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Regulation of tumor necrosis factor-alpha signaling in rheumatoid arthritis synovial fibroblasts by green tea polyphenol epigallocatechin-3-gallate

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A Thesis Entitled

Regulation of Tumor Necrosis Factor-alpha Signaling in Rheumatoid Arthritis Synovial Fibroblasts by Green Tea Polyphenol Epigallocatechin-3-gallate

By Sharayah Riegsecker

Submitted to the Graduate Faculty as a partial fulfillment of the requirement for the

Master of Science in Pharmacology and Toxicology

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May 2013

An Abstract of

Regulation of Tumor Necrosis Factor-alpha Signaling in Rheumatoid Arthritis Synovial
Fibroblasts by Green Tea Polyphenol Epigallocatechin-3-gallate

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Rheumatoid arthritis (RA) is a chronic inflammatory disease, which affects about 1% of

the population. It occurs when cytokines, such as interleukin (IL)-1β and tumor necrosis

factor (TNF)-α, stimulate synovial fibroblast proliferation leading to joint damage and

destruction. Epigallocatechin-3-gallate (EGCG) is a polyphenol found in green tea that

has been shown to have an anti-inflammatory effect in RA. Previous studies suggest that

EGCG blocks IL-1β signaling pathways to inhibit angiogenesis and tissue destruction in

RA. However, little research has been done to determine the effect of EGCG in the TNF-

mediated pathways in RA synovial fibroblasts. In these experiments, we investigated the

effects EGCG has on the TNF-α-induced signal transduction pathways in RA synovial

fibroblasts. In particular, we studied the effect of EGCG on the TNF receptors, TNF

Receptor 1 (TNF-R1) and TNF Receptor 2 (TNF-R2), as well as downstream proteins,

such as IL-6 and IL-8 in the TNF signaling pathways in RA synovial fibroblasts. We also

compared the acute and chronic effects of EGCG on TNF-α-mediated signaling pathways

in RA synovial fibroblasts. Results of the study showed that EGCG modestly decreased

TNF-R1 and increased TNF-R2 expression. Chronic low dose (100 nM – 1 μM) pretreatment of RA synovial fibroblasts resulted in a marked dose-dependent inhibition of TNF-α-induced AKT activation. We also observed that EGCG modestly inhibited JNK and p38 with no effect on ERK MAPK in RA synovial fibroblasts. Inhibition of TNF-α-induced downstream signaling pathways by EGCG resulted in decreases in IL-6 and IL-8 production by RA synovial fibroblasts. Overall, the results of this study suggest that EGCG decreases the inflammation and tissue invasion mediated by RA synovial fibroblast inflammatory cytokines via interfering with TNF-α signaling.

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Chapter 1

Introduction

1.1   Rheumatoid Arthritis:

Rheumatoid arthritis (RA) is a chronic inflammatory disease most commonly known for the inflammation of the joints, but this disease also has the ability to become systemic and impact other vital organs as well. In 2009, it was estimated that 0.6% (1.3 million) of the population of the United States had RA [1]. Of those diagnosed with RA, most of the patients are women [2, 3]. There is no known cause of RA, but it is believed that a combination of genetic and environmental factors may make a person more susceptible to the development of this disease. In RA, synovial fibroblasts are activated and as the disease establishes, these cells begin to hyperproliferate and destroy the cartilage and bone, which results in the joint destruction experienced by RA patients [1].

1.1.1 Risk Factors

As previously discussed, there are both genetic and environmental risk factors associated with the development of RA. Many cases of RA are believed to result from a

combination of both [4]. The following sections will discuss both types of risk factors in greater detail.

1.1.1.1 Genetic Risk Factors

The HLA-DRB1 allele is one of the most common genetic risk factors. These alleles encode the shared epitope (SE) [5], which has been linked, in some studies to the severity of RA. This was thought to be because of the rheumatoid factor (RF) in combination with the SE, but some studies have shown that both RF and SE alone can produce similar radiographic damage in patients who suffer from this disease long term [6]. Another study showed that the increased risk for RA in those with HLA-DR SE is only in those who are also positive for anti-citrulline antibodies [7].

Another gene that has come into the spotlight more recently for increasing a person's susceptibility to RA is Protein Tyrosine Phosphatase (*PTPN22*). The *PTPN22* gene encodes a lymphoid-specific phosphatase (Lyp), which then binds to the C-src tyrosine (Csk) kinase. Once this binding happens, T cell receptor binding is inhibited. If there is a single nucleotide polymorphism in this gene, the association between the Lyp and Csk does not happen and T cell receptor binding cannot be inhibited [8]. A study done by Begovich et al. showed that there was an association in white individuals with the single nucleotide polymorphism of *PTPN22* and susceptibility to RA [9]. Another study using Spanish caucasion study subjects showed that an association could be made between the

polymorphism of this gene and susceptibility to RA. It was suggested that *PTPN22* may even be used as a marker for systemic autoimmune diseases [8].

Another genetic risk factor that has recently emerged as a possible risk factor for RA is a single nucleotide polymorphism in the *TRAF1-C5* gene on chromosome 9 [10]. The *TRAF1* gene encodes a protein that mediates tumor necrosis factor (TNF) receptor 1 and 2 signal transduction. It is believed that a polymorphism in this gene could lead to an exaggerated T cell response. The *C5* gene is part of the complement pathway and a polymorphism in this gene could lead to an increase in complement activation in the affected joints [10]. Because it is unknown exactly how this risk factor makes a person more susceptible to RA, more research is being done in this area.

1.1.1.2 Environmental Risk Factors

Smoking has been shown to be the strongest environmental risk factor associated with RA [11]. Heavy smokers have twice the risk of developing RA than people who have never smoked and this increased risk continues for the smoker 20 or more years after cessation [11]. The increased susceptibility to RA faced by smokers is further increased 21-fold compared to non-smokers without the SE genes if the smoker has double copies of the susceptibility genes *HLA-DR*. These results are observed in those with anti-citrulline antibody-positive RA, but people who have SE genes are more likely to be positive for the presence of anti-citrulline antibodies [7]. Heavy smokers also are more likely to develop rheumatoid factor (RF) and seropositive RA [12].

Other environmental risk factors that have been found to be associated with RA include high birthweight, socioeconomic status, and geography. In 2009, a study was done using data from 1976 to 2002 that suggested that babies weighing more than ten pounds at birth were at a higher risk of developing RA although the biological mechanism is not yet known [13]. Socioeconomic status is another environmental factor that may play a role in the risk of RA. Studies show that people with more education and non-manual labor careers experienced a lower risk than those with less education and manual labor careers [11]. Geography also seems to play a role in the incidence of RA in women in the United States. Women who were born and remained in the Midwest through at least age 30 had a higher risk of developing RA than those who were born and remained in the West. RA risk was also found to be higher in the New England area [14].

A variety of environmental factors have been found to play a protective role against RA. Among these protective factors are oral contraceptives, alcohol consumption, and breastfeeding. Estrogen has been thought to be a risk factor in RA development because of the fact that most RA patients are women. Because of this, oral contraceptives (OC) were once thought to have a protective effect against this disease. Some recent studies have suggested that OC use is not as protective as originally predicted, but a study done in 2007 showed that healthy women who used OCs did not develop RF like those who did not use OCs [15]. Alcohol consumption has also been described as having a protective effect in RA. The results of two case-control studies showed that there is a

dose-dependent inverse relationship between alcohol consumption and RA [16].

Breastfeeding also seems to provide a protective effect to women. According to a study

done by Karlson *et al.,* with increased duration of breastfeeding, there was a decreased

risk of developing RA. Women in this study who had breast-fed for a lifetime total of

more than 24 months appeared to have a 50% decrease in risk [17].

1.2 Synovial Fibroblasts:

Synovial fibroblasts are found in the joints, with their major role being to provide plasma

proteins and lubricating molecules like hyaluronic acid to the joint cavity and cartilage

[18]. They also produce matrix-degrading enzymes as well as components that make up

the matrix in the process of matrix remodeling. When RA develops in the patient, the

synovial fibroblast activation drives inflammation by producing inflammatory cytokines

such as TNF-α, IL-6, and IL-8 and plays a major role in the degradation of the diseased

joint by producing matrix-degrading enzymes called matrix metalloproteinases (MMPs)

[18]. The synovial lining of a normal joint is made up of two or three layers of synovial

fibroblasts, but in a RA-diseased joint, hyperplasia of RA synovial fibroblasts result in a

multilayer lining and increased activation of inflammatory cytokines.

From the earliest phases of RA, synovial fibroblasts play a major role in disease

pathogenesis. Once the fibroblasts produce the cytokines and chemokines, macrophages,

T cells and neutrophils chemotaxis to the synovium. The increase in inflammatory cells

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in the joints further enhances the activation of the synovial fibroblasts [19]. Rheumatoid arthritis synovial fibroblasts can also directly damage cartilage by attaching to the cartilage by increasing the production of adhesion molecules and then producing MMP-2, thereby damaging bone and cartilage [20]. At these invasion sites, more of the fibroblasts avoid apoptosis through the upregulation of pro-survival proteins like Bcl-2, Mcl-1, AKT, and NFκB [21-24]. Since these cells are not regulated by contact inhibition, they constantly multiply and continue their invasion of the joint [25]. Angiogenic growth factors such as IL-8, vascular endothelial growth factor (VEGF), and transforming growth factor β (TGFβ) are also produced by these synovial fibroblasts [19], promoting new vessels to run through the increasing amount of fibroblasts to provide them with the nutrients needed for survival.

1.3 RA Classification Criteria:

Because RA is best treated when detected early in patients and early diagnosis has previously been shown to be quite difficult due to the non-specific symptoms experienced by the patient [4], a new set of classification criteria was developed in a collaborative effort by the American College of Rheumatology (ACR) and the European League Against Rheumatism. Prior to 2010, the criteria had not been updated since 1987. This former set of criteria had frequently been criticized because it was not sensitive enough to include those with early disease [26]. These new criteria first establish a target population of patients who should be tested for RA. These include patients who have at least one joint with clinical synovitis that cannot be better explained by another disease such as

gout, psoriatic arthritis, or systemic lupus erythematosus. These patients can then be

tested in four other criteria to determine whether they have definite RA. The following, is

the summary of the last four criteria involved in the classification:

1. **Number of Joints involved**

1 large joint	0
2-10 large joints	1
1-3 small joints (with or without swollen large joints)	2
4-10 small joints (with or without swollen large joints)	3
> 10 joints with at least one being a small joint	5

2. **Serology**

Negative Rheumatoid Factor (RF) and anti-citrullinated protein antibody (ACPA)	0
Low-positive RF or ACPA	2
High-positive RF or ACPA	3

3. **Acute-phase reactants**

Normal C-reactive protein (CRP) and erythrocyte sedimentation rate (ESR)	0
Abnormal CRP or ESR	1

4. **Duration of symptoms**

Less than 6 weeks	0
6 weeks or more	1

Each of the criteria have scores associated with them, with the total score, when scores from each criteria are added, ranging from 0 – 10. If the patient achieves a score greater than or equal to 6, that patient can be identified as having definite RA. Any score lower than 6 cannot be identified as having definite RA, but these patients can be periodically retested to make sure any definite cases of RA are detected as early as possible [26].

1.4 Current Pharmacotherapy for RA:

There is no cure for RA, but there are several different pharmaceutical options available to treat the symptoms of the disease as well to slow the progression. It has been found that these treatments are most effective when prescribed early in the disease. The pharmaceutical options include disease-modifying antirheumatic drugs (DMARDs), biologics, corticosteroids and nonsteroidal anti-inflammatory drugs (NSAIDs).

1.4.1 Disease-Modifying Antirheumatic Drugs

Methotrexate (MTX) is the most common DMARD and is often given in combination with other DMARDs. A low dose of MTX is more effective than the other DMARDs at treating the signs and symptoms as well as preventing further structural damage to reduce disability in RA. The specific mechanism of action of MTX in RA is unknown, but this drug is a known folic acid antagonist [27]. This drug can be administered orally, intramuscularly, or subcutaneously once a week at doses from 7.5 to 25 mg [28].

