Discovery of Small Molecule Modulators of TNF Receptor Signaling

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Abstract

Tumor necrosis factor (TNF) is a central regulator of immunity and plays a critical role in the pathogenesis of autoimmune inflammatory diseases. TNF interacts with two receptors called tumor necrosis factor receptor 1 (TNFR1) and TNFR2. Activation of TNFR1 leads to cell inflammation and cell death while TNFR2 signaling is generally associated with cell survival and protection. Overexpression or dysregulation of TNF can lead to many different diseases including rheumatoid arthritis, Crohn's disease, and ulcerative colitis. This resulted in anti-TNF biologics being developed to treat autoimmune inflammatory diseases. Currently there are five FDA approved TNF inhibitors used to treat these diseases. However, TNF inhibitors are known to cause side effects because they inhibit both TNFR1 and TNFR2 signaling by sequestering the ligand. Therefore, specifically targeting TNF receptors is a more attractive approach when treating these diseases. Previous work in our lab showed it's possible to inhibit TNF receptor signaling by specifically targeting the receptor-receptor interactions without blocking ligand binding. We developed a cellular time resolved fluorescence resonance energy transfer (TR-FRET)-based TNFR1 biosensor to monitor structural changes in TNFR1 oligomers upon activation and inhibition. We used this TNFR1 biosensor to screen a library of small molecules and identified a small molecule, zafirlukast, that inhibits TNFR1 function. In this current study, using biochemical and biophysical assays, we showed zafirlukast and its analogs do not affect ligand binding. Next, we developed a cell-based functional assay to monitor the effect these compounds have on TNFR2 function. This assay showed zafirlukast and its analogs do not affect TNFR2 function. We also sought to adapt the TR-FRET based biosensor technology to determine if it could be used to create a high throughput screening platform that can identify small molecule effectors of TNFR2 for treating certain autoimmune diseases, neurodegenerative diseases, and cancer. To do this, we created a TNFR2 biosensor using molecular biology and cloning techniques and tested its FRET efficiency and specificity using a fluorescence lifetime plate reader. In the future, a Selleck high throughput screen will be performed to identify TNFR2 binders. We will test the functionality and specificity of these TNFR2 lead compounds using cell-based functional assays. If the lead compounds for TNFR2 are shown to be potent and specific, then they can serve as a potential drug candidate for treating certain autoimmune diseases or neurodegenerative diseases.

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Chapter 1: Introduction

TNF Receptor Introduction

Tumor necrosis factor (TNF) is a cytokine which helps regulate autoimmunity and inflammation [1]. TNF can bind to and induce signaling in both tumor necrosis factor receptor 1 (TNFR1) and tumor necrosis factor receptor 2 (TNFR2). TNF has both a membrane and a soluble form. TNFR2 preferentially binds membrane TNF to induce signaling while both forms of TNF are able to bind to TNFR1 and induce signaling [1, 2]. Soluble TNF can also bind to TNFR2 but does not or weakly induce TNFR2 signaling. TNFR1 and TNFR2 signaling differ in that generally TNFR1 signaling is associated with cell death while TNFR2 signaling is associated with cell survival and protection. These receptors also differ in where they are expressed. TNFR1 is expressed in nearly every cell in the body while TNFR2 is expressed only in certain cells, such as T-regulatory cells (Tregs), neuron subtypes, endothelial cells, microglia, cardiac myocytes, oligodendrocytes, human mesenchymal stem cells and thymocytes [1].

TNFR2 Background

TNFR2 Signaling

In the absence of TNF, TNFR2 is able to self-assemble into homodimers or homotrimers which is mediated by the preligand assembly domain (PLAD) of TNFR2 [3]. The binding of TNF leads to the trimerization of TNFR2 [2, 3]. The binding of TNF to TNFR2 can also lead to the formation of oligomeric clusters or aggregates [3, 4]. Upon TNF binding to TNFR2 and trimerization of TNFR2, TNFR associated factor 2 (TRAF2) binds to the intracellular domain of TNFR2. Other signaling molecules including TRAF1, TRAF3, cellular inhibitor of apoptosis protein 1 (cIAP1) and cIAP2 then bind to TRAF2. These molecules interact with other signaling molecules which leads to the transcription of pro survival genes [1, 2]. The activation of the transcription factor nuclear factor κB (NF-κB) is one way TNFR2 signaling can lead to the transcription of pro survival genes [1].

There are two different NF-κB pathways that involve different NF-κB transcription factors. TNFR1 signaling activates the canonical NF-κB pathway while TNFR2 signaling activates

the non-canonical NF-κB pathway [5, 6]. The five NF-κB transcription factors include p105/p50, RELA, c-REL, p100/p52 and RELB [5, 7].

TNFR2 signaling can activate the non-canonical NF-κB pathway but TNFR1 signaling does not. The NF-κB transcription factors that participate in the non-canonical NF-κB pathway are p100/p52 and RELB. The transcription factor p100 is a precursor to p52. [5, 7]. At the start of the non-canonical NF-κB pathway, a receptor such as TNFR2 is activated. In the cytoplasm of the cell, NF-κB inducing kinase (NIK) is activated which leads to the phosphorylation and activation of Iκβ kinase α (IKKα). p100 is then phosphorylated which leads to p100 processing and the creation of p52. p52 and RELB form a dimer which undergoes nuclear translocation to induce gene transcription [5]. One study showed TNFR2 signaling led to an increase in NIK, p100 processing into p52 and nuclear translocation of RELB and p52. All these results confirm TNFR2 signaling activates the non-canonical NF-κB pathway [6].

Crosstalk between the TNFR1 and TNFR2 pathways can also occur on cells expressing both receptors. This is primarily done through TRAF2. TRAF2 is recruited to the intracellular domain of TNFR2 during TNFR2 signaling. However, TNFR2 signaling has also been shown to cause the degradation of TRAF2. TRAF2 also participates in the TNFR1 signaling pathway and it along with cIAP1 and cIAP2 are responsible for inhibiting cell death caused by TNFR1 signaling. Without TRAF2, cIAP1 and cIAP2 cannot bind to the TNFR1 signaling complex. This means when both TNFR1 and TNFR2 signaling occurs there is less TRAF2. This decrease in TRAF2 increases the cytotoxicity of TNFR1 signaling [2].

TNFR2 in Diseases

TNFR2 signaling is generally associated with cell survival and protection [1, 2]. However, abnormal regulation of TNFR2 could lead to several diseases. Many of these diseases are autoimmune diseases. TNFR2 is involved in some of these diseases because Tregs express a high level of TNFR2. Tregs are immunosuppressive cells that help maintain immune homeostasis. TNFR2 signaling has been shown to lead to Treg proliferation and an increase in their suppressive capabilities. Changes in the number or function of Tregs can lead to certain autoimmune diseases. A TNFR2 agonist would be useful in these contexts because

it would help increase Treg proliferation which would lead to an increased immunosuppressive effect [8, 9]. This is the case for type 1 diabetes. There are two subgroups of Tregs called activated Tregs or resting Tregs. Activated Tregs express a high level of TNFR2 and are more immunosuppressive compared to resting Tregs. In type 1 diabetes there is a decrease in the amount of activated Tregs and an increase in the amount of resting Tregs. This decrease in activated Tregs is associated with worse glycemic control and a lower level of residual insulin secretion. TNFR2 agonism would be beneficial because TNFR2 signaling is able to convert resting Tregs into activated Tregs. Another way TNFR2 signaling could be useful in treating type 1 diabetes is through CD8 T cells. In type 1 diabetes CD8 T cells destroy insulin secreting islets of Langerhans [10]. TNFR2 signaling has been shown to kill or suppress CD8 T cells but not CD4 T cells (Tregs) [11]. Therefore, TNFR2 agonism could be beneficial in type 1 diabetes by increasing the amount of activated Treg cells while also killing or suppressing CD8 T cells [10, 11]. Similar to type 1 diabetes, Tregs can also play a role in graft versus host disease (GVHD). Treg depletion made GVHD more severe in experimental mouse models of GVHD. TNFR2 agonists could be used to increase the proliferation and immunosuppressive effect of Tregs to help treat GVHD [12].

Other defects in TNFR2 have been found in autoimmune diseases. It has been shown that TNFR2 polymorphisms and the upregulation in TNFR2 can be involved in certain autoimmune diseases such as in Crohn's disease and colitis [1]. Also, it has been shown previously in Crohn's disease and a mouse colitis model there is upregulated TNFR2 expression on the lamina propria and peripheral blood T cells [13]. There are conflicting reports on what role TNFR2 signaling has in colitis and whether agonism or antagonism of TNFR2 is beneficial [13-21]. A more recent study suggests TNFR2 signaling is required to induce colitis. This study showed CD4+ Foxp3- effector T cells had an increased expression of TNFR2 which allowed them to proliferate and induce colitis in mice. Therefore, the inhibition of TNFR2 could be useful in treating colitis [13].

Another autoimmune disease where TNFR2 signaling could be targeted is in multiple sclerosis. One key feature of multiple sclerosis is demyelination [22]. In experimental autoimmune encephalomyelitis (EAE), which is an experimental model of multiple

sclerosis, TNFR2 signaling on oligodendrocytes was shown to promote oligodendrocyte remyelination and differentiation [23]. Also TNFR2 signaling on astrocytes was shown to promote oligodendrocyte maturation [24]. Therefore, TNFR2 agonism on oligodendrocytes and astrocytes could be used to help treat the demyelination caused by multiple sclerosis [22-24]. However, other studies have shown TNFR2 signaling on monocytes/macrophages or CD4+ T cells can help initiate or contribute to EAE [21, 25]. In this case the inhibition of TNFR2 on monocytes/macrophages and CD4+ T cells could also be beneficial for treating multiple sclerosis.

