A Comparison of Co-stimulatory HVEM Domains in Second Generation CAR-T Cells

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Accessibility
A Comparison of Co-stimulatory HVEM Domains in Second Generation CAR-T Cells

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A Thesis in the Field of Biology
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Abstract

Chimeric antigen receptor T cells (CAR-T) are T cells that have been genetically modified to express a receptor that recognizes a specific target molecule. When T cells bind to their target molecule, usually a surface antigen expressed on a tumor cell, they exhibit effector functions similar to T cells, including proliferation, cytokine secretion, and target cell killing. First-generation CAR-T cells contained only a synthetic receptor coupled to the CD3ζ signaling domain of the T cell receptor complex. Although first-generation CAR-T cells functioned well in vitro, they were generally unable to kill tumor cells effectively in mouse models. Second-generation CAR-T cells, which contain an intracellular co-stimulatory domain in tandem with the CD3ζ signaling domain, significantly improved the function of CAR-T cells and are now the basis for several approved therapies targeting lymphomas and leukemias. The choice of a costimulatory molecule (co-stim domain) is critical in ensuring CAR-T persistence and clinical response. As relapse rates in CAR-T recipients are still considerable, identifying new potential co-stim domains may be essential to improve clinical performance. The costimulatory domain of Herpes Virus Entry Mediator (HVEM) is a promising costimulatory domain in CAR-T cell models and might strike a “Goldilocks balance” between performance characteristics of the only co-stim domains in approved CAR-T therapies: CD-28 and 4-IBB. Mutated forms of the HVEM costimulatory domain were created to identify which components are critical to its function by generating HVEM variants with mutations in three key regions of the HVEM cytoplasmic domain (referred
to as M83): deletion of a membrane-proximal \( \alpha \) -helix, deletion of a small tail domain, and a point mutation in a potentially critical TRAF signaling domain.

Upon coculture of anti-CD19 CAR-T cells containing modified M83 domains with target cell lines, altered CAR-T activation responses emerged as a function of specific co-stim domain modifications. The loss of the membrane-proximal \( \alpha \)-helix severely ablated CAR activation. Mutating the M83 TRAF binding domain reduced the expression of the activation marker relative to wild-type M83 and CAR-T cells without a co-stim domain. Deleting the C-terminal tail domain produced a minimum impact on activation. However, interesting trends in the general responses of CAR-T culture to target cells can potentially indicate a change in the dynamics of TRAF signaling, suggesting increased non-canonical NF-\( \kappa \)B (ncNF\( \kappa \)B) signaling, which could encourage longer persistence of CAR-T cells. Although more work is needed to confirm these observations, this work strongly suggests that using a truncated M83 as the CAR-T co-stim domain may improve the persistence and efficacy of CAR-T cells.
Author's Biographical Sketch

After thirty years in pharmaceutical development, Derek Hennecke returned to school to participate in the exciting field of cell therapy. He has lived and worked in Germany, Canada, The Netherlands, Mexico, Egypt, and The United States.
Dedication

I dedicate this thesis to my wife, Marcea, who has been a constant support and encouragement as I return to school. We have created a full life together.

My appreciation starts with my friend Shailesh Maingi, whose vision of using HVEM in CAR-T allows me to complete this thesis during a global pandemic. I want to thank Dr. Warren Anderson for his regular and continuous patient hand in the lab over the last year. I do not know how he was so confident that I would get relevant results. I am grateful for Dr. Gail Bishop’s early reading and correspondence to catch some glaring errors in my understanding of TRAFs. Lastly, I would like to thank my supervisor, Dr. Mike Nicholson, for enthusiastically taking me into his lab and guiding me toward questions about HVEM and CAR-T cell co-stimulation.
# Table of Contents

Author's Biographical Sketch ........................................................................................................... v

Dedication ........................................................................................................................................ vi

List of Tables .................................................................................................................................... ix

List of Figures ................................................................................................................................... x

Chapter 1 Introduction .......................................................................................................................... 1

  Background of the Problem .............................................................................................................. 1

  TNFRs as Co-Stimulatory Domains ............................................................................................... 5

  HVEM .............................................................................................................................................. 5

  Co-stimulating and Co-inhibitory Effects of HVEM ................................................................. 6

  HVEM Expression ........................................................................................................................... 6

  HVEM Structure ............................................................................................................................. 8

TRAFs .................................................................................................................................................. 12

  Role of TRAFs ............................................................................................................................... 12

  TRAF Structure with TNFRs ......................................................................................................... 19

  TRAF/VEET Amino Acid Binding Sequence .............................................................................. 25

  The Length of a TRAF-Binding Site ............................................................................................ 32

  HVEM Amino Acids After the VEET Binding Site ................................................................. 32

  4-1BB Does Not Activate TRAF5 While HVEM Does .......................................................... 39

Chapter 2 Methods ............................................................................................................................. 41

  Plasmid Construction .................................................................................................................... 41
Primary Human T Cell Isolation and Culture .................................................................44
Lentiviral Vector Construction and Virus Preparation ...............................................45
T Cell Transduction ........................................................................................................45
Processing Samples for Flow Cytometry Analysis ....................................................46
Flow Cytometry ...............................................................................................................46
ELISA ..............................................................................................................................47
Data Processing and Visualization ................................................................................47

Chapter 3 Results ...........................................................................................................48
Loss of M83 Helix and TRAF Binding Domain Impacts CAR Expression ..............48
TRAF Binding Domain and Helix May Impact Activation ..........................................50
Change In CD4+ T Cells With M83NT .......................................................................57

Chapter 4 Discussion ....................................................................................................62
Loss of M83 Helix and TRF Binding Domain Impact CAR-T Expression ..............62
TRAF Binding Domain and Helix Are Needed For Activation .................................63
IL2 ..................................................................................................................................63
CCR7 and CD27 ............................................................................................................65
CD4+ T Cells Depend on TRAFs ..................................................................................66
CD4+ CAR-T Cells Increase and Slow Activation in Mutants ..................................69
Summary .......................................................................................................................72
Future Directions .........................................................................................................73

References .....................................................................................................................76

Appendix .........................................................................................................................94
Definition of Terms .......................................................................................................94
List of Tables

Table 1 TNFRs Interact Directly with Members of the TRAF Protein Family.................19
Table 2 Structures of the TRAF Binding Motif of the TRAF Receptor Complexes.........28
Table 3 Hydrogen Donor and Acceptor Atoms on Amino Acid Side Chains..................36
List of Figures

Figure 1 HVEM and 4-1BB................................................................. 8
Figure 2 Sequence Conservation of the 60 Amino Acids in M83....................... 10
Figure 3 TRAF Similarities. .................................................................. 13
Figure 4 Clinical Effect of TRAFs............................................................. 16
Figure 5 TRAF NF-κB Signaling Pathways............................................... 17
Figure 6 Model for the Activated TRAF–Receptor Complex........................... 20
Figure 7 Three-Dimensional Structure of the TRAF2–HVEM Complex........ 21
Figure 8 TRAF-trimer Configurations........................................................ 22
Figure 9 Seven TRAF Domain Organizations............................................ 24
Figure 10 Sequence Comparison of TRAF-Binding Regions......................... 25
Figure 11 Model of VEET.................................................................. 27
Figure 12 Recognition of HVEM / TRAF2................................................ 27
Figure 13 Surface Features of the TRAF2-HVEM Electrostatic Potential........ 30
Figure 14 The TRAF3 Binding Regions of LTβR, CD40 and HVEM............. 34
Figure 15 Ramachandran Plots of CD40 and HVEM.................................. 35
Figure 16 Relationship of T272 with Hydrogen Acceptors H283 and N282....... 36
Figure 17 HVEM Complexed with TRAF3................................................ 37
Figure 18 Proline Kink in HVEM............................................................. 39
Figure 19 Example Plasmid Construction............................................... 42
Figure 20 Transduction Efficiencies Achieved by Lentiviral Transduction....... 49
<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>21</td>
<td>CD19 Respond to Raji Co-Culture</td>
<td>50</td>
</tr>
<tr>
<td>22</td>
<td>Impact of Construct on Activation Markers</td>
<td>52</td>
</tr>
<tr>
<td>23</td>
<td>Construct has a Connection with CD69, CD25</td>
<td>53</td>
</tr>
<tr>
<td>24</td>
<td>Impact of Construct on Cytokines</td>
<td>54</td>
</tr>
<tr>
<td>25</td>
<td>Construct Connection with IL2 and IFNγ</td>
<td>55</td>
</tr>
<tr>
<td>26</td>
<td>IL2 Feedback on CD25 Expression</td>
<td>56</td>
</tr>
<tr>
<td>27</td>
<td>Impact of Construct on Differentiation Markers</td>
<td>57</td>
</tr>
<tr>
<td>28</td>
<td>CD4+ and CD8+ Markers in Constructs</td>
<td>58</td>
</tr>
<tr>
<td>29</td>
<td>Activation Markers on CD4+ and CD8+ T cells</td>
<td>60</td>
</tr>
<tr>
<td>30</td>
<td>Effect of Tail Loss on M83</td>
<td>61</td>
</tr>
<tr>
<td>31</td>
<td>Mechanism of No Tail Construct (M83NT) on NF-κB Pathways</td>
<td>73</td>
</tr>
</tbody>
</table>
Chapter 1

Introduction

Background of the Problem

In 2019 there were 23.6 million new cases worldwide and 10 million deaths from cancer (Collaboration, 2022). Although chemotherapy and small-molecule drugs can be effective for some tumor types, there is a clear trend for new treatment modalities.

Chimeric Antigen Receptor T cells (CAR-T) are an exciting new cell therapy for treating cancer. The immunologist Zelig Eshhar created the first CAR-T cells in the 1980s using a CAR that combined an antibody component with CD3ζ, a part of the natural T cell receptor (TCR) complex (Gross et al., 1989). Although very scientifically advanced, these 'first-generation CAR-T cells' were clinically unsuccessful and did not persist in patients. Michel Sadelain demonstrated that including a co-stimulatory molecule such as CD28 or 4-1BB in the CAR molecule resulted in CAR-T cells (referred to as “second generation CAR-T cells”) with superior function compared to first generation CAR-T cells (Krause et al., 1998). Unlike TCR, which requires interaction with a peptide/MHC complex for synapse formation and cytolytic activity, CARs work in an MHC-independent way. A major mechanism tumor cells use to escape native T-cells is the downregulation of MHC or related components. Thus, the MHC-independent nature of CAR-T cells allows them to bypass the mechanism of immune cell evasion.

Continuing on the success of second-generation CAR-T cells in vitro, groundbreaking research in 2003 demonstrated that human CD19-directed CAR-T cells
eradicated leukemia cells in mice (Hollyman et al., 2009). Years of research focusing on manufacturing and CAR optimization resulted in Science magazine naming CAR-T cells to treat B-ALL in humans as "Breakthrough of the year" in 2013 (Brentjens et al., 2013). The FDA approved two CAR-T therapies in 2017 for the treatment of the relapsed, refractory precursor B-ALL and diffuse large B cell lymphoma: Yescarta (axicabtagene ciloleucel) and Kymriah (tisagenlecleucel) (Boyiadzis et al., 2018; Brudno & Kochenderfer, 2018; Hopken & Rehm, 2019; Sadelain, 2017). CAR-T cells continue to lead the cell therapy pipeline, with a year-over-year increase of 24% and more than 1400 therapies in the pipeline (Saez-Ibanez et al., 2022).

CAR-T cell function is influenced by ligand-receptor interactions that are co-stimulatory or co-inhibitory, promoting or inhibiting CAR-T cell responses (Jacoby & Fry, 2020). Co-signaling molecules such as the tumor necrosis factor (TNF) receptor family (TNFR) and members of the immunoglobulin (Ig) superfamilies are important in determining T cell activity during an immune response (Locksley et al., 2001). CD28, CD27, 4-1BB, and OX-40 are some essential co-stimulatory molecules that increase the survival, growth and long-term maintenance of antigen-specific T lymphocytes (Bansal-Pakala et al., 2004). However, co-inhibitory molecules such as CTLA-4 and PD-1 slow T cell growth and effector responses (Wherry & Kurachi, 2015).

