ACIBADEM MEHMET ALI AYDINLAR UNIVERSITY

INSTITUTE OF HEALTH SCIENCES

DEVELOPMENT OF PROTEIN MIMICKING FUNCTIONAL PEPTIDES

BETÜL ZEHRA TEMUR

Ph.D. THESIS

DEPARTMENT OF MEDICAL BIOTECHNOLOGY

SUPERVISOR

Prof. Özge Can

SECONDARY SUPERVISOR

Prof. Tanıl Kocagöz

ISTANBUL-2025

ACIBADEM MEHMET ALI AYDINLAR UNIVERSITY

INSTITUTE OF HEALTH SCIENCES

DEVELOPMENT OF PROTEIN MIMICKING FUNCTIONAL PEPTIDES

BETÜL ZEHRA TEMUR

Ph.D. THESIS

DEPARTMENT OF MEDICAL BIOTECHNOLOGY

SUPERVISOR

Prof. Özge Can

SECONDARY SUPERVISOR

Prof. Tanıl Kocagöz

ISTANBUL-2025

DECLARATION

I declare that this thesis work is my own work, I had no unethical behavior at any stages from the planning to the writing of the thesis, I obtained all the information in this thesis in accordance with academic and ethical rules, I cited all the information and comments that were not obtained with this thesis work, and I provided resources in the list of references. I also declare that there was no violation of any patents and copyrights during the study and writing of this thesis.

22/05/2025

Betül Zehra Temur

PREFACE AND ACKNOWLEDGEMENT

Primordially, I would like to thank my supervisor, Prof. Özge CAN, for his constant support, trust, and guidance throughout my Ph.D. journey and daily life. His invaluable insights, knowledge, and constant encouragement have been the driving force behind the completion of this work. It has been an intense pleasure and honor to be his student. I would like to thank my esteemed advisor Prof. Dr. Tanıl KOCAGÖZ, who has provided me with all kinds of support since the day we met, who always believed and trusted that I would succeed. Many thanks to Turkish Scientific and Technical Research Council (TÜBİTAK) for their support through the TÜBİTAK 1003 project no 217S060, TÜBİTAK 1004 project no 22AG030, TÜBİTAK 2244-Industrial-Ph.D. Fellowship Program (Project No: 119C036) collaboration with Acibadem Mehmet Ali Aydinlar University and Atabay Kimya San. Ve Tic. A.Ş. and TÜBİTAK 2214-A International Research Fellowship Program for Ph.D. Students 2023/1 at Carnegie Mellon University. I am also grateful to Prof. Anne Skaja ROBINSON for her kind attitude, support, and hospitality. It was an honor to be a part of her research group. I would also like to thank Sarah SONBATI from the Robinson Lab. I would like to express my gratitude to Dr. Ahmet Emin ATİK for his support and trust. I would also like to thank him for being the hidden force behind my work. I would also like to thank Dr. Zeynep Zülfiye YILDIRIM KELEŞ for her endless support for my thesis. And, special thanks to my lab mates and especially Esma AYBAKAN.

My biggest thanks go to my family who have never withheld their support and have put up with me in every situation. I cannot repay their dedication and effort throughout my life. I would like to express my love to my mother and father, my greatest mentors, who raised me and have always supported me. My siblings, who have always believed in me, especially during the challenging times, have been a constant source of strength and motivation. I thank both of them for being there. Finally, I would like to express my deepest love, gratitude and thanks to my husband,

Sertaç TEMUR, who makes my life beautiful, gives me strength and supports me in every situation.

TABLE OF CONTENTS

ABBREVIATIONS AND SYMBOLS

%	Percentage
µg	Microgram
µl	Microliters
µM	Micromolar
C°	Celsius
DD	Death Domain
DMF	Dimethylformamide
DMSO	Dimethyl Sulfoxide
FBS	Fetal Bovine Serum
FDA	US Food and Drug Administration
F_{norm}	Normalized Fluorescence
HPLC	High Pressure Liquid Chromatography
K_d	Equilibrium Dissociation Constant
kDa	Kilodalton
LC-MS/MS	Chromatography-Tandem Mass Spectrometry
M	Molarity
mAb	Monoclonal Antibody
mg	Milligram

min	Minute
ml	Milliliter
mM	Millimolar
MS	Mass Spectrometry
MST	Microscale Thermophoresis
NF-κB	Nuclear Factor kappa B
PBS	Phosphate Buffered Saline
pH	Potential of Hydrogen
RA	Rheumatoid arthritis
SPPS	Solid Phase Peptide Synthesis
sTNFα	Soluble Form TNFα
TACE	TNFα Converting Enzyme
TFA	Trifluoroacetic Acid
TIS	Triisopropylsilane
tmTNFα	Transmembrane Form TNFα
TNFR1	Tumor Necrosis Factor Receptor 1
TNFR2	Tumor Necrosis Factor Receptor 2
TNFα	Tumor Necrosis Factor-Alpha
TRIC	Temperature Related Intensity Change

α	Alpha

LIST OF FIGURES

LIST OF TABLES

ABSTRACT

Development of Protein Mimicking Functional Peptides

In recent years, there has been increasing interest in the development of alternative molecules that can be used as TNF blockers. In this thesis we focused on peptides that mimic TNFα, TNFR1 and TNFR2. These peptides were designed to inhibit TNFα-receptor binding by different mechanisms. Six peptides (OB1, OB2, OB5, OB6, OB7 and OB8) with specific binding ability against TNFR1, TNFR2 and TNFα were designed and synthesized. Among these peptides, OB1 and OB2 exhibited a stronger binding affinity for TNFα with low dissociation constants (K_d) of 300 nM and 46.7 nM, respectively, compared to previously reported sequences. The synthesized peptides were able to inhibit TNFα both directly and indirectly in in vitro and were effective in L929 cells without causing cytotoxicity. In particular, the OB1 peptide showed a significant reduction in apoptosis in the presence of hTNFα. These results demonstrate that OB1 is a potent inhibitor of TNFα and has potential to inhibit cell death. The peptides developed in this study have the potential to be valuable tools for the therapeutic inhibition of TNF-α and offer a promising approach to the treatment of TNFα-related diseases.

Keywords: Biomimetic peptides, TNFα, TNFα-binding peptides, TNFR-binding peptides, TNFα inhibition

ÖZET

Protein Taklit Eden Fonksiyonel Peptitlerin Geliştirilmesi

Son yıllarda, TNF blokörleri olarak kullanılabilecek alternatif moleküllerin geliştirilmesine yönelik ilgi giderek artmaktadır. Bu çalışmada, TNFα, TNFR1 ve TNFR2'yi taklit eden peptitler üzerine yoğunlaşılmıştır. Bu peptitler, TNFα-reseptör bağlanmasını çeşitli mekanizmalarla inhibe etmek amacıyla tasarlanmıştır. Çalışmada, TNFR1, TNFR2 ve TNFα'ya özgü bağlanma yeteneği taşıyan toplam altı peptit (OB1, OB2, OB5, OB6, OB7 ve OB8) tasarlanmış ve sentezlenmiştir. Bu peptitlerden OB1 ve OB2, daha önce rapor edilen dizilerle karşılaştırıldığında, sırasıyla 300 nM ve 46.7 nM gibi oldukça düşük ayrışma sabitlerine (K_d) sahip olarak, TNFα'ya karşı daha güçlü bir bağlanma afinitesi sergilemiştir. Sentezlenen bu peptitler, in vitro ortamda hem doğrudan hem de dolaylı olarak TNFα'yı inhibe etmeyi başarmış ve L929 hücrelerinde sitotoksisiteye yol açmadan etkili olmuştur. Özellikle, OB1 peptiti, hTNFα'nın varlığında apoptozda önemli bir azalma göstermiştir. Bu bulgular, OB1'in TNFα'ya karşı güçlü bir inhibitör olduğunu ve hücre ölümünü engelleme potansiyeline sahip olduğunu ortaya koymaktadır. Çalışmada geliştirilen peptitlerin, TNFα'nın terapötik inhibisyonu için değerli araçlar olma potansiyeline sahip olduğu ve TNF ile ilişkili hastalıkların yönetilmesinde umut verici bir yaklaşım sunduğu düşünülmektedir.

Anahtar Sözcükler: Biyomimetik peptitler, TNFα, TNFα-bağlanan peptitler, TNFR-bağlanan peptitler, TNFα inhibisyonu

1 INTRODUCTION AND AIM

Tumor necrosis factor-alpha (TNFα) is a potent cytokine that has pro-inflammatory effects and regulates immune system responses. TNFα, which interferes with both acute and chronic inflammation, plays an important role in the pathogenesis of many autoimmune diseases. As autoimmune diseases are self-reactions against the body, overproduction of TNFα is an important factor in the progression of these diseases (1). Crohn's disease, cardiovascular disease, Rheumatoid arthritis (RA), psoriatic arthritis, cancer, Alzheimer's disease, sepsis and other neurodegenerative diseases have been linked to TNFα (2,3).

The effects of TNFα on cells are mainly mediated through cell surface receptors such as TNFR1 and TNFR2. While TNFR1 initiates pro-inflammatory pathways such as apoptosis (cell death), TNFR2 triggers more immunomodulatory and cell survival pathways. While TNFR1 has the ability to transduce apoptotic signals as it contains a death domain, TNFR2 has anti-apoptotic effects by ensuring the survival of immune cells (4,5).

The use of monoclonal antibodies, which now account for the largest share of the biopharmaceutical market, is increasing every day. The first monoclonal antibodies used in clinical practice were of murine origin, and these antibodies had a high potential to induce an immune response in humans. However, the transition from murine monoclonal antibodies to fully human antibodies was made in order to increase the efficacy of treatment and reduce side effects. This transition has led to a significant increase in the market share of monoclonal antibodies (6). Monoclonal antibodies are widely used, particularly in TNFα-neutralizing therapies. Drugs such as Etanercept, Infliximab and Adalimumab play an important role in the treatment of autoimmune diseases by inhibiting the action of TNFα (7). However, these treatments can cause various side effects. These side effects include the reactivation of tuberculosis, development of infections, and autoimmune diseases, and the risk of lymphoma. In addition, these drugs can cause serious toxic side effects such as fever, headache, vomiting, nausea, and low blood pressure (8).

In recent years, therapeutic peptides have become an important area of research. These peptides are effectively used as therapeutic agents due to their high specificity. Peptides produced by chemical and biological methods offer advantages such as high affinity, ability to cross cell membranes, easy to synthesize and modify. However, there are also challenges such as low stability of peptides in blood serum, short half-life and rapid elimination from the body (9). Several strategies have been developed to overcome these difficulties. Methods such as chemical modifications, polymer addition and delivery systems are used to increase the efficacy of peptides. Backbone modifications are used to increase stability and prolong the treatment period. In addition, modification methods such as polymer addition improve the pharmacokinetic profiles of peptides and increase treatment efficacy (10).

Therapeutic peptides are a promising area for the development of new treatment strategies. However, in order to use these peptides effectively, it is necessary to increase their stability and optimize their biological effects. The differences between monoclonal antibodies and peptides play an important role in determining treatment strategies.

The aim of this thesis is to develop functional peptide molecules that mimic proteins to identify candidate therapeutic peptides. As mentioned above, studies have been carried out on peptide sequences that may provide an alternative to monoclonal antibodies. Peptides have been designed to bind to inhibit TNFα and bind to TNFR1 and TNFR2 to prevent TNFα from interacting with these receptors. Peptides that can bind directly to TNFα act as TNFα inhibitors. Peptides that bind to TNFR1 provide indirect TNFα inhibition and are designed to respond to new treatments that ensure the clearance of sTNFR1 from the blood. This is an important step that will contribute to the development of a new generation of treatments. In addition, the TNFR2 receptor does not mediate the death signals associated with TNFα but instead promotes cell survival. Therefore, peptides that bind to TNFR2 indirectly target the elimination of TNFα-associated apoptosis, and these peptides should contribute to therapeutic strategies that specifically promote cell survival and prevent apoptosis.

2 BACKGROUND

2.1 Tumor Necrosis Factor- Alpha

TNFα is one of the most significant and well-characterized cytokines in the TNF superfamily and belongs to the type II transmembrane protein family (1). It is a potent pro-inflammatory cytokine with pleiotropic effects on a diverse range of cell types. It plays a central role in orchestrating a wide range of immune responses, including the induction of fever, the synthesis of acute phase proteins and the enhancement of vascular permeability. In addition, TNFα is involved in the activation and migration of T and B cells and modulates cellular processes such as proliferation and apoptosis. These diverse effects underscore its critical involvement in both the initiation and resolution of inflammation (11,12).

TNFα exists in two distinct forms: soluble and transmembrane forms (13). The transmembrane form of TNFα (tmTNFα), commonly referred to as tmTNFα, is initially synthesized as a precursor with a molecular weight of 26 kDa. This precursor form of tmTNFα is cleaved by the TNFα converting enzyme (TACE) encoded by the ADAM17 gene, resulting in the production of the soluble form (sTNFα) of 17 kDa. The sTNFα is secreted by various immune cells, including monocytes, macrophages and T cells, while the transmembrane form can remain anchored in the cell membrane. Large amounts of sTNF are released in response to lipopolysaccharides and other bacterial products. TNFα, together with other cytokines, is considered to be a key player in the development of septic shock. This widespread production and its role in inflammation highlight the importance of TNFα in both normal immune responses and pathological conditions such as septic shock. Both tmTNFα and sTNFα exert their biological effects through a trimeric structure mediated by interactions with two different receptors, tumor necrosis factor receptor 1 (TNFR1) and tumor necrosis factor receptor 2 (TNFR2) (4,5,14).

TNFα is a potent promoter of inflammation that triggers the production of several inflammatory molecules, including cytokines and chemokines, which are induced by immune cells (15). These TNFα induced chemokines play a critical role in

recruiting immune cells to sites of inflammation, thereby facilitating their accumulation in inflamed tissues. In addition, TNFα activates the Nuclear Factor kappa B (NF-κB) pathway, leading to the maturation of immature dendritic cells into mature antigen-presenting cells. As a result, TNFα is not only essential for the initiation of antigen-specific B and T cell responses but also plays a key role in promoting macrophage differentiation and inducing apoptosis in tumor cells. In addition to its inflammatory effects, TNFα signaling has pathological consequences, including promoting the growth of certain tumor cell types. In addition, TNFα is an important mediator of chronic inflammation, contributing to the onset and progression of several pathological conditions. Its over-activation has been identified as an important factor in the pathogenesis of several autoimmune diseases, where it exacerbates the inflammatory response (16).

The signal generated by TNFα binding to surface receptors triggers the translocation of NF-κB from the cytoplasm to the nucleus. This process regulates the cellular response to TNFα, contributing to the initiation of the inflammatory response. NF-κB proteins are involved in the transcriptional activation of many genes involved in inflammation in response to cytokines such as TNFα and IL-1, bacterial products and different forms of physical stress, including reactive oxygen species and UV radiation (11). The interaction between TNFα and NF-κB is critical for the regulation of inflammation and the body's defense mechanisms. However, when these processes become excessive or uncontrolled, they can contribute to disease progression. Recently, it has also been shown that NF-κB induces the expression of several anti-apoptotic factors that are critical for regulating TNFR1-mediated activation of the apoptotic machinery in the cell. These mechanisms have led to the investigation of NF-κB and TNFα pathways as key biological targets for the treatment of various diseases (17)

2.1.1 Tumor Necrosis Factor Receptor Superfamily: TNFR1 and TNFR2

Members of the TNF family perform their biological functions by interacting with their corresponding membrane receptors, which are part of the TNF receptor (TNFR) family (18). These receptors are characterized by the presence of one to six

cysteine-rich repeats in their extracellular domains, with each repeat typically comprising three cysteine bridges (19). Among the TNFR family, two key receptors, TNFR1 (also referred to as p55 or CD120a) and TNFR2 (also known as p75 or CD120b), bind both membrane-bound TNF (mTNF) and soluble TNF (sTNF), as well as the secreted homotrimeric lymphotoxin-a (LTa). Both TNFR1 and TNFR2 feature four cysteine-rich repeats in their extracellular regions. While the role of the 60kDa TNFR1 in mediating inflammation and immune responses is well-established, the contribution of TNFR2 (80kDa) is often underestimated, as this receptor is primarily activated by tmTNF (20).

In addition, the extracellular domains of both TNFR1 and TNFR2 can be cleaved by proteolytic cleavage to generate soluble fragments of the receptor with a potential neutralizing capacity (14). However, due to the lack of ability for cooperativity in ligand binding, the affinities of these soluble receptors are comparatively lower than those of their membrane-integrated counterparts. This discrepancy in receptor affinity underscores the complexity of TNFα signaling. It suggests that membrane-bound receptors may play a more critical role in the initiation of robust immune responses.

TNFα interacts with two specific cellular receptors, TNFR1 and TNFR2, each of which plays a different role in the immune response. Given the importance of selectively targeting these pathways, focusing on modulating the function of one receptor becomes a critical task. The mechanisms of TNFα-dependent signaling are well understood. TNFR1, which is expressed on almost all nucleated cells, plays a critical role in triggering pro-inflammatory immune responses and the development of inflammation (21). In contrast, TNFR2 is predominantly expressed on immune cells, endothelial cells and neurons, for example macrophages and regulatory T-cells, where it primarily plays an immunomodulatory role (22,23). Upon binding to these receptors, TNFα activates several keys signaling pathways, including activation of c-Jun N-terminal kinase (JNK), mitogen-activated protein kinase (MAPK), extracellular signal-regulated kinase (ERK) and the transcription factor NF-κB, all of which contribute to the complex cellular responses that drive inflammation and immune regulation (24).

There are marked differences within the intracellular domains of these receptors. Receptors contain an intracellular death domain (DD) that is critical for triggering apoptosis and other pro-inflammatory signals. Like other death receptors, TNFR1 can induce cell death in various cell lines through its cytoplasmic death domain, whereas TNFR2 lacks this domain (25). After binding TNFα, TNFR1 undergoes conformational changes that enable it to interact with other death domain containing proteins, such as TRADD (TNF receptor 1-associated death domain protein) (Figure 2.1). This interaction serves as a critical step in the formation of a complex signaling cascade and initiates downstream cellular responses. Binding of TRADD to TNFR1 then triggers the assembly of a multi-protein signaling complex that includes TNFR-associated factor 2 (TRAF2), cellular inhibitors of apoptosis proteins (cIAPs), IκBα kinase (IKK), receptor-interacting protein kinase 1 (RIP1) complex and linear ubiquitin chain association complex (LUBAC) (26). However, TNFR1 has a complex signaling capacity in which its contribution to the modulation of inflammation and the immune response is more critical than apoptosis (14,19). Activation of TNFR1 leads to activation of caspase-8, which in turn activates caspase-3/7, leading to cell death. This process plays an important role in the regulation of inflammation and immune responses, as TNFα can both induce cell death and promote the proliferation and activation of immune cells. The effect of TNFR1 on apoptosis may be critical for the proper functioning of the immune system in conditions such as cancer and autoimmune diseases. Thus, the effect of TNFR1 on apoptosis and inflammation is being investigated as a potential target for the treatment of various diseases.

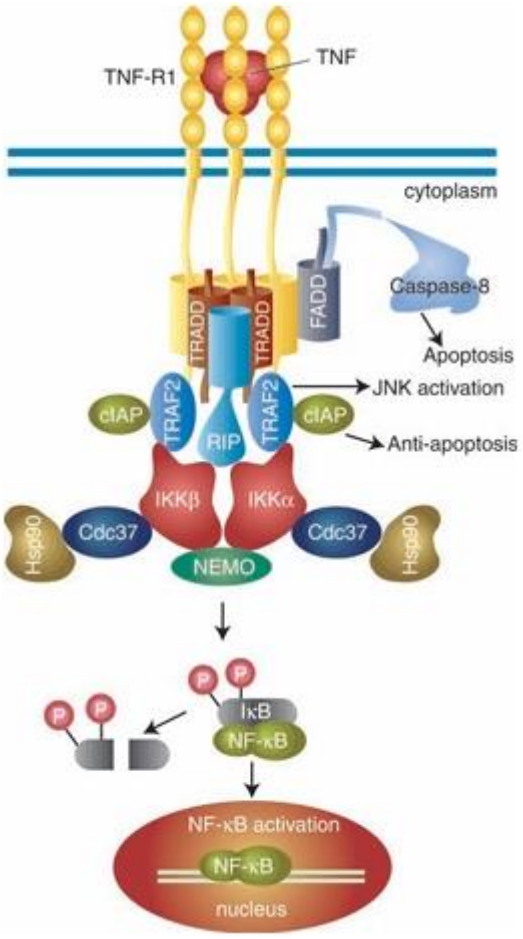

Figure 2.1 Signaling Pathway of TNFα-TNFR1 Interaction (27)

The default signaling pathway activated by TNFα binding to TNFR1 is the induction of the transcription factor NF-κB, which is pivotal for inflammation and cell survival. However, when NF-κB activation is inhibited, TNFR1 can instead induce cell death, including both apoptosis and necrosis. This highlights the dual role of TNFR1 in regulating both cell survival and death, depending on the cellular context and signaling environment (28). Regulation of cytokines and chemokines by TNFR1 is induced by both the tmTNFα and sTNFα forms of TNFα. This regulation in turn activates several downstream signaling pathways, with the most prominent effect being the migration of neutrophils to sites of inflammation (29). TNFR1 also induces the expression of molecules such as ICAM-1 and IL-6, which are involved in immune cell adhesion and inflammation (25,30). On the other hand, TNFR2 plays an essential role in the proliferation signaling of various cells, including thymic cells, natural killer

(NK) cells and lymphocytes (31). These differences highlight the distinct biological functions of each receptor, which are critical to their respective roles in the immune response.

TNFR2 has naturally occurred ligands which are mTNF, sTNF, homotrimer lymphotoxin α (LTα), and soluble and membrane-bound LTα2β heterotrimers. Both sTNF-α and mTNF-α are capable of activating TNFR2, but mTNF-α is a more efficient ligand for full activation of TNFR2. This suggests that TNFR2 primarily triggers cellular responses by binding more strongly to mTNF-α. These interactions can mediate pro-inflammatory effects, but they can also stimulate robust anti-inflammatory activities, depending on the cellular context. One of the key features of TNFR2 is that it lacks a death domain, which distinguishes it from other TNF receptors such as TNFR1, which can directly induce apoptosis. Instead, TNFR2 can interact with TNF receptor-associated factors (TRAFs), which are important in mediating signaling pathways that enhance cell activation and survival. As a TRAF-interacting receptor, TNFR2 favors the activation of cellular responses over the induction of cell death, contributing to its role in immune modulation and inflammation (32,33).