TNFR2 can also play a role in cancer. In some types of cancers TNFR2 is overexpressed on tumor cells which can promote the proliferation of those tumors. Because TNFR2 signaling usually leads to cell survival, this allows the tumor cells to survive and proliferate. Another way TNFR2 signaling can enhance cancer cell survival is through the interactions of Tregs and CD8+ effector T cells. TNFR2 is highly expressed on immunosuppressive Tregs and TNFR2 signaling leads to Treg expansion. Tregs can suppress subsets of CD8+ effector T cells which can be detrimental during cancer because CD8+ effector T cells are able to directly kill tumor cells. This Treg suppression of CD8+ effector T cells is another way tumor cells are able survive. Also, TNFR2 mutations have been found in cancer which leads to agonism of the receptor. These reasons make TNFR2 inhibition a potential treatment in cancer immunotherapy [26]. However, one study suggests TNFR2 agonism could also be beneficial in cancer treatment. TNFR2 agonism was shown to enhance CD8+ effector T cell tumor infiltration and IFN-γ production which inhibited tumor growth [27]. CD8+ effector T cells also express TNFR2. So an ideal approach to target TNFR2 in cancer treatment could be to simultaneously inhibit tumor cells expressing TNFR2, inhibit immunosuppressive Tregs and agonize CD8+ effector T cells [26].

TNFR2 signaling can also be beneficial in certain neurodegenerative diseases. TNFR2 signaling is known to be neuroprotective [28-30]. It also can be beneficial in treating neuropathic pain and in neuronal cell recovery [31, 32]. In Alzheimer's disease, it has been shown there is decreased expression of TNFR2 in brain tissue of Alzheimer's disease patients [28, 29]. Therefore, TNFR2 agonism could be used to help treat Alzheimer's

disease by promoting neuronal cell survival [28]. The involvement of TNFR2 in all these diseases show there is a need for both TNFR2 agonists and antagonists.

Current Modulators of TNFR2 Signaling

Currently peptides, antibodies and small molecules have been used as modulators of TNFR2 signaling [1]. One study showed certain TNFR2 binding peptides could be agonists or antagonists of TNFR2 signaling [33]. Multiple agonistic and antagonistic antibodies have been used to target TNFR2. Their binding and effects have mainly been studied in TNFR2 expressing tumor cells and TNFR2 expressing Treg cells [34-38].

Small molecules have also been used as modulators of TNFR2 signaling. Minocycline is a small molecule that has anti-inflammatory and immunomodulatory effects and has been used to treat acne, rheumatoid arthritis, and some sexually transmitted diseases. Docking studies of minocycline showed binding to the active sites of TNFR2, toll like receptor 4 (TLR-4) and TNF. Minocycline binding resulted in the inhibition of TNFR2, TLR-4 and TNF and was shown to have some beneficial effects in a mouse model of polycystic ovary syndrome [39]. Another small molecule called thalidomide was originally developed to treat morning sickness in pregnant women but has since been shown to have anti-inflammatory and immunomodulatory effects. Regarding TNFR2, thalidomide has been shown to inhibit the surface expression of TNFR2 by inhibiting intracellular TNFR2 transport to the surface of the cell. Thalidomide also has structural analogs. One thalidomide structural analog called lenalidomide together with azacitidine (a demethylating agent) was shown to lower TNFR2 expression in CD4 T cells and reduce the amount of Tregs expressing TNFR2 in patients with acute myeloid leukemia. A DNA alkylating agent called cyclophosphamide is used in cancer treatment as a cytotoxic chemotherapy but has also been shown to induce cell death in Tregs expressing TNFR2 and ki-67 [40]. A small molecule isolated from a Chinese herb called triptolide is an immunosuppressive compound that was shown to inhibit TNFR2 expression in a mouse colitis model [20, 40]. Another study showed TNFR2 was upregulated on neural stem cells treated with amiodarone HCl [41]. While there are many small molecules that effect TNFR2 signaling, only minocycline through a docking study was shown to bind to TNFR2 [39]. Therefore, there is a need for more small molecules that specifically bind to TNFR2.

High Throughput Screening and FRET Background

One way to identify small molecule binders to TNFR2 is through high throughput screening. Currently only one in silico screen of 400,000 small molecules to test binding to TNFR2 has been performed [42]. This means a high throughput screen with a TNFR2 biosensor could be novel and relevant in finding small molecules that bind to TNFR2. For this study, a high throughput screening platform for the discovery of small molecule modulators of TNFR2 signaling will be developed using time resolved fluorescence resonance energy transfer (TR-FRET). TR-FRET uses fluorescence lifetime measurements to detect changes in the intermonomeric spacing of a TNFR2 fluorescent biosensor. During the screen, small molecules could cause a change in the intermonomeric spacing of TNFR2 which would result in an increase or decrease in the FRET signal [43-45]. TR-FRET is a precise way of monitoring the changes in the intermonomeric spacing of the receptor. It is able to detect protein structural changes of 1 Å [43]. The lifetime measurements for TR-FRET are also 10-30 times more precise than intensity measurements performed at the same speed. [45]. These reasons make TR-FRET an ideal method to use when screening for small molecules that bind to TNFR2. The small molecules in the screen that cause a significant increase or decrease in FRET would be called lead compounds. After the screen, functional assays are used to determine if the lead compounds are agonists or antagonists of TNFR2 signaling.

TNFR1 Background

TNFR1 Signaling

The extracellular domain of TNFR1 contains PLAD which allows TNFR1 to self-assemble into homodimers or trimers [4, 46]. The binding of TNF is also able to lead to the formation of TNFR1 oligomers [4]. Once TNF binds to TNFR1, the inhibitory protein silencer of death domains (SODD) is released from the intracellular domain (ICD) of TNFR1. This allows TNF receptor associated death domain (TRADD) to bind to the ICD of TNFR1. Other adaptor proteins including TRAF2, receptor interacting protein (RIP) and fas associated death domain (FADD) are then recruited by TRADD. These proteins are able to recruit additional proteins or enzymes depending on the type of signaling [47]. TNFR1 signaling can lead to either a pro-apoptotic or pro-inflammatory pathway [48]. The pro-

apoptotic pathway is triggered when caspase-8 binds to FADD [47, 48]. While in the pro inflammatory pathway, TNFR1 signaling leads to the activation of NF-κB [48].

TNFR1 signaling can activate the canonical NF-κB pathway. The NF-κB transcription factors that participate in the canonical NF-κB pathway are p105/p50, RELA and c-REL. The transcription factor p105 is a precursor to p50 [5, 7]. In the canonical NF-κB pathway a receptor such as TNFR1 is activated. In the cytoplasm of the cell, TGFβ activated kinase 1 (TAK1) is activated. TAK1 phosphorylates and activates the Iκβ kinase (IKK) complex which consists of three subunits called IKKα, IKKβ and IKKγ. This phosphorylation occurs on IKKβ. The IKK complex then phosphorylates inhibitor of κB (IκB) molecules such as IκBα or p105. In the cytoplasm IκBα is bound to dimers of p50 and either RELA or c-REL, while p105 is bound to either p50, RELA or c-REL. The phosphorylation of IκBα causes it to degrade which allows the NF-κB dimer to undergo nuclear translocation. When IKK phosphorylates p105 it undergoes degradation or processing into p50 which allows it to form a dimer with either p50, RELA or c-REL and undergo nuclear translocation. The NF-κB dimers then induce gene transcription. The two dimers that most commonly undergo nuclear translocation in the canonical NF-κB pathway are p50-RELA or p50-c-REL while a p50-p50 dimer can also occur [5].

TNFR1 in Diseases

The inflammatory effect from TNF is mainly caused through TNFR1 signaling [49, 50]. An increase in TNF levels has been found in multiple autoimmune diseases including rheumatoid arthritis, multiple sclerosis, inflammatory bowel disease and psoriasis which can cause an increase TNFR1 signaling. One study showed mice overexpressing TNF developed chronic arthritis. The effect of this disease decreased in TNFR1 knockout mice and increased in TNFR2 knockout mice. In another study with an animal model, TNF inhibitors were shown to decrease the effects of rheumatoid arthritis similar to knocking out TNFR1. This shows TNFR1 signaling is harmful in rheumatoid arthritis while TNFR2 signaling may be beneficial. In a Crohn's disease mouse model, overexpression of TNF caused inflammation similar to the disease. Like in arthritis, when TNFR1 was knocked out it protected the mice from Crohn's disease. This shows TNFR1 signaling is also detrimental in Chron's disease [50].

Increased TNF levels have also been found in neurodegenerative diseases including Alzheimer's diseases, Parkinson's disease and multiple sclerosis [50]. In Alzheimer's disease, increased TNFR1 levels have also been observed in brains with Alzheimer's disease [28]. In multiple sclerosis the increase in TNF levels can lead to demyelination. Studies have also shown TNFR1 is necessary for the development of EAE (an animal model of multiple sclerosis). A polymorphism found in the TNFR1 gene has also been shown to increase the susceptibility of multiple sclerosis. This shows TNFR1 signaling is harmful in this disease [50]. TNFR1 can also be involved in cancer. TNFR1 expression has been shown to be increased in breast cancer tissue and cell lines [51]. Another study showed TNF promotes a positive feedback loop involving TNFR1 which promotes the growth of breast cancer [52]. Inhibiting TNFR1 signaling in these diseases would be beneficial which has led to the development of treatments to inhibit TNFR1 signaling [50].

Current TNFR1 Antagonists

Many methods have been used to target TNFR1 signaling. One approach used is to inhibit TNFR1 signaling by inhibiting TNF activity. Currently five TNF antagonists are used. These antagonists are etanercept, golimumab, infliximab, adalimumab and certolizumab pegol [53-56]. These antagonists inhibit TNF activity by binding to TNF to prevent its binding to TNFR1 and TNFR2 [54, 57]. Etanercept is a fusion protein while the other four are antibody-based [56]. While these molecules have had success in treating certain inflammatory diseases, there are some problems when using TNF antagonists to target TNFR1 signaling. Because TNF antagonists inhibit TNF function, they are not only inhibiting TNFR1 signaling but also TNFR2 signaling which can lead to side effects [55]. Adverse effects have been found when using TNF antagonists in diseases such as type 1 diabetes and multiple sclerosis [53]. An increase in infections and malignancies, the worsening of congestive cardiac failure, injection site reactions and development of new autoimmune diseases have also been reported when using TNF antagonists [55, 58]. Therefore selectively inhibiting TNFR1 is a better approach than inhibiting TNF [55].

Proteins, antibodies, and small molecules have also been used as TNFR1 antagonists [55]. Some examples for these include the equine herpesvirus type 2 E8 protein which has been shown to interfere with the caspase 8 and FADD interaction which blocks the TNFR1

apoptotic pathway [59]. As for antibodies, Atrosab is an antibody that binds to TNFR1 and competes with ligand binding [55]. Also an antibody fragment called IZI-06.1 was shown to compete with ligand binding to TNFR1 by binding to the cystine rich domain 1 of TNFR1 [60]. Previous work in our lab has shown that small molecules can be used as TNFR1 antagonists. The small molecule zafirlukast was shown to disrupt the PLAD interactions of TNFR1 to inhibit the TNFR1 activation of NF-κB [43].

