REDIRECTING T CELL SPECIFICITY TO IGE-EXPRESSING B CELL FOR THE MANAGEMENT OF SEVERE ALLERGIC ASTHMA

by

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A thesis submitted to the Faculty of the University of Delaware in partial fulfillment of the requirements for the degree of Master of Science in Biological Sciences

Fall 2017

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ACKNOWLEDGMENTS

My deepest appreciation goes to my advisor, Dr. Zhengyu (Mark) Ma for his unflinching support and guidance on the achievement of this milestone. His accessibility and hands on approach are attributes any student could wish to have in an advisor. His mentorship has helped develop my scientific skills and increased my knowledge in the field of Immunotherapy and has renewed my passion for precision and individualized medicine.

I want to thank my committee members – Dr. Donna Woulfe and Dr. Carlton Cooper for their support, suggestions and experimental insights. Their contributions were invaluable to the progress of my research.

My sincere gratitude goes to my lab members – Brittany Fay and Karen Christie. Their words of encouragement kept me going when research was at its low point. I want to specially thank Brittany for her initial help and guidance with experiments when I joined the Lab.

I want to thank Lisa Glazewski at the Nemours Biomolecular Core for her training in using the flow cytometer and always ensuring the instrument is always in a working order.

I am grateful to Betty Cowgill for her kind words and generous support throughout my program. She has been very efficient in helping me to keep track of departmental requirements and deadlines.

To my close friends Ibukun Dada and Oyindamola Aluko, thank you for always lending a listening ear when I needed to vent about my research.

I will also like to acknowledge the Potter's House Family for their affection, warm-heartedness and friendship. You all made acclimatizing to life in the US effortless. I am forever indebted to you all.

To my best friend and husband, Oluwasanmi Adeodu, thank you for being a shoulder to lean on, my worst critic and my greatest cheer leader! Thank you for believing in me and encouraging me to pursue my dreams.

This milestone would have been difficult to achieve without the support of my family. They are my greatest support system. Thank you for your unwavering love, and dedication to ensuring this dream became a reality.

I DEDICATE THIS THESIS TO THE HONOR OF MY GRANDMOTHER, OLUSOLA JULIANA BAJO, AND TO ALL WHO HAVE LOST THEIR LIVES DUE TO SEVERE ALLERGIC ASTHMA

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ABSTRACT

The prevalence of allergic diseases has dramatically increased in recent decades, affecting people of all ages. Severe allergic asthma affects a substantial proportion of the population and cannot be controlled by available medications. Patients often require large doses of corticosteroids in combination with other potentially toxic medications and still, may suffer from poorly controlled symptoms and frequent life-threatening asthma attacks and thus significantly impacting on their quality of life.

Allergic immune responses trigger the disease in two-thirds of patients with asthma and up to 50% of patients with severe asthma. IgE plays a central role in allergic immune response, therefore, targeting the source of IgE, the IgE-expressing B cells represents an attractive therapeutic approach. Adoptive cell immunotherapy using T cells engrafted with IgE-specific chimeric antigen receptors (CARs) has the potential to achieve long-term suppression of IgE through specific killing of IgE-expressing B cells.

Previously, first-generation FcεRIα-based CARs were constructed and efficiently expressed on the Jurkat T cell line. The wildtype (WT) and 4 low affinity mutant CARs expressed on Jurkat cells were able to bind IgE, and mediated potent and specific responses to target cells. However, the first-generation CARs showed limited persistence due to the lack of costimulatory signaling domains.

To improve CAR T cell persistence, we have constructed 2nd and 3rd generation FcεRIα-based CARs with costimulatory domains, expressed them on primary human CD8+ T cells through lentiviral transduction, tested their *in vitro* persistence and cytotoxicity towards IgE-expressing B cells. We show that the 2nd generation CARs

were robustly expressed on primary human CD8+ T cells and that these CARs mediated potent killing of target cells. CAR T cell *in vitro* persistence, however, was not improved by addition of co-stimulatory domains alone. After excluding the roles of IgE binding in culture system and excessive stimulation of T cells from the CD3/CD28 activation beads, we concluded that artificially high levels of CAR expression may lead to spontaneous signaling that is toxic to T cells. In support of this, modulating the expression level of CARs by using a lower expression platform increased CAR T cell *in vitro* persistence.

Taken together, these results demonstrate the effectiveness of the FcεRIα-based CARs in targeting IgE-expressing B cells in severe allergic asthma and addressed the issue of CAR expression persistence. Through detailed experimental analysis, we have advanced the understanding of factors critical for CAR T cell survival. We believe future studies building on the results of current research will lead to the successful development of adoptive T cell therapy for severe allergic diseases using FcεRIα-based CARs.

Chapter 1

INTRODUCTION

1.1 Targeting Immunoglobulin E (IgE) Antibody for Novel Asthma Therapies

Asthma is a chronic disease characterized by hyper-responsiveness of the airways and variable airflow obstruction, which is often reversible[1,2]. In the US, asthma affects nearly 25 million people[3]. In 2007, it was linked to nearly 3500 deaths[4]. Asthma is the leading cause of hospitalization among children; affecting more than 7 million children and resulting in more than 14 million lost school days[5]. Asthma therefore has a significant socio-economic impact as it affects a substantial population, imposes a burden in terms of treatment costs, loss of productivity and reduced quality of life[6].

Clinically, many different phenotypes of asthma have been recognized; however, allergic asthma affects a significant proportion of patients[2]. Allergic mechanisms have been implicated for asthma in two-thirds of the patients; of this population, approximately 50% of patients have a severe condition accounting for significant morbidity and mortality[7].

Although several medications are available, severe allergic asthma cannot be effectively controlled[8]. Patients require large doses of corticosteroids in combination with other potentially toxic medications and still, may have poor control of symptoms and frequent life-threatening asthma attacks as most of these drugs are palliative or are non-specific in their mode of action.

In a predisposed individual, an initial exposure to an allergen e.g., pollen, activates B cells to synthesize immunoglobulin E (IgE), an antibody that binds strongly

to its high affinity receptor, FcεRI, on certain immune cells (mast cells, basophils and eosinophils). This process is known as allergic sensitization[9]. Subsequent exposure of the individual to the same allergen causes allergen-induced crosslinking of the IgE-FcεRI cell surface complex, triggering degranulation of effector cells and release of both preformed and newly synthesized pro-inflammatory mediators (histamine, leukotrienes and cytokines) that are responsible for the symptoms observed in allergy (inflammation, bronchospasm, increased mucus secretion) **(Fig. 1)**[10]. IgE therefore plays a central role in the pathogenesis of allergic response and presents an attractive target for therapeutic intervention.

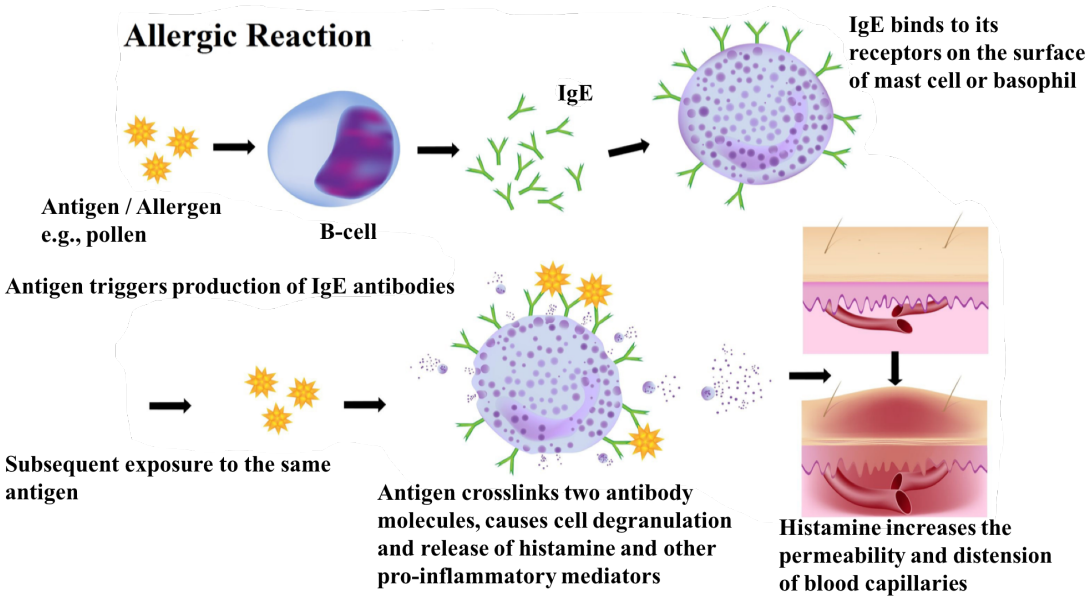


Figure 1: Pathophysiology of an allergic response. Allergic reactions are initiated when allergens cross-link specific IgE antibodies bound to the high-affinity receptor FcεRI on mast cells, basophils and eosinophils, thereby triggering degranulation that results in release of inflammatory chemical mediators (Image adapted from Rajiv Desai, 2013[10]).

1.2 Current Clinical Approach to Targeting IgE

The virtue of targeting IgE has been demonstrated by the success of omalizumab (Xolair®), a recombinant humanized IgE-specific monoclonal antibody that prevents the high affinity IgE-FcεRI binding thereby reducing circulating levels of free IgE available to trigger the allergic cascade[11,12]. In addition, omalizumab also blocks the IgE-dependent uptake of allergens by mature myeloid dendritic cells affecting the chronic allergic response by downregulating dendritic cell FcεRI expression[13,14].

Omalizumab is approved for the management of moderate to severe IgE-mediated (allergic) asthma[11]. Currently, omalizumab is the recommended treatment of severe allergic asthma in patients not responding to Step 4 of the GINA treatment approach, and need therapeutic upgrade to Step 5[15]. In the European Union, omalizumab is approved as an add-on therapy in adults, adolescents, and children (6 to <12 years of age) with severe persistent allergic asthma who have a positive skin test or *in vitro* reactivity to a perennial aeroallergen and who have reduced lung function (FEV1 <80%); as well as frequent daytime symptoms or night-time awakenings and who have had multiple documented severe asthma exacerbations despite daily high dose inhaled corticosteroids, plus a long-acting inhaled beta2-agonist[12].

Omalizumab therapy appreciably improves symptoms and is well tolerated in asthma patients. The drug reduces the frequency of asthma attacks and the need for high dosage of inhaled corticosteroids[16,17]. However, Omalizumab requires frequent administration at high doses (at least once every 4 weeks), and costs close to $20,000 annually[18]. This therefore limiting its range of clinical applications. An analysis from Harvard Medical School concluded that omalizumab was not cost-effective for adults with severe asthma[19]. It is therefore imperative that clinicians explore alternative therapies prior to initiating omalizumab. Furthermore, omalizumab is recommended

only for patients with IgE levels below 700 IU /ml and its use is associated with a minimal risk of systemic anaphylaxis[16,17,20].

1.3 T Cell Based Immunotherapy as an Alternative to Omalizumab (Xolair®)

According to the Still Fighting for Breath global survey, approximately 75% of asthma patients reported a severe asthma attack and required treatment in the emergency room. The survey also revealed that a significant number of adults and children with severe asthma still presents with uncontrolled symptoms; half of respondents did not consider their current medications effective, two-thirds disliked the cost, and 41% disliked the side-effects. 75% of respondents would prefer to have a non-drug treatment option for their asthma[21]. Therefore, an approach that has a long-lasting effect is highly desirable. This can be accomplished through the adoptive cellular therapy (ACT) based on the reprograming of cytotoxic T cells to recognize IgE expressing B cells.

Targeting the source of IgE, the IgE-expressing B cells can potentially overcome the limitations of omalizumab by eliminating the source of IgE production. IgE is produced by B cells that are class-switched with the help from type 2 helper T (Th2) cells. IgE-expressing B cells include germinal center B cells, plasmablasts, plasma cells, and memory B cells. These cells uniquely express a transmembrane form of IgE (mIgE) on their cell surface and presents as a molecular target for adoptively transferred T cells.

ACT has demonstrated potential in achieving absolute and long-lasting responses in some malignant and infectious diseases.[22-24]. In recent years, ACTs have made great strides in clinical trials for cancer immunotherapy. Clinical studies using chimeric antigen receptor (CAR) based ACTs have provided evidence of persistent anti-tumor activity in humans, and was chosen as Breakthrough of the Year for 2013 by *Science* magazine[25]. Recently, the US FDA in a historic action, approved the first CAR

T cell therapy to treat certain children and young adults with B cell acute lymphoblastic leukemia[26]. Although ACT has only been tested in terminally ill cancer patients, the good safety profile demonstrated in clinical trials and additional safety features being actively developed should justify its application, to less lethal but highly debilitating diseases such as severe asthma. In addition, T cells have the ability to differentiate into a memory phenotype that can survive and persist in an inactive state in the patient for years, thus, making this therapeutic approach feasible for patients with severe asthma as IgE level will be continuously depleted. Therefore, by reengineering T cells with mIgE-specific CARs to recognize and kill IgE-expressing B cells and exploiting T cell memory, adoptive T cell therapy may for the first time, offer an effective long-term control, or even a cure, of this devastating disease.

1.3.1 Adoptive T Cell Therapy – ACT

Adoptive immunotherapy is an emerging field in different stages of pre-clinical and clinical studies[27] and is being investigated in the treatment of cancers and chronic infections such as HIV. Although ACT has been around since the 1980s[28], it was not until recently that it gained increasing interest as a result of an improved understanding of T cell biology. In ACT, T cells are endowed with the ability to deliver specific cytotoxicity to cells that would otherwise be spared[29].

In adoptive immunotherapy **(Fig. 2)**, autologous T cells are isolated, expanded *ex vivo*, and genetically reprogrammed to express artificial immune receptors that redirect T cell specificity to the target molecule. The cells are further expanded before being transferred back to the patient. The engineered T cells can then specifically seek and destroy the target cells[29].

Prior to adoptive cell infusion in the clinics, patients are pre-conditioned by the administration of a lymphodepleting regimen. Chemotherapy-induced lymphodepletion has been shown to greatly enhance the expansion and persistence of transferred cells and improve anti-cancer effects[30]. Although lymphodepletion is not always required, the beneficial effects of lympho-depleting regimens have been attributed the following mechanism: elimination of T regulatory cells and/or myeloid-derived suppressor cells (MDSCs); elimination of endogenous lymphocytes, which may remove the competition for homeostatic cytokines such as IL-7 and IL-15; and activation of antigen-presenting cells, which increases antigen presentation to T cells, thus enhancing anticancer immunity[23,31].