The NF-κB signaling pathway is a important transcription factor that regulates a wide range of cellular responses and plays a pivotal role in inflammation, immune responses and cell survival. TNFR2 is one of the receptors that can activate this pathway. Upon activation, TNFR2 typically induces the transcription of anti-apoptotic and inflammation-related genes, leading to enhanced cell survival, proliferation and robust immune responses. This activation not only promotes immune cell survival but also enhances cellular resistance to apoptotic signals by increasing the expression of anti-apoptotic genes. The ability of TNFR2 to mediate these anti-apoptotic effects is particularly important for the survival of immune cells under stress conditions. Furthermore, these properties of TNFR2 could be utilized in therapeutic strategies, especially in contexts such as cancer treatment and autoimmune diseases, where the enhancement of immune cell survival and the modulation of inflammatory responses are crucial (17,34).

2.2 TNFα Related Disease

TNFα is a cytokine that plays a central role in both acute and chronic systemic inflammation through the initiation of immune responses. It is essential for the regulation of immune cell activation and its production is tightly controlled under normal conditions. However, excessive production or dysregulation of TNF-α has been implicated in the development and progression of a wide range of diseases. In addition to its role in inflammation, TNF-α also stimulates the production and release of other cytokines and chemokines that perpetuate and amplify the inflammatory response, leading to tissue damage and further complications in the body (35).

The immune system, which acts as the body's defense mechanism, can sometimes dysfunction and turn against the body itself, leading to serious illness or even death. This situation is known as autoimmune disease. Autoimmune diseases can affect any organ system in the body, although some organs are more commonly affected than others. Due to its potent proinflammatory and immunostimulatory activities, TNFα serves as a key mediator in the progression of autoimmune diseases. Overexpression of TNFα has been implicated in the development of several immune-related diseases, including RA, psoriatic arthritis, Crohn's disease, and inflammatory bowel disease (2,3). Furthermore, overexpression of TNFα is generally observed in other diseases, such as HIV and asthma, further highlighting its important role in a wide range of inflammatory diseases (36).

In autoimmune reactions following various types of brain trauma, the role of TNFα in the metabolic response is like its functions in other parts of the body. Some effects of TNFα lead to tissue damage. However, TNFα also has a neuroprotective effect in nerve tissue, partly through TNFR2, which contributes to tissue survival. The effects of TNFα on the brain are evident in several diseases in which TNFα plays a role, such as sleep disorders, pain, anorexia and high fever (37).

RA is a chronic autoimmune disease primarily mediated by T cells that results in inflammatory joint damage that is exacerbated by a variety of inflammatory mediators, particularly cytokines (38). In a similar manner, Crohn's disease (CD) is

another chronic inflammatory disease, also mediated by T cells, that affects parts of the gastrointestinal tract (39). TNFα is one of the critical cytokines involved in the progression of these and other inflammatory diseases. It plays a pivotal role by regulating the production of several inflammatory molecules, including IL-6 and IL-12, which contribute to the persistence and severity of inflammation. Because of its central role in the inflammatory process, the inhibition of TNFα has become one of the most widely used therapeutic strategies for the treatment of T-cell mediated diseases, with significant clinical benefits in diseases such as RA and CD (38,40). In addition, TNF-α is a proinflammatory cytokine implicated in a wide range of peripheral inflammatory diseases. A growing body of evidence has shown that TNF-α acts as a potent neuroinflammatory agent within the central nervous system (CNS), and its overproduction has been implicated in neurodegenerative diseases such as Parkinson's disease (PD) and Alzheimer's disease (AD) (41).

Notable examples include RA and inflammatory bowel disease, where significant clinical improvement has been reported when patients are treated with TNF inhibitor agents (e.g. Crohn's disease). Direct inhibition of TNF has become an important approach in the treatment of those diseases. These therapeutic agents have become critical in the treatment of diseases in which TNF-α plays a central role in driving inflammation and disease progression (14,42).

TNF plays a critical role in animal models of chemotherapy-induced septic shock and endotoxin-induced septic shock in advanced lung cancer patients. TNF α is one of the most promising drugs for the treatment of cancer. However, its use in the treatment of human melanoma and soft tissue sarcoma is limited to local perfusion (43,44).

2.3 Monoclonal Antibodies

Antibodies, also known as immunoglobulins (Ig), are used extensively in both research and industry to bind specific molecules. In nature, they have two primary functions: bind to and eliminate antigens such as bacteria or viruses, either individually or in clusters; and facilitate communication within the immune system by attaching to

proteins and activating cell surface receptors, thereby triggering cellular responses (45).

The mammalian IgG structure consists of a 24 kDa light chain and a 55 kDa heavy chain. These chains form three-dimensional structural domains, consisting of two Fab (antigen-binding region) and one Fc (crystallizable fragment) region connected by a region called the hinge. These regions have a structure that can be cleaved by proteases. The Fc region consists of a heavy chain dimer. The Fab region is formed by the combination of heavy and light chains. The heavy and light chain heterodimers are bound together by disulfide bonds (45).

Monoclonal antibodies (mAbs) are antibodies produced by B cells that are specific for a single epitope (46). mAbs which react to a specific antigen, were first produced using hybridoma technology.

Chimeric antibodies have been generated by genetic engineering. Human constant domains have been combined with mouse variable domains. The aim of this strategy is to reduce immunogenicity and immune response in humans. Fully human antibodies have been discovered and produced using this technology. The development of therapeutic antibodies has been further advanced by minimizing the immune response and increasing their efficacy in clinical applications (6,47).

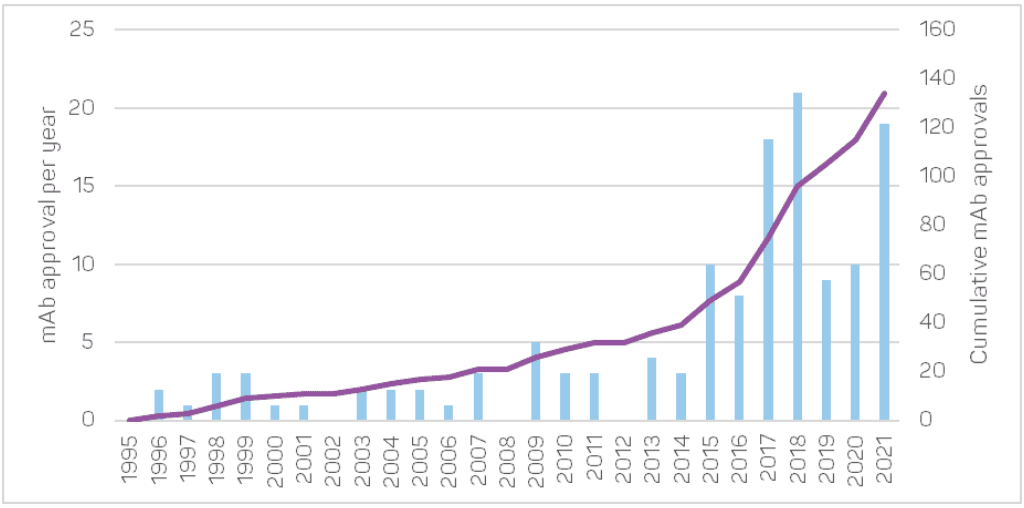

Figure 2.2 Therapeutic antibody approvals by 2021 from EMA

The number of approved antibody therapeutics for the treatment of diseases ranging from cancer to autoimmune and infectious diseases is on the rise. The global therapeutic mAb market has been growing over the years, with the development of new mAbs supporting the growth of this market. Muromonab was introduced in 1986 as the first therapeutic mAb approved by the FDA (48). The global biopharmaceutical mAb market is expected to increase at a growth rate of 11.5% and is estimated to be worth USD 292.22 billion by 2026 (49) (Figure 2.2).

To date, largely due to their unique advantages, including high specificity, biomacromolecular agents such as monoclonal antibodies have proven to be highly effective in the treatment of diseases such as inflammatory bowel disease and RA (50,51). Several important limitations have emerged despite these advantages. These include poor stability, the high cost of large-scale production and the challenge of crossing the blood-brain barrier. In response to these challenges, small-molecule chemical compounds are increasingly recognized as viable alternatives to overcome many of the drawbacks associated with macromolecular inhibitors. In addition, small molecules offer other clinical advantages, such as simpler preparation methods for oral administration, which can improve patient compliance and convenience (52,53).

2.3.1 TNFα inhibitors

In the early 1980s, the genetically engineered products of TNF-α were introduced into the clinical treatment of cancer. Specifically, TNF antibodies and TNFR1-Fc chimeras were developed to prevent TNFα from binding to its receptor, thereby blocking its pro-inflammatory effects (25). While these TNFα inhibitors have shown significant therapeutic potential, they are not without risk. These drugs can cause various side effects, including development of autoimmune diseases, reactivation of tuberculosis, and an increased risk of lymphoma (1). In addition, the use of TNFα inhibitors is associated with serious toxic effects such as fever, nausea, headache, vomiting and hypotension (54,55). Despite their efficacy, these adverse effects have raised concerns about the long-term safety of TNFα inhibitors and underscore the need for continued monitoring.

Several TNF-α antagonists, including Infliximab, Certolizumab, Etanercept, Golimumab, and Adalimumab, have been approved by the US Food and Drug Administration (FDA) and have revolutionized the therapeutic management of several autoimmune diseases, particularly rheumatic inflammatory diseases (7). These drugs work by binding to the TNFα dimer interface, forming a complex that blocks TNFα interaction with the receptor (24). This in turn stops the activation of downstream signaling pathways that typically induce inflammation and contribute to the progression of autoimmune responses. While these biologic agents have significantly improved treatment outcomes, they are primarily proteins or antibodies of higher molecular weight. As a result, they are associated with various side effects, including increased susceptibility to infection, allergic reactions and long-term safety concerns. Despite these challenges, TNFα antagonists remain a cornerstone in the treatment of autoimmune diseases, highlighting the need for continued research to improve their safety profile and efficacy (8,56).

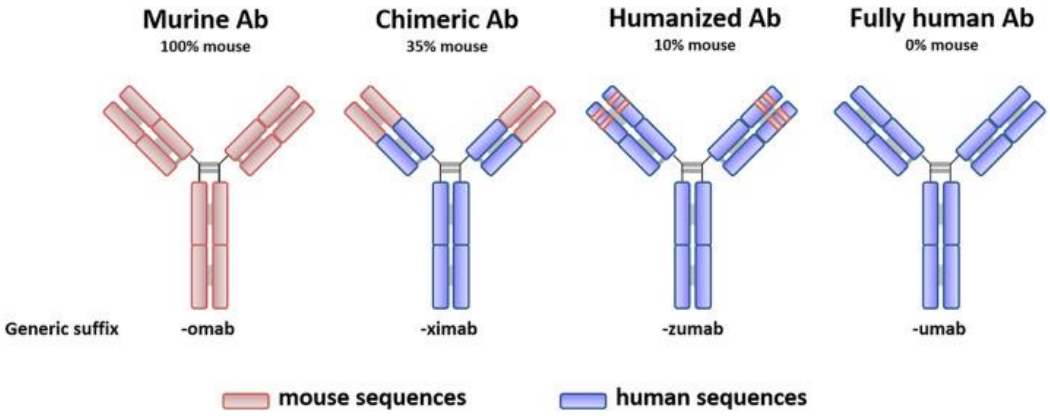

Figure 2.3 Generic Suffix and Sequence Sources of mAbs(57)

TNF-neutralizing therapies, involving the use of recombinant soluble TNFR2-Fc (Etanercept), mouse-human chimeric monoclonal antibodies (Infliximab) and fully human monoclonal antibodies (Adalimumab), have recently been introduced for the treatment of RA and other immune disorders (3). The transition from mouse-derived to fully human antibodies is largely driven by the need to reduce immunogenicity (Figure 2.3). This is due to the fact that mouse antibodies often elicit a stronger immune response in humans, leading to the formation of antibodies against therapeutic agents, which can reduce their efficacy and cause side effects. In contrast, fully human antibodies are more easily tolerated by the immune system and reduce the risk of immune-mediated side effects. In addition, fully human antibodies typically offer greater specificity and affinity for their targets, resulting in increased therapeutic efficacy (40).

The use of monoclonal antibodies has many advantages, but also significant limitations. Monoclonal antibodies are generally therapeutic options that have high production costs and require an advanced laboratory infrastructure, which can make it difficult to reach a large patient population. However, most monoclonal antibodies can trigger an immune response in the body, causing the immune system to react against the treatment. This can have a negative impact on the effectiveness of the treatment and lead to serious side effects. Also, the long half-life of monoclonal antibodies and the fact that they are mostly administered parenterally (by injection) can lead to difficulties with treatment compliance. In addition, some monoclonal antibodies are

only effective against specific targets and may have adverse effects on other biological processes, which requires careful monitoring during treatment. In addition, the use of monoclonal antibodies in the treatment of various diseases, such as cancer and autoimmune diseases, may make patients more susceptible to other infections during treatment (58).

2.4 Therapeutic Peptides

Peptides can act as active regulators and information carriers, making them ideal candidates for drug discovery and development (40,59). Over the past decade, significant progress has been made in peptide drug development thanks to advances in production, modification and analytical technologies. Both chemical and biological methods have been used to produce and modify peptides, along with novel design and delivery strategies. These innovations have helped to overcome the inherent challenges associated with peptide therapeutics, such as stability and bioavailability, and have facilitated the continued growth of the field. Since the synthesis of the first therapeutic peptide, insulin, in 1921, remarkable progress has been made, culminating in the approval of more than 80 peptide-based drugs worldwide (60). This growing portfolio highlights the increasing importance of peptides in modern medicine and their potential to treat a wide range of diseases.

Therapeutic peptides are short chains of amino acids, derived from naturally occurring peptides or synthesized by chemical processes, that exhibit high specificity in their target interactions. These peptides constitute a unique class of pharmaceutical agents typically with molecular weights ranging from 500 to 5000 Da. Due to their precise structure, therapeutic peptides can specifically target biological molecules, offering advantages such as reduced side effects and increased efficacy in the treatment of a wide range of diseases (60).

Therapeutic peptides offer several significant advantages over traditional proteins and antibodies. Their chemical and biological diversity, along with high affinity and specificity for molecular recognition, make them highly effective compared to conventional drugs. Additionally, peptides are small in size, easy to

synthesize and modify, and capable of penetrating cell membranes, which allows them to interact directly with cell surface receptors and trigger intracellular effects (9). These properties give therapeutic peptides a mode of action similar to biologics, including therapeutic proteins and antibodies. However, therapeutic peptides generally exhibit lower immunogenicity compared to biologics, making them a safer option in terms of immune system activation (61,62). When compared to monoclonal antibodies, therapeutic peptides have notable advantages, including lower production costs, reduced immunogenicity, superior tumor penetration, and greater flexibility in terms of structural modification and formulation. These benefits position peptides as an attractive alternative in the development of targeted therapies.

Therapeutic peptides have been extensively researched for their potential applications in areas such as cancer treatment and as antibiotic agents (63). Despite their promising therapeutic benefits, one of the major limitations of peptides is their low stability in blood serum, resulting in a short half-life and rapid clearance from the body. In addition, most therapeutic peptides are administered systemically, which often results in poor patient compliance due to the need for frequent dosing. To overcome these challenges, several strategies have been developed, including chemical modifications to increase stability and incorporation of peptides into specialized delivery systems, which can improve bioavailability and prolong therapeutic efficacy (40).

The identification of potential therapeutic peptides represents the initial stage in the development of peptide-based drugs. Following this, peptides are synthesized through either chemical or biological methods, with subsequent modifications to their sequences aimed at enhancing their pharmacological properties. Notably, the chemical synthesis of peptides, particularly via solid-phase peptide synthesis (SPPS) technology, pioneered by Merrifield in 1963, has undergone significant advancements over time (64). Compared to recombinant methods, peptides synthesized through SPPS are generally more uniform and devoid of unwanted biological impurities such as enzymes, DNA and RNA fragments, or unrelated proteins and peptides.

One of the main reasons for modifying the peptide backbone is to improve its proteolytic stability (10). Backbone modification strategies include the substitution of L-amino acids with D-amino acids (65,66), incorporation of methyl amino acids (65,67) and addition of peptoids (68) and β-amino acids (69). These non-natural amino acids are especially effective when introduced at proteolytic sites within the peptide sequence, as they significantly increase the stability and prolong the plasma half-life of peptide-based drugs. In addition, a common approach to prolonging the half-life of protein therapeutics is the addition of polymers, which can improve the pharmacokinetic profile and overall efficacy of the treatment (70).

3 MATERIALS AND METHODS

3.1 General Procedure For Solid Phase Peptide Synthesis

Functional peptides were chemically synthesized using peptide synthesizer (CEM, Liberty ™ Blue and CEM Discover ™, USA) following standard solid phase peptide synthesis protocol(64) at Acibadem University, Nanobiotechnology Laboratory.

SPPS, peptides were synthesized on resin in a directionality from the C-terminal to the N-terminal. Specifically, Rink Amide resin (CEM, USA), possessing a loading capacity of 0.70 mmol/g, was used for the synthesis of amide end peptides. To prevent unexpected reactions, amino acids which have Fmoc protecting groups (CEM, USA) were used with 0.2 M concentration in dimethylformamide (DMF, Sigma-Aldrich, Germany). To enhance the resin's binding capacity, it was swollen by incubating DMF for 1 hour before synthesis.

The first step of the synthesis, participated in the first amino acid was coupled to resin with its C-terminal (Figure 3.1). To bind the second amino acid, piperidine (Sigma-Aldrich, Germany) solution (1:5; Piperidine: DMF) was used to cleave the Fmoc protecting group from the N-terminal of first amino acid. Activation of the carboxyl group of the second amino acid was accomplished by using Oxyma® (Sigma-Aldrich, Germany) (1.0 M in DMF) and diisopropylcarbodiimide (DIC, Sigma-Aldrich, Germany) (0.5 M in DMF). In the concluding stage of the synthesis cycle, the N-terminal protecting group of the last amino acid was cleaved utilizing the established procedure.

3.1.1 Cleavage

After completion of the synthesis, the resin underwent a washing procedure with dichloromethane (DCM, Sigma-Aldrich, Germany) three times using the Razor (Rapid Peptide Cleavage System) device before cleavage. Subsequently, the peptides were dissociated from the resin, and the side chain protecting groups of amino acids were eliminated utilizing a trifluoroacetic acid (TFA, Sigma-Aldrich, Germany) /

water (H2O) / triisopropylsilane (TIS, Sigma-Aldrich, Germany) solution with a ratio of 95 / 2.5 / 2.5 (v/v/v) at a temperature of 37°C. The duration of cleavage varied based on the quantity of arginine residues within the peptide, ranging from 30 to 45 minutes. Peptides containing three or fewer arginine residues were cleaved for a duration of 30 minutes, while those containing more than three Arg residues required an additional 5 minutes of cleavage for Arg, with a maximum cleavage time of 45 minutes. The cleaved peptides were harvested by precipitation using cold diethyl ether (Sigma-Aldrich, Germany) at -20°C, followed by centrifugation at 4500 rpm for 3 minutes. This process resulted in crude peptide preparation free from residual chemicals. The precipitation procedure was repeated three times, and the final precipitated peptides were dried under a nitrogen stream to ensure their readiness for further purification.

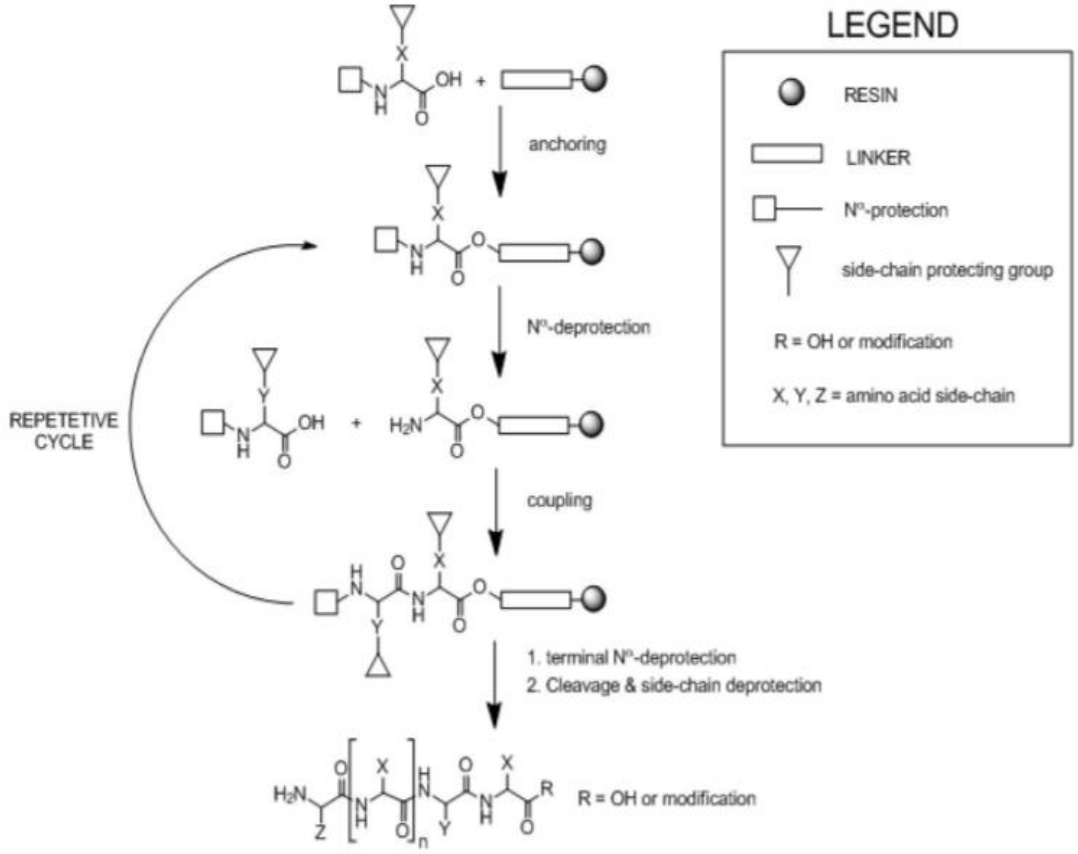

Figure 3. 1 Solid Phase Peptide Synthesis General Metod Diagram (71)

Table 3.1 Synthesized peptide list

Peptide	Sequence	Molecular Weight (g / mol)	Binding Partner
OB1	KGGGSGGGSHIHDDLLRYYGW	2453.62	rhTNFα
OB2	KGGGSGGGSGGGSCRKEMGQV	2074.26	rhTNFα
OB5	NHQVEEQLEWLSQRA	1866.02	TNFR1
OB6	NPQAEGQLQWLNRRA	1779.98	TNFR2
OB7	VLLTHTISRIAVSYQTKVNLL	2368.85	TNFR1
OB8	KRWSRYFWVDMTGTR	2210.52	rhTNFα

In this study, three of the peptides—OB5, OB6, and OB8—were designed and synthesized through molecular modeling, each consisting of 15 amino acids. The remaining three peptides, OB1, OB2, and OB7, were derived by modifying sequences previously reported in the literature and contain 21 amino acids. The peptide sequence 'HIHDDLLRYYGW,' which forms the foundation for OB1, was originally identified through phage display technology by Brunetti et al. (12). While the sequence was initially synthesized in a tetrameric form, we retained it in its linear form and incorporated a lysine residue along with a 'GGGSGGGS' linker at the N-terminus. This modification was made to enhance binding efficiency to an immobilized surface, providing a platform for the development of novel TNFα capture strategies. which is rich in glycine residues, is commonly used to improve peptide solubility. Furthermore, we aimed to standardize the peptide lengths by extending them to comparable sizes, which could potentially increase peptide stability. The use of longer and more flexible linkers is also expected to improve the positioning of the peptide, thereby facilitating more efficient interactions.