Specific Aims

This study seeks to develop a platform to identify small molecule modulators of TNFR2 signaling. Currently TNF antagonists inhibit both TNFR1 and TNFR2 signaling which can lead to side effects [55]. Also, modulators of TNFR2 signaling could be useful when treating different autoimmune diseases, cancer, and neurodegenerative diseases [1, 26-32]. Using small molecules to target receptors such as TNFR1 and TNFR2 have certain advantages over peptides and antibodies. Small molecules can produce a strong biological effect. They also can be manufactured in a cost-effective way and taken orally by patients which is important for healthcare systems. Many small molecules can also be quickly synthesized and screened for binding and specificity to a target [61]. While there have been multiple small molecules shown to modulate TNFR2 signaling, only minocycline through a docking study was shown to bind to TNFR2. However, in the same study minocycline was shown to also bind to TLR-4 and TNF [39]. Because of these reasons, there is a need for small molecules that specifically bind to TNFR2 and modulate TNFR2 signaling. High throughput screening can be used to screen many small molecules to determine if they bind to TNFR2 and currently only one in silico screen testing small molecule binding to TNFR2 has been performed [42]. This study seeks to fill this need for small molecule modulators of TNFR2 signaling through specific aim 1.

Specific aim 1: Develop a TR-FRET high throughput screening platform for the discovery of small molecule modulators of TNFR2 signaling.

To develop this high throughput screening platform, we created a TNFR2 biosensor. When creating the biosensor, the cytoplasmic domain of TNFR2 was removed and a fluorescent protein was inserted at the C terminus of TNFR2. ΔCD is used to denote the cytoplasmic domain was removed from TNFR2 in the TNFR2ΔCD biosensor. The cytoplasmic domain of TNFR2 was removed to help make sure the fluorescent proteins used in this biosensor were close enough together to produce a FRET signal. Using this biosensor in a high throughput screening platform, small molecule binders to TNFR2 can be discovered. These small molecule binders to TNFR2 would then be tested for their functionality, specificity, and mode of action. In this current study a high throughput screening platform was developed for TNFR2 but previously our lab used a TNFR1 biosensor in a high throughput screening platform to discover small molecule binders to TNFR1. Zafirlukast was discovered to be a TNFR1 binder using this TNFR1 high throughput screening platform [43]. This current study also investigated the specificity and mode of action of zafirlukast and its structural analogs.

The other focus of this study is to investigate the specificity and mode of action of TNFR1 antagonists. As stated previously TNF antagonists inhibit TNF function which effects both TNFR1 and TNFR2 signaling. Therefore, it is better to selectively inhibit either TNFR1 or TNFR2 signaling [55]. It has been shown previously in our lab that zafirlukast inhibits TNFR1 signaling. However, the potency of zafirlukast is low. Zafirlukast was shown to have an absolute inhibitory concentration (absolute IC_{50}) of 114 μM when inhibiting IκBα degradation and an absolute IC_{50} of 50 μM when inhibiting NF-κB activation [43]. Therefore, structural analogs of zafirlukast were created in our lab to test for increased potency in inhibiting TNFR1 signaling. Sixteen structural analogs were synthesized and their effect on NF-κB inhibition was investigated. Based on these results three analogs were chosen for further studies. The assays in this current study were used to investigate the specificity and mode of action of these three analogs which are referred to as CpCF3, MeCF3 and MeOEtCF3. Along with the zafirlukast structural analogs, a TNFR1-binding affibody created in our lab was also tested for specificity. Affibody molecules are proteins engineered to bind to a specific target and their structure usually contains a three-helix bundle domain framework. Affibodies have also shown the ability to block receptor signaling. One way affibodies could block receptor signaling is by blocking ligand binding

[62]. This project seeks to study these TNFR1 antagonists created in our lab through specific aim 2.

Specific aim 2: Investigate the specificity and mode of action of TNFR1 antagonists.

To test the specificity of these TNFR1 antagonists, we used human umbilical vein endothelial cells (HUVEC) that were transiently transfected with a membrane TNF plasmid to develop a western blot assay probing for RELB expression. This was done to test these antagonists' effect on TNFR2 signaling. Both the zafirlukast analogs and the TNFR1-binding affibody were tested for their effect on TNFR2 signaling using this assay. Another area of study for the zafirlukast analogs was to test their effect on ligand binding to determine their mode of action. This was done using anti-FLAG magnetic beads coated with FLAG tagged TNF in a TNFR1 pulldown assay.

Chapter 2: Methods

Cell Culture

Phenol red free Dulbecco's Modified Eagle Medium (DMEM) (Sigma) was used to culture human embryonic kidney 293 (HEK293) cells. This medium was supplemented with heat inactivated 10% fetal bovine serum (Sigma), 2 mM L-glutamine (Gibco), 100 μg/mL streptomycin and 100 U/mL penicillin (Gibco) [43]. EBM-2 medium (LONZA) was used to culture human umbilical vein endothelial cells (HUVEC). This medium was supplemented with heat inactivated 2% fetal bovine serum (Sigma), 100 μg/mL streptomycin and 100 U/mL penicillin (Gibco). All plates and flasks for culturing HUVEC cells were coated with 0.2% gelatin (Sigma). An incubator with 5% CO_2 (Forma Series II Water Jacket CO_2 Incubator, Thermo Fisher Scientific) was used to maintain the cell cultures at 37°C [43].

Development of a TNFR2ΔCD Biosensor

The two fluorescent proteins used in this biosensor were the green fluorescent protein (GFP) EGFP and the red fluorescent protein (RFP) TagRFP. Using plasmids containing

these fluorescent proteins and a TNFR2 plasmid, TNFR2ΔCD-GFP and TNFR2ΔCD-RFP plasmids were created to be used as the TNFR2ΔCD biosensor. Using Quikchange site directed mutagenesis, Nhe1 and BamH1 restriction sites were created at the N terminus and C terminus respectfully of the sequence encoding TNFR2ΔCD (amino acids 1-297) on a TNFR2 plasmid (Sino Biological). TNFR1ΔCD-GFP and TNFR1ΔCD-RFP plasmids previously made in our lab already contained Nhe1 and BamH1 restriction sites at the N terminus and C terminus of TNFR1ΔCD respectfully. Nhe1 (New England Biolabs) and BamH1 (New England Biolabs) restriction enzymes were used to cut out the cDNAs encoding TNFR2ΔCD from the TNFR2 plasmid. These restriction enzymes were also used to cut out TNFR1ΔCD from the TNFR1ΔCD-GFP and TNFR1ΔCD-RFP plasmids leaving EGFP and TagRFP plasmids. Ligation was performed to insert the cDNAs encoding TNFR2ΔCD (amino acids 1-297) at the N terminus of the EGFP and TagRFP vectors using a T4 DNA ligase (Promega). All plasmids were sequenced to confirm they were correct before use. Any mistakes in the sequences were corrected using Quickchange site directed mutagenesis. The EGFP vector contained the mutation alanine 206 to lysine (A206K) created by Quikchange site directed mutagenesis. This mutation prevented the dimerization and aggregation of EGFP [63].

Gel Electrophoresis

Plasmids to make the TNFR2ΔCD biosensor were digested using restriction enzymes for 1 hour at 37℃. 6X gel loading dye (New England Biolabs) was added to the digested plasmids and undigested plasmids. A 0.8% agarose (Invitrogen) gel containing sybr safe (Invitrogen) was poured and allowed to solidify at 4℃. 1X TAE buffer (Bio-Rad) was then poured over the gel. The digested plasmids and undigested plasmids were loaded in the gel and run at 100 V for 1-3 hours. Gels were imaged and the desired pieces of DNA were extracted from the agarose gel using a DNA gel extraction kit (New England Biolabs).

Fluorescence Microscopy

HEK293 cells were plated in 6 well plates (Sarstedt) and transiently transfected with TNFR2ΔCD-GFP and TNFR2ΔCD-RFP at a 1:6 plasmid ratio using lipofectamine 3000 (Invitrogen). After 6 hours of transfection, the medium and transfection reagent was removed and new DMEM medium was gently added to the cells. Cells were placed in an

incubator overnight. The next day cells were lifted with TrypLE (Gibco) and plated into 35 mm glass bottom dishes (MatTek). Cells were left in an incubator overnight. Cells were imaged for GFP and RFP expression using an ImageXpress Pico.

Flow Cytometry

HEK293 cells were transiently transfected with TNFR2ΔCD-GFP or TNFR2ΔCD-RFP using lipofectamine 3000 (Invitrogen). After 6 hours of transfection, the medium and transfection reagent was removed and new DMEM medium was gently added to the cells. Cells were placed in an incubator overnight. The next day cells were lifted with TrypLE (Gibco) and counted with an automatic cell counter (DeNovix CellDrop BF). For each sample, 3 million cells were washed 3 times with PBS (Sigma) containing 0.5% bovine serum albumin (Sigma) (PBSA). Each sample was then resuspended in 50 uL of PBSA and incubated with 1 μL of a TNFR2 antibody conjugated with phycoerythrin (Invitrogen) for 1 hour on ice. Cells were then washed 3 times with PBSA and filtered with a 70 μm cell strainer (Biologix). The fluorescence of the samples was analyzed using a BD Accuri C6 flow cytometer.

Preparation of Cells for Fluorescence Lifetime Measurements

HEK293 cells were plated in 6 well plates (Sarstedt) to be 70-80% confluent. The cells were placed in an incubator overnight. Cells were then transiently transfected with TNFR2ΔCD-GFP and pcDNA Empty Vector (EV) or TNFR2ΔCD-GFP and TNFR2ΔCD-RFP at a 1:6 plasmid ratio using lipofectamine 3000 (Invitrogen). After 6 hours of transfection the medium and transfection reagent was removed, and new DMEM medium was gently added to the cells. Cells were placed in an incubator overnight. The next day cells were lifted with TrypLE (Gibco), washed with 10 mL of phosphate buffered saline (PBS) (Sigma) two times and resuspended in 1 mL of PBS. Cells were then filtered with a 70 μm cell strainer (Biologix) and counted using a cell counter (DeNovix CellDrop BF). Cells were diluted with PBS to a concentration of 1 million cells/mL. Cells were plated into a black 384 well plate (Greiner bio-one) with 50 μL cells/well. For studies with zafirlukast, DMSO (Sigma) or 50 μM of zafirlukast were pipetted into the cells in the 384 well plate and incubated at room temperature for 1-2 hours before lifetime measurements.