Absorption of MTX takes place in the proximal jejunum and is unaffected by food, but its bioavailability decreases when higher oral doses are administered. Because of this, patients requiring higher doses receive parenteral treatment. Methotrexate is actively taken into cells such as erythrocytes, white blood cells, hepatocytes and synoviocytes via the reduced folate carrier (RFC). The half-life of MTX is about 6-8 hours and it takes about 6 hours after the start of treatment to reach steady state [28]. As with all drugs, there are some toxicities associated with MTX and these toxicities are classified into the following subcategories. *Type A toxicities* are dose-dependent effects that occur due to the effects of folate antagonism on cells that naturally undergo more rapid cell replication (such as bone marrow). *Type B toxicities* are strange or rare toxicities one wouldn't generally associate with a folate antagonist. The effects that are related to the long-term fibrogenesis associated with MTX, such as hepatotoxicity, are classified as *Type C toxicity*. *Type D toxicities* are the final subcategory that arise due to the effects that may occur after the patient has discontinued the use of the drug. Overall, the most common side effects of MTX are nausea and malaise with serious side effects only being reported in less than 10% of patients [28].

There are other DMARDs prescribed including hydroxychloroquine (HCQ), sulfasalazine (SSZ), and leflunomide. Gold salts, cyclosporine A, chloroquine, cyclophosphomide, and azathioprine are also DMARDs, but are less commonly prescribed. These other DMARDs are often given in combination with MTX as a means of more effective treatment of RA. For example, HCQ is given in combination with MTX to slow the clearance of MTX and thereby make the MTX treatment more effective [28]. A

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randomized, double-blind, placebo-controlled trial done over two years to compare the efficacy of different DMARDs in combination with MTX found that the efficacy of the drugs were highest when a triple therapy of MTX, SSZ, and HCQ is given. This triple therapy was also found to be well-tolerated by the patients [29].

1.4.2 Biologics

Biologics are protein-based or nucleic acid-based pharmaceutics that are produced by something other than the native/nonengineered biological source [28]. In RA, these biologic treatments are based on immunoglobulins and have been such a beneficial treatment option because they are able to inhibit cytokines rather than the small molecule DMARDs inhibit [30]. In theory, because these drugs are more targeted, their effects should be more specific and produce less adverse effects. There are five classes of biologics available for the treatment of RA: TNF inhibitors, Interleukin (IL)-1 receptor antagonists, B-cell inhibitors, T-cell costimulation inhibitors, and IL-6 inhibitors. The IL-1 receptor antagonist has been found to be less effective than the other four classes of biologics and is rarely used to treat RA [30].

There are five TNF inhibitors currently on the market to be used to treat RA. Infliximab is an anti-TNF-α antibody that binds to both membrane bound and soluble TNF-α preventing it from binding to its receptors. This drug also kills cells that express TNF-α. Infliximab is administered to patients intravenously (i.v.) at a dose of 3 mg/kg every 8

weeks [30]. Etanercept is another TNF inhibitor. This drug is a soluble TNF-receptor fusion protein that has two dimers linked to the Fc portion, each containing extracellular, ligand-binding portions of the TNF Receptor 2 (TNFR2). This protein can bind to both TNF-α and TNF-β, preventing them from binding to the TNF receptors. This drug is administered subcutaneously (s.c.) at doses of either 25 mg twice per week or 50 mg weekly [30]. Adalimumab is another TNF inhibitor that is given s.c. It is administered in the form of monoclonal antibody every two weeks and works by binding to TNF-α to prevent it from binding to its receptor as well as by lysing cells expressing TNF-α on their surface [30]. Certolizumab does not contain the Fc region as the other TNF-inhibitors do. This drug is composed of the antigen-binding domain of the TNF antibody added to a polyethylene glycol to increase its half-life. Like infliximab, certolizumab binds to both membrane-bound and soluble TNF-α. It is administered s.c. at a dose of 400 mg every two weeks for the first six weeks and 200 mg every two weeks following the six week loading dose [30]. Golimumab is the final TNF inhibitor. It is a monoclonal antibody that binds soluble and membrane-bound TNF-α. It is administered s.c. at a monthly dose of 100 mg [30].

The remaining three classes (IL-6 inhibitors and B-cell and T-cell modulators) are each made up of a single drug. Tocilizumab is the only IL-6 inhibitor available. It is a recombinant humanized anti-human IL-6 receptor monoclonal antibody, binding to both soluble and membrane-bound IL-6 receptors thus preventing IL-6 activation [30]. Rituximab is the B-cell modulating drug. It is a monoclonal antibody that targets cells with the CD20 surface marker. This drug is found to be most effective in RF-positive RA

patients, but the mechanism explaining why the elimination of B-cells improves RA is not proven [30]. Finally, the T-cell modulator, Abatacept, is a fusion protein that binds to the CD80/86 ligand and prevents it from interacting with CD28 on T-cells to prevent the activation of the T-cell. When T-cell activation is prevented, there is also a reduction in TNF, IL-1, and IL-6, key cytokines in the pathogenesis RA [30].

1.4.3 Corticosteroids and NSAIDs

Glucocorticoids and NSAIDs are given as more of a short-term treatment to reduce inflammation. They can also be administered in combination with DMARDs to aid in the reduction of joint erosion and systemic disease [30]. They should not be used as monotherapy for this disease because they do not have any effect of the progression of RA [27]. These drugs should only be used as a short-term treatment to alleviate some of the symptoms associated with RA, as the studies have shown that patients who used NSAIDs for 4 weeks or more observed a significant increase in blood pressure [31]. Because patients with RA are already at an increased risk for developing cardiovascular complications, they should be cautious about NSAID use.

1.5 Alternative Therapies for Rheumatoid Arthritis:

There are several complementary and alternative therapies available to RA patients. Some are nutritional options and others are activities patients can participate in including yoga, arthritis education or relaxation techniques. Yoga has been shown to be at least

somewhat effective in decreasing the pain and disability associated with RA. A study done in India enrolled RA patients in a one week yoga camp in which they participated in two yoga sessions per day equaling five hours of yoga. The study showed that, within the group, there was an increase in health assessment questionnaire score, a decrease in pain, an increase in hand grip strength and decreases in RF and C-reactive protein (CRP) levels [32]. A study reported in 2011 that relaxation and arthritis educational classes both improved the patient's pain levels and overall score on a RA symptom questionnaire as well as improvements in self-care and social activities [33].

There are also several herbal treatments that have been shown to have beneficial effects in RA patients. Borage oil, fish oil, *Tripterygium wilfordii* Hook-F (TWHF), and (-)-epigallocatechin-3-gallate (EGCG) are some of the complementary and alternative medicines available. Borage oil has been found to reduce TNF-α levels in RA patients. This oil can be extracted from the seeds of *Borago officinalis*. It is able to decrease TNF-α by increasing prostaglandin E_2 (PGE_2) levels. Because of the increased levels of PGE_2, this alternative therapy should not be used in individuals who rely on NSAIDs for pain relief or for women who are pregnant. Borage oil shouldn't be used in NSAID users because its effects would be diminished due to the NSAIDs decreasing PGE_2 levels and in pregnant women, PGE_2 induces labor, so it should not be administered to this population [34]. Fish oil is another alternative therapy that has been found useful in the RA patient population. Ten double-blind, placebo-controlled trials have shown that long term RA patients who take fish oil daily for three months experience less pain and fewer tender joints as well as a shorter duration of morning stiffness and less use of NSAIDs

[35]. Fish oil is also known for its positive cardiovascular effects, which is also beneficial to RA patients who are at an increased risk for cardiovascular problems. The Chinese herb TWHF is used as a way to regulate a wide range of mediators of the disease including TNF-α, IL-1, IL-17, MMP-3 and -13, adhesion molecules, cyclooxygenase-2, and E-selectin. Regulation of these factors resulted in reduced inflammation and increased apoptosis in RA synovial fibroblasts, which cause much of the invasive damage associated with the disease [36]. Epigallocatechin-3-gallate is a compound found in green tea and will be discussed further in the following section.

1.6 Epigallocatechin-3-gallate:

Green tea is a non-fermented tea and is produced by steaming and drying the leaves from *Camellia sinensis.* The leaves from this plant contain about 20-40% by weight polyphenols, most of which are catechins [37]. Epigallocatechin-3-gallate makes up about 63% of the catechins found in green tea [38]. Because green tea is one of the most popular beverages worldwide, it is an easy to find, relatively inexpensive alternative medicine option that provides the patient with between 60 and 125 mg catechins in each cup [38].

Phase I and phase II clinical trials have been performed on noncancer patients to determine the adverse effects related to the consumption of green tea and found that no adverse effects were apparent in any of the patients, even after 12 weeks of receiving the

highest dose [39]. Animal and *in vitro* studies have shown that when EGCG is itself administered, it does become toxic at high doses (200 mg/kg in mice and 100 μmol/L in noncancer cells) [40]. Pharmacokinetic studies performed in mice and rats showed that after intraperitoneal (i.p.) administration of EGCG, the highest plasma concentration occurred 30 minutes after administration. Another study found that dosing mice orally with EGCG resulted in a peak plasma concentration of 15-112 μg/ml at 2 hours after administration [41]. The half-life of EGCG in humans is between 5 – 5 ½ hours with the highest concentration occurring about 1.4 – 2.4 hours after ingestion [42]. One problem associated with EGCG as an alternative therapy option for RA patients is that EGCG consumed in green tea has a low absorption with less than 1% being absorbed. Because of this low absorption, the patient would have to drink about 7 or 8 cups of green tea to achieve plasma levels of EGCG high enough for any biologic activity [40].

Figure 1: Molecular Structure of EGCG
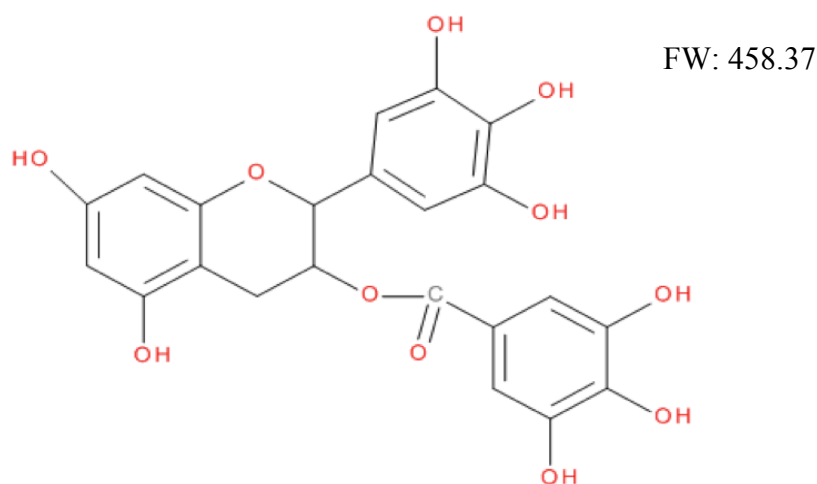
Fig. 1. The molecular structure of –(-) epigallocatechin-3-gallate

1.6.1 Beneficial Characteristics

The majority of the beneficial effects in the therapeutic use of green tea can be attributed to catechins, which are "polyphenols with a flavonoid structure" [43]. Epigallocatechin-3-gallate is the main catechin found in green tea and it is this catechin structure that gives EGCG its anti-oxidant classification. The C3 hydroxyl group on the flavonoid skeleton portion of the compound forms an ester with gallic acid to form EGCG. This compound can be oxidized to quinone structures by oxygen or enzymes, which allows it to have an anti-oxidant effect in the body [43].

1.6.2 Known Beneficial Effects of EGCG in RA

The effects of EGCG on the TNF-α-mediated effects of RA, especially in RA synovial fibroblasts, have not been fully studied. The effects of EGCG on other cytokines, including IL-1β, and other proteins and enzymes in RA have been better studied than TNF-α and can thus provide a window of insight into what might be expected when looking into the role EGCG plays in the TNF-mediated effects of the disease. There have been studies done which have shown that EGCG has both effects on bone-preservation as well as on synovial fibroblast regulation.

