMA, CS1, and/or CD38 expressed and did not create any antigen-less MM clones [265]. Nanomaterials used for TCEs for other cancers such as breast cancer is described elsewhere [267].

2.4 Conclusions

The development of TCEs for the treatment of MM is rapidly growing. There have been many findings regarding the activation of T cells and elimination of MM in vitro and in vivo; with many pharmacokinetic analyses in primates [268]. High potency and efficacy, safety, availability off-the-shelf, and low cost are all current advantages of TCEs. These traits allow TCEs to be very attractive as an immunotherapy for MM compared to CAR-T cell therapy. However, TCEs are not perfect and have many disadvantages associated. Disadvantages include poor pharmacokinetic profile, laborious and cumbersome to produce, and inability to target multiple antigens. The goal of creating a TCE that circumvents all current disadvantages while using antibody technology is still underway. Using nanomaterials to circumvent current TCE

www.manaraa.com

limitations have significant potential to advance TCE immunotherapy and be beneficial for the treatment of MM and patients in the near future.

www.manaraa.com

Chapter 3: Nanoparticle T cell Engagers as a Modular Platform for Cancer Immunotherapy

3.1 Introduction

Cancer immunotherapy improves the ability of the immune system to recognize and combat cancer cells, which enables long-term remission in cancer patients and is also in the forefronts of cancer therapy [214, 269]. T cell-based immunotherapies include CAR-T cells and BTCEs. CAR-T cells are autologous T cells obtained from individual patients and are genetically engineered to express an antibody scFv to recognize and kill cancer [270]. BTCEs are tandem scFv fragments connected by flexible linkers with one scFv targeting a T cell specific molecule such as CD3, while the other targets a tumor-associated antigen, which allows the BTCEs to redirect the T cell to the cancer cell, leading to T cell-redirected activation and tumor killing [188, 193, 271, 272].

T cell-based immunotherapy has shown promising clinical outcomes in many cancers including MM [271, 272] and Waldenstrom Macroglobulinemia (WM) [273]; however, these have significant limitations. CAR-T cells must be extracted from the patient, activated, expanded, genetically engineered, and purified ex vivo for reinjection into the patient [274, 275]. This process imposes technical challenges and significant expense [110]. BTCEs, on the other hand, have the advantage of being off-the-shelf for immediate use in patients [276]; however, they have a poor pharmacokinetic profile, with a half-life of around two hours [277], imposing compromised patient quality of life, and increased risk of infections-related deaths [62, 278-280]. Therefore, there is an urgent need to develop new forms of T cell immunotherapies that overcome these limitations.

www.manaraa.com

3.2 Methods

3.2.1 Materials and Reagents

All biotinylated and fluorescent antibodies, human CD138 microbeads, and Pan T Cell Isolation Kits were purchased from Miltenyi Biotec (Bergisch Gladbach, Germany). DMEM, RPMI-1640, L-glutamine, penicillin-streptomycin, and phosphate buffered saline (PBS) were purchased from Corning (Corning, NY). Fetal bovine serum, live-cell dyes, lipophilic tracers, collagenase, and counting beads were purchased from Life Technologies (Carlsbad, CA). 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC), 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[amino(polyethylene glycol)-2000] (DSPE-PEG2000), and polycarbonate membranes were purchased from Avanti Polar Lipids (Alabaster, AL). Cholesterol and chloroform were purchased from Millipore Sigma (Burlington, MA). Streptavidin conjugation kit was purchased from Abcam (Cambridge, United Kingdom). Human Cytokine Array Q1 was purchased from Raybiotech (Peachtree Corners, GA). All mice used in this study were NCG (strain: 572), female, 50-56 days old, and purchased from Charles River (Wilmington, MA). All mice experiments in this study were in compliance with the Institutional Animal Care and Use Committee at Washington University.

3.2.2 Cells

H929, MM.1S, and RPMI-8226 were purchased and authenticated by American Type Culture Collection (ATCC; Manassas, Virginia). All cell lines were tested for mycoplasma contamination. BCWM.1 and MWCL.1 were a gift from Irene Ghobrial. Primary bone marrow samples were isolated from MM patients at Washington University School of Medicine (IRB # 201102270) and subsequently selected for MM cells with the use of CD138 human microbeads.

www.manaraa.com

Informed consent was obtained from all individuals in accordance with the Declaration of Helsinki. Normal donor peripheral blood mononuclear cells (PBMCs) were isolated from healthy donors using Ficoll centrifugation [281] and subsequently separated for T cells using a human Pan T cell isolation kit (Miltenyi Biotec). Hs505.T cells were cultured in DMEM with 4.5 g/L glucose and L-glutamine with the addition of addition of 10% fetal bovine serum and 1% penicillin-streptomycin. The other cell lines were cultured in RPMI-1640 with the addition of 10% fetal bovine serum, 2 mM of L-glutamine, and 1% penicillin-streptomycin.

3.2.3 Preparation and Characterization of the nanoTCEs and nanoMuTEs

Nanoparticle T cell engagers (nanoTCEs) consisted of three components: cholesterol, DPPC, and DSPE-PEG2000 with a mass ratio equivalent to 30: 65: 5, respectively. Lipids were mixed and solubilized in chloroform and evaporated to form a thin film [282, 283]. Then, the film was hydrated with PBS, and the resulting suspension was extruded using the Avestin LiposoFast LF-50 (Ottawa, Ontario, Canada) with 100 nm polycarbonate membranes to yield unilamellar liposomes. Streptavidin was conjugated to the amine groups on the surface of the liposomes according to the protocol of the manufacturer (Abcam), to activate the liposomes. Biotinylated antibodies were added to bind to the streptavidin for targeting, as previously described [284]. For detailed amounts of each reagent used, please see **Table 2**. Malvern Zetasizer Nano ZS90 (Malvern, Worcestershire, United Kingdom) was used to determine zeta-potential, diameter, and polydispersity index of each preparation (see **Table 3** for details).

www.manaraa.com

Table 2. Amounts of Lipids and Antibodies Used to Make Each Liposome Formulation

	Lipids					Biotinylated Antibodies					
	DPPC	Cholesterol	DSPE-PEG-NH2	18:0 PC-Et-NH2	Streptavidin	CD3	Isotype	CD20	BCMA	CS1	CD38
Cat #	850355	C8667	880128	860376	ab102921	130-113-137	130-113-448	130-111-336	130-104-500	130-099-575	130-113-430
Concentration (mg/mL)						0.022	0.020	0.150	0.110	0.044	0.022
Unit	mg	mg	mg	mg	mg	µL	µL	µL	µL	µL	µL
Pegylated Liposomes	13	3.2	3.8		0.1						
CD3 Liposomes (1 ab/liposome)	13	3.2	3.8		0.1	874					
CD3 Liposomes (3 ab/liposome)	13	3.2	3.8		0.1	2622					
CD3 Liposomes (10 ab/liposome)	13	3.2	3.8		0.1	8740					
CD20 Liposomes (1 ab/liposome)	13	3.2	3.8		0.1			146			
CD20 Liposomes (3 ab/liposome)	13	3.2	3.8		0.1			438			
CD20 Liposomes (10 ab/liposome)	13	3.2	3.8		0.1			1460			
BCMA Liposomes (1 ab/liposome)	13	3.2	3.8		0.1				199		
BCMA Liposomes (3 ab/liposome)	13	3.2	3.8		0.1				597		
BCMA Liposomes (10 ab/liposome)	13	3.2	3.8		0.1				1990		
CS1 Liposomes (1 ab/liposome)	13	3.2	3.8		0.1					497	
CS1 Liposomes (3 ab/liposome)	13	3.2	3.8		0.1					1491	
CS1 Liposomes (10 ab/liposome)	13	3.2	3.8		0.1					4970	
CD38 Liposomes (1 ab/liposome)	13	3.2	3.8		0.1						994
CD38 Liposomes (3 ab/liposome)	13	3.2	3.8		0.1						2982
CD38 Liposomes (10 ab/liposome)	13	3.2	3.8		0.1						9940
Isotype/CD3 nanoTCEs	13	3.2	3.8		0.1	874	1092				
CD20/CD3 nanoTCEs	13	3.2	3.8		0.1	874		146			
BCMA/CD3 nanoTCEs	13	3.2	3.8		0.1	874			199		
CS1/CD3 nanoTCEs	13	3.2	3.8		0.1	874				497	
CD38/CD3 nanoTCEs	13	3.2	3.8		0.1	874					994
BCMA/CS1/CD38/CD3 nanoMuTEs	13	3.2	3.8		0.1	874			199	497	994
CD20/CD3 nanoTCEs (no PEG)	13	3.2		1.1	0.1	874		146			

www.manaraa.com

Table 3. Parameters for each nanoTCE or nanoMuTE.

Formulation	Mean Size (nm)	Polydispersity Index	Zeta Potential (mV)
Isotype/CD3	127.9 ± 3.6	0.066 ± 0.030	0.67 ± 0.32
CD20/CD3	128.1 ± 11.3	0.085 ± 0.034	1.41 ± 0.70
BCMA/CD3	133.2 ± 9.3	0.091 ± 0.017	-0.02 ± 0.05
CS1/CD3	123.4 ± 8.4	0.082 ± 0.040	0.61 ± 0.50
CD38/CD3	125.4 ± 8.3	0.107 ± 0.006	0.39 ± 0.38
BCMA/CS1/CD38/CD3	125.2 ± 0.5	0.047 ± 0.021	0.84 ± 0.34

3.2.4 Pharmacokinetics of nanoTCEs and nanoMuTEs

Each nanoTCE or nanoMuTE was stained with a fluorescent tracer (DiD and injected IV injection to NSG mice at .5 mg/mouse (n=3 for each formulation). Blood (50 □l) was taken from the tail vein of each mouse before treatment, and 0.25, 6, 24, 48, 72, and 96 hours after treatment. Fluorescence of whole blood or plasma was measured at 644/665 nm using a SpectraMax i3 plate reader (Molecular Devices, San Jose, CA). Half-life was calculated using polynomial regression.

3.2.5 Cell Surface Protein Expression Analysis

Cell lines or primary CD138+ MM cells were incubated with APC-anti-CD20, APC-anti-BCMA, APC-anti-CS1, or APC-anti-CD38 antibodies in 4°C for one hour; then washed, spun down, resuspended in 100 μl and analyzed by flow cytometry using MACSQuant Analyzer 10 with Ex= 635nm and Em= 655-730 nm [285]. Cells were gated using FSC and SSC, and analyzed for relative mean fluorescent intensity (RMFI) of APC using BD FlowJo Software [286].

www.manaraa.com

3.2.6 Liposome Binding and Binding Following Antigen Loss In Vitro

Each nanoTCE or nanoMuTE was stained with a fluorescent tracer DiO. Cell lines and primary cells (30,000 cells in 100 μl for each data point) were treated with or without Isotype/CD3, nanoTCEs, or nanoMuTEs (3.7 nM) for two hours at 37°C. In some cases (for mimicking antigen downregulation), cells were treated with 33.3 nM of anti-BCMA, CS1, and/or CD38 antibody of the same clone for one hour prior to the two-hour treatment with nanoTCEs or nanoMuTEs. Following the two-hour treatment with nanoTCEs or nanoMuTEs, the cells were stained with anti-BCMA, CS1, or CD38 of a different clone for one hour. Then, cells were spun down, washed with PBS, resuspended in 100 μl and analyzed by flow cytometry using MACSQuant Analyzer 10 with Ex= 488nm and Em= 525/50 nm. Cells were gated using FSC and SSC and analyzed for MFI of DiO using BD FlowJo Software.

3.2.7 3D Tissue-Engineered Bone Marrow (3DTEBM) Culture System

The culture's cellular content can be customized by inclusion of various cell populations. For testing patient samples, BM mononuclear cells were used as a whole, including the primary cancer cells and T cells. 3DTEBM was established by crosslinking fibrinogen in patient BM supernatant using $CaCl_2$, as previously described [287]. Briefly, for testing cell lines, 30,000 cancer cells were combined with 30,000 T cells; for primary cells 100,000 BM mononuclear cells were used as whole. Cells were suspended in BM supernatant which was then crosslinked with $CaCl_2$ to form the 3D matrix. The 3DTEBM was supplemented with media on top and incubated at 37°C for 4 days. At time of analysis, the scaffolds were digested with collagenase

www.manaraa.com

(Gibco, Life Technologies) for two hours at 37°C; cells were retrieved, washed, and subjected to flow cytometry analysis.

For the development of antigen-less populations, the above procedure was followed, and the remaining cells were incubated with APC-anti-BCMA, APC-anti-CS1, or APC-anti-CD38 antibodies in 4°C for one hour; then washed, spun down, resuspended in 100□l and analyzed by flow cytometry using MACSQuant Analyzer 10 with Ex= 635nm and Em= 655-730 nm. Cells were gated using FSC and SSC and analyzed for MFI of APC using BD FlowJo Software.

3.2.8 Cell Survival

Cell lines (prelabeled with fluorescent tracer DiO) and primary cells were incubated with T cells in 3DTEBM and treated with or without Isotype/CD3, nanoTCEs, or nanoMuTEs at a concentration of 3.7 nM for 4 days. Before digestion of the matrix, 5 µL of counting beads (Miltenyi Biotec) were added to the culture. The matrix was then digested, cells were retrieved, and analyzed by flow cytometry using MACSQuant Analyzer 10. For cell lines, the number of tumor cells analyzed as DiO+ cells and normalized to the number of counting beads using BD FlowJo Software. For primary cells, MM cells were identified as CD38+/CD3-/CD14-/CD16-/CD19-/CD123-, as previously described [288], and the number of MM primary cells was normalized to the number of counting beads using BD FlowJo Software. For the analysis of WM killing without T cells, the above procedure was mimicked except without including T cells in 3DTEBM.

3.2.9 Activation of T cells

Cells were in 3DTEBM and treated with or without Isotype/CD3, nanoTCEs, or nanoMuTEs at a concentration of 3.7 nM for 4 days. Then, cultures were digested, and the cells were retrieved

www.manaraa.com

and incubated with PE anti-CD3, FITC anti-CD4, Violet anti-CD8, and APC anti-CD69 antibodies for one hour in 4°C, washed with PBS, spun down, and suspended in PBS again. These samples were analyzed by flow cytometer using MACSQuant Analyzer 10 with Ex= 488, 488, 405, and 635 nm and Em= 585/40, 525/50, 450/50, 655-730 nm, respectively. Cells were gated using FSC and SSC followed by double positive CD3+/CD4+ or CD3+/CD8+, both of which were analyzed for % of cells positive for CD69 using BD FlowJo Software.

For cytokine secretion, the supernatant was kept and the 3DTEBM was digested for two hours using collagenase following the four-day incubation period. Once the 3DTEBM was digested and samples were spun down, the supernatant (with collagenase) was then added to the supernatant collected earlier. Subsequently, the samples were analyzed for cytokine presence following the manufacturer's protocol and scanned using the InnoScan 710 microarray fluorescence scanner (Innopsys) by the manufacturer of the cytokine array.

3.2.10 NanoTCE/nanoMuTE and T cell biodistribution, tumor efficacy, and survival in vivo

For all animal studies, mice were randomized into groups and no blinding was done in this study. For biodistribution, human MM.1S-CBR cells ($2x10^6$/mouse) were injected IV to NSG mice to generate the MM tumor models. PBMCs were isolated from healthy human donors using Ficoll centrifugation and subsequently separated for T cells using a human Pan T cell isolation kit (Miltenyi Biotec), as previously described [281]. T cells ($5x10^6$/mouse) were stained with calcein violet and injected IV to each mouse three weeks following propagation of the MM cells. One hour post T cell injection, mice were treated IV with Isotype/CD3, nanoTCEs, or nanoMuTEs stained with DiD (.5 mg/mouse). Organs were extracted 24 hours later and analyzed via flow cytometry.

www.manaraa.com

For tumor efficacy and survival, human BCWM.1-Luciferase cells or MM.1S-CBR cells ($2x10^6$/mouse) were injected IV to NSG mice to generate the WM or MM tumor models, respectively [289]. T cells ($5x10^6$/mouse) were injected IV to each mouse 7 days after the injection of tumor cells. One hour post T cell injection, mice were treated IV with Isotype/CD3, nanoTCEs, or nanoMuTEs (.5 mg/mouse), and weekly thereafter.

For tumor progression, mice were imaged weekly using bioluminescent imaging (BLI). Mice were injected with D-luciferin (150 ug/kg) intraperitoneally, and tumor burden was detected using an IVIS 50 bioluminescence imaging system (PerkinElmer, Waltham, MA) 10 minutes post luciferin injection, and images were analyzed using Living Image 2.6 software (PerkinElmer). For survival, mice were monitored on a daily basis to record survival.

3.2.11 Gene Expression Analysis

Gene expression data on MM patients were extracted from previously published literature [290] describing data from 600 newly diagnosed MM patients, in which plasma cells were subsequently selected using anti-CD138 beads and mRNA gene expression was performed using the Affymetrix U133 Plus 2.0 microarray platform (Santa Clara, CA) and analyzed using the Affymetrix Microarray Suite GCOS1.1. BCMA, CS1, and CD38 gene expression was analyzed and plotted using Python.

3.2.12 Statistical Analyses

All in vitro experiments in this study were independently replicated three times. Sample size for laboratory animals was estimated using published guidelines [291]. In vitro experiments were performed in quadruplicates, and in vivo experiments consisted of 7 mice each; data from in vitro and in vivo experiments were expressed as means ± standard deviation. Data normality was

www.manaraa.com

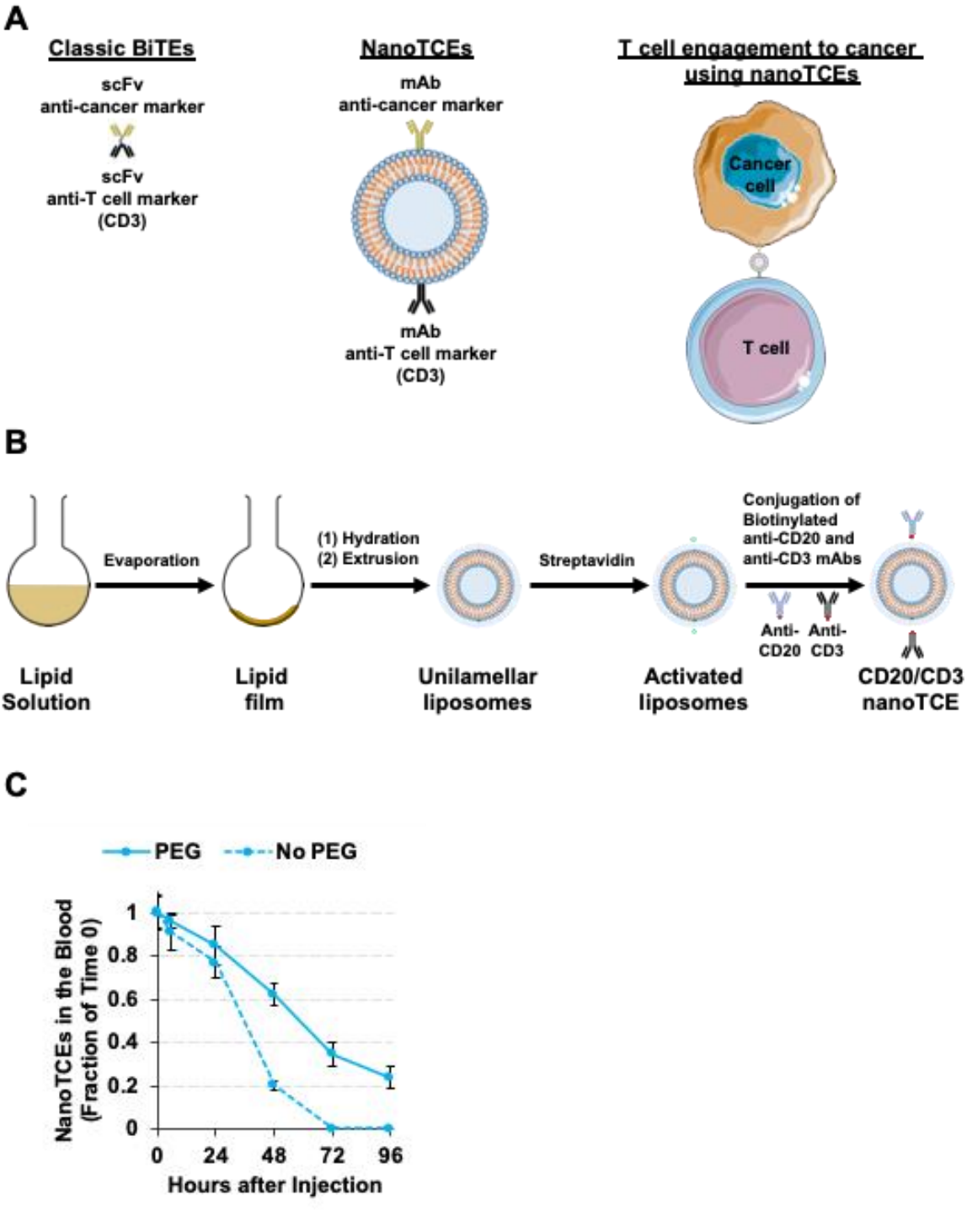
analyzed using residuals, and variance similarity across groups were also analyzed by examining the expected variance of each group. Statistical significance was analyzed using a Student's t-test, one-way, or two-way analysis of variance (ANOVA). Log-rank test was used to compare the Kaplan Meier curves. P values less than 0.05 were used to indicate statistically significant differences.