Fluorescence Lifetime Measurements

A fluorescence lifetime plate reader (Fluorescence Innovations, Inc., Minneapolis, MN) was used to perform fluorescence lifetime measurements for both donor only (GFP only) and donor-acceptor (GFP-RFP). A 473 nm microchip laser was used to excite GFP fluorescence. A 488 nm long pass filter and a 517/20 nm band pass filter were then used to filter the emission. Least squares minimization global analysis software (Fluorescence Innovations, Inc.) was used on the time resolved fluorescence waveforms produced for each well. This allowed the waveforms to be fit to single exponential decays. This gave the donor lifetime (τ_D) and donor-acceptor lifetime (τ_{DA}) which were used to calculate FRET efficiency percent (E) using the equation below [43, 63].

$$E = \left(1 - \left(\frac{\tau_{DA}}{\tau_D}\right)\right) * 100$$

Detection of RELB using Western Blotting

HUVEC cells were plated in a 6 well plate at 1 million cells per well and left in an incubator overnight. The next day the cells were transiently transfected with 0.5 μg of a plasmid encoding membrane TNF (Origene) using lipofectamine 3000 (Invitrogen). After 6 hours of transfection the medium and transfection reagent was removed, and new EBM-2 medium was gently added to the cells. At the same time cells were also treated with either the zafirlukast analogs or the TNFR1-binding affibody. For zafirlukast analog studies, cells were treated with DMSO or 250 nM of analogs or zafirlukast. For TNFR1-binding affibody studies, cells were treated with PBS or 0.01 μM of affibody. The cells were left in an incubator overnight and the next day cells were lifted with TrypLE (Gibco). Cells were washed three times with PBS (Sigma) and then lysed with native lysis buffer (Abcam) containing 1% protease inhibitor (ProteoGuard protease inhibitor cocktail, Takara). A bicinchoninic acid (BCA) assay (Thermo Fischer Scientific) was used to determine the total protein concentration of the lysate. For each sample, an equal amount of protein was mixed with 4x laemmli sample buffer (Bio-Rad) and boiled at 95℃ for 5 minutes. Western blotting was performed, and the blots were probed using anti-RELB (Cell Signaling Technology) and anti-β-actin (Cell Signaling Technology) antibodies. Three independent

trials were performed for this experiment. Images of the western blots were analyzed using Bio-Rad Image Lab version 6.1.0.

TNF-TNFR1 Pulldown Assay

In a 1.7 mL microcentrifuge tube (dot scientific), 10 μL of anti-FLAG magnetic beads (Thermo Fischer Scientific) were incubated with 30 μL of 25 μg/mL FLAG tagged TNF (ENZO) for two hours at 4℃. The TNF was then removed, and the magnetic beads were washed three times with PBS (Sigma) containing 0.5% bovine serum albumin (Sigma) (PBSA). Three confluent t225 flasks (Thermo Fischer Scientific) of HEK293 cells were previously lysed with 1.2 mL of native lysis buffer (Abcam) containing 1% protease inhibitor (ProteoGuard protease inhibitor cocktail, Takara). A BCA assay (Thermo Fischer Scientific) was used to determine the final protein concentrations of the lysate. For the three independent trials the total protein concentrations were between 14-17 μg/μL. After the magnetic beads were washed, the PBSA was removed and 250 μL of cell lysate was added to the magnetic beads. At the same time, DMSO (Sigma) or 250 nM of analog or zafirlukast treatment was also added to the magnetic beads. The beads were then incubated at 4℃ overnight on a rotator. The next day the beads were washed three times with PBSA. 10 μL of 1X laemmli Sample Buffer (Bio-Rad) was added to the 10 μL of beads and pipetted up and down 5 times in order to elute the proteins. Using a magnet, the 10 μL of dye was then removed and placed in a separate microcentrifuge tube. This elution step was repeated four times for each sample for a total of 40 μL of dye per sample. Western blotting was performed with each sample. A control well containing 30 μL of 25 μg/mL FLAG tagged TNF (ENZO) with 10 μL of 4X laemmli Sample Buffer (Bio-Rad) was also added to the western blot. Three independent trials were performed for this experiment. Western blots were probed using anti-TNFR1 (Cell Signaling Technology) and anti-FLAG (Cell Signaling Technology) antibodies. Images of the western blots were analyzed using Bio-Rad Image Lab version 6.1.0.

Statistical Analysis

All statistical analysis was performed in Excel using a two tailed unpaired t test.

Chapter 3: TR-FRET Based High Throughput Screening Platform for the Discovery of Small Molecule Modulators of TNFR2 Signaling

Development of the TNFR2ΔCD Biosensor

To make the TNFR2ΔCD biosensor, Nhe1 and BamH1 restrictions sites were created on a TNFR2 plasmid using Quikchange site directed mutagenesis at the ends of the TNFR2ΔCD sequence. Nhe1 and BamH1 restriction enzymes were then used on this TNFR2 plasmid along with TNFR1ΔCD-GFP and TNFR1ΔCD-RFP plasmids in order to extract the desired pieces from the plasmids. The digestion of the TNFR1ΔCD-GFP and TNFR1ΔCD-RFP plasmids was done to remove TNFR1ΔCD leaving the remaining EGFP or TagRFP plasmid. The digested plasmids were run in an agarose gel to separate the pieces of the plasmids (Figure 1).

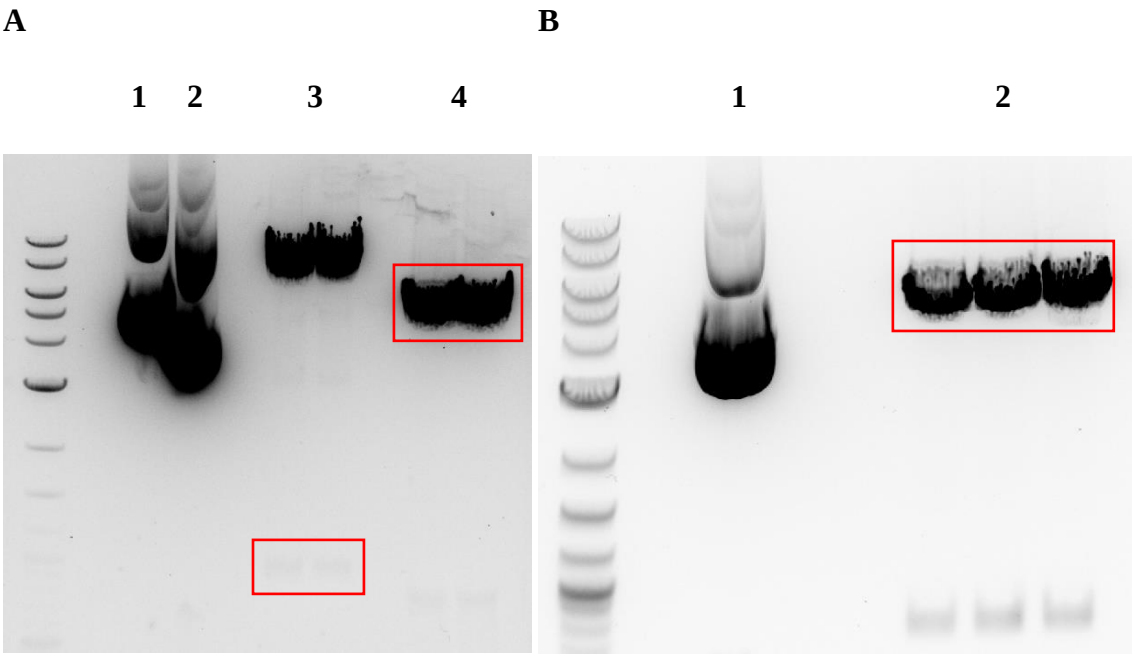

Figure 1: Agarose gels of digested plasmids. TNFR2 plasmid with created Nhe1 and BamH1 restriction sites, TNFR1ΔCD-GFP and TNFR1ΔCD-RFP plasmids were digested with Nhe1 and BamH1 restriction enzymes. (A) Digestion of TNFR2 plasmid with created Nhe1 and BamH1 restriction sites and digestion of TNFR1ΔCD-RFP. Samples are denoted by numbers. 1: undigested TNFR2. 2: undigested TNFR1ΔCD-RFP. 3: TNFR2 Digestion. 4: TNFR1ΔCD-RFP digestion. DNA bands outlined were extracted to be used in

ligation. Column 3 outline: TNFR2ΔCD. Column 4 outline: TagRFP plasmid. (B) Digestion of TNFR1ΔCD-GFP. Samples are denoted by numbers. 1: undigested TNFR1ΔCD-GFP. 2: TNFR1ΔCD-GFP digestion. DNA bands outlined were extracted to be used in ligation. Column 2 outline: EGFP plasmid.

TNFR2ΔCD, EGFP and TagRFP plasmids were extracted from the agarose gels. Ligation was used to insert TNFR2ΔCD into the EGFP and TagRFP plasmids. The final structure of the biosensor consisted of the extracellular domain and transmembrane domain of TNFR2 along with 10 amino acids of the cytoplasmic domain of TNFR2. At the C terminus of TNFR2, a linker of 7 amino acids in length connected TNFR2 to either GFP or RFP (Figure 2). The extracellular domain of TNFR2 contains four cystine rich domains with the first cystine rich domain being called PLAD. PLAD allows TNFR2 monomers to form homodimers or homotrimers in the absence of ligand [3]. This process of forming homodimers or homotrimers is important for the fluorescence lifetime readings because it brings the fluorescent proteins (GFP and RFP) close enough together to produce a FRET signal.