Epigallocatechin-3-gallate has been shown to have protective effects against bone destruction in RA. A study done by Tokuda *et al.* showed that EGCG was able to inhibit basic fibroblast growth factor-2 induced IL-6 production in osteoblasts, partially through

suppression of both the ERK1/2 and p38 pathways [44]. Another study showed that EGCG was able to reduce osteoclast formation by inhibiting the differentiation of osteoblasts [45]. Although EGCG prevents the differentiation of osteoblasts to osteoclasts, it allows the osteoblasts to remain viable and able to proliferate. Preventing osteoclast formation while allowing osteoblasts to continue their work is incredibly beneficial in preserving bone in RA.

The effects of EGCG on synovial fibroblasts have also been researched. Using RA synovial fibroblasts, Ahmed *et al.* showed that EGCG inhibits IL-1β-induced protein kinase Cδ and NF-κB pathways, which in turn led to the inhibition of chemokines MCP-1/CCL-2, RANTES, Gro-α, and ENA-78 as well as the IL-1β-induced activation of MMP-2 [46]. Epigallocatechin-3-gallate has also been shown to inhibit IL-1β-induced IL-6 production in synovial fibroblasts [47], which might be an additional method of bone preservation. One study showed that EGCG treatment resulted in decreased TNF-α-induced MMP-1 and MMP-3 production in synovial fibroblasts by preventing activation protein (AP)-1 from binding to the DNA [48]. Another study showed that EGCG was able to cause the degradation of Mcl-1, which sensitized the synovial fibroblasts to apoptosis [49]. This is an important effect to note because as previously stated, RA synovial fibroblasts resist apoptosis and become proliferative which results in pannus formation. Therapeutically, making these cells more sensitive to apoptosis may slow the progression and destruction of the disease.

Epigallocatechin-3-gallate has also been tested in animal models of RA to determine the effect on disease onset and severity. In rat or mouse collagen-induced arthritis (CIA), EGCG was shown to reduce bone resorption and, when administered i.p. at a dose of 20 mg/kg, EGCG inhibited inflammation [50]. The same dose of EGCG, when given to CIA mice, was effective in ameliorating the disease by reducing the number of macrophages entering the area as well as the number of osteoblasts producing the MCP-1/CCL-2 chemokine [51]. A recent study using CIA rats found that EGCG down-regulated the expression of the anti-apoptotic protein Bcl-$_{XL}$ while increasing the expression of Bim, a pro-apoptotic protein [52]. The beneficial effects of EGCG are also seen in adjuvant-induced arthritis (AIA) animals. Rats with AIA saw a reduction in inflammation with daily i.p. administration of EGCG at a dose of 100 mg/kg. This reduction in inflammation was attributed to the selective inhibition of IL-6 production in serum and the joints as well as the increase in the soluble gp130 production in these animals [47].

1.7 Tumor Necrosis Factor-α in Rheumatoid Arthritis:

1.7.1 Tumor Necrosis Factor-α

Along with IL-1β and IL-6, TNF-α is a major cytokine involved in the development and progression of RA. Tumor necrosis factor-α is a pro-inflammatory cytokine that is produced by many cell types, but macrophages and monocytes are the cell type primarily associated with the synthesis of this protein [53]. This protein is first produced as a transmembrane protein, which is then cleaved by the TNF-α converting enzyme (TACE)

[54]. Membrane bound TNF-α is a 27 kDa protein, but after it is cleaved from the

membrane, becoming soluble TNF-α, it is 17 kDa [55]. The 17 kDa TNF-α protomers

are made of two anti-parallel β-pleated sheets with anti-parallel β strands that form the

jelly roll β-structure that is typical in the TNF ligand family [54].

1.7.2 Tumor Necrosis Factor-α Signaling Pathway

In order to initiate signaling, TNF-α binds to either TNF receptor 1 (TNF-R1) also

known as p55 or p60 or TNF receptor 2 (TNF-R2), which is also known as p75 or p80

[55]. Soluble TNF-α primarily binds to TNF-R1 while the membrane-bound TNF-α

preferentially binds to TNF-R2. While the two pathways can work together, they each

have primary pathways that follow the binding of TNF-α.

Tumor necrosis factor receptor 1 is expressed in most tissues [54] and contains a

cytoplasmic death domain [55]. It is thought to initiate most of the biological activity

associated with TNF-α [56]. The TNF-R1 pathway eventually results in the activation of

the NF-κB and c-Jun transcription factors which are involved in cell growth and cell

death as well as stress, immune, and inflammatory responses [56], all important events in

the initiation and progression of RA. When the TNF-α trimer binds to TNF-R1, the

protein silencer of death domains (SODD) is released from the intracellular domain of

TNF-R1. This intracellular domain is recognized by the TNF receptor-associated death

domain (TRADD) adaptor protein, which then recruits other adaptor proteins including

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Fas-associated death domain (FADD), TNF-R-associated factor 2 (TRAF2), and receptor-interacting protein (RIP) [56]. FADD can recruit Caspase-8/10, which then activates Caspase-3. Caspase-3 activation results in apoptosis [57]. On the other hand, TRAF2 and RIP recruit the IκB kinase (IKK) complex, which leads to the degradation of phospho-IκBα and the release of NF-κB to be involved in transcription [56] of pro-survival genes. TRAF2 also activates c-Jun-N-terminal kinase (JNK) through dual-specificity mitogen-activated protein kinase kinase 3 (MKK3). Activation of JNK through phosphorylation also leads to cell survival [57].

Although not as well defined as the TNF-R1 pathway, TNF-R2 is known to be expressed on endothelial and immune cells like monocytes, macrophages, T cells, B cell and NK cells It is also known that TNF-R2 can only be fully activated by membrane bound TNF-α and not by soluble TNF-α. Because TNF-R2 lacks the cytoplasmic death domain found on TNF-R1, the TNF-R2 signaling pathway mainly results in cell survival through activation of NF-κB, c-Jun, and AP-1, but crosstalk between the TNF-R1 and TNF-R2 pathways does allow the TNF-R2 pathway to induce apoptosis [57]. The pathway, through which TNF-R2 activates pro-survival transcription factors is quite similar to the pathway used by TNF-R1. The only difference observed is that, in the TNF-R2 pathway, TRAF2 recruits mitogen-activated protein kinase kinase kinase (MEKK)1/7, which then results in the phosphorylation of JNK and activation of the AP-1 transcription factor [57].

These TNF receptors can work together when the cell is placed under certain conditions. For example when a cell is under stress, RIP recruited by TNF-R2 can also recruit FADD, which is generally seen in the TNF-R1 pathway and not the TNF-R2 pathway, to induce apoptosis [57]. It has also been shown that after 1-6 hours of TNF-R2 stimulation, there appears to be a shift from a pro-survival outcome to a pro-apoptotic outcome in cells. This is due to a depletion of the TRAF2 required for the pro-survival pathway of this receptor. Once the TRAF2 is depleted the signaling uses TRADD and FADD instead, resulting in cell death [58].

1.7.3 Tumor Necrosis Factor-α-Mediated Effects of RA

The fact that there are five drugs on the market to act as TNF-α antagonists in the treatment of RA indicates a major role played by TNF-α in the disease. Both TNF receptors are found in RA synovial fibroblasts, but TNF-R1, in particular, has been shown to induce the release of other pro-inflammatory cytokines such as IL-6 and IL-8 as well as PGE_2 and MMP-1, a collagenase MMP [59]. Tumor necrosis factor receptor 1 has also been implicated as being a key driving force in the development of the disease itself [60]. In a study using human TNF transgenic mice, mice without TNF-R1 were protected from the development of RA [61]. However, administration of TNF-R1 to these animals again helped the establishment of disease [61].

Tumor necrosis factor receptor 2 appears to have a beneficial effect in RA. In a study using TNF-R2 deficient mice the lack of TNF-R2 resulted in the development of more aggressive arthritis and more synovial hyperplasia than the control animals [62]. Bluml *et al.* showed that when TNF-R2 is absent from hematopoietic cells, there is an increase in the recruitment of inflammatory cells to the synovial membrane and increased osteoclastogenesis, which increases the bone destruction in this disease [63]. One hypothesis as to why TNF-R2 has a beneficial effect is that TNF-R2 may be cleaved and act as a scavenger of soluble TNF-α, thereby blocking the ability of TNF to bind to a membrane-bound TNF receptor [60]. This information suggests that selectively blocking TNF-R1 may be a good target for possible drug development against RA.

1.8 Aims and Objectives:

In the present studies, we intended to test the effects of acute and chronic EGCG treatment on the TNF-α-stimulated downstream signaling in RA synovial fibroblasts. Previous studies only suggested some of the acute effects of EGCG in RA synovial fibroblasts, but not much has been done to determine the chronic effects of EGCG on TNF-α-signaling effects. We used human synovial fibroblasts to determine what proteins in the TNF Receptor pathways, as well as some other apoptotic pathways, were affected by acute and chronic treatment with varying doses of EGCG. Using AIA rats, we also determined if EGCG was able to decrease the visible signs of RA. The joints from these animals were harvested upon termination of the study as well to determine the effect of EGCG administration on TNF-R1 and TNF-R2 in the joints of these animals.

Chapter 2

Methods and Materials

2.1 Antibodies and Reagents

All reagents used to prepare buffers used in the immunoblotting procedure were purchased from Sigma-Aldrich (St. Louis, MO). Many of the antibodies used in immunoblotting were purchased from Cell Signaling (Danvers, MA) and include rabbit monoclonal anti-Akt (#4685), rabbit monoclonal anti-TNF-R1 (#3736), rabbit polyclonal anti-TNF-R2 (#3727), rabbit monoclonal anti-p44/42 MAP Kinase (#4695), rabbit monoclonal anti-SAPK/JNK (#9258), rabbit monoclonal anti-p38 (#8690), rabbit polyclonal anti-phospho-Akt (Ser 473) (#9271), rabbit monoclonal anti-phospho-p44/42 MAPK (Erk1/2) (Thr 202/Tyr 204) (#4377), rabbit polyclonal anti-phospho-p38 (Thr 180/Tyr 182) (#9211), and rabbit polyclonal anti-phospho-SAPK/JNK (Thr 183/Tyr 185) (#9251). The TNF-R1 anitibody used in the joint homogenate study was a polyclonal anti-TNF-R1 antibody (#ab19139) purchased from Abcam (Cambridge, MA). The actin used to normalize the results was a rabbit anti-actin antibody from Sigma (#A2066). The secondary antibody used in the immunoblotting procedure was a goat anti-rabbit HRP-linked antibody (Cell Signaling #7074).

2.2 Rheumatoid Arthritis Synovial Fibroblast Culture

Cells were cultured from synovial tissue received from the National Disease Research Interchange or the Department of Orthopedic Surgery at the University of Toledo Medical Center. Synovium was obtained according to protocols approved by the Institutional Review Board (IRB) in compliance with the Helsinki Declaration, from RA patients who had undergone joint replacement surgery or synovectomy and fulfilled the criteria set by the ACR. After each synovial tissue was received, the media was aspirated from the shipping container and the tissue was washed with PBS. The tissue was transferred from the shipping container to a 100 mm dish containing a small amount of PBS to prevent the tissue from drying out. Any excess bone or fat was removed from the tissue and the tissue was cut into small (approximately 7 x 7 mm) pieces. These pieces were placed in another 100 mm dish containing PBS with pen/strep for about 10 minutes. Any pieces that were too small to be saved were kept to be digested for cell culture. Nalgene freezer tubes were labeled and the tissue pieces were placed in tubes with DMSO freezing media (90% FBS and 10% DMSO) and stored at -80° C overnight before being transferred to a liquid nitrogen tank for long-term storage.