The key component of the ACT are the artificial receptors expressed on T cells and the fundamental factors driving the development of ACT is the evidence that administered CAR^+ T cells transferred without prior host immunosuppression can undergo multi-log expansion, eradicate large tumor burdens and persist for more than a decade in humans[32]. The infused T cells can persist with a memory phenotype, which allows long-term monitoring and elimination of target cells[33]. Long-term persistence can be further enhanced by expressing CARs on *ex vivo* expanded central memory $CD8^+$ T cells[34].

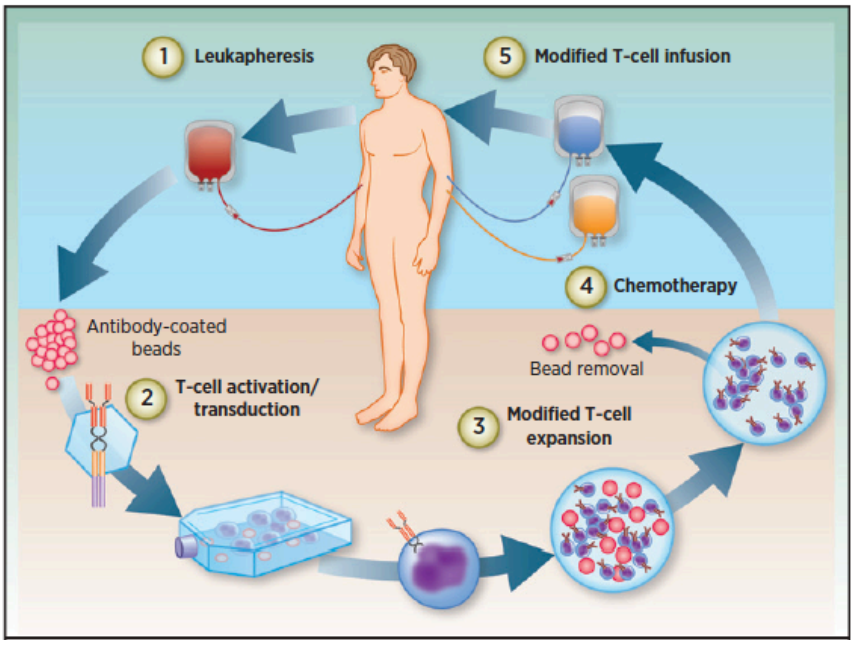


Figure 2: Overview of CAR T cell therapy in the clinic. (1) T cells are isolated through leukapheresis followed by **(2)** activation of the isolated T cells with antibody coated (CD3/CD28) beads serving as artificial dendritic cells. The activated T cells are then genetically reengineered *ex vivo* by transduction with a lentiviral vector construct encoding the artificial receptors and **(3)** the reengineered T cells are further expanded *ex vivo*. After the modified T cell product has been prepared and has passed all required quality testing, **(4)** the lympho-depleting chemotherapy and **(5)** the modified T cell infusion are administered to the patient. (Image adapted from Maus & June, 2016[29]).

1.4 Chimeric Antigen Receptors – CAR

Chimeric antigen receptors (CARs) are artificial immune receptors that redirect the specificity and function of T cells and other immune cells to specific diseases or tumor associated antigens[35]. Upon antigen binding, this artificial receptor transmits activating signals to T cells, which in turn trigger T cells to secrete various cytokines, perforins and granzymes as well as express FasL and tumor necrosis factor-related apoptosis inducing ligand (TRAIL) in order to eliminate target cells[36].

A major strength of the CAR platform is its modularity, with similar and structurally distinct options being available for each functional domain[37]. The rationale for the use of CARs in cancer immunotherapy is to rapidly generate T cells that target disease cells, which can bypass the barriers and incremental kinetics of active immunization[38,39].

Chimeric antigen receptors (CARs) incorporates an MHC non-restricted antigen binding extracellular domain fused with an extracellular spacer domain, a transmembrane domain and an intracellular signaling domains containing the immunoreceptor tyrosine-based activation motif (ITAM) designed to imitate the downstream signaling of T cell receptors (TCR) in natural T cells **(Fig. 3)**[40]. For most CARs, the extracellular binding domain is usually a single chain variable fragment (scFv) derived from a monoclonal antibody (mAb) while the intracellular signaling domain incorporates a CD3ζ chain, a component of the TCR/CD3 complex essential for T cell signaling[28].

One of the major benefits of equipping T cells with a non-MHC-restricted, antibody-derived specificity is that T cells are enabled to efficiently bypass the down regulation of MHC molecules - a major immune escape mechanisms of tumors[41]. Furthermore, the inclusion of signaling domains from different immune receptors can enhance the expansion and survival of engineered T cells. In addition, prospective targets structures are no longer limited to protein-derived peptides, but to all surface molecule on tumor cells and also to carbohydrates and glycolipid structures, making this approach applicable to a wide range of diseases[36].

Early CAR designs relied solely on the TCR zeta signal to activate T cells[42]. Although these so-called first-generation CARs were able to mediate targeted T cell

toxicity, they had limited *in vivo* activity due to the lack of persistence[35,37-39,43]. Recently, ACT based on CD19-specific CARs led to complete remission in 83% of patients enrolled in a clinical trial of B cell leukemia and lymphoma[22,24,44-46]. The favorable outcome of the clinical study has been attributed to the improved CAR signaling domain. In addition to the CD3ζ signaling domains from the costimulatory molecules CD28 and/or 4-1BB was added to create the second-generation CARs. The third generation CARs include signaling domains from both CD28 and 41BB[43]. The addition of a co-stimulatory signaling domain augments T cell activation leading to enhanced cytokine production, proliferation, differentiation and persistence[47-49]. However, several studies have shown that despite the incorporation of costimulatory domains, the majority of the CARs with the exception of anti-CD19 CARs, are unable to persist on a long-term on T cells[50,51]. One possible explanation is that the antigen binding domain in most CARs is an antibody in the scFvs format. The scFvs consists of the variable region of the immunoglobulin heavy chain (V_H) artificially linked to the variable region of the light chain (V_L). The existence of the scFvs in an unnatural configuration on the cell surface may result in low stability and tendency to self-aggregate leading to an undesired CAR clustering and spontaneous CAR signaling in the absence of antigen stimulation. Spontaneous CAR signaling has been linked to CAR-T cells exhibiting reduced functional activities such as proliferation, cytokine production and cytotoxicity, a phenomenon termed T cell exhaustion[37,50,52,53].

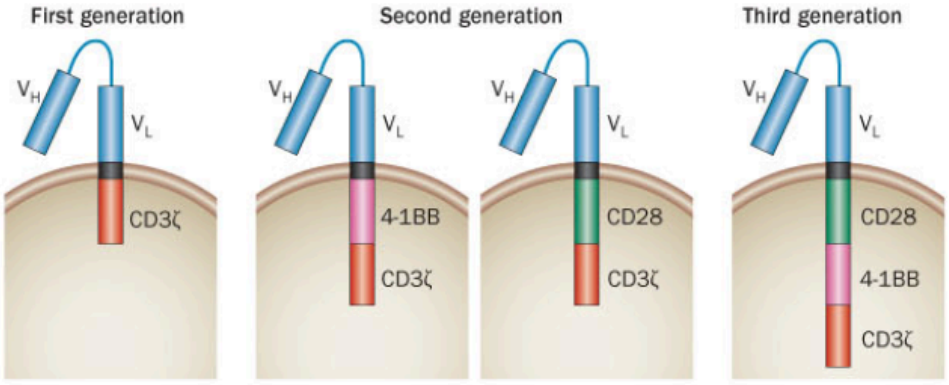


Figure 3: Chimeric antigen receptors. The first-generation CAR has a CD3ζ intracellular signaling domain (red) fused with a transmembrane domain (black) and an extracellular antigen binding domain (blue) usually comprising of an scFv from a mAb. All second and third generation CAR incorporates one or two co-stimulatory endo-domains in addition to the CD3ζ domain. Co-stimulatory domains have been shown to lead to enhanced expansion and persistence compared with CARs lacking a co-stimulatory domain. (Image adapted from Mackall *et al*, 2014[40]).

1.4.1 CARs Based on Natural IgE Receptors

Unlike most ACT approach employing CARs with scFvs for target recognition, our ACT based strategy for allergic asthma exploits the native IgE receptor for target recognition. Compared with the scFv-based CARs, CARs engineered with a natural receptor-derived binding domain has the potential advantage of increased stability and decreased clustering, thus lowering spontaneous signaling and enhancing persistence.

IgE has two known receptors, the high affinity receptor FcεRI and the low affinity receptor FcεRII (CD23)[54,55]. FcεRI consists of an α chain (FcεRIα) that binds to IgE Fc region with high affinity (Kd = 3.7 x 10^{-10} M), and β and γ chains containing the ITAM signaling domains. FcεRII forms trimers, binds to IgE and other class of molecules such as MHC class II, integrins and CD21[56] making it unsuitable for specific

targeting. Therefore, our CARs will be designed using the high affinity receptor FcεRI for specific targeting.

In our CAR design, the WT FcεRIα or a low affinity mutant of FcεRIα (FcεRIα') extracellular domain is fused with a CD3ζ intracellular signaling domain as in the first-generation CARs **(Fig. 4A)** or with the 41BB and/or CD28 costimulatory domain and a CD3ζ intracellular signaling domain as in the second **(Fig. 4B)** and third generation CARs **(Fig. 4C).**

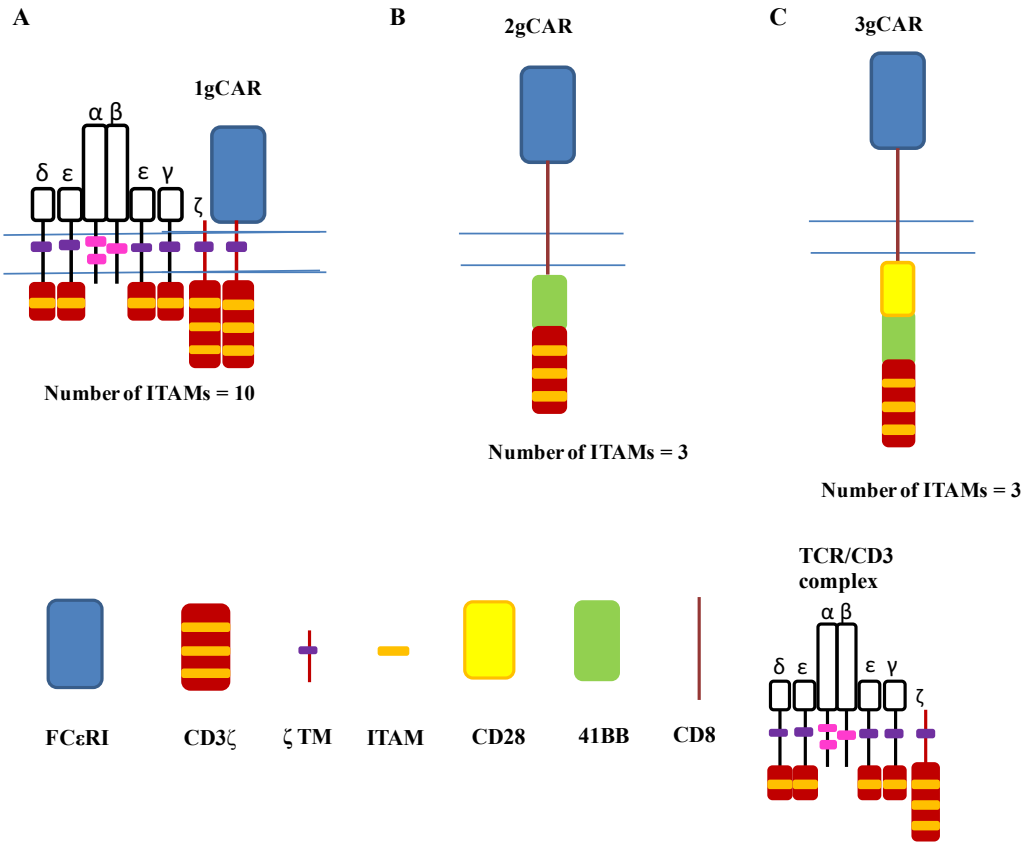


Figure 4: CARs based on FcεRI receptor. (A) The first-generation CAR associates with the TCR/CD3 complex through interaction between charged residues within their transmembrane domains. This increases the number of associated ITAMs. **(B)** The second-generation CAR incorporates a 41BB costimulatory domain. **(C)** The third-generation CAR includes both the CD28 and the 41BB costimulatory domain. In B & C, the polar charged zeta transmembrane domain has been replaced with a neutral CD8 transmembrane domain which does not associate with the TCR/CD3 complex therefore reducing the number of associated ITAMs.

1.5 Design Criteria for FcεRIα-Based CARs

For the FcεRIα-based CARs to be highly specific in their action, the CARs should satisfy four critical requirements. First, the CAR should bind mIgE with appropriate strength to trigger T cell activation and target cell killing (**Fig. 5A**). It has been shown that TCR signaling is normally triggered by low affinity ligand binding (Kd $\approx 10^{-6}$ M)[57], and CARs that bind ligands with Kd from 10^{-6} M to 10^{-9} M have been shown to function effectively[58]. Therefore, FcεRIα mutants of a wide range of affinities for mIgE will be evaluated for suitability as a CAR. Second, FcεRIα'-based CAR should not recognize cells with secreted IgE bound on cell surface through FcεRI (**Fig. 5B**). This prevents CAR^+ T cells from killing $FcεRI^+$ mast cells, eosinophils, basophils and Langerhans cells[54,59] and from activating these cells by crosslinking surface bound IgE and cause degranulation and associated side effects. This should not occur because there is only one FcεRIα binding site on IgE[60]. Third, the CAR should not recognize cells with secreted IgE bound on cell surface through FcεRII binding (**Fig. 5C**) thus, preventing CAR^+ T cells from targeting $FcεRII^+$ B cells and epithelial cells[61,62]. Although FcεRI and FcεRII bind IgE at different sites, FcεRII-IgE binding induces an Fc conformation that is incompatible with FcεRI-IgE binding and vice versa. In other words, the two bindings allosterically inhibit each other. Consequently, FcεRIα'-based CARs should not recognize and bind IgE that is already bound to FcεRII. Finally, the

recognition of mIgE^{+} target cells by the CAR should tolerate the presence of a high concentration of secreted IgE (**Fig. 5D**). Secreted IgE in circulation and tissues could bind CARs on the T cells and block their interaction with mIgE on the target cells. To alleviate this problem, a CAR based on low affinity FcεRIα mutant will be employed. This is because at a given concentration of secreted IgE, T cells expressing low affinity CARs will have a smaller proportion of CARs blocked by secreted IgE than cells expressing high affinity CARs. For example, the upper limit of patient serum IgE levels for omalizumab treatment is 700 IU/ml or 8.75 x 10^{-9} M, which would block 96% of CARs with wild type FcεRIα, but less than 1% of CARs with a low affinity FcεRIα of Kd = 10^{-6} M on a T cell.