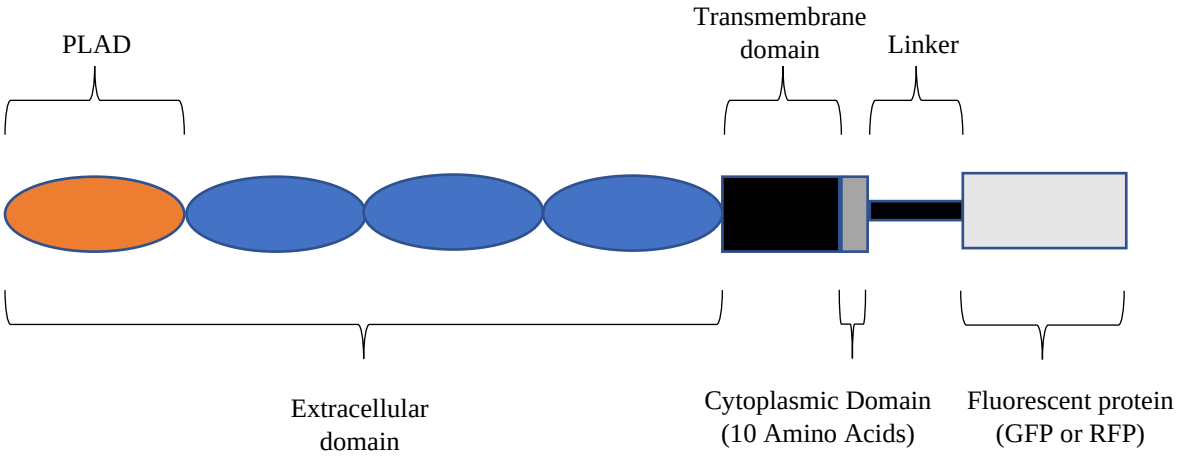

Figure 2: Schematic of the TNFR2ΔCD biosensor. The biosensor contains the extracellular and transmembrane domain of TNFR2, 10 amino acids of the cytoplasmic domain of TNFR2 and a 7 amino acid linker connecting the cytoplasmic domain to a fluorescent protein.

When treating the TNFR2ΔCD biosensor with small molecules the FRET signal could increase, decrease, or not change. When the distance between the two fluorescent proteins

decreases, the FRET signal increases. When the distance between the two fluorescent proteins increases, the FRET signal decreases. When the distance between the fluorescent proteins does not change, there is no change in the FRET signal (Figure 3). Small molecules that interact with the TNFR2ΔCD biosensor could change the FRET by either disrupting the PLAD-PLAD interactions of TNFR2 or by binding to and changing the conformation of TNFR2.

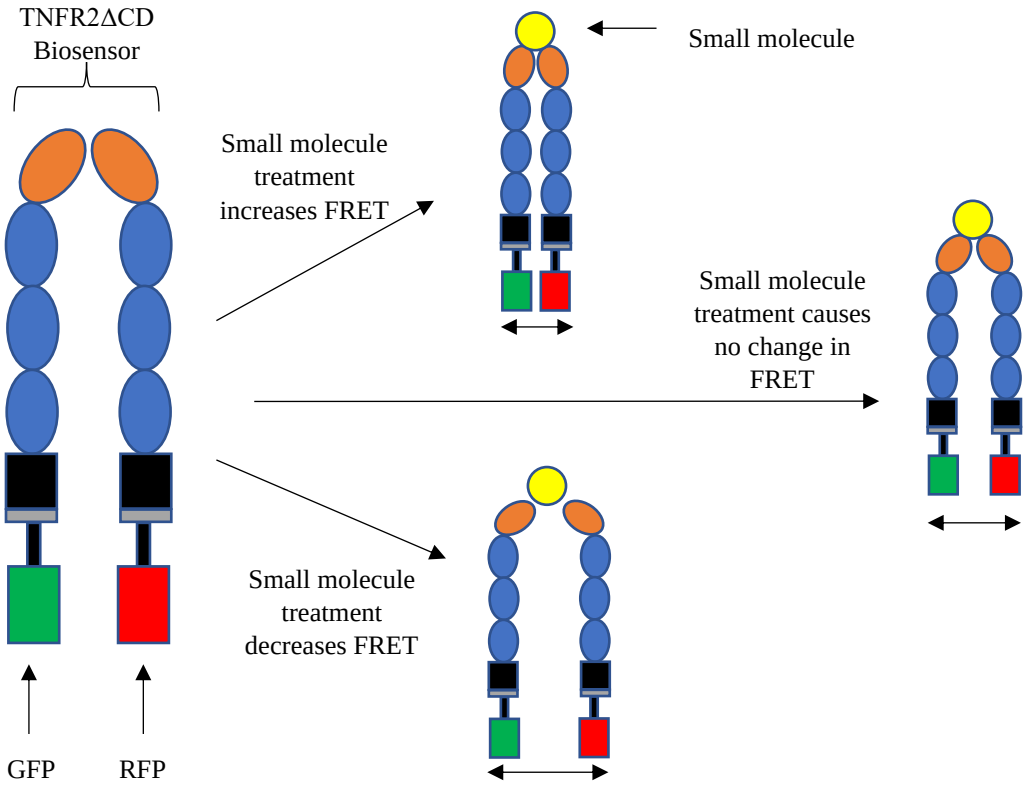

Figure 3: Schematic of small molecule treatment on the TNFR2ΔCD biosensor. Small molecule treatment could change the FRET signal of the TNFR2ΔCD biosensor by increasing or decreasing the distance between GFP and RFP. An increase in the distance decreases FRET. A decrease in the distance increases the FRET. Small molecule treatment could also cause no change in FRET.

TNFR2ΔCD Biosensor Characterization

The TNFR2ΔCD biosensor was characterized in multiple ways. Fluorescence microscopy was performed to observe the colocalization of the TNFR2ΔCD biosensor (Figure 4A). Fluorescence microscopy confirmed both GFP and RFP were expressing in cells transfected with the biosensor. Flow cytometry was performed using a TNFR2 antibody conjugated with phycoerythrin to determine if the TNFR2ΔCD-GFP and TNFR2ΔCD-RFP

biosensor constructs express the extracellular domain of TNFR2 when they are transiently transfected in HEK293 cells (Figure 4B). The phycoerythrin signal increased when HEK293 cells were transfected with either TNFR2ΔCD-GFP or TNFR2ΔCD-RFP meaning both constructs express the extracellular domain of TNFR2 at the surface of the cell. Fluorescence lifetime measurements were also performed on HEK293 cells transfected with either TNFR2ΔCD-GFP plus pcDNA empty vector (EV) or the TNFR2ΔCD biosensor (Figure 4C). The results show when both TNFR2ΔCD-GFP and TNFR2ΔCD-RFP are present the lifetime decreases compared to when only TNFR2ΔCD-GFP is present. This decrease in lifetime indicates FRET.

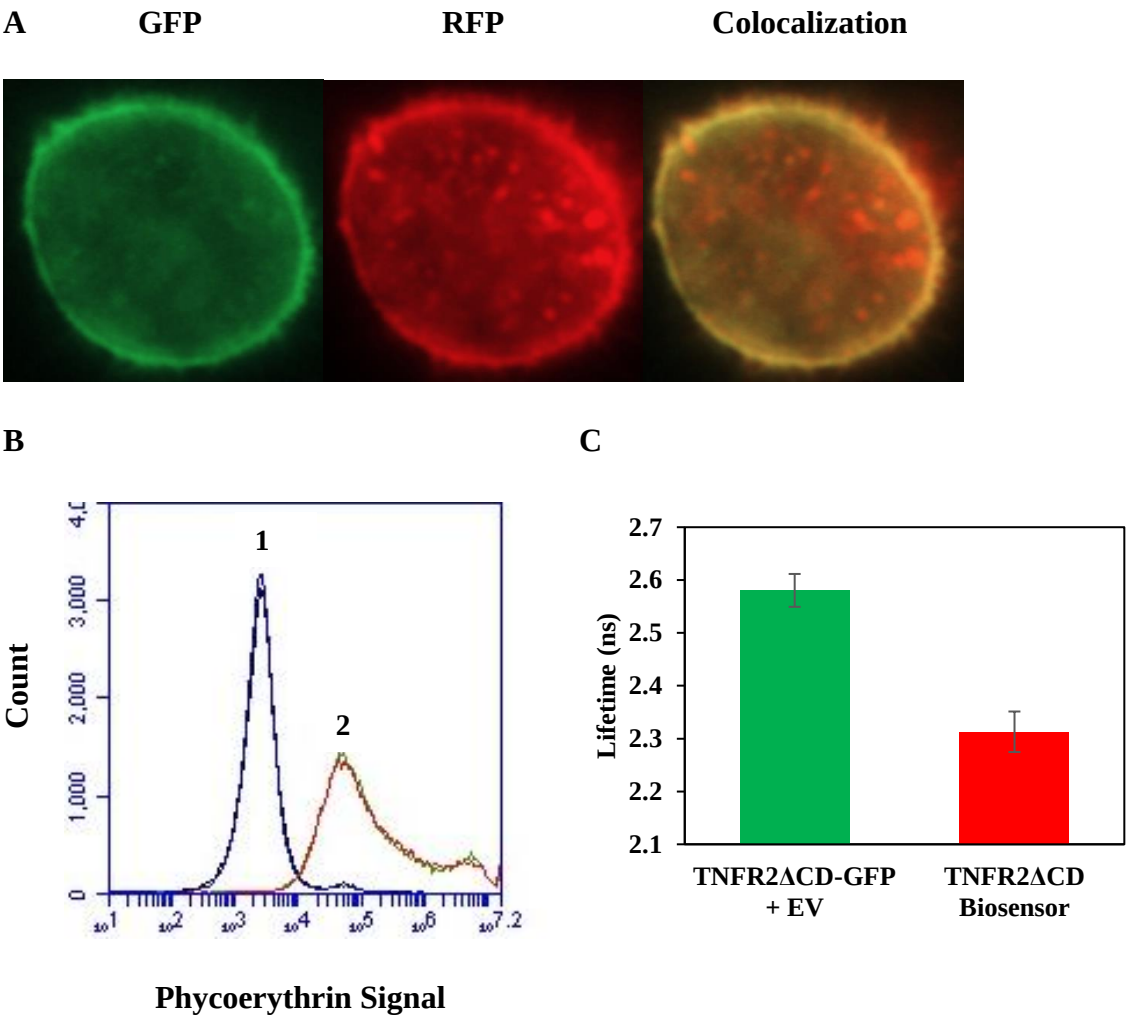

Figure 4: TNFR2 biosensor characterization. (A) Fluorescence microscopy images of TNFR2ΔCD-GFP and TNFR2ΔCD-RFP expressing in a HEK293 cell. 2 µg of TNFR2ΔCD-GFP and TNFR2ΔCD-RFP were transfected into HEK293 cells at a 1:6 ratio of donor to acceptor. All images were taken at 40x. (B) Flow

cytometry analysis of TNFR2 expression in HEK293 cells transfected with TNFR2ΔCD-GFP and TNFR2ΔCD-RFP plasmids. HEK293 cells were either non transfected, transfected with 2 μg of TNFR2ΔCD-GFP, or transfected with 2 μg of TNFR2ΔCD-RFP. The figure includes one line for each sample. Peak 1 is both the non-transfected HEK293 cells and the non-transfected HEK293 cells incubated with TNFR2 antibody. Peak 2 is HEK293 cells transfected with either TNFR2ΔCD-GFP or TNFR2ΔCD-RFP and incubated with TNFR2 antibody. (C) HEK293 cells were transfected with either TNFR2ΔCD-GFP and pcDNA EV or the TNFR2ΔCD biosensor. Both transfections were 2 μg of total DNA at 1:6 ratio of donor to acceptor/empty vector. Shown is the mean ± the standard deviation of 12 independent runs. Each run has between n=6 and n=13 trials.