3.3 Results and Discussion

We have developed nanoTCEs, which are liposomes decorated with anti-CD3 mAbs targeting T cells, and mAbs targeting the cancer antigen (**Figure 4A**). We hypothesized that the liposomal nature of nanoTCEs will have a prolonged half-life. We developed liposomes with or without stealth PEGylation conjugated to these anti-CD3 and anti-CD20 mAbs (CD20/CD3 nanoTCEs) (**Figure 4B**). We chose to target CD20 for targeting WM cells, since CD20 has been routinely and successfully used as a therapeutic target for WM [292, 293]. Non-PEGylated nanoTCEs improved the half-life to about 36 hours, while the PEGylated nanoTCEs had even a longer half-life of about 60 hours (**Figure 4C**). Therefore, we adopted the PEGylated nanoTCEs formulation for all upcoming experiments. The longer half-life enabled administration of the nanoTCEs once a week as an intravenous (IV) bolus injection for in vivo experiments. Clinically, the improved pharmacokinetic profile will be translated into a more convenient dosing regimen and therefore a dramatic improvement in the patient's quality of life and decrease risk of infections related to continuous infusion. Other solutions that have been established to circumvent the low pharmacokinetic profile include supplementing BTCEs with an Fc receptor or an anti-human serum albumin binding construct [294, 295]; both methods prevent the rapid elimination and degradation of BTCEs by the neonatal Fc receptor [296, 297]. There are currently multiple

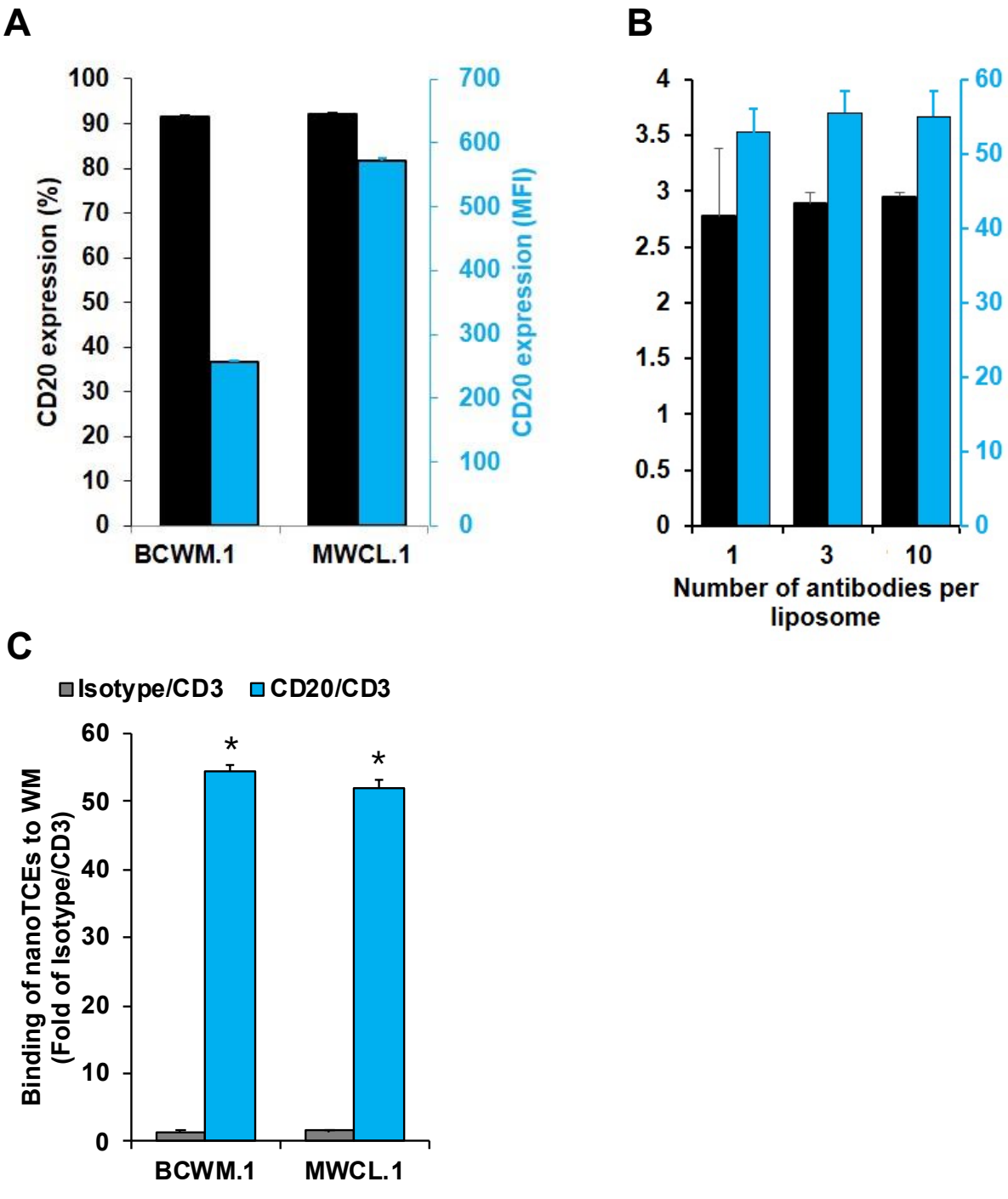
www.manaraa.com

ongoing clinical trials testing these newly designed BTCEs for efficacy and toxicity [240, 298-301].



www.manaraa.com

Figure 4. Development of nanoparticle T cell engagers. A. Schematic of classic BTCEs, nanoparticle T cell engagers, and the utilization of nanoTCE to engage T cells to cancer cells. **B.** A scheme of the production of the nanoTCE using thin-film evaporation method, followed by conjugation of mAbs of choice such as anti-CD20 and anti-CD3. **C.** The pharmacokinetic profile of nanoTCEs with or without PEGylation in vivo.



www.manaraa.com

Figure 5. Development of nanoTCEs for WM. A. RMFI and percent of CD20 protein expression on the surface of WM cells. **B.** The effect of the number of anti-CD20 mAbs conjugated to the liposome on the binding of the nanoTCEs to BCWM.1 cells, and the effect of the number of anti-CD3 mAbs conjugated to the liposome on the binding of the nanoTCEs to T cells. **C.** Binding of Isotype/CD3 and CD20/CD3 nanoTCEs to WM cells. Two-sided student t-test was used; statistical significance ($p<.05$) between CD20/CD3 and Isotype/CD3 was indicated by placing an asterisk.

First, we validated the use of CD20 as a target for the treatment of WM. We measured the percent of WM cells that express CD20. For both WM cell lines, CD20 is highly expressed and on approximately 90% of cells (**Figure 5A**). We then investigated the effect of the number of antibodies conjugated to the liposome. Increasing the number of antibodies conjugated to the liposomes did not increase the binding of the nanoTCEs to WM or T cells, which is shown in **Figure 5B**. Therefore, for all the upcoming experiments, we developed nanoTCEs with one CD3 and one CD20 mAb per liposome. We then tested the binding of the CD20/CD3 nanoTCEs to WM cells, compared to isotype and CD3 conjugated nanoTCEs (Isotype/CD3). The CD20/CD3 nanoTCEs bound to the WM cells about 50-fold greater than Isotype/CD3 nanoTCEs (**Figure 5C**).

To demonstrate the therapeutic efficacy of the nanoTCEs, we used our 3D Tissue Engineered Bone Marrow (3DTEBM) model [287] (**Figure 6A**), in which we used primary BM aspirates from patients to develop a 3D culture of the malignant BM niche. The model is developed using all the cells in the tumor microenvironment; not only tumor cells, but also other accessory cells including T cells. We used the BM supernatant from patients to create the 3D matrix by crosslinking fibrinogen naturally found in the marrow; the cellular fraction is also re-introduced into the scaffold. The 3DTEBM recapitulates cellular structures and oxygen gradients of the BM niche and allows proliferation of primary cells from various hematologic malignancies (such as WM and MM). It can be also used with cell lines in combination with the

www.manaraa.com

tumor microenvironment (without cancer cells) isolated from patients. We suggest this model as an optimal model for testing the effect of T cell-based immunotherapies in vitro.

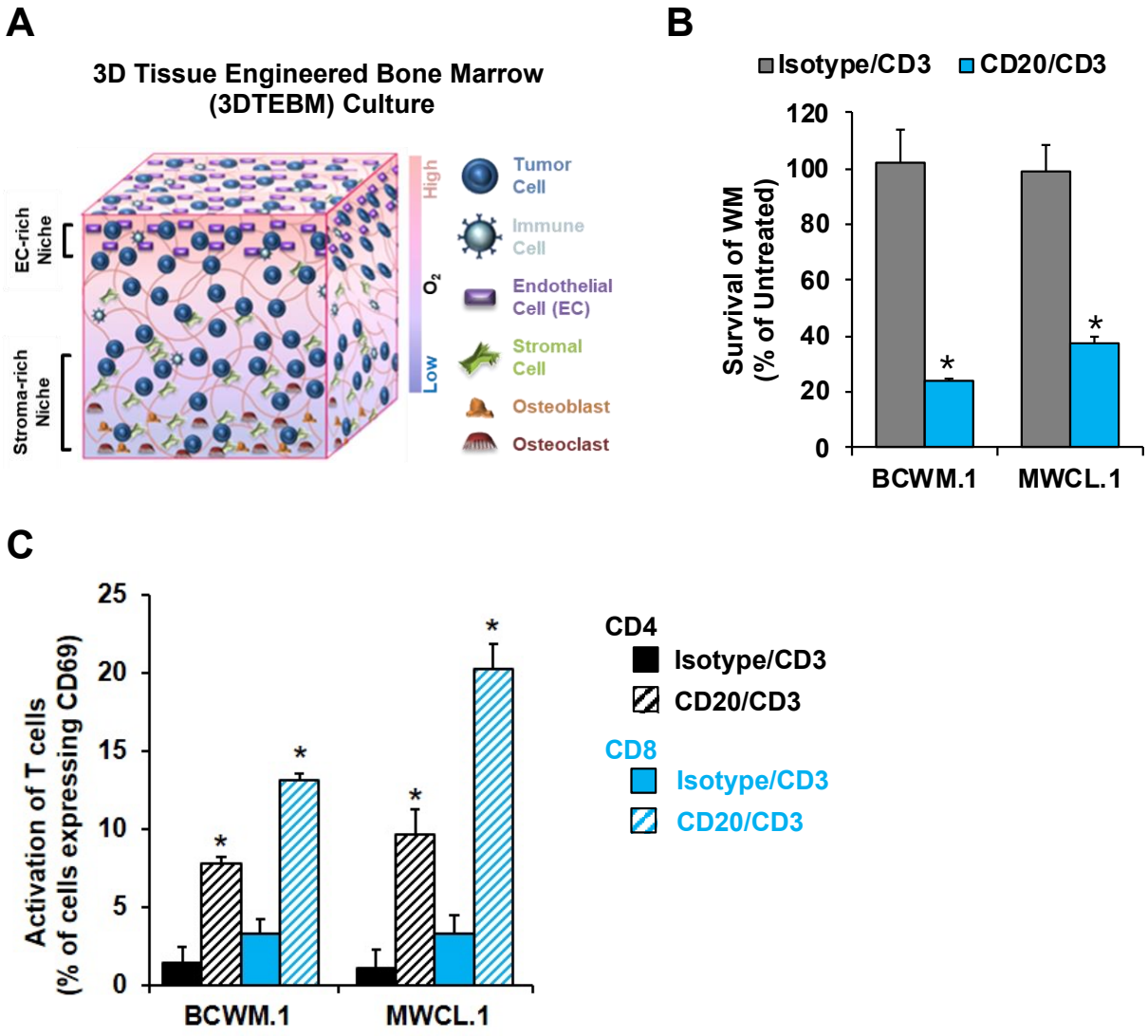


Figure 6. WM killing and T cell activation with nanoTCEs in vitro. A. A scheme of 3DTEBM cultures used to determine the effect of nanoTCEs on T cell activation and cancer cell killing in vitro. **B.** The effect of Isotype/CD3 and CD20/CD3 nanoTCEs on the killing of WM cells with T cells (n=4). **C.** The effect of Isotype/CD3 and CD20/CD3 nanoTCEs on the expression of CD69 on CD4+ and CD8+ T cells as a marker of T cell activation (n=3). Two-sided student t-test was used; statistical significance ($p<.05$) between CD20/CD3 and Isotype/CD3 was indicated by placing an asterisk.

www.manaraa.com

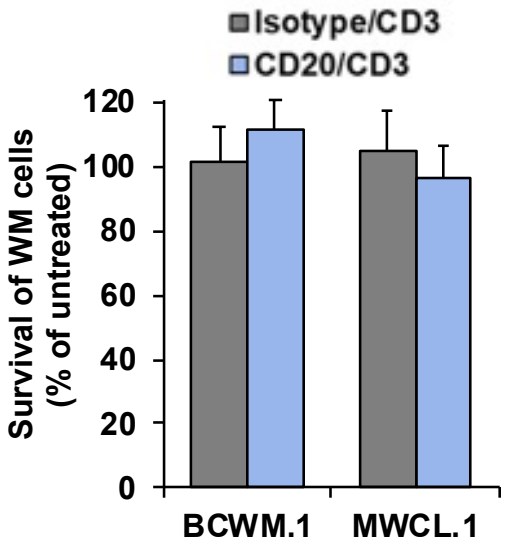


Figure 7. The Effect of Isotype and CD20 nanoTCEs on the killing of WM cells without T cells.

We tested the effect of CD20/CD3 nanoTCEs on the survival of WM cells in the 3DTEBM. CD20/CD3 nanoTCEs induced 60-70% killing of WM cells, while the Isotype/CD3 nanoTCEs did not induce any killing whatsoever (**Figure 6B**). We ensured that the MM cell lysis seen with nanoTCEs was T-cell mediated by incubating WM cells and nanoTCEs or Isotype/CD3 without T cells and observed no killing of WM cells as seen in **Figure 7**. In addition, we tested the activation of T cells by nanoTCEs in the 3DTEBM. CD69 expression, as a marker of T cell activation, in CD4 and CD8 T cells (**Figure 6C**) was higher after treatment with CD20/CD3 nanoTCE compared to Isotype/CD3. Moreover, the CD8 T cells showed higher activation compared to CD4 T cells. Secretion of cytokines is a hallmark of T cell activation. **Figure 8** shows the cytokine secretion of T cells following their activation with CD20/CD3 and Isotype/CD3 nanoTCEs in the 3DTEBM. The presence of IL-2, IL-6, IL-10, TNF-α, and IFN-γ is significantly greater when treated with CD20/CD3 compared to Isotype/CD3. These results indicate that the CD20/CD3 nanoTCEs are specific to WM cells and that the effect is only mediated via T cell engagement.

www.manaraa.com

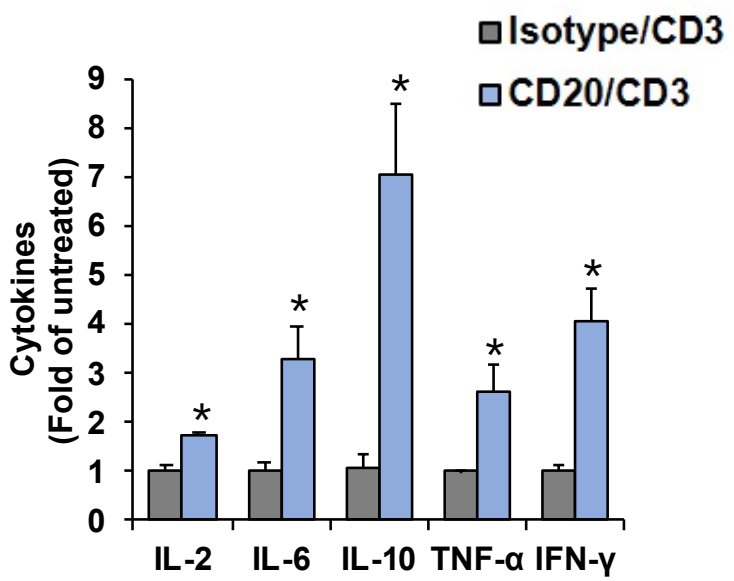


Figure 8. Cytokine secretions following treatment of WM cells with Isotype and CD20 nanoTCEs.

To demonstrate the therapeutic efficacy in vivo, we chose an aggressive xenograft WM model by injecting BCWM.1 cells IV (with humanized T cells), that kills the mice in less than 3 weeks following injection, if not treated; this represents the clinically aggressive/relapsed form of the disease. Mice treated with Isotype/CD3 nanoTCEs showed fast tumor progression and death of the entire cohort within 21 days. In contrast, mice treated with WM-targeting CD20/CD3 nanoTCE showed slower tumor progression at days 14 and 21, a significant reduction at day 28 (compared to day 21), and complete eradication of the tumor by day 35 (**Figures 9A** and **B**). The entire cohort survived with no signs of disease for as long as two months, which is when the experiment was stopped (**Figure 9C**). These results demonstrate that the CD20/CD3 nanoTCE immunotherapy has an outstanding potential to treat/cure even the most aggressive forms of WM.

www.manaraa.com

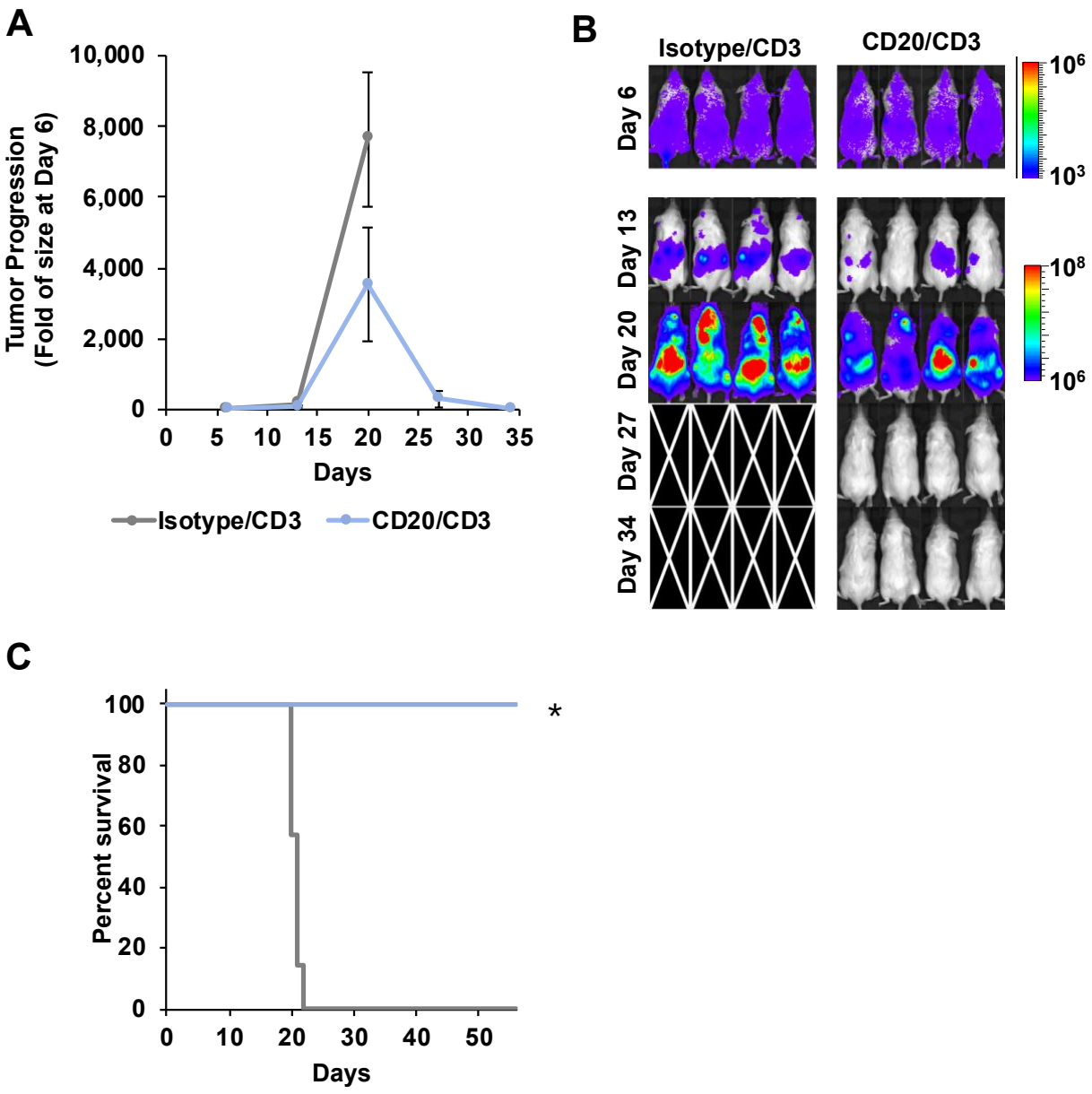


Figure 9. WM killing and T cell activation with nanoTCEs in vivo. A. Quantitative and **B.** qualitative analysis of the effect of Isotype/CD3 and CD20/CD3 nanoTCEs on the progression of WM tumors in vivo (n=7). **C.** The effect of Isotype/CD3 and CD20/CD3 nanoTCEs on the survival of WM-bearing mice (n=3). Log-rank test was used to compare the Kaplan Meier curves; statistical significance ($p<.05$) between CD20/CD3 and Isotype/CD3 was indicated by placing an asterisk.

www.manaraa.com

The second major limitation of BTCEs (and CAR-T cells) is that they are designed to target only one antigen on cancer cells. Preclinical studies have demonstrated that targeting multiple antigens by CAR-T cells or BTCEs are still technically challenging [302-306]. Especially in a multi-clonal disease like MM [307-310], these therapies confer the development antigen-less clones, causing tumor escape and relapse of the disease [271, 272].

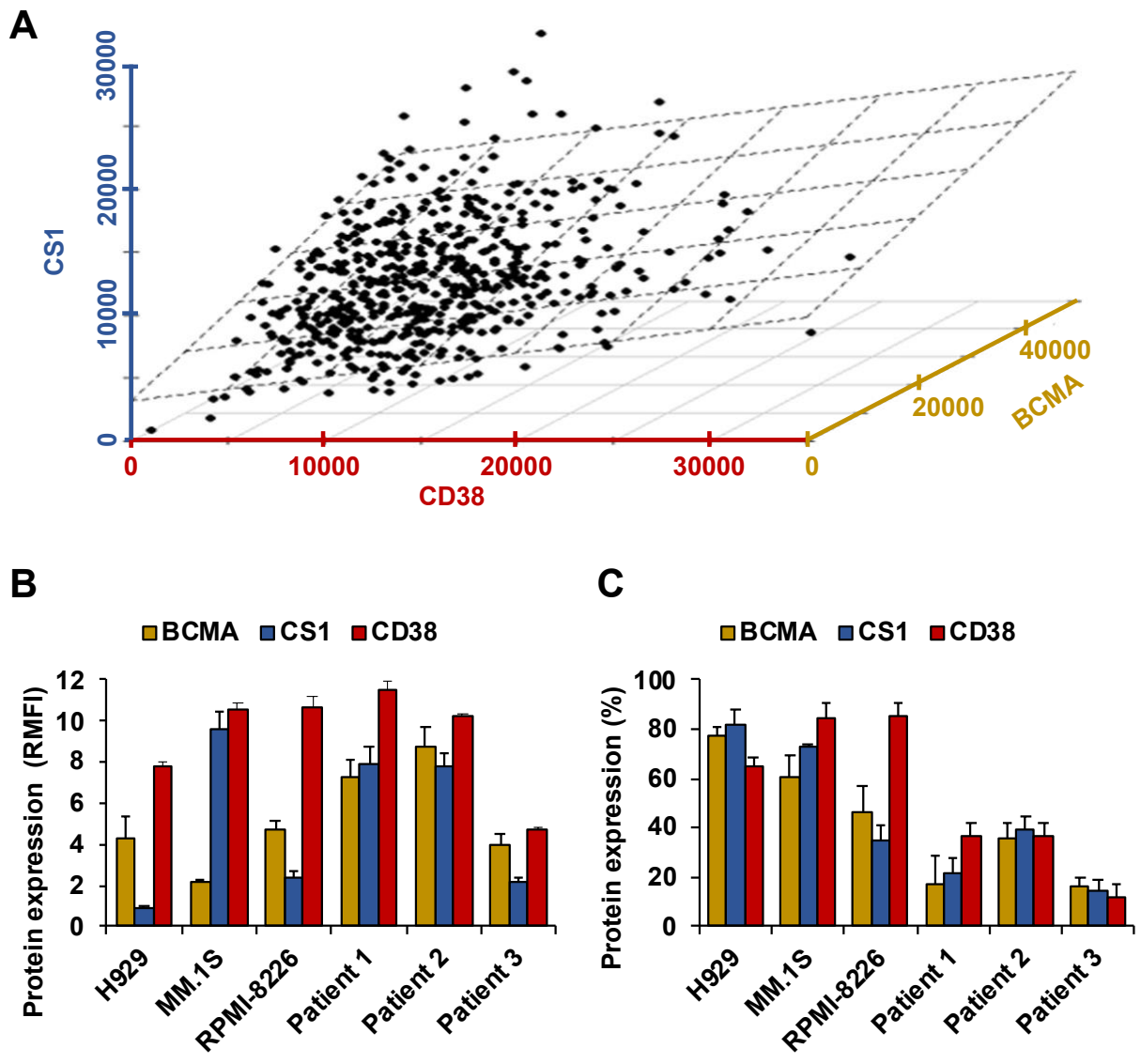


Figure 10. BCMA, CS1, and CD38 Expressions on Primary Multiple Myeloma (MM) and Cell Lines. A. mRNA gene expressions of BCMA, CS1, and CD38 in a cohort of 600 MM patients (n=600).

www.manaraa.com

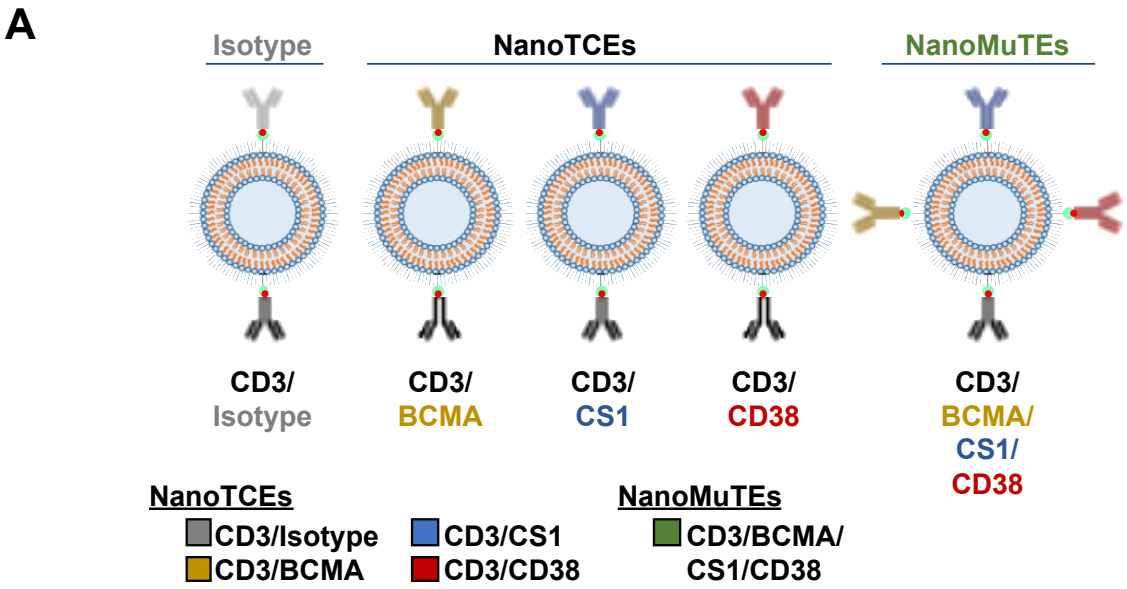
B. RMFI and **C.** percent protein expression of BCMA, CS1, and CD38 on MM cell lines and three patient primary cells.