Autologous CAR-T cell therapy uses CAR-T cells taken from blood from a patient, reprogrammed to express a CAR molecule by genetic engineering, and reinfused into the patient. Treatment with CAR-T cells has shown encouraging results in individuals with B-cell malignancies and, as of August 2022, the FDA had approved six autologous CAR-T therapies to treat lymphomas, some forms of leukemia, and, most
recently, multiple myeloma (Frey et al., 2020; Fujiwara et al., 2022; Melenhorst et al., 2022). The significant properties of CAR-T cells that shape the success of therapy are expansion, durability, and tumor cytotoxicity (Chaudhury et al., 2020). CAR molecules generally include an external ligand-binding domain, a transmembrane domain, and intracellular signaling domains. The extracellular ligand-binding domain provides target specificity, while intracellular signaling regions control the activities of CAR-T cells. An added co-stimulatory signaling domain supports CAR-T cell activation. Thus, CARs enable the expressing CAR-T cells to destroy specific tumor cells effectively and hopefully persist long-term to offer continued immune surveillance.

The TCR alone is insufficient to stimulate CAR-T cells to develop into effector and memory T cells (Chen & Flies, 2013; Shahinian et al., 1993). CAR-T cell activation requires a simultaneous co-stimulatory signal, which CD28 provides (van der Stegen et al., 2015). CD28 is part of the immunoglobulin superfamily of co-stimulatory and inhibitory receptors and is expressed in ninety-five percent of human CD4+ T cells and fifty percent of CD8+ T cells (Yamada et al., 1985). CD28's primary role is to enhance TCR signaling to stimulate cytokine production, clonal proliferation, differentiation, and survival (Viola & Lanzavecchia, 1996). CARs also need a second signal, for example, from CD-28 or 4-1BB, to fully activate the T cells. One drawback of a CAR that only activates CAR-T cells via chimeric CD3ζ is that activation of the signaling motif does not fully activate the genetically altered T cells, impairing antigen-dependent IL-2 generation, cell proliferation, and survival (Schwartz, 1992). Although CARs based on 4-1BB have the potential for long-term persistence, CD28-based CARs have an immediate
antitumor effect. CD-28 induces rapid stimulation of CAR-T cells, while 4-1BB acts slower, which appears to help prevent CAR-T cell exhaustion (Huang et al., 2020).

On the other hand, 4-1BB is expressed only in activated CAR-T cells and, when triggered by its ligand (4-1BBL), leads to additional stimulation of the CAR-T cell (Vinay & Kwon, 1998). Preclinical research shows that 4-1BB-containing CARs are less prone than CD28 to CAR-T cell depletion and lead to superior persistence of CAR-T cells in animal models (Sica & Chen, 1999). Third-generation CARs, which incorporate two co-stimulatory domains, add another degree of complexity to the equation and, in some studies, have been demonstrated to be superior to second-generation CD28-based CARs (Chaudhury et al., 2020). Third-generation CAR-T cells proliferated better in patients with MRD+ or a small burden of B cell malignancies at the time of infusion (da Silva et al., 2016; Enblad et al., 2018; Tang et al., 2016).

Utilizing 4-1BB in addition to CD28 can reduce CAR-T cell apoptosis, improve proliferation, and increase the NF-κB pathway (Dai et al., 2020). CAR-T cells combining the signaling domains 4-1BB and CD28 have higher activation and proliferation capacity than CD28 alone (Tang et al., 2016). However, the incidence of cytokine release syndrome (CRS, a potentially deadly side effect of CAR T treatment) varies between trials, with no apparent correlations between whether a CD28 or 4-1BB-containing CAR was administered (Cappell & Kochenderfer, 2021). Preclinical experiments with other co-stimulatory combinations boost CAR-T cells' tumor-killing activity and persistence better than just the combination of CD28 and 4-1BB co-stimulation domains (Guedan et al., 2018) but may result in more significant tonic signaling, contributing to more severe side effects and faster CAR-T cell exhaustion (Weinkove et al., 2019). Also, the rate of
CRS sometimes rises, possibly due to the repetitive signal supplied by two co-stimulation domains (Huang et al., 2020).

**TNFRs as Co-Stimulatory Domains**

Co-stimulatory receptors can be members of the Ig superfamily, such as CD28, or the TNFR superfamily, which includes 4-1BB, CD27, CD30, DR3, GITR, HVEM, OX40, and TNFR2 (Ha et al., 2009). Members of the TNFR superfamily can elicit a wide range of cellular responses after being activated by TNF and cytokine ligands, including proliferation, differentiation, and death. By activating downstream protein kinase cascades and eventually transcription factors in the NF-κB and AP-1 families, most TNFRs enhance cell survival by recruiting the TNFR-associated factor (TRAF) family of intracellular adapter molecules. These adapter molecules activate various genes in response to acute, immunological, and inflammatory phases and tumor development. Unlike CD28, which binds PI3K p85, molecules in the TNFR family do not directly bind protein kinases but transduce signals through TRAF (So et al., 2015).

**HVEM**

HVEM is a member of the TNFR superfamily that was first discovered as the main target receptor for the Herpes simplex virus to enter host cells (Sedy et al., 2005) and was found to be the same as previously discovered ATAR (Another TRAF-Associated Receptor) (Hsu et al., 1997).
Co-stimulating and Co-inhibitory Effects of HVEM

HVEM is an unusual TNFR as it has both stimulatory and inhibitory effects and at least five different ligands (Cai & Freeman, 2009; Del Rio et al., 2010; Granger & Rickert, 2003; Pasero & Olive, 2013; Sorobetea & Brodsky, 2018). The HVEM network comprises four separate known molecular interactions, making it a highly complicated pathway (Pasero et al., 2012). The stimulatory and inhibitory effects of HVEM depend on its ligands. For example, in numerous inflammatory disease models, the interaction of HVEM with the LIGHT ligand of the TNF family and lymphotoxin alpha (LTα) elicit a co-stimulatory signal, increasing T cell proliferation in vitro and forming effector and memory T cells (Del Rio et al., 2010). HVEM, on the other hand, can send an inhibitory signal to T cells when it binds to BTLA or CD160 (Pasero & Olive, 2013). The interaction between BTLA and HVEM can drive dominant inhibitory signaling over HVEM-mediated stimulatory signaling. The overexpression of BTLA/HVEM on T cells will contribute to T cell exhaustion (Ning et al., 2021; Shui et al., 2011). Tyrosine amino acids in the cytoplasmic region of BTLA are phosphorylated while binding to HVEM (Ritthipichai et al., 2017; Xu et al., 2020).

HVEM Expression

HVEM is broadly expressed in peripheral T and B cells and is present in resting T and B cells (Ning et al., 2021). HVEM expression is more potent in Treg cells than in effector cells, suggesting that Treg cells may mediate suppressor action (Del Rio et al., 2010). HVEM is constitutively expressed in naïve T cells. T cell activation down-
regulates HVEM and cells re-express HVEM later on effector and memory T cells (Del Rio et al., 2010).
HVEM Structure

Figure 1 HVEM and 4-1BB

The similarity of HVEM (left) and 4-1BB (right) showing the signal (red), extracellular (green), transmembrane (orange), and cytoplasmic (blue) domains. Alphafold (Jumper et al., 2021) calculated the three-dimensional structure and was colorized with Pymol (DeLano, 2002).

HVEM is structurally similar to 4-1BB in its signal, extracellular, transmembrane, and cytoplasmic domains (Figure 1). Both cytoplasmic domains have an α-helix, a TRAF domain, and a tail. Intracellular domains of TNF receptors can be as short as 36 residues in OX40 and as long as 188 residues in CD30. The cytoplasmic domain of HVEM, called M83 by Inceptor Bio, a company that uses it in its CAR-T platform, falls in the middle of this range at 60 residues. M83 and mouse HVEM share only 25% identity, but both interact with TRAF5 and TRAF2 (Hsu et al., 1997) (Figure 2). Adapter proteins, such as TRAFs, are important signaling intermediates downstream of TNFRs, serving as
convergent and divergent platforms for kinase activation, such as IκB kinases, leading to transcription factor activation, such as NF-κB (Zarnegar et al., 2008). T cell NF-κB activation controls tumors \textit{in vivo} and T cell priming requires activation of transcription factors AP-1, NFAT, and NF-κB downstream of TCR (Barnes et al., 2015).
Figure 2 Sequence Conservation of the 60 Amino Acids in M83

Seq2Logo (DTU Health Tech, Denmark) calculated the information content of each position with the relative frequencies at each base. A Hobohm identity threshold of 0.63 was applied. The probability-weighted Kullback-Leibler (KL) is a mixture of a regular KL and a Weighted KL (weight on prior is 200), where the height of the amino acids corresponds to their probability times their log-odds score, which is also the information contribution of the amino acid. The weighted KL logo gives an intuitive representation of amino acid enrichment and depletion in receptor-binding motifs. The negative axis reflects depleted (underrepresented) amino acids. Position of the amino acids of all 283 amino acids in the HVEM numbers shown along the x-axis. The α-helix and TRAF (VEET) domains are positioned above the sequence.

There are no discernible hydrophobic interactions in M83. M83 is similar to most other cytoplasmic TNFR domains with an α-helix, TRAF binding domain, and tail. In
human B cell lymphoma, missense mutations have been found in the cytoplasmic domain of HVEM, including three in the α-helix (G232S (COSMIC), Q242H (ICGC), E256Q (TCGA)) and two in the extended TRAF binding region (V267M (COSMIC) and T272I (COSMIC) (Forbes et al., 2017; Zhang et al., 2011).

M83 has only been studied once as a co-stimulatory domain in CAR-T cells, with M83 CAR-T cells producing more pro-inflammatory cytokines (such as IL-2, TNF-α, and IFN-γ) and anti-tumor cytotoxicity compared to control CD28 or 4-1BB CAR-T cells (Nunoya et al., 2019; Su, 2020). Furthermore, equal percentages of T-cell central memory (Tcm) and T-cell central memory (Tem) cells were also found, and CAR-T exhaustion decreased in M83 CAR-T cells (Nunoya et al., 2019). Tem, in contrast, were significantly enriched in the CD28 and Tcm subset enhanced in 4-1BB CAR-T cells compared to M83 CAR-T (Nunoya et al., 2019). Therefore, it appears that M83 CAR-T cells produce a more balanced phenotype CAR-T cells. Interestingly, M83 CAR-T cells in vitro showed increased rates of glycolysis and oxidative phosphorylation (Nunoya et al., 2019). Consequently, M83 co-stimulation appears to boost energy metabolism, a trait positively associated with decreased CAR-T cell exhaustion (Bengsch et al., 2016).

TNFRs do not have kinase activity and depend on the binding of adapter proteins to assemble signaling complexes to activate downstream pathways. However, two somewhat conserved cytoplasmic amino acids, S240 (DVVKIVSVQVRKQE) and N282 (SFTGRSPNH) in HVEM have been found in phosphorylation studies (Bian et al., 2014; Grimsrud et al., 2012; Mertins et al., 2016; Schwegge et al., 2013). S241 is inside the α-helix, and N274 is the second to last amino acid. S241 has a low-frequency level and is not crucial in HVEM binding (Figure 2).
TRAFs

Role of TRAFs

TRAFs are intracellular signaling adapters that link receptor activation events and intracellular signaling proteins. The TRAF proteins have two primary functions: an E3 ligase and scaffolding (Park, 2018; Walsh et al., 2015). The N-terminal RING finger domain of the TRAF protein is responsible for the activity of E3 ligases, while the C-terminal TRAF domain is responsible for scaffolding. TRAF domains also mediate interactions with upstream regulators and downstream effectors (Ha et al., 2009; Inoue et al., 2000; Wajant et al., 2001). Some TRAFs can bind to an activated TNFR and act as scaffold proteins, attracting additional regulatory proteins such as kinases. TRAFs can also restrict proteins in the cytoplasm, not only serving as scaffolds for membrane receptors (Wallis & Bishop, 2018; Wallis et al., 2017). TRAF2 and TRAF3 have been shown to have E3 ligase activity, while TRAF3 typically exerts this activity by recruiting TRAF2 (Yang & Sun, 2015).