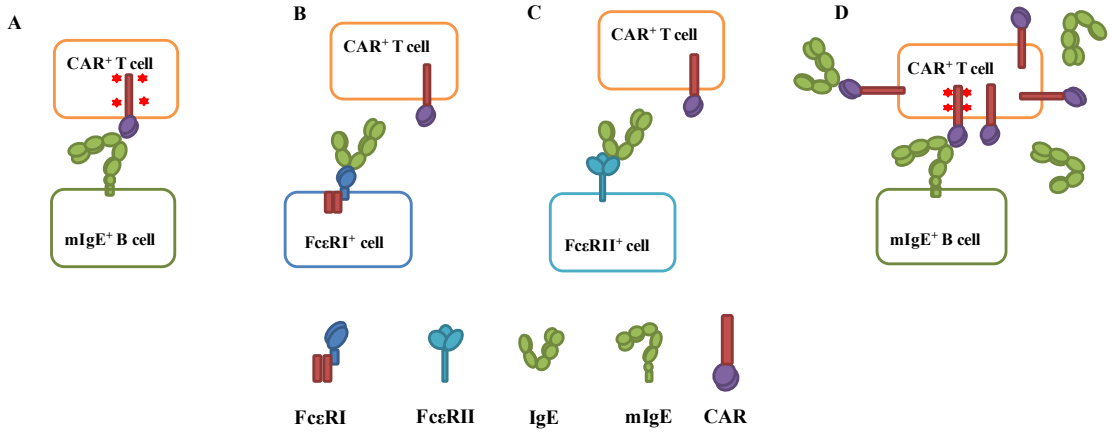


Figure 5: A CAR based on FcεRIα redirects T cell responses specifically to cells expressing mIgE. The CAR consists of the extracellular domain a low affinity mutant of FcεRIα (FcεRIα') fused to the co-stimulatory signaling domain of CD28 or 4-1BB and intracellular signaling domain of CD3ζ. **(A)** FcεRIα'-based CARs on a T cell bind mIgE with appropriate strength to trigger TCR signaling, T cell activation and target cell killing. TCR signaling is normally triggered by low affinity ligand binding (Kd ≈ 10^{-6} M). **(B)** CAR^+ T cells should not recognize cells with secreted IgE bound on cell surface through FcεRI because IgE has only one FcεRI binding site. **(C)** CAR^+ T cells should not recognize cells with secreted IgE bound through FcεRII because IgE-FcεRII binding allosterically inhibits IgE-FcεRI binding. **(D)** The recognition of $mIgE^+$ target cells by a CAR^+ T cell can tolerate the presence of secreted IgE at relatively high concentrations. CARs on T cells will only be partially blocked by secreted IgE because of the low affinity of FcεRIα'-IgE binding. The unoccupied CARs will still be able to recognize $mIgE^+$ on target cells as in A.

1.5.1 CARs Based on Low Affinity Mutants of FcεRIα

Based on previous mutagenesis studies on the critical residue necessary for high affinity binding[63-65], we designed and constructed seven CARs with WT FcεRIα or low affinity mutants for mIgE recognition. The six mutants CARs and fold reduction in affinity (in parentheses) are: K117D (27x), D159A (2x), Y131A (3x), W113A (5x), W87D (7x), V155A (10x). The combination of two mutations as shown in **Table 1** below is designed to increase the range of variations in CAR affinity.

Table 1: FcεRIα-based Mutants and their CAR Designation

CAR Designation	Mutants	Combined Mutants Fold Reduction
M1	K117D	27x
M2	K117D + D159A	27x; 2x
M3	K117D + Y131A	27x; 3x
M4	K117D + W113A	27x; 5x
M5	K117D + W87D	27x; 7x
M6	K117D + V155A	27x; 10x

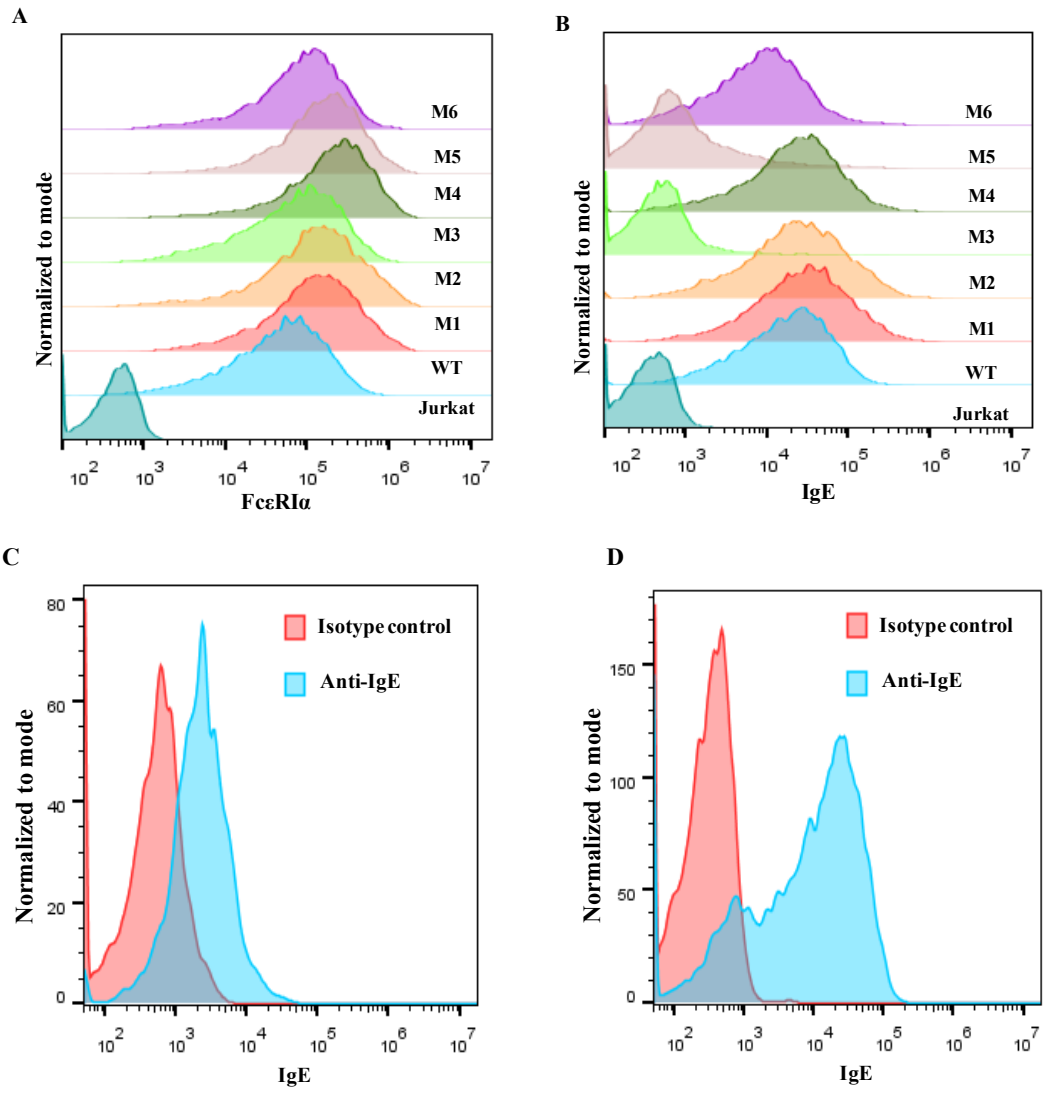
1.6 First-generation CARs Based on FcεRIα Mediate Potent and Specific Jurkat T Cell Responses to Target Cells

Previous studies in our group constructed first-generation FcεRIα based CARs and tested their abilities to recognize cells expressing the transmembrane form of IgE (mIgE). The ecto-domains of the wild type (WT) and six low affinity mutants of FcεRIα were fused with CD3ζ to form WT and mutant CARs. On transduction of Jurkat cells (an acute T cell leukemia cell line), with lentiviral vectors encoding the CARs, the results revealed that the WT and the six low affinity mutant CARs were stably expressed at similar levels **(Fig. 6A)**. We further showed that Jurkat cells expressing the WT and four low affinity mutants (M1, M2, M4, and M6) CARs bind IgE at high levels **(Fig. 6B)**, demonstrating the IgE-binding function of the FcεRIα component in the CAR. Further investigation of the low affinity mutant (M3 and M5) CARs that did not bind IgE was discontinued.

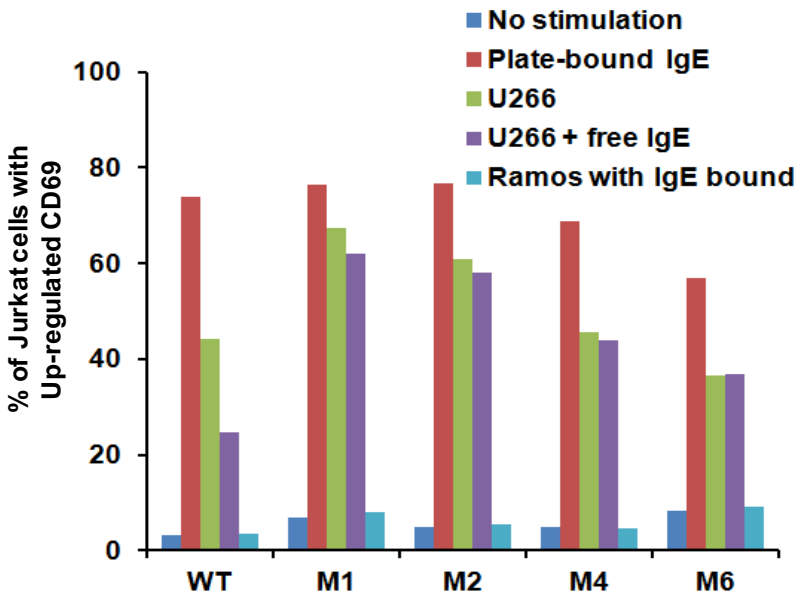
Since the FcεRIα component (extracellular domain) in the CAR has been shown to bind the target molecule, IgE, we sought to determine the potency and specificity of the CARs that bind IgE using target cell lines. The target cell lines used were the U266

myeloma cell line expressing the transmembrane form of IgE (main targets), LAD2 mast cell line expressing FcεRI and Ramos B cell line that express FcεRII (targets to avoid). Prior to the potency and specificity studies, we ascertained the level of mIgE expression and IgE binding by both U266 and Ramos cells respectively. The result showed that U266 cells express low levels of mIgE **(Fig. 6C)** while IL4 stimulated Ramos cells expressed FcεRII (data not shown) and bind IgE at high levels **(Fig. 6D).** Different stimuli were delivered to the CAR^+ Jurkat cells to establish if the CARs were able to mediate responses to the target cells. T cell response was quantified by the upregulation of CD69, a marker of T cell activation. The four low affinity mutant CARs mediated robust T cell responses to U266 myeloma cells expressing mIgE while the WT CAR mediated the lowest T cell responses **(Fig. 6E)**. This is consistent with reports that very high affinity TCR-ligand binding hinders T cell responses to low density ligands[66]. As expected, we found that the plastic plates with bound IgE stimulated stronger responses than U266 cells **(Fig. 6E)**. As predicted, the presence of free IgE significantly inhibited the activity of Jurkat cells expressing the high affinity WT CAR but responses by Jurkat cells expressing the low affinity mutant CARs were not affected **(Fig. 6E)** (free IgE concentration - 1.75μg/ml (700 IU/ml), the upper limit of serum IgE level for omalizumab prescription). This indicates that CARs with lower affinity are able to tolerate circulating levels of free IgE, consistent with the model proposed in **Fig. 5D.** Very importantly, we showed that CAR^+ Jurkat cells do not respond to cells with IgE bound through FcεRI or FcεRII (CD23) **(Fig. 6E & F)** and CAR^+ Jurkat cells do not lead to degranulation of LAD2 mast cells with IgE bound through FcεRI **(Fig. 6G)** suggesting that CAR^+ Jurkat cells were specific in their responses and do not target unwanted cells and cause side effects, consistent with the model proposed in **Fig. 5B** &

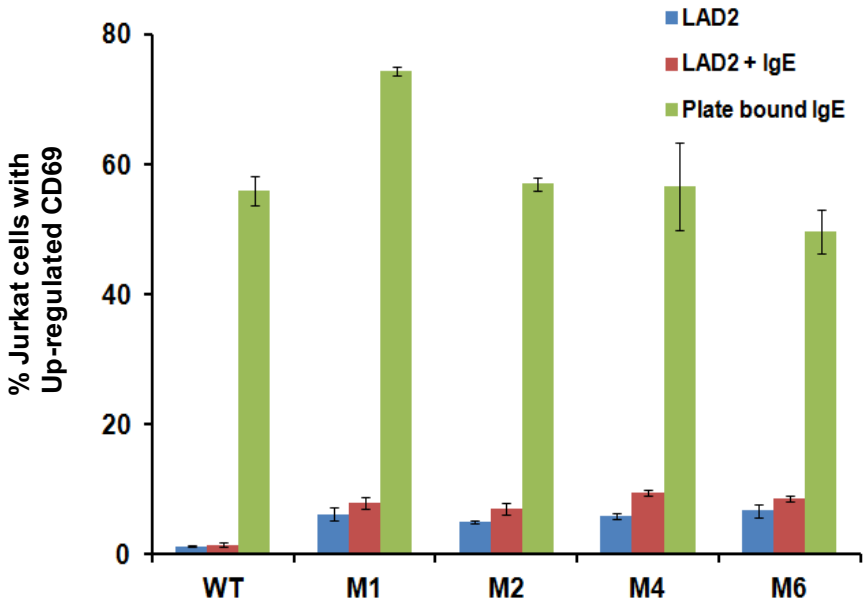
C respectively. Taken together, these results strongly indicate that CARs based on low affinity FcεRIα mutants can mediate potent and specific T cell responses to target cells expressing the transmembrane form of IgE even in the presence of free IgE, but not to cells with free IgE bound through FcεRI or FcεRII. These CARs therefore have the potency and specificity required for targeting IgE-expressing B cells in ACT for atopic diseases.