The interaction sites of TNFR1 and TNFR2 with TNFα have been the subject of extensive investigation and characterization in various studies. Specifically, the loop region of TNFR1, which spans from residues Arg^{77} to Gly^{81}, has been shown to interact with TNF-α across a broad surface area through Van der Waals forces (36,72,73).

These receptor-ligand binding dynamics indicate that the TNFR1 binding site may serve as a promising structural template for the development of TNFα inhibitors. Building on this structural understanding, OB2, which has sequence of KGGGSGGGSGGGSRKEMGQV, was designed in a manner similarly to OB1, with the incorporation of the RKEMGQV sequence to enhance its binding affinity.

Additionally, OB7 (VLLTHTISRIAVSYQTKVNLL) corresponds to a segment of the TNFα that interacts with TNFR1. Key residues within the TNFα sequence that have been identified as critical for TNFR1 binding include Ala^{84}, Val^{85}, Ser^{86}, Tyr^{87}, Gln^{88} and Thr^{89} (36). Furthermore, additional amino acids— Thr^{77}, His^{78}, Thr^{79}, Ser^{81}, Pro^{90}, Val^{91}, Asn^{92}, and Leu^{93} —have also been reported to play an essential role in TNFα's interaction with TNFR1 (74).

3.1.1 Purification and characterization of synthesized peptides

3.1.1.1. Purification

The peptides underwent analysis and purified using Agilent Technologies 1260 Infinity system. The UV-absorption was measured at 280 nm and 214 nm. The columns used to analyze and purify the peptides were as follows:

Analytical HPLC: Agilent 675950-902 AdvanceBio Peptide Plus, 2.1 x 150 mm, 2.7 µm, LC column.

Semi-preparative HPLC: C-18 column Agilent Technologies VariTide RPC, 250x10mm, 6 µm, ID column.

Analytical high-performance liquid chromatography (HPLC) was performed at a flow rate of 0.2 ml/min, while semi-preparative HPLC was carried out at a flow rate of 1 ml/min. Two distinct mobile phases were utilized during the process: Eluent A, which consisted of 0.05% trifluoroacetic acid (TFA) in water, and Eluent B, which contained 0.25% TFA in acetonitrile (ACN, Merck, Germany). Initially, synthesized peptides were subjected to analytical HPLC analysis over a 30-minute duration, employing a linear gradient from 5% to 80% of eluent B. Afterwards, purification was

undertaken by creating a new method, according to the retention % B. So, the gradient of the solvent was adjusted accordingly to each compound. Following separation, peptides were frozen at -80°C and subsequently lyophilized using a Labconco lyophilizer.

Table 3. 2 Analytical RP-HPLC analysis method

Metod	
Time (min)	Buffer B %
0-5	5
5-35	5-80
35-40	100

3.1.1.2 Characterization

All chemicals and reagents used were of analytical grade unless otherwise specified. HPLC-grade acetonitrile was sourced from Merck (Darmstadt, Germany). Sodium iodide (NaI, ≥99.5%) was obtained from Sigma-Aldrich, while leucine enkephaline (YGGFL-OH) was obtained from Waters Corporation (Milford, MA, USA). Ultra-pure water (18.2 MΩ·cm) was prepared in-house using a Milli-Q water purification system (Merck-Millipore, Darmstadt, Germany).

Liquid chromatography-tandem mass spectrometry (LC-MS/MS) was performed using an ultra-performance liquid chromatography system (Acquity H-Class Bio UPLC) combined with a Xevo® G2-XS QToF MS (Waters, Milford, MA, USA). The system was configured with an electrospray ionization (ESI) source and was operated in positive ion mode with MSE functionality. The mass range for both MS and MS/MS analysis was adjusted to m/z 50-2000. Data acquisition, system control, and processing were performed using UNIFI™ software (version 1.8.2). Prior to analysis, external calibration was performed using a sodium iodide buffer solution (2 μg/μL).

The ESI source was applied under the following conditions: a cone voltage of 50 V, a capillary voltage of 2 kV, a source temperature of 100°C and a desolation

temperature of 300°C. The sampling gas flow rate was maintained at 50 L/h, while the desolation gas flow rate was set at 600 L/h. For MS/MS analysis, the scan time was set to 0.5 seconds for both fragment and precursor ion detection.

For chromatographic separation, a 10 µL portion of the purified peptide sample was applied to a reversed-phase (RP) column (Waters Acquity UPLC Peptide CSH C18, 130 Å, 1.7 µm, 2.1 × 100 mm, PN: 186006937). The separation process used a mobile phase system consisting of ddH_2O (A), 100% ACN (B) and 1.0% formic acid in ddH_2O (C). The peptides were separated at a flow rate of 0.2 mL/min using a programmed linear gradient over 25 minutes. The system was initially held at 5% B for 2 minutes and then gradually increased to 70% B over the next 14 minutes. The column was then rinsed with 90% B for 4 minutes and then reconditioned at 5% B for an additional 5 minutes. Throughout the procedure, the temperature of column was maintained at 40°C and peptide elution was detected by monitoring UV absorbance at 214 nm.

Peptide identification was achieved by matching the measured molecular weight and MS/MS fragment ion patterns with the theoretical sequences. Using MSE acquisition mode, both fragment and precursor ions were simultaneously sampled by alternately applying low and high collision energies in the collision cell of the mass spectrometer. A mass tolerance of 8 ppm was applied for correct peptide assignment, with at least three confirming fragment ions required for verification.

3.2 Binding Affinity Analysis

The NanoTemper Monolith is an instrumental tool designed to measure molecular interactions using microscale thermophoresis (MST). In MST, a ligand-target binding interaction that has reached equilibrium is disrupted by the applying of thermophoresis through exposure with an infrared (IR) laser diode (Figure 3.2). The movement of molecules in response to a temperature gradient can alter to changes in the concentration of fluorescent molecules. The change of the fluorescence signal is measured over a specified duration. The strength of ligand-receptor binding is quantified in terms of the dissociation equilibrium constant (K_d). Non-covalent

intermolecular interactions such as electrostatic interactions, hydrogen bonding, hydrophobic forces and van der Waals forces between the molecules involved influence the binding affinity. This measurement was achieved by combining the fluorescently labeled target and the ligand at varying concentrations, utilizing the protein labeling kit (Red NHS 2nd generation-Amine Reactive). The mixture loaded into device-specific glass capillaries, and the temperature-dependent fluorescence alterations within the device are monitored.

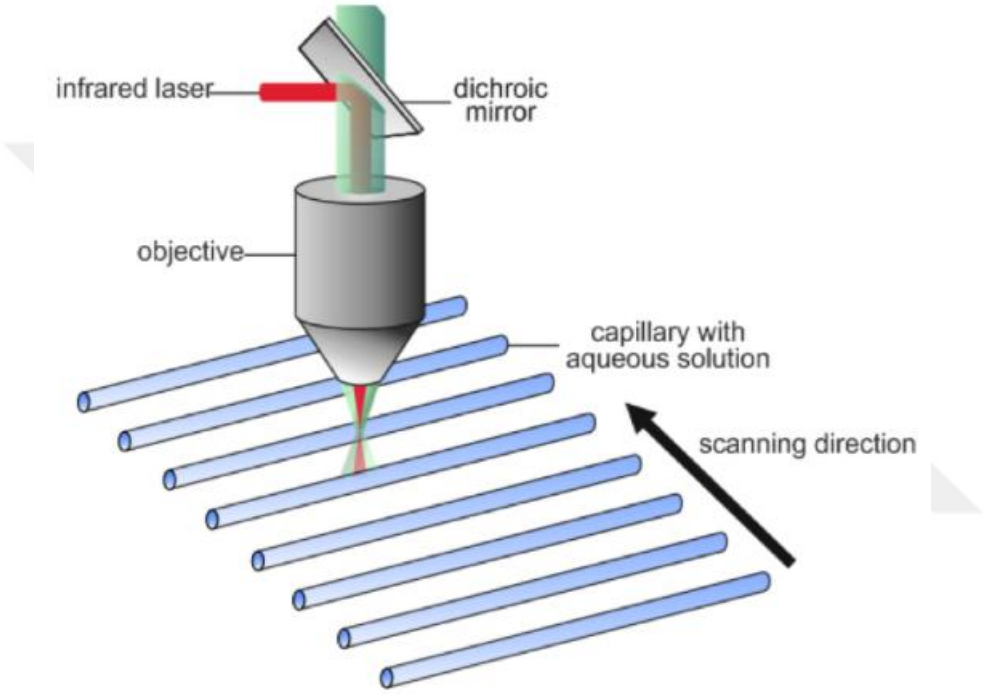

Figure 3.2 Working Principle of Monolith(75)

MST experiments were conducted utilizing a Monolith NT.115 Pico instrument (NanoTemper Technologies, Germany). The experiments employed using the Monolith Protein Labeling Kit RED-NHS 2nd Generation to labelled target molecules. Also, Monolith NT.115 Premium Capillaries (NanoTemper Technologies, Germany) used.

Ligand dependent changes in TRIC (Temperature Related Intensity Change) values are plotted as F_{norm} values versus ligand concentration for binding affinity

analysis. The subsequent Kd analysis is not affected by the 'direction' of the binding curve.

3.2.1 Ligand binding analysis

According to manufacturer guidelines, the fluorescent label was covalently attached to target molecules which could be rhTNFα, TNFR1 and TNFR2. Degree of labelling, which refers to dye: protein ratio, of used labelled proteins are between 0.5 to 1. PBS was used to prepare mixtures of labelled targets and functional peptides. Humira were used with specific citrate/phosphate buffered as a control. Targets were maintained at 20 nM, and the ligand concentrations adjust using the peptide concentration measurement kit (Pierce™ Quantitative Fluorometric Peptide Assay, Thermo Scientific™) and differ according to molecule with 16 serial dilutions. Target molecules and their ligand partners are listed in Table 3.3. Different incubation times were tested, and it was decided to incubate for 20 minutes. Following the incubation period, the prepared samples were transferred into premium glass capillaries, and microscale thermophoresis (MST) measurements were conducted using Pico-RED detection with 80% excitation power and medium MST power settings under thermophoretic conditions. The results were processed in the software M.O. Affinity Analysis v.2.3 (NanoTemper Technologies, Germany) (76,77).

Table 3.3 Target and Ligand Molecules in Binding Affinity Analysis

Target	Ligand
rhTNFα	Adalimumab
	TNFR1
	OB1
	OB2
	OB8
TNFR1	OB5

	OB7
TNFR2	OB6

3.2.2 Competitive binding analysis

The competitive binding assay was performed using the Monolith NT.115 Pico instrument. Unlike the binary binding assay, which uses a fluorescently labelled target and an unlabeled ligand, this two-step competitive binding assay employed a non-labelled target, a ligand and a fluorescently labelled competitor molecule.

In this study, TNFα was selected as the target molecule, TNFR1 as the competitive molecule and OB2 as the ligand. TNFR1 was fluorescently labelled using the Monolith Protein Labeling Kit RED-NHS 2nd Generation. The assay was conducted in two sequential stages. First, the binding affinity between TNFα and TNFR1 was determined. Unlike a traditional binary binding assay, serial dilutions (n = 16) of the unlabeled target (TNFα) were prepared while maintaining the fluorescently labelled competitive ligand (TNFR1) at a constant concentration. These mixtures were then loaded into premium glass capillaries and MST measurements were performed using the Pico-RED detection channel with 80% excitation power and medium MST power settings. Data analysis was performed using M.O. Affinity Analysis software (version 2.3, Nanotemper Technologies, Germany). The K_d obtained in this stage was then used to determine the optimal target-to-competitive ligand ratio for the second stage.

Table 3.4.1 Features of Molecules in Competitive Binding Assay

Unlabeled target	TNFα
Unlabeled ligand	OB2
Fluorescently labelled competitive	TNFR1

In the second stage, the unlabeled ligand OB2 was subjected to serial dilution and a constant concentration of the Target: Competitive Ligand complex, which had

been optimized previously, was added. The samples were then transferred into capillaries and analyzed under the same MST conditions. The half-maximal effective concentration (EC_{50}) was then determined. The inhibition constant (K_i) was then calculated using the K_d from the first stage and the EC_{50} from the second stage according to the following formula:

$$K_i = K_d \cdot \frac{\gamma}{2-\gamma} \cdot \left(\frac{EC_{50}}{[\mathrm{T}]_t - K_d \cdot \frac{\gamma}{2-\gamma} - 0.5 \cdot \gamma \cdot [\mathrm{C}]_T} - 1 \right)$$

$$\gamma = \frac{[\mathrm{T}]_t + [\mathrm{C}]_t + K_d - \sqrt{([\mathrm{T}]_t + [\mathrm{C}]_t + K_d)^2 - 4[\mathrm{T}]_t[\mathrm{C}]_t}}{2[\mathrm{C}]_t}$$

3.3 Cell Based Assays

Murine fibrosarcoma cells (L929) cell line (cat# CCL-1, ATCC) grow as an adherent cell. DMEM (Dulbecco's modified Eagle medium, cat# 11965092, Gibco™, USA) supplemented with 10% heat inactivated FBS (fetal bovine serum, ATCC 30-2020™, USA) and 1% Pen/Strep (penicillin/streptomycin, Gibco™, USA) was used for cell culture (72). L929 cells are used in TNFα related assays due to their TNFα sensitivity. In this assay, L929 cells were used for non-apoptotic and apoptotic cell death.

3.3.1 Morphological analysis of TNFα mediated cell cytotoxicity on L929 cell line

A microscopic examination was carried out to determine the concentration at which TNFα had an almost 100% lethal effect on the L929 cell line. The TNFα neutralizing capacity of the OB1 peptide, along with its potential cytotoxic effects, was further evaluated using fluorescence microscopy to visualize and assess cellular responses (Zeiss AxioVert A, Oberkochen, Germany). 5×10^4 cell/well were seeded on a 24-well plate. 1 µM OB1 peptide, 1 µM OB1, and 1 µM Adalimumab applied with different TNFα concentrations and 2 µg/mL D-Actinomycin (cat. #A9415-2MG, Sigma-Aldrich, USA) O.N.

3.3.2 Cytotoxicity assay of synthesized peptides

L929 cells were seeded with 100 µL DMEM consisting of 10% FBS and 1% Pen/Strep containing 3×10^4 cells in each well of 96-well tissue culture plate, then incubated at 37°C in 5% CO_2 incubator. All samples were diluted triplicate between 10 µM to 1 pM concentration and incubated at 37°C for 24 h in 5% CO_2 incubator. Peptide concentrations adjust using the Pierce™ Quantitative Fluorometric Peptide Assay. After 24 h incubation, cell cytotoxicity was assessed using the MTT cell proliferation assay kit (Cat. No. 11 465 007 001, Roche, Switzerland) according to the manufacturer's instructions. Absorbance was measured at 550 nm with a reference wavelength of 690 nm using a microplate reader for data analysis (Gen5 Synergy HT BioTek) (78).

3.3.3 Neutralization assay of hTNFα

The inhibition of the cytotoxic effect of TNFα by functional peptides was measured in L929 cell line. In the neutralization test 100 µL containing 3×10^4 cells were seeded in each well of a 96-well tissue treated culture plate. After 24 ± 2 h incubation, the culture medium was removed and replaced with medium with 2 µg/mL D-Actinomycin, TNFα (3rd WHO International Standard for cat. # 12/154 (NIBSC)) at 1 ng/mL, and different concentrations between 10 µM to 1 pM Humira and functional peptides for the neutralization test. Peptide concentrations adjust using the Pierce™ Quantitative Fluorometric Peptide Assay. After 24 ± 2 h incubation, the number of surviving cells was determined using the MTT assay as described by the manufacturer (79).

The percent viability was obtained by comparing the viability of unstimulated cells with that of cells stimulated in the absence of a cytotoxic agent. The results were expressed as cell viability (%) and calculated using the formula:

$$100\text{-}\ (100 \times ((\text{Treated cells}_{550nm\text{-}690nm} - \text{Negative control}_{550nm\text{-}690nm}) /$$

$$(\text{Untreated cells}_{550nm\text{-}690nm} - \text{Negative control}_{550nm\text{-}690nm})))$$

3.3.4 Neutralization of hTNFα induced apoptosis

The Caspase-Glo 3/7 Assay is an enzymatic analysis method used to measure the activity of caspase-3/7. The method works by caspase-3/7 cleaving their target substrates. This substrate, proluciferin, contains the DEVD sequence and when this region of the substrate is cleaved by caspase, a molecule called aminoluciferin is released. When aminoluciferin interacts with the luciferase enzyme, the luciferase reaction is initiated in the presence of ATP (Figure 3.3). This reaction results in the production of light. The amount of light produced is proportional to the activity of caspase-3/7, providing information on the degree of apoptosis in the cell. This method offers high sensitivity and accuracy in the measurement of caspase activity and is particularly used in studies investigating apoptotic responses.

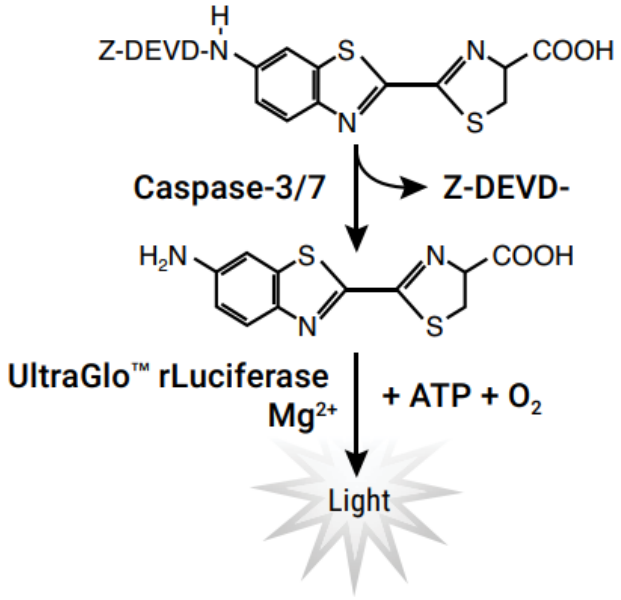

Figure 3.3 Schematic representation of the Caspase-Glo® 3/7 assay technology

The induction of cell apoptosis was evaluated using a luminescence-based assay with the Caspase-Glo 3/7 assay system (cat# G8090, Promega, USA). The L929 cell line was cultured, and 15 × 10^3 cells were seeded into a white 96-well tissue-treated culture plate. For this experiment, DMEM without phenol red and FBS was utilized. Cells were stimulated with 2 µg/mL D-Actinomycin and 1 ng/mL TNFα in the existence of peptides at concentrations of 10 µM, 1 nM, and 0.01 nM, with

subsequent incubation 4 hours at 37°C under 5% CO_2. After the incubation period, freshly prepared caspase-3/7 reagent was added 100 µL per well, and the plate was further incubated for 1 hour at RT. Luminescence readings were obtained with a plate reader (CLARIOstar Plus, BMG Labtech, Germany)(80,81). The fold change in apoptosis was determined by calculating the ratio of luminescence between the untreated cell control and cells treated with 1 ng/mL TNFα.

4 RESULTS

4.1 Purification and Characterization of Synthesized Peptides

The initial analysis of peptides synthesized using the SPPS method was conducted via reverse-phase HPLC (RP-HPLC). The chromatographic profiles of the synthesized peptides are displayed below. Retention times, along with the corresponding percentages of solution B, were established based on a linear gradient elution program. Subsequently, peptide purification was performed using a focused gradient approach, refining the scanned range of solution B to enhance separation efficiency.

The synthesized OB1 peptide sequence is 'KGGGSGGGSHIHDDLLRYYGW' which contains twenty-one amino acids. The HPLC chromatogram of the OB1 peptide is presented in Figure 4.1. The peak, corresponding to a retention time of 19.78 minutes, was successfully purified. During the purification process, only molecules associated with the primary peaks were collected, while minor fluctuations were eliminated, resulting in a 100% pure peptide sequence.

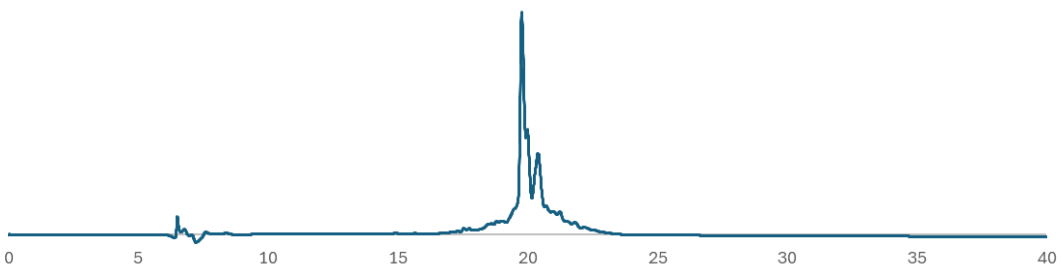

Figure 4.1 HPLC Chromatogram of OB1 peptide

The mass of the [M + H] $^{+}$ ion for the peptide was determined using UNIFI software, yielding an *m/z* value of 2231.0741. The theoretically calculated monoisotopic [M + H] $^{+}$ was 2231.0731, and the margin of error in mass measurement was -0.4 ppm. The MS/MS spectrum of the purified peak further confirmed the successful synthesis of the desired peptide (Figure 4.2).