Despite the similarities in the signaling pathways induced by the six distinct TRAF proteins, each TRAF appears to have unique physiological functions (Wu, 2007). TRAFs mediate interactions downstream of TNFR superfamily members, Toll-like receptors, T cell receptors, interleukin receptors, NOD-like receptors, RIGI-like receptors, IFNγ receptors, antigen receptors and TGF receptors, among others (Wu, 2007). These pathways are critical in many human disorders because they control inflammation, adaptive and innate immunity, and apoptosis. For various TRAFs, knockout experiments in mice have found effects ranging from embryonic death to
altered immunological function (Foight & Keating, 2016). All TRAFs other than TRAF4 regulate T cell function (Arkee & Bishop, 2020). Figure 3 shows that the amino acid sequences of TRAFs 1, 2, 3, and 5 are very similar.

![Figure 3 TRAF Similarities.](image)

(A) Sequence identities across the entire MATH domain. (B) Sequence identities in the core CD40 peptide binding site (Note. Adapted from Foight & Keating, 2016).

In response to TCR signaling, T-Traf1−/− mice show hyperproliferation and increased Th2 cytokine production (IL-4, IL-5, and IL-13) but poor responses in effector and memory CD8+ T cells (Arkee & Bishop, 2020). Constitutive activation of the canonical NF-κB2 pathway in TRAF1−/− T cells leads to hyperproliferation (McPherson et al., 2012). TRAF1 restricts the activation of the ncNFκB Pathway in antigen-activated CD8+ T cells. Therefore, TRAF1 plays opposite roles in prolonging T lymphocyte survival and restraining T cell proliferation until an appropriate co-stimulatory signal is received (McPherson et al., 2012; Yang & Sun, 2015).
Unlike other family members with the RING domain, TRAF3 lacks independent E3 ubiquitin ligase activity and recruits TRAF2 into heteromultimers for this purpose (Gardam et al., 2008). TRAF3 negative mice (CD4+CreTraf3^flox/flox, hereafter termed T-Traf3^+/−) show constitutive NF-κB activation through the ncNFκB pathway (Yi et al., 2015). T-Traf3^+/− mice produce more regulatory T cells (Treg), inadequate T-dependent IgG1 responses, and reduced T cell-mediated immunity to infection due to impaired TCR/CD28 signaling (Arkee & Bishop, 2020).

TRAF3 has various functions in regulating the proportions of different subsets of T cells, although it does not affect the total number of T cells (Arkee & Bishop, 2020). The absence of TRAF3 does not affect the ratio of CD4+ and CD8+ T cells, and deletion of TRAF3 from thymocytes does not affect the development and homeostasis of conventional CD4+ and CD8+ T cells (Xie et al., 2011). T-Traf3^+/− mice have fewer CD8+ Tcm cells (Xie et al., 2011). However, Tem cells and naïve T cells are not affected, indicating that TRAF3 plays a unique role in the production, proliferation and maintenance of Tcm cells (Yi et al., 2015).

Compared to normal mice, T-Traf3^+/− mice CD8+ Tcm cells undergo apoptosis sooner and do not respond well to IL-15 stimulation. T-Traf3^+/− mice have significantly fewer central memory cells than normal mice (Arkee & Bishop, 2020). However, there are no significant variations in the number of Tem or naïve T cells (Yi et al., 2015).

T-Traf3^+/− mice have double to triple Treg cell counts in all lymphoid organs compared to normal mice. Furthermore, more Treg cells that develop from the thymus in T-Traf3^+/− mice increase Treg cells rather than providing a survival or proliferation advantage (Yi, Lin, et al., 2014).
**T-Traf3−/−** mice exhibit more Treg cells in all lymphoid organs but fewer invariant natural killer T cells (iNKT) (Arkee & Bishop, 2020). iNKT cells are involved in the pathophysiology of autoimmune and inflammatory illnesses and play a role in antitumor immunity. **T-Traf3−/−** mice have ten times fewer iNKT cells in their spleen, liver, and thymus than normal mice. **T-Traf3−/−** mice had significantly lower proliferation and cytokine production, indicating that TRAF3 is vital in TCR CD28-mediated signaling (Yi et al., 2013).

The ability of TRAF3 to bind to individual TNFR members is receptor-dependent, with HVEM having the strongest interaction and CD40 having the weakest, according to co-expression studies (Hauer et al., 2005). Therefore, since TRAF3 is an inhibitor of TRAF2 5 activation by HVEM of the ncNFκB pathway, removing the last eight amino acids of HVEM could remove the involvement of TRAF3 while only slightly reducing TRAF2 binding and another modulation of the co-stimulatory function of HVEM.

TRAF2 and TRAF5 have functional and structural characteristics similar to those of TRAF3 and TRAF5 and can create mixed heterotrimers when they associate. **T-Traf5−/−** mice B lymphocytes had defective CD40 signaling, reduced proliferation, surface molecule upregulation, and in vitro immunoglobulin production (Nakano et al., 1999). TRAF5−/− T cells reduce CD27-mediated costimulatory signaling (Kraus et al., 2008). TRAFs 1, 2, and 3 affect CAR-T viability, proliferation, cytotoxicity, and cytokines by regulating NF-κB (Li et al., 2018). TRAF5 interacts with several TNFRs such as CD40 (Ishida et al., 1996), LTβR, CD30, GITR, HVEM, OX40, CD27 and RANK, but does not interact with 4-1BB (Zapata, 2003).
Targeting the activity of certain TRAFs could lead to novel techniques to restore normal immune system function (Figure 4).

Figure 4 Clinical Effect of TRAFs

Interaction of various TRAFs and their physiological function (Note. Adapted from Wu, 2007, p. 195).
TRAF2 and TRAF5 can activate and inhibit the canonical NF-κB and ncNFκB pathways. TRAF3 binds to TRAF2, and given the absence of a RING domain, TRAF1 plays both a stimulatory and a negative regulatory role. Canonical is green (right) and ncNFκB is blue (left). Ub, ubiquitination.

HVEM, through its TRAF domain, triggers both the canonical NF-κB pathway (RelA/p50, NF-κB1) (Cheung et al., 2009) and the ncNFκB pathway (RelB/p52, NF-κB2) (Hauer et al., 2005) similar to 4-1BB (Zapata et al., 2018) (Figure 5) The canonical NF-κB pathway activates NF-κB1 p50, RELA and c-REL, while the ncNFκB pathway activates only p100-sequestered NF-κB members, primarily NF-κB2 p52 and RELB. The ncNFκB pathway leads to a slower activation and is involved in B cell maturation,
osteoclast differentiation, and lymphoid organogenesis (Xiu et al., 2014; Yang et al., 2010). Specifically, HVEM can activate ncNFκB signaling to stimulate Th17 cell differentiation (Sakoda et al., 2016).

TRAF3 induces tumor cell apoptosis and acts as a negative regulator of NF-κB activation (Xie, 2013). The TRAF2/cIAP1/2 complex attracts TRAF3 and constitutively binds to the NF-κB inducing kinase (NIK) in the cytoplasm (Xie, 2013). This TRAF2/3/cIAP1/2 complex then works as an E3 ligase adaptor where cIAP1/2 induces constant ubiquitin and proteasomal degradation of NIK and prevents the activation of the ncNF-κB pathway. Upon stimulation of HVEM with its ligand, TRAF2 is recruited to the HVEM receptor domain, causing the cIAP1/2 proteins to ubiquitylate TRAF3 and stimulate its degradation. Subsequent accumulation of NIK in the cytoplasm mediates the activation of the ncNF-κB pathway by starting the process of converting p100 to p52 (Häcker et al., 2011).

Understanding HVEM and TRAF/NF-κB activation for use in CAR-T cells is vital, as CAR-T cells with 4-1BB require TRAF1, 2, and 3 (Li et al., 2018). To use HVEM as a co-stimulatory domain in CAR-T cells, it is important to understand how HVEM interacts with TRAF/NFKB machinery and how those interactions may differ from 4-1BB. Furthermore, constitutive or chronic activation of CAR-T cells by CAR-derived 4-1BB signaling can cause toxicity of CAR-T cells by constant activation of TRAF2-mediated NF-κB activation and increased FAS-dependent cell death. Therefore, it will be important to understand whether HVEM signaling results in similar outcomes (Gomes-Silva et al., 2017).
TRAF Structure with TNFRs

TRAFs form a mushroom-shaped trimer with a coiled-coil and a unique-sandwich domain after assembling with TNFRs. Each receptor peptide binds symmetrically to the side of the mushroom-shaped TRAF domain trimer and extends along the mushroom cap in the crystal structure of six distinct TRAF–TNFR complexes (Zapata, 2003). Table 1 describes the relationships between key receptors and TRAFs.

<table>
<thead>
<tr>
<th>Receptor</th>
<th>Interacting TRAF protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>4-1BB</td>
<td>1,2</td>
</tr>
<tr>
<td>CD27</td>
<td>2</td>
</tr>
<tr>
<td>CD30</td>
<td>1,2,3,5</td>
</tr>
<tr>
<td>CD40</td>
<td>1,2,3,5,6</td>
</tr>
<tr>
<td>EDAR</td>
<td>1,3</td>
</tr>
<tr>
<td>GITR</td>
<td>1,2,3,5</td>
</tr>
<tr>
<td>HVEM</td>
<td>1,2,3,5</td>
</tr>
<tr>
<td>IL-15R</td>
<td>2</td>
</tr>
<tr>
<td>IL-17R</td>
<td>6</td>
</tr>
<tr>
<td>LTβR</td>
<td>3,5</td>
</tr>
<tr>
<td>OX40</td>
<td>1,2,3,5</td>
</tr>
<tr>
<td>p75NTR</td>
<td>2,4,6</td>
</tr>
<tr>
<td>RANK</td>
<td>1,2,3,5,6</td>
</tr>
<tr>
<td>TACI</td>
<td>2,5,6</td>
</tr>
<tr>
<td>TAJ</td>
<td>1,2,3,5</td>
</tr>
<tr>
<td>TNF-R2</td>
<td>2</td>
</tr>
</tbody>
</table>

Table 1 TNFRs Interact Directly with Members of the TRAF Protein Family.

*Only the nondeath domain containing TNFRs can bind to one or more of the TRAF protein family members. A death domain allows receptors to signal apoptosis (Wajant et al., 2001; Xie et al., 2006).*
HVEM can bind to numerous members of the TRAF family, including TRAF1, TRAF2, TRAF3, and TRAF5, but not to TRAF6 (Marsters et al., 1997). The amino acid residues on the surface of TRAF involved in receptor interactions are conserved in TRAF1, 2, 3, and 5, which explains why these TRAFs have overlapping specificity for different receptors (Ye et al., 1999). Figure 7 shows a ribbon diagram of the three-dimensional structure of the TRAF2–HVEM complex.
Figure 7 Three-Dimensional Structure of the TRAF2–HVEM Complex.

TRAF2-HVEM using CD40's PDB entry 1QSC as a basis in PyMol (DeLano, 2002). (A) View along the trimer axis from below with the coiled-coil in the front. (B) Side view of TRAF2-HVEM binding sites with vertical trimer axis. The helix a2 is on the underside of the C domain and the C-terminus is on the top. TRAF2 forms a trimer (blue, yellow, and green ribbons), and HVEM peptides (space-filling; atom colors) bind to the rim of the three TRAF2 monomers. The sequences of the TRAF-N domain form a parallel coiled-coil, followed by the MATH domain, which adopts a unique β-sandwich. The HVEM peptides are ≈38 Å from the trimer axis and ≈54 Å from each other. The side chains of the conserved T/L/I motif that recruits NF-κB (Song et al., 1997) are colored orange.

