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Role of TNFα Antagonists and Genetic Polymorphisms in Modulating Susceptibility to Mycobacterial Infection among Patients with Crohn's Disease

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ROLE OF TNFα ANTAGONISTS AND GENETIC POLYMORPHISMS IN MODULATING SUSCEPTIBILITY TO MYCOBACTERIAL INFECTION AMONG PATIENTS WITH CROHN'S DISEASE

by:

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ABSTRACT

Tumor Necrosis Factor alpha antagonists (anti-TNFα) have been extensively used for Crohn's disease (CD) treatment. Even though they may control CD symptoms initially, treatment response varies among patients, which seems to depend on single nucleotide polymorphisms (SNPs) in TNFα receptors superfamily 1A and 1B (*TNFRSF1A/B*). Most importantly, *M. tuberculosis* infection has been strongly associated with these medications, but no studies have elucidated the effects of anti-TNFα on CD associated with MAP (*Mycobacterium avium* subspecies *paratuberculosis*; a possible causative agent of CD, and closely related to *M. tuberculosis*).

Here, we are investigating the effects of recombinant inflammatory cytokines and anti-TNFα therapeutics on macrophages infected with MAP isolated from CD patient. We also tested the prevalence of MAP and the significance of nine SNPs in *TNFα, TNFRSF1A* and *TNFRSF1B* from the blood of 54 CD and 50 healthy subjects by *IS900* nPCR.

Both PEGylated and non-PEGylated forms of anti-TNFα increased MAP viability by nearly 1.5 Log CFU/mL, while rIL-6 and rIL-12 induced MAP viability at 5.42 ± 0.25 and 4.79 ± 0.14 Log CFU/mL, respectively. In contrast, rTNFα reduced MAP survival in infected macrophages by 2.63 Log CFU/mL. Expression of *TNFα, IL-6,* and *IL-12* was upregulated by 3 folds following MAP or *M. tuberculosis* infection compared to other bacterial strains ($P<0.05$).

Four SNPs (*TNFα:rs1800629, TNFRSF1A:rs767455, TNFRSF1B:rs1061624 and TNFRSF1B:rs3397*) were overrepresented significantly ($P<0.05$) among CD patients compared to healthy controls. The *TNFRSF1A:rs767455* GG genotype was found in 15/54 CD patients (28%), while it was only found in 2/50 healthy controls (4%) [OR = 9.2, 95% CI: 1.98-42.83].

The *TNFRSF1B:rs3397* TT genotype was found in 15/54 CD patients (28%) compared to (4/50) healthy controls (8%) [OR = 4.4, 95% CI: 1.36-14.14]. Furthermore, the SNPs *TNFRSF1A:rs767455* and *TNFRSF1B:rs3397* were associated with downregulating their corresponding genes significantly ($P<0.05$). MAP infection was predominantly found among CD patients in comparison to healthy controls (57% vs 8%, respectively), which was also dependent on the SNPs *TNFRSF1A:rs767455* and *TNFRSF1B:rs3397*. Our SNP haplotype analysis of *TNFRSF1A:rs767455* and *TNFRSF1B:rs3397* indicates that the G – T haplotype is significantly distributed among CD patients (46%) and MAP infection susceptibility is also associated with this specific haplotype (31%).

The data indicate that MAP positive CD patients receiving anti-TNFα could result in favorable conditions for MAP infection, which explains the poor response of many CD patients to this treatment, leading to adverse outcomes ultimately.

This is dedicated to the memory of my father, Said Qasem, my mother, Mariam Qasem & all of my family and friends who continuously support me while I pursue my life ambitions.

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LIST OF ACRONYMS/ABBREVIATIONS

5-ASA: Aminosalicylates

ASD: Arcsine Differences

AZA: Azathioprine

CD: Crohn's Disease

CFU: Colony Forming Units

CRP: C-reactive Protein

DNA: Deoxyribonucleic Acid

EBC: Epidemiologically Based Correction

GM-CSF: Granulocyte Colony Stimulating Factor

HACAs: Human Anti-Chimeric Antibodies

HIV: human immunodeficiency virus

IBD: Inflammatory Bowel Disease

IFN-γ: Interferon Gamma

IgA: Immunoglobulin A

IgG: Immunoglobulin G

IgM: Immunoglobulin M

IL-1: Interleukin 1

IL-2: Interleukin 2

IL-2: Interleukin 2

IL-6: Interleukin 6

IL-8: Interleukin 8

IL-12: Interleukin 17

IL-17: Interleukin 17

IL-23: Interleukin 23

IL23R: Interleukin-23 Receptor

IS900: Insertion Sequence 900

JAK: Janus Kinases

K. pneumonaie: *Klebsiella pneumoniae*

L. monocytogenes: *Listeria monocytogenes*

LPS: Lipopolysaccharide

M. avium: *Mycobacterium avium subspecies avium*

MIC: Minimum Inhibitory Concentration

M. smegmatis: *Mycobacterium smegmatis*

M. tuberculosis: *Mycobacterium tuberculosis*

MAP: *Mycobacterium avium* subspecies *paratuberculosis*

Multiplex PCR: Multiplex Polymerase Chain Reaction

nPCR: Nested PCR

NSAIDs: Non-Steroid Anti-Inflammatory Drugs

OR: Odds Ratio

PBS: Phosphate Buffer Saline

PtpA: Protein Tyrosine Phosphatase

RA: Rheumatoid Arthritis

RCTs: Randomized Controlled Trials

RF: Rheumatoid Factor

RHB-104: Redhill Biopharma 104

RPMI: Roswell Park Memorial Institute Medium

RT-PCR: Real Time PCR

S.aureus: *Staphylococcus aureus*

SNPs: Single Nucleotide Polymorphisms

STAT-3: Signal Transducer and Activator of Transcription **3**

TB: Tuberculosis

TE: Tris-EDTA

TGF-β: Transforming Growth Factor beta

TNF-α: Tumor Necrosis Factor Alpha

TNFRSF1A: tumor necrosis factor-α receptor superfamily 1A

TNFRSF1B: tumor necrosis factor-α receptor superfamily 1B

UC: Ulcerative Colitis

UCF4: University of Central Florida strain 4

CHAPTER ONE: INTRODUCTION

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Overview of Crohn's Disease (CD)

Crohn's disease (CD) is a chronic relapsing form of inflammatory bowel disease (IBD) affecting the digestive tract. Patients diagnosed with this disease are suffering from abdominal pain, persistent diarrhea, and malnutrition. The prevalence of CD has been rapidly increasing in North America and other countries adapting western lifestyle over the recent decades [1]. Studies involved human genetics, animal models and clinical trials, have indicated new insights into CD pathogenesis [2]. We propose and support the hypothesis that dysregulated immune response against microbial environmental triggers in genetically susceptible subjects lead to development of CD [Figure 1]. Among the most debated and accepted microbial triggers in CD pathogenesis is *Mycobacterium avium* subspecies *paratuberculosis* (MAP) [3,4,5]. However, current standard treatment guidelines for CD do not primarily include anti-mycobacterial therapy. The ultimate goal is to suppress the abnormal inflammatory immune response by using several anti-inflammatory drugs and biologics, none of which have any meaningful effect to eradication of microbial triggers such as MAP. In some circumstances, CD patients might be prescribed with combinational therapy which includes a biologic and an immunomodulator. As with all therapy, there are many reported adverse effects of CD medications, especially those related to multiple

infections [6]. It is also essential to maintain a good nutritional status since CD patients have reduced ability to absorb proteins, carbohydrates, fat, water, vitamins and minerals.

Therapeutic Context of Targeting Cytokines in CD and Mycobacterial Infection

Contradicting Role of Cytokines in Mycobacterial Infection

Over-reactive immune response in CD patients includes significant elevation in Tumor Necrosis Factor alpha (TNFa). In normal state TNFa is produced by numerous cells, including macrophages, CD4+ and CD8+ T-cells, B-cells, neutrophils, endothelial cells, natural killer cells, smooth muscle cells, fibroblasts and osteoclasts, where its concentration is undetectable (<10fg/ml) [7]. In CD, macrophages secret high level of TNFa (>200 pg/ml) [8, 9] which causes upregulation in IFNγ, IL-6, IL-8, IL-1β and granulocyte-macrophage colony stimulating factors, leading to cell recruitment and formation and maintenance of granuloma [10]. [Figure 2]. The latter is an alternative approach selected by the immune system to overcome infection by isolating and neutralizing invasive microorganisms such as MAP [7, 11]. Lower TNFa level leads to impairment of the immune system in its effort to eradicate infection. Several studies demonstrated that granulomatous infection has increased significantly in TNFa-deficient animals [12, 13]. Granulocyte colony stimulating (GM-CSF) may suppress the growth of *M. avium* and *M. tuberculosis* by activating human macrophages [14]. The opposite is true; GM-CSF might also stimulate the growth of some other intracellular parasites [15]. *In vitro* studies have also shown that IL-6 increases the human macrophage susceptibility to *M. avium* infection which could explain susceptibility of patients with human immunodeficiency virus (HIV) to mycobacteria and others [16]. Collectively, cytokines might have bidirectional effect on intracellular microbial infections, and this could be either by stimulating or suppressing the

macrophages activity directly, or it could affect granuloma formation and maintenance leading to isolation of microbes from the whole system.