E


No stimulation
Plate-bound IgE
U266
U266 + free IgE
Ramos with IgE bound
% of Jurkat cells with Up-regulated CD69
100
80
60
40
20
0
WT
M1
M2
M4
M6

F


LAD2
LAD2 + IgE
Plate bound IgE
% Jurkat cells with Up-regulated CD69
80
60
40
20
0
WT
M1
M2
M4
M6

G

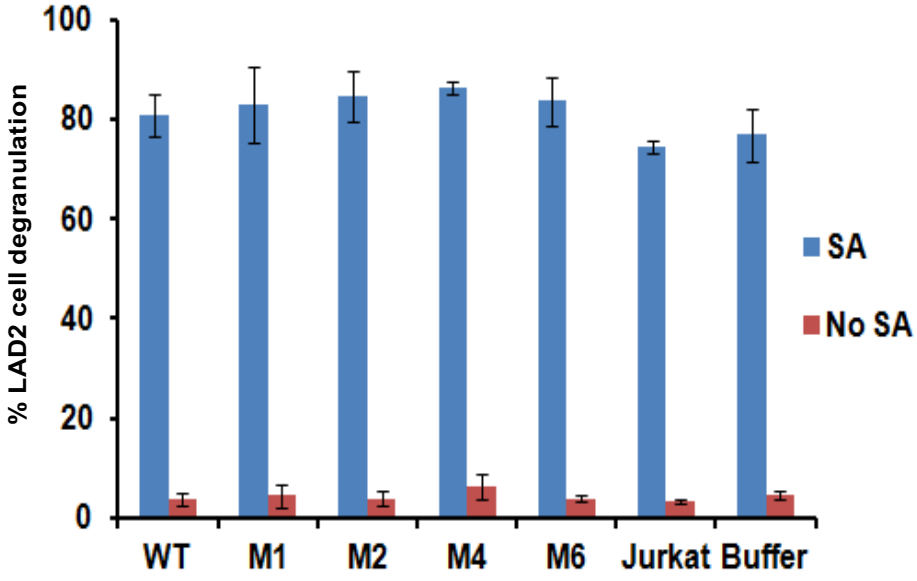

100
80
60
40
20
0
% LAD2 cell degranulation
WT
M1
M2
M4
M6
Jurkat
Buffer
SA
No SA

Figure 6: First-generation FcεRIα CARs mediate specific and potent Jurkat T cell responses to target cells in the presence of secreted IgE. (A) Jurkat cells transduced with lentiviral vectors for CARs based on WT and low affinity mutant FcεRIα were selected in medium containing puromycin. Cells were stained with anti-FcεRIα-PE antibody and analyzed by flow cytometry. Mock transduced Jurkat cells were used as a negative control. **(B)** Jurkat cells expressing FcεRIα based CARs bind IgE. Cells were stained with soluble IgE followed by fluorescently labeled anti-IgE antibody before flow cytometry analysis. Note that cells expressing M3 and M5 showed minimal levels of binding. **(C)** U266 cells express low levels of mIgE. Cells were stained with fluorescently labeled anti-FcεRIα antibody prior to flow cytometry analysis. **(D)** Ramos cells bind IgE through FcεRII. Ramos cells were upregulated with IL-4 overnight to upregulate FcεRII. IgE binding was determined as in B. **(E)** Jurkat cells expressing CARs based on WT FcεRIα and the mutants were tested for their responses to plate-bound IgE, U266 cells, U266 cells in the presence of soluble IgE (1.7μg/ml) and Ramos cells with IgE bound. Unstimulated Jurkat cells were used as a negative control (no stimulation). **(F)** FcεRI-based CARs do not mediate T cell responses to mast cells with soluble IgE captured through FcεRI. LAD2 cells were incubated with 1.7μg/ml of soluble IgE to bind with the FcεRI receptors on surface. In E & F, CAR^+ Jurkat cells were stimulated for 5hrs and the cells were stained with anti-CD69 antibody for flow cytometry. **(G)** FcεRI-based CARs do not mediate mast cell activation. LAD2 cells were coated with biotinylated IgE at 1.7μg/ml followed by incubation with CAR^+ Jurkat T cells for 30mins in the presence or absence of streptavidin. β-hexosaminidase was determined using PNAG as substrate, and percentage of degranulation was calculated. Data represent mean and standard deviation (n=3) (Images used with permission)[67].

1.7 Hypothesis and Aims

With results from preliminary studies supporting our overarching hypothesis that a CAR based on FcεRIα can specifically redirect T cell killing to IgE-expressing B cells, the next step is to test this hypothesis in more physiological conditions in order to align this study with the long-term goal of clinical application. Preliminary studies utilized Jurkat T cell line which tend to behave differently from primary human T cells and the first-generation CAR has an inherent limitation of poor *in vivo* efficacy and persistence[68]. Moreover, the first-generation CARs associate with the TCR/CD3 complex through interaction between charged residues within their transmembrane

domains **(Fig. 4A)**. This significantly increases the number of associated ITAM domains and the likelihood of T cell exhaustion and death due to enhanced spontaneous CAR signaling[37]. To address these issues, my thesis research focuses on upgrading the CAR design to the second and third-generation and test their potency and persistence. To this end, costimulatory domains will be added and zeta transmembrane domain will be replaced with that of CD8 **(Fig. 4B&C)**.

Specifically, we hypothesize that (1) higher generation CARs can mediate potent target cell killing by primary human CD8+ T cells despite the lower number of ITAM domains; and (2) higher generation CARs with co-stimulatory domains can be persistently expressed on primary human CD8+ T cells. To test these hypotheses, I will: **Aim 1:** Construct higher generation CARs with co-stimulatory domains. **Aim 2:** Express the CARs on primary human CD8 T cells. **Aim 3:** Determine the ability of the CARs to recognize and mediate the killing of mIgE+ target cells. **Aim 4:** Determine the duration of expression of the CARs on primary human CD8+ T cells. Together, these aims will address critical issues of the CAR design and accelerate its application in adoptive T cell therapy for severe allergic diseases.

Chapter 2

METHODS

2.1 Cell Lines

The multiple myeloma B cell line, U266, which naturally expresses the transmembrane form of IgE and the acute T cell leukemia line, Jurkat, that can be easily transduced with lentiviral vectors were obtained from the American Type Culture Collection (ATCC) and cultured in RPMI 1640 base medium (Corning®) complete with 10% FBS (Fetal Bovine Serum), 2mM L-glutamine, 100U/ml penicillin, 100ug/ml streptomycin and 2mmol/L Gentamycin. For *in vitro* specific cell lysis assay, the U266 cells were lentivirally transduced to stably express the firefly luciferase.

The Lenti-X 293T lentiviral packaging cell line was obtained from Clontech and cultured in Dulbecco Modified Eagle Medium (DMEM) (Corning®) supplemented with 10% FBS, 2mM L-glutamine, 100U/ml penicillin and 100ug/ml streptomycin.

All of the above media additives were purchased from Corning®. All cell lines were maintained at 37°C in a humidified atmosphere with 5% CO_2.

2.2 Construction of Plasmids

To construct the 3rd generation CARs, DNA sequences encoding the extracellular domains of WT FcεRIα or mutants (M1, M2, M4 & M6); CD8 hinge and CD8 transmembrane domain; CD28 and 41BB costimulatory domain; CD3ζ intracellular signaling domain were cloned into a pLVX lentiviral vector with an EF1α promoter (Clontech) or a z368 lentiviral expression vector with a CMV promoter via Gibson

Assembly® (New England Biolabs®). Second-generation CARs were made by deleting the CD28 costimulatory domain of the third-generation CARs using the Q5® Site Directed Mutagenesis (New England Biolabs®).

2.3 Lenti-vector Packaging

To package lentivectors, Lenti-X 293T (Clontech) cells were seeded on poly-L-lysine (Sigma-Aldrich) coated 10-cm plates (Corning) at 8.0×10^6 cells per plate in pre-warmed lentiviral packaging medium (Opti-MEM® Reduced-Serum medium, Glutamax™ Supplement (Gibco) supplemented with 5% FBS and 1 mM sodium pyruvate) and allowed to adhere for 16hrs. Transfections were performed using Lipofectamine™ 3000 reagent as per manufacturer's protocol. Briefly, 5.25µg of transfer vector plasmid, 5.25µg of viral enzymatic proteins, δ8.91, and 3.5µg of viral envelope proteins, VSV-G, were combined with 35µl P3000 reagent and 41µl of Lipofectamine™ 3000 reagent in Opti-MEM Reduced-Serum medium (Gibco) and used as transfection medium. At 6hrs post-transfection, the cells were washed with pre-warmed lentiviral packaging medium and fresh, pre-warmed lentiviral packaging medium was added and the cells were incubated for an additional 18hrs. Lenti-vector viral supernatant were collected at 24 and 52hrs post-transfection. The supernatants were combined and centrifuged at 2000 rpm for 10mins at room temperature to remove cellular debris. The cleared supernatant was then filtered through a 0.45µm bottle top filter (Autofill, USA Scientific) and concentrated by ultrafiltration using Centricon Plus-70 centrifugal filters (EMD Millipore) at 1500rpm for 2hrs at 15°C. Aliquots of the concentrated lentivirus were made and stored at -70°C prior to transduction.

2.4 Lenti-vector Transduction of Primary Human CD8+ T cell

Cryopreserved primary human CD8+ T cells were obtained from the Immunology Core of the University of Pennsylvania and cultured in RPMI-C. T cells were thawed and stimulated the same day with Human T-activator CD3/CD28 Dynabeads® (Life Technologies) at a 1:3 cell to bead ratio. Prior to transduction at 20hr, the T cell culture was supplemented with 300U/ml recombinant IL-2 (NIH) after which the T cells were exposed to the lentivector. 10ug/ml protamine sulphate (Sigma-Aldrich) was added to enhance transduction efficiency. Spinoculation was performed at 2500 rpm for 90mins at 32°C and T cells were incubated overnight at 37°C in a humidified atmosphere with 5% CO_2. The culture media was diluted 24hrs after transduction to reduce protamine sulfate concentration and culture media further supplemented with 300U/ml recombinant IL-2 (NIH). At day 4 after T cell stimulation, the Dynabeads® were removed and the T cells expanded until day 6 when they are used in assays. Transduction efficiency was measured on day 5 post transduction by flow cytometry.

2.5 Surface Immunostaining and Flow Cytometry

For FACS (Fluorescence-Activated Cell Sorting) analysis, primary human CD8+ T cells were harvested and washed twice in cold FACS buffer (DPBS/0.02% sodium azide with 0.5% BSA). To detect FcεRI CAR expression on the cell surface, T cells were stained with anti-human FcεRI-PE antibody (BioLegend) on ice for 30 mins.

To determine the level of IgE binding, T cells were incubated with 10ug/ml human IgE (Abcam) on ice for 1hr, washed twice before staining with anti-human IgE APC antibody at 4°C (BioLegend). Fluorescence was assessed using a NovoCyte 3000

(ACEA Biosciences) flow cytometer and all FACS data were analyzed using the FlowJo software.

2.6 Specific Cell Lysis Assay

Background lysis by the CD8+ T cells due to MHC class I-mediated alloreaction was blocked by incubating T cells with 10ug/ml anti-human CD8 antibody (Biolegend) for 30mins at 37°C before co-culturing with target cells. The cytotoxicity of CAR-transduced T cells was determined by standard luciferase based assay. Briefly, U266 cells naturally expressing membrane IgE and stably expressing firefly luciferase served as targets. The effector (E) and target (T) cells were co-cultured at a 1:1 E/T ratio in a white walled plate for 16hrs. U266 target cells alone were plated at the same density to determine the maximal luciferase expression (relative light units; RLUmax). Production of effluc was assessed with Bright-Glo® (Promega) luciferase assay system. Bioluminescence was measured with Perkin Elmer 2030 Multi-Label reader. Specific lysis was determined as [1 - (RLU sample) / (RLUmax)] X 100. The cytotoxicity was determined in triplicates and presented as Mean $\pm$ SD.

Chapter 3

RESULTS

3.1 Design and Construction of Higher Generation FcεRIα-Based CARs

The ability of low affinity FcεRIα-based CARs to mediate potent and specific killing of IgE-expressing B cells was clearly demonstrated using Jurkat cells expressing the first-generation CARs. However, the lack of persistence of first generation CARs limits their *in vivo* function[69]. Given the known anti-apoptotic and pro-survival effect of the 41BB and CD28 costimulatory endo-domains[70], the second and third generation WT and low affinity mutants FcεRIα based CARs were constructed for this study **(Table 2)**. The CARs were cloned into lentiviral transfer vectors for packaging lenti-vectors. Lenti-vectors were used because they have the ability to infect and integrate into non-dividing primary human cells. They also confer a decreased risk of insertional oncogenesis and are less susceptible to silencing by host restriction factors[71-74].

In order to alter the level of CAR expression on primary human CD8+ T cells, two different lentiviral transfer vectors were used. In previous studies, we have found that the pLVX-EF1α vector drives a high-level CAR expression while the z368 vector drives a lower expression through the CMV promoter.

For the second-generation CAR (2gCAR) with GFP (2gCAR-GFP), the green fluorescent protein (GFP) was fused to the tail of the WT 2gCAR to enable visualization of CAR expression without impacting functionality. A conventional WT 2gCAR without the zeta intracellular domain (WT-2gCARNZ) was constructed to evaluate the effect of spontaneous signaling.

Table 2: FcεRIα-based CAR Construct Generated

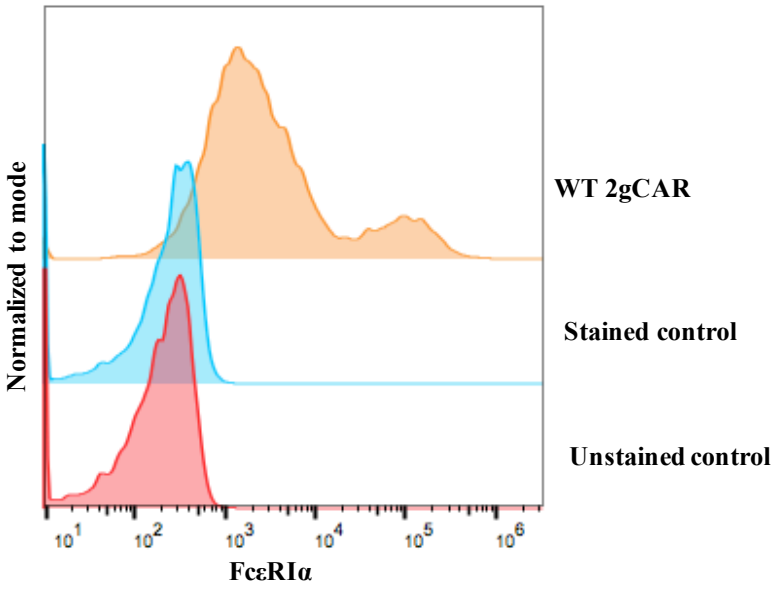
CAR Generation	pLVX Lentiviral Transfer Vector Constructs (EF1α Promoter)	z368 Lentiviral Transfer Vector Constructs (CMV Promoter)
2gCAR	WT & all Mutants, WT-GFP, WT-2gCARNZ	WT & all Mutants
3gCAR	WT & all Mutants	None

3.2 FcεRIα-based WT 2nd generation CAR Can Be Expressed on Primary Human CD8+ T Cells and Can Bind IgE

In order to test the functional expression of the higher generation CARs with costimulatory domains, primary human CD8+ T cells were stimulated with anti-CD3/CD28 beads followed by transduction with the pLVX-EF1α lentiviral vector encoding the WT 2gCAR. Mock transduced primary human CD8+ T cells were used as a control. CAR expression was detected by staining with anti-human FcεRIα-PE antibody and analyzed by flow cytometry 5 days post-transduction. The WT 2gCAR was robustly expressed on the cell surface of the CAR transduced T cells when compared to the control **(Fig. 7A)**.