The cytoplasmic domain of TNFR is in contact with a complementary groove on the rim of each TRAF monomer (McWhirter et al., 1999). Different TRAF trimers can compete to dock at the ligand-activated TNFR trimer, potentially resulting in TRAF trimers with different configurations held by nearby TNFR trimers in the same cell. Furthermore, other members of the TRAF family can form heterotrimers (Zapata et al., 2018) (Figure 8).
Figure 8 TRAF-trimer Configurations

(A) Different TRAF-trimer configurations might be recruited to active TRAF trimers. TRAF2-RING finger dimers are generated between the RING finger domains of two TRAF2 molecules from adjacent trimers. (B) CD-137-recruited TRAF trimers form a hexagonal network stabilized by RING finger domain dimers that form between adjacent trimer TRAF molecules or between the RING finger domains of contiguous cIAP1/2 molecules. cIAPs are critical arbiters of cell death and critical mediators of inflammation and innate immunity (Note. Adapted from Zapata et al., 2018).
The TRAF2 of the TNFRs plays a central role and complexes with several of the other TRAF2. At the C-terminus of the protein, TRAF2 has a coiled-coil followed by seven-eight antiparallel sheets, producing a coiled-coil and a Meprin and MATH homology domain at the top (Zotti et al., 2017). All TNFR receptor peptides bind to the mushroom-shaped MATH domain, which extends from the top to the bottom of the mushroom cap. In the active signaling complex, the directionality of the bound receptor peptides relative to the MATH domain brings the mushroom cap of the MATH domain trimer close to the membrane. Moving the MATH domain closer to the membrane allows the RING finger and zinc finger domains of the TRAF proteins, which are amino-terminal to the coiled-coil domain, to be exposed to the cytosol for interaction with downstream effector molecules (Zotti et al., 2017). The MATH domain binds to short linear motifs in the cytoplasmic tails of receptors. These connections are not strong and have only tens to hundreds of micromolar dissociation constants (Foight & Keating, 2016). To date, seven TRAFS have been discovered (Zotti et al., 2017). All except TRAF1 contain a RING finger domain localized at their amino terminus, followed by one or more zinc finger domains. TRAF7 does not interact with any TNFR and does not have a MATH domain (Zotti et al., 2017) (Figure 9).
Figure 9 Seven TRAF Domain Organizations

(Note. From Zotti et al., 2017).
Figure 10 Sequence Comparison of TRAF-Binding Regions
The sequence comparison of the TRAF-binding regions of several TNFRs shows the minimal consensus motifs for TRAF1,2,3, and 5 (Note. From Zapata, 2003).

Some conserved structural contacts preserve TRAF-binding sequences from different receptors (Figure 10). The general sequence motif for TRAF binding is (P/S/A/T)-X-(Q/E)-E (Ye et al., 1999). In CD27, the TRAF binding site is 246PIQED250 (Akiba et al., 1998; Yamamoto et al., 1998). OX40 has a TRAF binding site at 261TPIQEEQAD269 (Arch & Thompson, 1998). Several TNFRs have more than one TRAF binding site. 4-1BB has a TRAF binding site at 236QEED239 and 247EEEE250 (Arch & Thompson, 1998; Jang et al., 1998). CD30 has TRAF binding sites, 558PHYPEQET565 and 576MLSVEEEGKED586 (Gedrich et al., 1996). Additionally, GITR has two binding sites, 211STED214 and 221PEEE224 (Esparza & Arch, 2005; Kwon et al., 1999). HVEM follows this pattern with a TRAF binding site of 264VTTVAEEET272 (Hsu et al., 1997) (Figure 11). Different TRAFs can compete for receptor binding motifs, which agrees with the observation that other TRAFs than TRAF2 interact with the cytoplasmic domain of HVEM (McWhirter et al., 1999).
VEET is displayed in the default PyMol (DeLano, 2002) electron density map. This portion of the cytoplasmic HVEM domain produces an open poly-Pro II helix with no internal hydrogen bonding.

Figure 12 Recognition of HVEM / TRAF2.

The VEET recognition motif of HVEM (in teal) is bound to the three TRAF2 hotspots. Hotspot1 is green, hotspot2 is red, and hotspot3 is orange.
Table 2 Structures of the TRAF Binding Motif of the TRAF Receptor Complexes.

All residues critical for interaction with the VEET motif are well conserved in TRAF1, TRAF3, and TRAF5, except that A replaces an S residue in the S triad in TRAF1 (A369) (Note. From Park, 2018).

<table>
<thead>
<tr>
<th>Spot</th>
<th>TRAF 1</th>
<th>TRAF 2</th>
<th>TRAF 3</th>
<th>TRAF 5</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>F325</td>
<td>F410</td>
<td>F475</td>
<td>Y462</td>
</tr>
<tr>
<td></td>
<td>F347</td>
<td>L432</td>
<td>L496</td>
<td>L484</td>
</tr>
<tr>
<td></td>
<td>F362</td>
<td>F447</td>
<td>F512</td>
<td>F499</td>
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<tr>
<td></td>
<td>F371</td>
<td>F456</td>
<td>F521</td>
<td>F508</td>
</tr>
<tr>
<td></td>
<td>C391</td>
<td>C469</td>
<td>C534</td>
<td>C521</td>
</tr>
<tr>
<td>2</td>
<td>S368</td>
<td>S453</td>
<td>S518</td>
<td>S505</td>
</tr>
<tr>
<td></td>
<td>A369</td>
<td>S454</td>
<td>S519</td>
<td>S506</td>
</tr>
<tr>
<td></td>
<td>S370</td>
<td>S455</td>
<td>S520</td>
<td>S507</td>
</tr>
<tr>
<td>3</td>
<td>R308</td>
<td>R393</td>
<td>R458</td>
<td>R445</td>
</tr>
<tr>
<td></td>
<td>Y310</td>
<td>Y395</td>
<td>Y460</td>
<td>Y447</td>
</tr>
<tr>
<td></td>
<td>D314</td>
<td>D399</td>
<td>D464</td>
<td>D451</td>
</tr>
</tbody>
</table>
Other members of the TNFR family, such as CD30, CD40, Ox40, and TANK, have a typical binding pattern with TRAFs 1,2,3,5 (Park, 2018). TRAFs have three regions, known as binding hot spots, to form tight interactions with their TNFR (Table 2) (Figure 12). Similarly, TRAF2 residues that create hot spot 1 (F410, L432, F447, F456, and C469) could cause van der Waals interactions with V267, A268, and V269 in HVEM. E252 could interact with the residues from the hot spot 2 (S triad, S453, S454, and S455). In HVEM, E270 can only establish one hydrogen bond with the three S residues, but a Q at this position (as seen in many other TNFRs) can form hydrogen bonds with all three. Hot spot 3 formed by residues R393, Y395, and D399 in TRAF2 remains involved in the interaction with E271. The negatively charged E271 creates an ion-pair interaction with the positively charged R393. In hotspot 3, the carboxylate moiety of the E271 residue interacts with the guanidinium group of the side chain of R393. E271 also forms a hydrogen bond with Y395 in TRAF2 through an ion-pair interaction. When combined, the surface characteristics of the TRAF2-HVEM complex show a positive electrostatic potential on the top facing the cell membrane (Figure 13). The trimer polarizes with a positive potential facing the membrane. A negative potential rings the coiled-coil motif.
Figure 13 Surface Features of the TRAF2-HVEM Electrostatic Potential.

*The potential (−5 to +5 kt/e) was calculated with APBS electrostatics and displayed in blue (positive), white (neutral), and red (negative). (A) View along the trimer axis looking onto the "top" of the TRAF structure. Large indentations in the molecular profile mark the subunit interfaces. (B) Oblique view of the "underside" of the TRAF2-HVEM complex with the coiled-coil pointing to the bottom right. The position of the HVEM is indicated and is located just below the rim. The positive potential characterizes the sides of the canyons that exit the bowl and the interfaces of the subunits.*

The HVEM sequence fits securely at the TRAF2-binding crevice due to the HVEM alternating pattern of large and small side chains in the VEET sequence. The VEET peptide fits into a small solvent pocket in the TRAF2 binding crevice. The overall dimensions of this cave are 17.5 x 7.5 x 8 Å. A sequence alignment based on the receptor peptide structure reveals that the third position of the VEET-like sequence is almost always occupied by an E residue and has a very high degree of sequence conservation (Ye et al., 1999). Although LMP1 has an A in the third position, E occupies this position in the main TRAF binding motif. The carboxylate moiety of the E residue makes a
bidentate ion pair contacts with the side chain guanidinium group of R393 and an extra hydrogen bond with the hydroxyl of Y395 (Ye et al., 1999). Because other residues are too short to reach R393 and Y395 in TRAF2, these hydrogen-bonding interactions require E specifically. LMP1 and TANK have an A and a C, respectively, instead of E at the third position and cannot participate in ion pair and hydrogen bonding interactions. Instead, LMP1 can create new interactions with the R393 and Y395 residues by having an N residue much further down (where the position I273 is in HVEM). The side chain of R393 conforms to connect N to R393.

Although VEET does not contain prolines, the domain forms the shape of a polyproline II helix (PPII) (Adzhubei et al., 2013). PPII is a somewhat twisted strand that allows for a three-turn side-chain periodicity. Protein–peptide interactions, such as recognition of peptides by SH3 domains and class II MHC molecules, often adopt a PPII conformation. The peptide chain can twist in this shape to optimize the interaction of its side chains with a protein surface (Ye et al., 1999). PPII allows for the burying of VEET in a complex with TRAF2 (390Å/603Å=64%).

Most other TNFRs replace A268 in HVEM with a P. P at this position makes extensive van der Waals contacts with TRAF2 and reinforces the PPII conformation. A268 in HVEM and 4-1BB lowers the affinity of the peptides and produces a weaker electron density in the complex. The side chains of the remaining receptor peptide residues are exposed on the surface of the complex, but these residues have little sequence conservation among the TNFRs. The V269 residue appears to be essentially a spacer. In TRAF2, the side chain of the T272 residue is close to D399, and T272 forms hydrogen-bonding interactions with the side chain of D399. Surprisingly, some receptor
peptides from other TNFRs include an acidic residue (D or E) at this location, but none have a basic residue (L or R).

The Length of a TRAF-Binding Site

The corresponding residue "G271" in TNF-R2 has five ordered residues at the carboxyl terminus. On the other hand, the CD40 complex has ordered residues five places after its corresponding "G271" at the amino end. These findings imply that a complete TRAF2 binding sequence could have up to 11 residues (5+1+5) to cover the entire span of one MATH domain surface face. The side chain chemistry determines the conformation, and the lengths of the TRAF binding area differ from receptor to receptor. A view of the HVEM/TRAF2 complex in PyMol (DeLano, 2002) suggests that the actual size of HVEM could be VAVEET of six amino acids long.

HVEM Amino Acids After the VEET Binding Site

The removal of the last 15 residues from the C-terminus of the cytoplasmic portion of CD40 completely removed the binding of TRAF3 to CD40 and reduced the binding to TRAF2 (Leo et al., 1999). As a result, CD40 binds to TRAF2 and TRAF3 in different ways to modulate the intensity of the connections. TRAF3 interacts with CD40 and LTβR receptor residues, creating a hairpin essential for TRAF3 contact (Ni et al., 2000) (Figure 14). When CD40 is linked to TRAF3, the two strands that flank the reverse turn of CD40 do not engage in the usual hydrogen bonding in β-hairpins. Instead, hydrogen bonds involving T254 in the consensus sequence PxQxT stabilize the structure. On the next strand, the threonine side chain forms intrapeptide hydrogen connections.
with the main chains of D265 and E264. This hairpin does not happen in CD40-TRAF2 studies, and CD40’s T254 moves to a position to hydrogen bond with TRAF2’s D399 instead (Ni et al., 2000).