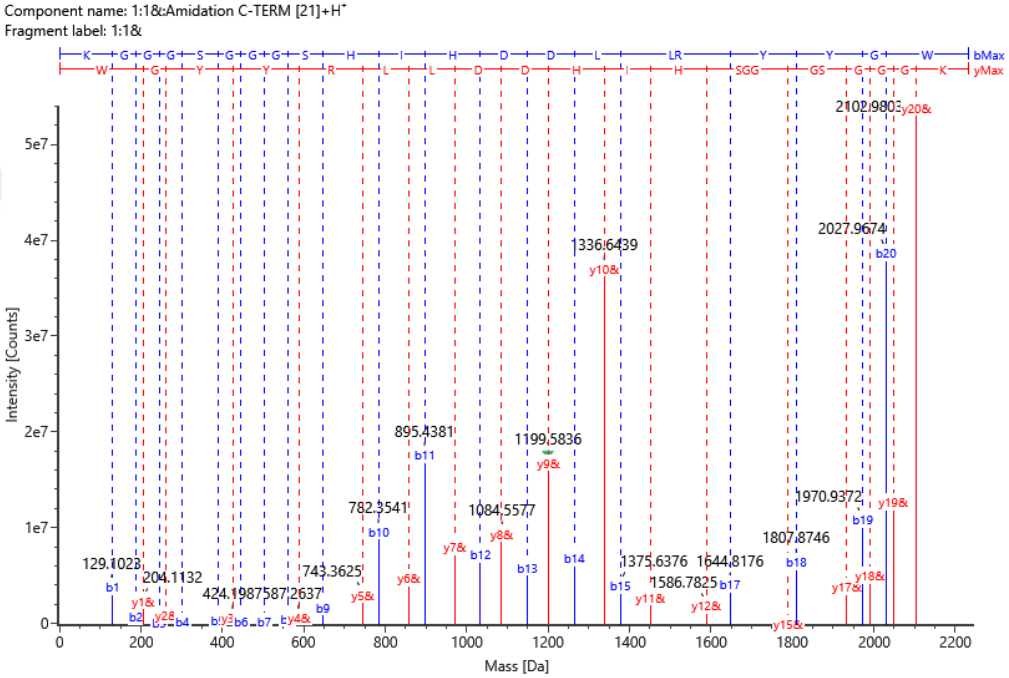

Figure 4.2 MS/MS Spectrum of OB1 Peptide

The synthesized OB2 peptide sequence is 'KGGGSGGGSGGGSRKEMGQV' which contains twenty amino acids. The HPLC chromatogram of the OB2 peptide is presented in Figure 4.3. The peak, corresponding to a retention time of 17.77 minutes, was successfully purified. During the purification process, only molecules associated with the primary peaks were collected, while minor fluctuations were eliminated, resulting in a 100% pure peptide sequence.

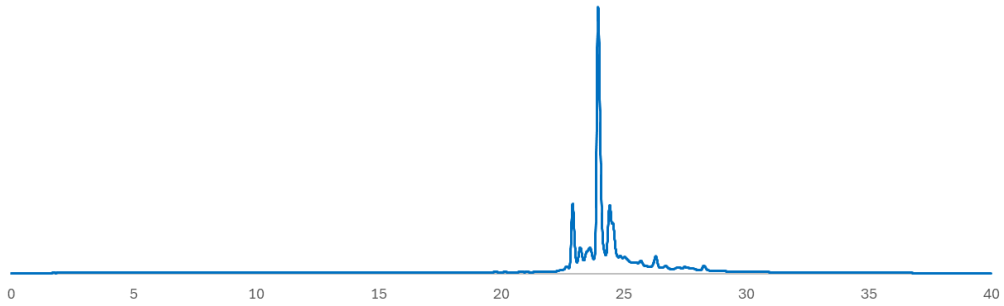

Figure 4.3 HPLC Chromatogram of OB2 peptide

The mass of the [M + H] $^{2+}$ ion for the peptide was determined using UNIFI software, yielding an *m/z* value of 874.92. The theoretically calculated monoisotopic [M + H] $^{+}$ was 1748.84, and the margin of error in mass measurement was 1.0 ppm. The fragmentation ions (b and y ions) obtained as a result of gas-phase cleavage of the peptides (MS / MS) yielded the amino acid sequence of the OB2 peptide (Figure 4.4).

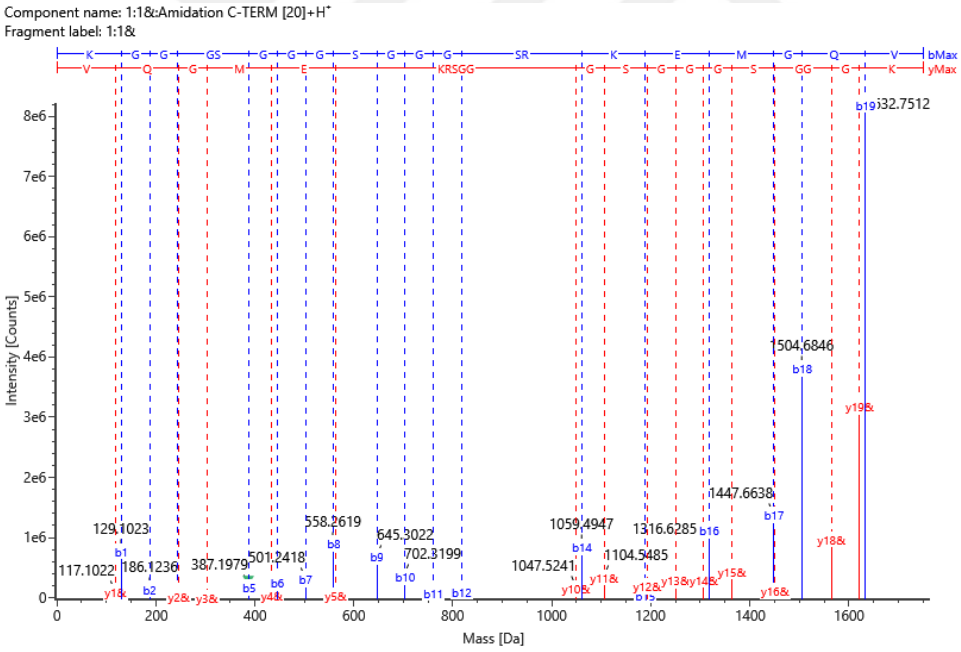

Figure 4.4 MS/MS Spectrum of OB2 Peptide

The synthesized OB5 peptide sequence is 'NHQVEEQLEWLSQRA' which contains fifteen amino acids. The HPLC chromatogram of the OB5 peptide is presented in Figure 4.5. The peak, corresponding to a retention time of 18.75 minutes, was successfully purified. During the purification process, only molecules associated

with the primary peaks were collected, while minor fluctuations were eliminated, resulting in a 100% pure peptide sequence.

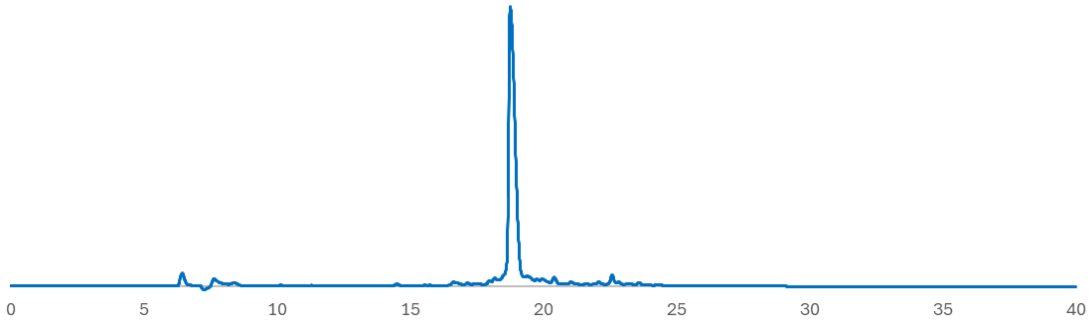

Figure 4.5 HPLC Chromatogram of OB5 peptide

The mass of the $[M + H]^{2+}$ ion for the peptide was determined using UNIFI software, yielding an *m/z* value of 933.95. The theoretically calculated monoisotopic $[M + H]^{+}$ was 1866.90, and the margin of error in mass measurement was -4.6 ppm. The fragmentation ions (b and y ions) obtained as a result of gas-phase cleavage of the peptides (MS / MS) yielded the sequence of the OB5 peptide (Figure 4.6).

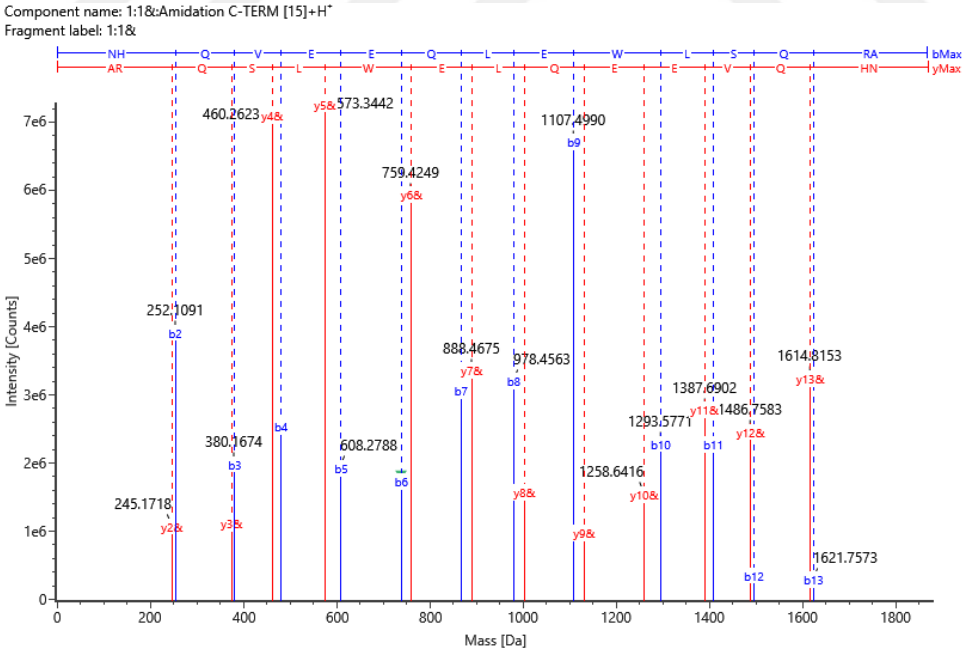

Figure 4.6 MS/MS Spectrum of OB5 Peptide

The synthesized OB6 peptide sequence is 'NPQAEGQLQWLNRRA' which contains fifteen amino acids. The HPLC chromatogram of the OB6 peptide is presented in Figure 4.7. The peak, corresponding to a retention time of 17.65 minutes, was successfully purified. During the purification process, only molecules associated with the primary peaks were collected, while minor fluctuations were eliminated, resulting in a 100% pure peptide sequence.

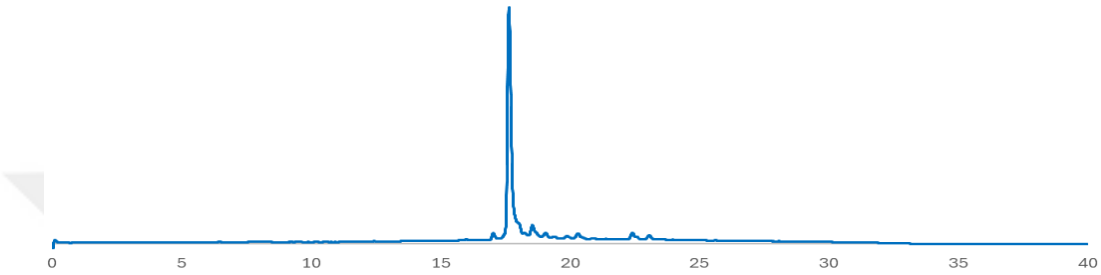

Figure 4.7 HPLC Chromatogram of OB6 peptide

The mass of the $[M + H]^{2+}$ ion for the peptide was determined using UNIFI software, yielding an *m/z* value of 890.47. The theoretically calculated monoisotopic $[M + H]^{+}$ was 1779.93, and the margin of error in mass measurement was 0.1 ppm. The fragmentation ions (b and y ions) obtained as a result of gas-phase cleavage of the peptides (MS / MS) yielded the sequence of the OB6 peptide (Figure 4.8).

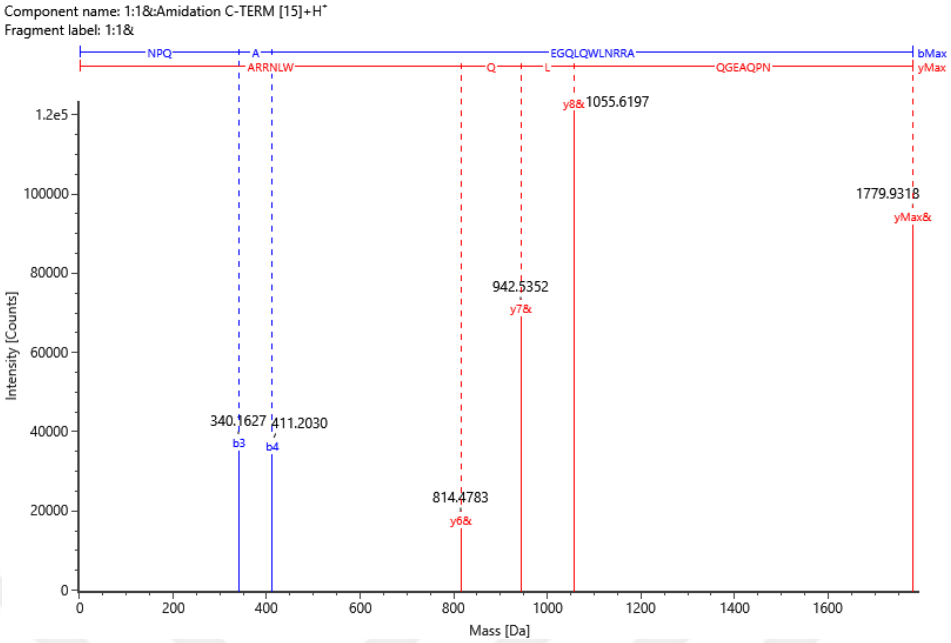

Figure 4.8 MS/MS Spectrum of OB6 Peptide

The synthesized OB7 peptide sequence is 'VLLTHTISRIAVSYQTKVNLL' which contains twenty-one amino acids. The HPLC chromatogram of the OB7 peptide is presented in Figure 4.9. The peak, corresponding to a retention time of 19.65 minutes, was successfully purified. During the purification process, only molecules associated with the primary peaks were collected, while minor fluctuations were eliminated, resulting in a 100% pure peptide sequence.

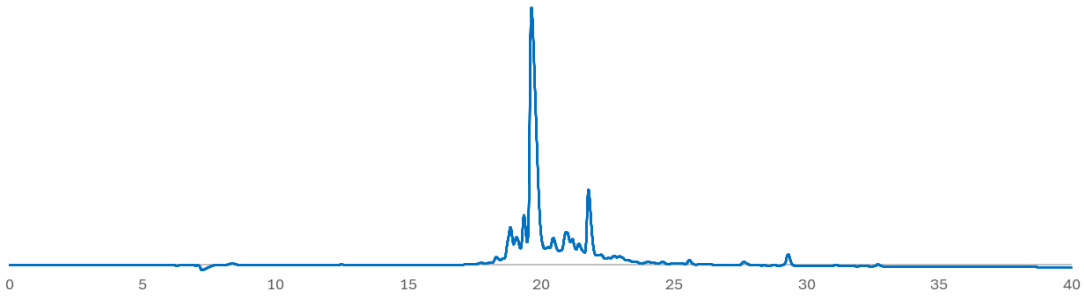

Figure 4.9 HPLC Chromatogram of OB7 peptide

The mass of the $[M + H]^{2+}$ ion for the peptide was determined using UNIFI software, yielding an *m/z* value of 1184.70. The theoretically calculated monoisotopic

[M + H] $^{+}$ was 2368.40, and the margin of error in mass measurement was -4.4 ppm. The fragmentation ions (b and y ions) obtained as a result of gas-phase cleavage of the peptides (MS / MS) yielded the sequence of the OB7 peptide (Figure 4.10).

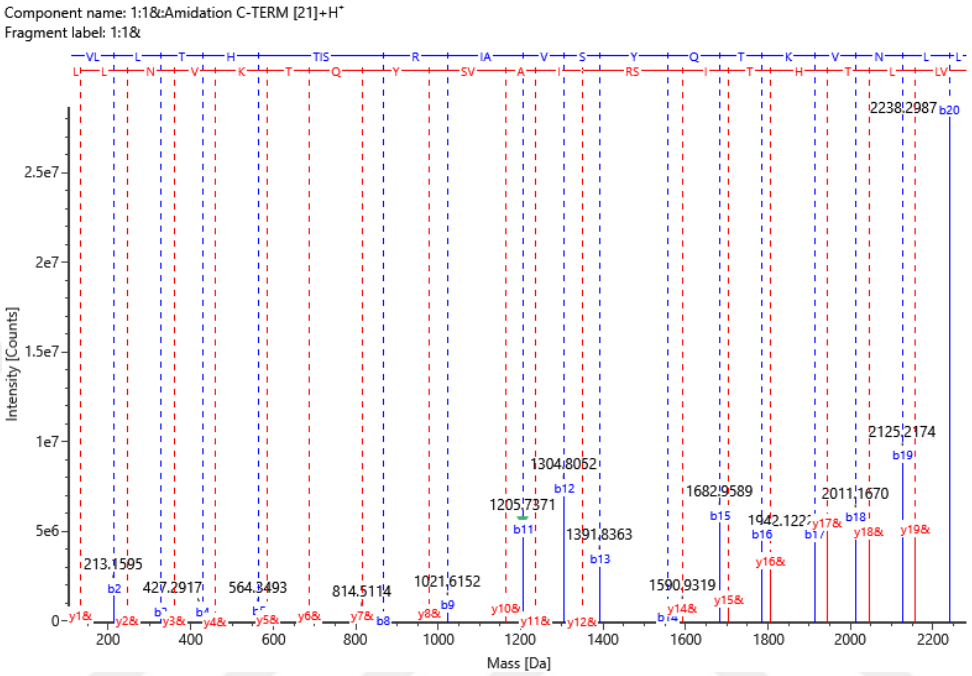

Figure 4.10 MS/MS Spectrum of OB7 Peptide

The synthesized OB8 peptide sequence is 'KRWSRYFWVDMTGTR' which contains fifteen amino acids. The HPLC chromatogram of the OB8 peptide is presented in Figure 4.11. The peak, corresponding to a retention time of 23.40 minutes, was successfully purified. During the purification process, only molecules associated with the primary peaks were collected, while minor fluctuations were eliminated, resulting in a 100% pure peptide sequence.

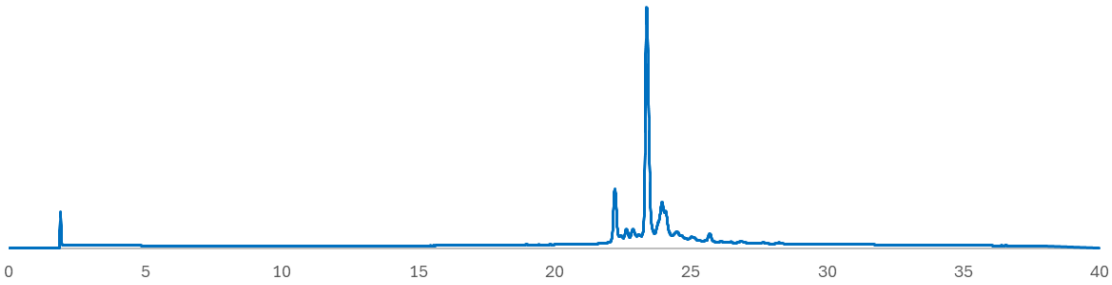

Figure 4.11 HPLC Chromatogram of OB8 peptide

The mass of the $[M + H]^{3+}$ ion for the peptide was determined using UNIFI software, yielding an *m/z* value of 663.34. The theoretically calculated monoisotopic $[M + H]^{+}$ was 1988.00, and the margin of error in mass measurement was 0.9 ppm. The fragmentation ions (b and y ions) obtained as a result of gas-phase cleavage of the peptides (MS / MS) yielded the amino acid sequence of the OB8 peptide (Figure 4.12).

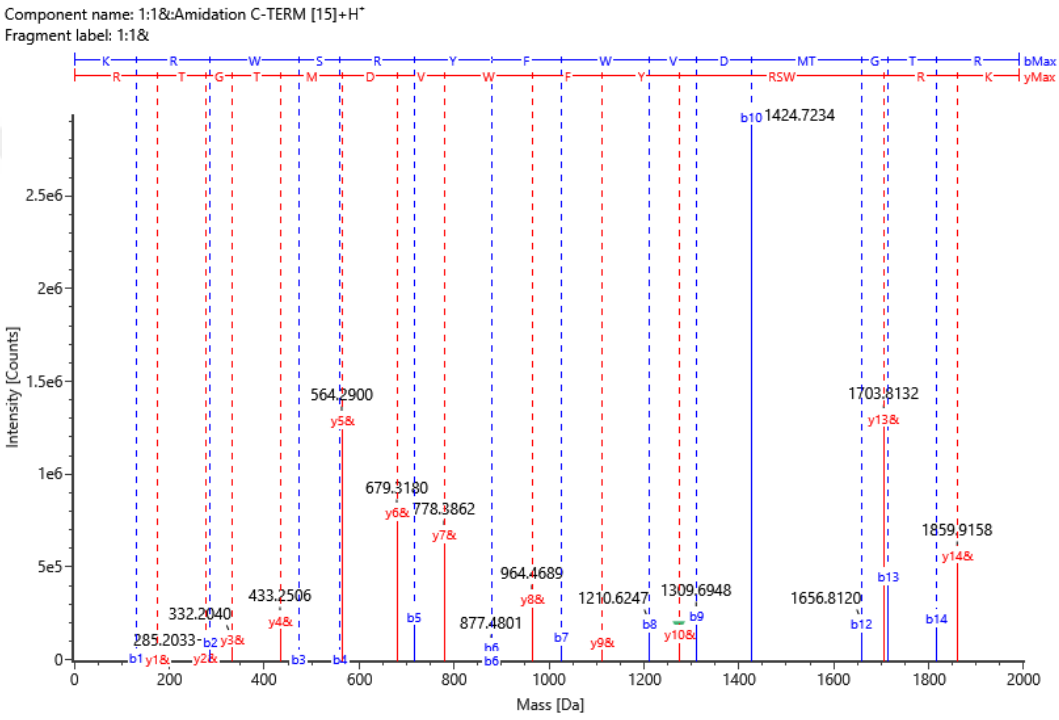

Figure 4.12 MS/MS Spectrum of OB8 Peptide

4.2 Binding Affinity Analysis

4.2.1 Interaction of designed peptides with ligands

The binding affinities of the designed peptides and ligands were determined by MST as described in the Methods section. Target molecules and their ligand partners are listed in Table 3.3. Instead of synthesized peptide molecules, rhTNFα, TNFR1 and TNFR2, which are large molecules and can bind to more than one molecule, are fluorescently labelled. MST data were processed and analyzed using M.O. Affinity

Analysis software version 2.3. Dissociation constant (K_d) for the specified interactions is reported as the mean ± standard deviation (SD) of replicate measurements and shown in Table 4.1. The binding curve is modelled by a sigmoidal dose-dependent binding curve, illustrating changes in signal relative to ligand concentration. The binding curves, representing the mean ± SD of three independent replicates, are presented in Figure 4.7, Figure 4.8, Figure 4.9, and Figure 4.10.