Tissue pieces that were saved for digestion and subsequent isolation of synovial fibroblasts were placed on a 100 mm dish and minced into a pulp. This pulp was evenly distributed on a 100 mm dish with RPMI 1640 media (Corning cellgro, Manassas, VA), 2% pen/strep (Corning cellgro), and 1 mg/ml type 2 collagenase (Worthington Biochemical Corporation, Lakewood, NJ) and left 24 hours at 37° C for the digestion to

occur. The digest mixture was put through a 70 μm nylon cell strainer (BD Falcon) into a
T75 cm^2 flask with 10% FBS RPMI media supplemented with 2% L-glutamine and 1%
pen/strep. The 100 mm dish was rinsed with 10% FBS RPMI 1640 media and was
transferred to the T75 cm^2 flask. The flask was marked as passage zero and was placed at
37° C until the cells were confluent, changing the media every 2-4 days. After the cells
were confluent, they were split into three flasks and replated until passage four where
only the fibroblast population survived in culture. Synovial fibroblasts were then plated
in six-well plates and grown to confluency. The cells were treated with 5, 10, and 20 μM
EGCG (Sigma-Aldrich) for 24 hours for acute studies or with a daily dose of 100 nM,
250 nM, 500 nM, and 1 uM EGCG, and 100 nM AT101 (#3367) (R&D Systems/Tocris
Bioscience, Minneapolis, MN) for 7 days to determine the chronic effect. AT101
(100nM) was used in the repetitive, low-dose EGCG treatment as a control to observe
cryostatic effects on these cells.

2.3 Viability Study

RA synovial fibroblasts were plated in 24 well plates, grown to confluency, and exposed
daily to EGCG and AT101 according to the concentrations mentioned above. After seven
days of treatment, 50 μl MTT solution (5 mg thizolyl blue tetrazolium bromide (Sigma)
per 1 ml PBS) was added to each well and the plates were returned to the 37° C
incubator. After two hours, media was aspirated from each well and 500 μl DMSO
(Research Organics, Cleveland, OH) was added to solubilize MTT crystals. The plates
were covered with aluminum foil and placed on an orbital shaker to shake for five

minutes before being placed in the oven at 37° C for 10 minutes. The plates were read

using a spectrophotometer at 570 nm. The results were presented as the percent control of

untreated (NS) samples.

2.4 Immunoblotting:

2.4.1 Protein Collection and Sonication

Lysis buffer was prepared by mixing 100mM Tris HCl pH 7.4, 100 mM NaCl, 1 mM

EGTA, 1mM EDTA, 1 mM NaF, 2.5 mM sodium pyrophosphate (Thermo Fisher

Scientific, Inc. Waltham, MA), 2 mM sodium orthovanadate (Thermo Fisher Scientific,

Inc.), 1% Triton X-100, 10% glycerol, 0.1% SDS, 0.5% sodium deoxycholate, 0.1%

DTT, 1mM PMSF, a protease inhibitor tablet (Roche Diagnostics, Indianapolis, IN), and

deionized water.

At the termination of the experiment, the cells were removed from the incubator and the

conditioned media was collected and stored at -80°C. Lysis buffer was added (200

μl/well) and the 6-well plate was placed on ice for 15 minutes with occasional shaking to

keep the cells covered. A cell scraper was then used to detach the fibroblasts from the

plate and the lysate was collected in a 1.5 ml Eppendorf tube. The samples were placed

on ice and the cells were lysed by passing them several times through a 20 gauge syringe.

After the cells had been on ice for five minutes, the lysates centrifuged at 10,000 rpm for

10 minutes at 4°C. The supernatant obtained was placed in a new, labeled tube and stored at -80°C.

2.4.2 Protein Quantification Assay

The amount of protein in each sample was determined by BCA assay using the commercially available Pierce BCA Protein Assay Kit (Thermo Fisher Scientific, Inc.). The protein quantification values were used to calculate the volume of lysate to ensure the equal loading of protein in each Western blot. Bovine serum albumin (0.0625 - 2 mg/ml, Thermo Fisher Scientific, Inc.) was used as the standard. Serial dilutions of BSA were made to produce a linear standard curve. The working reagent for the assay was made by combining 1 part Reagent B with 50 parts Reagent A. The known BSA standards or samples (10 μl) were loaded in the 96-well plate and 100 μl working reagent was added to each well. The plate was incubated at 37°C for 30 minutes. The absorbance was read at 570 nm wavelength using a BioTek Synergy HT microplate reader and Gen5 Microplate software. The standard curve, regression, and line equation were obtained. The concentration of the protein in the samples was then calculated using the optical density and the standard curve. The protein concentration was then used to calculate the amount of protein to be used for Western blotting.

2.4.3 Western Immunoblotting

2.4.3.1 Preparation of samples

The samples were thawed on ice a 5X Reducing sample buffer (10% SDS, 20% glycerol, 0.2 M Tris-HCl pH 6.8, 10 mM DTT, 0.05% bromophenol blue) was added to the sample according to the calculations done based on the volume of the sample. The samples were then boiled for seven minutes before storing at -80°C.

2.4.3.2 Electrophoresis:

SDS-Polyacrylamide gels (10%; 1.0mmX12 well) were prepared using Bio-Rad reagents (Hercules, CA)(deionized water, Tris pH 8.8, Tris pH 6.8, 30% acrylamide, 10% APS, 20% SDS, and TEMED) were placed in the electrophoresis apparatus (Bio-Rad) and immersed with 1X running buffer, which was prepared with 25 mM Tris (basic), 190 mM glycine, 0.1% SDS, and deionized water. The cell lysates were loaded in each well at a concentration of 20-25 μg protein per well. Electrophoresis was performed at 120 volts for 1.5 hours.

2.4.3.3 Transfer of proteins:

After electrophoresis, the gels with the separated protein were transferred onto supported nitrocellulose membranes (Bio-Rad) in a Trans-blot SD Semi-Dry transfer cell (Bio-Rad). The gels were placed directly on the nitrocellulose membrane previously soaked in

transfer buffer. The gel and membrane were sandwiched between extra thick blot paper (Bio-Rad), which had also been previously soaked in transfer buffer (25 mM Tris (base), 0.2M glycine, 0.01% SDS, 20% methanol, and deionized water). The cell was run at 12 volts for 40 minutes.

2.4.3.4 Primary antibody

 The membranes were placed in boxes and blocked with blocking buffer made of 5% non-fat dry milk and 1X TBST (137 mM sodium chloride, 20 mM Tris base, 0.1% Tween-20, and deionized water at pH adjusted to 7.6) on a rocker for 2 hours at room temperature. After 2 hours, the primary antibody was added at a 1:1000 dilution. The boxes were again placed on the rocker and incubated at 4° overnight.

2.4.3.5 Secondary antibody

The blocking buffer with primary antibody was removed and the membranes were washed with 1X TBST three times for ten minutes each wash. The membranes were then incubated with the appropriate horseradish peroxidase (HRP)-labeled secondary antibody at 1: 1000 dilution for 1.5 hours. The membranes were washed again with 1X TBST three times for ten minutes each wash.

2.4.3.6 Developing

Excess liquid was dried off the membranes before being used for chemiluminiscent detection. They were incubated with a mixture of SuperSignal West Dura Luminol enhancer solution and SuperSignal West Dura stable peroxide Buffer solution (Thermo Fisher Scientific, Inc.) in 1:1 ratio for about three minutes. The membranes were then placed in an autoradiography cassette (Thermo Fisher Scientific, Inc.) and exposed HyBlot ES autoradiography film (Denville Scientific, Inc. South Plainfield, NJ) in a cassette for varying amounts of time in order to achieve clear band intensity(s). The developed films were used for the quantitative analysis of the band intensities.

2.4.4 Normalization of Protein loading

After each specific protein, blot was stripped or a new gel was re-run to detect β-Actin to be used to normalize results.

2.4.5 Analysis of the Western blots

The developed films were scanned into the computer and the images were quantified using ImageJ software (NIH). The bands were measured and normalized with β-actin. The ratios of the protein/β-actin were calculated and analyzed.

2.5 ELISA Procedure

The levels of IL-6 and IL-8 were determined in the conditioned media obtained from different treatment groups. IL-6 and IL-8 ELISA kits were obtained from R&D Systems and ELISAs were performed according to the protocol given in the kit. The conditioned media was diluted 1:5 and 1:10 with the reagent diluent for the IL-6 and IL-8 kits, respectively.

2.6 Animal Study (Rat Adjuvant-Induced Arthritis)

2.6.1 Treatment

Female Lewis (LEW/SsNHsd) rats were acquired from Harlan Laboratories and quarantined in the University of Toledo DLAR for three days. These animals were divided into 3 groups with n=8 for each group. The three groups were naïve (healthy), adjuvant induced arthritis (AIA) (untreated), and AIA with EGCG treatment). After quarantine, these animals were weighed and all but the naïve group were injected subcutaneously at the base of the tail with 300 μl inactivated *Mycobacterium butyricum* (5 mg/ml) in mineral oil to induce arthritis. Seven days after the injection of the bacteria, the animals began receiving i.p. injections of EGCG at doses of 50 mg/kg. Weights, articular index scores and ankle circumference were measured periodically throughout the treatment. On day 17, the animals were euthanized and blood and ankles were collected from each animal. The ankles were snap frozen in liquid nitrogen and stored at -

80° C until they were homogenized. The study was approved by the University of Toledo IACUC committee.

2.6.2 Joint Homogenization

Skin was removed from the ankles. Each ankle was placed on ice in a 50 ml tube containing a PBS buffer with a protease inhibitor tablet. The ankles were then homogenized using an Omni International homogenizer with stainless steel probe. The tubes were then centrifuged at 3,000 rpm for 10 minutes at 4° C. The supernatant was removed from the tube and placed in 1.5 ml Eppendorf tubes. The centrifugation step was repeated and the supernatant was again saved in 1.5 ml Eppendorf tubes. The 1.5 ml tubes containing the joint homogenates were stored at -80° C until needed.

2.7 Statistical Analysis

Student's *t*-tests were performed to calculate statistical differences between the means of the different variables obtained in both the *in vitro* and *in vivo* studies. *P* values less than 0.05 (2-tailed) were considered significant.

Chapter 3

Results

3.1 The Effect of Repeated Exposure to low concentrations of Epigallocatechin-3-gallate on Rheumatoid Arthritis Synovial Fibroblast Viability

After seven days of treatment with varying low doses of EGCG, the viability of the RA synovial fibroblasts was measured using an MTT viability assay. AT101 was used as a control based on its cytostatic effect. The results of this assay show that increasing concentrations of EGCG also had a cytostatic effect on the RA synovial fibroblasts (Figure 3-1). Because the untreated (NS) cells grow unchecked, the cytostatic effect of EGCG results in a decrease in cell number relative to the NS group because the EGCG-treated cells are not replicating at the same rate the NS cells are. The cells being administered the highest dose of EGCG in this study slowed their growth so that the well contained about 18% fewer cells than the NS well. The AT101 control well contained 45% fewer cells than the NS well.
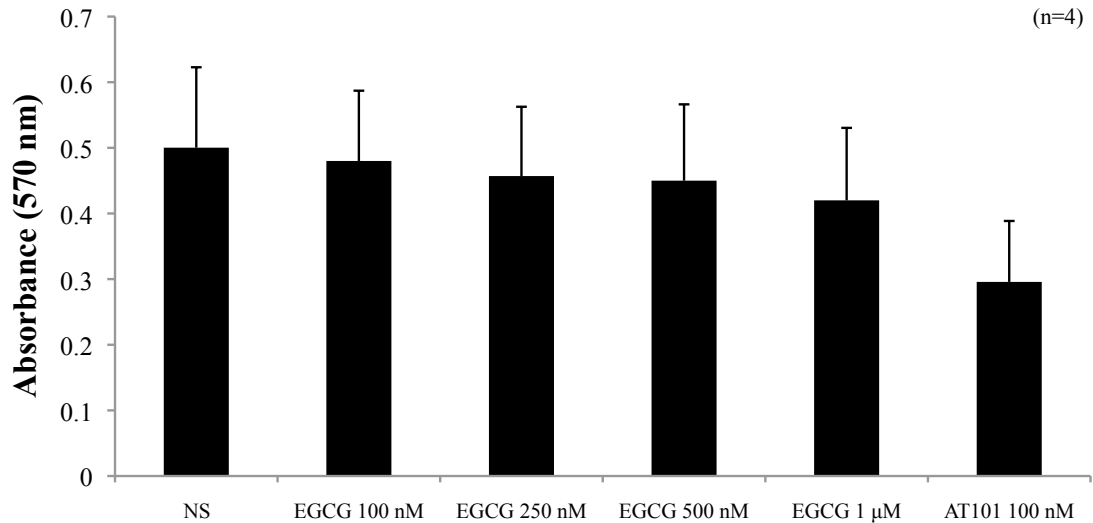
Figure 3-1

Fig 3-1. Viablilty of RA synovial fibroblasts after repeated exposure to low concentrations of EGCG. After daily treatment of RA synovial fibroblasts with low doses of EGCG, none of the dosages resulted in significant cytotoxicity or death, but EGCG appears to have a slight cytostatic effect in these cells.