As with CD40, HVEM’s tail can also produce a structurally stable hairpin as shown in Figure 15 by the Ramachandran plot and graphically depicted in Figure 14. A Ramachandran plot helps to analyze protein folding; Phi and Psi values are used to map the allowed structures of transition states and intermediates in the folding, binding, and conformational transitions of proteins (Fersht & Sato, 2004).
The TRAF3 binding crevice is on the right in each image. LTβR (left) and CD40 (middle) (Note. From Li et al., 2003). For HVEM (right), the author took the energy minimization and simulated the annealing result in the SWISS-PDB viewer (Biozentrum, Basel) in vacuo utilizing a GROMOS96 energy minimization (Guex & Peitsch, 1997). The resulting pdb file was manipulated using PyMol (DeLano, 2002). The simulation consisted of 30 cycles, which reached 2,000 heating and annealing steps. The steps cycled between a temperature of 300 K / mol and 1000 K / mol. The TRAF3 binding crevice is on the right. As in CD40 and LTβR, the amino acids after the TRAF3 binding domain in HVEM could reverse course.
Ramachandran plots of the tails of CD40 (left) and HVEM (right) after interacting with TRAF3. The plot shows amino acids within acceptable regions of the Phi and Psi angles. The CD40 data are from the cocrystallization of CD40 and TRAF3 (Ni et al., 2000) and show 4 amino acid outliers (phi, psi): A249 (94.2, -157.4); P250 (-109.9, -111.6); L255 (158.6, 146.9); S258 (-164.2, -0.3). HVEM is the last 20 homologous annealed amino acids and has three outliers (phi, psi): V249 (92.8, -163.9); I255 (163.7, 137.7); F258 (-164.1, 1.9). Both Pdb's were run through the MolProbity Ramachandran analysis tool from Duke University (Lovell et al., 2003).

Similarly, for CD40 and LTβR, the last eight amino acids of HVEM could also double over VEET when they are bound to TRAF3 (Figure 17). When CD40 links to TRAF3, HVEM's T272 in the PxQxT consensus sequence could stabilize a reverse-stranded structure. T-272 could form intrapeptide hydrogen connections on the next strand with N282 and H283. However, because P281 does not have a hydrogen in the amino group when in a peptide bond, it cannot give a hydrogen bond to T272 and S280 is too far away to participate (6.4 Å) (Figure 16) and (Table 3).
Figure 16 Relationship of T272 with Hydrogen Acceptors H283 and N282.

Distances in Å.

<table>
<thead>
<tr>
<th>Amino acids</th>
<th>Hydrogen donor atoms</th>
<th>Hydrogen acceptor atoms</th>
</tr>
</thead>
<tbody>
<tr>
<td>T272</td>
<td>OG1</td>
<td>OG1 (2)</td>
</tr>
<tr>
<td>N282</td>
<td>ND2 (2)</td>
<td>OD1 (2)</td>
</tr>
<tr>
<td>H283</td>
<td>ND1, NE2</td>
<td>ND1, NE2</td>
</tr>
<tr>
<td>S280</td>
<td>OG</td>
<td>OG (2)</td>
</tr>
</tbody>
</table>

Table 3 Hydrogen Donor and Acceptor Atoms on Amino Acid Side Chains

The table shows the number in parentheses of "sp hydrogens" that a donor atom can donate or the number of hydrogen bonds that an acceptor atom can accept. Atoms within 5 Å are colored.
Many proteins require histidine to function correctly (Brosnan & Brosnan, 2020). Because the histidine side chain pKa lies within physiological pH, it can act as both an acid and a basic. It is possible that local pH changes H283 and its impact on the VEET domain.

Because proline is an imino acid with a five-membered ring, rotation around the N-C(alpha) bond is sterically constrained, giving it a Phi value of approximately -63 ±15 degrees. Proline can disrupt or stabilize regular secondary structural components and cause kinks (Morgan & Rubenstein, 2013). Proline (which has two carbons attached to N) has a smaller energy difference between the cis and trans states than other amino acid
residues (with an H atom and a C atom attached to the peptide N), creating a conformationally relevant peptide (Morgan & Rubenstein, 2013). Pro274 and Pro281 are highly conserved and lie across from each other in the HVEM tail and pull the bottom of the loop away from TRAF 3 domain (roughly an additional 8 Å). The cis and trans conformations of proline peptide bonds are thermodynamically stable (Vitagliano et al., 2001). In HVEM, the two prolines can sit comfortably in either a trans configuration with Phi and Psy angles of (-75, 145) or a cis configuration of (-75, 160). A trans-to-cis isomerization of the two proline peptide bonds is a potential mechanism to move the HVEM tail away from the VEET (Figure 18).
Figure 18 Proline Kink in HVEM

*HVEM in cyan, TRAF2 in green, and trans prolines in red.*

4-1BB Does Not Activate TRAF5 While HVEM Does.

Interestingly, although HVEM binds the same set of TRAFs as CD30, HVEM signaling in T cells predominantly induces TH1 cell-associated cytokines (Cheung et al., 2009). Therefore, HVEM as a co-stimulatory molecule could provide the following advantages over 4-1BB in a CAR-T system (Arkee & Bishop, 2020).

1. Help CD27-mediated signaling rescue CD8+ CAR-T cells from apoptosis
2. Decrease Th2 cell differentiation and decrease non-inflammatory cytokine production

3. Decrease IL-6R signaling

4. Decrease IL-6-mediated Th17 differentiation

5. Increase NF-κB1, p38, and ERK activation

6. Increase IL-2 production
Chapter 2

Methods

Three DNA constructs were designed using Snapgene 5.3.2 software (Insightful Science) and synthesized by Azenta Life Sciences.

1. The 40 amino acids on the N-terminal side that form an α-helix in M83 were removed to test with only a peptide from V264 to H283 (hereafter called M83NH):
   a. VTVAVEETIPSFTGRSPDH

2. M83 which substitutes the second E with A at the TRAF binding site, VEET.
   Replacement of E for A at position 271 was the only point mutation that abolished the HVEM interaction with the TRAF2 and TRAF 5 proteins (Hsu et al., 1997). This approach of introducing a point mutation of E271A was used again in 2019 to determine whether the loss of HVEM's ability to recruit TRAF2/5 affected germinal center B cell participation (Mintz et al., 2019). A is similarly negatively charged as E. This mutant is hereafter called M83E/A:
   a. CVKRRKPRGDVVKIVSVQRKRQEAEGATVIEALQAPPD
      VTVAVEATIPSFTGRSPDH

3. M83 minus the last eight amino acid tail. This construct was named M83NT.
   a. CVKRRKPRGDVVKIVSVQRKRQEAEGATVIEALQAPPD
      VTVAVEETIPS

Plasmid Construction
Figure 19 Example Plasmid Construction

*The plasmid construct for construct M83E/A. Here, the substitution of E for A with a GCG codon changes VEET to VEAT.*

Figure 19 shows an example of the construction of the plasmid for M83E/A. The M83 region of the anti-CD19 CAR-T lentiviral plasmid construct GMCSF.M83.FMC63 (Nunoya et al., 2019) was digested with EcoRV and SalI, and PCR products containing CD3ζ through GFP and one of the three modified M83 constructs were inserted. The plasmid used EF1a-short promoter (EFS) to express an anti-CD19-modM83-CD3ζ CAR. This CAR is separated from an EGFP marker by a P2A ribosomal self-skipping peptide, allowing the fluorescence identification of CAR-expressing cells.

The 5' LTR is a promoter to transcribe the viral genome, and the 3' LTR has a polyadenylation signal to terminate the upstream transcript. A packaging signal, HIV psi, is needed to efficiently package the viral genome in the vector (Kim et al., 2012).
HIV-1 Rev response element, RRE, allows for Rev-dependent mRNA export from the nucleus to the cytoplasm of viral genomic RNA that has not been spliced and boosts titers. Gp41 is a transmembrane protein required for lentiviral infection of CD4+ T cells (Sakuma et al., 2012). HIV-1’s central polypurine tract and central termination sequence, cPPT/CTS, create a "DNA flap" that promotes viral genome nuclear importation during target-cell infection, improving vector integration and transduction efficiency. The eukaryotic translation elongation factor, EF1α, can be a better promoter than CMV (Wang et al., 2017). When the CAR is expressed inside a T cell, an N-terminal GMCSF signal peptide is added to the ScFv, and the protein product is guided to the endoplasmic reticulum, then to the cell surface and is expressed. FMC63 is the most prevalent ectodomain component of CD19-specific CARs used in immunotherapy B-lineage leukemias. The transmembrane domain, derived from CD8, provides stability to the receptor. The intracellular domain of the CAR-T cell receptor-CD3ζ chain acts as a stimulatory molecule to activate the immune response mediated by T cells.

Enhanced Green Fluorescent Protein, EGFP, is a standard green fluorescent protein with good brightness, photostability, and pH stability to report CAR expression. The post-transcriptional regulatory element of the Woodchuck hepatitis virus (WPRE) is a DNA sequence that enhances expression by forming a tertiary structure when transcribed. WPRE prevents readthrough of the poly(A) site, enhances RNA processing and maturation, and boosts RNA nuclear export. It improves vector packaging and increases titer in genomic transcripts. WPRE increases transgene expression in transduced target cells by promoting mRNA transcript maturation. In viral RNA transcription during packing, the early polyadenylation signal of Simian virus 40, the
origin of replication, favors transcriptional termination after the 3’ LTR. SV40 improves the viral titer by increasing the amount of functional viral RNA in cell packaging. An ampicillin resistance gene, AmpR, allows the plasmid to be maintained by ampicillin selection in E. coli. Plasmids carrying the Ori origin of replication exist in high copy numbers in E. coli. Various promoters and binding sites, such as the CAP binding site, the Lac promoter/ operator, the M13 rev, and the T3 RNA promoter. These plasmids were assembled following the instructions of NEBuilder HiFi Assembly (New England Biolabs, Ipswich, MA) and transformed into E. coli.

Primary Human T Cell Isolation and Culture.

Total CD3+ T cells were isolated from two deidentified and healthy donors’ human peripheral blood obtained from STEMCELL Technologies using negative selection beads (EasySep™ Human T Cell Isolation Kit from STEMCELL Technologies). T cells were cryopreserved in Cryostor CD10 (BioLife Solutions) at 10^6 cells per ml in 1 ml aliquots. After thawing, T cells were maintained at 37 °C and 5% CO₂ in human T cell medium consisting of RPMI with ATCC Modification (Gibco A1049101) plus 10% heat-inactivated FBS with 10 ng/mL IL-2 (STEMCELL Technologies). T cells were resuspended in human T cell medium without IL-2 immediately prior to all assays. Before coculture with target cells, T cells were thawed and diluted with untransduced/expanded T cells from the same donor, bringing all cultures to 10% CAR+ (except M83E/A cells). For co-culture assays, T cells and target cells were cultured in a medium formulated with ATCC RPMI plus 10% heat-inactivated FBS without IL-2.
Lentiviral Vector Construction and Virus Preparation

Following the manufacturer's instructions, lentivirus was produced using the LV-MAX™ Lentiviral Production System (Thermo Fisher) to produce 13 ml of Lenti Supernatant for each construct. The virion content was purified and measured with the Lenti-X™ qRT-PCR Titration Kit (Takara) and the QuantStudio™ 3 Real-Time PCR System (Thermo Fisher).