To assess the binding capability of the extracellular domain of the CAR in binding the target molecule, IgE, control T cells and CAR transduced T cells were incubated with human IgE followed by staining with anti-human IgE-APC antibody. We observed that the WT 2gCAR showed a level of IgE binding that closely corresponded to the level of FcεRIα expression detected on CAR+ T cells **(Fig. 7B)**.

A


Normalized to mode
WT 2gCAR
Stained control
Unstained control
10^1 10^2 10^3 10^4 10^5 10^6
FcεRIα

B

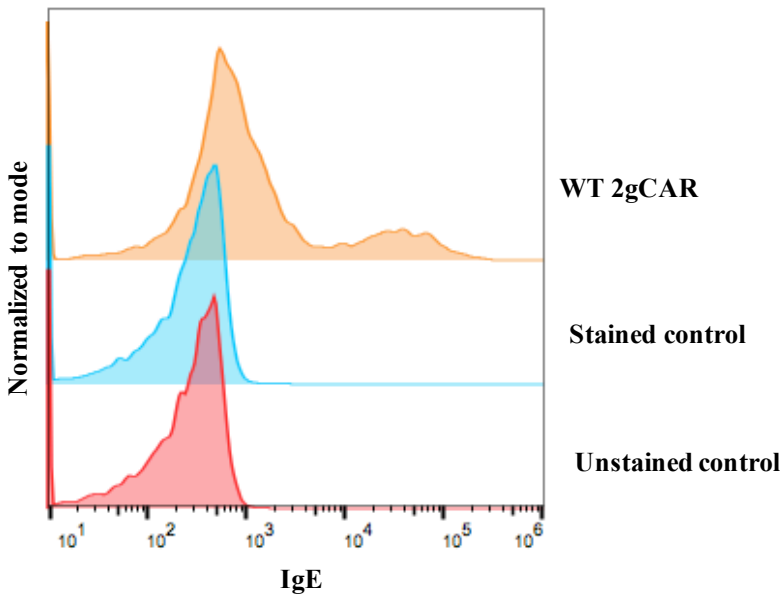

Normalized to mode
WT 2gCAR
Stained control
Unstained control
10^1 10^2 10^3 10^4 10^5 10^6
IgE

Figure 7: WT 2gCAR can be expressed on primary human CD8+ T cells. Primary human CD8+ T cells were activated and transduced with pLVX lentiviral vector encoding the WT 2gCAR and expanded ex vivo. On day 5 post transduction, both control and CAR transduced T cells were (A) stained with anti-human FcεRIα PE to detect CAR expression on the cell surface (B) incubated with human IgE followed by staining with APC conjugated anti-human IgE to assess the function of the extracellular domain of the CAR in binding the target molecule.

3.3 Primary Human CD8+ T Cells Expressing Higher Generation CARs Can Mediate Potent Responses to Target Cells Despite Lower Numbers of ITAM Domain.

With the IgE-binding function of the extracellular domain (FcεRIα component) in the CAR demonstrated, we sought to determine the ability of the CD8+ T cells expressing the WT 2gCAR to recognize and kill target cells. U266 cells, a myeloma B cell line expressing low levels of IgE was used as target cells. The U266 cells were co-cultured with the 2gCAR transduced T cells at a 1:1 effector to target cell ratio. Mock transduced T cells were used as a control. The WT 2gCAR transduced T cells were able to recognize and mediate potent killing of target cells at a statistically significant level (48.67% ± 10.72%) compared to the mock transduced T cells ($p < 0.005$). As expected, mock transduced T cells only produced background levels of lysis (13.11% ± 1.93%) **(Fig. 8)**.

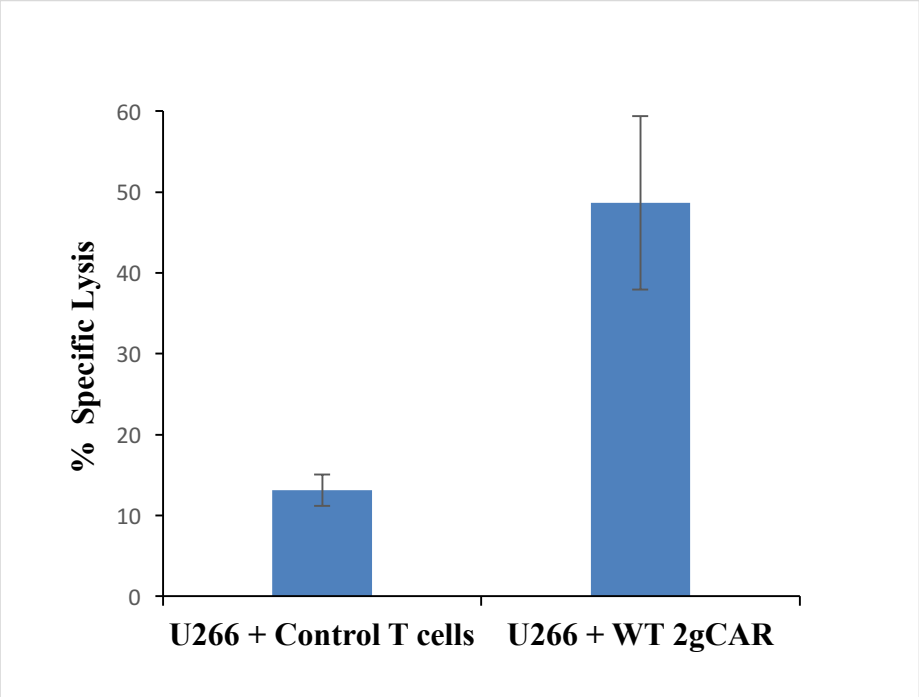


Figure 8: WT 2gCAR mediate potent and specific lysis of U266 target cell line expressing membrane IgE. Control primary human CD8+ T cells and T cells transduced with WT 2gCAR were co-cultured for 16hrs at 37°C with U266 target cell line expressing low levels of mIgE at a 1:1 effector to target cell ratio and cytotoxity was measured using a luciferase based assay. Assay were performed using T cells on day 5 post transduction. (n = 3; Mean ± SD; $p < 0.005$ using a two-tailed student's t test).

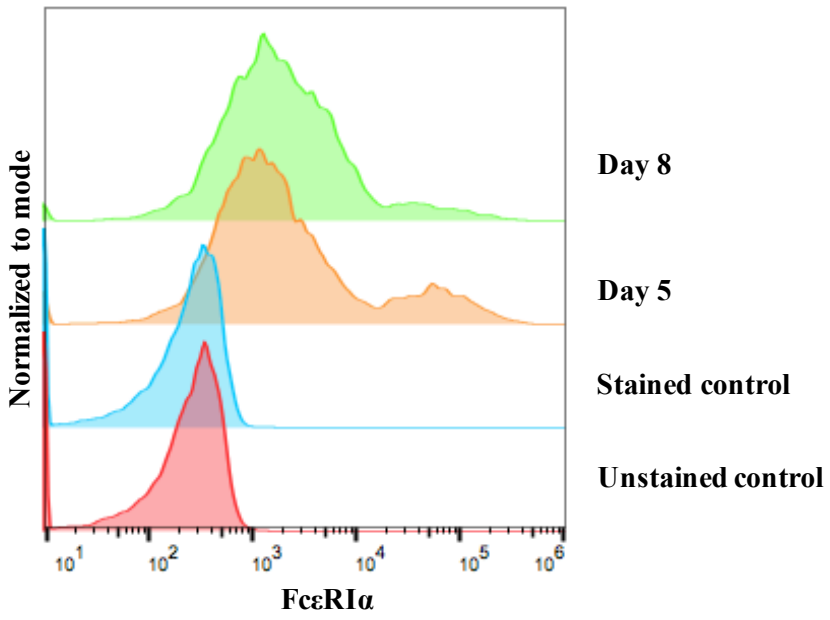
3.4 The Lack of Persistence of CAR Expression on Primary Human CD8+ T Cells is only Partially Corrected by the Incorporation of Co-Stimulatory Domains in the 2nd and 3rd Generation CARs

The long-term persistence of T cells expressing the second and third generation CARs has been attributed to the incorporation of costimulatory signaling domains[47-49,70]. To determine whether the addition of costimulatory domains in the FcεRIα-based CARs enhances persistence, we observed the duration of persistence of the WT 2gCAR expression on primary human CD8+ T cells. CAR expression level and IgE binding of primary human CD8+ T cells were measured with flow cytometry on day 5 and day 8

post transduction. Surprisingly, the level of receptor expression declined **(Fig. 9A),** which is mirrored by the level of IgE binding **(Fig. 9B)**. The 2gCAR incorporates 41BB co-stimulatory domain and upon CAR engagement, 41BB provides signals that augment T cell proliferation and survival. In addition, 41BB receptor engagement has been reported to inhibit activation-induced cell death particularly in CD8+ T cells[53]. Nevertheless, the incorporation of 41BB signaling domain did not prevent the expression decline in our FcεRIα-based CARs. We therefore reasoned that 41BB signaling domain alone may not provide sufficient survival signal to the transduced T cells and a third generation CAR with an additional costimulatory domain may have improved persistence. CD28 and 41BB has been shown to be functionally additive when combined within a single CAR[70]. Accordingly, we incorporated the CD28 domain upstream of the 41BB domain and generated third-generation CARs (3gCARs) **(Fig. 4C)**.

WT 3gCAR was expressed on activated primary CD8+ T cells and the level of expression was monitored overtime by flow cytometry. The results showed a robust CAR expression on day 5 post transduction but a decline on day 8 post transduction **(Fig. 9C)** indicating that the addition of CD28 signaling domain does not enhance persistence. The results also suggest that insufficient co-stimulatory signaling is not the reason for the lack of persistence of second-generation CARs. There have been conflicting reports on which costimulatory domain, CD28 or 41BB, is better at helping CAR T cell survival. Our results are consistent with recent reports by Long *et al.* that CD28 actually negatively impacts persistence[53].

A


Normalized to mode
Day 8
Day 5
Stained control
Unstained control
10^1
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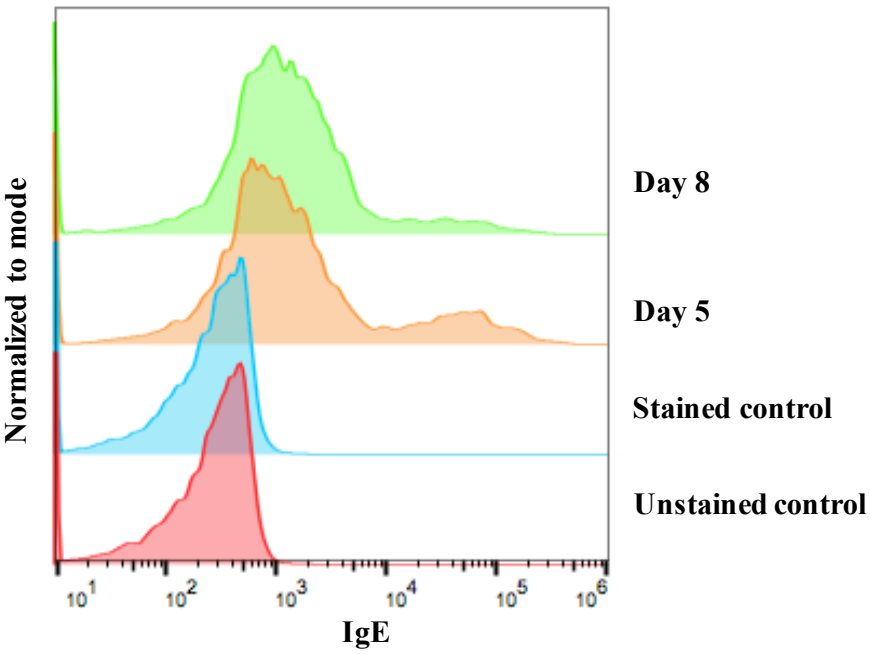
10^4
10^5
10^6
FcεRIα

B


Normalized to mode
Day 8
Day 5
Stained control
Unstained control
10^1
10^2
10^3
10^4
10^5
10^6
IgE

C

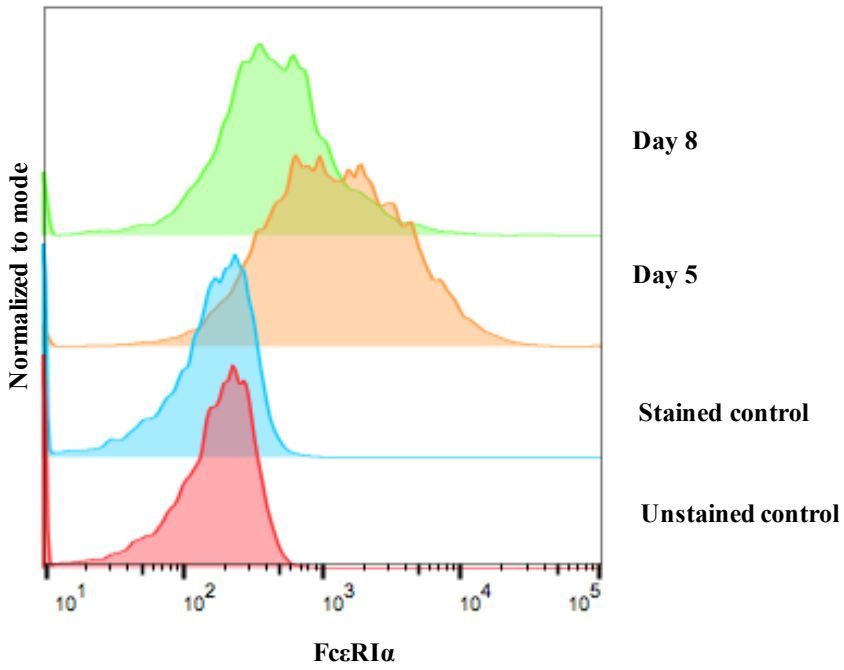


Figure 9: Inclusion of co-stimulatory domain did not improve the persistence of the FcεRIα-based CARs. To monitor CAR persistence *in vitro*, **(A)** 2gCAR transduced T cells were stained with anti-human FcεRIα PE to determine the level of CAR expression on the cell surface on day 5 & 8 post transduction. **(B)** 2gCAR transduced T cells were incubated with human IgE followed by staining with APC conjugated anti-human IgE to determine the level of IgE binding on day 5 & 8 post transduction. **(C)** The CD28 co-stimulatory endo-domain was incorporated into the CAR construct (3gCAR) and expressed on primary human CD8+ T cells. CAR transduced T cells were stained with anti-human FcεRIα PE to detect CAR expression on the cell surface on day 5 & 8 post transduction.