3.2 The Effect of Epigallocatechin-3-gallate on Tumor Necrosis Factor Receptors 1 and 2

3.2.1 Repeated *in vitro* exposure to low concentrations of Epigallocatechin-3-gallate and Tumor Necrosis Factor Receptor Expression

After the RA synovial fibroblasts are dosed with EGCG for 8 days, these cells were lysed and prepared for Western blotting. Results of repeated EGCG exposure with 24 hours of

20 ng/ml TNF-α stimulation (n=3) showed that treatment with EGCG at low doses for 8 days and 24 hour TNF-α stimulation appears to increase slightly the expression of TNF-R1 in RA synovial fibroblasts (Figure 3-2). Using the two-tail t-test, it was determined that there were no significant changes in TNF-R1 expression in these samples. The same treatment method was used to determine the effect of repeated exposure to low concentrations of EGCG with 24 hour TNF-α stimulation on TNF-R2 (n=5). Using a two-tail t-test, it was determined that this treatment resulted in only a slight increase in TNF-R2 expression as well (Figure 3-2B).

Figure 3-2
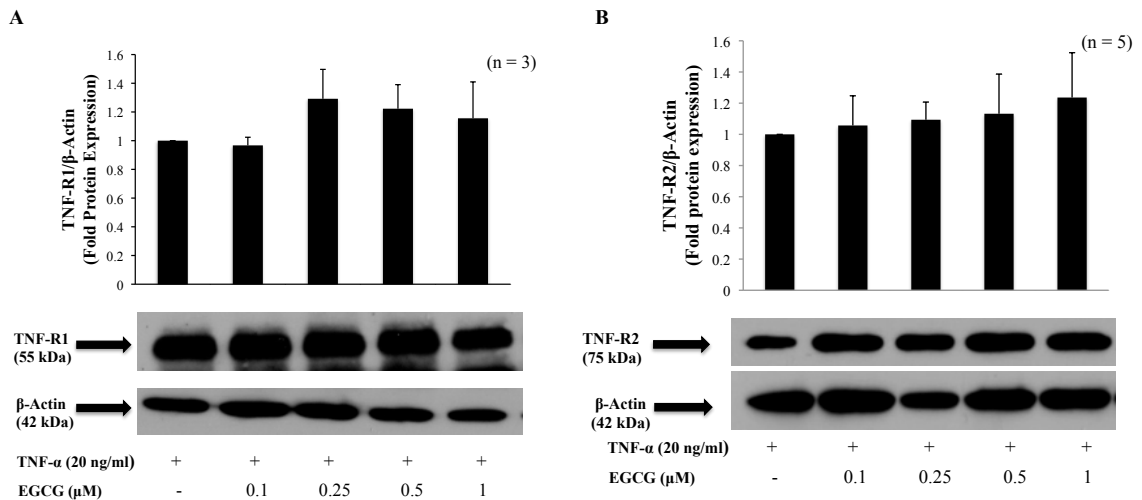
Fig 3-2. Effect of repeated exposure to low concentrations of EGCG on TNF-R1 and TNF-R2. **A.** After seven days of EGCG treatment and one day of EGCG treatment with TNF-α stimulation (20 ng/ml), there were slight changes seen in TNF-R1 expression (n=3). **B.** RA synovial fibroblasts repeatedly exposed to EGCG with 24 hour TNF-α stimulation showed only a slight increase in TNF-R2 expression (n=5).

After seeing the results of the study using repeated EGCG exposure with 24 hour TNF-α stimulation, we repeated this study without the TNF-α stimulation to see what the effect of EGCG itself had on these cells. These results showed that repeated exposure to low

concentrations of EGCG alone also resulted in increases in TNF-R1, but there was no

significant increase seen in the expression of TNF-R1 without TNF-α stimulation (n=5).

The trend in the expression of TNF-R2 in the RA synovial fibroblasts was similar to what

was seen in TNF-R1, but there was a significant increase in TNF-R2 at a dose of EGCG

250 nM (p<0.05) (n=3) (Figure 3-3).

Figure 3-3
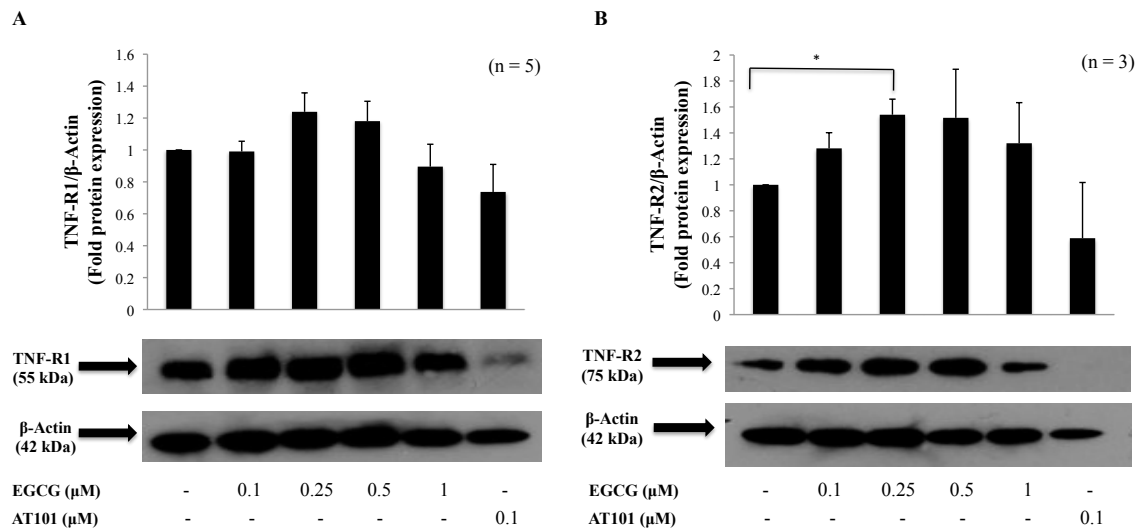
Fig 3.3. Effect of repeated exposure to low concentrations of EGCG alone on TNF-R1 and TNF-R2. **A.** After repeated exposure of RA synovial fibroblasts to low concentrations of EGCG without additional TNF-α stimulation, there was a significant increase in TNF-R1 expression (n=5) **B.** Repeated exposure to low concentrations of EGCG alone resulted in a significant increase in TNF-R2 expression (n=3) (* = p < 0.05).

3.2.2 Single exposure, *in vitro,* to high concentrations of Epigallocatechin-3-gallate and Tumor Necrosis Factor Receptor Expression

We also treated RA synovial fibroblasts for 24 hours only using high concentrations of

EGCG and TNF-α stimulation to determine if a single exposure to high concentrations of

EGCG had an effect on TNF receptor expression. In these studies, we found that TNF-R1 decreased in cells treated with up to 10 μM EGCG, but at 20 μM EGCG, the expression of TNF-R1 begins to increase again (n=3). Interestingly, TNF-α-stimulated cells expressed slightly less TNF-R1 expression than NS (Figure 3-4A). When treated with high doses of EGCG for 24 hours with TNF-α stimulation, TNF-R2 expression was significantly decreased, specifically at 10 μM ($p < 0.05$). When these cells were not stimulated with TNF-α, there was also a significant decrease between NS cells and cells treated with 20 μM EGCG ($p < 0.01$) (n=4) (Figure 3-4B). The data was analyzed using a two-tail t-test.

Figure 3-4
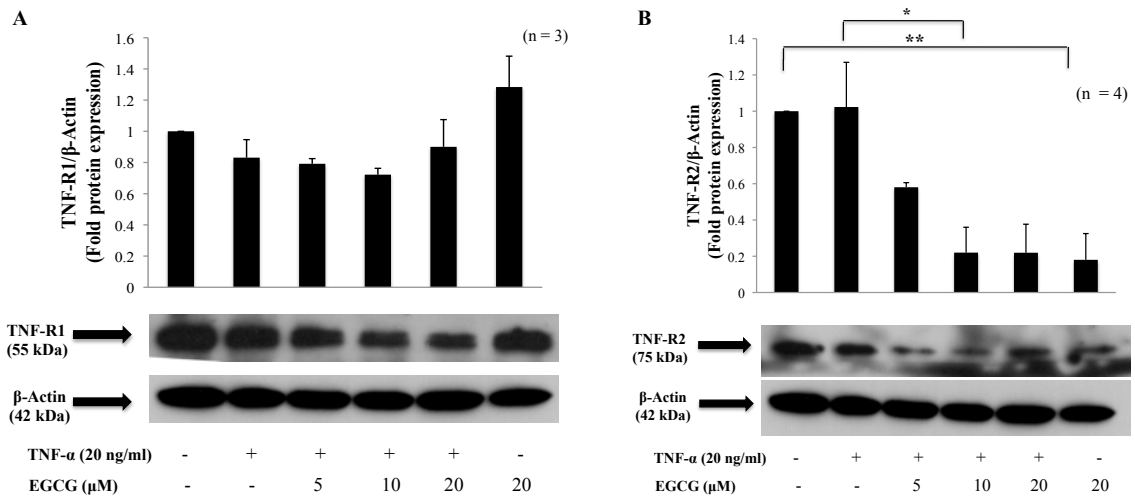
Fig 3-4. Effect of a single exposure to high concentrations of EGCG on TNF-R1 and TNF-R2. **A.** After 24 hour exposure to high concentrations of EGCG and stimulation with TNF-α, there was no significant change in the expression of TNF-R1 (n=3). **B.** RA synovial fibroblasts showed significant decrease in TNF-R2 expression when exposed to single, high concentrations of EGCG with TNF-α stimulation (n=4) (* = $p < 0.05$ and ** = $p < 0.01$).

3.3 Regulation of downstream signaling proteins with repeated exposure to low concentrations and single exposure to high concentrations of RA synovial fibroblasts with epigallocatechin-3-gallate

We treated synovial fibroblasts with low concentrations of EGCG for seven days with 30 minutes of TNF-α stimulation (20 ng/ml) on the final day to determine the effect of EGCG on downstream signaling proteins. We first looked into the different MAPKs in TNF receptor signaling. After western blotting, it was discovered that this repeated exposure to EGCG did not result in any significant change in phospho-JNK in these animals (Figure 3-5A)(n=4). The phosphorylation seemed to slightly increase with the lowest doses of EGCG administered, but with 1 μM, we saw an approximate 10% decrease in pJNK in these RA synovial fibroblasts. We also were able to determine that EGCG does not impact the phosphorylation of ERK (Figure 3-5B). There was a significant effect of repeated exposure to low concentrations of EGCG on the phosphorylation of p38 MAPK (Figure 3-5C). Our results showed that the 250 nM dose was most effective in decreasing the phosphorylation of p38, but the 1 μM dose of EGCG also resulted in a significant decrease in p38 phosphorylation.

Figure 3-5

Fig 3-5. Effect of repeated exposure to low concentrations of EGCG on MAPKs. **A.** No significant change in pJNK was seen in the EGCG-treated RA synovial fibroblasts after repeated exposure to EGCG and 30 minute TNF-α stimulation. (n=4) **B.** No change was seen in phosphorylation of ERK with repeated exposure of RA synovial fibroblasts with EGCG. (n=4) **C.** Phosphorylation of p38 MAPK was significantly decreased by repeated exposure to low concentrations of EGCG in these cells (n=3) (* = $p < 0.05$) (** = $p < 0.01$).