T Cell Transduction

T cell activation was carried out using 25 µl/10⁶ per ml of incomplete media. To activate T cells, 25 µL/mL of ImmunoCult Human CD3/CD28 T Cell Activator (STEMCELL Technologies) was added to the cell suspension. Cells were incubated at 37°C and 5% CO2 for 3 days. The T cells were expanded by taking a viable cell count and adjusting the viable cell density every 2 - 3 days by adding fresh complete ImmunoCult-XF T Cell Expansion Medium (STEMCELL Technologies) to the cell suspension. The cells were incubated at 37°C and 5% CO2 until the desired cell number was obtained.

T cells were transduced with concentrated lentivirus 24 h after activation with CAR-T constructs at various MOIs to produce transduction rates between 5 and 20%. Forty-eight hours after Lentivirus transductions, cell cultures were washed and given fresh media to remove residual virus. Transduced CAR-T cells were then expanded in a human T cell medium at a density of 1-2⁶ cells/ml for ten days. After a 13-day expansion, the transduced CAR-T cell cultures were frozen in CS-10 medium (BioLife Solutions). Anti-CD19 CAR-T plasmid construct (GMCSF.M83. FMC63) containing a complete M83 domain or a first generation CAR-T (GEN 1) containing a single CD3ζ intracellular
domain without additional co-stimulatory domains were used to account for the stimulatory activity of the zeta chain alone, to see where the activity of the new constructs would fall between the GEN1 and the entire M83 construct. A sixth construct containing VRC01 (Control), an scFv against an HIV antigen, served as an irrelevant binder control. CD19-positive Raji lymphoma cells were purchased from the American Type Culture Collection and kept in log phase growth in RPMI 1640 medium supplemented with 10% heat-inactivated FBS (Thermo Fisher) in a humidified atmosphere of 5% CO2 at 37 °C.

Processing Samples for Flow Cytometry Analysis.

Before coculture with target cells, CAR-T cells were thawed and diluted with untransduced/expanded T cells from the same donor, bringing all cultures to 10% CAR+ (except M83E/A cells). For coculture assays, CAR-T cells and target cells were cultured in a medium formulated with ATCC RPMI plus 10% heat-inactivated FBS without IL-2. Samples were transferred from a cell culture incubator to a biosafety cabinet with the biosafety cabinet lights turned off. All subsequent sample processing was performed with the light within the biosafety cabinet used for processing turned off to prevent fluorophore photobleaching. Before further processing, the samples were transferred to a 96-well round-bottom tissue culture plate (Thermo Fisher).

Flow Cytometry

Staining was performed with DAPI and cells were labeled with fluorescent Abs to CCR7- BV421, CD4+-BV605, CD4+5RO-BV785, surface CAR-PE, CD25-PE-CF594,
CD27-PerCP/Cy5.5, CD69-APC, CD8-APCcy7 (all purchased from BioLegend), and CD3-BUV496 (BD Biosciences). Analyses were performed using a CytoFLEX LX flow cytometer (Beckman Coulter) and analyzed with FlowJo v10.8.1 (BD Biosciences).

**ELISA**

DuoSet ELISA to measure human IL-2 concentration of supernatants was from R&D Systems and performed as the manufacturer recommended. The concentration of IFNγ in cell culture supernatants was determined with the Human IFNγ DuoSet ELISA kit (R&D Systems), according to the manufacturer's instructions, and measured on a Varioskan LUX with SkanIt Software RE for Microplate Readers RE, ver. 6.1.1.7 (Thermo Fisher).

**Data Processing and Visualization**

Data processing was performed in R (Team, 2013). The plots were generated using ggplot2 (Wickham et al., 2016).
Chapter 3

Results

Loss of M83 Helix and TRAF Binding Domain Impacts CAR Expression.

CAR-T cells containing the three M83 mutations described above were compared with a first-generation CAR-T with only CD3ζ (GEN1) and a nonbinding control (Control). CD3+ CAR-T cells were isolated, activated, and transduced with lentivirus described above. Flow cytometric analysis revealed that the transduction rates of CAR-T cells with different lentiviral vectors varied by construct, despite a similar multiplicity of infection (Figures 20 and 21).
Figure 20 Transduction Efficiencies Achieved by Lentiviral Transduction.

Representative dot plots of CAR-transduced donor CAR-T cells are shown. Flow cytometry determined transmission efficiencies by measuring GFP expression (x-axis). The numbers in the plots show the percentages of each cell population.
Figure 21 CD19 Respond to Raji Co-Culture

The bar graph shows the percentage of GFP+ transducer cells that are positive for CAR expression of each of the indicated constructs. The control is a non-targeting CAR. The mean and first and third quartiles are represented by the lower and upper hinges, respectively. The whiskers extend a 1.5 * interquartile range from the hinge. Data beyond the whiskers' end are shown separately (n = 16). Example p-values are shown (paired Wilcoxon).

Surprisingly, the percentage of GFP+ cells also expressing CAR was considerably lower in CAR-T cells with the M83NH or M83E/A mutation (Figure 21).

TRAF Binding Domain and Helix May Impact Activation
After generating multiple populations of anti-CD19 CAR-T cells using the M83 costimulatory domain or mutated variations of it, the impact of mutations on effector function was examined when these CAR-T cells were co-cultured with CD19+ target cells. CAR-T populations with Raji cells in multiple effector-to-target ratios were co-cultured to do this. An anti-CD19 CAR-T cohort lacking any costimulatory domain (GEN1) was included, allowing us to compare the impact of mutated M83 domains with CAR-T cells with wild-type M83 or without M83 signaling. Twenty-four hours after coculture, culture supernatants and cells were cultured by flow cytometry.

The expression of CD69 and CD25, markers of T cell activation, was investigated in transduced GFP+ donor T cells and controls (Figures 23). A significant reduction in CD69 was observed only in M83NH (Figure 22B), and CD25 was reduced in both M83E/A and M83NH (Figure 22C). Most of the impact of these activation markers is found on the first day (Figure 22D). All constructs reach their maximum CD69 on the first day before decreasing expression. CD25 activation occurs mainly on the first day and increases on the second day. M83NH, as mentioned above, has the lowest expression levels of both markers. These early markers correlate with CAR's level of expression (Figure 23).
Figure 22 Impact of Construct on Activation Markers

(A, B, C) Early activation markers change most when M83 loses its α-helix (M83NH) or with mutated binding domain M83E/A TRAF. (D) Evolution of the constructs from CD69 to CD25 over two days. Example p-values are shown (paired Wilcox).
Figure 23 Construct has a Connection with CD69, CD25

(A) Frequency of CD69 and (B) CD25 as a percentage of cells expressing CAR. The Khachiyan algorithm is used to estimate the surrounding ellipses, using the default tolerance level of 0.01 (Todd & Yıldırım, 2007). M83 Deletions Impact Cytokine Secretion and Expression of Other Markers Associate with T Cell Function

To further elucidate how M83 variants impact CAR-T cells co-cultured with target cells, the expression of several cytokines and other cell makers associated with effector function was measured. IL2 and IFN were drastically reduced in M83E/A and M83NH compared to the other variants (Figure 24A&B). As was the case for activation makers described above, the level of cytokine expression is correlated with the level of CAR expression (Figure 25). There is a positive feedback loop between IL-2 levels and CD25 expression (Busse et al., 2010) and in this experiment, we see a direct correlation between CD25 and IL-2 levels (Figure 26).
Figure 24 Impact of Construct on Cytokines

The early activation markers change the most when M83 loses its α-helix (M83NH) or the mutated binding domain of M83E/A TRAF (A, B). There is less production of the cytokines IL2 and IFNγ. Example p-values are shown (paired Wilcox).
Figure 25 Construct Connection with IL2 and IFNγ

Cytokine secretion by CAR-T cells after 48 hours of Raji co-stimulation. (A) IL2 concentration as a function of the percentage of cells expressing CAR (B). IFNγ concentration as a function of the percentage of cells expressing CAR. The Khachiyan algorithm is used to estimate the surrounding ellipses, using the default tolerance level of 0.01 (Todd & Yildirim, 2007).
Figure 26 IL2 Feedback on CD25 Expression

*IL2 expression is plotted against the median fluorescence intensity of CD25 for the first day of all constructs. The shaded band is the 95% confidence level interval for predictions from a best-fitting linear model. The correlation analysis was evaluated using the two-tailed Pearson correlation test.*

The expression of CD27 and CCR7, a later stage cell marker, and a homing receptor, respectively, was investigated in transduced GFP+ donor T cells and controls (Figures 28). M83NH showed a significant increase in the expression of CCR7 (Figure 27B), and the expression of CD27 increased in M83E/A and M83NH (Figure 27C). Most
of the impact of these activation markers is found on the first day (Figure 27D). All constructs increase both markers rapidly on the first day and decrease on the second day of the experiment. Constructs with a low percentage of CAR expression (M83E/A, M83NH, Control) actually have higher levels of the late-stage markers CCR7 and CD27.

Figure 27 Impact of Construct on Differentiation Markers

*The early activation markers change the most when M83 loses its α-helix (M83NH) or mutates the binding domain of M83E/A TRAF (A, B). The signal from CCR7 increases (C) and CD27 is not removed (D). Example p-values are shown (paired Wilcoxon).*

Change In CD4+ T Cells With M83NT
To better understand the phenotype of CAR-T cells carrying various M83 variants, the CD4+/CD8+ T cell ratio was compared. Two T lymphocyte phenotypes, CD4+ helper/inducer cells and CD8+ cytotoxic/suppressor cells, are distinguished by different surface markers (Amadori et al., 1995). As seen in Figure 28, M83NH showed a substantially higher CD4+/CD8+ ratio than the other constructs. M83E/A showed a trend towards a higher CD4+/CD8+ ratio, but was not significantly different from GEN1 or M83.

![Figure 28 CD4+ and CD8+ Markers in Constructs](image)

*Figure 28 CD4+ and CD8+ Markers in Constructs*

*Comparisons between constructs of CD4+ and CD8+ markers. Example p-values are shown (paired Wilcox).*

To better understand possible differences in CD4+ and CD8+ T cells, the expression of activation markers in these subsets was examined individually rather than
in the total population of T cells (Figure 29). As shown above, the expression of CD69 and CD25 is decreased in M83NH compared to the other variants or the GEN1 control, and Figure 29 shows that this decrease is consistent in both CD4+ and CD8+ T cells (Figure 29 A, B). Similarly, CD25 is expressed lower in M83NH and affects both CD4+ and CD8+ T cells equivalently (Figure 29 C, D). Interestingly, CD27 is expressed higher in M83NH and M83E/A compared to the other variants and the GEN1 control, but only significantly in CD4+ T cells (Figure 29 E, F).
Figure 29 Activation Markers on CD4+ and CD8+ T cells

CD4+ with various other markers is plotted to investigate CD4+ T cells further. Example p-values are shown (paired Wilcox).

Loss of the M83 tail has three impacts. First, M83NT has more CD4+ T cells (Figure 30A), the frequency of proliferation of CD4+ T cells (Figure 30B), and the intensity of GFP of GFP + cells (Figure 30C).
Figure 30 Effect of Tail Loss on M83
Chapter 4
Discussion

HVEM is an unusual TNFR in that it has both stimulatory and inhibitory effects and at least five different ligands (Cai & Freeman, 2009; Del Rio et al., 2010; Granger & Rickert, 2003; Pasero & Olive, 2013; Sorobetea & Brodsky, 2018). HVEM is broadly expressed in peripheral T and B cells and is present in resting T and B cells (Ning et al., 2021). HVEM expression is more potent in Treg cells than in effector cells, probably identifying it as a mechanism of immune regulation (Del Rio et al., 2010). T cell activation down-regulates HVEM, though HVEM is expressed later on effector and memory T cells (Del Rio et al., 2010). HVEM signaling enhances expression of IFNγ, IL-2R, and CD69, and supports CD4+ Th1 cells, CD8+ effector T cells, and Treg hyperproliferation and survival (Miyagaki et al., 2013; Murphy et al., 2006; Murphy & Murphy, 2009). The cytoplasmic portion of HVEM, referred to as “M83” by Inceptor Bio, has recently been investigated as a co-stimulatory domain for CAR-T and was shown to have several properties that could make it a valuable alternative to other widely used co-stimulatory domains, including 4-1BB (Nunoya et al., 2019; Su, 2020). However, the specific role of structural domains in M83 signaling was not investigated.