3.5 Lack of CAR Persistence is Ligand Independent; Enhanced Spontaneous CAR signaling is not due to CARs Binding to Secreted IgE in Culture Medium

T cells transduced to express CARs were cultured in medium with 10% FBS which may contain IgE that is capable of binding to CARs. We therefore hypothesized that the lack of persistence may be due to continuous signaling triggered by CAR

engagement by IgE in the medium. To test this hypothesis, we transduced T cells to express the M6 mutant CAR with the weakest affinity for IgE and analyzed the cells over a time period. We found that M6 CAR expression declined in a similar fashion to the WT CAR **(Fig. 10)**, suggesting that binding to soluble IgE in the medium does not contribute to the lack of CAR persistence.

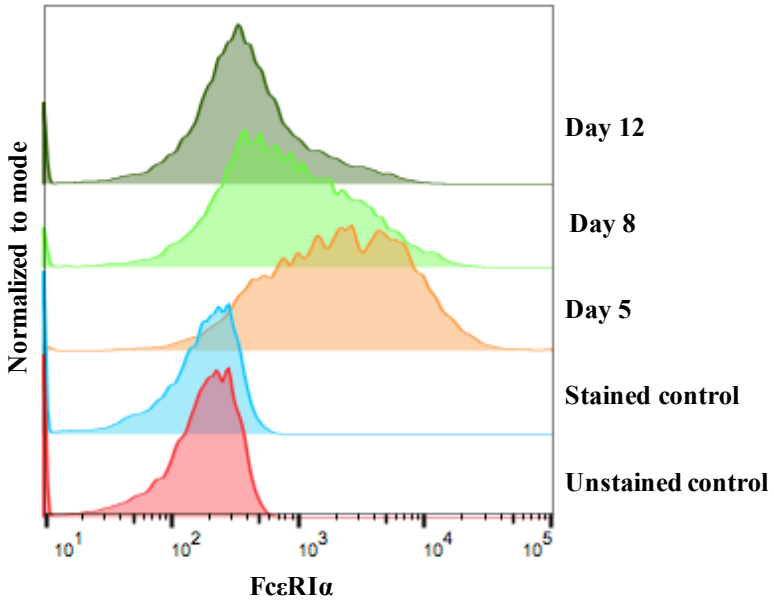


Figure 10: Lack of CAR persistence is not due to CARs binding to IgE in culture medium. M6, the CAR with the weakest affinity for IgE was expressed as a 3gCAR on primary human CD8+ T cells. M6 transduced T cells were stained with anti-human FcεRIα-PE to detect CAR expression on the cell surface on day 5 post transduction and CAR expression level was monitored over time.

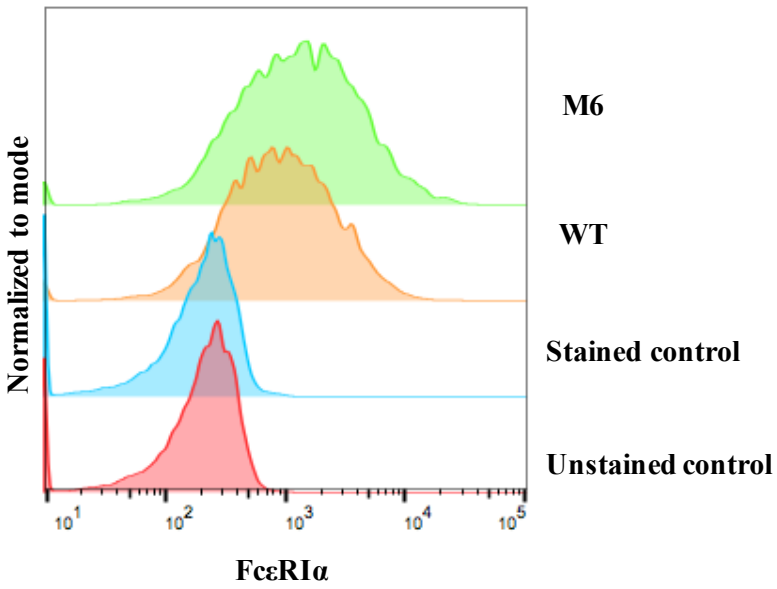
3.6 CAR Expression Decline is not due to Excessive Stimulation from CD3/CD28 Beads during Primary Human CD8+ T Cell Activation

Primary human CD8+ T cells are usually in a resting state after thawing and requires *ex vivo* stimulation with the CD3/CD28 beads (acting as artificial dendritic

cells) to get them into the G1 cell cycle phase to facilitate transduction and CAR expression. We hypothesized that the 96-hour stimulation period may exacerbate activation induced cell death (ACID) of CAR T cells and contribute to the lack of persistence.

To test this hypothesis, the duration of *ex vivo* activation of primary human CD8+ T cells was reduced by half (96 to 48hrs). Then, T cells were transduced to express the WT or the M6 3gCARs. We observed a robust expression of both CARs on day 5 post transduction **(Fig. 11A)** but subsequent analysis on day 8 **(Fig. 11B)** revealed a decline in the expression levels of both the WT and M6 3gCAR. We concluded that CAR expression decline is not resulting from excessive stimulation of T cells during the activation step.

A



B

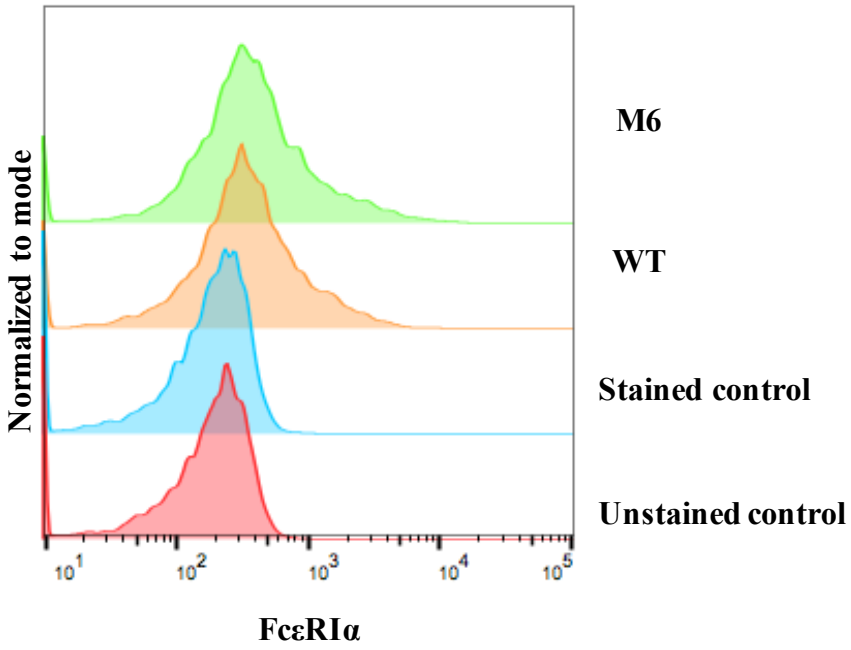


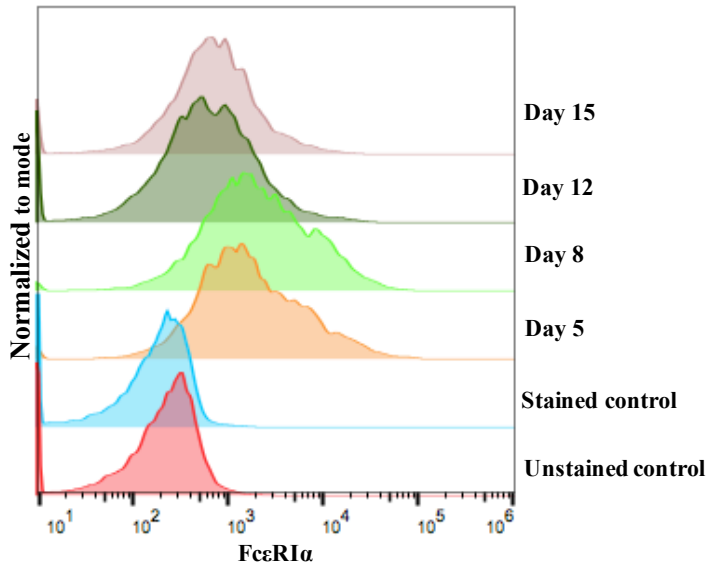
Figure 11: Lack of CAR persistence is not due to excessive stimulation from CD3/CD28 beads. Both WT and M6 3gCAR were expressed on primary human CD8+ T cells after 48hours of activation with Human T-activator CD3/CD28 Dynabeads®. CAR transduced T cells were stained with anti-human FcεRIα PE to determine CAR expression level on **(A)** day 5 and **(B)** day 8 post transduction.

3.7 High CAR Expression on the Cell Surface is Partially Responsible for the Lack of CAR Persistence.

Salmon *et al.* reported that lentiviral vectors containing an EF1α promoter induce high levels of transgene expression in primary T cells[75], and high CAR expression on the cell membrane have been reported to cause spontaneous ligand-independent clustering of CAR molecules and result in spontaneous signaling[51-53], we therefore hypothesized that reducing CAR expression level may enhance expression persistence.

To alter the level of CAR expression, we inserted our 2gCAR cassettes in a z368 lentiviral vector in which a CMV promoter drives a lower expression of the CARs. The WT 2gCAR was then expressed on primary human CD8+ T cells and its expression profile monitored over time. Our results showed a robust and sustained CAR expression profile until day 8 post transduction, thereafter, a steady decline in the CAR expression level was observed **(Fig. 12A).** We speculate that decreasing CAR expression reduced spontaneous signaling of CARs which might be occurring as a result of cluster formation of CAR molecules. We observed a similar trend in T cells transduced with the most potent CAR, M2 **(Fig. 12B)** suggesting that high EF1α CAR expression contributes to T cell exhaustion and that reduced CAR expression attenuates ligand-independent signaling. Considering z368, the lower expression vector system showed a sustained CAR expression level till day 8 post transduction, we concluded that a low basal level expression may be beneficial but not sufficient for CAR persistence.

A



B

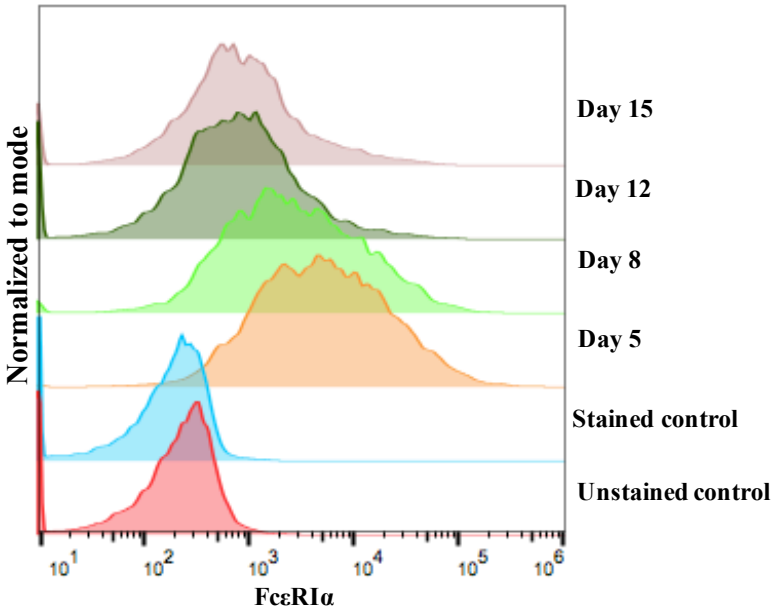


Figure 12: Lower expression level is beneficial but not sufficient for CAR persistence. Primary human CD8+ T cells were activated and transduced with z368 lentiviral vector encoding the WT and M2 2gCAR. Both groups of CAR-T cells were stained with anti-human FcεRIα PE to detect CAR expression on the cell surface. Both **(A)** WT and **(B)** M2 2gCAR are robustly expressed on primary human CD8+ T cells on day 5 with a sustained expression level on day 8. CAR expression level begins to decline after day 8.

3.8 CAR Protein is Properly Folded, Efficiently Targeted and Uniformly Distributed across the Cell Membrane

Several studies have described *in vitro* oligomerization of CAR extracellular binding domains and attributed this phenomenon to the lack of CAR expression persistence resulting from T cell exhaustion[75-77]. Oligomerization may be a result of, or facilitated by unnatural tertiary structure of CARs. Therefore, we hypothesized that FcεRIα-based CAR proteins might be improperly folded, leading to accumulations of misfolded proteins in the endoplasmic reticulum and/or CAR aggregations on the cell

surface and consequently, culminating to toxicity and cell death. To visualize CAR distribution in T cells, DNA sequence encoding the green fluorescence protein (GFP) was fused to the c-terminal of the WT 2gCAR to recreate fusion protein that retained CAR functionality. Under the microscope, GFP fluorescence was most observed on the cell surface, with small amount of intracellular accumulation. This suggests that CAR protein is likely to be correctly folded and targeted on to the plasma membrane efficiently. Moreover, GFP fluorescence is uniformly distributed across the T cell plasma membrane **(Fig. 13)**. Although the limited resolution of optical microscope does not allow us to determine the spatial relationship between CAR on the cell surface at the molecular level, this result does not support CARs forming large aggregates on the cell surface.