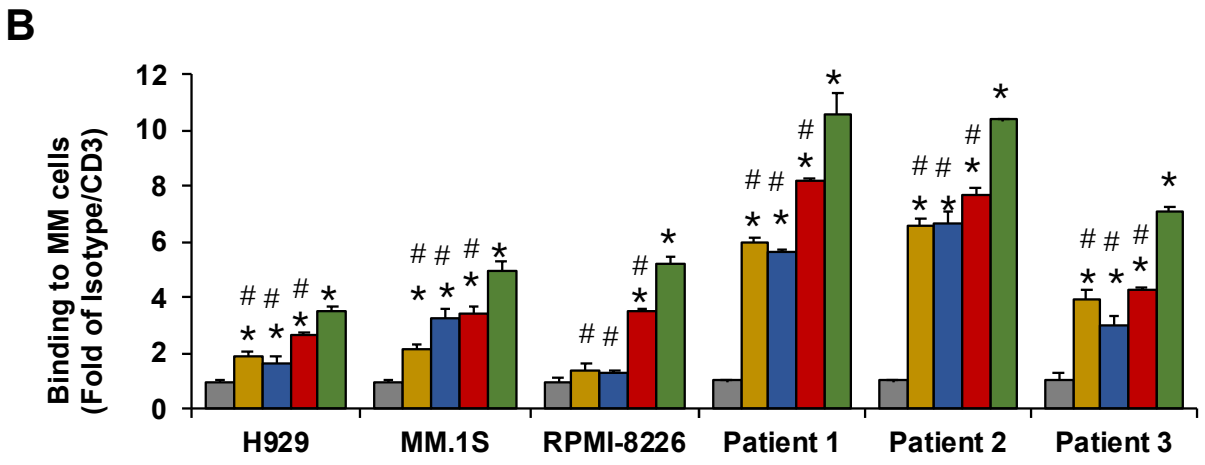
Several antigens were used previously as targets for T cell-based immunotherapy in MM, including B cell maturation antigen (BCMA), CD38, and SLAMF7 (CS1) [186, 187, 208, 311, 312]. Gene expression analysis of these antigens in MM patients showed that the expression of each marker was highly variable, emphasizing the heterogeneity of the expression of these genes in MM patients (**Figure 10A**). We also tested the surface protein expression of these antigens on MM cells, which further showed and emphasized the variability and presence of expression (**Figures 10B** and **C**). Such heterogeneous expression presents a challenge for the efficacy of any immunotherapy that targets any of these antigens as a single target.

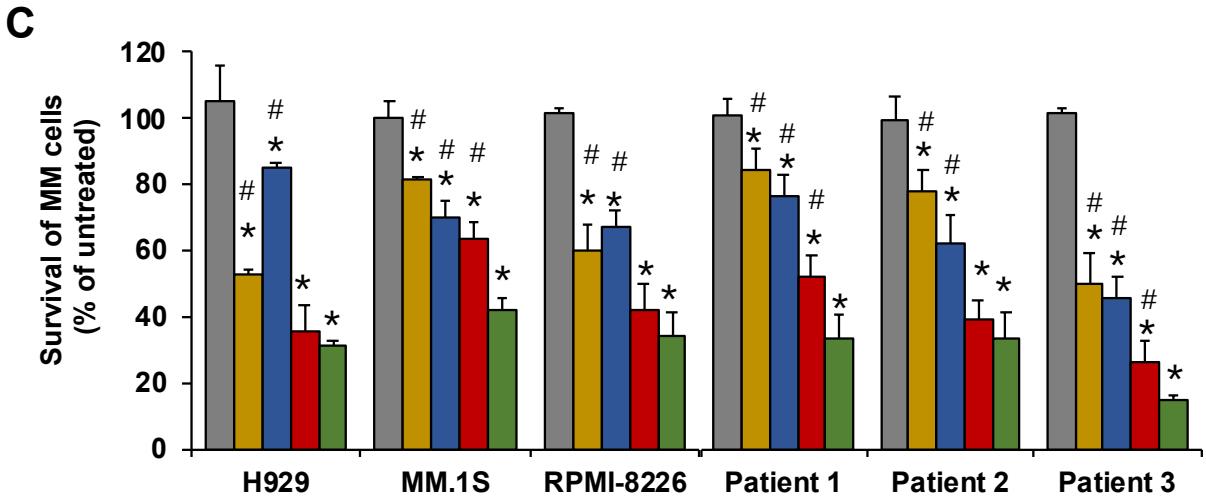
Therefore, we developed a nanoparticle that targets multiple cancer antigens simultaneously by conjugating multiple mAbs against multiple cancer antigens for T cell engagement (nanoMuTEs; **Figure 11A**). We hypothesized that nanoMuTEs will target multiple clones simultaneously, prevent antigen-less tumor escape, and be more efficacious than targeting individual antigens.

We tested the binding of three different BCMA/CD3, CS1/CD3, and CD38/CD3 nanoTCEs (each targeting one antigen) and BCMA/CS1/CD38/CD3 nanoMuTEs (targeting all three antigens) to MM cells. Each nanoTCE bound to MM cells more than the Isotype/CD3-nanoTCE, in correlation with the surface expression of each antigen; nanoMuTEs showed higher binding compared to each nanoTCE alone (**Figure 11B**).

www.manaraa.com







www.manaraa.com

Figure 11. Development and biological function of nanoparticle Multispecific T cell Engagers (nanoMuTEs). A. Illustrations of the Isotype/CD3, BCMA/CD3, CS1/CD3, CD38/CD3 nanoTCEs and BCMA/CS1/CD38/CD3 nanoMuTEs. **B.** Binding of the nanoTCEs and nanoMuTEs to MM cell lines and primary cells (n=3). **C.** The effect of nanoTCEs and nanoMuTEs on the killing of MM cells by T cells (n=4). One-way and two-way ANOVA was used; statistical significance ($p<.05$) was indicated using two symbols (* and #); specifically, * represents significance between the nanoTCE and Isotype/CD3, and # represents significance between the nanoTCE and nanoMuTE.

We further tested T cell-induced killing of MM cells by nanoTCEs and nanoMuTEs in the 3DTEBM. Each nanoTCEs induced more MM killing compared to Isotype/CD3, while nanoMuTEs induced more MM killing compared to each nanoTCE (**Figure 11C**). We also tested T cell activation. Activation of CD4 and CD8 T cells (**Figures 12Ai** and **ii**, respectively) was higher after treatment with each nanoTCE compared to Isotype/CD3, while activation after treatment with nanoMuTEs was higher than each nanoTCE. CD8 T cells showed higher activation compared to CD4 T cells when treated with any of the nanoTCEs or nanoMuTEs. In addition, we investigated the presence of cytokines following treatment with nanoTCEs or nanoMuTEs (**Figures 12Bi** and **ii**). The presence of IL-2, IL-6, IL-10, TNF-α, and IFN-γ is significantly greater when treated with each nanoTCE compared to Isotype/CD3; nanoMuTEs induced greater secretion than the nanoTCEs.

www.manaraa.com

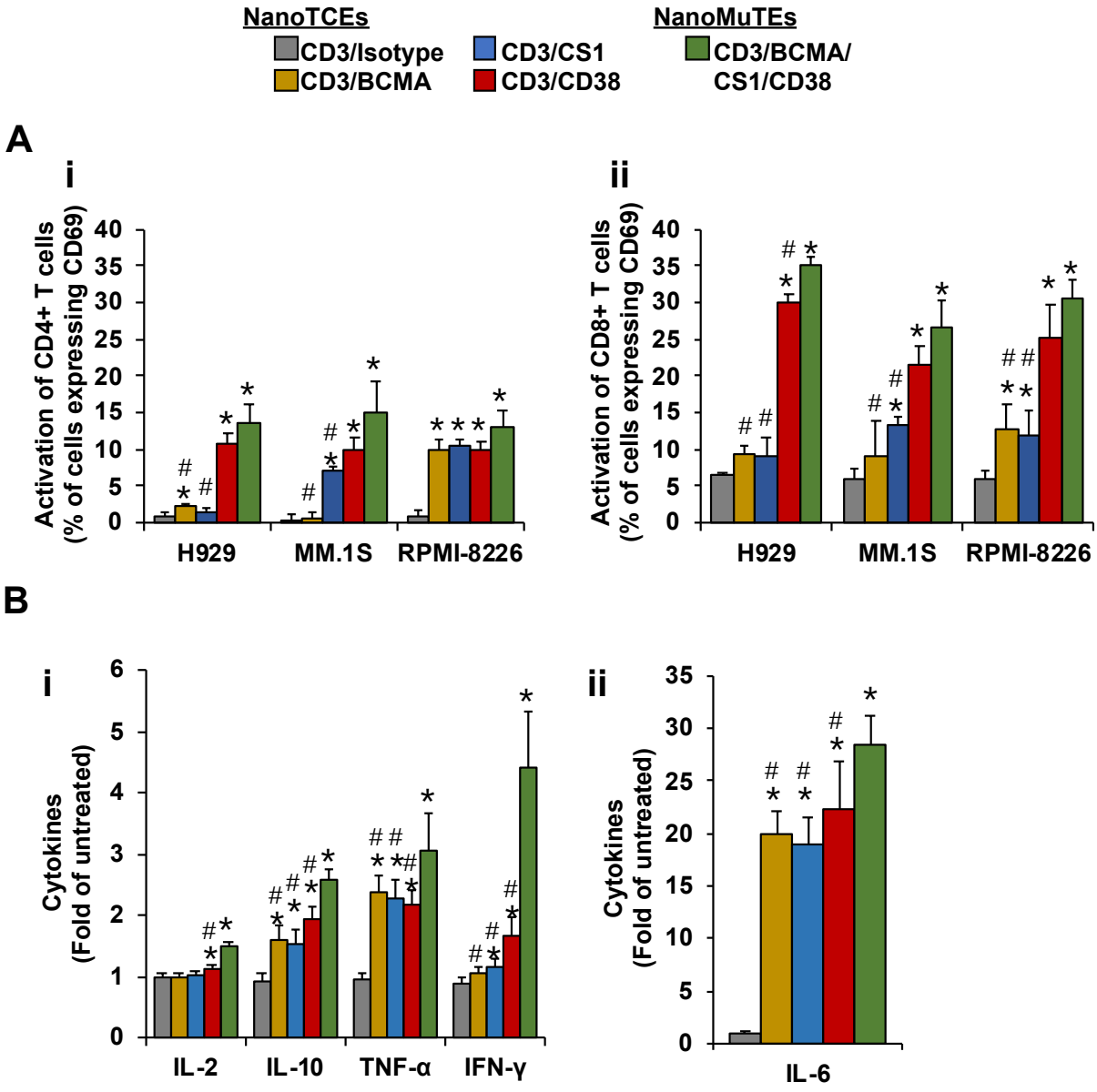


Figure 12. Activation of T cells using nanoTCEs and nanoMuTEs. Ai. and **ii.** The effect of nanoTCEs and nanoMuTEs on the expression of CD69 on CD4+ and CD8+ T cells, respectively (n=4). **B. i.** and **ii.** Cytokine secretions following treatment of MM with nanoTCEs or nanoMuTEs (n=5).

www.manaraa.com

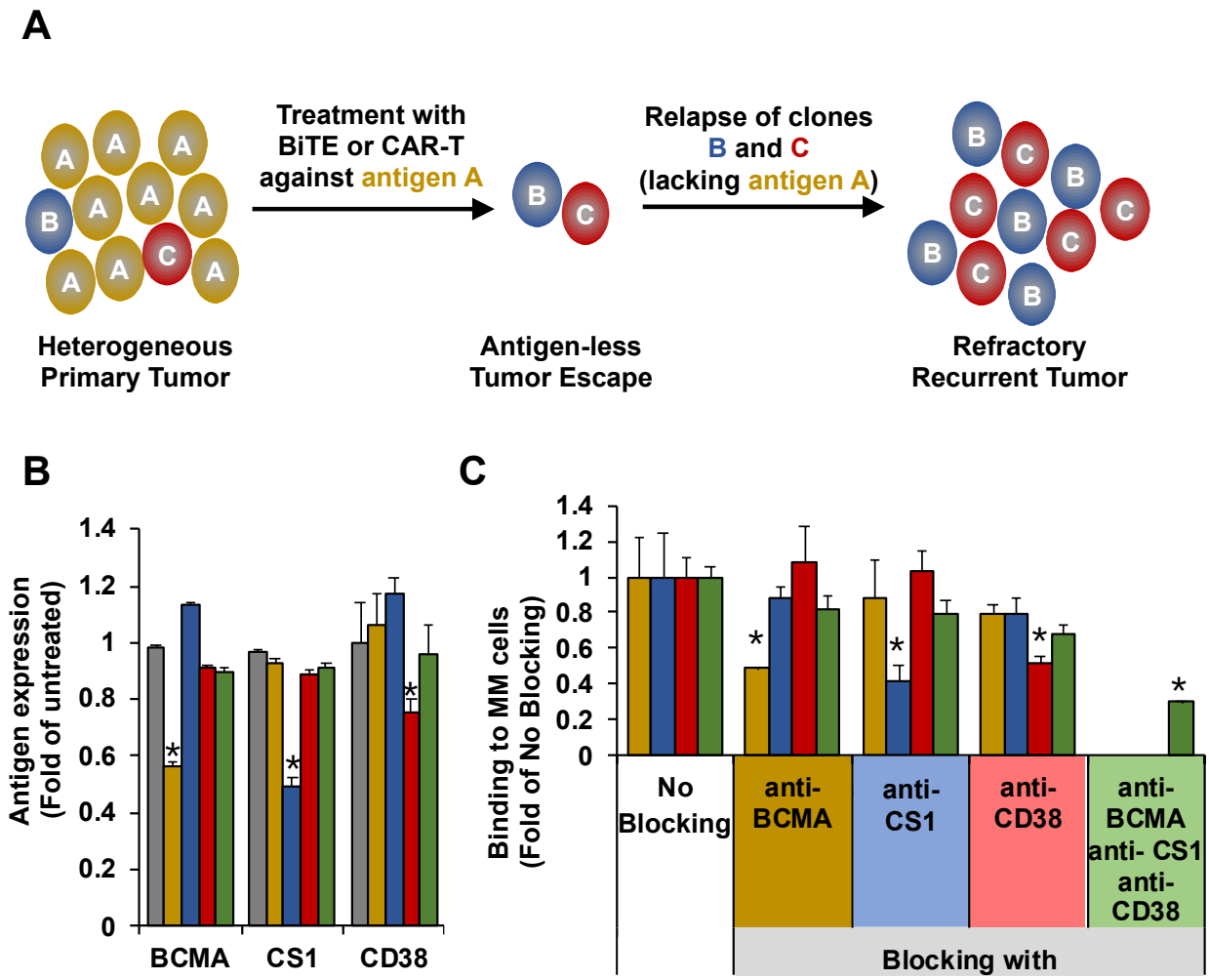


Figure 13. Circumventing antigen-less tumor escape with nanoMuTEs. A. Schematic of the mechanism of tumor escape after treatment with immunotherapies targeting one antigen due to development of antigen-less tumor clones which cause relapse of the disease. **B.** The effect of nanoTCEs and nanoMuTEs on the expression of BCMA, CS1, and CD38 on MM cells remaining following treatment (n=4). **C.** The effect of blocking tumor antigens (BCMA, CS1, and CD38) on the binding of nanoTCEs and nanoMuTEs to MM cells (n=3). One-way and two-way ANOVA was used; statistical significance ($p<.05$) was indicated using two symbols (* and #); specifically, * represents significance between the nanoTCE and Isotype/CD3, and # represents significance between the nanoTCE and nanoMuTE.

www.manaraa.com

Next, we developed antigen-less clones by testing the effect of nanoTCEs and nanoMuTEs on the expression of antigens on MM cells (**Figure 13A**). When treated with BCMA/CD3, CS1/CD3, or CD38/CD3, the expression of BCMA, CS1, and CD38 in the whole MM cell population was decreased, respectively (**Figure 13B**), but not affected by the nanoTCEs with other targets. The decrease can be attributed to killing of the population with high expression of the specific antigen or downregulation of the specific antigen on the cells, both of which contribute to the development of antigen-less populations. In contrast, the treatment with nanoMuTEs did not generate a population with lower expression of any of the three antigens, which suggests that treatment with nanoMuTEs will not cause antigen-less tumor escape and create a better therapeutic strategy.

We next investigated the effect of blocking (as a model for downregulation) of BCMA, CD38, and CS1 on the binding of nanoTCEs and nanoMuTE to MM cells. The binding of each of the nanoTCEs was reduced when the antigens on the cells were blocked with the respective blocking antibody against the antigen that it is targeting. In contrast, no significant decrease of the binding of nanoMuTEs was observed when treated with any of the antibodies blocking alone; likely because the binding was facilitated through other antigens. Binding of nanoMuTEs was decreased when treated with a combination of the three blocking antibodies (**Figure 13C**). This demonstrates that downregulation (or loss) of an antigen will reduce the binding (and hence the efficacy) of the nanoTCE, as observed clinically with the treatment with CAR-T cells and BTCEs, but did not affect the binding of nanoMuTEs, which creates a better therapeutic strategy.

www.manaraa.com

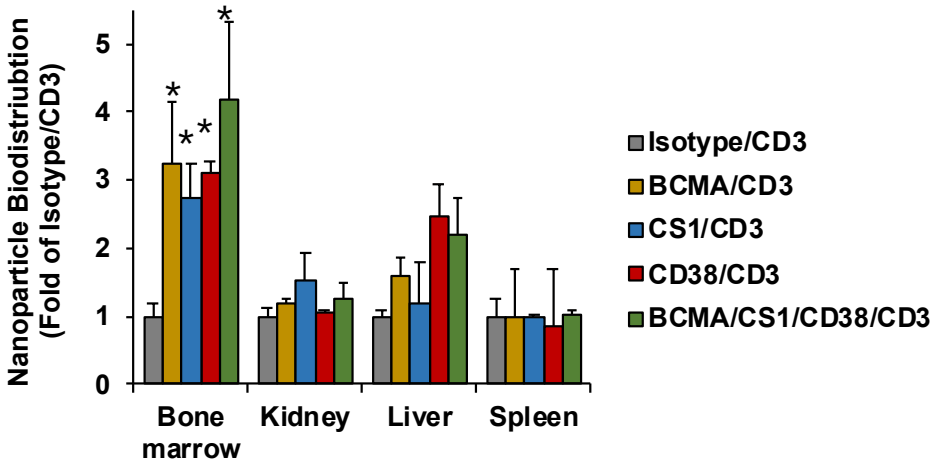
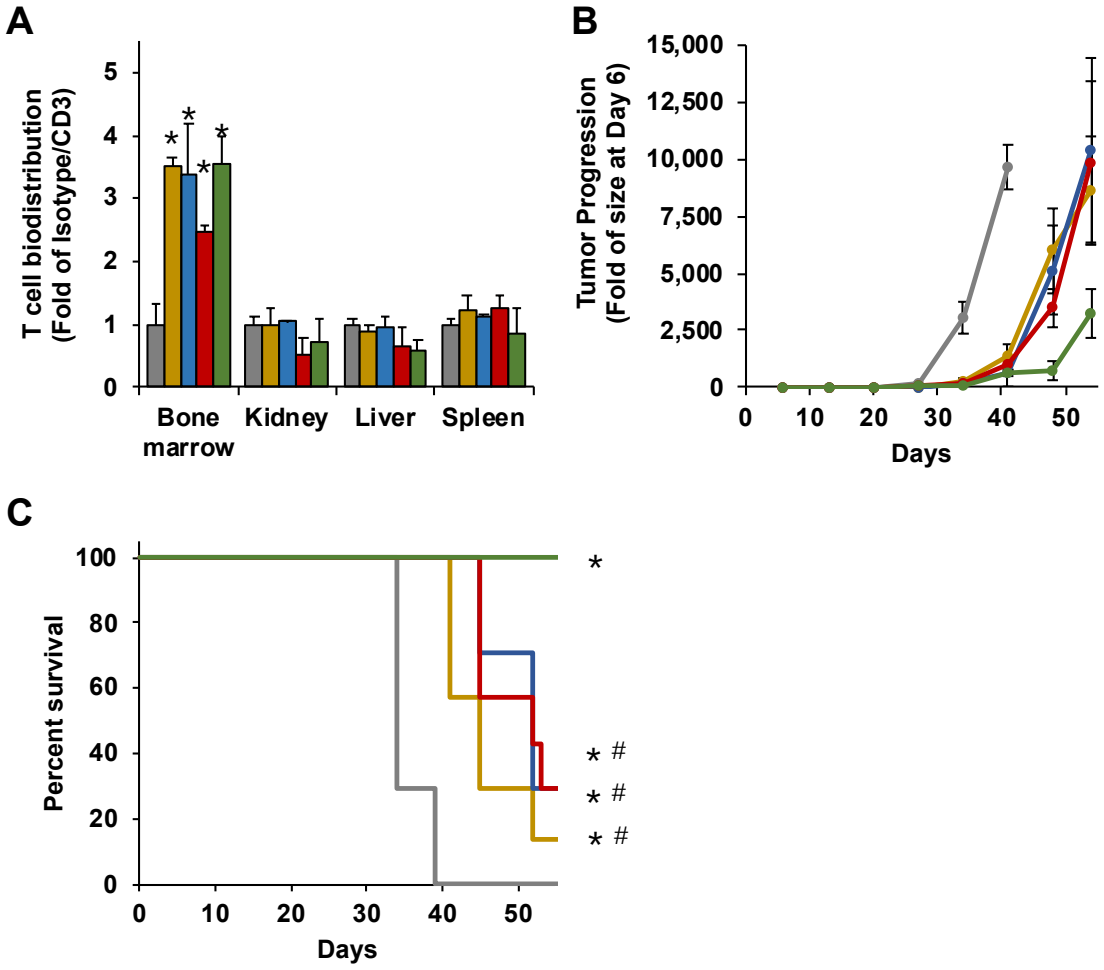


Figure 14. Biodistribution of nanoTCEs/nanoMuTEs.



www.manaraa.com

Figure 15. Circumventing antigen-less tumor escape with nanoMuTEs. A. Biodistribution of T cells following 24 hours in vivo (n=3). **B.** The effect of nanoTCEs and nanoMuTEs on the progression of MM tumors in vivo (n=7). **C.** The effect of nanoTCEs and nanoMuTEs on the survival of MM-bearing mice (n=7). One-way and two-way ANOVA was used to assess Figure 15A. Log-rank test was used to compare the Kaplan Meier curves; statistical significance ($p<.05$) was indicated using two symbols (* and #); specifically, * represents significance between the nanoTCE and Isotype/CD3, and # represents significance between the nanoTCE and nanoMuTE.