The TNFR2ΔCD biosensor was also tested for its specificity. One way this was done was by performing lifetime measurements on HEK293 cells transfected with either the TNFR2ΔCD biosensor or TNFR2ΔCD-GFP and TNFR1ΔCD-RFP (Figure 5A). The FRET signal from cells transfected with TNFR2ΔCD-GFP and TNFR1ΔCD-RFP was significantly lower than the FRET signal from cells transfected with the TNFR2ΔCD biosensor. This result suggests TNFR2ΔCD-GFP and TNFR1ΔCD-RFP don't interact with each other to produce a FRET signal. This means the FRET signal coming from the TNFR2ΔCD biosensor is specific to the interaction of TNFR2 between the two constructs (TNFR2ΔCD-GFP and TNFR2ΔCD-RFP) of the biosensor and not from the fluorescent proteins (GFP and RFP).

Another way the specificity of the TNFR2ΔCD biosensor was tested was by treating the biosensor with zafirlukast. Zafirlukast has been shown to decrease FRET in a TNFR1ΔCD FRET biosensor with a half maximal effective concentration (EC_{50}) value of 18 μM [43]. HEK293 cells were transfected with the TNFR2ΔCD biosensor and plated into a 384 well plate. The cells were then treated with either DMSO or 50 μM of zafirlukast and the fluorescence lifetime measurements were performed (Figure 5B). This result shows zafirlukast did not change the FRET signal of the TNFR2ΔCD biosensor even with zafirlukast treatment above the EC_{50} value for the TNFR1ΔCD biosensor. This shows zafirlukast is specific to TNFR1 while also showing the specificity of the TNFR2ΔCD biosensor.

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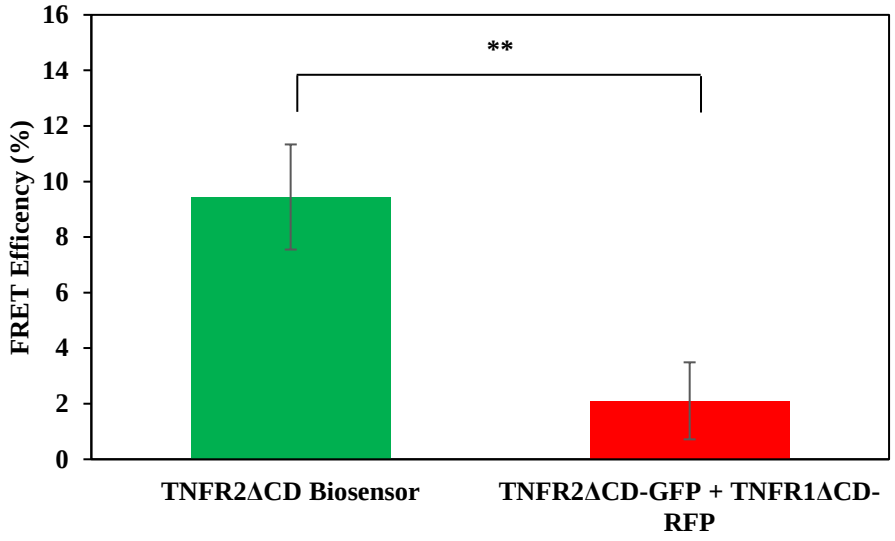

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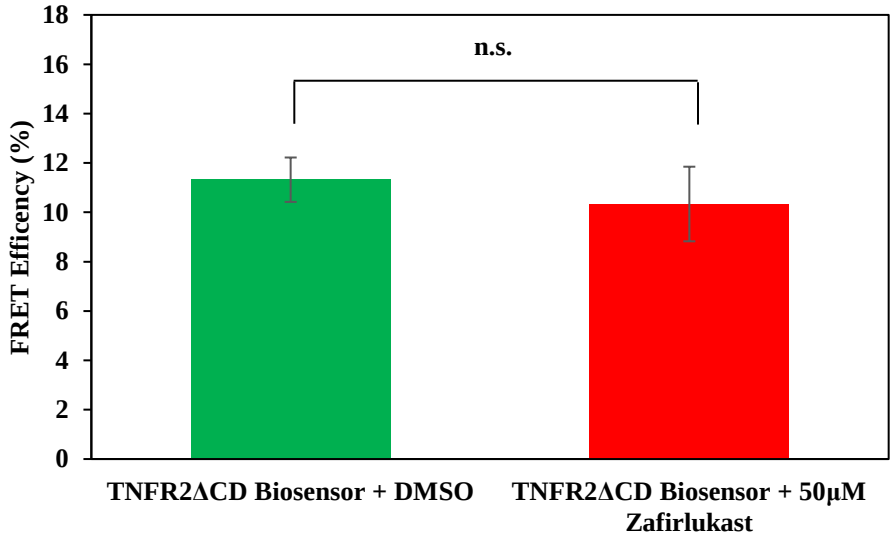

Figure 5: Specificity of TNFR2ΔCD biosensor. (A) HEK293 cells were transfected with either the TNFR2ΔCD biosensor or TNFR2ΔCD-GFP and TNFR1ΔCD-RFP. Both transfections were 2 μg of total DNA at 1:6 ratio of donor to acceptor. Shown is the mean ± the standard deviation of the FRET efficiency of three independent runs. Each run has n=13 trials. A two-tail unpaired t test was performed which produced a p value of 0.0056. (B) HEK293 cells were transfected with 2 μg of TNFR2ΔCD biosensor at a 1:6 ratio of donor to acceptor. The cells were either treated with DMSO or 50 μM of zafirlukast. Shown is the mean ± the standard deviation of the FRET efficiency of three independent runs with each run having n=7 trials. A two-tail unpaired t test was performed and produced a p value of 0.3871.

Chapter 4: Investigating the Specificity and Mode of Action of TNFR1 antagonists

Specificity of TNFR1 Antagonists

To test the specificity of TNFR1 antagonists, we developed a western blot assay to determine if they effect TNFR2 signaling. This was done by probing for the TNFR1 antagonists' effect on the expression of the NF-κB transcription factor RELB from the non-canonical NF-κB pathway [5, 7]. Knowing their effect on TNFR2 signaling is important for determining whether these antagonists are specific to TNFR1. For this assay, HUVEC cells were transiently transfected with membrane TNF. HUVEC cells endogenously express TNFR2 and TNF treatment has been shown to increase the expression of RELB in HUVEC cells [64]. The transfected HUVEC cells were treated with TNFR1 antagonists to determine if they effect the expression of RELB and therefore TNFR2 signaling. Western blots were run to probe for RELB and β-actin with β-actin being used as a control for total protein loaded in the gel. The TNFR1 antagonists tested were the zafirlukast analogs (Figure 6) and the TNFR1-binding affibody (Figure 7). Three independent runs were performed for both the zafirlukast analogs and the TNFR1-binding affibody. All three runs were quantified and RELB was normalized to β-actin.

The results for both the zafirlukast analogs and TNFR1-binding affibody show HUVEC cells transfected with membrane TNF saw an increase in RELB expression which is consistent with the literature. Both results also show no significant difference in the RELB expression with TNFR1 antagonist treatment. This means they do not affect TNFR2 signaling.

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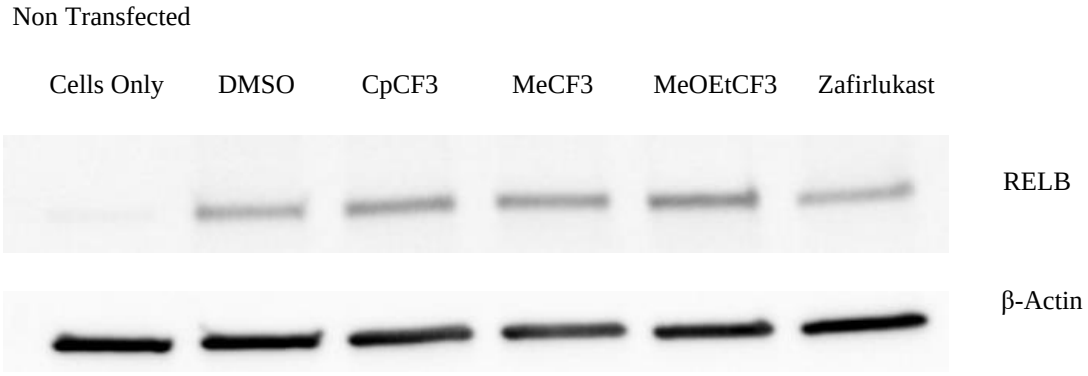