Dual Effect of Anti-TNFa and other Immunomodulators in Crohn's Disease

There are several drugs being prescribed to CD patients which are mainly designed to lower the TNFa in order to reduce inflammation and achieve remission. Etanercept, infliximab, adalimumab and certolizumab pegol are just examples of anti-TNFa IgG monoclonal antibodies which are widely used for treatment of CD patients [11]. Anti-TNFa therapeutic agents indirectly inhibit the production of IFNγ produced by activated T-cells [17]. A contradicting effect is expected in cases associated with microorganisms such as MAP, since lowering IFNγ reduces T-cell response, formation of granuloma and isolation of infecting pathogens [18].

Recent *in vitro* culture studies reported that some drugs used in CD standard treatment have shown anti-MAP growth activity [19-21]. Altered MAP growth in culture treated with azathioprine (AZA), 6-mercaptopurine (6-MP), cyclosporine A, tacrolimus and rapamycin has been reported [19,20]. Methotrexate (MTX) inhibited MAP growth in higher potency than 6-MP [21]. MTX could potentially affect MAP growth and survival since it reduces folate generation, which interferes with bacterial DNA replication. MTX in lower doses is also known to downregulate pro-inflammatory cytokines, which results in clinical improvement in CD patients [22, 23]. As shown in Table 1, other drugs used in CD standard treatment have contradicting effect on MAP. Aminosalicylates (5-ASA) intensified MAP growth in culture when the bacteria were exposed to concentrations higher than 25ug/ml [19]. The negative effect of some of these drugs on MAP growth suggest that response of CD patients to treatment with AZA, 6-MP, cyclosporine A, tacrolimus and rapamycin may be due, in part, to the detrimental effect of the

drugs on MAP in these patients. On the other hand, the favorable effect of 5-ASA on MAP growth is alarming to CD patients treated with 5-ASA. However, these *in vitro* direct effects might be different from how the drug might interfere with MAP survival under physiological conditions.

While treatment of CD with anti-inflammatories and biologics is necessary to control inflammation and to provide short term benefit to the patients, a strategy to eradicate infection in cases associated with microorganism such as MAP should be included before selection of treatment. The anti-MAP effect of these non-antibiotics drugs should not be misleading. Bacteriostatic or bactericidal of any drug requires optimum doses at or above the minimum inhibitory concentration (MIC) of the drug. Therefore, alternative combined therapy plan should be considered for long term remission and possibly a cure from this disease. Basically, an alternative strategy should combine targeted immunotherapy and effective antibiotics where together they can block the source and the signs of inflammation. Effective antibiotics treatment includes selection of specific anti-MAP drugs, the necessary treatment duration and enforced compliance by the patients. RHB-104 is an investigational anti-MAP formulation consisting of clarithromycin, rifabutin and clofazimine which is currently being used in an FDA-approved phase III international clinical trial to treat moderate to severe cases of CD [24,25]. It is effective *in vitro* against large number of microorganism including several MAP clinical strains, and others. The ingredients of RHB-104 have demonstrated effective healings in CD patients when administered individually or in combination with other antibiotics [26,27,28].

Effects of Immunomodulators on MAP Proteins Immunogenicity

The mycobacterial protein tyrosine phosphatase (PtpA) in MAP shares 90% homology to *M. tuberculosis* PtpA [29]. This protein inhibits phagosome-lysosome fusion in human macrophages by dephosphorylating the vacuolar protein sorting 33B (VPS33B) in the host, also it has shown efficacy in inhibiting phagosome acidification [30,31]. By this mechanism, the pathogen will be able to avoid containment lysis in order to establish a successful infection. Recently, the level of antibodies against PtpA has been found to be significantly elevated in the serum of CD patients in comparison to healthy subjects [32], and this level was decreased after infliximab treatment [33]. *Xia et al.* proposed that PtpA nurtures the survival of MAP inside the host cells as in the case of *M. tuberculosis*, and that's why PtpA antibodies are expected to be higher in CD patients serum [34]. CD patients treated with AZA showed a significant reduction in PtpA antibodies level [34]. However, when AZA treatment was combined with other medications such as steroids or/and 5-ASA this significant reduction was lost, and there was no significant difference in PtpA antibodies level in patients treated with 5-ASA or steroids alone or in combination [34].

Anti-TNFa Therapeutics Increase Granulomatous Infection

The potential role of anti-TNFa therapeutic agents in developing granulomatous infections varies among different treatment options. This might be attributed to several factors, such as differences in their pharmacokinetics. Etanercept for instance has a shorter serum half-life than infliximab or adalimumab, which results in an extended suppression of TNF following treatment with longer half-lives TNF antagonists [35]. Studies have shown that the incidence of developing tuberculosis infection (TB) is higher after using infliximab in comparison to etanercept [36, 37, 38]. Adalimumab is similar to infliximab in its pharmacokinetics, but the incidence of

developing TB has been reported to be greater with infliximab use [7]. This could be justified by differences in the bioavailability and the peak concentration since infliximab is given intravenously while adalimumab is administered as a subcutaneous injection. Anti-TNFa agents increased the risk of granulomatous infection, especially *M. tuberculosis* in a dose dependent manner [36]. For instance, patients receiving higher dosages of adalimumab (40 mg per week) have developed active Tuberculosis [39]. Other granulomatous infections are listed in Table 2.

Anti-TNFa Treatment is Linked to Cytotoxicity and Autoimmunity

Cytotoxicity of anti-TNF-a therapeutics

The long-term consequences of anti-TNFa medications and their effects at cellular level needs further investigation. Infliximab had no effect on THP-1 cells apoptosis *in vitro* at concentrations as high as 100ug/ml, however, etanercept and pirfenidone showed a significant reduction in cellular viability at 0.5 and 300ug/ml, respectively [40].

Treating animal models with monomeric or synthetic dimeric soluble TNF receptors increased serum TNFa after LPS stimulation in comparison to LPS alone [41]. Grattendick et al. *in vitro* study agrees with this result since Etanercept but not Infliximab elevated cell-associated TNFa up to 6 folds in THP-1 cells following LPS treatment compared to cells treated with LPS alone at 0.1ug/ml [40]. However, the amount of secreted TNFa was neutralized in the same study following treatment with infliximab and etanercept at 0.1 and 0.01ug/ml, respectively [40]. Pirfenidone is capable of inhibiting TNFa synthesis at the translational level, and it showed significant reduction of secreted TNFa at 33ug/ml and cell-associated TNFa at 100ug/ml in THP-1 cells stimulated by LPS [40, 42].

Autoimmunity and Anti-TNFa Therapeutics

The immunogenicity of TNF inhibitors is crucial since unintended consequences such as autoimmunity might occur [43]. Various factors influence the rates of immunogenicity such as age, gender, genetics, route of administration, drug dose and clearance rate. In clinical trials, more than 35% of IBD and Rheumatoid arthritis (RA) patients have developed resistance or side effects to anti-TNFa therapeutic agents due to the development of neutralizing antibodies which antagonize the therapeutic effects of these medications [43,44]. Since infliximab has been used for a long time, it is the most extensively studied anti-TNFa therapeutic agent. Infliximab is a chimeric antibody with 25% murine sequence which makes it more susceptible to induce immune reactions characterized by secretion of anti-chimeric antibodies known as (HACAs) [45]. Those antibodies target Infliximab by neutralizing its ability to inhibit TNFa and by enhancing its clearance due to immune precipitation, which prevents the drug from reaching the sites of inflammation [46]. HACAs therefore will be able to hinder the clinical efficacy of infliximab by affecting its pharmacokinetics, pharmacodynamics and the bioavailability [47]. Antibodies against etanercept have been detected in less than 5% of patients receiving this treatment, however, those antibodies unlike Infliximab antibodies are non-neutralizing [48]. Although adalimumab has a humanized structure, antibodies against it were detected in 12% of treated patients [49]. Furthermore, patients treated with certulizumab pegol has developed antibodies against the drug but the clinical aspect remains unknown [45]. Researchers have evaluated the presence of specific types of antibodies by quantifying IgM and IgG produced against anti-TNFa therapeutic agents [49]. IgM antibodies were more common in patients treated with infliximab, etanercept and adalimumab, however the clinical response was not correlated

with the level of antibodies produced, suggesting that the type of antibody (IgG, IgM or IgA) plays a significant role in developing adverse reactions or altering the therapeutic effects [50].

Reported Adverse Effects of Anti-TNFa Therapeutics

As previously reported, the most common problem with anti-TNFa therapy is frequent infections [6]. There is a strong affiliation between mycobacterial infection and anti-TNFa therapeutic agents, since the risk for developing TB is higher compared to placebo controls [51]. TNFa-deficient animal models were more susceptible to develop mycobacterial infections in comparison to wild-type controls, although they had no difference in survival rate in a healthy environment [52]. TNFa might have a critical role in the immune defense against mycobacterial infections, however, the link between blocking TNFa and macrophage mycobactericidal activity needs further investigation. Indeed, other types of infections have been reported after prescribing anti-TNFa agents, such as meningitis, sepsis, histoplasmosis and pneumonia [53, 54, 55,58]. Infusion site reactions have been reported after the use of anti-TNFa agents. It could happen at the infusion time or within one hour of discontinuation, however delayed hypersensitivity reactions were reported within 3 to 12 days after infusion in CD patients but it was infrequent [6]. Other reported side effects include malignancy, heart failure, neurologic disorders and diabetes mellitus in young individuals [6]. Most recently approved humanized monoclonal antibodies targeting integrins for CD treatment such as vedolizumab has shown lower adverse effects incidence compared to other traditional Anti-TNFa therapeutics, although natalizumab carries a significant risk of leukoencephalopathy [63].