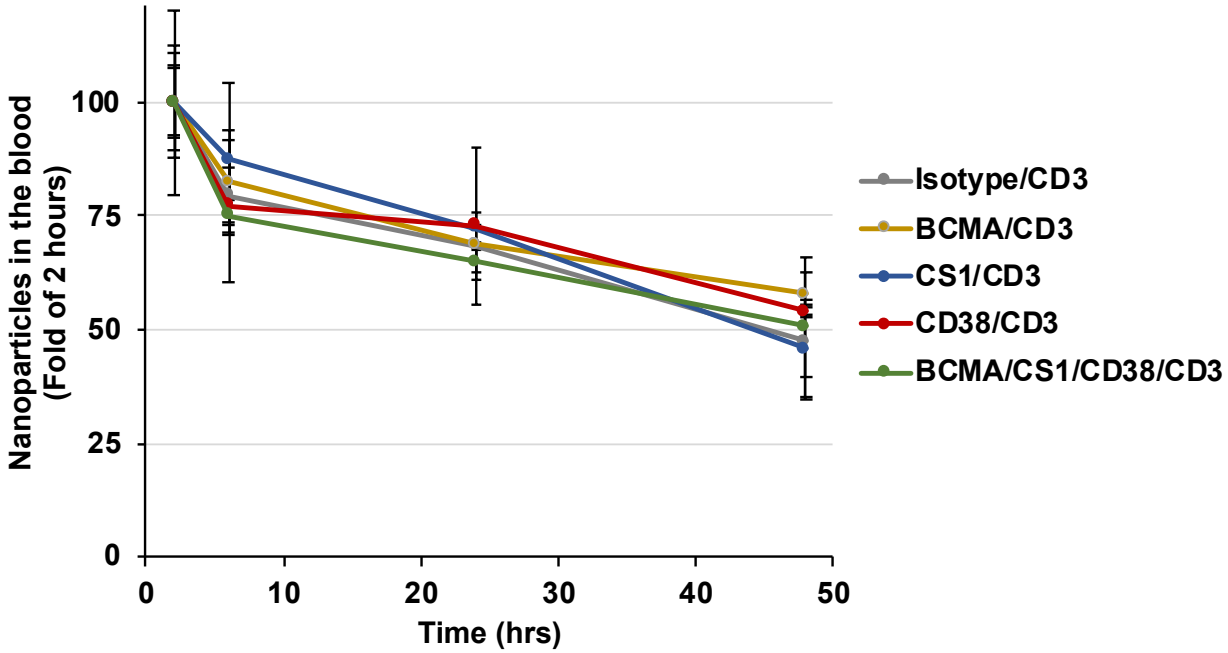


Figure 16. Pharmacokinetics of nanoTCEs and nanoMuTEs in vivo.

We also investigated the biodistribution of nanoTCEs/nanoMuTEs and T cells following 24 hours (**Figures 14** and **15A**, respectively). We see specific accumulation of nanoTCEs and nanoMuTEs at the tumor site (BM) compared to Isotype/CD3. Consequently, T cells were specifically engaged to the tumor site following treatment with nanoTCEs or nanoMuTEs compared to Isotype/CD3. Next, the pharmacokinetic profile of each of the nanoTCEs and nanoMuTEs was similar to the CD20/CD3 nanoTCEs with a half-life of approximately 50-60 hours (**Figure 16**). To demonstrate the therapeutic efficacy of the nanoTCEs and nanoMuTEs in vivo, we used an aggressive xenograft MM model by injecting MM.1S IV (with humanized T

www.manaraa.com

cells), that kills the mice in less than 4-5 weeks after injection, if not treated, and represents the clinically aggressive/relapsed form of the disease. Treatment with Isotype/CD3 nanoTCEs showed fast tumor progression and death of the cohort within 40 days (**Figures 15B** and **17**). Treatment of each of the nanoTCEs targeting one antigen (BCMA, CS1 or CD38) resulted in delayed tumor progression and prolonged survival, while the treatment with the nanoMuTEs induced longer tumor progression delay and resulted in survival of the entire cohort till 55 days (**Figure 15C**).

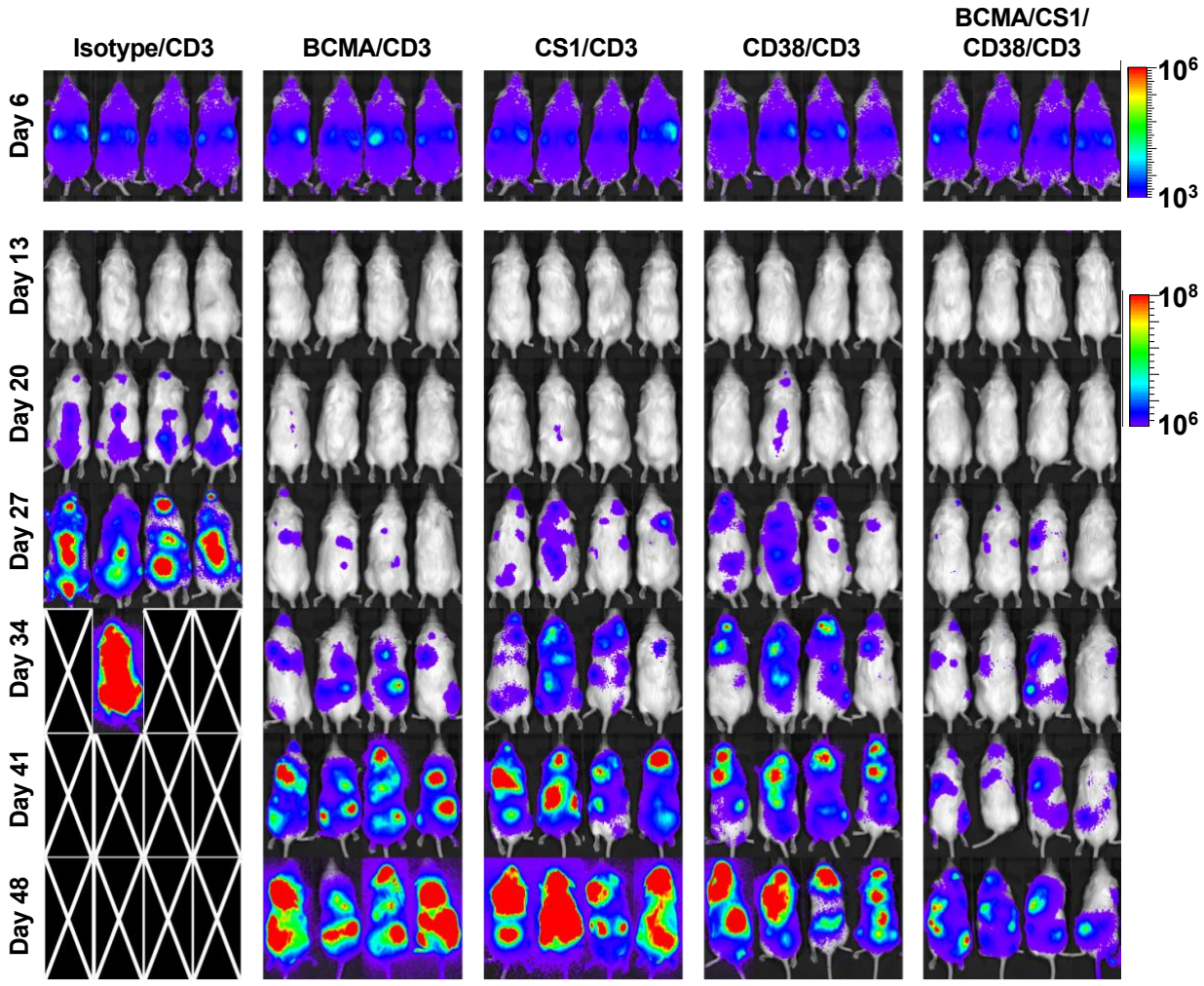


Figure 17. The effect of nanoTCEs and nanoMuTEs on the progression of MM tumors in vivo.

www.manaraa.com

Our study successfully shows the proof-of-concept of redirecting T cells to cancer using nanoparticles. The nanoTCEs/nanoMuTEs used for WM and MM were able to induce T cell-mediated cancer cell killing. The effect of the CD20/CD3 nanoTCEs for the treatment of WM was significantly more profound than the nanoTCEs/nanoMuTEs used for MM; CD20/CD3 cured the WM xenograft murine model whereas the nanoTCEs/nanoMuTEs prolonged survival of MM mice by only 10-20 days. This is, likely, due to the difference in antigen level and presence on each cancer type; expression of CD20 was prevalent in the vast majority of WM cells, while the expression of BCMA, CS1 and CD38 was variable on MM. Moreover, the intensity of the expression of CD20 on WM cells was 2-3 orders of magnitude higher than BCMA, CS1 and CD38 on MM.

In conclusion, the nanoTCE/nanoMuTE platform uses nanotechnology to provide a relatively easy-to-make and off-the-shelf solution to circumvent the major limitations of the current immunotherapy technologies (CAR-T cells and BTCEs). It takes advantage of the established high specificity of mAbs to better navigate the robust immune response to eliminate cancer. In this instance, it would be easy to modify this system to generate a new nanoTCE as an immunotherapy to target any cancer type by using existing or new mAbs that have the ability to specifically bind to the cancer cells of interest. The flexibility of the nanoparticle-based immuno-engaging technology provides a general platform with groundbreaking translational potential for developing easy-to-make, specific, and efficacious immunotherapy for cancer in general.

www.manaraa.com

Chapter 4: Nanoparticle T cell Engagers for the Treatment of Acute Myeloid Leukemia

4.1 Introduction

AML is the most common type of leukemia and characterized by the overproduction of immature myeloid stem cells in the bone marrow that has a 5-year survival rate of around 25% [313, 314]. The survival curves for AML patients have remained stagnant in the past decades due to the lack of newly approved therapies for AML. However recently, novel therapeutics and technologies are actively being developed and have shown promising results in preclinical and clinical settings [315-317].

Exciting immunotherapy technologies that are being investigated for AML including CAR-T cells and bispecific T cell engagers (TCEs). CAR-T cells are autologous T cells that have been virally transfected to express an engineered CAR construct, containing a synthesized fragment that targets the desired surface antigen on the target cell. Several studies have shown promising preclinical and clinical results with the use of this technology [318]. The main disadvantages of this technology relative to traditional therapies include toxicity, the long-term safety profile of the viral vector, the need to perform quality control testing frequently throughout the production of CAR-T cells, the high costs associated with this technique due to the need of extensive labor and expensive facility equipment, complex production, and the inability to target multiple tumor antigens with one CAR-T cell [275, 319].

In addition to CAR-T cells, T cell-based therapy can be pursued with TCEs. TCEs consists of two single chain variable fragments which are connected by a protein linker. One of the domains recognizes a tumor-associated surface antigen, while the other recognizes the T cell using the CD3 receptor [320]. This enables the TCE to redirect the T cell to the tumor and induce

www.manaraa.com

subsequent activation and expansion of the T cell. TCEs have exploited the use of endogenous T cells while also demonstrating high potency and efficacy against tumor cells [217, 321, 322]. This immunotherapeutic option has been shown to be successful for both solid and liquid tumors but is mostly known for the treatment of hematological malignancies [323]. TCEs demonstrate high potency and efficacy against tumor cells and exploit the use of endogenous T cells, circumventing the limitation of genetically engineering extracted patient T cells to express CARs. The disadvantages of TCEs, however, include toxicity, expensive costs with regards to its labor and production, complex production, short pharmacokinetics, and the inability to target multiple cancer surface markers [324, 325].

We have previously developed a nanoparticle-based T cell engagers (nanoTCEs) technology that is based on conjugation of two monoclonal antibodies to the surface of a liposomal nanoparticle; one antibody is against a cancer antigen and the other is against the CD3 receptor in T cells [265]. NanoTCEs utilize existing monoclonal antibodies which we conjugate to the surface of a nanoparticle, therefore taking advantage of the high specificity of existing monoclonal antibody-therapies, to engage and direct the potency and robust response of the immune system (T cells). NanoTCEs have been shown to circumvent the disadvantages of both CAR-T cells and TCEs; prolong pharmacokinetic profile, use endogenous T cells, and target multiple tumor and immune cell antigens while also inducing T cell activation and T cell-induced cancer cell lysis in vitro and in vivo. In addition, nanoTCEs are simple to make; in which the production of activated nanoparticle and the chemical conjugation of existing and clinically proven antibody against a specific disease takes only few hours. Therefore, the nanoTCE technology provides an easy to make platform for development of T-cell engaging immunotherapy using any existing anti-cancer monoclonal antibody.

www.manaraa.com

In the past, CD33 has been the target of immunotherapies for AML due to its presence on the majority of AML cells, and its expression correlates with stage of disease [314]. Therapy options that have used CD33 as a target and have been rendered successful includes the antibody-drug conjugate, gemtuzumab ozogamicin [326]. CD33-targeted TCEs in the realm of AML have also been proven to be effective and safe ex vivo, and these studies have led to the creation of a phase I clinical study with Amgen's TCE, AMG 330 (NCT02520427) [327]. In this study, we sought to create a nanoTCE targeted to CD33 for the treatment of AML.

4.2 Methods

4.2.1 Materials and Reagents

Antibodies and Pan T Cell Isolation Kits were purchased from Miltenyi Biotec (Bergisch Gladbach, Germany). RPMI-1640, .25% trypsin, L-glutamine, and penicillin-streptomycin were purchased from Corning (Corning, NY). Fetal bovine serum, lipophilic tracers, collagenase, and counting beads were purchased from Life Technologies (Carlsbad, CA). 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC), 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[amino(polyethylene glycol)-2000] (DSPE-PEG2000), and membranes were purchased from Avanti Polar Lipids (Alabaster, AL). Cholesterol was purchased from Millipore Sigma (Burlington, MA). Streptavidin conjugation kit was purchased from Abcam (Cambridge, United Kingdom). Mice were NCG (strain: 572), female, 50-56 days old, and purchased from Charles River (Wilmington, MA), and all experiments using these rodents were in compliance with the Institutional Animal Care and Use Committee at Washington University.

www.manaraa.com

4.2.2 Cells

K052, MOLM-14, NOMO-1, and THP-1 were all obtained from the lab of John DiPersio. Normal donor peripheral blood mononuclear cells (PBMCs) were isolated from healthy donors using Ficoll and separated for T cells using a Pan T cell kit. Cell lines were cultured in RPMI-1640 supplemented with 10% fetal bovine serum, 2 mM of L-glutamine, and 1% penicillin-streptomycin and cultured in NuAire water jacket incubators (NuAire, Plymouth, MN) at 37 °C and in 5% CO2.

4.2.3 Creation of nanoTCEs

The procedure of making nanoTCEs has been described [265]. Briefly, nanoTCEs were made up of three components: cholesterol, DPPC, and DSPE-PEG2000 with a mass ratio equivalent to 30: 65: 5, respectively. Lipids were mixed and solubilized in chloroform and evaporated. The film was then hydrated, and the resulting suspension was extruded using an extruder with 100 nm polycarbonate membranes [283, 328]. The biotinylated antibodies were conjugated to the liposomes using streptavidin and biotin reaction [289]. Malvern Zetasizer Nano ZS90 (Malvern, Worcestershire, United Kingdom) was used to determine zeta potential, diameter, and polydispersity index.

4.2.4 Protein Expression

Cells were incubated with anti-CD33 APC antibody in 4°C for one hour, washed, and analyzed by flow cytometry using MACSQuant Analyzer 10 with an Ex/Em of 635/655-730 nm. Cells were gated using forward and side scatter and analyzed for relative mean fluorescent intensity (MFI) of APC using BD FlowJo Software [285, 286].

www.manaraa.com

4.2.5 Liposomal Binding

Each nanoTCE was stained with a fluorescent tracer DiO. Cell lines and T cells were treated with Isotype/Isotype or CD33/CD3 nanoTCEs (3.7 nM) for two hours at 37°C. Cells were spun down, washed, and analyzed by flow cytometry with Ex/Em of 488/ 525±25 nm. Cells were gated using forward and side scatter and analyzed for MFI of DiO using BD FlowJo Software.

4.2.6 Activation of T cells in vitro

Cells were cultured in the 3D tissue engineered bone marrow (3DTEBM) [287] and treated with Isotype/CD3 or CD33/CD3 nanoTCEs at a concentration of 3.7 nM for 4 days. Then, cultures were digested, and cells were retrieved and incubated with anti-CD3 PE, anti-CD4 FITC, anti-CD8 Violet, and anti-CD69 APC antibodies for one hour in 4°C. These samples were analyzed by flow cytometer with Ex/Em of 488/585±20, 488/525±25, 405/450±25, and 635/655-730 nm, respectively. Cells were gated using forward and side scatter followed by double positive CD3+/CD4+ or CD3+/CD8+, both of which were analyzed for % of cells positive for CD69 using BD FlowJo Software.

4.2.7 T cell-mediated Killing of AML in vitro

Cell lines (pre-labeled with DiO) were incubated with T cells in the 3DTEBM and treated with Isotype/CD3 or CD33/CD3 nanoTCEs at a concentration of 3.7 nM for 4 days. Before digestion of the matrix, counting beads were added to the culture. The matrix was then digested, cells were retrieved, and analyzed by flow cytometry. Number of AML cells were analyzed as DiO+ cells and normalized to the number of counting beads using BD FlowJo Software.

www.manaraa.com

4.2.8 T cell-mediated Killing of AML in vivo

Mice were randomized into groups and no blinding was done in this study. Human THP-1 CBR cells ($1x10^6$/mouse) were injected intravenously, and T cells ($5x10^6$/mouse) were injected intravenously seven days post-injection of AML cells. One hour following injection of T cells, mice were treated intravenously with Isotype/CD3 or CD33/CD3 nanoTCEs (0.5 mg/mouse) and weekly thereafter for four weeks. These mice were then imaged weekly using bioluminescent imaging. Mice were injected with D-luciferin (150 ug/kg) intraperitoneally, and tumor burden was detected using an IVIS 50 bioluminescence imaging system (PerkinElmer, Waltham, MA) 10 minutes post-luciferin injection, and images were analyzed using Living Image 2.6 software (PerkinElmer). Mice were monitored on a daily basis to record survival.

4.2.9 Statistical Analyses

All experiments were independently replicated three times and performed in quadruplicates, and animal experiments consisted of seven mice per group; data from in vitro and in vivo experiments were expressed as means ± standard deviation. Statistical significance was analyzed using a Student's t-test, one-way, or two-way analysis of variance. Log-rank test was used to compare the Kaplan Meier curves. P-values less than 0.05 were used to indicate statistically significant differences.

4.3 Results and Discussion

CD33 is a valuable target for the treatment of AML, therefore, we first validate the presence of the marker in our experimental setup. We measured the fluorescent intensity and percent of CD33 in four different AML cell lines. For all cell lines, CD33 was expressed in high levels (**Figure 18A**) and uniformly on 90 – 100% of the cells (**Figure 18B**).

www.manaraa.com

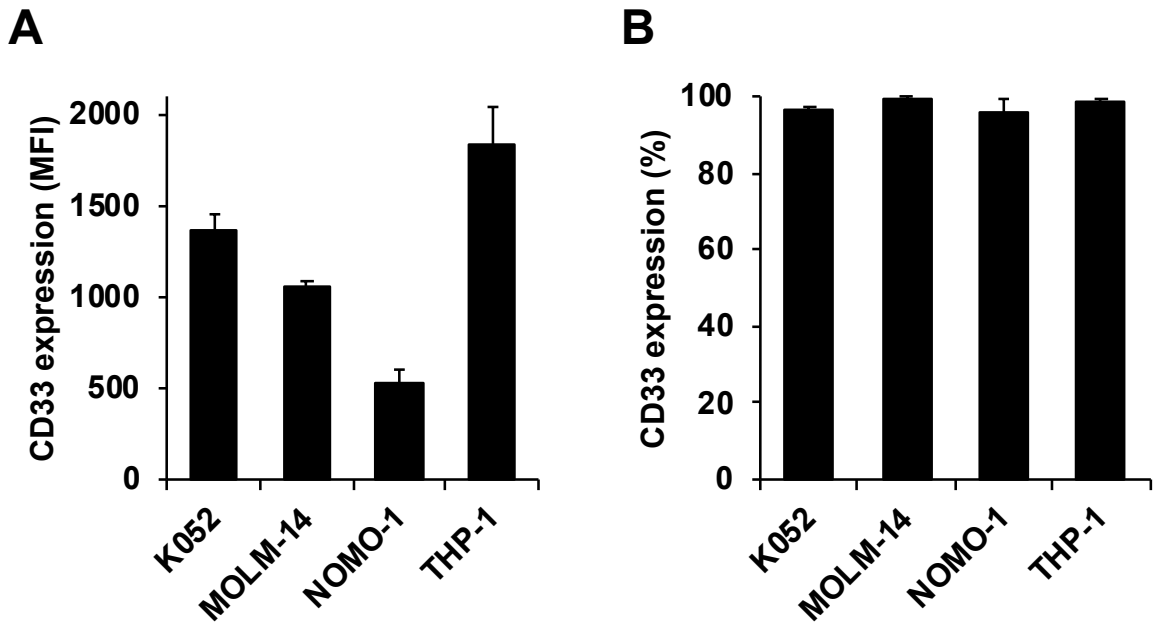


Figure 18. CD33 expression on AML cell lines. A. Mean fluorescent intensity and **B.** percent expression on K052, MOLM-14, NOMO-1, and THP-1.

Once we validated CD33 as a target in our systems, we developed CD33/CD3 nanoTCEs for the treatment of AML and used Isotype/CD3 nanoTCEs as control. We characterized the physicochemical properties of these nanoTCEs such as diameter, polydispersity index, and zeta potential which are shown in **Table 4**. We found that the size of the nanoTCEs was about 140 nm, with low polydispersity index indicating the uniformity of the particle size, and with close to neutral net charge.

Table 4. Parameters for CD33 nanoTCE.

Formulation	Size (nm)	Polydispersity Index	Zeta Potential (mV)
Isotype/CD3	140.5 ± 1.0	0.11 ± 0.02	0.9 ± 0.2
CD33/CD3	141.3 ± 1.0	0.07 ± 0.01	1.1 ± 0.1

www.manaraa.com

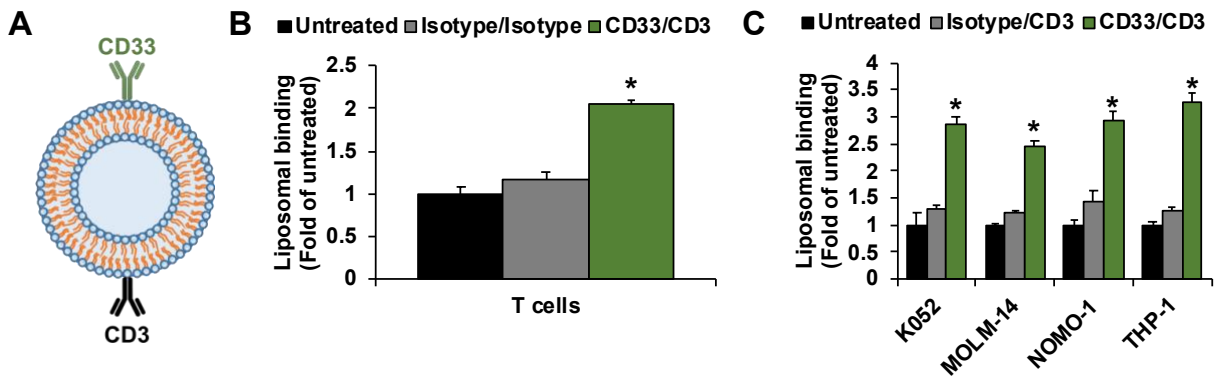


Figure 19. Development of nanoTCEs for AML. A. Schematic of CD33/CD3 nanoTCEs. Liposomal binding of Isotype and CD33/CD3 nanoTCEs to **B.** T cells and **C.** AML cell lines.