Table 4.1 K_d of indicated rhTNFα interactions representing mean ± SD of three independent replicates

Binding Partners	**K_d Value (M)**
Adalimumab – rhTNFα	21.13 ± 3.6E-12
OB1 – rhTNFα	300± 4.85E-09
OB2 – rhTNFα	46.7 ± 5.46E-09
OB8 – rhTNFα	3.89 ± 4.5E-06

Figure 4.7 shows the binding curve of Adalimumab – rhTNFα interaction as a standard for comparison. As a result of analysis of the binding curve, the K_d value was calculated as 21.13 ± 3.6E-12 M (Table 4.1.). This value indicates that the binding between Adalimumab – rhTNFα is of high affinity.

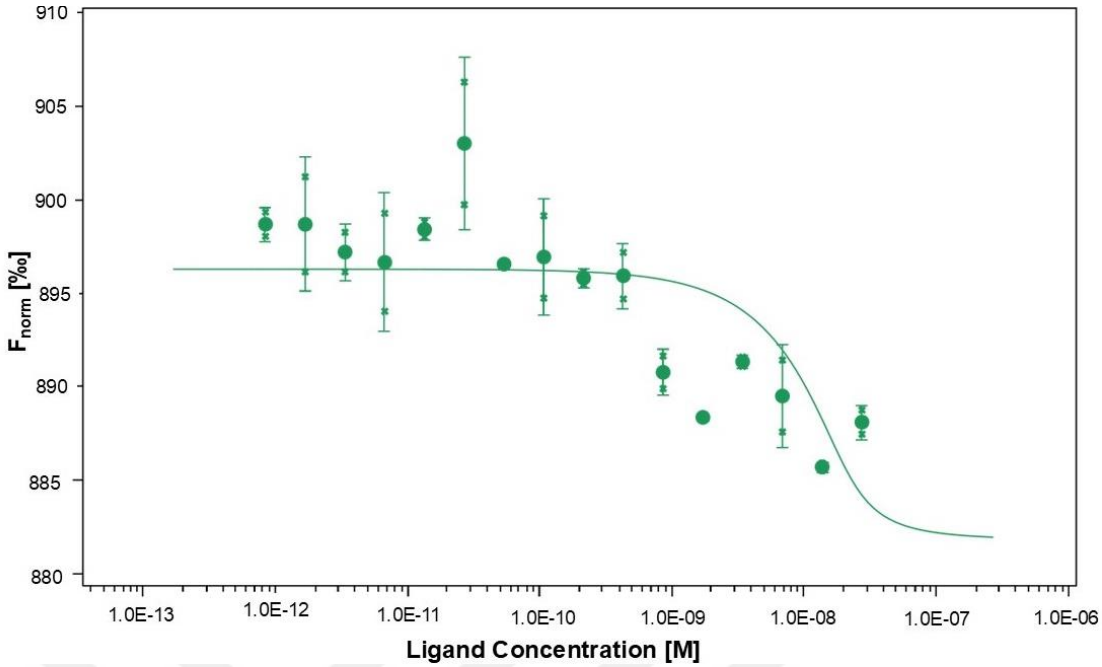

Figure 4.13 Binding curve of the Adalimumab towards rhTNFα via microscale thermophoresis.

Figure 4.8 shows the binding curve of OB1 – rhTNFα interaction as a standard for comparison. As a result of analysis of the binding curve, the K_d value was calculated as 300± 4.85E-09 M (Table 4.1). This value indicates that the binding between OB1 – rhTNFα is of medium affinity.

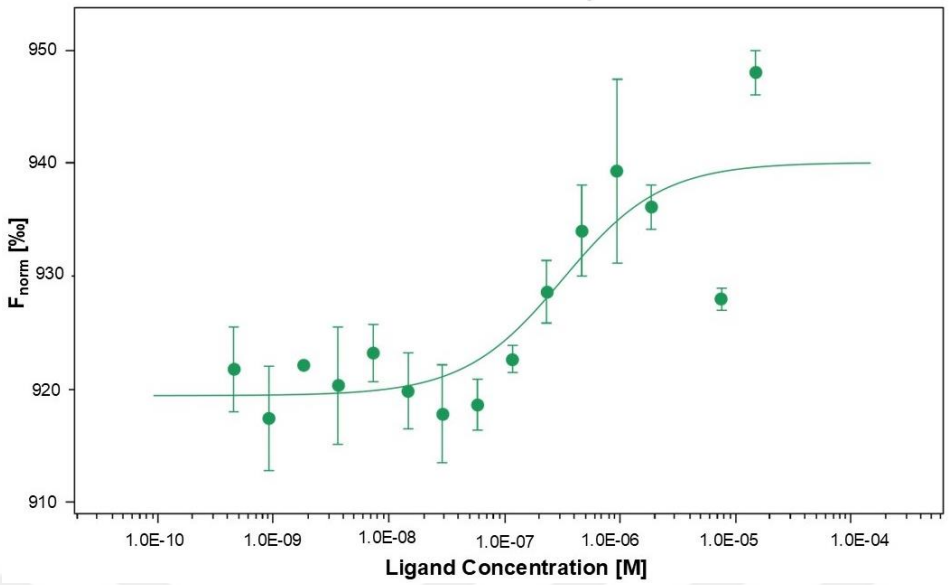

Figure 4.14 Binding curve of the OB1 towards rhTNFα via microscale thermophoresis

Figure 4.9 shows the binding curve of OB2 – rhTNFα interaction as a standard for comparison. As a result of analysis of the binding curve, the K_d value was calculated as 46.7 ± 5.46E-09 M (Table 4.1). This value indicates that the binding between OB2 –rhTNFα is of high affinity.

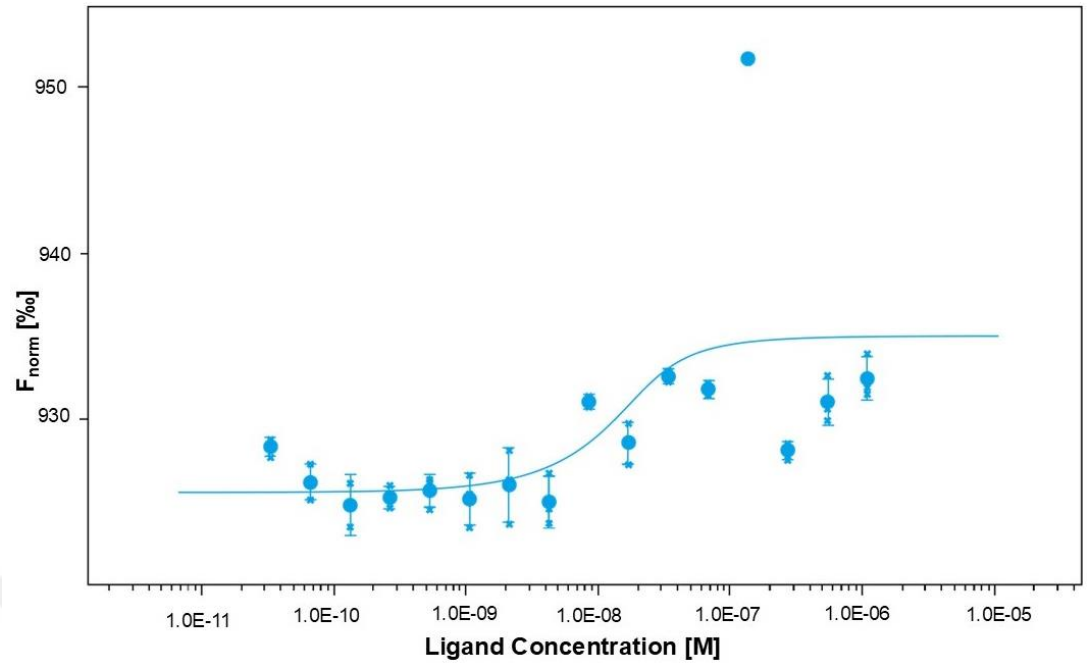

Figure 4.15 Binding curve of the OB2 towards rhTNFα via microscale thermophoresis

Figure 4.10 shows the binding curve of OB8 –rhTNFα interaction as a standard for comparison. As a result of analysis of the binding curve, the K_d value was calculated as 3.89 ± 4.5E-06 M (Table 4.1). This value indicates that the binding between OB8 –rhTNFα is of low affinity.

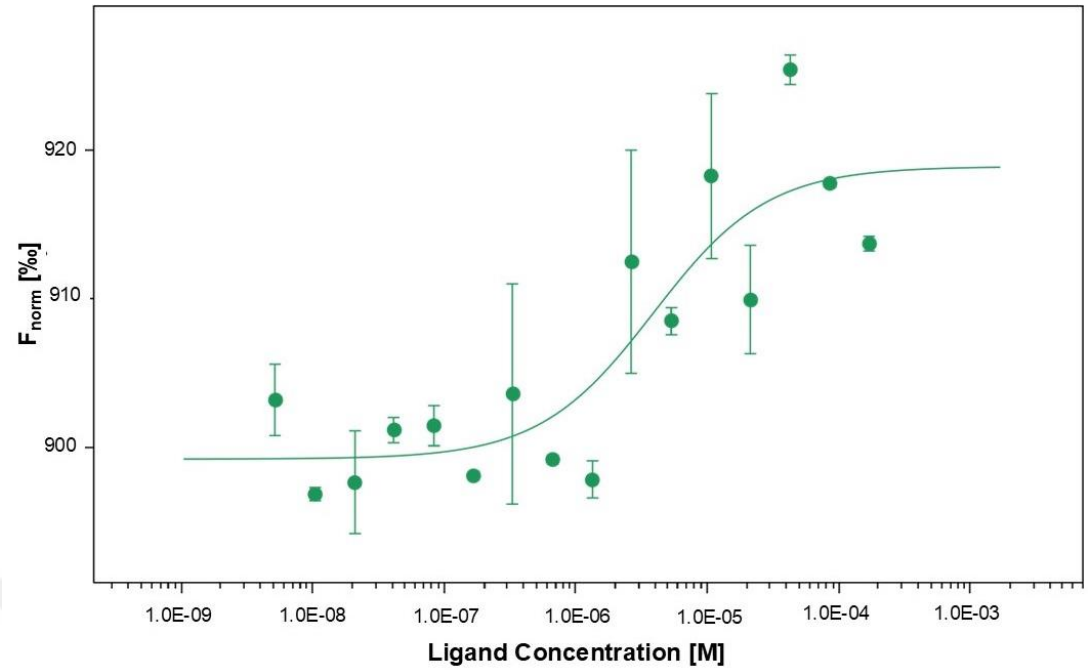

Figure 4.16 Binding curve of the OB8 towards rhTNFα via microscale thermophoresis

Table 4.2 K_d of indicated TNFR interactions representing mean ± SD of three independent replicates

Binding Partners	**K_d Value (M)**
rhTNFα – TNFR1	4.94 ± 3.92E-09
OB5 – TNFR1	204.28 ± 4.32E-09
OB6 – TNFR2	3.9 ± 49.95E-06
OB7 – TNFR1	8.6 ± 17.4E-06

Figure 4.11 shows the binding curve of rhTNFα – TNFR1 interaction as a standard for comparison. As a result of analysis of the binding curve, the K_d value was calculated as 4.94 ± 3.92E-09 M (Table 4.2). This value indicates that the binding between rhTNFα – TNFR1 is of high affinity.

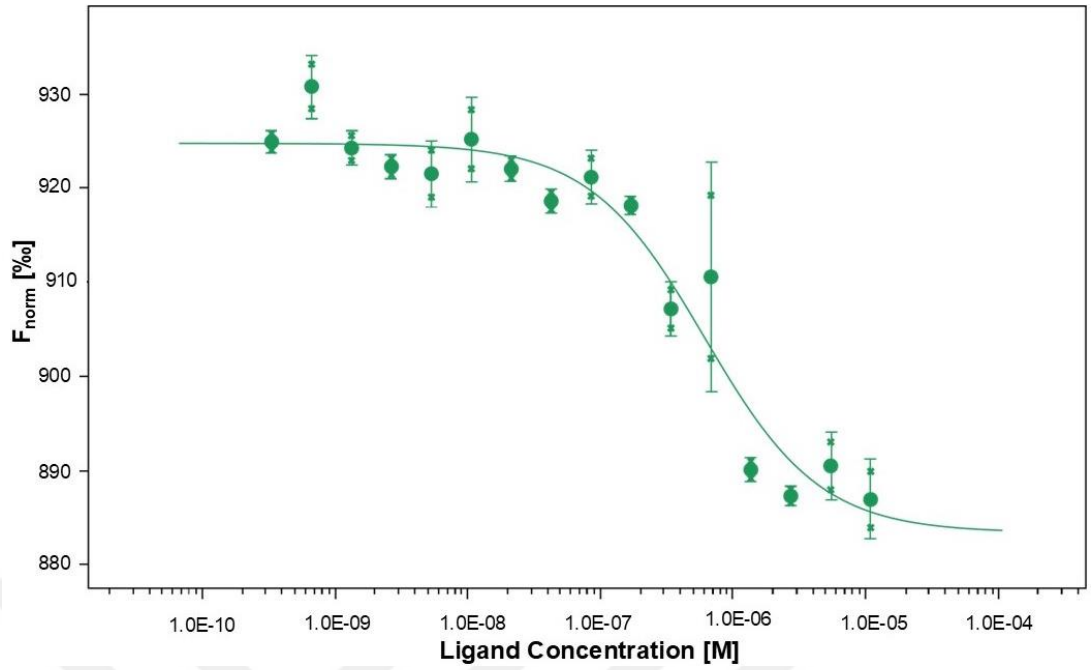

Figure 4.17 Binding curve of the rhTNFα towards TNFR1 via microscale thermophoresis

Figure 4.12 shows the binding curve of OB5 –TNFR1 interaction as a standard for comparison. As a result of analysis of the binding curve, the K_d value was calculated as 204.28 ± 4.32E-09 M (Table 4.2). This value indicates that the binding between OB5 –TNFR1 is of low affinity.

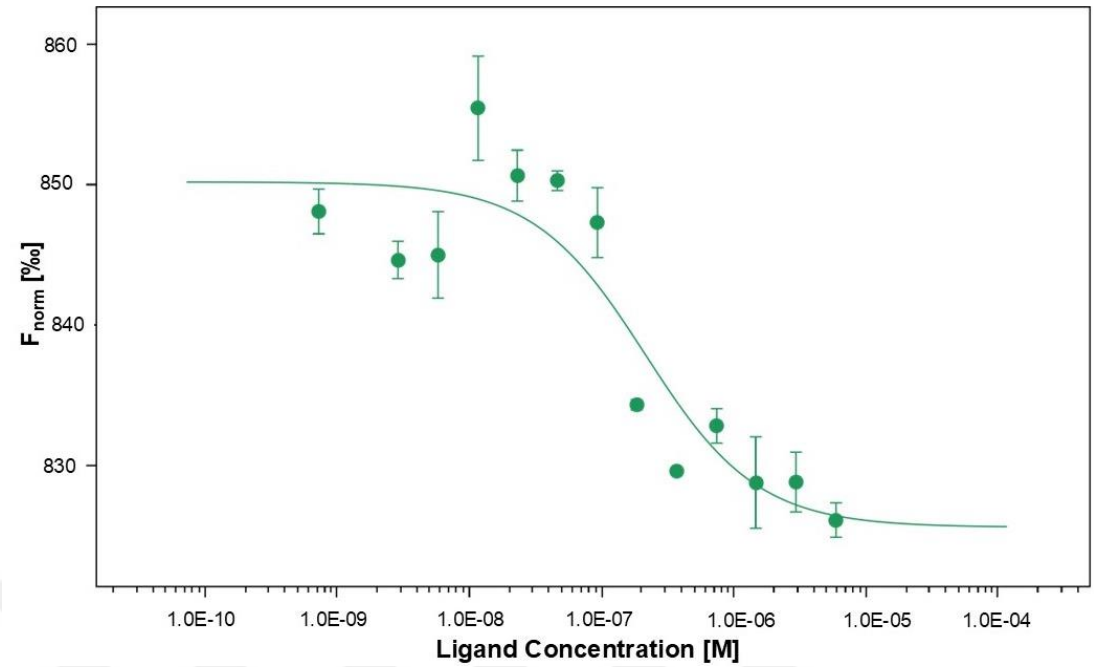

Figure 4.18 Binding curve of the OB5 towards TNFR1 via microscale thermophoresis

Figure 4.13 shows the binding curve of OB6 –TNFR2 interaction as a standard for comparison. As a result of analysis of the binding curve, the K_d value was calculated as 3.9 ± 49.95E-06 M (Table 4.2). This value indicates that the binding between OB6 –TNFR2 is of low affinity.

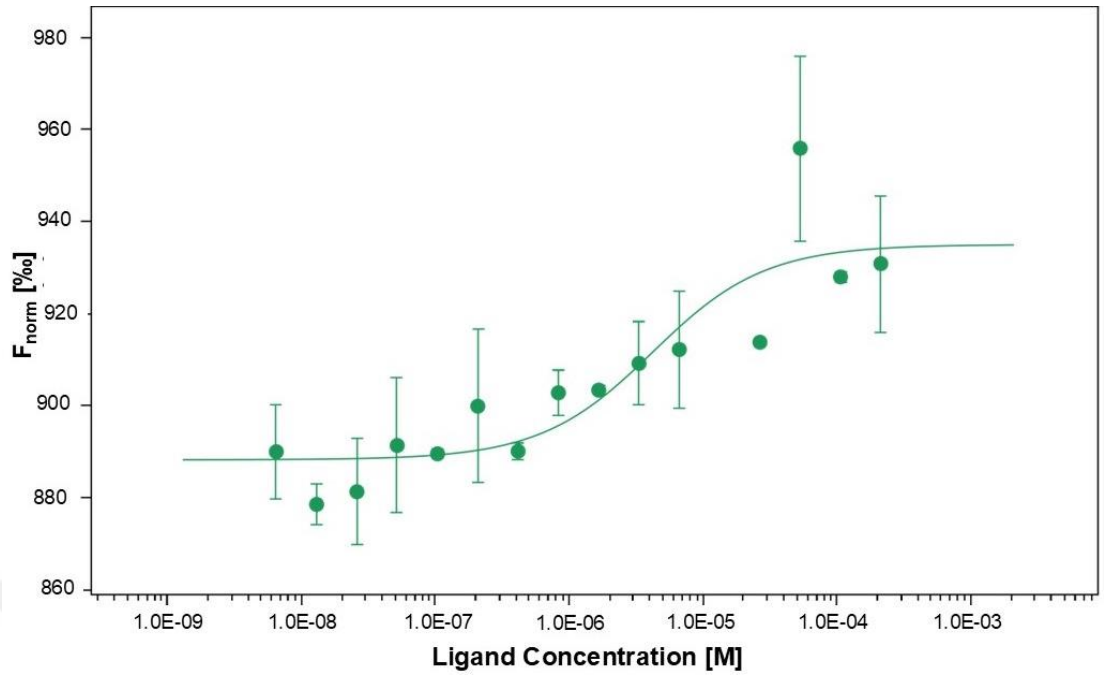

Figure 4.19 Binding curve of the OB6 towards TNFR2 via microscale thermophoresis

Figure 4.14 shows the binding curve of OB7–TNFR1 interaction as a standard for comparison. As a result of analysis of the binding curve, the K_d value was calculated as 8.6 ± 17.4E-06 M (Table 4.2). This value indicates that the binding between OB7–TNFR1 is of low affinity.