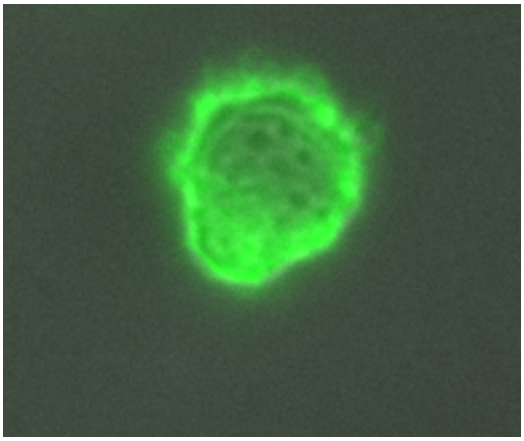
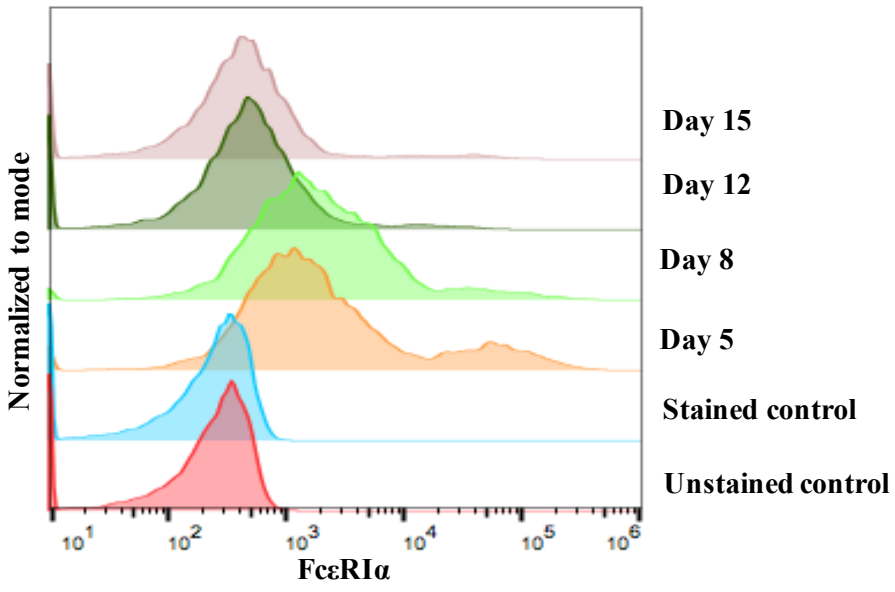


Figure 13: CAR protein is efficiently targeted and uniformly distributed across the T cell plasma membrane. Fluorescence microscopy of T cells transduced with green fluorescent protein (GFP)-tagged CAR; GFP was fused to the tail of the WT 2gCAR; WT 2gCAR is uniformly distributed on the cell membrane.

3.9 CAR Expression Decline is Partially due to Spontaneous CAR Signaling

Finally, to definitively determine whether spontaneous CAR signaling contributes to rapid CAR expression decline, we removed the CD3ζ signaling domain to construct the 2gCARNZ CAR by deleting the CD3ζ signaling domain sequences from the WT 2gCAR via site directed mutagenesis. This enabled the 2gCARNZ to serve as a non-signaling control. The CAR expression profile of the 2gCARNZ **(Fig. 14B)** was then compared with that of an intact 2gCAR over time **(Fig. 14A)**. We observed a steady expression decline in both the intact 2gCAR and the non-signaling control, 2gCARNZ. However, the expression decline was at a slower rate in the non-signaling control, 2gCARNZ **(Fig. 14B)**. Together with the observation that lower levels of CAR expression enhance persistence, the data supports our hypothesis that the lack of persistent CAR expression is at least partially due to spontaneous CAR signaling, which is exacerbated by CAR over-expression.

A



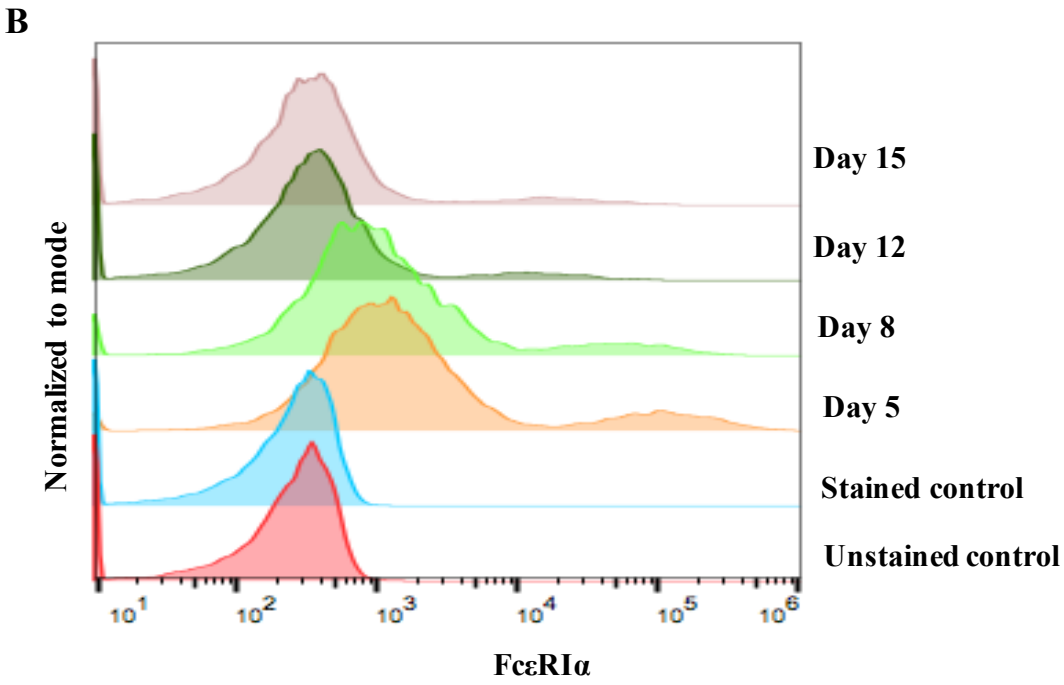


Figure 14: Elimination of CD3ζ signaling domain enhanced CAR persistence. The CD3ζ intracellular signaling domain of the pLVX-WT 2gCAR was knocked out in **(B)** and compared with an intact pLVX-WT 2gCAR in **(A)**. CAR persistence was monitored over time by surface immunostaining and flow cytometry.

Chapter 4

DISCUSSION

4.1 Justification of Adoptive Immunotherapy in the Management of Severe Allergic Asthma Patients

The rationale for the use of the ACT approach in severe allergic asthma derive from the fact that T cells are able to move throughout the body to many disease tissues[78-80]. CAR T cells can therefore be targeted to kill IgE-expressing B cells with the goal of suppressing the level of IgE in the body. Moreover, due to T cell memory phenotype, T cells have the capacity to persist in the body and generate long-lived effect on diseases thus preventing recurrence over the course of years or a lifetime[33,81].

Severe allergic asthma, mediated by IgE, affects approximately 50% of patients with allergic asthma and accounts for significant morbidity and mortality[4]. Despite the availability of several medications, severe allergic asthma remains uncontrollable[8]. Patients require large doses of corticosteroids in combination with other potentially toxic medications and still, may have poor control of symptoms and frequent life-threatening asthma attacks. This greatly impacts on patients' quality of life. Furthermore, an asthma survey revealed that 75% of respondents would prefer a non-drug treatment option for their asthma, therefore, an approach that has a long-lasting effect is highly desirable. Engrafting T cells with IgE-specific CARs in ACT has the potential to achieve long-term suppression of IgE through specific killing of IgE-expressing B cells.

4.2 History and Evolution of CAR-based Immunotherapy

The concept of CARs arose from the cloning of TCR CD3ζ chain and the observation that the CD3ζ chain could activate T cells independently of the rest of the TCR complex[82-84]. The first-generation CARs were created by fusing a scFv directly to the CD3ζ chain[85]. In preclinical cancer models, these CARs provided potent antigen-specific, effector function against tumor cells. However, in human trials, results of clinical studies were disappointing as T cells expressing the first-generation CARs showed no anti-tumor effect, limited expansion, and relatively short persistence[69,86,87]. This possibly resulted from the failure of the CAR to fully activate T cells after antigen engagement on tumor cells, especially when the tumor cells lack expression of costimulatory molecules that are required for sustained T cell activation, growth, and survival[88]. Additionally, prolonged *in vitro* expansion of T cells is often associated with downregulation of the receptors for the costimulatory ligands[88] . In order to become fully activated, a naïve T cell requires costimulatory signals induced by neighboring cells[43].

Over the years, the CAR design has evolved to include more activating signals to enhance full T cell activation, efficacy, and persistence. Costimulatory endo-domains (CD28, 4-1BB, or OX40) have been incorporated into the CAR design (second-generation CARs) to provide the co-stimulation lacking in tumor cell targets with a view to overcoming the limitations inherent in the first-generation CARs[48,49]. A combination of two costimulatory domains (third-generation CARs)[89,90] or three costimulatory domains[91] (fourth-generation CARs) has also been included in the CAR design to optimize efficacy and persistence.

Several groups testing the second-generation CAR T cells equipped with CD28 or 41BB signaling domains in patients with B cell malignancies have reported potent

antitumor effects[47-49]; ACT using CD19-specific CARs led to complete remission in 83% of patients enrolled in clinical studies of B cell leukemia and lymphoma[22,24,44-46]. The major advancement that enabled the success of these clinical studies has been attributed to the improved CAR signaling endo-domain, which greatly enhanced the potential of transferred T cells to expand and persist *in vivo*[43].

However, there are conflicting reports about which costimulatory endo-domain, CD28 or 41BB, is better at enhancing CAR T cell survival. Reports from studies at the University of Pennsylvania and the Memorial Sloan Kettering Cancer Center demonstrated a significantly enhanced persistence of CD19 CARs incorporating the 41BB costimulatory domain compared with those including the CD28 domain[22,24,33,46]. A possible explanation is that CAR T cells with CD28 signaling domains lack long-term engraftment and persistence[88]. Long *et al*. provided a biological basis for the differential *in vivo* persistence of CD19 CAR T cells with CD28 versus the 41BB costimulatory domains by demonstrating that CARs incorporating the CD28 endo-domain induced spontaneous signaling and promoted antigen-independent expansion of T cells *in vitro,* however, the expanded CAR T cells had inferior anti-tumor activity and limited persistence *in vivo*[53]. They further showed that 41BB endo-domain ameliorated the functional exhaustion associated with continuous CAR signaling[53]. However, a recent study by Diogo *et al*. has shown that continuous 41BB co-stimulation can have contrasting effects on T cells under different conditions[92]. The group identified that under conditions of high CAR expression, CARs incorporating 41BB can induce spontaneous antigen-independent signaling and can produce toxicity in T cells. Therefore, 4-1BB co-stimulation of T cells may not be generally favorable as the magnitude of CAR expression may undermine its anti-apoptotic effect[92].

The CAR design has also evolved to include immune checkpoint inhibitors to enhance the efficacy of CAR-T cell therapy in solid tumors[29]. The solid tumor microenvironment is composed of a complex desmoplastic matrix consisting of immune cells, extracellular proteins, stellate cells and immunosuppressive cytokines[29]. This microenvironment plays a role in negative regulatory signaling and reduces trafficking of modified T cells to solid tumor mass which limits CAR T-cell efficacy[29]. Furthermore, T cells have been engineered to express CARs along with an inducible cytokine gene cassette for universal cytokine-mediated killing (TRUCKs). Consequently, immune stimulatory cytokines such as IL12 are secreted upon CAR engagement[93]. In addition, an inverted cytokine receptor that can convert the inhibitory effect of a cytokine into an immunostimulatory effect has been developed to reverse the immunosuppressive tumor microenvironment and enhance the efficacy of CAR-T cell therapy in solid tumors[94]. All these and many more significant innovations in active development resulted in the landmark approval of gene therapy in US clinics[26]. The CAR T cell platform will no doubt revolutionize medicine and create a turning point in the management of patients with debilitating diseases.

4.3 Potential Issues in Adoptive Immunotherapy and Solutions

While CAR T-cell therapy has demonstrated sterling efficacy in clinical trials, a number of issues could limit its widespread clinical application. One major safety concern is the use of viral vectors for stable CAR gene transfer. These vectors have the ability to generate replication competent viruses or lead to virus integration-related insertional mutagenesis and cellular transformation. Lentiviral vectors can integrate into both dividing and non-dividing primary human cells, confer a reduced risk of insertional oncogenesis, and thus, are preferred to retroviral vectors. Lentiviral vectors are also less

susceptible to silencing by host restriction factors.[71-74,95,96]. Regarding cellular transformation, no insertional mutagenesis or oncogenic events have been reported so far in clinical trials employing T lymphocytes and hematopoietic stem cells reprogrammed with lentiviral vectors[97-101], indicating that lentiviral manipulation of mature T cells is fundamentally safe.

The rapid development of non-viral gene integration techniques through transposon/transposase systems may eliminate the risk of replication competent viruses[102]. Studies have shown that DNA transposons can efficiently deliver gene cassettes into the host genome.[103-105]. An example of this technique is the Sleeping Beauty (SB) transposon system, which has produced modified T cells of acceptable standards for clinical studies [106,107]. Furthermore, several types of reprogramed T cells (e.g., CD19 CAR-T cells[108], and Epstein–Barr virus (EBV)-specific HER2-CAR T cells[109]) have been generated by an alternative approach called the PiggyBac transposon system.

An emerging strategy in gene delivery is the electroporation of engineered T cells. Electroporation enables generation of non-integrating constructs thus eliminating the risk of insertional oncogenesis. Although the risk of insertional oncogenesis is eliminated, due to rapid degradation, electroporated mRNA is transiently expressed on modified cells compared to integrated constructs which have been observed to persist for more than a decade after transfer of modified cells[32 ,110-112]. Together with the development of electroporation techniques, the efficiency of non-integrating methods of gene modification are promising as a complement to viral vector-based methods. However, the possibility of insertional oncogenesis still remains with both viral and non-viral methods of gene integration[113].

Another major safety concern is the potential for severe toxicity of cellular therapeutics stemming from the lack of precise control over the activity of the adoptively transferred T cells once they are infused into patients[114]. Off tumor cross-reaction of therapeutic T cells can lead to the killing of non-tumor cells[115]. Even with successful on-tumor targeting, the rapid rise in the overall T cell activity heightened by CAR signaling during treatment can lead to systemic life-threatening side effects such as the cytokine release syndrome or tumor lysis syndrome resulting from rapid and excessive tumor clearance in a short period of time[114]. These conditions can trigger multiple organ failures requiring urgent medical intervention[116]. Rapid improvement in the engineering of T cells with regulatory systems that allow for control over the dose, location, and timing of T cell function has greatly reduced these risks[117-119]. T cells have been engineered with combinatorial antigen sensing capabilities that enhance the recognition of diseased target tissues with high precision and specificity[118]. This dual receptor circuit restricts the expression of CARs to the tumor microenvironment and has the potential to overcome the problem of off tumor cross-reaction that can occur with convention CARs when the target antigen is also present in bystander cells[118]. Furthermore, therapeutic T cells can be engineered with a protease-sensitive linker and a masking peptides that block the antigen binding site; proteases commonly active in the tumor microenvironment can then cleave the linker and disengage the masking peptide thereby enabling T cells to recognize target antigens only at the tumor site[119]. In addition, adoptively transferred T cells can also be engineered with molecular suicide switches that allows elimination of the infused T cells once the toxic effect begins to get out of control[120]. An example is an inducible, caspase-9-based suicide mechanism which can be incorporated into modified T cells as a fail-safe switch[121]. Alternatively, T cells

can be engineered with negative regulatory co-receptors that can override killing responses when a specific ligand is encountered on T cells[122,123]. Moreover, rapid apoptosis of adoptively transferred T cells can be triggered by administering a small molecule drug to quickly terminate any unexpected adverse effects caused by these cells. Recently, split synthetic receptors in which the antigen binding and intracellular signaling components of the CAR assemble only in the presence of an exogenous molecule has been developed[117]. This split receptor design allows for positive regulation and gradual titration of activity to appropriate therapeutic levels, enabling physicians to precisely control the timing, location and dosage of T cell activity thereby mitigating toxicity[117].