A schematic of the nanoTCEs is shown in **Figure 19A**. The CD33/CD3 nanoTCEs bound preferentially to the T cells and AML cell lines which are shown in **Figures 19B** and **19C**., respectively, compared to Isotype. To demonstrate the therapeutic efficacy of nanoTCEs in vitro, we used our 3DTEBM model, in which is a 3D cell culture that mimics the leukemic bone marrow niche, in which it recapitulates the tumor microenvironment and drug resistance better than classic 2D cultures [265]. We investigated the effect of nanoTCEs on activation of T cells and T cell-mediated killing of AML cell lines. In **Figure 20A** and **20B**, activation of T cells was observe as increase in CD69 upregulation in CD4 and CD8 T cells, respectively, following co-culture of T cells with AML cell lines in the presence of CD33/CD3 nanoTCEs, but not Isotype/CD3 TCEs. We have shown previously that the nanoTCE is not able to activate T cells alone; this is shown by the use of the Isotype/CD3. T cells do not activate following the binding of the nanoTCE alone. It only works following the engagement of the T cell and the target cell via nanoTCE which aligns with the kinetic segregation model for T cell receptor triggering [203, 265]. Consequently, no T cell-mediated killing of AML cells was observed following treatment with Isotype/CD3 TCEs, while 50-75% killing was observed following treatment with CD33/CD3 nanoTCEs (**Figure 20C**).

www.manaraa.com

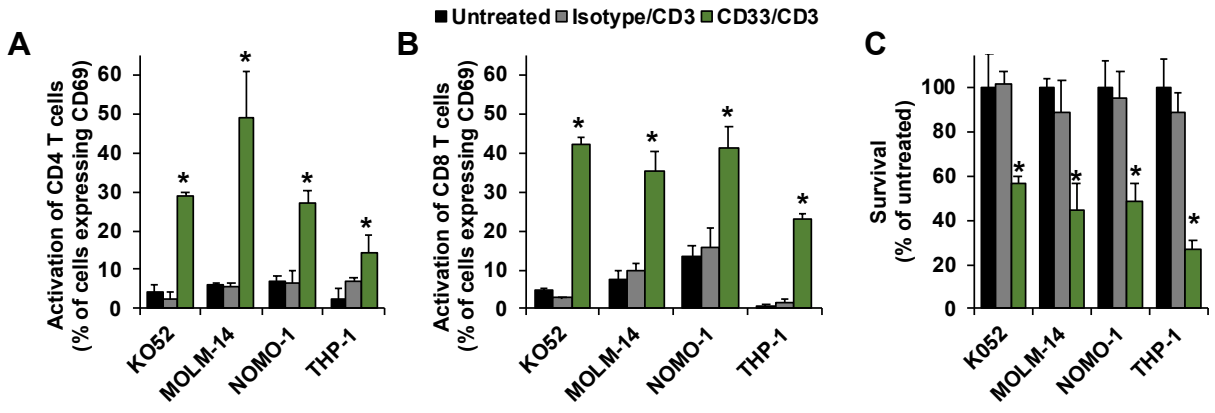


Figure 20. Efficacy of CD33 nanoTCEs in vitro. The effect of Isotype/CD3 and CD33/CD3 nanoTCEs on activation of **A.** CD4 and **B.** CD8 T cells, and on **C.** survival of AML cell lines.

To demonstrate the therapeutic efficacy of nanoTCEs in vivo, we injected human AML THP-1 cells genetically engineered to express luciferase in an NCG immunocompromised mice. Then we injected human primary T cells at Day 7 to humanize the T cells in the mouse and treated with nanoTCEs weekly thereafter. Mice treated with Isotype/CD3 TCEs had high tumor burden and experienced 60% death at Day 62, and the whole cohort died around Day 67. In contrast, CD33/CD3 nanoTCE-treated group had significantly lower tumor burden at all time points (not including Day 6), and 100% of the cohort was alive while all the Isotype/CD3 cohort was dead on Day 66 (**Figure 21A** and **21B**).

www.manaraa.com

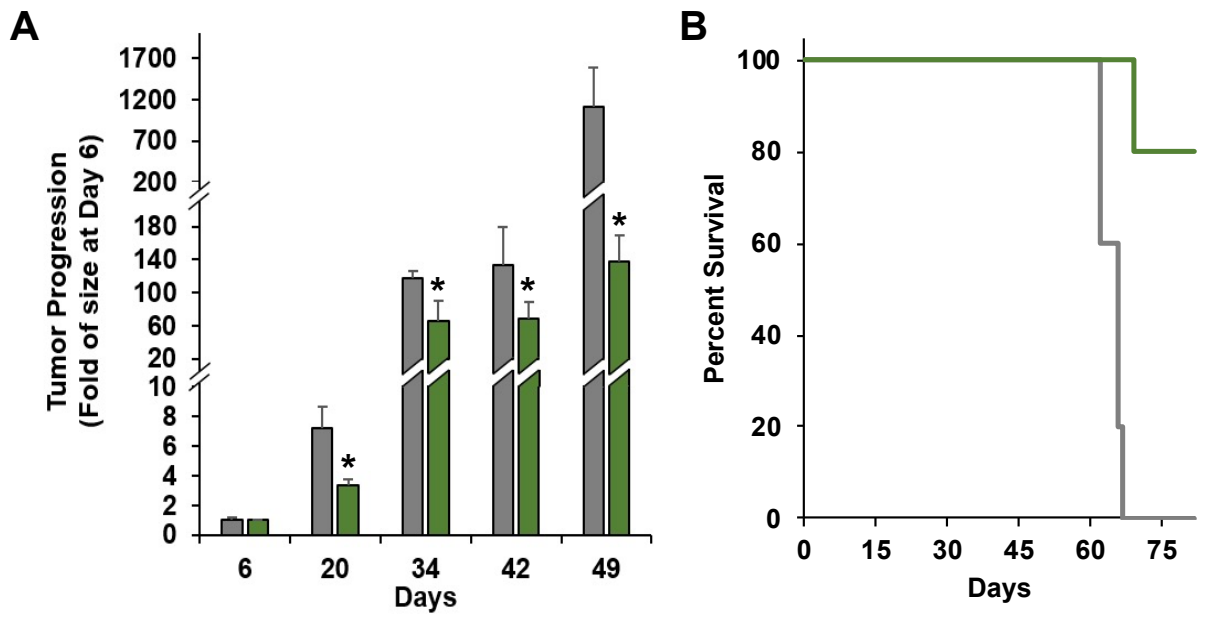


Figure 21. Efficacy of CD33 nanoTCEs in vivo. A. Tumor progression and **B.** percent survival of mice treated with Iso-type/CD3 or CD33/CD3 nanoTCEs.

We have demonstrated the successful use of our nanoTCE for the treatment of AML. The nanoTCE was able to target CD33, a very abundant and relevant marker on AML, to induce cytotoxic activity [314]. The CD33/CD3 nanoTCE bound preferentially to AML and T cells; this enables specific binding to only these cells and prevents binding to other hematopoietic cells to reduce off-target toxicities. T cell activation and T cell-mediated AML cell lysis was induced following the use of the nanoTCEs in vitro and in vivo. This shows that the CD33 nanoTCE is a potent and efficacious immunotherapy treatment for AML which circumvents current limitations of TCEs including the laborious and complex procedures that are involved in producing TCEs. The uniqueness of our nanoTCE technology allows the creation of an immunotherapeutic technique that is simple, reproducible, and quick to make which are all important traits to have while pursuing an immunotherapy option for AML.

www.manaraa.com

4.4 Conclusions

All in all, the nanoTCE platform shown here uses nanoparticles to create a relatively simple, reproducible, and off-the-shelf solution to overcome the major limitations of current immunotherapy techniques such as TCEs and CAR-T cells. The CD33 nanoTCE targets each antigen with the high specificity of monoclonal antibodies which enables the creation of a more robust and customizable immunotherapy technology to take advantage of the immune system for an effective response. Our system enables the customization of the nanoTCE as an immunotherapy with the use of existing monoclonal antibodies for the targeting of any desired cancer or immune cell antigen. This simple, customizable, specific, translational, and efficacious nanoTCE platform provides the flexibility to engage any immune cell for the treatment of the cancer of interest.

www.manaraa.com

Chapter 5: Liposomal Phytohemagglutinin: In Vivo T Cell Activator as a Novel Pan-Cancer Immunotherapy

T cell-based immunotherapy is a promising approach for manipulating T cells to combat disease. Multiple clinical trials are investigating different aspects of T cell immunotherapy and have been rendered successful with impressive clinical outcomes. Examples of T cell immunotherapy include chimeric antigen receptor (CAR)-T cells which are T cells extracted from the patient, genetically engineered with a CAR vector that targets a specific antigen on a target cell, activated and multiplied, and finally injected back into the patient [317]. CAR-T cells are highly activated and eliminate the target cells in one single injection; however, there are many limitations to this immunotherapeutic approach such as its complex and tedious production; expensive cost due to the fact that labor and equipment needed are highly costly; requires frequent quality control testing throughout production, the long-term safety profile of the viral vector, inability to target multiple targets, and toxicity [275, 319].

A solution that circumvents most of the limitations of CAR-T cells are bispecific T cell engagers (TCEs). TCEs are two single chain variable fragments connected by a protein linker which bind the target cell and T cell by using the desired cell surface antigen and CD3, respectively [317]. TCEs are highly efficacious and activate T cells endogenously which eliminates the need to genetically engineer extracted primary T cells. However, there are disadvantages to using TCEs such as short pharmacokinetic half-life, requires extensive labor for tedious production, inability to target multiple targets, and toxicity [324, 325]. In addition, a major disadvantage TCEs have is that their T cell activation and persistence is much weaker than CAR-T cells, which is why CAR-T cells have a much greater anti-tumor response compared to

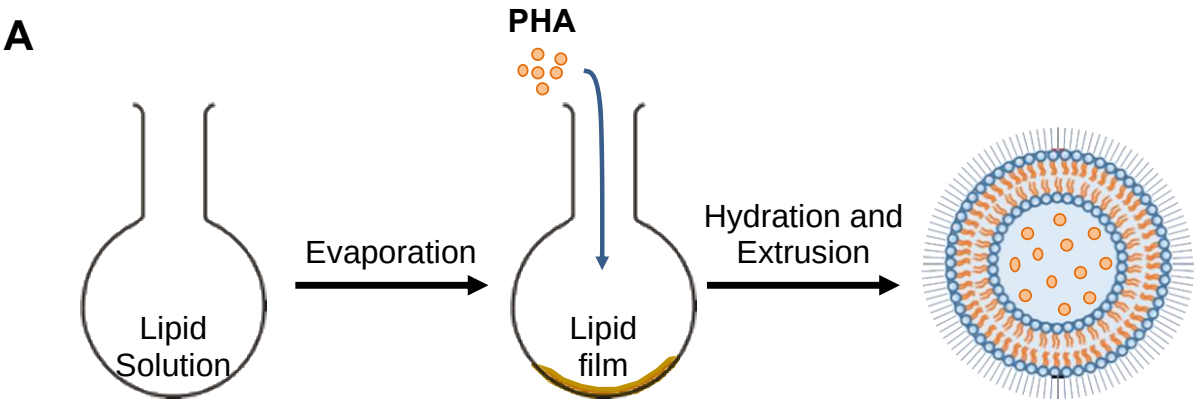
www.manaraa.com

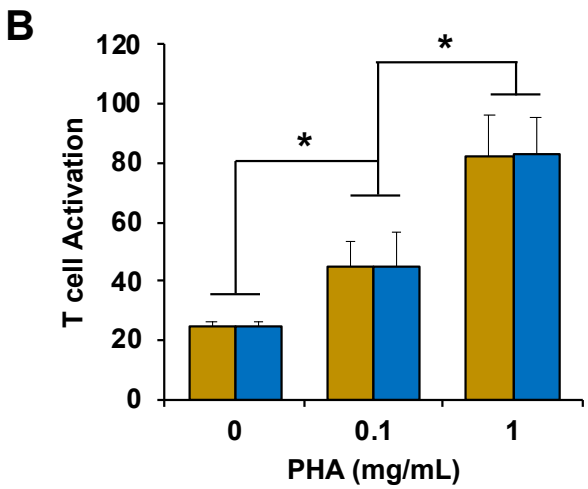
TCEs [329]. Therefore, alternative solutions to induce greater activation and persistence of T cells during TCE immunotherapy.

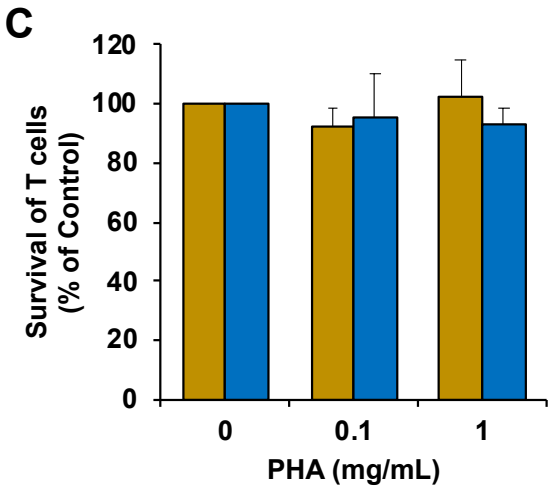
Methods to activate T cells include the use of small molecules and lectins, such as phorbol 12-myristate 13-acetate (PMA), ionomycin, concanavalin A, and phytohemagglutinin (PHA) [330], are commonly used for research purposes ex vivo, but not in vivo [331]. PMA and ionomycin stimulate T cells by activating protein kinase C [332], however their use is limited by their carcinogenic potential [333, 334]. Concanavalin A and PHA, both lectins, stimulate T cells by binding to glycoproteins on the T cell receptor [332, 335, 336]; however, PHA stimulates T cells at lower concentrations and induces greater T cell activation compared to concanavalin A [337]. Yet, PHA has not been used to activate T cells in vivo, for immunotherapy, due to its biological instability and toxicity. The instability stems from its protein-nature, which causes its degradation and short bioavailability profile in the blood [216], and toxicity (agglutination of red and white blood cells) leading to death [336]. Therefore, in order to take full advantage of PHA for the use as an immune activator, an approach of circumventing the limitations of PHA while also preserving function is needed. In this study, we report the encapsulation of PHA in a liposome to increase stability, reduce toxicity, and create an immunotherapeutic that is able to activate T cells for the use in future immunotherapies to circumvent current obstacles in immunosuppression and T cell exhaustion.

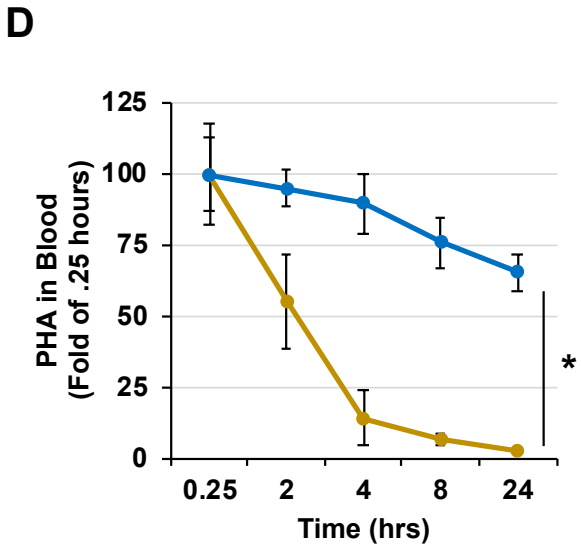
First, we created liposomes using three types of lipids: DPPC, cholesterol, and 16:0 PEG2000 PE at a mass ratio equal to 60:30:10, respectively. These lipids were then prepared as previously published [265, 283, 328]. In brief, the lipids were dissolved in chloroform, evaporated, hydrated with PHA in PBS and extruded as shown in **Figure 22A**.

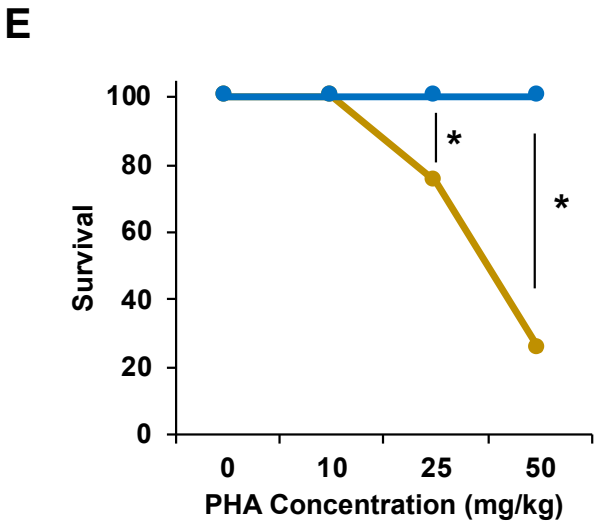
www.manaraa.com











www.manaraa.com

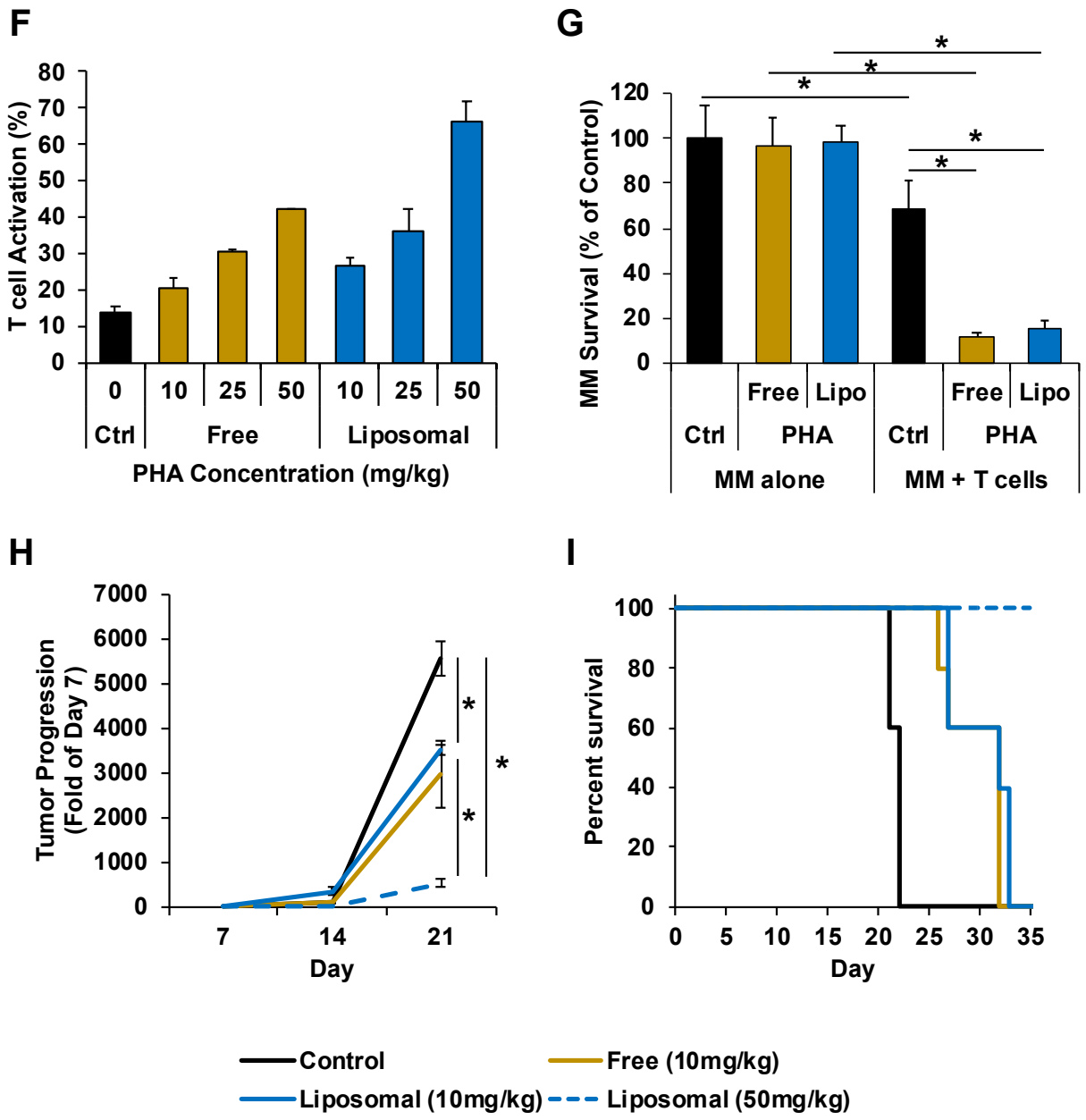


Figure 22. Liposomal Phytohemagglutinin (PHA) Has Similar T Cell Activation, Increases Pharmacokinetic Profile, and Reduces Toxicity Compared to Free. A. Schematic of creation of liposomal PHA. **B.** Activation of T cells at increasing concentrations of free or liposomal PHA in vitro. **C.** Survival of T cells at increasing concentrations of free or liposomal PHA. **D.** Pharmacokinetic profile of free and liposomal PHA. **E.** Survival of mice at increasing concentrations of free or liposomal PHA. **F.** Activation of T cells at increasing concentrations of free or liposomal PHA in vivo. **G.** Multiple myeloma (MM) survival at increasing concentrations of free or liposomal PHA. **H.** Tumor progression for four groups of mice with MM burden (control, free – 10 mg/kg, liposomal – 10 mg/kg, and liposomal – 50 mg/kg). **I.** Survival for four groups of mice with MM burden (control, free – 10 mg/kg, liposomal – 10 mg/kg, and liposomal – 50 mg/kg).

www.manaraa.com

Then, we investigated the ability of the PHA-loaded liposomes to activate T cells compared to free PHA. Peripheral blood mononuclear cells (PBMCs) were incubated for 24 hours in 0, 0.1, or 1 mg/mL of free or liposomal PHA. CD25 expression on CD3 T cells was analyzed via flow cytometry, as a marker for activation of T cells. CD25 expression was increased in correlation with PHA concentration, regardless of its formulation, free or liposomal. Around 90% of T cells had an increase in CD25 expression when incubated with 1 mg/mL of free or liposomal PHA (**Figure 22B**). Next, we examined the effect of PHA on T cell survival, as a marker for toxicity. PBMCs were incubated for 24 hours with free or liposomal PHA and analyzed for survival via flow cytometry (**Figure 22C**). No change in T cell survival was observed for free and liposomal PHA. These results demonstrate that the liposomal formulation maintained the desired effect of activation of T cells without inducing additional toxicities in vitro.

However, we are aware that the main limitation of the use of PHA is not the in vitro/ex vivo, rather it is the low bioavailability and toxicity in vivo. Therefore, we investigated the effect of the liposomal formulation on PHA's pharmacokinetic profile and toxicity in vivo. PHA was labeled with a fluorescent dye Alexa Fluor 647 (AF647). In brief, 25 mg of PHA was dissolved in 500 microliters of 0.1 M sodium carbonate, excess of AF647 was added, and left for stirring for one hour at room temperature, and unbound Alexa Fluor 647 was removed with dialysis. AF-647-PHA was used free or encapsulated in liposomes. Free or liposomal AF647-PHA (10 mg/kg) were injected intravenously to C57BL/6 mice (n=3), and blood serum was analyzed at 0.25, 2, 4, 8, and 24 hours using a fluorescent plate reader (Ex/Em=644/665). As expected, free PHA was degraded rapidly and had an elimination half-life of around two hours. On the contrary, liposomal PHA showed a significantly longer half-life of about 50 hours (**Figure 22D**). These

www.manaraa.com

results demonstrate that the liposomal formulation of PHA overcame its first limitation of low biological stability in vivo.

We then investigated the effect of the liposomal formulation on the toxicity of PHA in vivo. It has been previously reported that PHA-induced death of animals happens within few hours after injection, and no adverse effects are observed in long term [336]. C57BL/6 mice (n=4 per concentration, per condition) were injected with increasing dosed (0, 10, 25 and 50 mg/kg) of free and liposomal PHA, and closely monitored for survival for three days. Mice treated with free PHA showed decline in survival with increasing the PHA concentration; while 10mg/kg did not show toxicity, 25 mg/kg induced death of 1 of 4 animals, and 50 mg/kg induced death of 3 of 4 animals, over 50% of the group, where the dose escalation was stopped. On the contrary, liposomal PHA induced no death of any of the treated animals at any of the doses. These results demonstrate that the liposomal formulation of PHA circumvented the most limiting factor of PHA use in vivo, in which it improved its toxicity profile dramatically (**Figure 22E**).