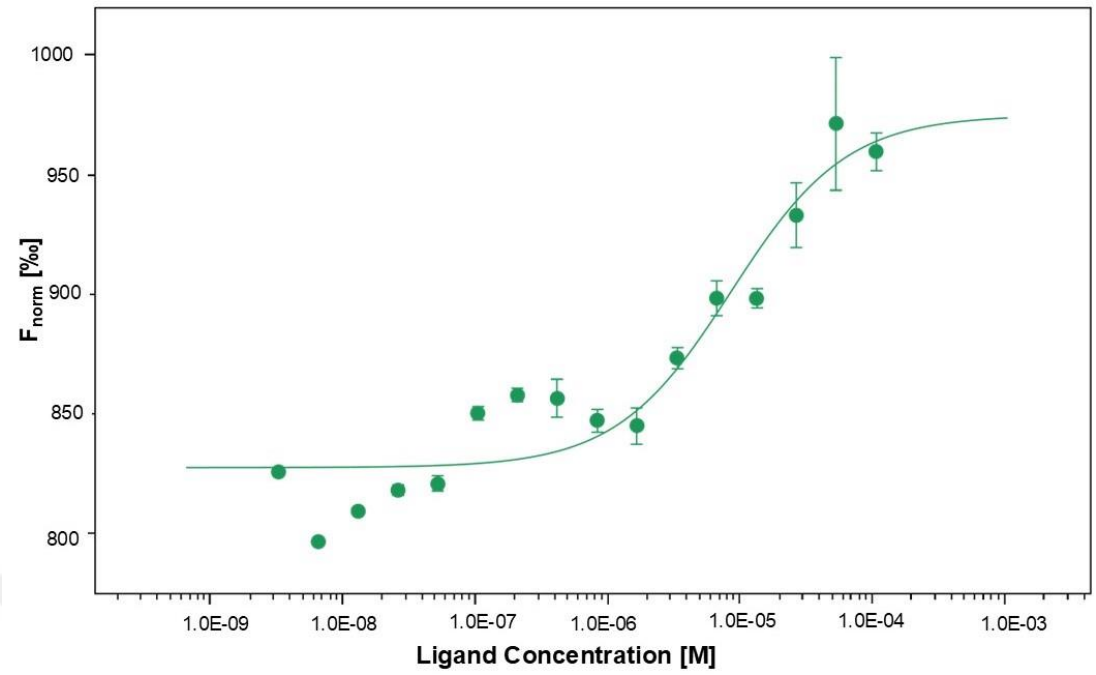

Figure 4.20 Binding curve of the OB7 towards TNFR1 via microscale thermophoresis

4.2.2 Competitive binding analysis of OB2

The competitive binding affinities of the OB2 peptide and TNFR1 were determined by MST as described in the Methods section. Table 3.4 shows the molecules used and their properties. MST data were processed and analyzed using M.O. Affinity Analysis software version 2.3. Dissociation constant (K_d) for the specified interactions is reported as the mean ± standard deviation (SD) of replicate measurements. The binding curve is modelled by a sigmoidal dose-dependent binding curve, illustrating changes in signal relative to ligand concentration. The binding curves, representing the mean ± SD of three independent replicates, are presented in Figure 4.21, Figure 4.22.

Figure 4.21 indicates that binding affinity of labelled competitive TNFR1 and unlabeled target TNFα as a first stage of competitive binding. K_d was calculated 4.9 nM from this interaction.

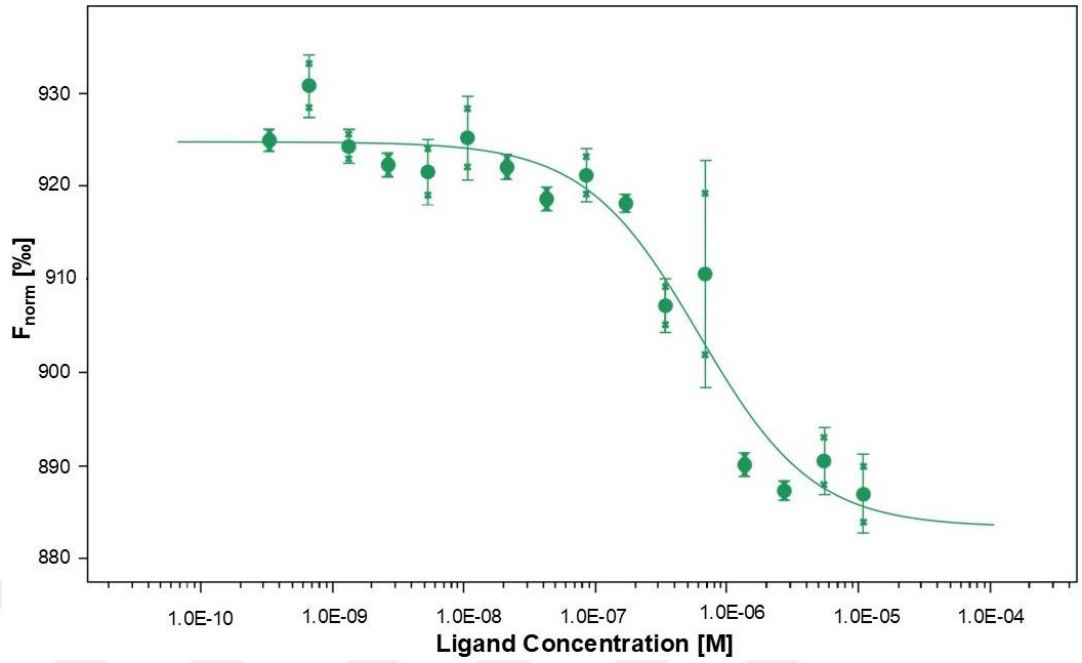

Figure 4.21 Binding curve of the TNFR1 towards TNFα via microscale thermophoresis

In the second stage, OB2 was expected to replace the TNFR1 molecules and bind to TNFα. The EC50 value was determined to be 59nM. The K_i value was calculated from the K_d and EC_{50} values (K_i = 29.3nM). According to these results, OB2 is competitive with the TNFR1 molecule.

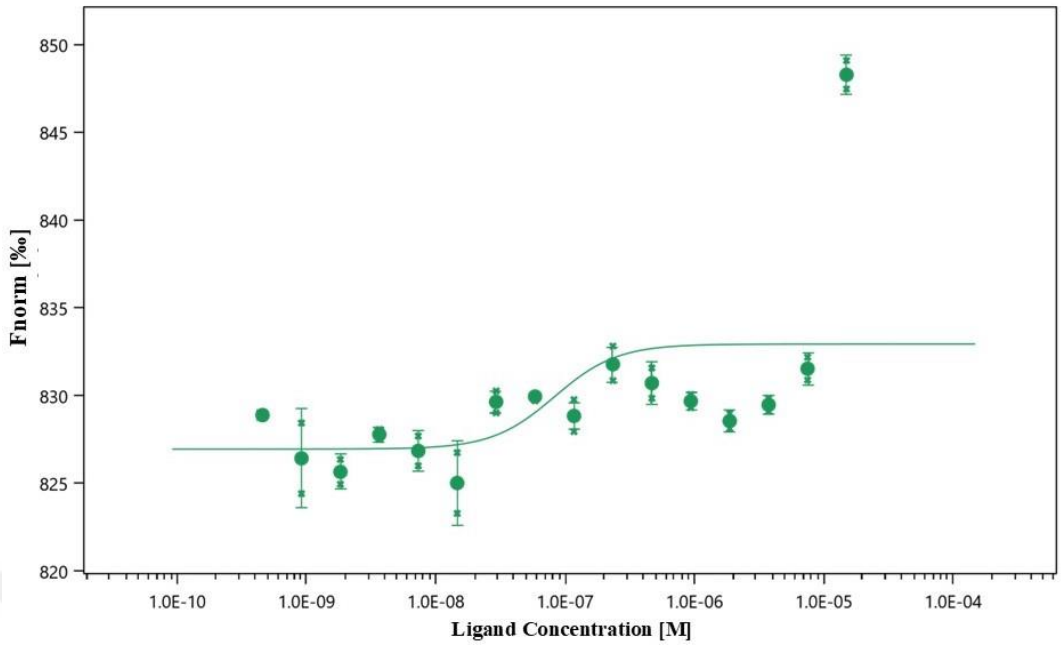

Figure 4.22 Binding curve of the competitive binding of OB2 towards TNFα via microscale thermophoresis in competition with TNFR2

4.3 Cell Based Assays of hTNFα Neutralization

The binding affinities of the synthesized peptides to their respective targets were assessed using microscale thermophoresis (MST). In further experiments, it needed to be shown that these peptides also bind their target in cell culture on L929 cell line.

4.3.1 Morphological analysis of TNFα mediated cell cytotoxicity on L929 cell line

Morphological analysis of L929 cell line for performed experiments was examined with fluorescence microscope. Microscopic examination was first used to determine the concentration at which TNFα was almost 100% lethal to the L929 cell line. In Figure 4.21, DMEM treated cells are given as control image of 100% survival.

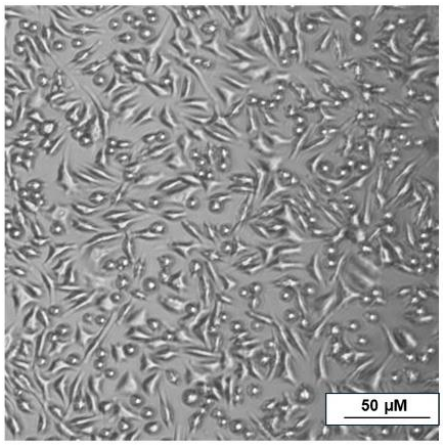

Figure 4.23 DMEM treated L929 control cells

A concentration of 1ng/ml was found to be almost 100% lethal among the different concentrations of TNFα. Figure 4.22 shows 1 ng/ml TNFα and 2 µg/mL D-Actinomycin applied L929 cell line.

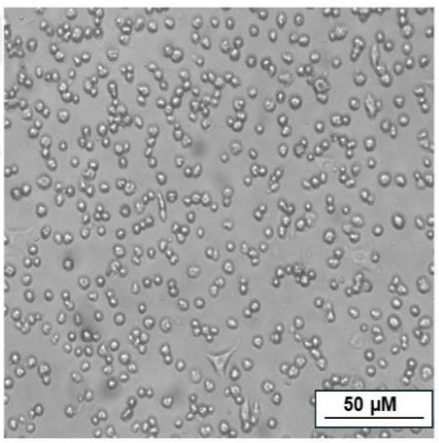

Figure 4.24 1 ng/ml TNFα treated L929 cell line

1 µM Adalimumab caused almost 100% cell survival when administered with 1 ng/ml TNFα and 2 µg/mL D-Actinomycin (Figure 4.23).

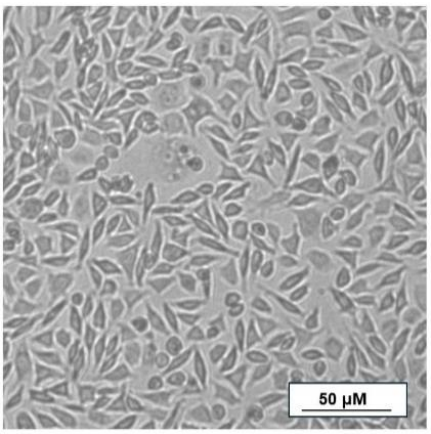

Figure 4.25 1 µM Adalimumab and 1 ng/ml TNFα treated L929 cell line

To see the effects of OB1 on the L929 cell line, first the cytotoxic effect of OB1 was investigated (Figure 4.24). 1 µM OB1 peptide kills less than 50% of seeded cells. The same concentration of OB1 was applied to see its neutralizing effect when applied with 1ng/ml TNFα and 2 µg/mL D-Actinomycin. Almost all the lethal effects of 1ng/ml TNFα were abolished by 1 µM OB1 peptide (Figure 4.25).

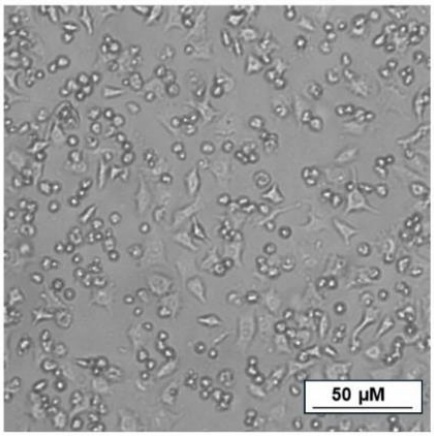

Figure 4.26 1 µM OB1 treated L929 cell line

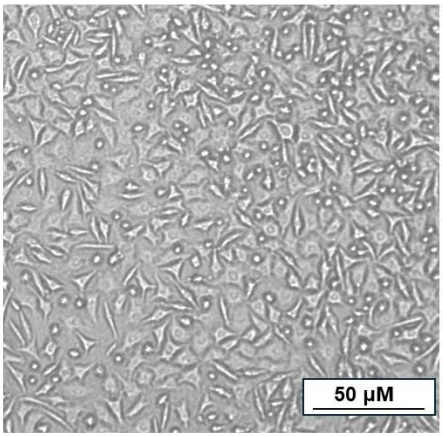

Figure 4.27 1 µM OB1 and 1 ng/ml treated L929 cell line

4.3.2 Cytotoxic effect of the synthesized peptides on the L929 cell line

Cytotoxic profiles of synthesized peptides by chemical production on L929 cell line were evaluated at different concentrations using MTT assay.

Figure 4.26 displays the cytotoxicity of hTNFα blocking peptides, OB1, OB2, and OB8, and Adalimumab. OB1 and OB2 peptides showed dose-dependent cytotoxic effects between 100 to 10000 nM concentrations. Cytotoxic effects of these peptides increased with respect to concentrations. According to the results in Figure 4.26, OB1 and OB2 cytotoxic effects are still under 50% cytotoxicity even at 10000 nM concentration. Adalimumab and OB8 showed non-toxic effect even at highest concentration 10000 nM.

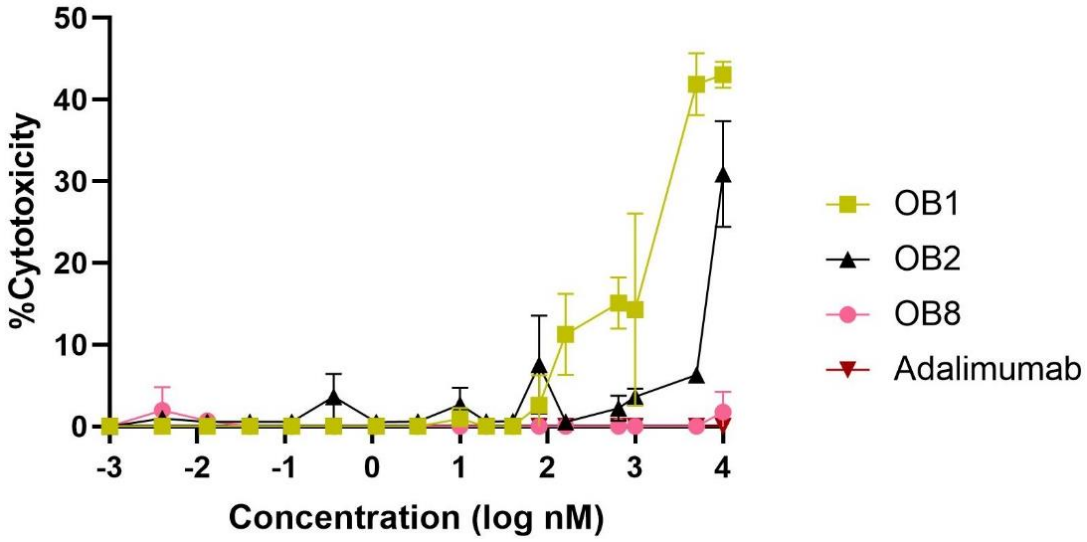

Figure 4.28 Cytotoxic Effect of hTNFα binding peptides and Adalimumab on L929 cell line

Figure 4.27 displays the cytotoxicity of TNFR1 and TNFR2 binding peptides, OB5, OB6, and OB7. OB5, OB6 and OB7 peptides showed dose-dependent increased cytotoxic effects between 100 to 10000 nM concentrations. According to the results, in Figure 4.27, OB5, OB6 and OB7 cytotoxic effects are still under 50% cytotoxicity even at 10000 nM concentration.

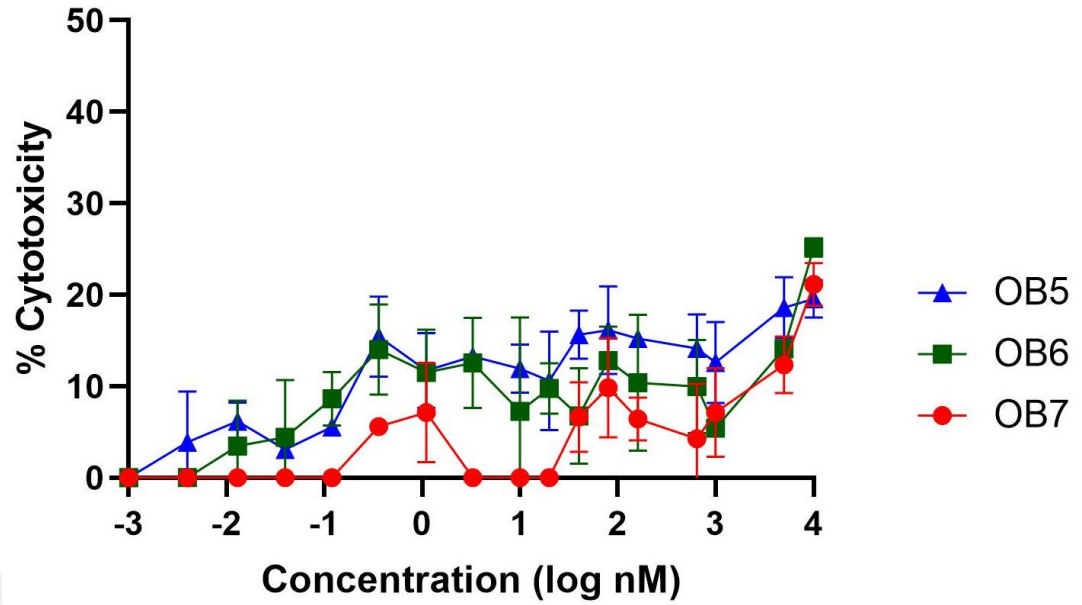

Figure 4.29 Cytotoxic Effect of TNFR binding peptides and Adalimumab on L929 cell line

4.3.3 Inhibition of hTNFα-induced cytotoxicity and TNFR interaction with designed peptides

L929 cell line has TNFR1 and TNFR2 receptors located on cell surface. TNFα can bind these receptors and induced hTNFα-mediated cell cytotoxicity. The designed peptides are expected to reduce hTNFα mediated cell cytotoxicity by inhibiting TNFα-TNFR interactions by binding TNFα or TNFR. Neutralizing level of hTNFα-mediated cell cytotoxicity, percent survival, was calculated relative to the viability of non-TNFα-stimulated cells. Table 4.3 demonstrates the TNFα mediated IC50 values of synthesized peptides and Adalimumab.

Table 4.3 TNF-α-Induced IC50 Measurements of Synthesized Peptides and Adalimumab

Molecule Name	IC50 (nM)
Adalimumab	3.85
OB1	4.6
OB2	0.081
OB5	79.5
OB6	15000
OB7	0.72
OB8	7500

Figure 4.28 displays the inhibition of hTNFα-mediated cell cytotoxicity via TNFα binding peptides and Adalimumab. Adalimumab reduced the hTNFα-induced cell death with dose-dependent manner (IC50=3.85 nM determined with MTT assay in our experiments). OB1 and OB2 peptides showed similar hTNFα neutralizing effect approximately %80 survival at 10000 nM concentration, similar to Adalimumab. OB2 (IC50= 0.081 nM) was more effective at lower concentrations than other TNFα binding molecules tested in this study (Table 4.3).

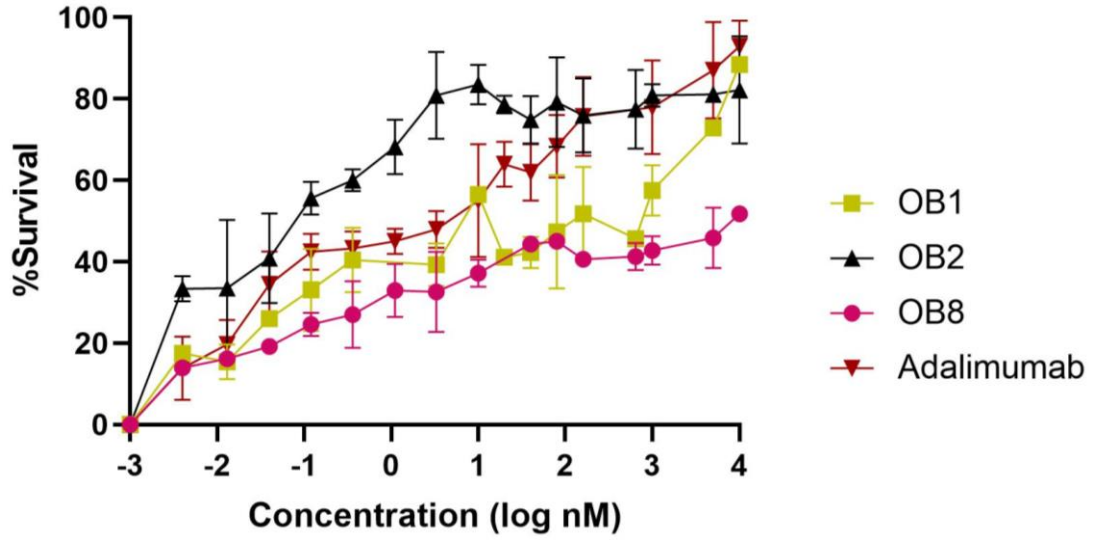

Figure 4.30 Inhibition of TNFα-mediated Cell Cytotoxicity by TNFα binding peptides and Adalimumab on L929 cell line

Figure 4.29 displays the inhibition of hTNFα-mediated cell cytotoxicity via TNFR binding peptides. OB5 and OB7, which are TNFR1 binding peptides, showed similar hTNFα neutralizing effect approximately %80 survival at 10000 nM concentration (IC50 of OB5 is 79.5 nM, and OB7 is 0.72 nM). According to IC50 results, OB7 is more effective than OB5 at low concentrations. OB6, which is TNFR2 binding peptide, was less effective than other TNFR binding molecules tested in this study (IC50= 15000 nM). OB6 and OB8 showed similar TNFα neutralising activities, despite having different targets (IC50 of OB8 is 7500 nM).