Expert Commentary

Targeting TNF-a has initially shown that antagonizing single cytokine pathway may control the symptoms of CD or IBD in general in addition to other autoimmune disease such as RA. However, Anti-TNFa therapy was insufficient to induce response in many CD patients, which indicates that there are other factors influencing CD pathophysiology. Consequently, Anti-TNFa therapy could worsen the condition of these particular non-responding patients with inducing multiple infections especially those affiliated with granuloma maintenance and several unwanted adverse effects [6]. Studies have reported that about 10 to 30% of IBD patients have shown no initial response to Anti-TNFa and almost half of the patients who showed an initial response have lost it over time [59]. Therefore, there is an extensive search for alternative therapeutic targets such as IL-6, IL-12, and IL-23, in addition to novel inhibition of selective pathways such as using antisense oligonucleotide to target SMAD-7 and small molecules inhibiting JAK-1/JAK-3 pathways [60].

Although many of these investigational medications seem to be promising candidates for effective treatment of CD, none of them is targeting MAP the hypothesized causative pathogen of CD except the combinational antibiotic treatment which is currently known as RHB-104 [24,25]. There is an increasing supportive evidence showing the role gut microbiota in CD pathogenesis [61]. Using antibiotics such as metronidazole in combination with ciprofloxacin and rifaximin has been recommended for active luminal disease involving the colon in some CD patients [61]. Furthermore, one meta-analysis has suggested that combining broad spectrum antibiotics with immunomodulators improves clinical outcome of CD patients [62]. Another meta-analysis has demonstrated similar efficacy between using immunomodulators or antibiotics

in fistulizing CD with lower incidence of severe adverse effects in patients receiving antibiotics [63]. In contrast, antibiotics might carry a risk for inducing microbial resistance and before using them for CD treatment, MAP infection must be proven as a strong causative microbial agent affiliated with CD. In addition to that, CD patients must be informed for drug compliance in order to avoid antibiotic nonadherence.

For many decades, CD has been widely known as an autoimmune disorder which warranted treatment with anti-inflammatory and immunosuppressant drugs. Like other autoimmune disorders, multi-factors have been associated with disease pathogenesis. There is a compelling evidence pointing to MAP as a major bacterial infectious agent involved in this disease. The progressive understanding of the immunopathogenesis of CD has opened more avenues into targeted therapy. However, two issues remain to be addressed:

- Screening programs should cover all CD patients in order to detect any persistent bacterial infection.
- Positive status for MAP infection should be eradicated with antibiotics targeting this pathogen.

It is likely that in the next five years, screening programs will be more efficient to detect MAP infection in the blood or intestinal tissues of CD patients. Once the patient is diagnosed with MAP infection, it is highly recommended to start him/her on anti-MAP therapy which is currently in phase III international clinical trial. Successful treatment of CD with antibiotics might induce disease remission instead of controlling chronic symptoms with immunomodulators, especially when combining them with new novel CD therapeutics targeting other cytokine pathways aside from TNFa.

Systematic review and meta-analysis on the association of tuberculosis in Crohn's disease patients treated with TNFα inhibitors

Aims and methods

To perform a meta-analysis on the risk of developing TB infection in CD patients treated with TNFα inhibitors.

A meta-analysis of randomized, double-blind, placebo-controlled trials of TNFα inhibitors for treatment of CD in adults was conducted. Arcsine transformation of TB incidence was performed to estimate risk difference. A novel epidemiologically-based correction (EBC) enabling inclusions of studies reporting no TB infection cases in placebo and treatment groups was developed to estimate relative odds.

Results

Twenty-three clinical trial studies were identified, including 5669 patients. Six TB infection cases were reported across 5 studies, all from patients receiving TNFα inhibitors. Eighteen studies reported no TB infection cases in placebo and TNFα inhibitor treatment arms. TB infection risk was significantly increased among patients receiving TNFα inhibitors, with a risk difference of 0.028 (95%CI: 0.0011-0.055). The odds ratio was 4.85 (95%CI: 1.02-22.99) with EBC and 5.85 (95%CI: 1.13-30.38) without EBC.

The risk of TB infection is higher among CD patients receiving TNFα inhibitors. Understanding the immunopathogenesis of CD is crucial, since using TNFα inhibitors in these patients could favor mycobacterial infections, particularly *Mycobacterium avium* subspecies *paratuberculosis* (MAP), which ultimately could worsen their clinical condition.

Discussion

One of the most common complications following the use of TNFα inhibitors is increasing frequency of opportunistic infections [64]. Particularly, there is strong evidence linking mycobacterial infection to TNFα inhibitors, and TB infection risk is higher among patients receiving infliximab in comparison to controls [65]. Interestingly, TNFα-deficient animal models were more susceptible to mycobacterial infections compared to wild-type controls, although there was no survival rate difference in a healthy environment [66]. This indicates that TNFα plays a critical role in the immune response against mycobacterial infections.

Several studies have shown that there is a microbial factor affecting CD patients, and MAP was isolated from intestinal tissues, blood, and milk samples of not only CD patients but also patients with RA and type 1 diabetes [67, 68]. Since MAP shares molecular homology and activity similar to TB, inducing TB infection susceptibility is an alarming sign for the immune response against MAP infection [29, 31].

This study advances knowledge and awareness of the association between TNFα inhibitors and TB among CD patients. First, a non-biased estimation of TB infection risk associated with TNFα inhibitors for CD treatment was performed through arcsine transformation of TB incidence, which enabled the inclusion of all qualified studies including double-zero studies in the analysis. Second, a novel, epidemiologically-based background correction to adjust for zero counts was developed to enable the inclusion of double-zero studies into the estimation of the relative effect (odds ratio in this study). Lastly, with the use of these analytical approaches, a significant increase of TB infection risk associated with using TNFα inhibitors to treat CD was shown from existing evidence, challenging findings of previous studies.

In our study, all 23 qualified trials were included. Among these 23 studies, 18 (78%) did not report

TB cases from either the anti-TNFα treatment or the control group; these double-zero studies would have been excluded if we had followed the methods that previous meta-analyses in this area took. The double-zero observation was not a surprise. TB infection was rare in the Americas, Europe, Japan, Austria, and South Africa, where these RCTs were conducted. The median sample size of the control group across these 23 studies was 73 people; the median follow-up duration was 30 wk. Mathematically, only about 0.0084 TB cases would be expected in these control patients if the background TB infection incidence was 20 cases/100,000 person-years as reported by Aberra *et al*[24]. If TNFα inhibitors had increased the TB infection risk by 5 times, there might have been about a 4% chance to observe 1 TB case in the anti-TNFα treatment arm. Meta-analysis provides excellent opportunities to pool multiple studies together to improve the estimation of the chance of observing a TB case. Discarding these double-zero studies (78% of the studies in our analysis) might decrease the value of meta-analysis.

We regarded the risk difference calculated after arcsine transformation of incidence as the primary results. The ASD method does not call for any correction for zero counts. Additionally, the robustness of an ASD estimate is not contingent on the effect size and the treatment-to-control balance of sample size. These analytical features provided a distinct advantage over either the Yusuf-Peto method or the Mantel-Haenszel method. However, from a biological perspective, it is possible that the TB infection risk from the use of TNFα inhibitors in patients with CD may be better described on a multiplicative scale (relative scale). Thus, a risk difference of 0.028 could be translated into increasing the risk of TB by a factor of 8, as the number needed to harm was calculated at 565 patients compared to the background number needed to harm of 5000 patients (Table 3). However, the 8 times increased risk of TB (based off ASD) only applies if the incidence of TB remains constant (20 cases/100000 person-years). We thus performed further analysis to

better describe this multiplicative scale, for which we chose the Yusuf-Peto method because, as compared to the Mantel-Haenszel method, it can handle single-zero studies [70]. Unfortunately, the Yusuf-Peto method cannot handle double-zero studies.

We proposed an epidemiologically-based background correction (*i.e.*, EBC) to mathematically replace zeros. If metrics other than ASD (*e.g.*, odds ratio, hazard ratio or rate ratio) have to be estimated and the risk of interest is so rare that even one occurrence is not expected, we recommend that EBC be used for continuity correction instead of adding 0.5 (or a similar number depending on the ratio of sample size between treatment and control groups) or statistical-model based estimates [71, 72]. The latter approaches lack biological considerations, and in the case of adding a number around 0.5, artificially make a much larger background incidence than there actually is (it would have boosted the background incidence by ~60 times in this meta-analysis).

There are major limitations with the use of EBC. The EBC was based off TB incidence rate in the UK IBD populations. Although the RCTs in this study were largely conducted in Western countries, the TB incidence of Crohn's patients in the UK may not represent the TB incidence of the countries in which the clinical trials were conducted, let alone the patients who participated in the clinical trial. Furthermore, the TB incidence rate was found in populations with IBD, which may not be representative of TB incidence in the population with CD.

Aside from the analytical approach to avoid Simpson's paradox, the validity to pool results from individual studies in this meta-analysis largely resides in the fact that each study had a placebo-treatment arm. The impact from the difference of study populations was therefore minimized, as the end point (risk difference or odds ratio) mainly reflected the effect of TNFα inhibitors [the effect of confounders was either subtracted out (for ASD) or normalized (for ORs)]. Thus, common factors restricting the use of meta-analysis, such as geographic location, population

characteristics, exposure, maintenance *vs* induction trials, status (*e.g.*, phase 3 *vs* phase 4) of the clinical trials, secular trend, and TB screening methods could be assumed to be not of major concern.