As we showed that the liposomal formulation of PHA improved its stability and toxicity in vivo, we tested if it maintained the efficacy of PHA in activation of T cells. C57BL/6 mice (n=4 per concentration, per condition) were injected intravenously with 10, 25, or 50 mg/kg of free and liposomal PHA, and three days following injection, blood was extracted, and T cells activation was measured as the downregulation of CD62L expression on CD3+ T cells. Activated T cells were defined as total CD3 T cells minus the T cells with high expression CD62L. Free and liposomal PHA both induced T cell activation in a dose-dependent manner, however, liposomal PHA showed higher T cell activation compared to free PHA at all doses (**Figure 22F**). These results demonstrate that the liposomal formulation of PHA not only overcame the stability and toxicity limitations of PHA in vivo, but also maintained, even

www.manaraa.com

improved, the efficacy of PHA in activation of T cells, most likely due to the improved bioavailability in vivo, and not due to other biological mechanisms, since this effect was not observed in vitro.

We then investigated the effect of free or liposomal PHA on the survival of multiple myeloma (MM) cells, directly or in directly as an immune activator of T cells. Fluorescently labeled MM cells (OPM2) as a monoculture, or in co-culture with T cells, were treated in vitro with 1 mg/mL of free or liposomal PHA for 24 hours, and the survival of MM cells was analyzed using flow cytometry and normalized using counting beads. Neither free nor liposomal PHA had any direct effects or toxicity on MM cells when the treatment was performed in the absence of T cells. However, when co-cultured with T cells, both free and liposomal abolished the MM survival in vitro (**Figure 22G**). These results emphasize that the robust effect of PHA is mediated by T cell activation.

Finally, to demonstrate the efficacy of liposomal PHA as an immune activator for the treatment of cancer, we tested its efficacy in immunocompetent MM mouse model using C57BL/KaLwRij mice. One million luciferase-expressing 5TGM1 cells were injected per mouse (n=20) at Day 0. Mice were randomly divided into 4 groups of five mice each and treated with intravenous injection once a week, starting at Day 7 post cell injection, of: (a) vehicle control; (b) the maximal tolerated dose of free PHA which did not cause any deaths in vivo (10 mg/kg); (c) a comparable dose of liposomal PHA (10 mg/kg); and (d) with the highest tested dose of liposomal PHA which did not show toxicity in vivo (50 mg/kg), no comparable dose was use of free PHA, since this induced immediate death of more than 50% of the animals. In **Figure 22H**, tumor progression was significantly lower than control for both 10 mg/kg free and liposomal PHA, no statistical difference was found between free and liposomal PHA. However, the higher dose of

www.manaraa.com

liposomal PHA (50 mg/kg) abrogated the tumor progression, significantly lower control than 10 mg/kg free and liposomal PHA. The control cohort died within Day 22, 10 mg/kg free and liposomal PHA had 60% of mice survive past Day 31; whereas 50 mg/kg liposomal PHA-treated mice had 100% survival up to day 35 (**Figure 22I**).

In conclusion, liposomal PHA is a promising cancer immunotherapy that manipulates T cells in situ. Liposomal PHA can be used as a highly effective, stand-alone, T cell immunotherapy, which in opposite to CAR-Ts and TCEs does not included specific targeting to a specific tumor, which makes it available for use in pan-cancer fashion. Moreover, unlike CAR-Ts, liposomal PHA can be used off the shelf without over-personalization of the therapy, without the needs of complex manufacturing and safety concerns. In addition, antigen-targeted T cell therapies, such as CAR-Ts and TCEs, were previously shown to induce antigen-less tumor escape and cause relapse of the disease [227, 338-342], therefore, and since it is not targeted against a single antigen, we expect that the liposomal PHA will not induce antigen-less tumor escape and relapse of the disease. Further studies are needed to investigate the use of these PHA liposomes with different tumors and in combination with other T cell therapies, and traditional chemo and biological therapies.

www.manaraa.com

References

1. Weiner, L.M., J.C. Murray, and C.W. Shuptrine, *Antibody-based immunotherapy of cancer.* Cell, 2012. **148**(6): p. 1081-4.

2. Kimiz-Gebologlu, I., S. Gulce-Iz, and C. Biray-Avci, *Monoclonal antibodies in cancer immunotherapy.* Molecular Biology Reports, 2018. **45**(6): p. 2935-2940.

3. Weiner, L.M., R. Surana, and S. Wang, *Monoclonal antibodies: versatile platforms for cancer immunotherapy.* Nature Reviews Immunology, 2010. **10**(5): p. 317-27.

4. Osterborg, A., H. Mellstedt, and M. Keating, *Clinical effects of alemtuzumab (Campath-1H) in B-cell chronic lymphocytic leukemia.* Medical Oncology, 2002. **19 Suppl**: p. S21-6.

5. Weiner, G.J., *Rituximab: mechanism of action.* Seminars in Hematology, 2010. **47**(2): p. 115-23.

6. Hudis, C.A., *Trastuzumab--mechanism of action and use in clinical practice.* New England Journal of Medicine, 2007. **357**(1): p. 39-51.

7. Guziewicz, N., et al., *Lyophilized silk fibroin hydrogels for the sustained local delivery of therapeutic monoclonal antibodies.* Biomaterials, 2011. **32**(10): p. 2642-50.

8. Andrew, J.S., et al., *Sustained Release of a Monoclonal Antibody from Electrochemically Prepared Mesoporous Silicon Oxide.* Advanced Functional Materials, 2010. **20**(23): p. 4168-4174.

9. Aliperta, R., et al., *Cryogel-supported stem cell factory for customized sustained release of bispecific antibodies for cancer immunotherapy.* Scientific Reports, 2017. **7**: p. 42855.

10. Melero, I., et al., *Immunostimulatory monoclonal antibodies for cancer therapy.* Nature Reviews Cancer, 2007. **7**(2): p. 95-106.

11. Tutt, A.L., et al., *T cell immunity to lymphoma following treatment with anti-CD40 monoclonal antibody.* Journal of Immunology, 2002. **168**(6): p. 2720-8.

www.manaraa.com

12. Todryk, S.M., et al., *CD40 ligation for immunotherapy of solid tumours.* Journal of Immunology Methods, 2001. **248**(1-2): p. 139-47.

13. Advani, R., et al., *Phase I study of the humanized anti-CD40 monoclonal antibody dacetuzumab in refractory or recurrent non-Hodgkin's lymphoma.* Journal of Clinical Oncology, 2009. **27**(26): p. 4371-7.

14. Jackaman, C., et al., *CD40-activated B cells contribute to mesothelioma tumor regression.* Immunology Cell Biology, 2011. **89**(2): p. 255-67.

15. Gu, L., et al., *Multivalent porous silicon nanoparticles enhance the immune activation potency of agonistic CD40 antibody.* Advanced Materials, 2012. **24**(29): p. 3981-7.

16. Kwong, B., H. Liu, and D.J. Irvine, *Induction of potent anti-tumor responses while eliminating systemic side effects via liposome-anchored combinatorial immunotherapy.* Biomaterials, 2011. **32**(22): p. 5134-47.

17. Dubrot, J., et al., *Treatment with anti-CD137 mAbs causes intense accumulations of liver T cells without selective antitumor immunotherapeutic effects in this organ.* Cancer Immunology Immunotherapy, 2010. **59**(8): p. 1223-33.

18. Zhang, Y., et al., *Nanoparticle anchoring targets immune agonists to tumors enabling anti-cancer immunity without systemic toxicity.* Nature Communications, 2018. **9**(1): p. 6.

19. Topalian, S.L., C.G. Drake, and D.M. Pardoll, *Immune checkpoint blockade: a common denominator approach to cancer therapy.* Cancer Cell, 2015. **27**(4): p. 450-61.

20. Sharma, P. and J.P. Allison, *Immune checkpoint targeting in cancer therapy: toward combination strategies with curative potential.* Cell, 2015. **161**(2): p. 205-14.

21. Brahmer, J.R., et al., *Safety and activity of anti-PD-L1 antibody in patients with advanced cancer.* New England Journal of Medicine, 2012. **366**(26): p. 2455-65.

22. Naidoo, J., et al., *Toxicities of the anti-PD-1 and anti-PD-L1 immune checkpoint antibodies.* Annals of Oncology, 2015. **26**(12): p. 2375-91.

23. Rahimian, S., et al., *Polymeric microparticles for sustained and local delivery of antiCD40 and antiCTLA-4 in immunotherapy of cancer.* Biomaterials, 2015. **61**: p. 33-40.

www.manaraa.com

24. Li, Y., et al., *Hydrogel dual delivered celecoxib and anti-PD-1 synergistically improve antitumor immunity.* Oncoimmunology, 2016. **5**(2): p. e1074374.

25. Ye, Y., et al., *Synergistic Transcutaneous Immunotherapy Enhances Antitumor Immune Responses through Delivery of Checkpoint Inhibitors.* ACS Nano, 2016. **10**(9): p. 8956-63.

26. Quail, D.F. and J.A. Joyce, *Microenvironmental regulation of tumor progression and metastasis.* Nature Medicine, 2013. **19**(11): p. 1423-37.

27. Shekarian, T., et al., *Pattern recognition receptors: immune targets to enhance cancer immunotherapy.* Annals of Oncology, 2017. **28**(8): p. 1756-1766.

28. Pickup, M., S. Novitskiy, and H.L. Moses, *The roles of TGFbeta in the tumour microenvironment.* Nature Reviews Cancer, 2013. **13**(11): p. 788-99.

29. Gigante, M., L. Gesualdo, and E. Ranieri, *TGF-beta: a master switch in tumor immunity.* Current Pharmaceutical Design, 2012. **18**(27): p. 4126-34.

30. Schmid, D., et al., *T cell-targeting nanoparticles focus delivery of immunotherapy to improve antitumor immunity.* Nature Communications, 2017. **8**(1): p. 1747.

31. Park, J., et al., *Combination delivery of TGF-beta inhibitor and IL-2 by nanoscale liposomal polymeric gels enhances tumour immunotherapy.* Nature Materials, 2012. **11**(10): p. 895-905.

32. Wang, T., et al., *Regulation of the innate and adaptive immune responses by Stat-3 signaling in tumor cells.* Nature Medicine, 2004. **10**(1): p. 48-54.

33. Liao, D., et al., *Targeted therapeutic remodeling of the tumor microenvironment improves an HER-2 DNA vaccine and prevents recurrence in a murine breast cancer model.* Cancer Research, 2011. **71**(17): p. 5688-96.

34. Lee, S. and K. Margolin, *Cytokines in cancer immunotherapy.* Cancers (Basel), 2011. **3**(4): p. 3856-93.

www.manaraa.com

35. He, T., et al., *Interferon gamma stimulates cellular maturation of dendritic cell line DC2.4 leading to induction of efficient cytotoxic T cell responses and antitumor immunity.* Cell Molecular Immunology, 2007. **4**(2): p. 105-11.

36. Ahmed, S. and K.R. Rai, *Interferon in the treatment of hairy-cell leukemia.* Best Practice & Research Clinical Haematology, 2003. **16**(1): p. 69-81.

37. Sun, T., et al., *Inhibition of tumor angiogenesis by interferon-gamma by suppression of tumor-associated macrophage differentiation.* Oncology Research, 2014. **21**(5): p. 227-35.

38. Rosenberg, S.A., *IL-2: the first effective immunotherapy for human cancer.* Journal of Immunology, 2014. **192**(12): p. 5451-8.

39. Trinchieri, G., *Interleukin-12 and the regulation of innate resistance and adaptive immunity.* Nature Reviews Immunology, 2003. **3**(2): p. 133-46.

40. Yan, W.L., et al., *Recent progress in GM-CSF-based cancer immunotherapy.* Immunotherapy, 2017. **9**(4): p. 347-360.

41. Alconcel, S.N.S., A.S. Baas, and H.D. Maynard, *FDA-approved poly(ethylene glycol)–protein conjugate drugs.* Polymer Chemistry, 2011. **2**(7).

42. Mero, A., et al., *Selective conjugation of poly(2-ethyl 2-oxazoline) to granulocyte colony stimulating factor.* Journal of Controlled Release, 2012. **159**(3): p. 353-61.

43. Shimizu, T., et al., *Nanogel DDS enables sustained release of IL-12 for tumor immunotherapy.* Biochemical and Biophysical Research Communications, 2008. **367**(2): p. 330-5.

44. Wang, Y., et al., *Polymeric nanoparticles promote macrophage reversal from M2 to M1 phenotypes in the tumor microenvironment.* Biomaterials, 2017. **112**: p. 153-163.

45. Hanes, J., et al., *Controlled local delivery of interleukin-2 by biodegradable polymers protects animals from experimental brain tumors and liver tumors.* Pharmaceutical Research, 2001. **18**(7): p. 899-906.

www.manaraa.com

46. Sun, W., et al., *Transformable DNA nanocarriers for plasma membrane targeted delivery of cytokine.* Biomaterials, 2016. **96**: p. 1-10.

47. Takeuchi, O. and S. Akira, *Pattern recognition receptors and inflammation.* Cell, 2010. **140**(6): p. 805-20.

48. Kawai, T. and S. Akira, *The role of pattern-recognition receptors in innate immunity: update on Toll-like receptors.* Nature Immunology, 2010. **11**(5): p. 373-84.

49. Engel, A.L., G.E. Holt, and H. Lu, *The pharmacokinetics of Toll-like receptor agonists and the impact on the immune system.* Expert Reviews of Clinical Pharmacology, 2011. **4**(2): p. 275-89.

50. Krieg, A.M., *Toll-like receptor 9 (TLR9) agonists in the treatment of cancer.* Oncogene, 2008. **27**(2): p. 161-7.

51. Bourquin, C., et al., *Targeting CpG oligonucleotides to the lymph node by nanoparticles elicits efficient antitumoral immunity.* Journal of Immunology, 2008. **181**(5): p. 2990-8.

52. Zhang, L., et al., *Self-Assembled DNA Immunonanoflowers as Multivalent CpG Nanoagents.* ACS Applied Materials & Interfaces, 2015. **7**(43): p. 24069-74.

53. Nuhn, L., et al., *pH-degradable imidazoquinoline-ligated nanogels for lymph node-focused immune activation.* Proceedings of the National Academy of Sciences of the United States of America, 2016. **113**(29): p. 8098-103.

54. Owens, B., *Faster, deeper, smaller-the rise of antibody-like scaffolds.* Nature Biotechnology, 2017. **35**(7): p. 602-603.

55. Pluckthun, A., *Designed ankyrin repeat proteins (DARPins): binding proteins for research, diagnostics, and therapy.* Annual Reviews of Pharmacology & Toxicology, 2015. **55**: p. 489-511.

56. Pollaro, L., et al., *Bicyclic peptides conjugated to an albumin-binding tag diffuse efficiently into solid tumors.* Molecular Cancer Therapy, 2015. **14**(1): p. 151-61.

57. Huehls, A.M., T.A. Coupet, and C.L. Sentman, *Bispecific T-cell engagers for cancer immunotherapy.* Immunology Cell Biology, 2015. **93**(3): p. 290-6.

www.manaraa.com

58. Loffler, A., et al., *A recombinant bispecific single-chain antibody, CD19 x CD3, induces rapid and high lymphoma-directed cytotoxicity by unstimulated T lymphocytes.* Blood, 2000. **95**(6): p. 2098-103.

59. Topp, M.S., et al., *Safety and activity of blinatumomab for adult patients with relapsed or refractory B-precursor acute lymphoblastic leukaemia: a multicentre, single-arm, phase 2 study.* The Lancet Oncology, 2015. **16**(1): p. 57-66.

60. Goebeler, M.E., et al., *Bispecific T-Cell Engager (BiTE) Antibody Construct Blinatumomab for the Treatment of Patients With Relapsed/Refractory Non-Hodgkin Lymphoma: Final Results From a Phase I Study.* Journal of Clinical Oncology, 2016. **34**(10): p. 1104-11.

61. Kantarjian, H., et al., *Blinatumomab versus Chemotherapy for Advanced Acute Lymphoblastic Leukemia.* New England Journal of Medicine, 2017. **376**(9): p. 836-847.

62. Topp, M.S., et al., *Safety and activity of blinatumomab for adult patients with relapsed or refractory B-precursor acute lymphoblastic leukaemia: a multicentre, single-arm, phase 2 study.* The Lancet Oncology, 2015. **16**(1): p. 57-66.

63. Maher, J. and E.T. Davies, *Targeting cytotoxic T lymphocytes for cancer immunotherapy.* British Journal of Cancer, 2004. **91**(5): p. 817-21.

64. Rosenberg, S.A., et al., *Adoptive cell transfer: a clinical path to effective cancer immunotherapy.* Nature Reviews Cancer, 2008. **8**(4): p. 299-308.

65. Duong, C.P., et al., *Cancer immunotherapy utilizing gene-modified T cells: From the bench to the clinic.* Molecular Immunology, 2015. **67**(2 Pt A): p. 46-57.

66. Kim, J.V., et al., *The ABCs of artificial antigen presentation.* Nature Biotechnology, 2004. **22**(4): p. 403-10.

67. Munn, D.H. and V. Bronte, *Immune suppressive mechanisms in the tumor microenvironment.* Current Opinions in Immunology, 2016. **39**: p. 1-6.

68. Turtle, C.J. and S.R. Riddell, *Artificial antigen-presenting cells for use in adoptive immunotherapy.* Cancer Journal, 2010. **16**(4): p. 374-81.

www.manaraa.com

69. Eggermont, L.J., et al., *Towards efficient cancer immunotherapy: advances in developing artificial antigen-presenting cells.* Trends in Biotechnology, 2014. **32**(9): p. 456-65.

70. Lu, X., et al., *Adoptive transfer of pTRP2-specific CTLs expanding by bead-based artificial antigen-presenting cells mediates anti-melanoma response.* Cancer Letters, 2008. **271**(1): p. 129-39.

71. Jiang, X., et al., *HLA Tetramer Based Artificial Antigen-Presenting Cells Efficiently Stimulate CTLs Specific for Malignant Glioma.* Clinical Cancer Research, 2007. **13**(24): p. 7329-34.

72. Schilbach, K., et al., *Cytotoxic minor histocompatibility antigen HA-1-specific CD8+ effector memory T cells: artificial APCs pave the way for clinical application by potent primary in vitro induction.* Blood, 2005. **106**(1): p. 144-9.

73. Oelke, M., et al., *Ex vivo induction and expansion of antigen-specific cytotoxic T cells by HLA-Ig-coated artificial antigen-presenting cells.* Nature Medicine, 2003. **9**(5): p. 619-24.

74. Sunshine, J.C. and J.J. Green, *Nanoengineering approaches to the design of artificial antigen-presenting cells.* Nanomedicine (Lond), 2013. **8**(7): p. 1173-89.

75. Hickey, J.W., et al., *Biologically Inspired Design of Nanoparticle Artificial Antigen-Presenting Cells for Immunomodulation.* Nano Letters, 2017. **17**(11): p. 7045-7054.

76. Herrmann, S.H. and M.F. Mescher, *Secondary cytolytic T lymphocyte stimulation by purified H-2Kk in liposomes.* Proceedings of the National Academy of Sciences of the United States of America, 1981. **78**(4): p. 2488-92.

77. Giannoni, F., et al., *Clustering of T cell ligands on artificial APC membranes influences T cell activation and protein kinase C theta translocation to the T cell plasma membrane.* Journal of Immunology, 2005. **174**(6): p. 3204-11.

78. Ashley, C.E., et al., *The targeted delivery of multicomponent cargos to cancer cells by nanoporous particle-supported lipid bilayers.* Nature Materials, 2011. **10**(5): p. 389-97.

79. Lambert, L.H., et al., *Improving T Cell Expansion with a Soft Touch.* Nano Letters, 2017. **17**(2): p. 821-826.

www.manaraa.com

80. Steenblock, E.R. and T.M. Fahmy, *A comprehensive platform for ex vivo T-cell expansion based on biodegradable polymeric artificial antigen-presenting cells.* Molecular Therapy, 2008. **16**(4): p. 765-72.

81. Sunshine, J.C., et al., *Particle shape dependence of CD8+ T cell activation by artificial antigen presenting cells.* Biomaterials, 2014. **35**(1): p. 269-277.

82. Fadel, T.R., et al., *Enhanced cellular activation with single walled carbon nanotube bundles presenting antibody stimuli.* Nano Letters, 2008. **8**(7): p. 2070-6.

83. Fadel, T.R., et al., *Clustering of stimuli on single-walled carbon nanotube bundles enhances cellular activation.* Langmuir, 2010. **26**(8): p. 5645-54.

84. Rabinovich, G.A., D. Gabrilovich, and E.M. Sotomayor, *Immunosuppressive strategies that are mediated by tumor cells.* Annual Reviews Immunology, 2007. **25**: p. 267-96.

85. Kalaitsidou, M., et al., *CAR T-cell therapy: toxicity and the relevance of preclinical models.* Immunotherapy, 2015. **7**(5): p. 487-97.

86. Almasbak, H., T. Aarvak, and M.C. Vemuri, *CAR T Cell Therapy: A Game Changer in Cancer Treatment.* Journal of Immunology Research, 2016. **2016**: p. 5474602.

87. Bonifant, C.L., et al., *Toxicity and management in CAR T-cell therapy.* Molecular Therapy Oncolytics, 2016. **3**: p. 16011.

88. Hasani-Sadrabadi, M.M., et al., *Mechanobiological Mimicry of Helper T Lymphocytes to Evaluate Cell-Biomaterials Crosstalk.* Advanced Materials, 2018. **30**(23): p. e1706780.

89. Stephan, M.T., et al., *Therapeutic cell engineering with surface-conjugated synthetic nanoparticles.* Nature Medicine, 2010. **16**(9): p. 1035-41.

90. Kojima, R., L. Scheller, and M. Fussenegger, *Nonimmune cells equipped with T-cell-receptor-like signaling for cancer cell ablation.* Nature Chemical Biology, 2018. **14**(1): p. 42-49.

91. Sadelain, M., I. Riviere, and S. Riddell, *Therapeutic T cell engineering.* Nature, 2017. **545**(7655): p. 423-431.

www.manaraa.com

92. Rosenberg, S.A., P. Spiess, and R. Lafreniere, *A new approach to the adoptive immunotherapy of cancer with tumor-infiltrating lymphocytes.* Science, 1986. **233**(4770): p. 1318-21.

93. Itzhaki, O., et al., *Establishment and large-scale expansion of minimally cultured "young" tumor infiltrating lymphocytes for adoptive transfer therapy.* Journal of Immunotherapy, 2011. **34**(2): p. 212-20.

94. Rosenberg, S.A., et al., *Use of tumor-infiltrating lymphocytes and interleukin-2 in the immunotherapy of patients with metastatic melanoma. A preliminary report.* New England Journal of Medicine, 1988. **319**(25): p. 1676-80.

95. Rosenberg, S.A. and N.P. Restifo, *Adoptive cell transfer as personalized immunotherapy for human cancer.* Science, 2015. **348**(6230): p. 62-8.

96. Dudley, M.E., et al., *Adoptive cell transfer therapy following non-myeloablative but lymphodepleting chemotherapy for the treatment of patients with refractory metastatic melanoma.* Journal of Clinical Oncology, 2005. **23**(10): p. 2346-57.

97. Chapuis, A.G., et al., *Transferred WT1-reactive CD8+ T cells can mediate antileukemic activity and persist in post-transplant patients.* Science Translational Medicine, 2013. **5**(174): p. 174ra27.

98. Morgan, R.A., et al., *Cancer regression in patients after transfer of genetically engineered lymphocytes.* Science, 2006. **314**(5796): p. 126-9.

99. Parkhurst, M.R., et al., *T cells targeting carcinoembryonic antigen can mediate regression of metastatic colorectal cancer but induce severe transient colitis.* Molecular Therapy, 2011. **19**(3): p. 620-6.

100. Johnson, L.A., et al., *Gene therapy with human and mouse T-cell receptors mediates cancer regression and targets normal tissues expressing cognate antigen.* Blood, 2009. **114**(3): p. 535-46.