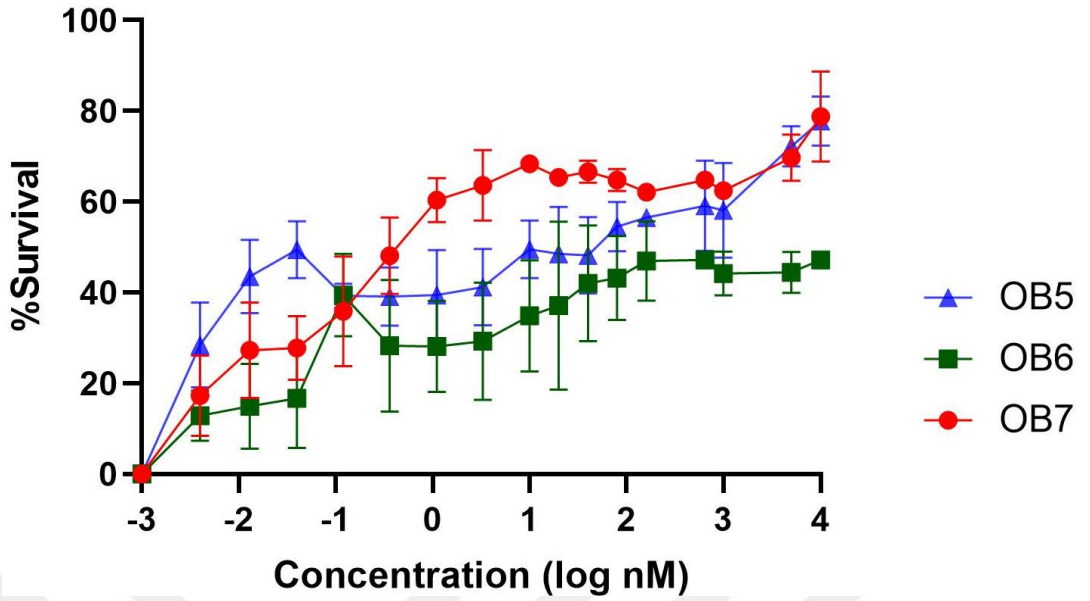

Figure 4.31 Inhibition of TNFα-mediated Cell Cytotoxicity by TNFR1 and TNFR2 binding peptides on L929 cell line

4.3.4 Inhibition of hTNFα-induced apoptosis with designed peptides

The L929 cell line is known for its sensitivity to TNFα-induced apoptosis. TNFα initiates the apoptotic cascade primarily through the activation of caspase-8, which subsequently triggers the downstream activation of caspase-3 and caspase-7, key executors of apoptosis.

As illustrated in Figure 4.30, the application of TNFα-binding peptides resulted in the modulation of Caspase-3/7 activity. Specifically, the peptides OB1, OB2, and OB8 demonstrated the ability to exhibit concentration-dependent inhibition of TNFα-induced apoptosis relative to the TNF-α-stimulated control group. Among these, OB1 exhibited the most pronounced inhibitory effect on the apoptotic pathway, showing greater efficacy than both OB2 and OB8 in suppressing caspase activation.

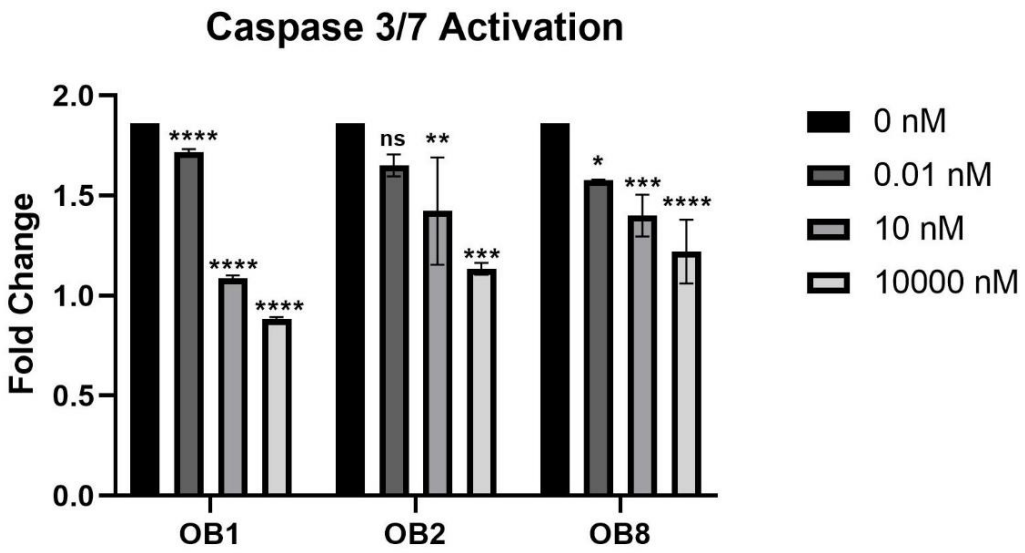

Figure 4.32 The effect of TNFα binding peptides on caspase 3/7 activation. Data are expressed as mean values ± Standard Error of the Mean (SEM). Statistical significance is indicated as follows: ns = not significant; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p \leq 0.0001$, relative to the control group.

Figure 4.31 illustrates the assessment of caspase-3/7 activity in L929 cells treated with TNFR1. TNFR1 was applied at concentrations of 0.1 nM, 10 nM, and 100 nM, alongside a constant concentration of TNFα (1 ng/mL). At 10 nM and 100 nM, TNFR1 significantly reduced caspase-3/7 activation compared to lower concentrations. Notably, at 10 nM, the inhibitory effect of TNFR1 on caspase-3/7 activity was comparable to that observed with OB1, the most potent TNFα-binding peptide identified in this study.

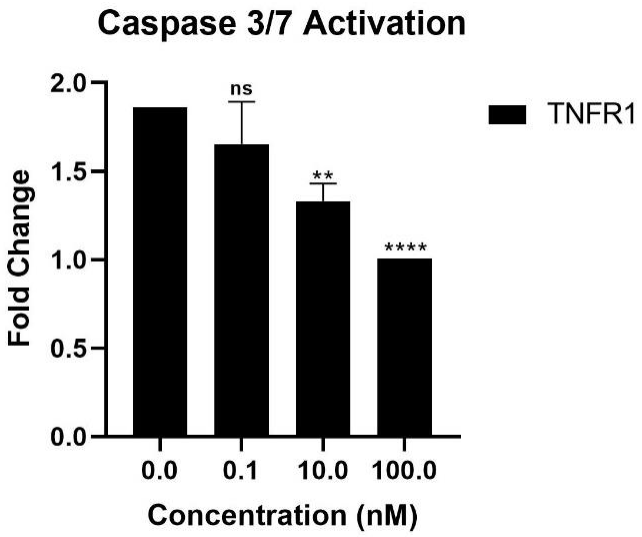

Figure 4.33 The effect of TNFR1 applied with TNFα on Caspase 3/7 activity. Data are expressed as mean values ± SEM. Statistical significance is indicated as follows: ns = not significant; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p \leq 0.0001$, relative to the control group.

Figure 4.32 displays the caspase-3/7 activity levels in cells exposed to peptides designed to interact with TNFR, illustrating the impact of these peptides on apoptotic signaling. OB5, OB6, and OB7 peptides inhibited TNFα-induced apoptosis in a concentration-dependent relative to the TNFα-treated controls. OB6 which is TNFR2 binding peptide and has lower TNFα neutralizing activity (Figure 4.29), was considerably more effective than OB5 and OB7 in inhibiting the apoptosis pathway.

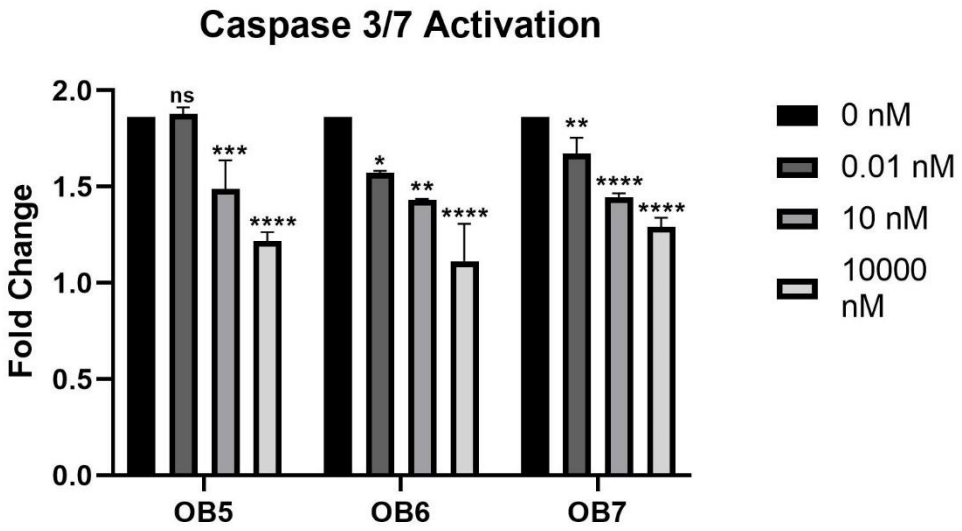

Figure 4.34 The effect of TNFR binding peptides on caspase 3/7 activation. Data are expressed as mean values ± SEM. Statistical significance is indicated as follows: ns = not significant; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p \leq 0.0001$, relative to the control group.

5 DISCUSSION

TNFα serves as a key regulatory molecule in the pathogenesis of autoimmune diseases by either inducing programmed cell death (apoptosis) or activating the nuclear factor kappa B (NF-κB) signaling pathway, which in turn promotes the expression of multiple pro-inflammatory mediators associated with disease progression. TNFα exists in two biologically active forms, soluble and membrane-bound, both of which are capable of binding to its receptors, TNFR1 and TNFR2. TNFα triggers complex intracellular signaling pathways that amplify inflammatory responses and contribute to disease progression through these interactions with both receptors (82). Considering the safety-related risks and high economic burden associated with mAb-based treatments for autoimmune diseases, the present study aimed to investigate the therapeutic potential of peptide-based inhibitors targeting TNFα. These alternative agents were evaluated as cost-effective and potentially safer candidates for modulating inflammatory pathways. In this study, six candidate peptides, named OB1, OB2, OB5, OB6, OB7, and OB8, were identified as potential modulators of the TNF signaling cascade. These peptides have demonstrated a wide range of biomedical potential, exhibiting properties that suggest their applicability not only in the therapeutic management of autoimmune diseases, but also in the promotion of tissue regeneration and the enhancement of wound healing processes.

Among the synthesized TNFα- blocking peptides evaluated in this thesis—namely OB1, OB2, and OB8—distinct differences were observed in terms of their binding affinities and capacities to neutralize TNFα activity. OB1 exhibited the highest affinity for TNFα, with a dissociation constant (K_d) of 300 nM (Table 4.1), and demonstrated potent inhibitory activity, as evidenced by an IC50 value of 4.6 nM (Table 4.3). This strong interaction enabled OB1 to effectively function as a TNFα antagonist by blocking its binding to TNFR1, thereby attenuating downstream inflammatory signaling. In contrast, OB8, a novel peptide designed as part of this study, displayed a considerably weaker interaction (Kd = 3.89 μM) and a much higher IC50 value (7500 nM), indicating substantially reduced neutralization efficiency relative to OB1 and OB2 (Figure 4E).

Peptides, characterized in this study, have a different mechanism of action to conventional TNFR antagonists in that they target and bind directly to TNFα, rather than interfering with TNFR function itself. By binding to TNFα, these peptides competitively inhibit its interaction with TNFR1, thereby reducing the pool of free TNFα available for receptor activation and downstream signaling. This indirect mode of inhibition suggests that the peptides may prevent the effective initiation of TNFR1-mediated inflammatory pathways. Importantly, OB1 exhibited high affinity for TNFα and successfully suppressed TNFR1 activation, as evidenced by its potent inhibition of TNFα-induced cell death. Interestingly, OB2, despite its comparatively lower binding affinity, showed superior neutralization efficiency and significantly reduced caspase-3/7 activation, indicating a potent anti-apoptotic effect. In comparison, OB8 exhibited a relatively limited impact on caspase activity, which aligns with its lower efficiency in neutralizing TNFα.

Adalimumab is currently recognized as one of the most potent clinically approved inhibitors of TNFα, exerting its therapeutic effect by directly binding to soluble TNFα (sTNFα) and preventing its interaction with the cell surface receptors TNFR1 and TNFR2 (82). The binding affinity of Adalimumab for TNFα is remarkably high, with a K_d of 21.13 pM, as shown in Table 4.1 and Figure 4.13. In comparison, OB1 peptide emerged as a promising alternative candidate for TNFα neutralization, with an IC50 of 4.6 nM - which is comparable to that of Adalimumab (IC50 = 3.85 nM) - highlighting its potential as a cost-effective and efficient peptide-based therapeutic agent (Table 4.13).

The peptide sequence 'HIHDDLLLRYYYGW' was previously synthesized in a tetra-branched form by Brunetti et al. who reported that their multivalent anti-TNFα construct exhibited a K_d of approximately 6 μM (12). In the present study, a monomeric and structurally extended version of this sequence, named OB1, exhibited a significantly higher binding affinity with a Kd of 300 ± 4.85 nM, as shown in Table 4.1 and Figure 4.14. Morphological assessments confirmed that OB1 attenuated TNFα-induced cell death (Figure 4.25). In addition, OB1 effectively inhibited TNFα activity by over 80% at a concentration of 10 μM, with an IC50 of 4.6 nM (Table 4.3), while exhibiting no cytotoxic effects on L929 fibroblast cells (Figure 4.26). Notably,

while the tetra-branched peptide of Brunetti et al. inhibited the TNFα - TNFR2 complex with an IC50 of 4.3 μM (12), OB1 demonstrated markedly higher potency, exhibiting an IC50 value of 4.6 nM, which represents a difference of approximately three orders of magnitude compared to less effective counterparts. This discrepancy may be due to differences in experimental methodology; the current study used a cell-based bioassay to assess inhibitory activity, whereas Brunetti et al. used surface plasmon resonance (SPR). Although tetra-branched peptide architectures are well known for their enzymatic stability and extended half-life, the peptides described in this study, such as OB1, may also benefit from future structural optimization. The adoption of multivalent or modified configurations could improve their protease resistance and pharmacokinetic profiles, further enhancing their therapeutic applicability.

OB2, a peptide rationally designed to mimic the TNFR1 binding domain, exhibited a K_d of 46.7 nM, indicating moderate binding affinity to TNFα. Despite this, OB2 demonstrated high neutralizing capacity with an IC50 value of 0.081 nM, suggesting a efficient mechanism of action. This discrepancy between binding affinity and inhibitory potency suggests that OB2 may function analogous to sTNFR1, acting as a receptor that effectively captures TNFα and prevents its interaction with membrane-bound TNFR1. By competitively binding to TNFα, OB2 can thus attenuate receptor-mediated inflammatory signaling with remarkable efficiency, reinforcing its potential as a potent therapeutic peptide.

C87 is a well-characterized small molecule inhibitor designed based on the loop structure of TNFR1, specifically the amino acid sequence spanning Arg77 to Val83 (RKEMGQV), which enables it to interfere with TNFα signaling by binding directly to the cytokine (79). Functionally analogous to the OB2 peptide, C87 is often used as a positive control in studies evaluating TNFα inhibitors. SPR studies have reported a Kd of 110 nM for C87. In comparison, OB2, which contains the same critical loop motif, showed a stronger binding affinity with a Kd of 46.7 nM. Furthermore, OB2 exhibited significantly greater inhibitory potency, with an IC50 of only 0.081 nM in TNFα-mediated cytotoxicity assays, in contrast to IC50 of 8.73 μM of C87. These results indicate that although C87 has a remarkable binding capacity,

OB2 is much more effective in suppressing TNFα activity in biological systems, even at significantly lower concentrations (Figure 4.28). Given that TNFα-induced apoptosis is primarily initiated by its interaction with TNFR1 and subsequent activation of caspase pathways, the functional relevance of TNFα inhibition extends to apoptotic modulation. While C87 has been shown to inhibit caspase-3 and caspase-8 activation (79), our results show that OB2 also significantly suppressed caspase activation at a concentration of 10,000 nM, supporting its role as a potent modulator of TNFα-induced apoptotic signaling (Figure 4.30). In a competitive binding assay, it was also shown that OB2 can replace TNFR1 with competition.

The peptide sequences OB5 and OB6 were identified through molecular docking simulations in different project, which predicted their ability to interact selectively with TNFR1 and TNFR2, two receptors known to mediate TNFα signaling through different mechanisms. Computational modelling indicated favorable binding trends for both peptides to their respective receptor targets. Empirical validation revealed Kd of 204.2 nM for OB5-TNFR1 and 3.9 μM for OB6-TNFR2 interactions (Figure 5E). Notably, OB5 exhibited a significantly higher neutralizing potency with an IC50 of 79.5 nM compared to OB6 which exhibited an IC50 of 15,000 nM (Figure 4.35). This difference in inhibitory potency may be due to fundamental differences in the activation mechanisms of TNFR1 and TNFR2. TNFR1 can be activated by both sTNFα and tmTNFα, whereas TNFR2 responds primarily to tmTNFα (83). Consequently, peptides targeting TNFR1 - such as OB5 - may be more effective at blocking TNFα-mediated biological functions by interfering with sTNFα interactions, thereby reducing both apoptotic and inflammatory signaling. Nevertheless, OB6 was able to inhibit approximately 50% of TNFα activity at elevated concentrations, despite its lower binding affinity. This observation suggests a possible indirect modulatory effect on TNFR2-mediated pathways, possibly involving signaling regulation rather than direct receptor antagonism. In addition, as TNF receptors are typically activated by trimeric TNFα, these small peptides may act as receptor-specific agonists or antagonists by targeting discrete receptor domains, further influencing TNF signaling outcomes.

Findings from the caspase-3/7 activation assay provide additional evidence supporting the hypothesis that TNF receptors possess distinct functional roles in the regulation of apoptotic processes. Although both TNFR1 and TNFR2 are transmembrane receptors involved in TNFα signaling, only TNFR1 contains an intracellular death domain that enables it to initiate apoptotic cascades. In contrast, TNFR2 lacks this domain and is instead associated with the activation of anti-apoptotic pathways that promote cell survival (84). The observed reduction in caspase-3/7 activity upon OB6 treatment indicated a potential enhancement of TNFR2-mediated pro-survival signaling (Figure 4.36). While OB6 demonstrated limited TNFα neutralizing capacity, its ability to reduce apoptotic markers suggests a mechanistic effect that may be exerted through alternative pathways such as NF-κB or PI3K/Akt signaling. These pathways are known to inhibit apoptosis and promote cell survival. Therefore, despite its low binding affinity and inhibitory potency, OB6 may exert indirect regulatory effects on apoptosis via TNFR2 activation. Further detailed studies are essential to uncover the specific molecular pathways through which OB6 exerts its anti-apoptotic activity.

OB7, a peptide representing a small binding fragment generated from TNFα, exhibited a Kd of 8.6 μM in its interaction with TNFR1, which is significantly weaker than the binding affinity of full-length TNFα to TNFR1 (Kd = 4.94 nM) (Table 4.2 and Figure 4.37). This marked difference in binding strength suggests that OB7 has a limited ability to competitively inhibit TNFα-TNFR1 interactions. Consistent with this observation, functional assays revealed that OB7 had minimal effects on inhibiting apoptosis, as evidenced by the relatively small reduction in caspase-3/7 activity (Figure 4.38).

When compared to two other TNFR1-blocking peptides, OB5 and OB7, OB5 showed a higher affinity for TNFR1 with a Kd of 204.2 nM, while OB7 showed a much lower binding affinity (Kd = 8.6 μM) (Table 4.2). Interestingly, despite this difference in binding strength, OB7 outperformed OB5 in terms of TNFα neutralization efficiency, exhibiting an IC50 value of 0.72 nM compared to OB5's 79.5 nM (Figure 4.39). This suggests that OB7 is more effective in competing with TNFα for TNFR1 binding, potentially preventing both apoptotic and inflammatory pathways

associated with TNFα-TNFR1 interaction. In contrast, OB5, while still able to bind TNFR1, did not completely inhibit TNFα-TNFR1 interaction, resulting in partial receptor activation. These findings suggest that OB7 may act by a direct mechanism, blocking TNFR1 activation, whereas OB5 is likely to exert its effect by modulating the availability of TNFα without completely preventing receptor activation.

While the peptides showed promising in vitro activities, their ability to effectively penetrate cellular membranes remains unclear. The incorporation of a CPP (cell-penetrating peptide) at N or C terminus might be required to enhance cellular uptake and should be investigated in future studies.

In the treatment of autoimmune disorders, peptide-based inhibitors provide distinct benefits over monoclonal antibody (mAb) approaches. These include lower production costs, shorter manufacturing times and improved tissue penetration due to their smaller size and reduced immunogenicity. However, challenges such as limited binding affinity, rapid degradation and poor systemic bioavailability hinder their clinical development. Addressing these challenges is crucial for the successful translation of peptide therapeutics into clinical applications, positioning them as a viable alternative to existing mAb-based treatments.

6 CONCLUSION

This study demonstrates the capability of peptide-based TNF-α inhibitors as promising alternatives to mAb therapies for the modulation of autoimmune and inflammatory processes. The peptides designed within the scope of this research displayed different levels of binding affinity and neutralization capacity toward TNFα, with OB1 and OB2 emerging as the most effective inhibitory candidates. Furthermore, OB5 and OB6, which were engineered to interact specifically with TNFR1 and TNFR2, respectively, offered important insights into receptor-selective regulation mechanisms. These findings suggest that targeting TNF-α signaling pathways through peptide-based approaches may hold considerable therapeutic promise, particularly by enabling fine-tuned modulation of receptor-mediated responses. The results of this study indicate that peptide-based TNFα inhibitors have strong potential to serve as next-generation therapeutic agents for the treatment of inflammatory diseases. Nevertheless, realizing their full clinical utility will require further investigation, particularly in the areas of pharmacokinetic profiling, in vivo efficacy, and the development of efficient targeted delivery systems. Addressing these challenges will be essential for progressing peptide therapeutics toward clinical translation and establishing them as a practical and effective alternative to current monoclonal antibody-based therapies.