We also exposed these RA synovial fibroblasts to single, high concentrations of EGCG followed by 30 minutes of TNF-α stimulation (20 ng/ml) to determine the effect of higher concentrations of EGCG on the MAPKs. In this study, we found that there was no

significant change in the 46 kDa isoform of pJNK in samples (n=4) where the synovial fibroblasts received the 30 min TNF-α stimulation. There was a significant decrease ($p < 0.01$), however, in the phosphorylation of this isoform of JNK in the samples where the synovial fibroblasts received the high dose of EGCG, but no TNF-α stimulation (Figure 3-6A). Unlike the results of the pJNK experiment, there was a significant increase in phosphorylation of ERK when these cells were stimulated with TNF-α (n=4) ($p < 0.05$). The results of the pERK experiments showed that even at high concentrations, EGCG did not impact the phosphorylation of ERK in cells that received 30 minute TNF-α stimulation. There was, again, a significant decline between the non-stimulated samples and the sample from the cells treated with EGCG 20μM without the additional TNF-α stimulation ($p < 0.01$) (Figure 3-6B). Immunoblotting for p-p38 showed that there was no significant difference in any of the concentrations in RA synovial fibroblasts exposed to a single, high concentrations of EGCG (n=3) (Figure 3-6C).
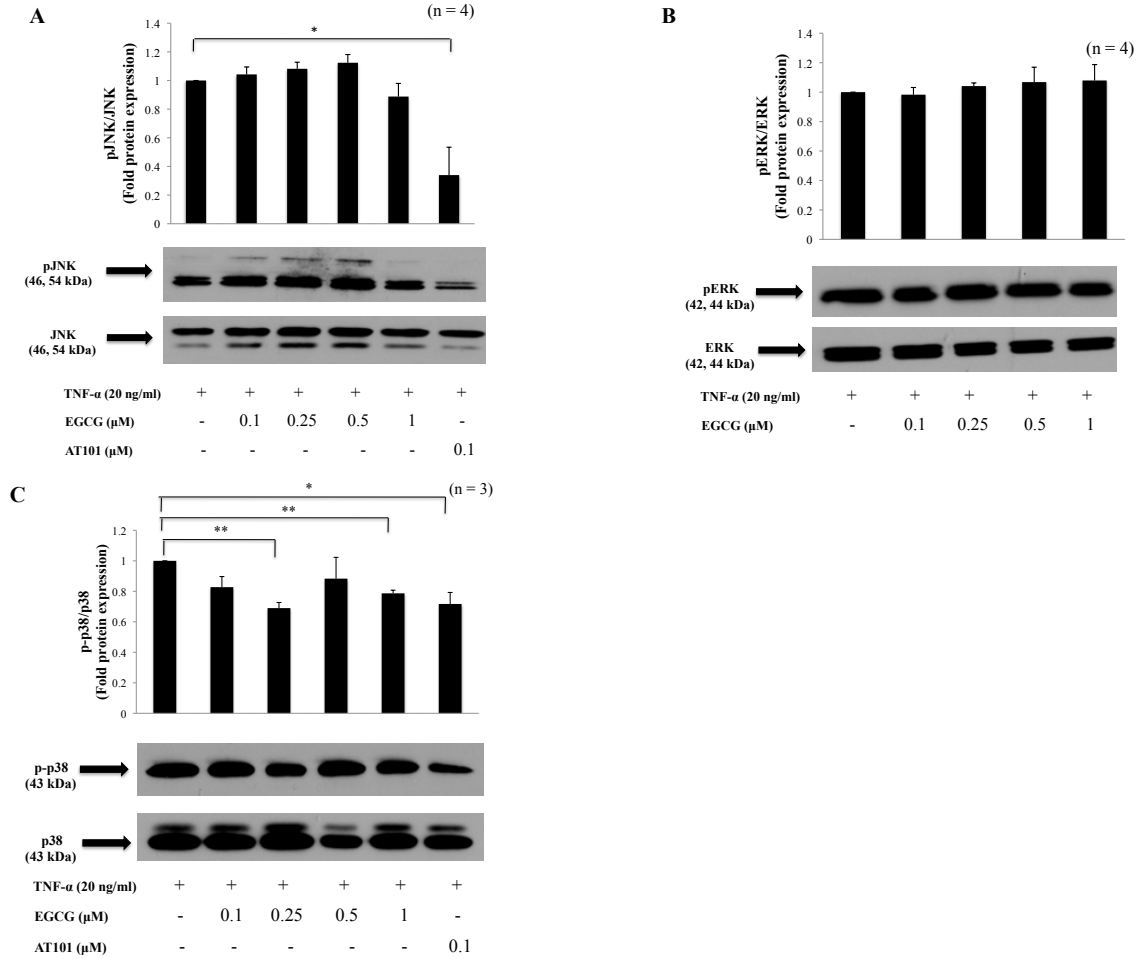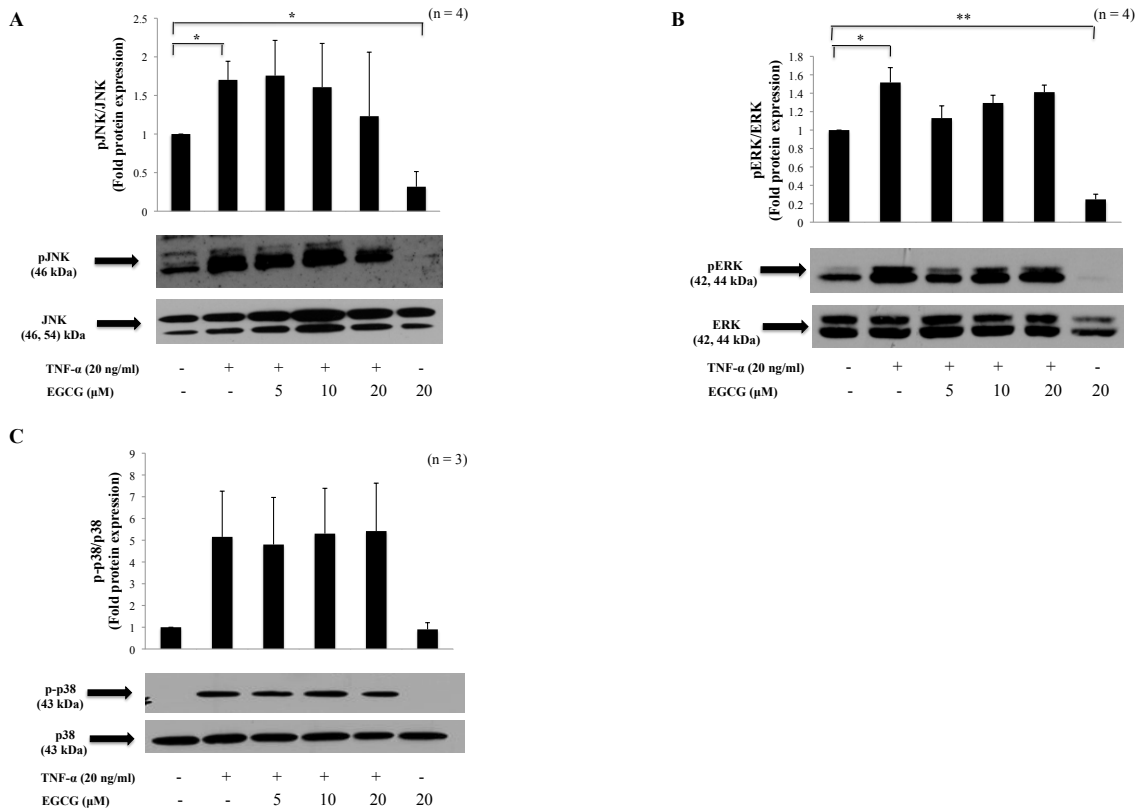
Figure 3-6

Fig 3-6. Effect of a single exposure to high concentrations of EGCG on MAPKs. **A.** No significant change in pJNK was seen in TNF-α-stimulated synovial fibroblasts treated with a single exposure to EGCG (n=4). **B.** There was also no significant change in pERK among TNF-α-stimulated single exposure EGCG treatments (n=4). **C.** Single exposures to high concentrations of EGCG with TNF-α-stimulation did not result in any change in p-p38 (n=3) (* = p<0.05) (** = p<0.01).

After looking at the effects of EGCG on the MAPKs, we wanted to look into whether EGCG had an impact on the phosphorylation of AKT, another protein involved in cell survival. As with the MAPKs, we treated the RA synovial fibroblasts for seven days with EGCG and then stimulated these cells with TNF-α (20 ng/ml) for 30 minutes before lysing the cells. We found that repeated exposure to low concentrations of EGCG did not significantly affect the phosphorylation of AKT (n=3) (Figure 3-7). There was only a modest decrease in this phosphorylation at concentrations of 500 nM and 1 μM EGCG.

Figure 3-7
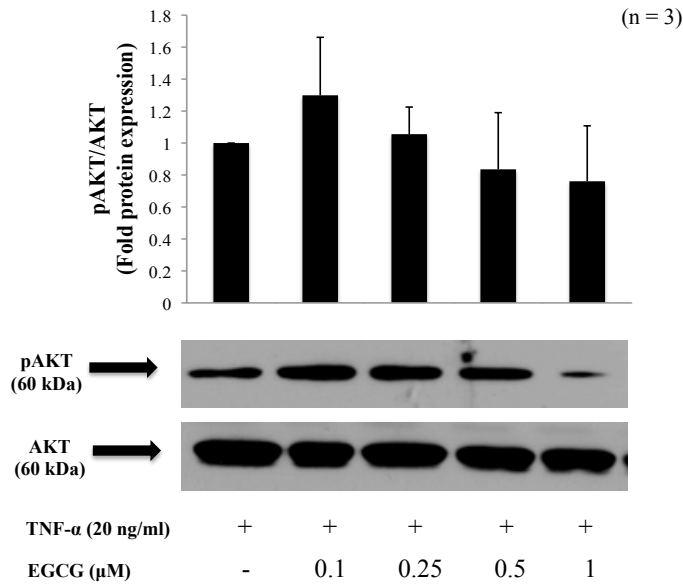
Fig. 3-7. Effect of repeated exposure to low concentrations of EGCG on pAKT. There was no significant change in AKT phosphorylation in RA synovial fibroblasts treated for seven days with EGCG and stimulated for 30 minutes with TNF-α (n=3).

We repeated this experiment using a single exposure to high concentrations of EGCG with 30 minute TNF-α stimulation (n=3). With this single exposure, we found that there was a significant increase in pAKT in cells stimulated with TNF-α as compared to non-stimulated cells. Among the samples that received the 30-minute TNF-α stimulation, there was no significant change in pAKT with increasing doses of EGCG. There was a significant decrease in pAKT seen between the non-stimulated RA synovial fibroblasts and cells receiving a 20 μM dose of EGCG without additional TNF-α stimulation (Figure 3-8)

Figure 3-8
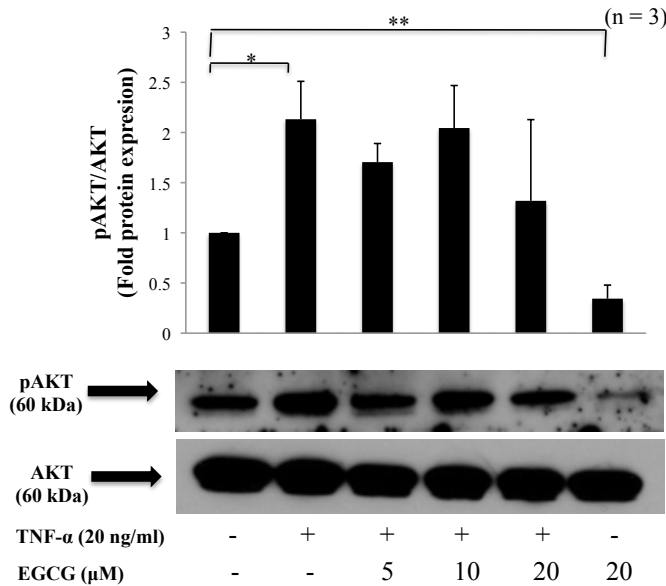
Fig. 3-8. Effect of single exposure to high concentrations of EGCG on pAKT. After RA synovial fibroblasts are exposed to a single, high concentration of EGCG and 30 minutes of TNF-α stimulation, EGCG had no significant effect on TNF-α stimulated cells treated with EGCG, but without additional TNF-α stimulation, EGCG significantly decreased pAKT (n=3) (* = $p < 0.05$) (** = $p < 0.01$).