101. Davis, J.L., et al., *Development of human anti-murine T-cell receptor antibodies in both responding and nonresponding patients enrolled in TCR gene therapy trials.* Clinical Cancer Research, 2010. **16**(23): p. 5852-61.

www.manaraa.com

102. Morgan, R.A., et al., *Cancer regression and neurological toxicity following anti-MAGE-A3 TCR gene therapy.* Journal of Immunotherapy, 2013. **36**(2): p. 133-51.

103. Rapoport, A.P., et al., *NY-ESO-1-specific TCR-engineered T cells mediate sustained antigen-specific antitumor effects in myeloma.* Nature Medicine, 2015. **21**(8): p. 914-921.

104. Marincola, F.M., et al., *Escape of human solid tumors from T-cell recognition: molecular mechanisms and functional significance.* Advances in Immunology, 2000. **74**: p. 181-273.

105. Sadelain, M., R. Brentjens, and I. Riviere, *The basic principles of chimeric antigen receptor design.* Cancer Discovery, 2013. **3**(4): p. 388-98.

106. Stephan, M.T., et al., *T cell-encoded CD80 and 4-1BBL induce auto- and transcostimulation, resulting in potent tumor rejection.* Nature Medicine, 2007. **13**(12): p. 1440-9.

107. Zhong, X.S., et al., *Chimeric antigen receptors combining 4-1BB and CD28 signaling domains augment PI3kinase/AKT/Bcl-XL activation and CD8+ T cell-mediated tumor eradication.* Molecular Therapy, 2010. **18**(2): p. 413-20.

108. Levine, B.L., et al., *Global Manufacturing of CAR T Cell Therapy.* Molecular Therapy: Methods & Clinical Development, 2017. **4**: p. 92-101.

109. Waehler, R., S.J. Russell, and D.T. Curiel, *Engineering targeted viral vectors for gene therapy.* Nature Reviews Genetics, 2007. **8**(8): p. 573-87.

110. Guedan, S., M. Ruella, and C.H.J.A.r.o.i. June, *Emerging Cellular Therapies for Cancer.* 2018.

111. Kalos, M., et al., *T cells with chimeric antigen receptors have potent antitumor effects and can establish memory in patients with advanced leukemia.* Science Translational Medicine, 2011. **3**(95): p. 95ra73.

112. Zhang, W.-y., et al., *Treatment of CD20-directed chimeric antigen receptor-modified T cells in patients with relapsed or refractory B-cell non-Hodgkin lymphoma: an early phase IIa trial report.* 2016. **1**: p. 16002.

www.manaraa.com

113. Wang, C.M., et al., *Autologous T Cells Expressing CD30 Chimeric Antigen Receptors for Relapsed or Refractory Hodgkin Lymphoma: An Open-Label Phase I Trial.* Clinical Cancer Research, 2017. **23**(5): p. 1156-1166.

114. Wang, Q.S., et al., *Treatment of CD33-directed chimeric antigen receptor-modified T cells in one patient with relapsed and refractory acute myeloid leukemia.* Molecular Therapy, 2015. **23**(1): p. 184-91.

115. Cohen, A.D., et al., *B cell maturation antigen–specific CAR T cells are clinically active in multiple myeloma.* The Journal of clinical investigation, 2019. **129**(6): p. 2210-2221.

116. Giavridis, T., et al., *CAR T cell–induced cytokine release syndrome is mediated by macrophages and abated by IL-1 blockade.* 2018. **24**(6): p. 731.

117. Lee, D.W., et al., *Current concepts in the diagnosis and management of cytokine release syndrome.* 2014. **124**(2): p. 188-195.

118. Cooper, M.L., et al., *An "off-the-shelf" fratricide-resistant CAR-T for the treatment of T cell hematologic malignancies.* 2018. **32**(9): p. 1970.

119. Ren, J., et al., *Multiplex genome editing to generate universal CAR T cells resistant to PD1 inhibition.* 2017. **23**(9): p. 2255-2266.

120. Stephan, S.B., et al., *Biopolymer implants enhance the efficacy of adoptive T-cell therapy.* 2015. **33**(1): p. 97.

121. Smith, T.T., et al., *Biopolymers codelivering engineered T cells and STING agonists can eliminate heterogeneous tumors.* 2017. **127**(6): p. 2176-2191.

122. Gumperz, J.E. and P.J.N. Parham, *The enigma of the natural killer cell.* 1995. **378**(6554): p. 245.

123. Miller, J.S., et al., *Successful adoptive transfer and in vivo expansion of human haploidentical NK cells in patients with cancer.* 2005. **105**(8): p. 3051-3057.

124. Chu, J., et al., *CS1-specific chimeric antigen receptor (CAR)-engineered natural killer cells enhance in vitro and in vivo antitumor activity against human multiple myeloma.* 2014. **28**(4): p. 917.

www.manaraa.com

125. Boissel, L., et al., *Retargeting NK-92 cells by means of CD19-and CD20-specific chimeric antigen receptors compares favorably with antibody-dependent cellular cytotoxicity.* 2013. **2**(10): p. e26527.

126. Zhang, G., et al., *Retargeting NK-92 for anti-melanoma activity by a TCR-like single-domain antibody.* 2013. **91**(10): p. 615.

127. Arai, S., et al., *Infusion of the allogeneic cell line NK-92 in patients with advanced renal cell cancer or melanoma: a phase I trial.* 2008. **10**(6): p. 625-632.

128. Zhang, C., et al., *Chimeric antigen receptor-engineered NK-92 cells: an off-the-shelf cellular therapeutic for targeted elimination of cancer cells and induction of protective antitumor immunity.* 2017. **8**: p. 533.

129. Leong, J.W., et al., *Preactivation with IL-12, IL-15, and IL-18 induces CD25 and a functional high-affinity IL-2 receptor on human cytokine-induced memory-like natural killer cells.* 2014. **20**(4): p. 463-473.

130. Chanmee, T., et al., *Tumor-associated macrophages as major players in the tumor microenvironment.* 2014. **6**(3): p. 1670-1690.

131. Stevenson, H.C., et al., *Ex vivo activated monocytes and adoptive immunotherapy trials in colon cancer patients.* 1986. **211**: p. 75.

132. Green, D.S., et al., *A Phase 1 trial of autologous monocytes stimulated ex vivo with Sylatron®(Peginterferon alfa-2b) and Actimmune®(Interferon gamma-1b) for intra-peritoneal administration in recurrent ovarian cancer.* 2018. **16**(1): p. 196.

133. Morrissey, M.A., et al., *Chimeric antigen receptors that trigger phagocytosis.* 2018. **7**: p. e36688.

134. Klichinsky, M., et al., *Chimeric antigen receptor macrophages (CARMA) for adoptive cellular immunotherapy of solid tumors*. 2017, AACR.

135. Brode, S. and P.A.J.I. Macary, *Cross-presentation: dendritic cells and macrophages bite off more than they can chew!* 2004. **112**(3): p. 345-351.

www.manaraa.com

136. Guermonprez, P., et al., *Antigen presentation and T cell stimulation by dendritic cells.* 2002. **20**(1): p. 621-667.

137. Porgador, A. and E.J.J.o.E.M. Gilboa, *Bone marrow-generated dendritic cells pulsed with a class I-restricted peptide are potent inducers of cytotoxic T lymphocytes.* 1995. **182**(1): p. 255-260.

138. Kantoff, P.W., et al., *Sipuleucel-T immunotherapy for castration-resistant prostate cancer.* 2010. **363**(5): p. 411-422.

139. Schwartzentruber, D., et al., *A phase III multi-institutional randomized study of immunization with the gp100: 209–217 (210M) peptide followed by high-dose IL-2 compared with high-dose IL-2 alone in patients with metastatic melanoma.* 2009. **27**(18_suppl): p. CRA9011-CRA9011.

140. Murphy, G., et al., *Phase I clinical trial: T-cell therapy for prostate cancer using autologous dendritic cells pulsed with HLA-A0201-specific peptides from prostate-specific membrane antigen.* 1996. **29**(6): p. 371-380.

141. Brossart, P., et al., *Virus-mediated delivery of antigenic epitopes into dendritic cells as a means to induce CTL.* 1997. **158**(7): p. 3270-3276.

142. Zhong, L., et al., *Recombinant adenovirus is an efficient and non-perturbing genetic vector for human dendritic cells.* 1999. **29**(3): p. 964-972.

143. Draube, A., et al., *Dendritic cell based tumor vaccination in prostate and renal cell cancer: a systematic review and meta-analysis.* 2011. **6**(4): p. e18801.

144. Squadrito, M.L., et al., *EVIR: chimeric receptors that enhance dendritic cell cross-dressing with tumor antigens.* 2018. **15**(3): p. 183.

145. Palucka, K. and J.J.N.R.C. Banchereau, *Cancer immunotherapy via dendritic cells.* 2012. **12**(4): p. 265.

146. Ghafouri-Fard, S. and S.J.I. Ghafouri-Fard, *siRNA and cancer immunotherapy.* 2012. **4**(9): p. 907-917.

www.manaraa.com

147. Schlee, M., V. Hornung, and G.J.M.T. Hartmann, *siRNA and isRNA: two edges of one sword.* 2006. **14**(4): p. 463-470.

148. Poeck, H., et al., *5′-Triphosphate-siRNA: turning gene silencing and Rig-I activation against melanoma.* 2008. **14**(11): p. 1256.

149. Khairuddin, N., et al., *siRNA-induced immunostimulation through TLR7 promotes antitumoral activity against HPV-driven tumors in vivo.* 2012. **90**(2): p. 187.

150. Kanasty, R., et al., *Delivery materials for siRNA therapeutics.* 2013. **12**(11): p. 967.

151. Wang, J., et al., *Delivery of siRNA therapeutics: barriers and carriers.* 2010. **12**(4): p. 492-503.

152. Sahay, G., et al., *Efficiency of siRNA delivery by lipid nanoparticles is limited by endocytic recycling.* 2013. **31**(7): p. 653.

153. Cubillos-Ruiz, J.R., et al., *Polyethylenimine-based siRNA nanocomplexes reprogram tumor-associated dendritic cells via TLR5 to elicit therapeutic antitumor immunity.* 2009. **119**(8): p. 2231-2244.

154. Leuschner, F., et al., *Therapeutic siRNA silencing in inflammatory monocytes in mice.* 2011. **29**(11): p. 1005.

155. Conde, J., et al., *Dual targeted immunotherapy via in vivo delivery of biohybrid RNAi-peptide nanoparticles to tumor-associated macrophages and cancer cells.* 2015. **25**(27): p. 4183-4194.

156. Bryers, J.D., et al., *Engineering biomaterials to integrate and heal: the biocompatibility paradigm shifts.* 2012. **109**(8): p. 1898-1911.

157. Burnett, J.C., J.J. Rossi, and K.J.B.j. Tiemann, *Current progress of siRNA/shRNA therapeutics in clinical trials.* 2011. **6**(9): p. 1130-1146.

158. Senzer, N., et al., *Phase I trial of "bi-shRNAifurin/GMCSF DNA/autologous tumor cell" vaccine (FANG) in advanced cancer.* 2012. **20**(3): p. 679-686.

www.manaraa.com

159. Fenton, O.S., et al., *Advances in biomaterials for drug delivery.* 2018. **30**(29): p. 1705328.

160. Riley, R.S., et al., *Delivery technologies for cancer immunotherapy.* 2019: p. 1.

161. Fotin-Mleczek, M., et al., *Messenger RNA-based vaccines with dual activity induce balanced TLR-7 dependent adaptive immune responses and provide antitumor activity.* 2011. **34**(1): p. 1-15.

162. Ziegler, A., et al., *A new RNA-based adjuvant enhances virus-specific vaccine responses by locally triggering TLR-and RLH-dependent effects.* 2017. **198**(4): p. 1595-1605.

163. Benteyn, D., et al., *mRNA-based dendritic cell vaccines.* 2015. **14**(2): p. 161-176.

164. Van Tendeloo, V.F., et al., *Highly efficient gene delivery by mRNA electroporation in human hematopoietic cells: superiority to lipofection and passive pulsing of mRNA and to electroporation of plasmid cDNA for tumor antigen loading of dendritic cells.* 2001. **98**(1): p. 49-56.

165. Melhem, N.M., et al., *High-level antigen expression and sustained antigen presentation in dendritic cells nucleofected with wild-type viral mRNA but not DNA.* 2008. **15**(9): p. 1337-1344.

166. De Temmerman, M.-L., et al., *mRNA-Lipoplex loaded microbubble contrast agents for ultrasound-assisted transfection of dendritic cells*. 2011. **32**(34): p. 9128-9135.

167. Bontkes, H., et al., *Dendritic cells transfected with interleukin-12 and tumor-associated antigen messenger RNA induce high avidity cytotoxic T cells.* 2007. **14**(4): p. 366.

168. Bontkes, H.J., et al., *Tumor associated antigen and interleukin-12 mRNA transfected dendritic cells enhance effector function of natural killer cells and antigen specific T-cells.* 2008. **127**(3): p. 375-384.

169. Pen, J., et al., *Interference with PD-L1/PD-1 co-stimulation during antigen presentation enhances the multifunctionality of antigen-specific T cells.* 2014. **21**(3): p. 262.

170. Pruitt, S.K., et al., *Enhancement of anti-tumor immunity through local modulation of CTLA-4 and GITR by dendritic cells.* 2011. **41**(12): p. 3553-3563.

www.manaraa.com

171. Boczkowski, D., et al., *Dendritic cells engineered to secrete anti-GITR antibodies are effective adjuvants to dendritic cell-based immunotherapy.* 2009. **16**(12): p. 900.

172. Van Lint, S., et al., *Intratumoral delivery of TriMix mRNA results in T-cell activation by cross-presenting dendritic cells.* 2016. **4**(2): p. 146-156.

173. Wilgenhof, S., et al., *A phase IB study on intravenous synthetic mRNA electroporated dendritic cell immunotherapy in pretreated advanced melanoma patients.* 2013. **24**(10): p. 2686-2693.

174. Maus, M.V. and C.H. June, *Making better chimeric antigen receptors for adoptive T-cell therapy*. 2016, AACR.

175. Maus, M.V., et al., *T cells expressing chimeric antigen receptors can cause anaphylaxis in humans.* 2013. **1**(1): p. 26-31.

176. Foster, J.B., et al., *Purification of mRNA Encoding Chimeric Antigen Receptor Is Critical for Generation of a Robust T-Cell Response.* 2018.

177. Moffett, H., et al., *Hit-and-run programming of therapeutic cytoreagents using mRNA nanocarriers.* 2017. **8**(1): p. 389.

178. Eggers, R., et al., *Primary T cells for mRNA-mediated immunotoxin delivery.* 2018. **25**(1): p. 47.

179. Kreiter, S., et al., *Mutant MHC class II epitopes drive therapeutic immune responses to cancer.* 2015. **520**(7549): p. 692.

180. Laubach, J., P. Richardson, and K. Anderson, *Multiple myeloma.* Annu Rev Med, 2011. **62**: p. 249-64.

181. Richardson, P.G., et al., *Interpreting clinical trial data in multiple myeloma: translating findings to the real-world setting.* Blood Cancer J, 2018. **8**(11): p. 109.

182. Siegel, R.L., K.D. Miller, and A. Jemal, *Cancer statistics, 2020.* CA Cancer J Clin, 2020. **70**(1): p. 7-30.

www.manaraa.com

183. Minnie, S.A. and G.R. Hill, *Immunotherapy of multiple myeloma.* J Clin Invest, 2020. **130**(4): p. 1565-1575.

184. Sadelain, M., I. Rivière, and S. Riddell, *Therapeutic T cell engineering.* Nature, 2017. **545**(7655): p. 423-431.

185. Rosenblatt, J. and D. Avigan, *Targeting the PD-1/PD-L1 axis in multiple myeloma: a dream or a reality?* Blood, 2017. **129**(3): p. 275-279.

186. Mikkilineni, L. and J.N. Kochenderfer, *Chimeric antigen receptor T-cell therapies for multiple myeloma.* Blood, 2017. **130**(24): p. 2594-2602.

187. *BiTE Therapy Active in Multiple Myeloma.* Cancer Discov, 2019. **9**(2): p. 157-158.

188. Huehls, A.M., T.A. Coupet, and C.L. Sentman, *Bispecific T-cell engagers for cancer immunotherapy.* Immunol Cell Biol, 2015. **93**(3): p. 290-6.

189. Sedykh, S.E., et al., *Bispecific antibodies: design, therapy, perspectives.* Drug Des Devel Ther, 2018. **12**: p. 195-208.

190. Wang, Q., et al., *Design and Production of Bispecific Antibodies.* Antibodies (Basel), 2019. **8**(3).

191. Inbar, D., J. Hochman, and D. Givol, *Localization of antibody-combining sites within the variable portions of heavy and light chains.* Proc Natl Acad Sci U S A, 1972. **69**(9): p. 2659-62.

192. Baeuerle, P.A., C. Reinhardt, and P. Kufer, *BiTE: a new class of antibodies that recruit T-cells.* Drugs Future, 2008. **33**(2): p. 1-11.

193. Baeuerle, P.A., P. Kufer, and R. Bargou, *BiTE: Teaching antibodies to engage T-cells for cancer therapy.* Curr Opin Mol Ther, 2009. **11**(1): p. 22-30.

194. Baeuerle, P.A. and C. Reinhardt, *Bispecific T-cell engaging antibodies for cancer therapy.* Cancer Res, 2009. **69**(12): p. 4941-4.

www.manaraa.com

195. Dreier, T., et al., *Extremely potent, rapid and costimulation-independent cytotoxic T-cell response against lymphoma cells catalyzed by a single-chain bispecific antibody.* Int J Cancer, 2002. **100**(6): p. 690-7.

196. Davis, S.J. and P.A. van der Merwe, *The kinetic-segregation model: TCR triggering and beyond.* Nat Immunol, 2006. **7**(8): p. 803-9.

197. Mustelin, T., K.M. Coggeshall, and A. Altman, *Rapid activation of the T-cell tyrosine protein kinase pp56lck by the CD45 phosphotyrosine phosphatase.* Proc Natl Acad Sci U S A, 1989. **86**(16): p. 6302-6.

198. Trowbridge, I.S. and M.L. Thomas, *CD45: an emerging role as a protein tyrosine phosphatase required for lymphocyte activation and development.* Annu Rev Immunol, 1994. **12**: p. 85-116.

199. Cordoba, S.P., et al., *The large ectodomains of CD45 and CD148 regulate their segregation from and inhibition of ligated T-cell receptor.* Blood, 2013. **121**(21): p. 4295-302.

200. Davis, M.M., et al., *Ligand recognition by alpha beta T cell receptors.* Annu Rev Immunol, 1998. **16**: p. 523-44.

201. Offner, S., et al., *Induction of regular cytolytic T cell synapses by bispecific single-chain antibody constructs on MHC class I-negative tumor cells.* Mol Immunol, 2006. **43**(6): p. 763-71.

202. Burroughs, N.J., Z. Lazic, and P.A. van der Merwe, *Ligand detection and discrimination by spatial relocalization: A kinase-phosphatase segregation model of TCR activation.* Biophys J, 2006. **91**(5): p. 1619-29.

203. Li, J., et al., *Membrane-Proximal Epitope Facilitates Efficient T Cell Synapse Formation by Anti-FcRH5/CD3 and Is a Requirement for Myeloma Cell Killing.* Cancer Cell, 2017. **31**(3): p. 383-395.

204. Ellerman, D., *Bispecific T-cell engagers: Towards understanding variables influencing the in vitro potency and tumor selectivity and their modulation to enhance their efficacy and safety.* Methods, 2019. **154**: p. 102-117.

www.manaraa.com

205. Slaney, C.Y., et al., *CARs versus BiTEs: A Comparison between T Cell-Redirection Strategies for Cancer Treatment.* Cancer Discov, 2018. **8**(8): p. 924-934.

206. Cohen, A.D., et al., *How to Train Your T Cells: Overcoming Immune Dysfunction in Multiple Myeloma.* Clin Cancer Res, 2020. **26**(7): p. 1541-1554.

207. Yuraszeck, T., S. Kasichayanula, and J.E. Benjamin, *Translation and Clinical Development of Bispecific T-cell Engaging Antibodies for Cancer Treatment.* Clin Pharmacol Ther, 2017. **101**(5): p. 634-645.

208. Hipp, S., et al., *A novel BCMA/CD3 bispecific T-cell engager for the treatment of multiple myeloma induces selective lysis in vitro and in vivo.* Leukemia, 2017. **31**(8): p. 1743-1751.

209. Stieglmaier, J., J. Benjamin, and D. Nagorsen, *Utilizing the BiTE (bispecific T-cell engager) platform for immunotherapy of cancer.* Expert Opin Biol Ther, 2015. **15**(8): p. 1093-9.

210. Trinklein, N.D., et al., *Efficient tumor killing and minimal cytokine release with novel T-cell agonist bispecific antibodies.* MAbs, 2019. **11**(4): p. 639-652.

211. Zhukovsky, E.A., R.J. Morse, and M.V. Maus, *Bispecific antibodies and CARs: generalized immunotherapeutics harnessing T cell redirection.* Curr Opin Immunol, 2016. **40**: p. 24-35.

212. Roy, A., et al., *Estimating the Costs of Therapy in Patients with Relapsed and/or Refractory Multiple Myeloma: A Model Framework.* Am Health Drug Benefits, 2015. **8**(4): p. 204-15.

213. Strohl, W.R. and M. Naso, *Bispecific T-Cell Redirection versus Chimeric Antigen Receptor (CAR)-T Cells as Approaches to Kill Cancer Cells.* Antibodies (Basel), 2019. **8**(3).

214. Alhallak, K., et al., *Biomaterials for cancer immunotherapy*, in *Biomaterials for Cancer Therapeutics*. 2020, Elsevier. p. 499-526.

215. Lobo, E.D., R.J. Hansen, and J.P. Balthasar, *Antibody pharmacokinetics and pharmacodynamics.* J Pharm Sci, 2004. **93**(11): p. 2645-68.

www.manaraa.com

216. Roopenian, D.C. and S. Akilesh, *FcRn: the neonatal Fc receptor comes of age.* Nat Rev Immunol, 2007. **7**(9): p. 715-25.

217. Zhu, M., et al., *Blinatumomab, a Bispecific T-cell Engager (BiTE(®)) for CD-19 Targeted Cancer Immunotherapy: Clinical Pharmacology and Its Implications.* Clin Pharmacokinet, 2016. **55**(10): p. 1271-1288.

218. Wesche, H., et al., *TriTACs are novel T cell-engaging therapeutic proteins optimized for the treatment of solid tumors and for long serum half-life*. 2018, AACR.

219. Leenaars, M. and C.F. Hendriksen, *Critical steps in the production of polyclonal and monoclonal antibodies: evaluation and recommendations.* Ilar j, 2005. **46**(3): p. 269-79.

220. Vazquez-Lombardi, R., et al., *Transient expression of human antibodies in mammalian cells.* Nat Protoc, 2018. **13**(1): p. 99-117.

221. Kuan, C.T., et al., *125I-labeled anti-epidermal growth factor receptor-vIII single-chain Fv exhibits specific and high-level targeting of glioma xenografts.* Clin Cancer Res, 1999. **5**(6): p. 1539-49.

222. Clackson, T., et al., *Making antibody fragments using phage display libraries.* Nature, 1991. **352**(6336): p. 624-8.

223. Mack, M., et al., *Biologic properties of a bispecific single-chain antibody directed against 17-1A (EpCAM) and CD3: tumor cell-dependent T cell stimulation and cytotoxic activity.* J Immunol, 1997. **158**(8): p. 3965-70.

224. Choi, B.D., et al., *Systemic administration of a bispecific antibody targeting EGFRvIII successfully treats intracerebral glioma.* Proc Natl Acad Sci U S A, 2013. **110**(1): p. 270-5.

225. Foltz, S.M., et al., *Evolution and structure of clinically relevant gene fusions in multiple myeloma.* Nat Commun, 2020. **11**(1): p. 2666.