Perhaps the fact that only studies in English were included in the analysis may limit the generalizability of the results, considering that the demographics and trends of CD and TB infection differ among different regions or different populations [73]. The included studies were mainly EMA- and FDA-regulated clinical trials conducted in western countries. In fact, only one study that contained strictly Asian populations was included in this meta-analysis [74]. Thus, caution should be taken when extrapolating the results from this analysis to predict TB infection risk of TNFα inhibitors treating CD in non-western countries.

Additional attention should be paid to TB screening. Patients could have had either latent TB infection that was reactivated or acquired TB infection through exposure. The screening methods of trials varied and often went unreported. Furthermore, screening out patients based on a positive tuberculin skin test may have different impacts on the TB infection occurrence due to the different practices of Bacillus Calmette-Guérin vaccinations [75]. Lastly, two studies did not report screening methods [76, 77]. Close examination of the additional details of TB screening may provide further insight on the nature of TB infection – whether it was acquired or reactivated.

We conclude that there is sufficient evidence to assert that using TNFα inhibitors increases the risk of developing TB infection in patients with CD. Twenty-three studies were analyzed, and multiple statistical methods repeatedly gave significant risk. To our knowledge, these 23 studies represented all appropriate literature available for the topic at hand, with an extensive and careful review conducted. No studies were excluded, provided that they used a placebo control and were randomized and masked. The randomization minimized potential confounding such as age,

duration of IBD, and disease activity. These results challenge findings of previous studies, which reported no significantly increased risk of TB infection when TNFα inhibitors were used to manage patients with CD [78, 79]. Based on the risk difference found in this study, on average 565 patients treated with TNFα inhibitors may result in 1 patient getting infected with TB, *vs* 5000 patients not treated with TNFα inhibitors producing 1 case of TB, if the background incidence of TB infection in moderately severe CD is similar to the rates found in the UK IBD population.

The etiology of CD remains uncertain. Evidence suggests that CD may be caused by an immune response to commensal enteric bacteria [2]. Recent research also suggests that CD is intimately linked to MAP, which is a TB-like bacterium [3, 25]. The use of TNFα inhibitors in these patients could favor MAP infection and worsen the patient condition. It is currently difficult to come to conclusions considering that the RCTs did not test for MAP infection – much less reported it. Further research could be done on looking at patient outcomes and determining which patients had MAP infection and what their susceptibility to infection was.

Figures

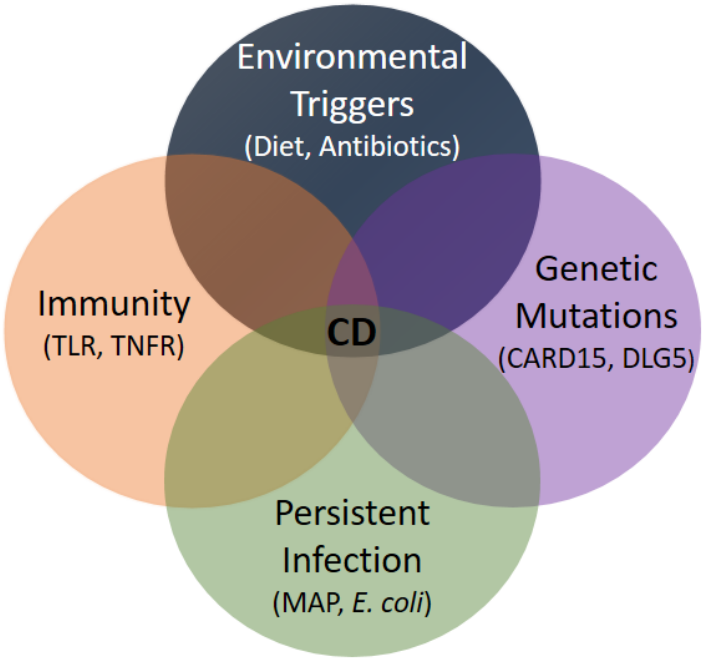

Figure 1: Interplay of genetics, environmental triggers and immune response in Crohn's disease pathogenesis.

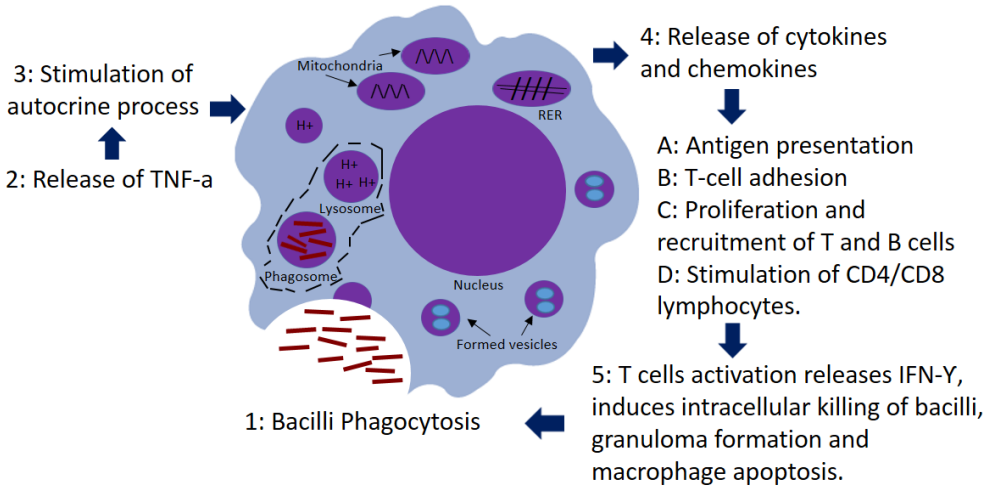

Figure 2: Role of TNFα in Crohn's disease-associated with *Mycobacterium avium* subspecies *paratuberculosis* (MAP) infection

Tables

Table 1: In vitro effect of immunomodulators on MAP culture.

Drug	Mechanism of action	Susceptibility S: Sensitive R: Resistant	Minimum concentrations inhibited MAP growth (ug/mL)	Reference
6-Mercaptopurine	Inhibition of purine synthesis	S	32.0	18
5-ASA	Scavenger of free radicals	R	>64.0	18
Sulfasalazine	Reduces synthesis of eicosanoids	R	>64.0	18
Sulfapyridine	Inhibition of folic acid synthesis	R	>64.0	18
Azathioprine	Antagonizing purine metabolism	R	>32.0	18
Cyclosporine A	Inhibition of calcineurin	S	32.0	19
Rapamycin	Inhibition of mTOR complex	S	64.0	19
Tacrolimus	Inhibition of calcineurin phosphatase	S	64.0	19
Methotrexate	Inhibition of folic acid synthesis	S	4.0	20

Table 2: Other reported causative agents of granulomatous infections after using Anti-TNFa therapeutics.

Granulomatous infection causative agent	Reference
Histoplasma capsulatum	56
Listeria monocytogenes	57
Cryptococcus neoformans	35
Coccidioides immitis	35

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CHAPTER TWO: TNFα INHIBITORS EXACERBATE MYCOBACTERIUM PARATUBERCULOSIS INFECTION IN TISSUE CULTURE: A RATIONAL FOR POOR RESPONSE OF CROHN'S DISEASE PATIENTS TO CURRENT APPROVED THERAPY

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Introduction

Crohn's disease (CD) is described as a complex idiopathic inflammation, which can affect any part of the digestive tract. Patients diagnosed with this chronic form of inflammatory bowel disease (IBD) suffer from persistent diarrhea, abdominal pain, and malnutrition [1]. The prevalence of CD in the western countries increased recently, which carries a huge economic burden on healthcare cost; since 50% of CD patients require surgical intervention within 10 years of diagnosis [1,2,3].

The etiology of CD involves various components including genetic susceptibility, altered microbiota and environmental triggers [4]. One of the most investigated pathogens associated with CD is *Mycobacterium avium* subspecies *paratuberculosis* (MAP) [5,6]. However, current treatment guidelines do not consider this bacterial infection as a source of inflammation. Although the standard CD therapy has shifted from general immunosuppressants such as corticosteroids and thiopurines, toward more specific targets including Tumor Necrosis Factor alpha (TNFα), patients are usually unable to reach full remission or at least maintain a sustainable clinical response to this kind of therapeutic approach [7,8,9].

Introducing anti-TNFα biologics (infliximab, adalimumab, certolizumab pegol) to CD treatment has shown that targeting a specific cytokine could be helpful to control CD symptoms and reduce flare-ups [7]. Unfortunately, sustainable remission is very limited to a small group of patients [9]. It has been reported that 10-30% of CD patients, have no initial response to anti-TNFα therapeautics, and over 50% of initial responders lose their response to treatment over time [10]. Additionally, about 40% of CD patients are at risk of disease relapse after anti-TNFα treatment discontinuation [11].

Moreover, blocking TNFα carries several adverse effects, including higher risk for malignancy, heart failure, and multiple infections [4]. There is a well-established evidence supporting the role of anti-TNFα therapeutics in increasing the incidence for mycobacterial infections including *mycobacterium tuberculosis,* which is due to the importance of TNFα in granuloma formation and containment of *M. tuberculosis* [12]. Since MAP and *M. tuberculosis* share molecular similarities and they both avoid phagosome-lysosome fusion in infected macrophages [13, 14, 15], TNFα remains the essential cytokine required for containment and eradication of MAP. It is, therefore, alarming for CD patients who are infected with MAP to receive anti-TNFα therapeutics, and an extensive search for alternative CD therapeutic drug targets is required. Nevertheless, anti-TNFα treatment might still be considered for CD patients who do not have MAP infection, and who are genetically more likely to respond to this treatment [16].