7 REFERENCES

1. Fischer R, Kontermann RE, Maier O. Targeting sTNF/TNFR1 Signaling as a New Therapeutic Strategy. Antibodies 2015, Vol 4, Pages 48-70 [Internet]. 2015 Mar 6 [cited 2025 Apr 8];4(1):48–70. Available from: https://www.mdpi.com/2073-4468/4/1/48/htm
2. Shealy DJ, Visvanathan S. Anti-TNF antibodies: lessons from the past, roadmap for the future. Handb Exp Pharmacol [Internet]. 2008 [cited 2025 Apr 14];181(181):101–29. Available from: https://pubmed.ncbi.nlm.nih.gov/18071943/
3. Hu S, Liang S, Guo H, Zhang D, Li H, Wang X, et al. Comparison of the Inhibition Mechanisms of Adalimumab and Infliximab in Treating Tumor Necrosis Factor α-Associated Diseases from a Molecular View. Journal of Biological Chemistry. 2013 Sep 20;288(38):27059–67.
4. Richter F, Zettlitz KA, Seifert O, Herrmann A, Scheurich P, Pfizenmaier K, et al. Monovalent TNF receptor 1-selective antibody with improved affinity and neutralizing activity. MAbs [Internet]. 2019 Jan 2 [cited 2025 Apr 8];11(1):166–77. Available from: https://www.tandfonline.com/doi/abs/10.1080/19420862.2018.1524664
5. Mahdavi Sharif P, Jabbari P, Razi S, Keshavarz-Fathi M, Rezaei N. Importance of TNF-alpha and its alterations in the development of cancers. Cytokine [Internet]. 2020 Jun 1 [cited 2025 Apr 8];130. Available from: https://pubmed.ncbi.nlm.nih.gov/32208336/
6. Morrison SL, Johnson MJ, Herzenberg LA, Oi VT. Chimeric human antibody molecules: mouse antigen-binding domains with human constant region domains. Proc Natl Acad Sci U S A [Internet]. 1984 [cited 2025 Apr 15];81(21):6851–5. Available from: https://pubmed.ncbi.nlm.nih.gov/6436822/
7. Taylor PC. Anti-TNFα Therapy for Rheumatoid Arthritis: an Update. Internal Medicine. 2003 Jan 1;42(1):15–20.
8. Dixon WG, Hyrich KL, Watson KD, Lunt M, Galloway J, Ustianowski A, et al. Drug-specific risk of tuberculosis in patients with rheumatoid arthritis treated with anti-TNF therapy: Results from the British Society for Rheumatology Biologics Register (BSRBR). Ann Rheum Dis. 2010 Mar;69(3):522–8.
9. Torres MDT, Sothiselvam S, Lu TK, de la Fuente-Nunez C. Peptide Design Principles for Antimicrobial Applications. J Mol Biol [Internet]. 2019 Aug 23 [cited 2025 Apr 19];431(18):3547–67. Available from: https://pubmed.ncbi.nlm.nih.gov/30611750/
10. Miranda LP, Holder JR, Shi L, Bennett B, Aral J, Gegg C V., et al. Identification of potent, selective, and metabolically stable peptide antagonists to the calcitonin gene-related peptide (CGRP) receptor. J Med Chem [Internet]. 2008 Dec 25 [cited 2025 Apr 19];51(24):7889–97. Available from: https://pubs.acs.org/doi/abs/10.1021/jm8009298
11. Baud V, Karin M. Signal transduction by tumor necrosis factor and its relatives. Trends Cell Biol. 2001 Sep 9;

12. Brunetti J, Lelli B, Scali S, Falciani C, Bracci L, Pini A. A novel phage-library-selected peptide inhibits human TNF-α binding to its receptors. Molecules. 2014;19(6):7255–68.
13. Black RA, Rauch CT, Kozlosky CJ, Peschon JJ, Slack JL, Wolfson MF, et al. A metalloproteinase disintegrin that releases tumour-necrosis factor-alpha from cells. Nature [Internet]. 1997 Feb 20 [cited 2025 Apr 8];385(6618):729–33. Available from: https://pubmed.ncbi.nlm.nih.gov/9034190/
14. Wajant H, Pfizenmaier K, Scheurich P. Tumor necrosis factor signaling. Cell Death Differ [Internet]. 2003 [cited 2025 Apr 9];10:45–65. Available from: www.nature.com/cdd
15. Wajant H, Pfizenmaier K, Scheurich P. Tumor necrosis factor signaling. Cell Death & Differentiation 2003 10:1 [Internet]. 2003 Mar 25 [cited 2025 Apr 8];10(1):45–65. Available from: https://www.nature.com/articles/4401189
16. Chen G, Goeddel D V. TNF-R1 signaling: a beautiful pathway. Science [Internet]. 2002 May 31 [cited 2025 Jan 8];296(5573):1634–5. Available from: https://pubmed.ncbi.nlm.nih.gov/12040173/
17. Verma IM, Stevenson JK, Schwarz EM, Van Antwerp D, Miyamoto S. Rel/NF-kappa B/I kappa B family: intimate tales of association and dissociation. Genes Dev [Internet]. 1995 Nov 15 [cited 2025 Apr 12];9(22):2723–35. Available from: https://pubmed.ncbi.nlm.nih.gov/7590248/
18. Locksley RM, Killeen N, Lenardo MJ. The TNF and TNF receptor superfamilies: integrating mammalian biology. Cell [Internet]. 2001 Feb 23 [cited 2025 Apr 9];104(4):487–501. Available from: https://pubmed.ncbi.nlm.nih.gov/11239407/
19. Naismith JH, Sprang SR. Modularity in the TNF-receptor family. Trends Biochem Sci [Internet]. 1998 Feb [cited 2025 Apr 9];23(2):74–9. Available from: https://pubmed.ncbi.nlm.nih.gov/9538693/
20. Grell M, Douni E, Wajant H, Löhden M, Clauss M, Maxeiner B, et al. The transmembrane form of tumor necrosis factor is the prime activating ligand of the 80 kDa tumor necrosis factor receptor. Cell [Internet]. 1995 Dec 1 [cited 2025 Apr 9];83(5):793–802. Available from: https://pubmed.ncbi.nlm.nih.gov/8521496/
21. McCoy MK, Tansey MG. TNF signaling inhibition in the CNS: implications for normal brain function and neurodegenerative disease. J Neuroinflammation [Internet]. 2008 Oct 17 [cited 2025 Apr 9];5. Available from: https://pubmed.ncbi.nlm.nih.gov/18925972/
22. Perik-Zavodskaia O, Alrhmoun S, Perik-Zavodskii R, Zhukova J, Lopatnikova J, Volynets M, et al. Knockouts of TNFRSF1A and TNFRSF1B Genes in K562 Cell Line Lead to Diverse Long-Lasting Responses to TNF-α. Int J Mol Sci [Internet]. 2023 Dec 1 [cited 2025 Apr 9];24(24):17169. Available from: https://pmc.ncbi.nlm.nih.gov/articles/PMC10742831/
23. Yurkina DM, Romanova EA, Tvorogova A V., Naydenysheva ZK, Feoktistov A V., Yashin D V., et al. The 12-Membered TNFR1 Peptide, as Well as the 16-Membered and 6-Membered TNF Peptides, Regulate TNFR1-Dependent Cytotoxic Activity of TNF. Int J Mol Sci. 2024 Apr 1;25(7).

24. Zia K, Ashraf S, Jabeen A, Saeed M, Nur-e-Alam M, Ahmed S, et al. Identification of potential TNF-α inhibitors: from in silico to in vitro studies. Sci Rep. 2020 Dec 1;10(1).
25. Ma Y, Zhao S, Shen S, Fang S, Ye Z, Shi Z, et al. A novel recombinant slow-release TNF α-derived peptide effectively inhibits tumor growth and angiogensis. Sci Rep. 2015 Sep 4;5.
26. Walczak H. TNF and ubiquitin at the crossroads of gene activation, cell death, inflammation, and cancer. Immunol Rev [Internet]. 2011 Nov [cited 2025 Apr 13];244(1):9–28. Available from: https://pubmed.ncbi.nlm.nih.gov/22017428/
27. Chen G, Goeddel D V. TNF-R1 signaling: A beautiful pathway. Vol. 296, Science. 2002. p. 1634–5.
28. Pobezinskaya YL, Liu Z. The role of TRADD in death receptor signaling. Cell Cycle [Internet]. 2012 Mar 1 [cited 2025 Apr 9];11(5):871–6. Available from: http://www.ncbi.nlm.nih.gov/pubmed/22333735
29. Locksley RM, Killeen N, Lenardo MJ. The TNF and TNF receptor superfamilies: integrating mammalian biology. Cell [Internet]. 2001 Feb 23 [cited 2025 Apr 9];104(4):487–501. Available from: https://pubmed.ncbi.nlm.nih.gov/11239407/
30. Duerrschmid C, Crawford JR, Reineke E, Taffet GE, Trial JA, Entman ML, et al. TNF receptor 1 signaling is critically involved in mediating angiotensin-II-induced cardiac fibrosis. J Mol Cell Cardiol [Internet]. 2013 Apr [cited 2025 Apr 9];57(1):59–67. Available from: https://pubmed.ncbi.nlm.nih.gov/23337087/
31. Cabal-Hierro L, Lazo PS. Signal transduction by tumor necrosis factor receptors. Cell Signal [Internet]. 2012 Jun [cited 2025 Apr 9];24(6):1297–305. Available from: https://pubmed.ncbi.nlm.nih.gov/22374304/
32. Medler J, Kucka K, Wajant H. Tumor Necrosis Factor Receptor 2 (TNFR2): An Emerging Target in Cancer Therapy. Vol. 14, Cancers. MDPI; 2022.
33. Bodmer JL, Tschopp J, Schneider P. The molecular architecture of the TNF superfamily. Trends Biochem Sci [Internet]. 2002 Jan;27(1):19–36. Available from: http://tibs.trends.com
34. Moatti A, Cohen JL. The TNF-α/TNFR2 Pathway: Targeting a Brake to Release the Anti-tumor Immune Response. Vol. 9, Frontiers in Cell and Developmental Biology. Frontiers Media S.A.; 2021.
35. Carbone M, Ly BH, Dodson RF, Pagano I, Morris PT, Dogan UA, et al. Malignant mesothelioma: facts, myths, and hypotheses. J Cell Physiol [Internet]. 2012 Jan [cited 2025 Apr 14];227(1):44–58. Available from: https://pubmed.ncbi.nlm.nih.gov/21412769/
36. Chen S, Feng Z, Wang Y, Ma S, Hu Z, Yang P, et al. Discovery of Novel Ligands for TNF-# and TNF Receptor-1 through Structure-Based Virtual Screening and Biological Assay [Internet]. 2017. Available from: http://pubs.acs.org
37. Dantzer R. Cytokine-induced sickness behavior: mechanisms and implications. Ann N Y Acad Sci [Internet]. 2001 [cited 2025 Apr 14];933:222–34. Available from: https://pubmed.ncbi.nlm.nih.gov/12000023/

38. Mackay F, Kalled SL. TNF ligands and receptors in autoimmunity: An update. Curr Opin Immunol [Internet]. 2002 Dec 1 [cited 2025 Apr 14];14(6):783–90. Available from: https://pubmed.ncbi.nlm.nih.gov/12413530/
39. Adegbola SO, Sahnan K, Warusavitarne J, Hart A, Tozer P. Anti-TNF Therapy in Crohn's Disease. Int J Mol Sci [Internet]. 2018 Aug 1 [cited 2025 Apr 14];19(8). Available from: https://pubmed.ncbi.nlm.nih.gov/30065229/
40. Sahu D, Gupta C, Yennamalli RM, Sharma S, Roy S, Hasan S, et al. Novel peptide inhibitor of human tumor necrosis factor-α has antiarthritic activity. Sci Rep. 2024 Dec 1;14(1).
41. Chadwick W, Magnus T, Martin B, Keselman A, Mattson MP, Maudsley S. Targeting TNF-alpha receptors for neurotherapeutics. Trends Neurosci [Internet]. 2008 Oct [cited 2025 Apr 15];31(10):504–11. Available from: http://www.ncbi.nlm.nih.gov/pubmed/18774186
42. Blam ME, Stein RB, Lichtenstein GR. Integrating anti-tumor necrosis factor therapy in inflammatory bowel disease: current and future perspectives. Am J Gastroenterol [Internet]. 2001 Jul [cited 2025 Apr 10];96(7):1977–97. Available from: https://pubmed.ncbi.nlm.nih.gov/11467623/
43. Ma Y, Zhao S, Shen S, Fang S, Ye Z, Shi Z, et al. A novel recombinant slow-release TNF α-derived peptide effectively inhibits tumor growth and angiogensis. Sci Rep. 2015 Sep 4;5.
44. Asher A, Mulé JJ, Reichert CM, Shiloni E, Rosenberg SA. Studies on the anti-tumor efficacy of systemically administered recombinant tumor necrosis factor against several murine tumors in vivo. Journal of Immunology. 1987 Feb 1;138(3):963–74.
45. Stanfield RL, Wilson IA. Antibody Structure. Antibodies for Infectious Diseases [Internet]. 2015 May 26 [cited 2025 Apr 15];49–62. Available from: https://onlinelibrary.wiley.com/doi/full/10.1128/9781555817411.ch3
46. Lu RM, Hwang YC, Liu IJ, Lee CC, Tsai HZ, Li HJ, et al. Development of therapeutic antibodies for the treatment of diseases. J Biomed Sci [Internet]. 2020 Jan 2 [cited 2025 Apr 15];27(1). Available from: https://pubmed.ncbi.nlm.nih.gov/31894001/
47. Boulianne GL, Hozumi N, Shulman MJ. Production of functional chimaeric mouse/human antibody. Nature 1984 312:5995 [Internet]. 1984 [cited 2025 Apr 15];312(5995):643–6. Available from: https://www.nature.com/articles/312643a0
48. Kaplon H, Chenoweth A, Crescioli S, Reichert JM. Antibodies to watch in 2022. MAbs. 2022;14(1).
49. Global Monoclonal Antibodies (MAbS) Market Report 2023: [Internet]. [cited 2025 Apr 15]. Available from: https://www.globenewswire.com/news-release/2023/03/07/2622442/28124/en/Global-Monoclonal-Antibodies-MAbS-Market-Report-2023-Major-Players-Include-Merck-AbbVie-Amgen-GlaxoSmithKline-Norvatis-and-Pfizer.html

50. Nam J, Emery P. Aspects of TNF inhibitor therapy in rheumatoid arthritis. Mod Rheumatol [Internet]. 2010 Aug [cited 2025 Apr 15];20(4):325–30. Available from: https://pubmed.ncbi.nlm.nih.gov/20195684/
51. Ma L, Gong H, Zhu H, Ji Q, Su P, Liu P, et al. A novel small-molecule tumor necrosis factor α inhibitor attenuates inflammation in a hepatitis mouse model. Journal of Biological Chemistry. 2014;289(18):12457–66.
52. Hasegawa A, Takasaki W, Greene M, Murali R. Modifying TNFalpha for therapeutic use: a perspective on the TNF receptor system. Mini Rev Med Chem [Internet]. 2001 Mar 25 [cited 2025 Apr 15];1(1):5–16. Available from: https://pubmed.ncbi.nlm.nih.gov/12369987/
53. Goldbach-Mansky R, Lipsky PE. New concepts in the treatment of rheumatoid arthritis. Annu Rev Med [Internet]. 2003 [cited 2025 Apr 15];54:197–216. Available from: https://pubmed.ncbi.nlm.nih.gov/12359827/
54. Moritz T, Niederle N, Baumann J, May D, Kurschel E, Osieka R, et al. Phase I study of recombinant human tumor necrosis factor α in advanced malignant disease. Cancer Immunol Immunother [Internet]. 1989 Jun [cited 2025 Apr 19];29(2):144. Available from: https://pmc.ncbi.nlm.nih.gov/articles/PMC11038471/
55. Lejeune FJ, Rüegg C, Liénard D. Clinical applications of TNF-alpha in cancer. Curr Opin Immunol [Internet]. 1998 [cited 2025 Apr 19];10(5):573–80. Available from: https://pubmed.ncbi.nlm.nih.gov/9794839/
56. Debandt M, Vittecoq O, Descamps V, Le Loët X, Meyer O. Anti-TNF-alpha-induced systemic lupus syndrome. Clin Rheumatol [Internet]. 2003 Feb [cited 2025 Apr 19];22(1):56–61. Available from: https://pubmed.ncbi.nlm.nih.gov/12605321/
57. Randárová E, Kudláčová J, Etrych T. HPMA copolymer-antibody constructs in neoplastic treatment: an overview of therapeutics, targeted diagnostics, and drug-free systems. Journal of Controlled Release. 2020 Sep 10;325:304–22.
58. Shepard HM, Phillips GL, D Thanos C, Feldmann M. Developments in therapy with monoclonal antibodies and related proteins. Clin Med (Lond) [Internet]. 2017 Jun [cited 2025 Apr 19];17(3):220–32. Available from: https://linkinghub.elsevier.com/retrieve/pii/S1470211824019274
59. Trier N, Hansen P, Houen G. Peptides, Antibodies, Peptide Antibodies and More. Int J Mol Sci [Internet]. 2019 Dec 2 [cited 2025 Apr 19];20(24). Available from: https://pubmed.ncbi.nlm.nih.gov/31847088/
60. Wang L, Wang N, Zhang W, Cheng X, Yan Z, Shao G, et al. Therapeutic peptides: current applications and future directions. Signal Transduction and Targeted Therapy 2022 7:1 [Internet]. 2022 Feb 14 [cited 2025 Apr 19];7(1):1–27. Available from: https://www.nature.com/articles/s41392-022-00904-4

61. Fosgerau K, Hoffmann T. Peptide therapeutics: current status and future directions. Drug Discov Today [Internet]. 2015 [cited 2025 Apr 19];20(1):122–8. Available from: https://pubmed.ncbi.nlm.nih.gov/25450771/
62. Davda J, Declerck P, Hu-Lieskovan S, Hickling TP, Jacobs IA, Chou J, et al. Immunogenicity of immunomodulatory, antibody-based, oncology therapeutics. J Immunother Cancer [Internet]. 2019 Apr 15 [cited 2025 Apr 19];7(1). Available from: https://pubmed.ncbi.nlm.nih.gov/30992085/
63. Jones SD, Castillo FJ, Levine H. Advancesinthe Development of Therapeutic Monoclonal Antibodies. 2007;
64. Merrifield RB. Solid Phase Synthesis (Nobel Lecture)**. 1985.
65. Werner HM, Cabalteja CC, Horne WS. Peptide Backbone Composition and Protease Susceptibility: Impact of Modification Type, Position, and Tandem Substitution. Chembiochem [Internet]. 2016 Apr 15 [cited 2025 Apr 19];17(8):712–8. Available from: https://pubmed.ncbi.nlm.nih.gov/26205791/
66. Wei X, Zhan C, Chen X, Hou J, Xie C, Lu W. Retro-inverso isomer of Angiopep-2: a stable d-peptide ligand inspires brain-targeted drug delivery. Mol Pharm [Internet]. 2014 Oct 6 [cited 2025 Apr 19];11(10):3261–8. Available from: https://pubmed.ncbi.nlm.nih.gov/24673510/
67. Chatterjee J, Rechenmacher F, Kessler H. N-methylation of peptides and proteins: an important element for modulating biological functions. Angew Chem Int Ed Engl [Internet]. 2013 Jan 2 [cited 2025 Apr 19];52(1):254–69. Available from: https://pubmed.ncbi.nlm.nih.gov/23161799/
68. Liskamp RMJ, Rijkers DTS, Kruijtzer JAW, Kemmink J. Peptides and proteins as a continuing exciting source of inspiration for peptidomimetics. Chembiochem [Internet]. 2011 Jul 25 [cited 2025 Apr 19];12(11):1626–53. Available from: https://pubmed.ncbi.nlm.nih.gov/21751324/
69. Cheloha RW, Watanabe T, Dean T, Gellman SH, Gardella TJ. Backbone Modification of a Parathyroid Hormone Receptor-1 Antagonist/Inverse Agonist. ACS Chem Biol [Internet]. 2016 Oct 21 [cited 2025 Apr 19];11(10):2752–62. Available from: https://pubmed.ncbi.nlm.nih.gov/27533344/
70. Jevševar S, Kunstelj M, Porekar VG. PEGylation of therapeutic proteins. Biotechnol J [Internet]. 2010 Jan [cited 2025 Apr 19];5(1):113–28. Available from: https://pubmed.ncbi.nlm.nih.gov/20069580/
71. Fields GB. Introduction to Peptide Synthesis. Curr Protoc Protein Sci. 2001 Dec;26(1).
72. Sawai H. Induction of apoptosis in TNF-Treated L929 cells in the presence of necrostatin-1. Int J Mol Sci. 2016 Oct 1;17(10).
73. Mukai Y, Nakamura T, Yoshikawa M, Yoshioka Y, Tsunoda SI, Nakagawa S, et al. Solution of the structure of the TNF-TNFR2 complex. Sci Signal. 2010 Nov 16;3(148).
74. Saddala MS, Huang H. Identification of novel inhibitors for TNFα, TNFR1 and TNFα-TNFR1 complex using pharmacophore-based approaches. J Transl Med. 2019 Jul 2;17(1).
75. User Manual Monolith ® NT.115.

76. Contreras MA, Macaya L, Manrique V, Camacho F, González A, Montesinos R, et al. A trivalent TNF-R2 as a new tumor necrosis factor alpha-blocking molecule. Proteins: Structure, Function and Bioinformatics. 2021 Nov 1;89(11):1557–64.
77. Manrique-Suárez V, Macaya L, Contreras MA, Parra N, Maura R, González A, et al. Design and characterization of a novel dimeric blood–brain barrier penetrating TNFα inhibitor. Proteins: Structure, Function and Bioinformatics. 2021 Nov 1;89(11):1508–21.
78. Jia F, Zhang Y, Wang J, Peng J, Zhao P, Zhang L, et al. The effect of halogenation on the antimicrobial activity, antibiofilm activity, cytotoxicity and proteolytic stability of the antimicrobial peptide Jelleine-I. Peptides (NY) [Internet]. 2019;112:56–66. Available from: https://doi.org/10.1016/j.peptides.2018.11.006
79. Ma L, Gong H, Zhu H, Ji Q, Su P, Liu P, et al. A novel small-molecule tumor necrosis factor α inhibitor attenuates inflammation in a hepatitis mouse model. Journal of Biological Chemistry. 2014;289(18):12457–66.
80. Abdulrahman N, Honda TJ, Ali A, Abdulrahman N, Vrinceanu D, Shishodia S. Impacts of Indoor Dust Exposure on Human Colonic Cell Viability, Cytotoxicity and Apoptosis. Toxics. 2023 Jul 1;11(7).
81. Napiórkowska M, Kumaravel P, Amboo Mahentheran M, Kiernozek-Kalińska E, Grosicka-Maciąg E. New Derivatives of 1-(3-Methyl-1-Benzofuran-2-yl)Ethan-1-one: Synthesis and Preliminary Studies of Biological Activity. Int J Mol Sci. 2024 Feb 1;25(4).
82. Lee JAJ, Yang J, Lee C, Moon Y, Ahn S, Yang J. Demonstration of functional similarity of a biosimilar adalimumab SB5 to Humira®. Biologicals. 2019 Mar 1;58:7–15.
83. Zhang N, Wang Z, Zhao Y. Selective inhibition of Tumor necrosis factor receptor-1 (TNFR1) for the treatment of autoimmune diseases. Cytokine Growth Factor Rev. 2020 Oct 1;55:80–5.
84. Moatti A, Cohen JL. The TNF-α/TNFR2 Pathway: Targeting a Brake to Release the Anti-tumor Immune Response. Front Cell Dev Biol [Internet]. 2021 Oct 12 [cited 2025 Feb 14];9:725473. Available from: www.frontiersin.org

8 CURRICULUM VITAE