226. Dunn, G.P., et al., *Cancer immunoediting: from immunosurveillance to tumor escape.* Nat Immunol, 2002. **3**(11): p. 991-8.

www.manaraa.com

227. Cohen, A.D., et al., *B cell maturation antigen-specific CAR T cells are clinically active in multiple myeloma.* J Clin Invest, 2019. **129**(6): p. 2210-2221.

228. June, C.H., et al., *Role of the CD28 receptor in T-cell activation.* Immunol Today, 1990. **11**(6): p. 211-6.

229. Guedan, S., et al., *Enhancing CAR T cell persistence through ICOS and 4-1BB costimulation.* JCI Insight, 2018. **3**(1).

230. Wu, L., et al., *Trispecific antibodies enhance the therapeutic efficacy of tumor-directed T cells through T cell receptor co-stimulation.* Nature Cancer, 2020. **1**(1): p. 86-98.

231. Maura, F., et al., *Genomic landscape and chronological reconstruction of driver events in multiple myeloma.* Nat Commun, 2019. **10**(1): p. 3835.

232. Muz, B., et al., *A CD138-independent strategy to detect minimal residual disease and circulating tumour cells in multiple myeloma.* Br J Haematol, 2016. **173**(1): p. 70-81.

233. Miller, A., et al., *High somatic mutation and neoantigen burden are correlated with decreased progression-free survival in multiple myeloma.* Blood Cancer J, 2017. **7**(9): p. e612.

234. Cho, S.F., K.C. Anderson, and Y.T. Tai, *Targeting B Cell Maturation Antigen (BCMA) in Multiple Myeloma: Potential Uses of BCMA-Based Immunotherapy.* Front Immunol, 2018. **9**: p. 1821.

235. O'Connor, B.P., et al., *BCMA is essential for the survival of long-lived bone marrow plasma cells.* J Exp Med, 2004. **199**(1): p. 91-8.

236. Shah, N., et al., *B-cell maturation antigen (BCMA) in multiple myeloma: rationale for targeting and current therapeutic approaches.* Leukemia, 2020. **34**(4): p. 985-1005.

237. Schuh, E., et al., *Human Plasmacytoid Dendritic Cells Display and Shed B Cell Maturation Antigen upon TLR Engagement.* J Immunol, 2017. **198**(8): p. 3081-3088.

238. Topp, M.S., et al., *Anti-B-Cell Maturation Antigen BiTE Molecule AMG 420 Induces Responses in Multiple Myeloma.* J Clin Oncol, 2020. **38**(8): p. 775-783.

www.manaraa.com

239. Goldstein, R.L., et al., *AMG 701 induces cytotoxicity of multiple myeloma cells and depletes plasma cells in cynomolgus monkeys.* Blood Adv, 2020. **4**(17): p. 4180-4194.

240. Cooper, D., et al., *Safety and preliminary clinical activity of REGN5458, an anti-Bcma x Anti-CD3 bispecific antibody, in patients with relapsed/refractory multiple myeloma*. 2019, American Society of Hematology Washington, DC.

241. Law, C.-L., et al., *Preclinical and nonclinical characterization of HPN217: A Tri-Specific T Cell Activating Construct (TriTAC) targeting B cell maturation antigen (BCMA) for the treatment of multiple myeloma.* Blood, 2018. **132**(Supplement 1): p. 3225-3225.

242. Costa, F., B. Dalla Palma, and N. Giuliani, *CD38 Expression by Myeloma Cells and Its Role in the Context of Bone Marrow Microenvironment: Modulation by Therapeutic Agents.* Cells, 2019. **8**(12).

243. Chillemi, A., et al., *CD38 and bone marrow microenvironment.* Front Biosci (Landmark Ed), 2014. **19**: p. 152-62.

244. Malavasi, F., et al., *Evolution and function of the ADP ribosyl cyclase/CD38 gene family in physiology and pathology.* Physiol Rev, 2008. **88**(3): p. 841-86.

245. Lonial, S., et al., *Daratumumab monotherapy in patients with treatment-refractory multiple myeloma (SIRIUS): an open-label, randomised, phase 2 trial.* Lancet, 2016. **387**(10027): p. 1551-1560.

246. Frerichs, K.A., et al., *CD38-targeting antibodies in multiple myeloma: mechanisms of action and clinical experience.* Expert Rev Clin Immunol, 2018. **14**(3): p. 197-206.

247. Zuch de Zafra, C.L., et al., *Targeting Multiple Myeloma with AMG 424, a Novel Anti-CD38/CD3 Bispecific T-cell-recruiting Antibody Optimized for Cytotoxicity and Cytokine Release.* Clin Cancer Res, 2019. **25**(13): p. 3921-3933.

248. Richter, J.R., et al., *Phase 1, multicenter, open-label study of single-agent bispecific antibody T-cell engager GBR 1342 in relapsed/refractory multiple myeloma.* J Clin Oncol, 2018. **36**(15 suppl): p. TPS3132.

www.manaraa.com

249. Ollier, R., et al., *CD3/CD38 T CELL RETARGETING HETERO-DIMERIC IMMUNOGLOBULINS AND METHODS OF THEIR PRODUCTION*. 2019, US Patent App. 16/159,231.

250. Elkins, K., et al., *FcRL5 as a target of antibody-drug conjugates for the treatment of multiple myeloma.* Mol Cancer Ther, 2012. **11**(10): p. 2222-32.

251. Polson, A.G., et al., *Expression pattern of the human FcRH/IRTA receptors in normal tissue and in B-chronic lymphocytic leukemia.* Int Immunol, 2006. **18**(9): p. 1363-73.

252. Ise, T., et al., *Elevation of soluble CD307 (IRTA2/FcRH5) protein in the blood and expression on malignant cells of patients with multiple myeloma, chronic lymphocytic leukemia, and mantle cell lymphoma.* Leukemia, 2007. **21**(1): p. 169-74.

253. Brentjens, R.J., E.L. Smith, and C. Liu, *Chimeric antigen receptors targeting fc receptor-like 5 and uses thereof.* 2016, Google Patents.

254. Ovacik, A.M., et al., *Single cell-produced and in vitro-assembled anti-FcRH5/CD3 T-cell dependent bispecific antibodies have similar in vitro and in vivo properties.* MAbs, 2019. **11**(2): p. 422-433.

255. Depoil, D., et al., *CD19 is essential for B cell activation by promoting B cell receptor-antigen microcluster formation in response to membrane-bound ligand.* Nat Immunol, 2008. **9**(1): p. 63-72.

256. Hajek, R., S.A. Okubote, and H. Svachova, *Myeloma stem cell concepts, heterogeneity and plasticity of multiple myeloma.* Br J Haematol, 2013. **163**(5): p. 551-64.

257. Tembhare, P.R., et al., *Flow cytometric differentiation of abnormal and normal plasma cells in the bone marrow in patients with multiple myeloma and its precursor diseases.* Leuk Res, 2014. **38**(3): p. 371-6.

258. Boucher, K., et al., *Stemness of B-cell progenitors in multiple myeloma bone marrow.* Clin Cancer Res, 2012. **18**(22): p. 6155-68.

259. Jen, E.Y., et al., *FDA Approval: Blinatumomab for Patients with B-cell Precursor Acute Lymphoblastic Leukemia in Morphologic Remission with Minimal Residual Disease.* Clin Cancer Res, 2019. **25**(2): p. 473-477.

www.manaraa.com

260. Dufner, V., et al., *Long-term outcome of patients with relapsed/refractory B-cell non-Hodgkin lymphoma treated with blinatumomab.* Blood Adv, 2019. **3**(16): p. 2491-2498.

261. Gharbaran, R., *Advances in the molecular functions of syndecan-1 (SDC1/CD138) in the pathogenesis of malignancies.* Crit Rev Oncol Hematol, 2015. **94**(1): p. 1-17.

262. Hayashida, K., et al., *Molecular and cellular mechanisms of ectodomain shedding.* Anat Rec (Hoboken), 2010. **293**(6): p. 925-37.

263. Akhmetzyanova, I., et al., *Dynamic CD138 surface expression regulates switch between myeloma growth and dissemination.* Leukemia, 2020. **34**(1): p. 245-256.

264. Zou, J., et al., *Immunotherapy based on bispecific T-cell engager with hIgG1 Fc sequence as a new therapeutic strategy in multiple myeloma.* Cancer Sci, 2015. **106**(5): p. 512-21.

265. Alhallak, K., et al., *Nanoparticle T-cell engagers as a modular platform for cancer immunotherapy.* Leukemia, 2021.

266. Ding, L., Y. Hu, and H. Huang, *Novel progresses of chimeric antigen receptor (CAR) T cell therapy in multiple myeloma.* Stem Cell Investig, 2021. **8**: p. 1.

267. Gong, N., et al., *Nanomaterials for T-cell cancer immunotherapy.* Nat Nanotechnol, 2021. **16**(1): p. 25-36.

268. Caraccio, C., et al., *Bispecific Antibodies for Multiple Myeloma: A Review of Targets, Drugs, Clinical Trials, and Future Directions.* Front Immunol, 2020. **11**: p. 501.

269. Irvine, D.J. and E.L. Dane, *Enhancing cancer immunotherapy with nanomedicine.* Nature Reviews Immunology, 2020: p. 1-14.

270. June, C.H. and M. Sadelain, *Chimeric antigen receptor therapy.* New England Journal of Medicine, 2018. **379**(1): p. 64-73.

271. Kantarjian, H., et al., *Blinatumomab versus chemotherapy for advanced acute lymphoblastic leukemia.* New England Journal of Medicine, 2017. **376**(9): p. 836-847.

www.manaraa.com

272. Raje, N., et al., *Anti-BCMA CAR T-Cell Therapy bb2121 in Relapsed or Refractory Multiple Myeloma.* New England Journal of Medicine, 2019. **380**(18): p. 1726-1737.

273. Bansal, R., et al., *Chimeric antigen receptor T cells for treatment of transformed Waldenström macroglobulinemia.* Leuk Lymphoma, 2020. **61**(2): p. 465-468.

274. Guedan, S., M. Ruella, and C.H. June, *Emerging cellular therapies for cancer.* Annual review of immunology, 2018.

275. Levine, B.L., et al., *Global Manufacturing of CAR T Cell Therapy.* Mol Ther Methods Clin Dev, 2017. **4**: p. 92-101.

276. Velasquez, M.P., C.L. Bonifant, and S. Gottschalk, *Redirecting T cells to hematological malignancies with bispecific antibodies.* Blood, 2018. **131**(1): p. 30-38.

277. Huehls, A.M., T.A. Coupet, and C.L. Sentman, *Bispecific T-cell engagers for cancer immunotherapy.* Immunology and cell biology, 2015. **93**(3): p. 290-296.

278. Wang, Q., et al., *Design and production of bispecific antibodies.* Antibodies, 2019. **8**(3): p. 43.

279. Klinger, M., et al., *Immunopharmacologic response of patients with B-lineage acute lymphoblastic leukemia to continuous infusion of T cell–engaging CD19/CD3-bispecific BiTE antibody blinatumomab.* Blood, The Journal of the American Society of Hematology, 2012. **119**(26): p. 6226-6233.

280. Zhu, M., et al., *Blinatumomab, a Bispecific T-cell Engager (BiTE(®)) for CD-19 Targeted Cancer Immunotherapy: Clinical Pharmacology and Its Implications.* Clinical pharmacokinetics, 2016. **55**(10): p. 1271-1288.

281. Azab, A.K., et al., *CXCR7-dependent angiogenic mononuclear cell trafficking regulates tumor progression in multiple myeloma.* Blood, 2014. **124**(12): p. 1905-1914.

282. Luderer, M.J., et al., *Thermal Sensitive Liposomes Improve Delivery of Boronated Agents for Boron Neutron Capture Therapy.* Pharmaceutical Research, 2019. **36**(10): p. 144.

www.manaraa.com

283. Federico, C., et al., *Tumor microenvironment-targeted nanoparticles loaded with bortezomib and ROCK inhibitor improve efficacy in multiple myeloma.* Nat Commun, 2020. **11**(1): p. 6037.

284. Pidgeon, C. and C.A. Hunt, *Calculating number and surface area of liposomes in any suspension.* Journal of pharmaceutical sciences, 1981. **70**(2): p. 173-176.

285. Sun, J., et al., *Targeting CD47 as a Novel Immunotherapy for Multiple Myeloma.* Cancers (Basel), 2020. **12**(2).

286. Muz, B., et al., *CXCR4-targeted PET imaging using (64)Cu-AMD3100 for detection of Waldenström Macroglobulinemia.* Cancer Biol Ther, 2020. **21**(1): p. 52-60.

287. de la Puente, P., et al., *3D tissue-engineered bone marrow as a novel model to study pathophysiology and drug resistance in multiple myeloma.* Biomaterials, 2015. **73**: p. 70-84.

288. Muz, B., et al., *A CD 138-independent strategy to detect minimal residual disease and circulating tumour cells in multiple myeloma.* British journal of haematology, 2016. **173**(1): p. 70-81.

289. de la Puente, P., et al., *Enhancing proteasome-inhibitory activity and specificity of bortezomib by CD38 targeted nanoparticles in multiple myeloma.* J Control Release, 2018. **270**: p. 158-176.

290. Zhan, F., et al., *The molecular classification of multiple myeloma.* Blood, 2006. **108**(6): p. 2020-2028.

291. Festing, M.F. and D.G. Altman, *Guidelines for the design and statistical analysis of experiments using laboratory animals.* Ilar j, 2002. **43**(4): p. 244-58.

292. Till, B.G., et al., *CD20-specific adoptive immunotherapy for lymphoma using a chimeric antigen receptor with both CD28 and 4-1BB domains: pilot clinical trial results.* Blood, 2012. **119**(17): p. 3940-3950.

293. Wang, Q.-s., et al., *Treatment of CD33-directed chimeric antigen receptor-modified T cells in one patient with relapsed and refractory acute myeloid leukemia.* Molecular Therapy, 2015. **23**(1): p. 184-191.

www.manaraa.com

294. Caraccio, C., et al., *Bispecific Antibodies for Multiple Myeloma: A Review of Targets, Drugs, Clinical Trials, and Future Directions.* Frontiers in Immunology, 2020. **11**(501).

295. Müller, D., et al., *Improved pharmacokinetics of recombinant bispecific antibody molecules by fusion to human serum albumin.* J Biol Chem, 2007. **282**(17): p. 12650-60.

296. Wu, B. and Y.N. Sun, *Pharmacokinetics of Peptide-Fc fusion proteins.* J Pharm Sci, 2014. **103**(1): p. 53-64.

297. Pyzik, M., et al., *The Neonatal Fc Receptor (FcRn): A Misnomer?* Frontiers in Immunology, 2019. **10**(1540).

298. Usmani, S.Z., et al., *Phase I study of teclistamab, a humanized B-cell maturation antigen (BCMA) x CD3 bispecific antibody, in relapsed/refractory multiple myeloma (R/R MM)*. 2020, American Society of Clinical Oncology.

299. Richter, J.R., et al., *Phase 1, multicenter, open-label study of single-agent bispecific antibody T-cell engager GBR 1342 in relapsed/refractory multiple myeloma.* J Clin Oncol, 2018. **36**(5 Suppl).

300. Cohen, A.D., et al., *GO39775: A multicenter phase I trial evaluating the safety, pharmacokinetics, and activity of BFCR4350A, a FcRH5/CD3 T-cell dependent bispecific antibody, in patients with relapsed or refractory multiple myeloma*. 2020, American Society of Clinical Oncology.

301. Buelow, B., et al., *TNB383B. 0001: A Multicenter, Phase 1, Open-Label, Dose-Escalation Andexpansion Study of TNB-383B, a Bispecific Antibodytargeting BCMA in Subjects with Relapsed or Refractorymultiple Myeloma*. 2019, American Society of Hematology Washington, DC.

302. Strohl, W.R. and M. Naso, *Bispecific T-Cell Redirection versus Chimeric Antigen Receptor (CAR)-T Cells as Approaches to Kill Cancer Cells.* Antibodies, 2019. **8**(3): p. 41.

303. Kriz, A., et al., *A plasmid-based multigene expression system for mammalian cells.* Nature communications, 2010. **1**(1): p. 1-6.

304. Glockshuber, R., et al., *A comparison of strategies to stabilize immunoglobulin Fv-fragments.* Biochemistry, 1990. **29**(6): p. 1362-1367.

www.manaraa.com

305. Le Gall, F., et al., *Effect of linker sequences between the antibody variable domains on the formation, stability and biological activity of a bispecific tandem diabody.* Protein Engineering Design and Selection, 2004. **17**(4): p. 357-366.

306. Karste, K., M. Bleckmann, and J. van den Heuvel, *Not limited to E. coli: Versatile expression vectors for mammalian protein expression*, in *Heterologous Gene Expression in E. coli*. 2017, Springer. p. 313-324.

307. Bolli, N., et al., *Heterogeneity of genomic evolution and mutational profiles in multiple myeloma.* Nature communications, 2014. **5**: p. 2997.

308. Boussi, L. and R. Niesvizky, *Advances in immunotherapy in multiple myeloma.* Curr Opin Oncol, 2017. **29**(6): p. 460-466.

309. Panea, R.I., et al., *The whole-genome landscape of Burkitt lymphoma subtypes.* Blood, The Journal of the American Society of Hematology, 2019. **134**(19): p. 1598-1607.

310. Papaemmanuil, E., et al., *Genomic classification and prognosis in acute myeloid leukemia.* New England Journal of Medicine, 2016. **374**(23): p. 2209-2221.

311. Ormhoj, M., et al., *CARs in the Lead Against Multiple Myeloma.* Curr Hematol Malig Rep, 2017. **12**(2): p. 119-125.

312. Maruta, M., et al., *Direct comparison of target-reactivity and cross-reactivity induced by CAR- and BiTE-redirected T cells for the development of antibody-based T-cell therapy.* Sci Rep, 2019. **9**(1): p. 13293.

313. Siegel, R.L., K.D. Miller, and A. Jemal, *Cancer statistics, 2016.* CA Cancer J Clin, 2016. **66**(1): p. 7-30.

314. Pelcovits, A. and R. Niroula, *Acute Myeloid Leukemia: A Review.* R I Med J (2013), 2020. **103**(3): p. 38-40.

315. Guy, D.G. and G.L. Uy, *Bispecific Antibodies for the Treatment of Acute Myeloid Leukemia.* Curr Hematol Malig Rep, 2018. **13**(6): p. 417-425.

316. Tabata, R., et al., *Emerging Immunotherapy for Acute Myeloid Leukemia.* Int J Mol Sci, 2021. **22**(4).

www.manaraa.com

317. Alhallak, K., et al., *Biomaterials for cancer immunotherapy.* Biomaterials for Cancer Therapeutics, 2020: p. 499-526.

318. Stanchina, M., et al., *Advances in Acute Myeloid Leukemia: Recently Approved Therapies and Drugs in Development.* Cancers (Basel), 2020. **12**(11).

319. Bedoya, F., M.J. Frigault, and M.V. Maus, *The Flipside of the Power of Engineered T Cells: Observed and Potential Toxicities of Genetically Modified T Cells as Therapy.* Mol Ther, 2017. **25**(2): p. 314-320.

320. Goebeler, M.E. and R.C. Bargou, *T cell-engaging therapies - BiTEs and beyond.* Nat Rev Clin Oncol, 2020. **17**(7): p. 418-434.

321. Topp, M.S., et al., *Safety and activity of blinatumomab for adult patients with relapsed or refractory B-precursor acute lymphoblastic leukaemia: a multicentre, single-arm, phase 2 study.* Lancet Oncol, 2015. **16**(1): p. 57-66.

322. Pishvaian, M., et al., *Phase 1 Dose Escalation Study of MEDI-565, a Bispecific T-Cell Engager that Targets Human Carcinoembryonic Antigen, in Patients With Advanced Gastrointestinal Adenocarcinomas.* Clin Colorectal Cancer, 2016. **15**(4): p. 345-351.

323. Middelburg, J., et al., *Overcoming Challenges for CD3-Bispecific Antibody Therapy in Solid Tumors.* Cancers (Basel), 2021. **13**(2).

324. Huston, J.S., et al., *Protein engineering of single-chain Fv analogs and fusion proteins.* Methods Enzymol, 1991. **203**: p. 46-88.

325. Mallender, W.D. and E.W. Voss, Jr., *Construction, expression, and activity of a bivalent bispecific single-chain antibody.* J Biol Chem, 1994. **269**(1): p. 199-206.

326. Loke, J., et al., *Mylotarg has potent anti-leukaemic effect: a systematic review and meta-analysis of anti-CD33 antibody treatment in acute myeloid leukaemia.* Ann Hematol, 2015. **94**(3): p. 361-73.

327. Laszlo, G.S., et al., *Cellular determinants for preclinical activity of a novel CD33/CD3 bispecific T-cell engager (BiTE) antibody, AMG 330, against human AML.* Blood, 2014. **123**(4): p. 554-61.

www.manaraa.com

328. Luderer, M.J., et al., *Thermal Sensitive Liposomes Improve Delivery of Boronated Agents for Boron Neutron Capture Therapy.* Pharm Res, 2019. **36**(10): p. 144.

329. Stone, J.D., et al., *A sensitivity scale for targeting T cells with chimeric antigen receptors (CARs) and bispecific T-cell Engagers (BiTEs).* Oncoimmunology, 2012. **1**(6): p. 863-873.

330. Abdouh, M., et al., *Transcriptional mechanisms for induction of 5-HT1A receptor mRNA and protein in activated B and T lymphocytes.* J Biol Chem, 2001. **276**(6): p. 4382-8.

331. Reusch, U., et al., *A tetravalent bispecific TandAb (CD19/CD3), AFM11, efficiently recruits T cells for the potent lysis of CD19(+) tumor cells.* MAbs, 2015. **7**(3): p. 584-604.

332. Kay, J.E., *Mechanisms of T lymphocyte activation.* Immunol Lett, 1991. **29**(1-2): p. 51-4.

333. Wingett, D., et al., *pim-1 proto-oncogene expression in anti-CD3-mediated T cell activation is associated with protein kinase C activation and is independent of Raf-1.* J Immunol, 1996. **156**(2): p. 549-57.

334. Van Duuren, B.L., A.C. Smith, and S.M. Melchionne, *Effect of aging in two-stage carcinogenesis on mouse skin with phorbol myristate acetate as promoting agent.* Cancer Res, 1978. **38**(3): p. 865-6.

335. Imose, M., et al., *Leflunomide protects from T-cell-mediated liver injury in mice through inhibition of nuclear factor kappaB.* Hepatology, 2004. **40**(5): p. 1160-9.

336. Epstein, L.B. and C.W. Smith, *The in vivo induction of mouse lymphocyte transformation by phytohemagglutinin.* J Immunol, 1968. **100**(2): p. 421-35.

337. Lindahl-Kiessling, K., *Mechanism of phytohemagglutinin (PHA) action. V. PHA compared with concanavalin A (Con A).* Exp Cell Res, 1972. **70**(1): p. 17-26.

338. Raje, N., et al., *Anti-BCMA CAR T-Cell Therapy bb2121 in Relapsed or Refractory Multiple Myeloma.* N Engl J Med, 2019. **380**(18): p. 1726-1737.

www.manaraa.com

339. Yan, Z., et al., *A combination of humanised anti-CD19 and anti-BCMA CAR T cells in patients with relapsed or refractory multiple myeloma: a single-arm, phase 2 trial.* Lancet Haematol, 2019. **6**(10): p. e521-e529.

340. Xu, J., et al., *Exploratory trial of a biepitopic CAR T-targeting B cell maturation antigen in relapsed/refractory multiple myeloma.* Proc Natl Acad Sci U S A, 2019. **116**(19): p. 9543-9551.

341. Ali, S.A., et al., *T cells expressing an anti-B-cell maturation antigen chimeric antigen receptor cause remissions of multiple myeloma.* Blood, 2016. **128**(13): p. 1688-700.

342. Zhao, W.H., et al., *A phase 1, open-label study of LCAR-B38M, a chimeric antigen receptor T cell therapy directed against B cell maturation antigen, in patients with relapsed or refractory multiple myeloma.* J Hematol Oncol, 2018. **11**(1): p. 141.

www.manaraa.com