For refractory cases of CD, there are a few current medications with a novel therapeutic pathway known as integrin inhibitors, such as natalizumab (Tysabri®) and vedolizumab (Entyvio®). Although these two medications have shown a clinical efficacy in CD treatment, however they increased the risk for Progressive Multifocal Leukoencephalopathy (PML) in multiple clinical trials [17, 18, 19]. Besides, several pro-inflammatory cytokines are emerging as possible

therapeutic targets for CD such as IL-6, IL-12, and IL-23 [20]. However, the effect of these medications on mycobacterial infection is unknown. Additionally, the effect of MAP infection on upregulating pro-inflammatory cytokines in CD needs further investigation.

In this study, we focused on elucidating the effects of non-PEGylated and PEGylated anti-TNFα monoclonal antibodies on MAP survival in infected human-derived macrophages. We also evaluated the ability of recombinant cytokines (TNFα, IL-6, IL-12, IL-23, and IFN-Y) to modulate MAP survival *in vitro.* Finally, we evaluated the effects of MAP infection on the gene expression level of these pro-inflammatory cytokines.

Materials and Methods

Effect of anti-TNFα monoclonal antibodies and recombinant TNFα on MAP in culture

BD Bactec™ MGIT™ Para-TB medium (Sparks, MD) system was used to determine the bactericidal effects of anti-TNFα therapeutics against clinical MAP strain (UCF4; isolated from intestinal biopsy of CD patient), and other microorganisms as controls. MGIT-para media with supplements were used to culture mycobacteria as described previously [21]. For other microorganisms, nutrient broth media replaced MGIT-para media in MGIT tubes. All tubes contained a fluorescent molecule embedded in an oxygen sensitive silicone, where fluorescence is detected in the presence of active respiring bacteria. Anti-TNFα therapeutics were tested in concentrations between 0 and 200 ug/mL. Recombinant *TNFα (rTNFα) was* obtained as sterile filtered lyophilized powder (Gemini®), where each 1.0ug of powder had 2500 Units. A 50ug was dissolved in 500uL of sterile water in order to prepare 0.1mg/mL stock solution, which was stored at -20^{o}C. Then, rTNFα was added to bacterial cultures at final concentrations between 0 and 1000U/mL. The microorganisms included in this study are *MAP* UCF4, *Listeria*

monocytogenes ATCC 19112, *Klebsiella pneumoniae* ATCC 13883, and Recombinant *Escherichia coli. All culture tubes were incubated in* BD Bactec™ MGIT™ 320 Analyzer *at 37*ºC. Mycobacterial cultures were read weekly whereas other microorganisms cultures were read daily. Bacterial growth was visualized under the UV by using Andromeda BioSens SC 645 UV illuminator.

Effect of anti-TNFα monoclonal antibodies and recombinant TNFα on THP-1 cell viability and measurement of apoptosis

THP-1 cells (ATCC® TIB-202™) were cultured in RPMI-1640 medium containing 9.8% FBS (Sigma life science®) and 0.09% BME (Gibco life technologies®), while maintained in a humidified 5% CO2 incubator at 37ºC. Cells were grown until confluency in tissue culture plates was reached. Then, cells were distributed in 1 mL aliquots in a 24-well tissue culture plate. THP-1 cells were then treated with recombinant TNFα or anti-TNFα monoclonal antibodies at final concentrations between 0 and 50ug/mL, followed by incubation of 24, 48 and 72 hours. Cell viability was determined by trypan blue exclusion assay. Briefly, 10uL of cell suspension was mixed with 10uL of 0.4% trypan blue solution. Following five minutes of incubation at room temperature, 10ul of mixture were injected on a hemocytometer, and the percentage of stained (viable) cells was counted by binocular microscope. The apoptotic activities of recombinant TNFα and anti-TNFα monoclonal antibodies were determined by caspase-3 colorimetric assay (abcam®). At each time point, 500uL of each cell suspension was mixed with 50uL of chilled lysis buffer and incubated on ice for 10 minutes. Mixture was centrifuged for 1 minute at 10,000*g*. The cytosolic extract was transferred to another tube and protein concentration was measured and adjusted to 100ug per 50uL of samples. Caspase reaction mix was prepared by mixing 50uL of reaction buffer with 0.5uL of 1,4-dithiothreitol (DTT) for each reaction in

duplicates. Each sample was mixed with 50uL of reaction buffer and 5uL of 4 mM DEVD-*p*-NA substrate on a 96-well microplate. Following 90 minutes of incubation at 37^{o}C**,** the output was measured at 405nm wavelength by using a microplate reader. Fold increase in caspase-3 activity was determined by comparing treated samples with the untreated control.

Infection of monocyte-derived macrophages with MAP

THP-1 cells (ATCC® TIB-202™) were cultured as described earlier, then they were differentiated into monocyte-derived macrophages by adding 50ng/mL of PMA (Sigma-Aldrich®) for 24 hours of incubation. Monocyte-derived macrophages were then infected with *1x10^{7} bacteria/mL. Microorganisms used include* viable *M. tuberculosis* HR237, *MAP* UCF4, *and M. smegmatis* ATCC 27199, and heat inactivated *M. tuberculosis* HR237, *MAP* UCF4, *and M. smegmatis* ATCC 27199. Non-mycobacterial microorganisms included *L. monocytogenes* ATCC 19112, *K. pneumonia* ATCC 13883, and Recombinant *E. coli.* All microorganisms were used at a final concentration of 1x10^{7} viable bacteria/mL, while maintained at same conditions for 24 hours.

Measurement of TNFα, IL-6, IL-12, IL-23 and IFN-ϒ expression in infected macrophages

RNA was isolated from 1.0 mL of each sample suspension post 24 hours of infection, and used for cDNA synthesis in order to analyze expression of *TNFα, IL-6, IL-12, IL-23* and *IFN-ϒ* via RT-PCR. Briefly, cells were centrifuged at 100*g* for 5 minutes. Pellets were suspended in 1.0 mL of TRIzol® reagent (Invitrogen) for 15 minutes, and then mixed with 0.2 mL of chloroform. Following three minutes of incubation at room temperature, samples were then centrifuged at 12,100*g* for 15 minutes at 4°C. The RNA was transferred from the upper aqueous colorless part

into a new 2.0 mL microcentrifuge tube. Each sample was mixed with 0.5 mL of 100% isopropanol following 10 minutes of incubation at room temperature. Then, each sample was centrifuged at 12,100*g* for 10 minutes at 4°C, and mixed with 1.0 mL of 75% ethanol, followed by centrifugation at 7000*g* for 5 minutes at 4°C. After RNA pellets were air-dried for 15 min, samples were suspended in 20ul of RNase free water, and finally heated at 60°C for 10 minutes. A total amount of 600 ng of each RNA sample was added to 0.2 mL of PCR reaction, 4uL of iScript™ Reverse Transcription (Bio-Rad®), and up to 20 uL RNase free water for cDNA synthesis. All samples were transferred to thermal cycler (MyGene™ Series Pelteir Thermal Cycler), where reaction was performed for 5 minutes at 25°C, 20 minutes at 46°C and 1 minute at 95°C. A total volume of 1 uL of cDNA (30 ng/uL) was mixed with 10uL of Fast SYBR Green Mastermix (Thermofisher Scientific®), 1 uL of either IL-6, IL-12, IL-23 or IFN-Ɣ PrimePCR SYBER Green Assay mix (Bio-Rad®), in addition to 8 uL of molecular biological grade sterile water in a 96-well microamp RT-PCR reaction plate. The 18s RNA gene oligonucleotide primers (forward primer: 5'-GTA ACC CGT TGA ACC CCA TT-3'; reverse primer: 5'-CCA TCC AAT CGG TAG TAG CG-3') were used as controls to get baseline CT values. The RT-PCR reaction was performed using 7500 Fast Real-Time PCR System (Applied Biosystems®). Relative mRNA expression levels were calculated by using the equation ($2^{\wedge(-\Delta CT)} \times 1000$), where ΔCT = Sample RT-PCR CT value - 18s CT baseline value.

Measurement of TNFα, IL-6, IL-12, IL-23 and IFN-Ɣ protein level in infected macrophages

Following 24 hours of infection with different microorganisms, cells were centrifuged at 100*g* for 5 minutes and supernatant was collected. Protein levels were quantified by using ELISA sandwich assays for TNFα, IL-6, IL-12, IL-23 and IFN-Ɣ (Invitrogen®), according to

manufacturer's instructions. Briefly, all samples and reagents were brought up to room temperature. Six different concentrations of standards were provided and they were added to a pre-coated plate, in addition to samples and controls (25 µL of each). A volume of 100 µL of anti-cytokine-horseradish peroxidase enzyme conjugate was added to each well followed by 2 hours of incubation at room temperature. The plate was washed 3 times then 100 µL of chromogen solution was added into each well, followed by 30 minutes of incubation time at room temperature. Finally, 100 µL of stop solution was added into each well and absorbance was read by a plate reader at 450 nm wavelength, then concentrations were calculated from the generated standard curve equation.

Modulating MAP viability in infected macrophages pulsed with exogenous recombinant cytokines and anti-TNFα monoclonal antibodies

Recombinant cytokines (r*TNFα, rIL-6, rIL-12*, *rIL-23*, and rIFN-ϒ) were obtained as sterile filtered lyophilized powders (Gemini®), where each 1.0ug of powder had 2500 Units. 50ug of each powder was dissolved in 500uL of sterile water in order to prepare 0.1mg/mL stock solution, which was stored at -20^{o}C. Recombinant cytokines at final concentrations between 0 and 1000U/mL were added to monocyte-derived macrophages infected with MAP strain UCF4. Similarly, two anti-TNFα monoclonal antibodies (PEGylated and non- PEGylated forms) at final concentrations between 0 and 50ug/mL were also evaluated. Then, MAP viability was tested after 24, 48 and 72 hours.