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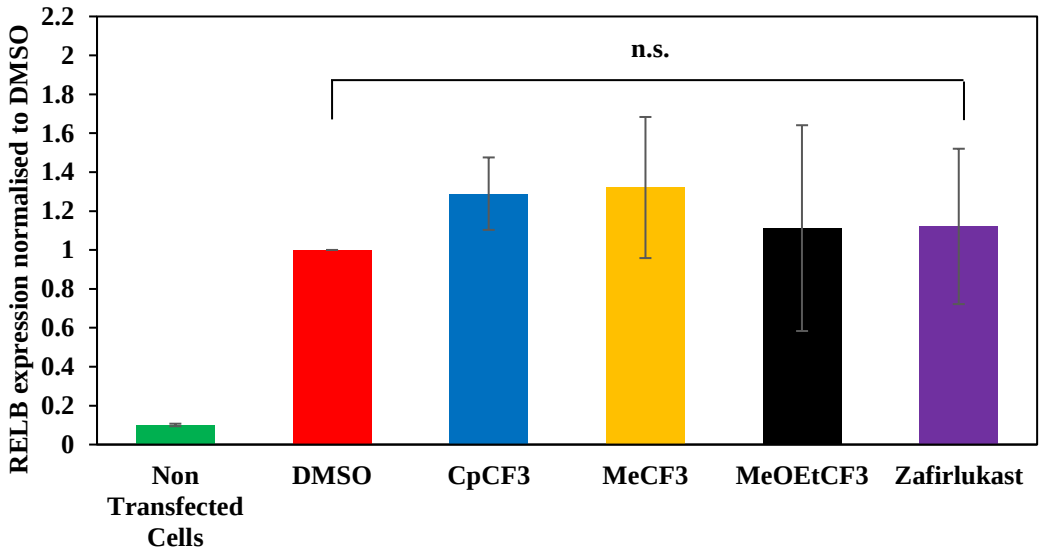

Figure 6: Effect of zafirlukast analogs on TNFR2 signaling. HUVEC cells were transfected with membrane TNF and treated with DMSO or 250nM of analogs or zafirlukast. Cells were lysed and western blot analysis was run probing for RELB and β-actin. (A) Representative image of western blots. (B) Densitometry analysis of western blot bands with RELB normalized to β-actin. All samples were normalized to the DMSO treatment for their respective trial. Graph shows the mean ± the standard deviation for the three independent trials. A two-tail unpaired t test was performed between DMSO and the analogs and zafirlukast. This produced p values of 0.518, 0.491, 0.887 and 0.947 for CpCF3, MeCF3, MeOEtCF3 and zafirlukast respectively.

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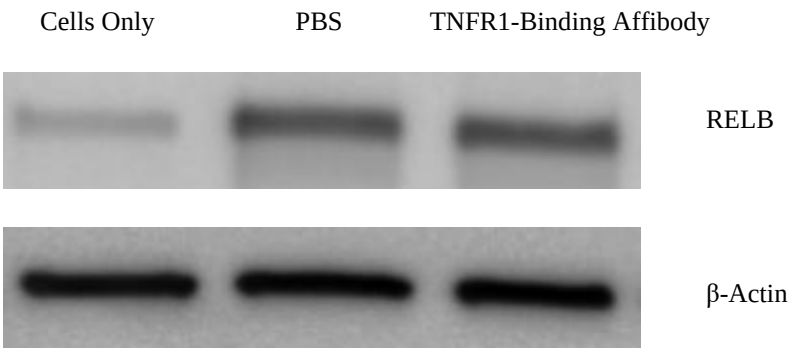

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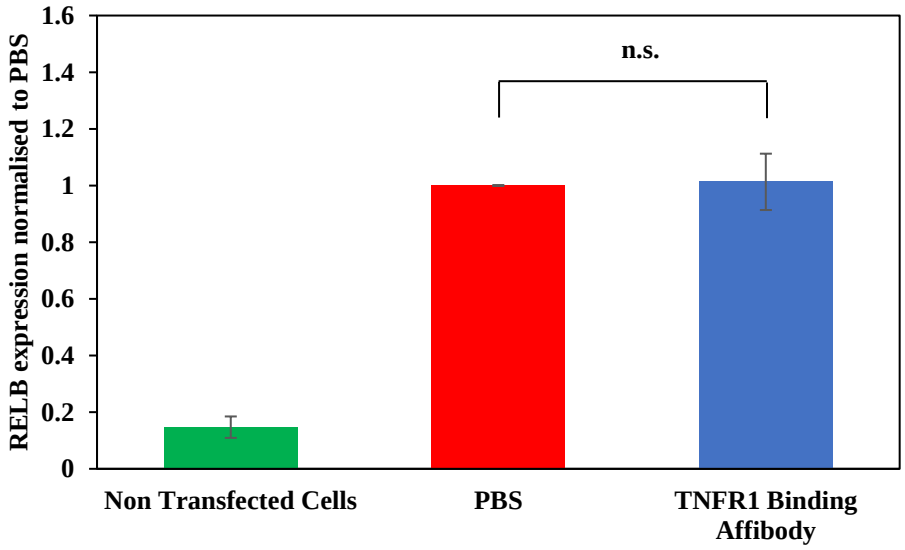

Figure 7: Effect of TNFR1-binding affibody on TNFR2 signaling. HUVEC cells were transfected with membrane TNF and treated with PBS or 0.01 μM of the TNFR1-binding affibody. Cells were lysed and western blot analysis was run probing for RELB and β-actin. (A) Representative image of western blots. (B) Densitometry analysis of western blot bands with RELB normalized to β-actin. All samples were normalized to the PBS treatment for their respective trial. Graph shows the mean ± the standard deviation for the three independent trials. A two-tail unpaired t test was performed between PBS and the affibody and produced p value of 0.892.

The specificity of the zafirlukast analogs was also shown by testing their effect on the TNFR2ΔCD biosensor. The TNFR2ΔCD biosensor was treated with DMSO or 10 μM of zafirlukast analog. The results show no significant difference between the DMSO and analog treatments (Figure 8).

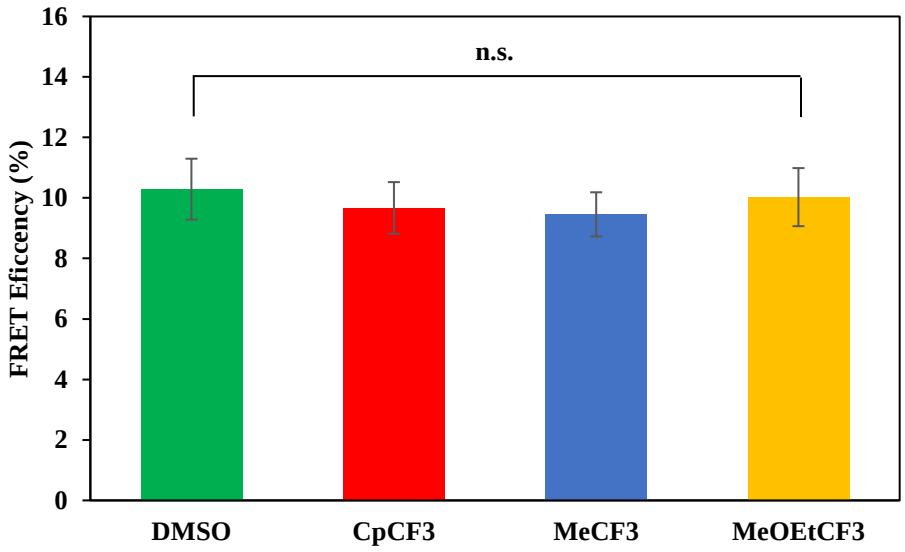

Figure 8: Effect of zafirlukast analogs on the FRET of the TNFR2ΔCD biosensor. HEK293 cells were transfected with 2 μg of TNFR2ΔCD biosensor at a 1:6 ratio of donor to acceptor. The cells were treated with either DMSO or 10 μM zafirlukast analogs. Shown is the mean ± the standard deviation of the FRET efficiency of three independent runs for a total of n=14 trials for DMSO and n=17 trials for analogs. A two-tail unpaired t test was performed between DMSO and the analogs and produced p values of 0.462, 0.311 and 0.76 for CpCF3, MeCF3 and MeOEtCF3 respectively.

Mode of Action of Zafirlukast Analogs

The mode of action of the zafirlukast analogs were tested to see if they disrupt ligand binding. In this assay TNF with a FLAG tag was incubated in anti-FLAG magnetic beads. This coated the magnetic beads with TNF. HEK293 cell lysate was then incubated in the TNF coated magnetic beads with or without the zafirlukast analogs. If the zafirlukast analogs disrupt ligand binding, the amount of TNFR1-binding to the magnetic beads will change. The beads were washed with PBS and the proteins attached to the beads were eluted. A western blot was run probing for TNFR1 and FLAG (Figure 9A). Three independent runs were performed, and all three runs were quantified with TNFR1 being

normalized to FLAG (Figure 9B). The results show no significant difference between DMSO treatment or any of the zafirlukast analog treatments. This means the zafirlukast analogs do not disrupt ligand binding.