Loss of M83 Helix and TRF Binding Domain Impact CAR-T Expression

Surprisingly, the levels of CAR expression on the cell surface of GFP+ cells differed considerably and were the lowest in CAR-T cells with the M83NH or M83E/A mutation (Figure 23). There are several possibilities. The lack of an α-helix may have
caused misfolding of the CAR, preventing localization to the membrane, or it could have decreased stability at the surface through multiple potential mechanisms. The surface expression of CAR might need some separation from the membrane for it to function. The lack of an α-helix also may have interfered with the CD3ζ signaling of the CAR. M83E/A showed slightly lower CAR expression, but possibly maintained most of the function of the untouched CD3ζ chain more like GEN1 (Figure 21).

TRAF Binding Domain and Helix Are Needed For Activation

M83NH showed a significant reduction in CD69 expression compared to the other variants and the GEN1 control (Figure 22B), and both M83E/A and M83NH showed lower levels of CD25 (Figure 22C). The first day after co-cultures are established is when these activation signals are expressed the highest (Figure 22D). On the first day, all the constructs reach their maximum CD69 before beginning to retract. The first day is when CD25 activation occurs mainly. CD69 is an early marker of lymphocyte activation and appears on the plasma membrane within hours after stimulation, but decreases shortly thereafter (Cibrian & Sanchez-Madrid, 2017).

It is possible that the decrease in the expression of these activation markers in M83NH is just due to decreased levels of CAR expression. However, because M83E/A shows a similar expression of CD69, the decrease in M83NH could be due to the lack of the TRAF binding domain and helix despite having less CAR expression.
Culture supernatants showed that the secretion of IL-2 and IFN by transformed donor CAR-T cells with CAR was induced by co-culture with Raji cells. IL-2 is not universally administered in the clinic with CAR-T treatments, as high doses of IL-2 can cause temporary adverse effects such as general malaise, fever, nausea, and vomiting (Rosenberg et al., 1989; Rosenberg, 1987). CAR-T culture medium was not supplemented with IL-2; all identified IL-2 was a product of CAR-T activation due to recognition of the target antigen in Raji cells. Although some CAR-T manufacturing processes include IL-2 in cell media, our process does not; therefore, we can be sure that all identified IL-2 was the product of Raji target CAR-T activation. M83E/A and M83NH are less activated and their levels of IL2 and IFN were significantly reduced (Figure 23A&B). The CD25 (alpha chain of the IL-2 receptor) expression increases as IL2 increases (Figure 26). A STAT5-dependent positive feedback loop results in up-regulation of CD25 following IL-2 binding (Lin & Leonard, 1997; Malek, 2008; Malek et al., 2001).

In non-CAR modified T cells, after stimulating the TCR / CD3 complex, IL2 is up-regulated in the first 24 hours and remains elevated for a few days (Jackson et al., 1990; Reddy et al., 2004). Eventually the level of IL2 will rise high enough to change from a microenvironment, autocrine function to a more paracrine Treg system (CD25+ CD4+ Foxp3+) (Busse et al., 2010; Fuhrmann et al., 2016). At some point, CD25 ceases to respond to IL-2 secretion and is not necessarily representative of the total level of activation/effecter function. The effects of the M83 variants are probably more complex than what we see in just these surface markers.
CCR7 and CD27

Only M83NH showed a significant increase in CCR7 expression, while M83E/A and M83NH both showed an increase in CD27 expression compared to the other variants (Figure 24C). The first day after the establishment of the co-culture is when these activation signals have the most significant influence (Figure 24D). On the first day, all variants of M83 result in a rapid increase in expression of both markers before decreasing. The most pronounced increases in these late-stage markers are observed in constructs with low or no CAR expression (M83E/A, M83NH, Control).

Of all mutations made within the M83 costimulatory subunit of our anti-CD19 CAR construct, M83NH created the most extensive changes of all mutants tested, possibly by moving both the TRAF binding domain and scFv closer to the cytoplasmic membrane. Loss of the M83 tail also decreased the number of cells that differentiated into CD4+ T cells, decreased the frequency of proliferation of CD4+ T cells, and reduced the intensity of GFP of GFP + cells. A reduced intensity of the GFP intensity may be an indicator of a reduced global translation of mRNA.

The loss of the α-helix could have affected folding and the ability to integrate within the membrane. However, the possible interference of the membrane on the binding of TRAF would be surprising since binding of TRAF6 close to the membrane domain on the C-terminal side of CD40 is possible (Pullen et al., 1999).

It is possible that a TRAF complex cannot form close to the membrane. Although an α-helix is very compact, 40 amino acids were removed, which is a substantial structural element. Each amino acid in an α-helix advances 1.5 angstrom units (Å) down the helix axis, giving each full turn 3.6 amino acid residues (Branden & Tooze, 2012). A
full turn advances by 5.4 Å, so the α-helix removed measures about 216 Å. To put this in perspective, the overall dimensions of the cave on TRAF2 that binds to receptors are only 17.5 x 7.5 x 8 Å.

Furthermore, the top of the TRAF trimer polarizes with a positive potential charge facing the membrane (Figure 13). The interior of the cytoplasmic membrane is negatively charged. Bringing the TRAF top to the membrane surface might lead to membrane depolarization. Although membrane depolarization may not imply cell death, it does decrease cell activity (Moo-Young, 2019). It is possible that the positive charge of the TRAF trimer could be enough to depolarize the membrane. The M83NH mutant had significantly reduced CAR surface expression compared to other mutants and showed several reduced activation markers after coculture with a target. As activation readouts within our Gen1 CAR, which lacked any costimulatory domain or TRAF binding site, were not nearly as affected, most likely, the lack of an α-helix might have also interfered with CD3ζ signaling, or the CAR lacking a helix may never have made it to the cell membrane.

CD4+ T Cells Depend on TRAFs

When the tail is removed, there is a noticeable increase in the CD4+ T cell population. Other M83 constructs did not exhibit the same behavior (Figure 28A). The same pattern was observed in the CD8+ T cell population (Figure 28B). Fewer CD4+ T cells were found in the M83E/A and M83NH mutants (Figure 28B).

M83NH is the only cell with lower early activation marker CD69 and CD25 in CD4+ and CD8+ T cells (Figure 29 A,B,C,D). However, the expression of CD27 shows
that M83NH and M83E/A primarily impact CD4+ T cells (Figure 29 E, F). Furthermore, in populations transduced with the M83NH or the M83E/A construct, there is a considerable increase in CD4+ T cell count (Figure 30).

Various TNFRs have different CD4/CD8 T cell dynamics. The impact of HVEM on the dynamics of CD4+/CD8+ T cells has not been studied. Other TNFRs have been compared. OX40 activity has been revealed to be crucial for activated CD8+ T cell survival, proliferation, and cytokine production (Taraban et al., 2002). OX40 is believed to be more active in CD4+ T cells and 4-1BB in CD8+ T cells (even independently of CD4+ T cell involvement) (Bansal-Pakala et al., 2004; Fujita et al., 2006). In mice, the knockout of TRAF2 and TRAF3 in T cells affects the number and types of CD4+ and CD8+ T cells differently. For example, T-Traf2−/− mice have an average number of CD4+ T cells but 50% fewer CD8+ T cells (Villanueva et al., 2015). The development of CD4+ T cell subtypes depends on TRAF2, while CD8+ T cells are not affected by the loss of TRAF2 for optimal activation and proliferation downstream of TCR signaling (Villanueva et al., 2017). T-Traf3−/− mice have normal numbers of total CD4+ and CD8+ T cells (Xie et al., 2011; Yi, Stunz, et al., 2014) but have an increased frequency of effector/memory CD4+ T cells (Xie et al., 2011; Xie et al., 2007; Yi, Lin, et al., 2014; Yi, Stunz, et al., 2014). This impact of TRAF 3 and TRAF 2 on CD4+ T cells could be due to regulation of the ncNFκB as T-Traf5−/− mice have normal proportions of CD4+ and CD8+ T cells but skew CD4+ T cells toward an activated phenotype (Nakano et al., 1999) (Figure 5).

This work suggests that M83NT mutants could preferentially signal through the ncNFκB pathway due to the loss of TRAF3 and/or TRAF5 binding. Several researchers
have thought that the reduction in the number of naïve CD4+ T cells in T-Traf3−/− mice is due to the loss of Treg-mediated suppression of effector/memory CD4+ T cells (Chang et al., 2014; Yi, Stunz, et al., 2014).

It is possibly not due to the impact of Treg mediation, as the experiment ended two days before many Treg cells could develop. However, it is also possible that the CD4/8 T cell ratios are different in M83E/A because this mutant did not express the same level of CAR as in wild-type GEN 1 or M83.

The E3 ubiquitin ligases are the final enzymes in transferring ubiquitin to substrate proteins, which decides the fate of the modified protein. The E3 ubiquitin ligases add ubiquitin to proteins by covalently modifying one or more lysine residues. E3 ligases play such an important role that their activity must be strictly regulated to ensure that E3 ligases act only when needed. Numerous recent experiments have focused on the inactivation of E3 ligases and the ensuing effects at the molecular or cellular level. Inactivation of E3 ligases has been described using site-specific mutations of critical residues involved in protein association, substrate recognition, or ubiquitin transfer (Garcia-Barcena et al., 2020). However, predicting which mutations prevent a ligase from catalyzing is not always straightforward. Even with an accurate method to determine which residues are ubiquitinated, such as mass spectrometry, it can be difficult. When a ubiquitinated lysine is mutated, another lysine residue within the protein of interest may become ubiquitinated. Also, lysines may be important for protein folding, protein/protein interactions, or as a target for various post-translational changes in addition to ubiquitination (Stringer & Piper, 2011). Mutating a lysine to eliminate ubiquitination in
M83 could function similarly to M83NT because M83NT also lacks lysines (Dejardin, 2006).

**CD4+ CAR-T Cells Increase and Slow Activation in Mutants.**

There are three significant effects resulting from the removal of the tail of M83 from these CAR constructs. First, Figures 32A and 32B show that, in populations that harbor tail constructs, there is an increase in the frequency of CD4+ T cells. The enrichment in CD4+ T cells could be because they are proliferating better, or maybe CD8+ T cells are dying. Second, constructs without tails show a decreased intensity of GFP (Figure 30C). The level of GFP could be construct-specific but also may reflect the extent of mRNA translation worldwide. Third, M83NT has slower activation and progresses slower from naïve CD4+ T cells to later subsets.

Removing the last 15 residues from the C end of the cytoplasmic portion of CD40 completely removed the binding of TRAF3 to CD40 (Leo et al., 1999). As a result, CD40 binds to TRAF2 and TRAF3 in different ways to modulate the intensity of the connections. TRAF3 interacts with CD40 and LTβR receptor residues, creating a hairpin essential for contact with TRAF3 (Ni et al., 2000). Notwithstanding the above, it should be noted that the second generation CAR-T, M83, is followed by CD3ζ, which could change the conformation and function of the M83 tail. In CD40 and wild-type HVEM, the protein's tail is the C terminus. Therefore, the wild-type tail can conformationally move and rotate, while in the M83 CAR-T, the tail is restricted in its movement by CD3ζ.

In many situations, such as Epstein-Barr virus infection (Eliopoulos et al., 2003), cancer (Cormier et al., 2013; Ranuncolo et al., 2012; Vallabhapurapu et al., 2015; Wharry
et al., 2009), and normal lymphoid growth under the supervision of TNFRs (Claudio et al., 2002; Shinkura et al., 1999; Willmann et al., 2014), the ncNFκB pathway increases cell survival. Additionally, ncNFκB signaling was induced by 4-1BB CARs and not by a CD28 CAR after ligand interaction (Philipson et al., 2020). Furthermore, reduced ncNFκB signaling was associated with a significant increase in the abundance of Bim pro-apoptotic isoforms (Philipson et al., 2020). This evidence highlights the importance and non-redundant function of ncNFκB signaling in increasing the survival of CAR-T cells of 4-1BB CAR-T cells (and probably other TNFRs), despite the possibility that there are additional crucial components in exhaustion.