Measurement of bacterial viability in infected macrophages

After infecting monocyte-derived macrophages with MAP for 24 hours, cells were washed to remove extracellular bacilli, and then recombinant cytokines/anti-TNFα monoclonal antibodies

were added to the culture accordingly. Monocyte-derived macrophages were collected at three time points (24, 48 and 72 hours) and lysed with 200uL of M-PER Mammalian reagent (ThermoScientific) for bacterial viability detection using Live/Dead™ Baclight™ (ThermoFisher Scientific). Briefly, five different proportions of live and dead MAP were prepared (0:100, 10:90, 50:50, 90:10, 100:0), then 100uL of each bacterial suspension was pipetted in triplicates into separate wells of 96-well flat-bottom microplate and mixed 100uL of staining reagent mixture. Following 15 minutes of incubation at room temperature in the dark, fluorescence intensity was measured at 530nm, which indicates live bacteria reading (green), and again at 630nm for the dead bacteria reading (red). Data were analyzed by dividing the fluorescence intensity of the stained bacterial suspensions (Ratio = Emission 1(green)/Emission 2 (red)). The least-squares fit line was generated and the equation was used to calculate the bacterial viability from the infected cells post-treatment.

Statistical Analysis

Data are expressed as mean ± standard error of the mean by using GraphPad® Prism 7.02 (GraphPad®, CA, USA). Two tailed student's t-test was used. The difference between samples and controls was considered statistically significant at a level of $^{*}P<0.05$.

Results

Anti-TNFα therapeutics and recombinant TNFα have no direct bactericidal activity against MAP or other tested microorganisms

In order to rule out the direct bactericidal effects of anti-TNFα monoclonal antibodies and rTNFα, we tested them in supratherapeutic levels against MAP and non-mycobacterial *MGIT cultures.* Both PEGylated and non-PEGylated anti-TNFα monoclonal antibodies have demonstrated no direct bactericidal activity when they were tested against MAP in addition to 3 non-mycobacteria strains (*L. monocytogenes* ATCC 19112, *K. pneumonia* ATCC 13883, and Recombinant *E. coli).* All bacterial strains we tested showed resistance to anti-TNFα treatment at concentrations as high as 200ug/mL (4X Cmax). Similarly, all strains we tested were resistant to rTNFα treatment at concentrations as high as 1000U/mL. Figure 3 shows the growth florescence of MGIT tubes incubated with MAP after 14 days of exposure to anti-TNFα, while Figure 4 shows the growth fluorescence of MGIT tubes incubated with non-mycobacteria strains following 72 hours of anti-TNFα exposure.

Anti-TNFα therapeutics demonstrate similar cytotoxic effects to recombinant TNFα on uninfected macrophages

To further establish the cytotoxic role of TNFα and anti-TNFα monoclonal antibodies on monocyte apoptosis and whether these alone are able to modulate monocyte apoptosis independently of MAP infection, we treated uninfected THP-1 cells with different concentrations of exogenous rTNFα and therapeutic levels of anti-TNFα antibodies. Following 24 hours of incubation with 250U/mL of rTNFα, THP-1 cell viability has decreased from 100% to 77.2%, which continued to decline over time until it reached 63.7% after 72 hours. In a concentration-dependent manner, 500U/mL of rTNFα lead to decrease in THP-1 cell viability from 100% to

52.6%, while it reached 45.9% with 1000U/mL, following 72 hours of incubation ($P<0.05$) [Figure 5A]. Caspase-3 activity has confirmed these findings, since it has shown higher activity in THP-1 cells treated with rTNFα. At 250U/mL, caspase-3 activity was 2.47 folds higher than control, while it was 6.49 and 8.92 folds higher in cells treated with 500 and 1000U/mL of rTNFα following 72 hours of incubation, respectively ($P<0.05$) [Figure 6A]. Surprisingly, anti-TNFα monoclonal antibodies showed similar cytotoxic effects as rTNFα in a dose-dependent manner. The viability of THP-1 cells treated with 5ug/mL of non-PEGylated anti-TNFα started to decrease over 72 hours of incubation, when it reached 70.6%. Higher concentrations have shown further decline in cell viability, when it reached 62.8%, 57% and 48% following 72 hours of incubation with 10, 20, and 50ug/mL, respectively ($P<0.05$) [Figure 5B]. The PEGylated form of anti-TNFα was less cytotoxic, but it still demonstrated decline in THP-1 cell viability following 72 hours of incubation, when cell viability was 74.7%, 68.8%, 62.6% and 57.9%, treated with 5, 10, 20, and 50ug/mL, respectively ($P<0.05$) [Figure 5C]. Similarly, caspase-3 activity was 4.9 and 4.4 folds higher than control following 72 hours of treatment with 50ug/mL of non-PEGylated and PEGylated anti-TNFα therapeutics, respectively ($P<0.05$) [Figure 6B and 6C].

MAP induces expression of TNFα, IL-6 and IL-12 in infected macrophages

We have found that MAP and *M. tuberculosis* induce *TNFα* expression in infected macrophages significantly (3.2 ± 0.23 and 2.8 ± 0.13, respectively), compared to *M. smegmatis* (0.8 ± 0.17) and other non-mycobacteria species following 24 hours of infection [Figure 7A]. Similarly, we found that MAP and *M. tuberculosis* both had about two times higher IL-6 expression (2.9 ± 0.41 and 2.6 ± 0.19, respectively) compared to *M. smegmatis (1.3* ± 0.12) or other non-

mycobacterial pathogens (P<0.05). Furthermore, when *M. tuberculosis* and *MAP* were both heat inactivated (80°C for 20 minutes), they have lost their activity to induce *IL-6* expression (0.35 ± 0.14 and 0.29 ± 0.11, respectively) (P<0.05) [Figure 7B]. Additionally, *IL-12* expression was also significantly higher in MAP or *M. tuberculosis* infected macrophages (1.6 ± 0.36 and 1.5 ± 0.29, respectively), but only in comparison to non-mycobacteria species (P<0.05) [Figure 7C]. However, the expression of other pro-inflammatory cytokines (*IFN-Y* and *IL-23*) was not significantly higher in MAP or *M. tuberculosis* infected macrophages [Figure 7D and 7E]. Similarly, protein level of TNFα, IL-6 and IL-12 was significantly elevated following MAP or *M. tuberculosis* infection, in comparison to other bacterial strains [Figure 8].

Recombinant TNFα decreases MAP survival in infected macrophages

First, we tested the effects of adding exogenous rTNFα into MAP infected macrophages at different concentrations between 0 and 1000U/mL. We found that MAP viability drops with time significantly in a concentration-dependent manner. At 500U/mL, MAP viability has declined from 7.0 (Log. CFU/mL) to 3.95 ± 0.22, 3.31 ± *0.34* and 2.11 ± 0.17, following 24, 48, and 72 hours of incubation, respectively (P<0.05) [Figure 9A]. At the maximum concentration we have tested, adding rTNFα at 1000U/mL to MAP infected macrophages, had decreased MAP viability from 7.0 (Log. CFU/mL) to 1.50 ± 0.15, following 72 hours of incubation (P<0.05) [Figure 9A].

Anti-TNFα therapeutics increase MAP survival in infected macrophages

In contrast, treating MAP infected macrophages with anti-TNFα monoclonal antibodies, increases MAP survival in a dose-dependent manner. The non-PEGylated form of anti-TNFα at 50ug/mL, had MAP viability of 5.09 ± 0.11 (Log. CFU/mL), while the untreated control had

4.02 ± 0.16 (Log. CFU/mL) (P<0.05) [Figure 9B]. Similarly, the PEGylated anti-TNFα had increased MAP viability up to 5.63 ± 0.14 (Log. CFU/mL) at 50ug/mL following 72 hours of incubation (P<0.05) [Figure 9C].

Recombinant IL-6 promotes MAP survival in infected macrophages

Since we found that MAP modulates some pro-inflammatory cytokine gene expression in infected macrophages, it was worthy to test the effects of exogenous recombinant cytokines on MAP survival. We tested rIL-6 at different concentrations between 0 and 1000U/mL. Interestingly, rIL-6 has promoted MAP survival significantly in a concentration-dependent manner. Following 72 hours of incubation, MAP viability was 3.42 ± 0.39, 4.25 ± 0.23, 4.82 ± 0.19 and 5.42 ± 0.25 (Log. CFU/mL), at 0, 250, 500, 1000U/mL of rIL-6, respectively (P<0.05) [Figure 10A]. Similarly, rIL-12 had some positive effect on MAP viability in infected macrophages; however, it was only significant at a concentration of 1000U/mL, when MAP viability was 4.79 ± 0.14 compared to 3.73 ± 0.33 (Log. CFU/mL), following 72 hours of incubation (P<0.05) [Figure 10B]. On the other hand, both *rIL-23* and rIFN-ϒ had similar effects to rTNFα, where they had detrimental effects on MAP survival. MAP viability has declined from 7.0 to 2.76 ± 0.16 and 2.95 ± 0.19 (Log. CFU/mL), following 72 hours of incubation with 1000U/mL of *rIL-23* and rIFN-ϒ, respectively [Figure 10C and 10D]. The effects of IL-6 neutralizing receptors (Anti-IL-6) and IL-12 neutralizing receptors (Anti-IL-12) have reduced MAP viability in concentration dependent manner. Following 72 hours of incubation, MAP viability was 4.76±0.23, 3.83±0.27 and 3.14±0.17 (Log. CFU/mL), at 0, 20 and 50ug/mL, respectively (P<0.05) [Figure 10E]. In parallel, MAP viability following 72 hours of incubation

with anti-IL-12 was 5.11±0.27, 4.19±0.14 and 3.49±0.29 (Log. CFU/mL), at 0, 20 and 50ug/mL, respectively ($P<0.05$) [Figure 10F].