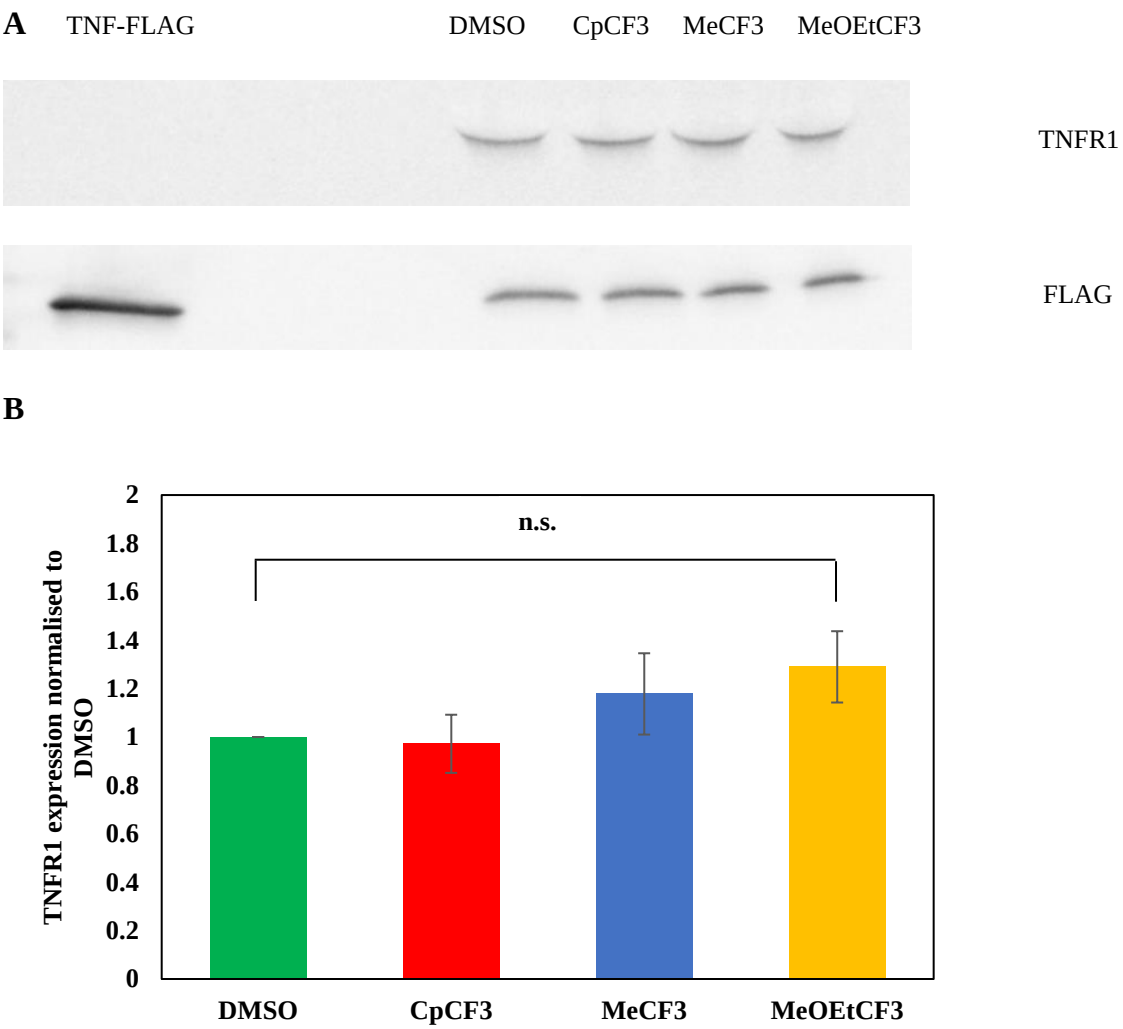

Figure 9: Effect of zafirlukast analogs on ligand binding. Anti-FLAG magnetic beads were incubated with FLAG tagged TNF for 2 hours at 4°C. The magnetic beads were washed three times with PBSA and then incubated overnight at 4°C with HEK293 cell lysate and DMSO or 250 nM of zafirlukast analogs. The beads were again washed three times with PBSA, and the protein was eluted from the beads. Western blot analysis was performed. (A) Representative image of western blots. (B) Densitometry analysis of western blot bands with TNFR1 normalized to FLAG. All samples were normalized to the DMSO treatment for their respective trial. Graph shows the mean ± the standard deviation for the three independent trials. A two-tail unpaired t test was performed between DMSO and the analogs and produced p values of 0.907, 0.548 and 0.317 for CpCF3, MeCF3 and MeOEtCF3 respectively.

Chapter 5: Discussion

Currently five FDA approved TNF antagonists are used to inhibit the inflammatory effects of TNFR1 signaling [54]. These antagonists act by binding to TNF and inhibiting its ability to bind to TNFR1 [54, 57]. However, by binding to TNF to inhibit TNFR1 signaling, these antagonists also inhibit TNF binding to TNFR2 which can create severe side effects [55]. Adverse effects have been found when using TNF antagonists to treat diseases such as type 1 diabetes and multiple sclerosis [53]. Many other adverse effects have been reported when using TNF antagonists including an increase in infections and malignancies, the worsening of congestive cardiac failure, and injection site reactions [58]. Therefore, it is better to selectively target either TNFR1 or TNFR2 signaling [55]. Previous work in our lab sought to discover small molecules that selectively target TNFR1 signaling. Selectively targeting TNFR1 signaling would not affect TNFR2 activity. We showed a TR-FRET high throughput screening platform could be used to discover small molecule binders to TNFR1. Using this screening method, zafirlukast was discovered to be a TNFR1 inhibitor. However, the potency of zafirlukast was low with an absolute IC_{50} of 114 μM when inhibiting IκBα degradation and an absolute IC_{50} of 50 μM when inhibiting NF-κB activation [43].

This previous work done in our lab to discover small molecule modulators of TNFR1 signaling has led to two new areas of study. One of these areas being to discover small molecule modulators of TNFR2 signaling. Because the TR-FRET based high throughput screening platform used previously in our lab was successful in discovering a new TNFR1 inhibitor, the same methods were applied to TNFR2. Modulators of TNFR2 signaling would be useful because TNFR2 can be involved in different autoimmune diseases, cancer, and neurodegenerative diseases [1, 26-32]. The other new area of study was to improve the potency of the TNFR1 lead compound zafirlukast. This was done by creating new structural analogs of zafirlukast. Improving the potency of zafirlukast will help make it a better TNFR1 inhibitor by reducing nonspecific interactions. TNFR1 inhibitors could be useful when treating certain inflammatory diseases, neurodegenerative diseases, and cancer [50-52].

The first goal of the current study was to develop a TR-FRET based high throughput screening platform for the discovery of small molecule modulators of TNFR2 signaling. The development of this screening platform involved creating and testing a TNFR2ΔCD biosensor. The results of this study showed the TNFR2ΔCD biosensor constructs expressed both the TNFR2 extracellular domain and either GFP or RFP. The biosensor was also shown to produce a FRET signal specifically because of the interactions between the TNFR2 of the TNFR2ΔCD-GFP and TNFR2ΔCD-RFP constructs. Knowing the FRET signal from the biosensor is specific to TNFR2 interactions is important because during a high throughput screen the biosensor will be able to identify any small molecule that interacts with the structure or conformation of TNFR2. These small molecules identified in the screen can then be studied further to investigate whether they modulate TNFR2 signaling.

The other goal of this study was to investigate the specificity and mode of action of TNFR1 antagonists. The specificity of these compounds was investigated by developing an assay to test their effect on RELB expression. TNFR2 signaling can activate the non-canonical NF-κB pathway while TNFR1 signaling cannot [5, 7]. Therefore, investigating the effect these compounds have on RELB expression corresponds to their effect on TNFR2 signaling. Both the zafirlukast analogs and TNFR1-binding affibody did not affect TNFR2 signaling. This shows their specificity to TNFR1. Also, the zafirlukast analogs did not affect TNF binding to TNFR1. This means the analogs are not binding to the ligand binding site of TNFR1. Both the specificity and mode of action results are promising. Because both antagonists do not affect TNFR2 signaling, they would not cause the same side effects as TNF inhibitors. Also, the zafirlukast analogs not disrupting ligand binding could help reduce side effects. This is because if the zafirlukast analogs did disrupt ligand binding the concentration of the ligand would change outside the cell since TNF would bind with a different affinity to TNFR1. A change in the concentration of ligand could create undesired effects such as different amounts of TNF being available to bind to TNFR2. However, since they do not disrupt ligand binding the amount of TNF binding to TNFR1 will not change.

While the results from this study are promising there are some limitations to the screening methods. While performing this TR-FRET high throughput screen, it's possible some small molecules could cross the plasma membrane of the cell. Small molecules can cross the plasma membrane based on their size, polarity, and charge. The larger the molecule is the less permeable it is. The plasma membrane is also impermeable to almost all charged molecules and large uncharged polar molecules. However, small and moderately polar molecules can cross the plasma membrane [65]. If small molecules cross the plasma membrane into the cell, they could interact with the fluorescent proteins or other parts of the intracellular domain of the biosensor which could affect the FRET signal. Also, some small molecules may be fluorescent which would change the FRET signal. These problems could create false positives. However, the false positives would be able to be identified when doing functional assays because they would not affect TNFR2 signaling.

The results of this current study also create the opportunity for future studies. One future study could be to further develop the RELB assay. The RELB assay used in this study probed for total RELB expression. However, TNFR2 signaling has been shown to trigger the nuclear translocation of RELB [6]. Investigating whether molecules effect the nuclear translocation of RELB is another way of determining their effect on TNFR2 signaling. More nuclear translocation of RELB would indicate more TNFR2 signaling. In the future, an assay probing for nuclear and cytoplasmic RELB could be useful in studying the effect these small molecules have on TNFR2 signaling. Another possible method for testing TNFR2 signaling could also be by investigating p100 and p52 expression. TNFR2 signaling has been shown to trigger p100 processing and the nuclear translocation of p52 [6]. Using this information, assays could be developed to probe for the ratio of total p100 and p52 expression and the amount of nuclear and cytoplasmic p52. These new assays could then be used to test the functionality of the lead compounds found from high throughput screen with the TNFR2ΔCD biosensor and to investigate the specificity of the TNFR1 antagonists.

Along with more assays to investigate the effect different molecules have on TNFR2 signaling, other future studies will be needed. These future studies primarily focus on the discovery of small molecule modulators of TNFR2 signaling. In the future, a Selleck high

throughput screen will be performed to screen for small molecule binders to TNFR2 using the TNFR2ΔCD biosensor created in this study. The small molecules that significantly increase or decrease the FRET signal will be identified as lead compounds. These lead compounds would then be tested for their cytotoxicity, effect on the TNFR2 signaling pathway through functional assays, mode of action through ligand binding assays, and their specificity to TNFR2. Many of these assays have previously been performed in our lab. These include the assays used in this study to investigate the specificity and mode of action of the TNFR1 antagonists. Another future study with the TNFR2 lead compounds could be testing their effect on other FRET biosensors such as the TNFR1ΔCD biosensor. TNFR1 and TNFR2 have similar structural features such as an extracellular domain containing four cystine rich domains and an α-helical transmembrane domain. The main differences between the two receptors come from their cytoplasmic domain. TNFR1 has a death domain while TNFR2 does not [66]. If the TNFR2 lead compounds do not affect the FRET of the TNFR1ΔCD biosensor it would further show this method of screening small molecules is effective.

The methods used in our lab to discover small molecule modulators specific to TNFR1 or TNFR2 signaling have been shown to be effective. Using FRET biosensors in high throughput screening platforms and testing the lead compounds in different assays following the screen has led to the discovery of new TNFR1 inhibitors and could lead to new modulators of TNFR2 signaling. These lead compounds could be promising therapeutic candidates for treating diseases involving TNFR1 or TNFR2. In the future, this strategy could also be used to discover small molecules specifically targeting other receptors besides TNFR1 and TNFR2.

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