3.4 Regulation of Tumor Necrosis Factor-α-induced IL-6 and IL-8 by repeated exposure to low concentrations and single exposure to high concentrations of Epigallocatechin-3-gallate to Rheumatoid Arthritis synovial fibroblasts *in vitro*

The conditioned media was collected after both repeated and single exposures of RA synovial fibroblasts with EGCG with 24 hour TNF stimulation. This conditioned media was then used to determine the amounts of IL-6 and IL-8 released by the synovial fibroblasts with varying doses of EGCG. We found that with repeated exposure to low

concentrations of EGCG, there was a significant decrease in IL-6 production by RA

synovial fibroblasts at doses of 500 nM or 1 μM EGCG (n=6) (Figure 3-9). The single

exposure to high concentrations of EGCG on these RA synovial fibroblasts resulted in a

larger decrease in IL-6 in the conditioned media than the repeated exposure to EGCG
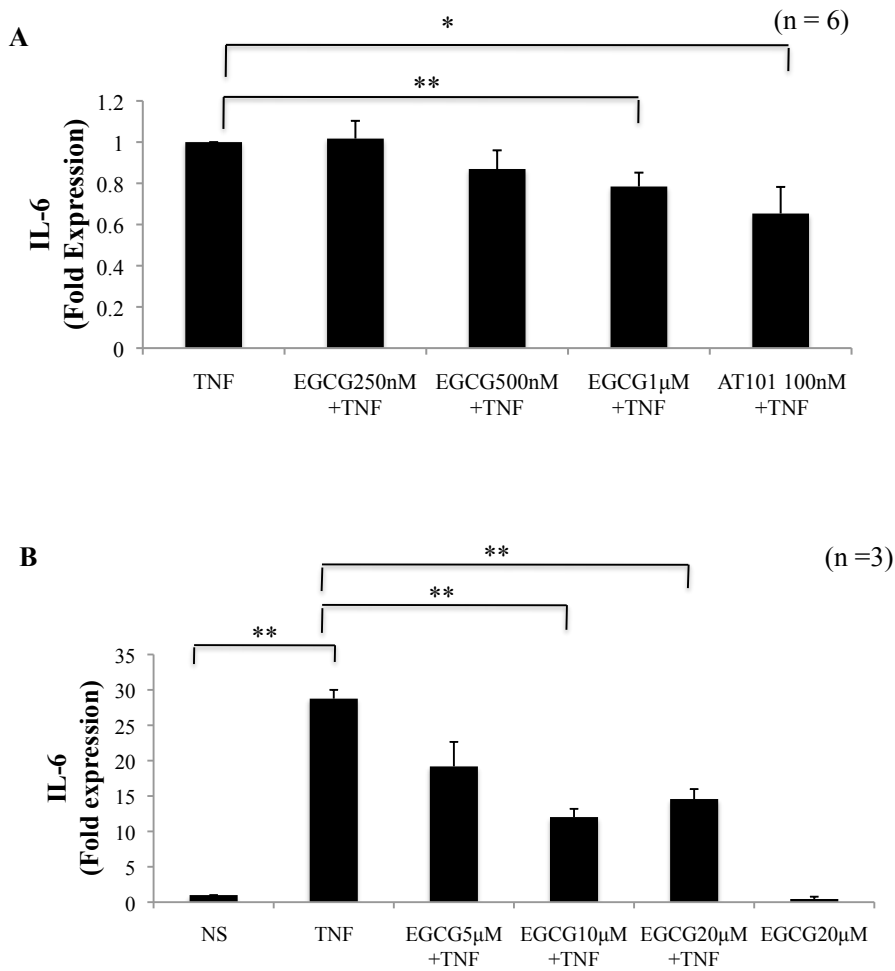
(n=3).

Figure 3-9

Fig. 3-9. Effects of repeated exposure to low concentrations and single exposure to high concentrations of EGCG on IL-6. IL-6 expression in RA synovial fibroblasts exposed to **B.** higher concentrations of EGCG for 24 hours was decreased more than **A.** repeated exposure to low concentrations of EGCG (* = p<0.05 and # = p<0.01).

The results of the IL-8 ELISAs showed that repeated exposure to low concentrations of EGCG in RA synovial fibroblasts leads to modest decreases in IL-8 concentrations in conditioned media (n=3) (Figure 3-10). The decrease that does occur appears to be dose-dependent. In the RA synovial fibroblasts receiving a single exposure to high concentrations of EGCG, there was a much more significant decrease in IL-8 concentration in the conditioned media (n=3). There was about a 70% decrease in IL-8 concentration in the 10 µM EGCG treated cells compared to the untreated TNF-α-stimulated group.

Figure 3-10
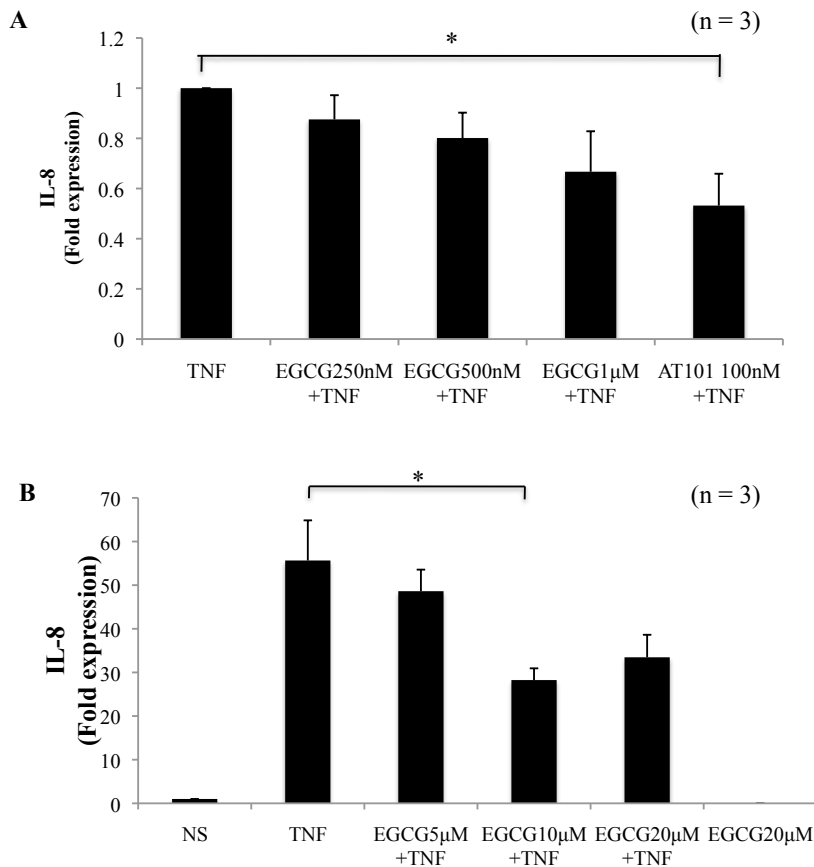
Fig. 3-10. Effects of repeated exposure to low concentrations and a single exposure to high concentrations of EGCG on IL-8. **A.** Repeated exposure to low concentrations of EGCG resulted in a more modest decrease in IL-8 production compared to the **B.** significant decreases in IL-8 production seen in conditioned media from RA synovial fibroblasts exposed only once to high concentrations of EGCG (* = $p < 0.05$ and # = $p < 0.01$).

3.5 The Effects of repeated exposure to Epigallocatechin-3-gallate on Tumor Necrosis Factor-α signaling *in vivo* using Adjuvant-Induced Arthritis Rat Joint Homogenates

After the ankles of the animals in the AIA study were collected and homogenized, the samples were prepared for use in western blotting. Using western immunoblotting, we were able to successfully acquire results for TNF-R1 (n=7). These results showed that in EGCG (50 mg/kg) treated AIA rats, there was a slight increase in TNF-R1 expression in the joint homogenates compared to the joint homogenates of the untreated AIA animals (Figure 3-11). This increase in TNF-R1 expression is similar to what is seen in TNF-R1 expression in human RA synovial fibroblasts treated repeatedly with EGCG alone (Figure 3-3A). Attempts at probing for TNF-R2 proved to be unsuccessful. This could be due to the fact that the joint homogenate sample was a mixture of several different cell types and not synovial fibroblasts alone. Because TNF-R2 is not found on most cells as TNF-R1 is, the amount of TNF-R2 in the sample was likely too diluted to be detected via western blot.

Figure 3-11
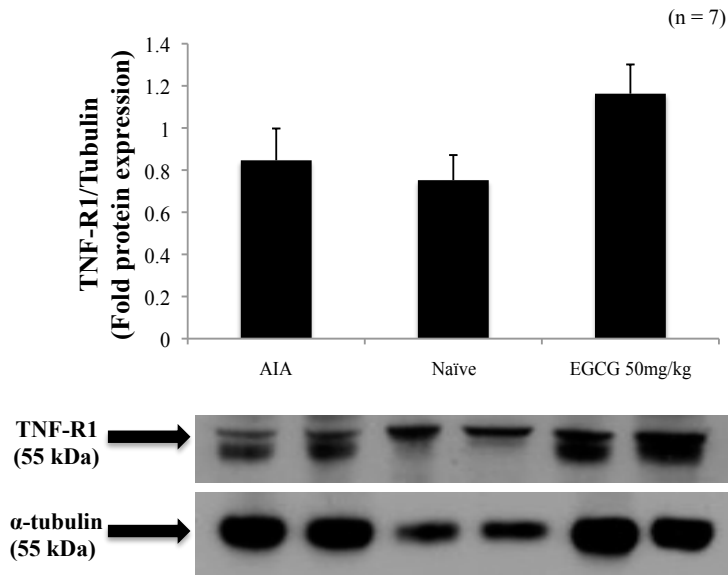
Fig 3-11. Effect of EGCG on TNF-R1 in rat joint homogenates. Ankles were taken from AIA (n=7), naïve (n=7), and AIA rats being treated with 50 mg/kg EGCG (n=7) and homogenized. Samples from two ankles in each group were loaded on a SDS-polyacrylamide gel for western blotting. The results show that there is no significant change between the TNF-R1 expression in the EGCG-treated AIA rats and the untreated AIA rats.

We wanted to determine if what we were seeing in the phosphorylation of JNK in the synovial fibroblasts *in vitro* was also happening *in vivo* in the AIA, Naïve and EGCG treated rats. The joint homogenates were used to determine the protein expression of pJNK in these animals. The result of this study showed that in naïve rats in the AIA rat animal study, the rats displayed lower amounts of pJNK than in AIA rats. Arthritic rats receiving daily EGCG treatment (50 mg/kg) experienced a slightly lower level of pJNK than untreated arthritic rats, but the amount of pJNK was still higher than in naïve animals (Figure 3-12).

Figure 3-12
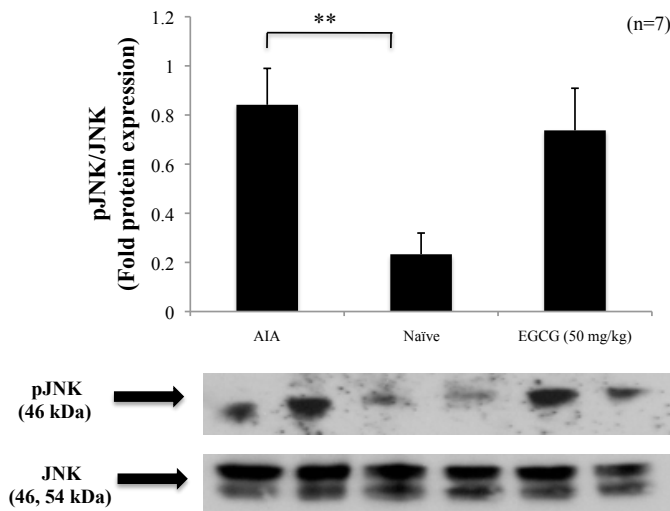
Fig.3-12. Effect of EGCG on pJNK expression in rat joint homogenates. Joint homogenates from the AIA rat study (n=7) were used to determine the expression of pJNK in the ankles of these animals. Expression of pJNK was significantly higher in AIA untreated animals and AIA EGCG-treated animals than it was in Naïve (healthy) animals. It does appear that EGCG treatment did result in a slight decrease in pJNK expression in these animals (** = $p < 0.01$).