Discussion

Macrophages exhibit several mechanisms in order to eradicate intracellular pathogens, such as releasing reactive oxygen intermediates, changing phagolysosomal acidity, and production of pro-inflammatory cytokines [22]. TNFα is one of those cytokines, which has a prominent role during mycobacterial infection, resulting in a formation of a complex circuit of other cytokines able to modulate T-cells and macrophages response to infection and granuloma formation [23]. Consequently, blocking TNFα function in animal models induced mycobacterial proliferation and reduced granuloma formation, indicating that TNFα is a primary cytokine for protection against mycobacterial infection [24,25,26]. However, current treatment guidelines for many inflammatory disorders of supposed non-infectious origin, recommend blocking TNFα pathway in order to suppress the hyperactive immune response and inflammation [27].

Although there is a strong evidence demonstrating the involvement of MAP in CD, there is no recommendations of using antibiotics for CD treatment so far [21]. On the contrary, the clinical use of anti-TNFα therapeutics has increased the risk for multiple infections including tuberculosis [28, 29, 30]. Thus, assessment of latent tuberculosis infection status is highly recommended in order to determine if any patient intended to initiate anti-TNFα therapy has a risk for development of active disease [31].

This study was concerned with identifying the detrimental ability of recombinant TNFα, IL-6, IL-12, IL-23 and IFN-ϒ on MAP survival in infected macrophages and if blocking TNFα

function by anti-TNFα monoclonal antibodies modulates MAP viability *in vitro.* Additionally, our goal was to identify which pro-inflammatory cytokines are highly expressed by macrophages following MAP infection. A recent study has reported that infliximab treatment increases MAP viability in infected macrophages from CD patients by predominant induction of MAP dormant form [32]. Our data shows that both PEGylated and non-PEGylated forms of anti-TNFα therapeutics do not have any direct bactericidal effects against MAP or other non-mycobacterial strains at supratherapeutic concentrations (>200mg/mL). However, these medications increased MAP survival in infected macrophages in a dose-dependent manner, which indicates that CD patients receiving such treatment are at a higher risk for MAP growth if they had MAP infection before initiation of therapy. In contrast, MAP viability declined in infected macrophages pulsed with exogenous rTNFα in a concentration-dependent manner, which shows that TNFα plays a significant role in protection against MAP infection.

Furthermore, anti-TNFα therapeutics demonstrated similar cytotoxicity level to rTNFα at therapeutic concentrations, which explains why these medications increase the risk for infections once they induce apoptosis in macrophages. In addition, we measured expression level of *TNFα, IL-6, IL-12, IL-23,* and *IFN-Y* in infected macrophages following 24 hours. We found that *TNFα, IL-6* and *IL-12* are also expressed significantly in MAP or *M. tuberculosis* infected macrophages, which shows that a high level of these cytokines in CD patients could be a result of MAP infection. Interestingly, MAP survival was induced significantly when exogenous rIL-6 was added to infected macrophages in a concentration-dependent manner. However, rIL-23 and IFN-Y had a similar effect to rTNFα, where they reduced MAP viability significantly with higher concentrations.

Newly emerging monoclonal antibodies indicated for CD treatment have shifted from targeting TNFα into more selective targets such as anti-IL-6 (PF-04236921), anti-IL-23 (AMG-139) and anti-IL-12/IL-23 (ustekinumab) [20]. Indeed, IL-6 is highly expressed in CD patients [33, 34, 35]. Therefore, blocking IL-6 pathway is anticipated to reduce the hyperactive immune response among CD patients. Moreover, our data suggests that IL-6 promotes MAP survival in infected macrophages. Thus, targeting this cytokine in specific will lead to decline in MAP viability, which could replace anti-TNFα treatment eventually. Additionally, in *M. tuberculosis* infected macrophages, IL-6 was found to inhibit IFN-Ɣ responsive genes at the transcriptional level selectively, which also results in inhibition of MHC class II induction [36]. Clinical studies are needed to offer a proof of principle for this new CD drug target with dual effect on MAP infection and inflammation.

Figures

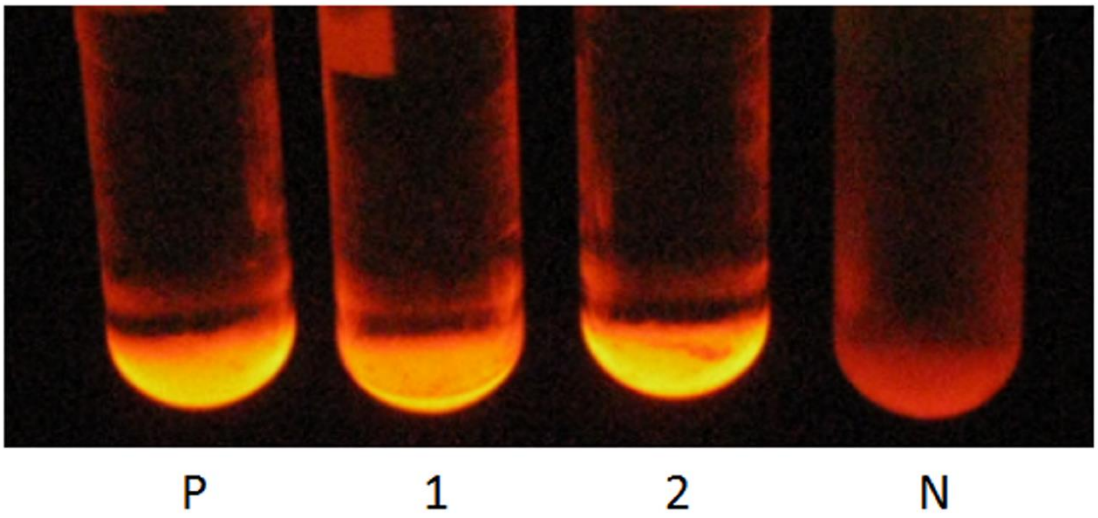

Figure 3: Direct bactericidal activity of non-PEGylated anti-TNFα (1) and PEGylated anti-TNFα (2) therapeutics against MAP growth in MGIT fluorescence system at 200ug/mL, following 14 days of incubation. (P) Indicates positive control (0ug/mL) and (N) is a negative control.

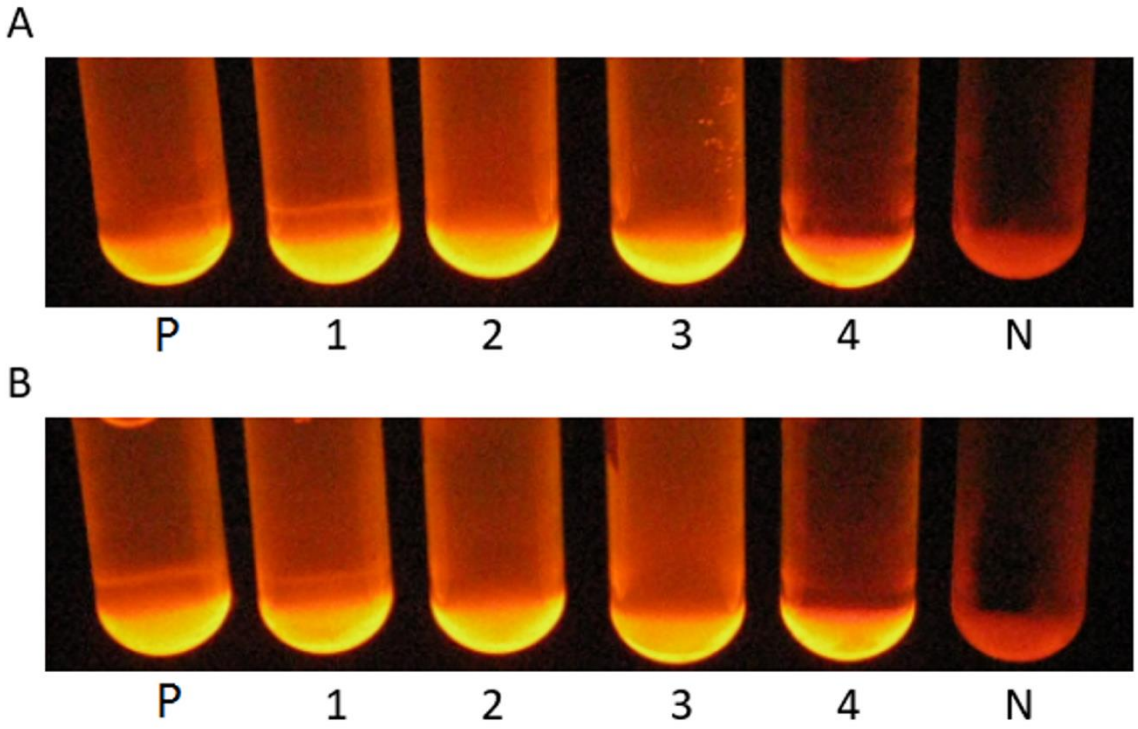

Figure 4: Direct bactericidal activity of non-PEGylated anti-TNFα (A) and PEGylated anti-TNFα (B) therapeutics against bacterial growth in MGIT fluorescence system at 200ug/mL, following 72 hours of incubation. (1): S. aureus, (2): Recombinant E. coli, (3): *K. pneumonia* and (4): *L. monocytogenes*. (P) Indicates positive control (0ug/mL) and (N) is a negative control.