Activation of the ncNFκB pathway is slow (hours scale) compared to the canonical NF-κB pathway, and the ncNFκB pathway requires new protein synthesis to progress (Dejardin, 2006). The ncNFκB pathway contrasts with TNFR receptor-mediated activation of the canonical NF-κB route, which occurs in minutes and does not require new protein synthesis (Vallabhapurapu et al., 2008; Zarnegar et al., 2008). Furthermore, the ncNFκB pathway generally maintains more CD4+ naïve T cells, and its slower metabolism preserves the naïve T phenotype (Sun, 2017). CARs that encourage more and longer persistence of naïve CD4+ T cells could be better CARs than those generated from effector T cells. Therefore, T cells that use the ncNFκB pathway will activate slower and persist longer. Although the experiments described in the current work were not designed to measure persistence, M83NT appears to activate slower (Figure 30).

CD4+ T cells produce more cytokines than those obtained from CD4+ Tcm cells and Tem cells or CD8+ T cells (Sommermeyer et al., 2016). CD4+ T cells dominate and eventually overwhelm the T cell population in long-term investigations of CAR patients
In a recent study using single-cell analysis of two patients ten years after receiving CAR-T cells transduced with mouse FMC63-based CAR that carry 4-1BB and CD3ζ signaling domains and an anti-CD19 scFv, a highly active CD4+ T cell population emerged and became dominant over time, indicating that CAR-T cells evolved. After an initial phase in which CD8+ T cells and gamma delta T cells predominated, CD4+ T cells took over in the remission phase. In one patient, CD4+ T cells finally made up 99.6% of cells, while in the other, they made up 97.6% (Melenhorst et al., 2022). The findings imply that responses to CAR-T cell therapy in these patients can be divided into two phases: the early phase dominated by killer T cells and the long-term remission controlled by CD4+ T cells. These CD4+ T cells continued to kill tumor cells and proliferate with time.

We must be careful not to generalize. It should be noted that this article only looked at two long-term CLL survivors who received FMC63-41BB CARs. There was no control for the composition of the T cell input (i.e., CD4+ to CD8+ T cells after transfusion). How responses proceed in patients with CAR-T cells associated with less persistence, such as CAR-T cells that use CD28 as their co-stimulatory domain, can differ.

M83, in this way, might behave more like 4-1BB. A CD4+ T cell such as that perhaps seen in M83NT could ensure long-term tumor remission if it did not jeopardize the early benefit of CD8+ T cells. M83NT has lower activation and progresses slower from naïve CD4+ T cells to later subsets. Removal of the tail would decrease the number of ready naïve T cells (Figure 30A), reduce the frequency of proliferation of T cells (Figure 30B), and generally slow their metabolism (Figure 30C).
Summary

Three domains of the M83 costimulatory domain are noted to play unique roles and have a critical relevance in their use in a chimeric antigen receptor. A point mutation of E to A in the M83 TRAF binding domain (M83E/A) was sufficient to alter the wild-type phenotype of M83. M83E/A showed poor CAR expression but possibly maintained most of the function of the CD3ζ chain as seen in GEN1. Unexpectedly, removing the long α-helix severely reduced the CAR's function. Finally, removal of the C-terminal tail domain (M83NT) resulted in a functional but nuanced CAR phenotype, as shown by slower activation of wild-type M83 (Figure 30).

There are several possible explanations for the M83NT phenotype. As represented in Figure 31, it is possible that the lack of a tail in M83 acts similarly to CD40 and LTβR. Presumably, the lack of the tail prevents the formation of the TRAF2/TRAF3/cIAP1/cIAP2 complex. This structure would ordinarily limit the activation of the ncNFκB pathway by constitutive NIK proteasomal degradation. The molecular complex that includes NIK does not have to be disrupted by degradation of TRAF3 because TRAF2 and TRAF3 are not recruited to the TNFRs and NIK is constitutively generated. NIK then phosphorylates IKK, which then phosphorylates p100, causing p100 to be processed into p52 and NFκB dimers (such as p52-RelB) to be translocated to the nucleus. TRAF2 or TRAF3 inhibits NIK activity and lowers ncNFκB movement through the pathway in a normal M83 T cell (Vallabhapurapu et al., 2008; Zarnegar et al., 2008).
Figure 31 Mechanism of No Tail Construct (M83NT) on NF-κB Pathways

Ub, ubiquitination.

Future Directions

Coculture of anti-CD19 CAR-T cells bearing modified M83 domains with target cell lines can alter CAR-T activation responses that emerged as a function of specific costimulatory domain modifications. Loss of the membrane-proximal α-helix severely inhibited CAR activation. Mutation of the M83 TRAF binding domain reduced the expression of activation markers relative to wild-type M83 and CAR-T cells without a
costimulatory domain. Deleting the C-terminal tail domain yielded a minimum impact on activation. However, interesting trends in the overall responses of CAR-T culture to target cells can potentially indicate a change in the dynamics of TRAF signaling, suggesting increased non-canonical ncNFκB signaling, which could encourage longer persistence of CAR-T cells. This work indicates that using a truncated M83 as a CAR-T costimulatory domain may improve the persistence and efficacy of CAR-T, but more work is needed.

In addition to the challenges of the variability of culture conditions, reagents, and equipment, there is also variability in donor T cell responses. These experiments showed dramatic donor-derived variation in cytokine expression levels and kinetics. M83 may have more stem or memory-like phenotype generation than GEN1 CAR depending on the donor. To remove this donor variability would require an impractical number of donors. The donor variability we see mirrors a significant challenge of autologous cell therapies in that every patient, and therefore every manufacturing run, has starting material unique from every other patient.

More studies with M83NT mutants should be performed to determine the presence of a population of CD8+ T cells from Tcm (CD4+4hiCD62Lhi). If M83NT reduces the binding of TRAF3, we should expect fewer Tcm cells. Tcm CD8+ T cells have better antitumor immunity and durability than Tem and Teff cells (Wherry et al., 2003). Furthermore, the Tcm/Teff ratio is a biomarker for immunological responses to some malignancies (Reboursiere et al., 2018). It would also be interesting to see if IL-7 and IL-15 can similarly encourage Tcm-like CD8+ T cells, as has been developed for immunotherapy (Araki et al., 2009; Carrio et al., 2004).
Killing of target B cells (Raji) was not determined. The purpose was to study the impact of altered costimulation on the phenotype of T cells, not necessarily the overall performance of the T cell, and to determine which residues of M83 are critical to its enhancement of T cell signaling. Further studies should investigate the targeted killing of Raji by M83NT to see if this mutant can extend the persistence and killing of CAR-T. Furthermore, studies could be performed on the extent of M83 exhaustion by tracking longer-term markers such as PD-1, TIM-3, and LAG-3. Finally, TRAFs affect T-Regs, so they could also be explored.

Furthermore, further studies could be performed to determine whether there is a constitutive nuclear localization of the NF-κB proteins p52 and RelB in M83NT mutants. Finally, it would be interesting to see if M83 without a tail has a slower metabolism, as this could improve the required persistence characteristics of the CAR.
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Appendix

Definition of Terms

• "4-IBB" or "CD137": Molecule of the tumor necrosis factor receptor (TNFR) superfamily expressed primarily in activated T cells.

• "Adoptive T-cell transfer": Adoptive T cell transfer involves the isolation and reinfusion of T-cells into patients to treat disease.

• "Antigen-presenting cells (APCs)": Cells that display antigen complexes with major histocompatibility complexes (MHC) on their surfaces.

• "AP-1": Activator protein-1

• "BLTA": An inhibitory coreceptor of the B cell receptor (BCR) signaling pathway that attenuates the activation of HVEM-recognized B cells.

• "CD-160": Cell surface receptor expressed in NK cells, CD8+ T cells.

• "CD-19": A biomarker for normal and neoplastic B cells. CD19 establishes intrinsic B cell signaling thresholds.

• "CD-28": Protein expressed on T cells that provide co-stimulatory signals required for T cell activation.

• "CD3ζ" or "CD3 zeta": Part of the T cell receptor (TCR) TCR-CD3 complex, which is found on the surface of T-lymphocytes and plays an essential role in the adaptive immune response. TCR-mediated signals are conveyed across the cell membrane by CD3ζ when antigen-presenting cells activate TCR.
• "CD4+ T cells": A kind of T cell that assists other immune cells by detecting foreign antigens and secreting cytokines that activate T and B cells in the immunological response.

• "CD8+ T cells": Type of T cell that binds to other cells through MHC and induces the target cells to undergo programmed cell death.

• "Chemokines": A subfamily of cell signaling molecules or cytokines secreted by cells to induce chemotaxis in nearby cells.

• "Chimeric antigen receptor (CAR)": A recombinant receptor designed to bind to specific antigens in cancer cells and activate downstream signaling that leads to CAR-T cell activation.

• "Construct": Recombinant domain encoding a specific CAR.

• "Co-stimulatory domain": Specific physical region or amino acid sequence in a protein associated with the co-stimulatory signal required for full CAR-T cell activation.

• "CRD": Cysteine-rich domain

• "Cytokine release syndrome (CRS)": an adverse effect that may occur after immunotherapy treatment caused by a significant and rapid release of cytokines (small signaling molecules) into the blood, causing an immune reaction.

• "HVEM", “CD270” or “TNFRSF14”: Herpesvirus entry mediator, TNFRSF14, CD270

• "ICOS": Inducible T cell co-stimulator structurally and functionally related to CD28
• "Lentiviral vector": A modified lentivirus used in genetic engineering to deliver genetic material into a host cell, but it cannot proliferate and cause disease in the host.

• "LIGHT": Secreted protein of the TNF superfamily recognized by HVEM

• "M83": 83 Amino-Acid HVEM cytoplasmic domain

• "MHC": Major histocompatibility complex

• "NF-κB": Nuclear factor kappa-light-chain-enhancer of activated B cells

• "Single-chain variable fragment (scFv)": Fusion proteins of the variable regions of the heavy and light chains of an antibody (VH and VL) connected with a short-linker peptide of 10 to 25 amino acids

• "TNF": Tumor necrosis factor, TNFSF2

• "cIAPs": Cellular inhibitors of apoptosis proteins are critical arbiters of cell death and essential mediators of inflammation and innate immunity.

• "GITR": Glucocorticoid-induced TNFR-related protein

• "LTα": Lymphostxin α recognized by HVEM

• “LTβR”: Lymphostxin-β receptor, TNFRSF3

• "NFAT": Nuclear factor of activated T cell

• "NIK": NF-κB inducing kinase

• "RING": Really Interesting New Gene. Part of the TRAF

• "TANK": Modulator of TRAF signaling that serves as an enhancer or inhibitor of TRAF-mediated signaling pathways.
• “TCR”: T cell receptor is a protein complex found on the surface of T cells that is responsible for recognizing antigen fragments as peptides bound to major histocompatibility complex molecules.

• “TLR2”: Toll/interleukin-1 receptor domain of Toll-like receptor 2)

• “TNFR”: Tumor Necrosis Factor Receptor

• “TNFR-SF”: TNFR superfamily

• "TRAF": TNFR-associated factor. A family of conserved intracellular adapter molecules containing a C-terminal domain that interacts with the tumor necrosis factor receptor superfamily and an N-terminal domain that links receptors to downstream signaling events such as cell survival by activating downstream protein kinase cascades like NF-κB.

• "T-Traf3–/– mice": TRAF3 (CD4+CreTraf3flox/flox) mice conditionally deficient in the indicated TRAF molecule in T cells