To better solidify the *in vitro* results we obtained with pAKT, we used joint homogenate samples from the AIA rat study (n=4). The results of this *in vivo* study showed that, like the *in vitro* studies done in synovial fibroblasts, there was no significant difference in AKT phosphorylation in the joint homogenates of the naïve and AIA animals. There was also no significant effect in the phosphorylation of AKT when these animals were treated with 50 mg/kg EGCG (Figure 3-13).

Figure 3-13

Fig. 3-13. Effect of EGCG treatment on pAKT in rat joint homogenates. There was no significant difference in phosphorylation of AKT in any of the groups of animals in the rat AIA study (n=4).

Chapter 4

Discussion

This study provides evidence that repeated exposure to EGCG at nanomolar concentrations may modulate TNF-R1 and TNF-R2-mediaed TNF-α signaling pathways in both human RA synovial fibroblasts and rat AIA. Importantly, our results sugges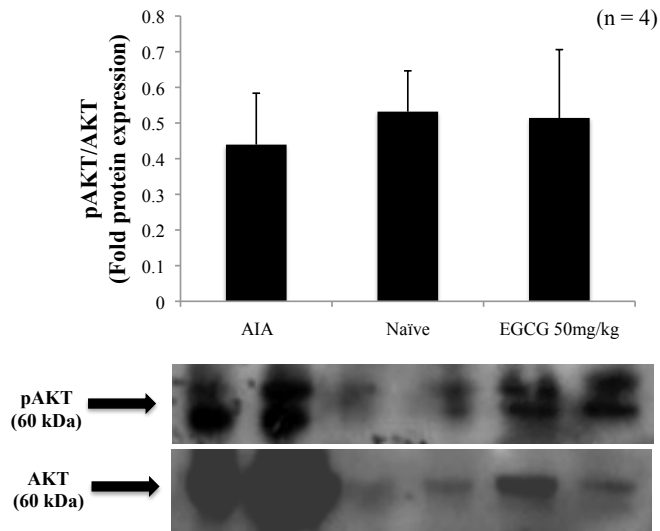t that by regulating TNF-R1 and TNF-R2 expression, low doses of EGCG were capable of inhibiting IL-6 and IL-8 production in RA synovial fibroblasts. Based on these results, one can argue that chronic consumption may provide a sufficient systemic concentration of EGCG to produce similar beneficial effects in RA patients.

The results show that repeated exposure to low concentrations of EGCG result in modest increases in TNF-R1 expression up to 250 nM dose of EGCG daily. Further increases in the concentration begin to show a decrease in the expression seen with the lowest doses of EGCG administered. Interestingly, when we treated the RA synovial fibroblasts with single, high doses of EGCG, we observed that the effect on TNF-R1 expression in these RA synovial fibroblasts appeared to be a continuation of the effect we began to see with 500 nM and 1 μM concentrations of EGCG administered daily to RA synovial fibroblasts. This trend continues until the RA synovial fibroblasts are treated with 20 μM EGCG and we again see a slight increase in the expression of TNF-R1. This increase in TNF-R1

expression by EGCG was shown to occur in the presence or absence of TNF-α stimulation in RA synovial fibroblasts.

To correlate these findings, the joint homogenates from the AIA rat study were also tested for TNF-R1 expression. Results from this experiment showed the expression of TNF-R1 is significantly increased in arthritic rats as compared to the healthy control animals. We also observed a modest increase in TNF-R1 expression in the EGCG-treated animals when compared to the untreated arthritic animals. This would suggest that the repeated exposure to EGCG at low concentrations is the most true to what is actually happening inside the body. While this increase in TNF-R1 is modest, being able to chronically maintain TNF-R1 expression with low, physiologically attainable doses of EGCG could be beneficial in conjunction with the modulation of TNF-R2 expression used in RA.

The expression of TNF-R2 also increased in RA synovial fibroblasts repeatedly exposed to low concentrations of EGCG. In the study requiring repeated exposure to low concentrations of EGCG with additional TNF-α stimulation, we saw a dose-dependent increase in TNF-R2 expression in the RA synovial fibroblasts. Without the additional stimulation, this repeated exposure results in a trend similar, but more pronounced and statistically significant for TNF-R2, to that seen in the expression of TNF-R1 in cells being repeatedly exposed to EGCG. There is an increase in expression at the lower doses of EGCG, but at 500 nM EGCG, we begin to see a decline in TNF-R2 expression in the RA synovial fibroblasts. The decrease in expression at these doses still resulted in greater TNF-R2 expression than TNF-R1 expression in these cells, which is believed to be beneficial in

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the treatment of arthritis. We also found that, in RA synovial fibroblasts, a single exposure of EGCG at high concentrations resulted in TNF-R2 expression being significantly reduced. This result was more interesting because in both of the studies we performed with repeated EGCG exposure in the synovial fibroblasts, we found that there was a slight increase in TNF-R2 expression in comparison to the untreated control group. In the single EGCG exposure study, the decline in TNF-R2 expression was much more drastic than that which was seen in TNF-R1 expression as well. This significant decrease in expression levels of TNF-R2 could be the result of potential cleavage of this receptor from the cells, which has also been suggested as a possible mechanism for the beneficial effects of TNF-R2 in RA patients [60]. One of the limitations of our study was a very low or almost undetectable level of TNF-R2 expression in the joint homogenates of AIA rats. Future experiments will be done to determine the effect of EGCG on TNF-R2 at the mRNA level in the joint homogenates of these animals.

When evaluating the downstream signaling proteins involved in TNF receptor signaling, we found that the repeated exposure of RA synovial fibroblasts to high concentrations EGCG was more effective than the single exposure to high EGCG concentrations in down-regulating TNF-α-stimulated MAPK activation. Our results showed that phosphorylation of JNK was slightly increased dose-dependently with repeated exposure to EGCG, but at 1 μM EGCG, the pJNK expression decreased. Single exposure to high EGCG concentrations also resulted in a decrease of pJNK. It appeared that the maximum effect of EGCG on pJNK was reached with the 10 μM concentration of EGCG. The decrease seen in JNK phosphorylation with repeated exposure to EGCG is encouraging because in RA, the JNK

pathway has been shown to mediate collagenase gene expression that regulates cartilage destruction [64]. Because of this link, a decrease in JNK phosphorylation may result in a protective effect against the destruction of the extracellular matrix in the arthritic patient, slowing the joint destruction resulting from this disease. In the AIA rat joints, treatment with EGCG (50 mg/kg, daily) resulted in a slightly decreased pJNK expression. These results suggest that inhibition of arthritis by EGCG may b independent of the JNK pathway and may involve some other critical pathway.

As observed in a study done using osteoarthritis chondrocytes by Singh *et al*. [65], EGCG did not have any inhibitory effect on the phosphorylation of ERK MAPK. It appeared that in the study in which the RA synovial fibroblasts were exposed overnight to high concentrations of there was a slight decrease in phosphorylation. This was not the case when these cells were repeatedly exposed to low concentrations of EGCG, where we observed almost no change in the phosphorylation of ERK at any concentration of EGCG administered to the synovial fibroblasts.

Synovial fibroblasts are one of the major cell types in which activation of the p38 MAPK occurs. In animal models, inhibition of p38 activation resulted in reduced inflammation in the animal as well as a decrease in the production of inflammatory cytokines such as IL-1 and IL-6 [66]. In our study, we found that repeated exposure to low concentrations of EGCG proved effective in significantly decreasing the phosphorylation of p38 in RA synovial fibroblasts. This repeated exposure approach proved to be more effective in down-regulating p38 phosphorylation than administering high concentrations of EGCG to

these synovial fibroblasts for 24 hours as the single exposure had no effect on the phosphorylation of p38. Because there were so many cell types combined to make up the joint homogenate and p38 activation is limited to few cell types, we were unable to verify these results *in vivo*. Nonetheless, reduction in synovial p38 phosphorylation may be an interesting and promising therapeutic outcome.

After observing the inhibitory effect that EGCG had on JNK and p38 phosphorylation, we decided to look into what effect, if any, EGCG had on the phosphorylation of AKT in RA synovial fibroblasts. AKT is an important survival protein that helps RA synovial fibroblasts in uncontrolled proliferation [67]. In the studies with both repeated and single exposures to EGCG, we saw a slight decrease in phosphorylation of AKT with increasing concentrations of EGCG, but there was no significant decrease in either treatment. The same was true when we performed Western blotting to see if EGCG treatment of AIA rats had any effect on AKT phosphorylation. The amount of pAKT in the joint homogenate samples was nearly the same for all three groups. Because phosphorylation of AKT has a pro-survival effect in synovial fibroblasts by protecting them from Fas-induced apoptosis, it can be postulated that EGCG may not have any marked effect on Fas-induced apoptosis.

The pro-inflammatory cytokine IL-6 is a major cytokine involved in the progression of RA. It promotes MMP production, autoantibody production and the differentiation of Th17 cells. We performed ELISAs using conditioned media from the repeated and single exposure studies to determine what effects each treatment had on TNF-α-induced production of IL-6. The results of the IL-6 ELISA using the conditioned media from the

cells repeatedly exposed to EGCG showed a dose-dependent decrease in IL-6 production by the synovial fibroblasts. The RA synovial fibroblasts treated with 1 μM EGCG had about 20% less IL-6 production than the untreated cells. A single exposure of RA synovial fibroblasts to 10 or 20 μM EGCG resulted in a 40% decrease in the amount of IL-6 in the conditioned media. These results further attest to previous publications showing the efficacy of EGCG in regulating IL-1β-induced IL-6 production [47]. Because IL-6 plays such a major role in RA, even a modest decrease in IL-6 levels could be quite beneficial, especially when the fact that this decrease would be coming as an effect of a supplemental therapy.

In RA, synovial fibroblasts produce IL-8, a pro-inflammatory cytokine, which recruits more T-cells, neutrophils and basophils to the already inflamed joint. When we determined the TNF-α-induced IL-8 production by the RA synovial fibroblasts, we found that the repeated exposure to EGCG resulted in a dose-dependent decrease in IL-8 production in cells treated with 250 nM – 1 μM EGCG. This decrease was amplified in the study in which the RA synovial fibroblasts were exposed for 24 hours to high concentrations of EGCG. The conditioned media from the RA synovial fibroblasts treated with 10 and 20 μM EGCG showed almost 60% inhibition in IL-8 production in the conditioned media compared to the untreated synovial fibroblasts. The results showing a decrease in both IL-6 and IL-8 production may support a study by Suzuki *et al.,* in which, inhibition of phosphorylation of the p38 MAPK in RA synovial fibroblasts resulted in the suppression of IL-6 and IL-8 production [68]. Overall, these results suggest that the decrease in IL-6

and IL-8 production in the conditioned media of the EGCG-treated samples, may be at least partially due to the inhibition of p38 MAPK and JNK phosphorylation.

In conclusion, these studies suggest that EGCG is able to play a beneficial role in TNF-α-mediated signaling in RA synovial fibroblasts. Repeated exposure of RA synovial fibroblasts to low concentrations of EGCG results in no marked change in TNF-R1 expression. However, a marked increase in TNF-R2 expression by cells suggest that enhanced TNF-R2 expression may engage TNF-α to stimulate signaling through this receptor, thereby providing a possible method of decreasing inflammation. In addition, repeated exposure to low concentrations of EGCG may be able to prevent uncontrolled cell growth and help in slowing the progression of joint destruction by the synovial fibroblasts. In summary, the results of this study suggest that EGCG may be a beneficial supplemental adjunct treatment option for RA. However, further studies are required to validate the effect of repeated exposure to low concentrations of EGCG on the NF-κB pathway as a result of TNF-R1 and TNF-R2 modulation.

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