Finally, the process of *ex vivo* expansion and genetic manipulation of T cells has become highly efficient and streamlined, which should reduce the cost and further improve safety; synthetic DNA nano-carriers that can reprogram circulating T cells *in situ* are actively being developed[124]. These polymeric DNA nanoparticles are easy to manufacture in a stable form which simplifies storage and reduces cost[124]. Taken together, we believe that the safety and efficacy of the CAR-based ACT have progressed to an exciting stage where it can be exploited for treating less lethal but incapacitating and refractory diseases such as severe allergic asthma.

4.4 Extending Adoptive Immunotherapy to Severe Allergic Asthma

Although ACT has only been evaluated in critically ill cancer patients, the remarkable safety demonstrated in clinical trials and the ability of T cells to form memory cells and provide sustained functional immunity justifies its application in severe allergic asthma.

To take advantage of the rapid development and maturation of the CAR-based ACT approach for severe allergic diseases, we designed a novel FcεRIα-based CAR to recognize mIgE on IgE-expressing B cells. Unlike most CARs with scFvs, our FcεRIα-based CAR design for severe allergic asthma employs the natural IgE receptor for target recognition. CARs engineered with a natural receptor-derived binding domain have the potential advantage of increased stability and decreased clustering, thus reducing spontaneous CAR signaling and enhancing persistence. Our FcεRIα-based CAR design takes advantage of the uniqueness of the high affinity FcεRI in binding solely to IgE, allowing for specific targeting. The low affinity FcεRII (CD23) receptor binds IgE in addition to other molecules such as the MHC class II, integrin and CD21[56], making it unsuitable for specific targeting. Moreover, the recent finding of reciprocal allosteric inhibition between IgE-FcεRI and IgE-FcεRII[65] binding benefits our FcεRIα-based CAR design in preventing nonspecific targeting of cells with secreted IgE captured on the surface, a key roadblock in ACT targeting of IgE expressing cells. This approach may also be employed for other atopic diseases, such as chronic urticaria, allergic dermatitis and severe food allergy.

A potential problem with long-term suppression of IgE-expressing B cells using ACT is that increased incidents of parasitic infection or malignancy may occur as a result of reduced level of systemic IgE[11]. Although IgE is capable of mediating parasite killing, its role in controlling parasitic infection has been debated[12]. In addition, in a study of subjects at high risk of helminth infection, omalizumab was not associated with increased morbidity[16,17]. The effect of IgE in cancer immune surveillance is controversial. A long-term study showed no increase in incidents of malignancy in patients treated with omalizumab[16]. Furthermore, our ACT approach targets only IgE-

expressing B cells, which makes up a very small fraction of total B cells. IgE has the lowest abundance of the immunoglobulins isotypes; concentration of free serum IgE are ~50-200ng per ml of blood in healthy individuals compared with ~ 1-10mg per ml of blood for other immunoglobulin isotypes[125]. Therefore, IgE depletion should not significantly impact the overall humoral immunity, which is mediated mostly by IgG antibodies[126]. These findings suggest that long-term suppression of IgE using ACT is relatively safe. The concept of utilizing ACT approach in the management of severe allergic asthma has a great potential to revolutionize patients' treatment. It may for the first time, offer an effective long-term control of this debilitating disease.

4.4.1 Second-generation FcεRIα-based CARs Mediate Potent Primary Human CD8+ T Cell killing of U266 Target Cells

The ability of low-affinity FcεRIα-based CARs to mediate potent and specific killing of IgE-expressing B cells was clearly demonstrated using Jurkat cells expressing the first-generation CARs. However, the first-generation CARs have limited *in vivo* function due to the lack of CAR expression persistence.

The lack of persistence has been linked to the association of the first-generation CAR to TCR/CD3 complex through interactions between charged residues within their transmembrane domains which significantly increases the number of associated ITAM domains and the likelihood of T cell exhaustion and death resulting from enhanced spontaneous CAR signaling. The second and third-generation CARs incorporate costimulatory domains known to enhance CAR persistence[70,127] and also has the zeta transmembrane domain replaced with that of CD8, thus, reducing the number of ITAM domain and hence, spontaneous CAR signaling. Furthermore, to be in alignment with

the long-term goals of clinical application, this favorable outcome of the cell line studies needs to be demonstrated using primary human T cells.

Given the known anti-apoptotic and pro-survival effect of the 41BB costimulatory endo-domain[53], we constructed a second-generation FcεRIα-based CARs with lower ITAM domains and expressed on primary human CD8+ T cells. Our result showed that primary human CD8+ T cells expressing the second-generation FcεRIα-based CARs reliably mediated potent killing of U266 target cells despite the lower number of ITAMs. The robust response of the FcεRIα based CARs to U266 cells is particularly noteworthy, as it suggests that low IgE-expressing plasma cells may be targeted *in vivo*. The second-generation FcεRIα-based CARs, therefore have the potency required for targeting IgE-expressing B cells in ACT for severe allergic asthma.

4.4.2 Improving Persistence of FcεRIα-based CARs

The second-generation FcεRIα-based CAR incorporates 41BB costimulatory domain. 41BB domain provides signals that augment T cell proliferation and survival upon CAR engagement. In addition, 41BB receptor engagement has been reported to inhibit activation-induced cell death, particularly in CD8+ T cells[53]. Nevertheless, the incorporation of 41BB signaling domain did not prevent the expression decline in our FcεRIα-based CARs **(Fig. 9A)**.

While the inclusion of the costimulatory 41BB endo-domain in some CAR constructs has improved their clinical success[128,129], the incorporation of 41BB in the FcεRIα CAR design is likely inadequate to enhance CAR persistence on primary human T cells. Our results are in line with a recent report that CARs incorporating 41BB can induce spontaneous antigen-independent CAR signaling, thus, limiting CAR+ T cell expansion[92]. This study demonstrated that under conditions of high expression, toxicity

can arise from tonic 41BB signaling via continuous TRAF2-dependent activation of the nuclear factor κB (NF-κB) pathway and augmented FAS-mediated apoptosis in CAR T cell suggesting that by stably upregulating apoptotic target genes of NF-κB, the beneficial effect of 41BB co-stimulation in CARs expressed by T cells may be compromised[92].

A number of preclinical studies have reported higher *in vitro* activation status and superiority of third-generation CARs over the second-generation in promoting cytokine release and *in vivo* T-cell survival via the induction of PI_3Kinase/Akt activation and Bcl-X_L expression, resulting in the low level of apoptosis in transduced T cells[70,130]. Therefore, to enhance persistence of the FcεRIα-based CARs, we included the CD28 costimulatory domain in our CAR design and generated third-generation CARs. However, on expressing the third-generation FcεRIα-based CARs on primary human CD8+ T cells, the T cells showed limited clonal expansion and high level of apoptosis **(Fig. 9C)**. Our results are in contrast with these reports as the third-generation FcεRIα-based CARs also showed limited persistence. This results further suggest that the lack of sufficient co-stimulation in the second-generation FcεRIα-based CARs is not the reason for the lack of persistence. CD28 and 41BB costimulatory signaling have been shown to influence the functional characteristics of CAR-T cells differently; 41BB-based CARs have the capacity for long-term persistence whereas CD28-based CARs have been shown to direct an immediate antitumor potency in clinical trials[70]. The conflicting reports about the superiority of either the third or second-generation CARs suggest that the resulting persistent effect of combining two costimulatory domains may be dependent on the influence of the CAR on the metabolic characteristics of the CAR-

T cell and the balance of response towards either long-term persistence (long-lived memory) or immediate antitumor potency (short-lived effector cells)[70,93,130].

Some studies have identified that spontaneous signaling of some CARs may be associated with ligand engagement resulting in increased rate of apoptosis and lack of persistence[53]. The lack of persistent expression of the M6 mutant CAR with the weakest affinity for IgE **(Fig. 10)** suggest that CAR binding by soluble IgE in the medium does contribute to the lack of persistence. Furthermore, our first-generation low-affinity FcεRIα-based CARs functioned in the presence of soluble IgE **(Fig. 6E)**, suggesting that spontaneous signaling by FcεRIα-based CAR is independent of soluble IgE.

Prolonged TCR stimulation has been reported to increase differentiation and promote spontaneous signaling and exhaustion of CAR-T cells via aggravation of activation-induced cell death (ACID) and contribute to the lack of persistence[131]. ACID is triggered by prolonged TCR stimulation which can induce the transcription of pro-apoptotic genes (e.g., Fas, FasL, Bad and Bax) and decrease the expression of anti-apoptotic molecules (e.g., Bcl-2 and Bcl-xL) thereby, creating an intracellular environment that is conducive for apoptosis[132]. Our results that reduction of the stimulation period of T cells with CD3/CD28 beads did not enhance persistence **(Fig. 11)** however suggests that the lack of FcεRIα CAR-T cell persistence is not due to excessive TCR stimulation.

The phenomenon of spontaneous signaling has been reported for multiple CAR constructs, with high surface density CAR expression correlating with increased spontaneous signaling and T cell exhaustion[37,51-53]. In an effort to reduce spontaneous signaling, we modulated CAR expression by constructing our CARs in a vector with lower promoter activity. This is because high CAR expression is not always required

for T cell activation[92]. Our result demonstrates an enhanced CAR persistence **(Fig. 12)** and is consistent with previous studies where attenuating CAR expression by regulating promoter activity alleviated spontaneous CAR signaling and enhanced their antitumor function[51,92]. In addition, our results are consistent with recent reports that spontaneous signaling of CARs incorporating 41BB is vector-dependent and both the translated and non-translated region of vectors can contribute to spontaneous signaling and hence, limit CAR-T cell persistence[92].

Several studies have reported that the ecto-domains of CARs incorporating the scFvs frequently form oligomers, raising the possibility of CAR cluster formation and spontaneous CAR signaling[50,53]. Our FcεRIα-based CAR design for severe allergic asthma employs the natural IgE receptor for target recognition. CARs engineered with a natural receptor-derived binding domain have the potential advantage of increased stability and decreased clustering, thus reducing spontaneous CAR signaling and enhancing persistence. Although our CAR uses the natural IgE receptor for target antigen recognition, oligomerization may be as a result of or facilitated by unnatural tertiary structures of CARs arising from mis-folding of CAR protein. Using CAR-GFP fusion construct that retained functionality, we observed the uniform distribution of GFP on the cell surface with an only small amount of intracellular accumulations **(Fig. 13)**. This suggests that CARs are efficiently targeted onto the plasma membrane. Although the limited resolution of optical microscope does not allow us to determine the spatial relationship between CAR on the cell surface at the molecular level, this result does not support CARs forming large aggregates on the cell surface.

Finally, to definitively determine the mechanism by which spontaneous signaling occur in FcεRIα-based CARs, we removed the CD3ζ signaling sequence to

construct a non-signaling CAR. Our results from CD3ζ signaling knock out suggest that CD3ζ signaling partially contributes to the spontaneous signaling activities of CARs. The decline in CAR expression was at a reduced rate in the non-signaling CAR-T cells **(Fig. 14B)**. Though elimination of CD3ζ signaling enhanced persistence, the CAR-T cells still continue to show signs of exhaustion and apoptosis. Together with the observation that lower levels of CAR expression enhanced persistence, the results support our hypothesis that the lack of persistence of CAR expression is at least partially due to spontaneous CAR signaling.

4.5 Conclusion

Adoptive immunotherapy is a rapidly evolving field in the treatment of cancer. Due to ongoing rigorous research and good safety profile demonstrated in clinical trials, this technology may soon be exploited for the treatment of less lethal diseases such as severe allergic asthma.

We have designed and constructed the first generation FcεRIα-based CARs for the management of severe allergic asthma and have upgraded them to second and third-generation CARs. We have shown that despite the lower number of ITAM domains, the second-generation CARs can mediate potent killing of target cells expressing mIgE. These results demonstrate the effectiveness of the FcεRIα-based CARs in targeting IgE-expressing B cells in severe allergic asthma.

However, the lack of CAR expression persistence still remains a challenge of the FcεRIα-based CARs. The incorporation of 41BB costimulatory domain in the second-generation CAR did not enhance persistence. Through extensive investigations and in-depth analysis, we determined that the lack of persistence is at least partially due to spontaneous CAR signaling exacerbated by high CAR expression. We also conclude

that spontaneous CAR signaling is not due to lack of sufficient co-stimulation, excessive stimulation of T cells, IgE binding in culture or accumulation of misfolded CAR protein. Our work has advanced the understanding of factors critical for CAR T cell survival. We believe future studies building on the results of current research will accelerate the successful development of adoptive T cell therapy for severe allergic diseases using FcεRIα-based CARs.

Chapter 5

FUTURE DIRECTION

We have identified that the lack of persistence is partially due to spontaneous CAR signaling exacerbated by high CAR expression. To further identify the mechanism by which spontaneous signaling occurs in FcεRIα-based CARs, we will:

1. Mutate one or both cysteine amino acid residues in the CD8 hinge region to serine residues. The cysteine residues in the CD8 hinge region has the potential to promote CAR aggregation and spontaneous signaling by forming disulfide bonds with the ones in the neighboring CARs.
2. Target the FcεRIα-based CARs to the T-cell receptor α constant (TRAC) locus. Eyquem *et al*. recently demonstrated that targeting a CAR to the TRAC locus averted spontaneous CAR signaling by reducing expression, therefore, delaying effector T-cell differentiation and exhaustion[51].
3. Mutate TRAF binding site on the 41BB endo-domain of the FcεRIα-based CARs. Recently, Diogo *et al*. demonstrated that disruption of TRAF binding site on the 41BB endo-domain eliminated pro-apoptotic signaling arising from 41BB[92].
4. Assess spontaneous intracellular signaling downstream of FcεRIα-CAR / TCR-CD3ζ. The expression of genes associated with T cell exhaustion and apoptosis will be quantified to isolate pathways that may be manipulated for enhanced T cell survival and CAR persistence.

5. Reduce the potential negative impacts of artificial components in the CAR i.e., the CD8 hinge by fusing FcεRIα extracellular domain directly with the FcεRIβ chain. The FcεRIβ chain naturally exists in close proximity with the FcεRIα chain. Therefore, fusing the FcεRIα and the FcεRIβ may facilitate natural folding of CARs, reduce CAR aggregation and spontaneous signaling and thus, improve CAR persistence.

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