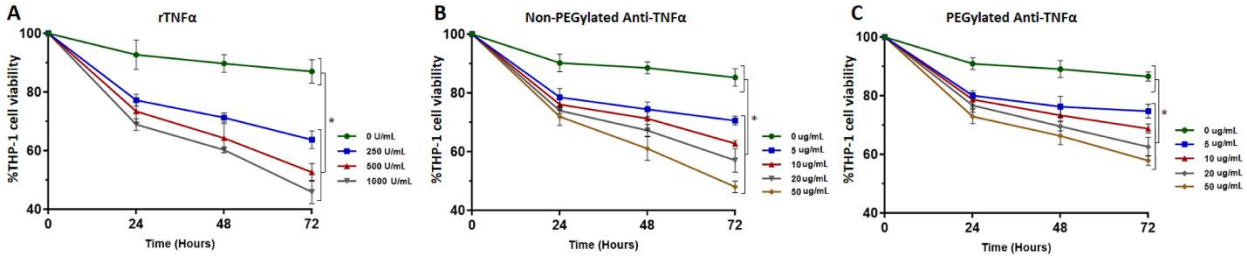

Figure 5: Cytotoxicity of rTNFα (A), non-PEGylated anti-TNFα (B) and PEGylated anti-TNFα (C) therapeutics determined by Trypan blue exclusion assay, following 24, 48 and 72 hours of incubation.

***P<0.05**

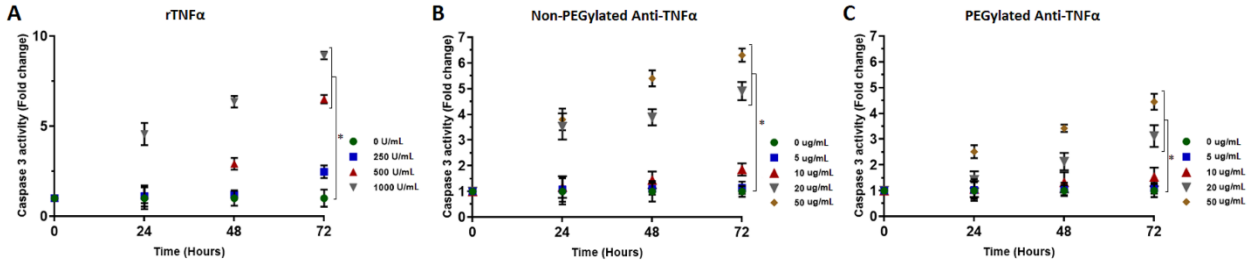

Figure 6: Cytotoxicity of rTNFα (A), non-PEGylated anti-TNFα (B) and PEGylated anti-TNFα (C) therapeutics determined by Caspase-3 activity assay, following 24, 48 and 72 hours of incubation.

***P<0.05**

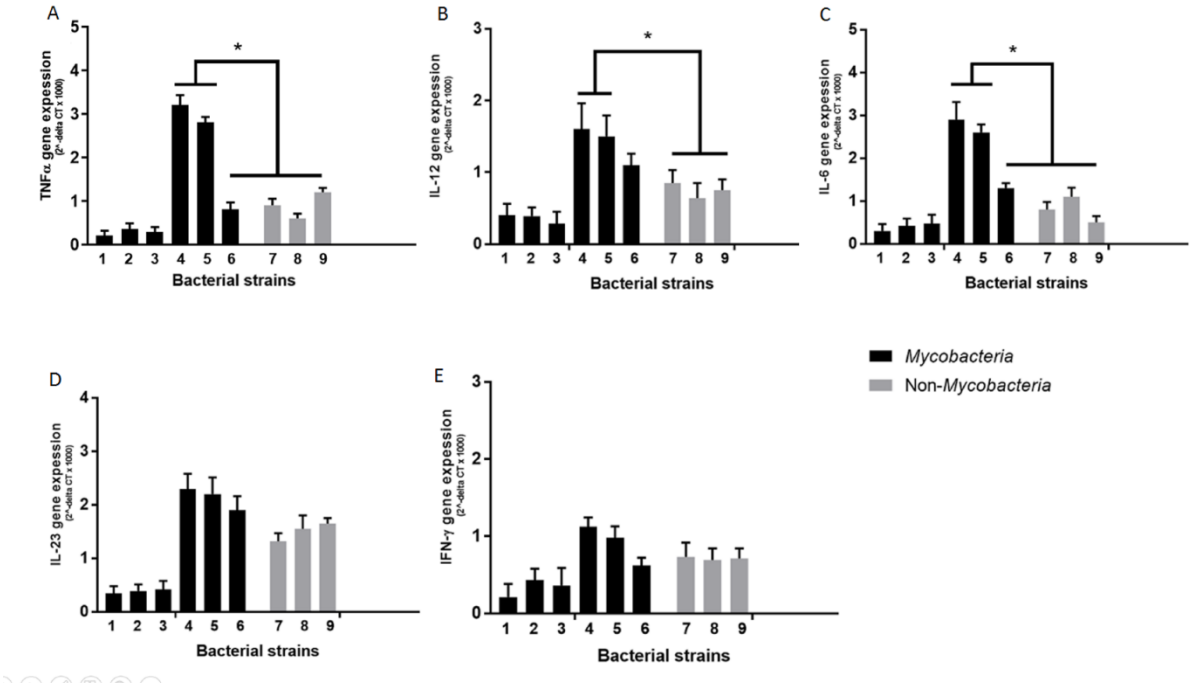

Figure 7: Expression of *TNFα* (A), *IL-6* (B), *IL-12* (C), *IL-23* (D) and *IFN-Y* (E) in monocyte-derived macrophages following 24 hours of bacterial infection. Bacterial strains presented in the X-axis are 1: Control (without infection), 2: *M. tuberculosis* (heat inactivated), 3: *MAP* UCF4 (heat inactivated), 4: *M. tuberculosis*, 5: *MAP* UCF4, 6: *M. smegmatis, 7:K. pneumonia*, 8: *L. monocytogenes,* 9: Recombinant *E. coli.*

***P<0.05**

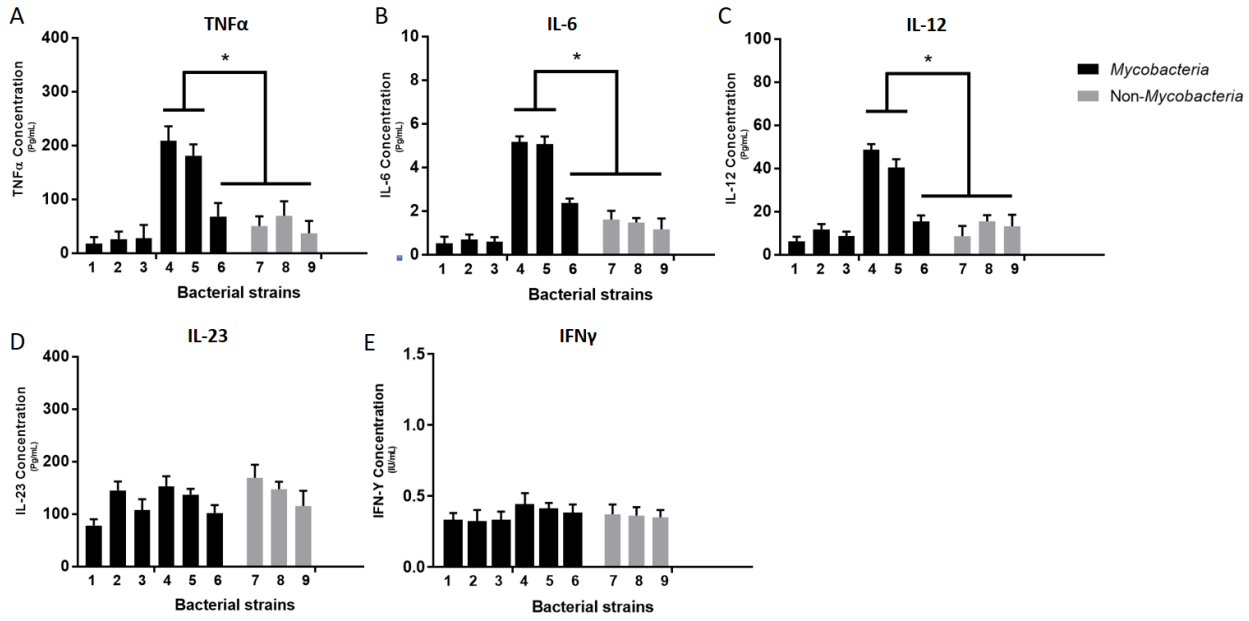

Figure 8: Level of TNFα (A), IL-6 (B), IL-12 (C), IL-23 (D) and IFN-γ (E) in monocyte-derived macrophages following 24 hours of bacterial infection. Bacterial strains presented in the X-axis are 1: Control (without infection), 2: *M. tuberculosis* (heat inactivated), 3: *MAP* UCF4 (heat inactivated), 4: *M. tuberculosis*, 5: *MAP* UCF4, 6: *M. smegmatis, 7:K. pneumonia*, 8: *L. monocytogenes*, 9: Recombinant *E. coli*.

***P<0.05**

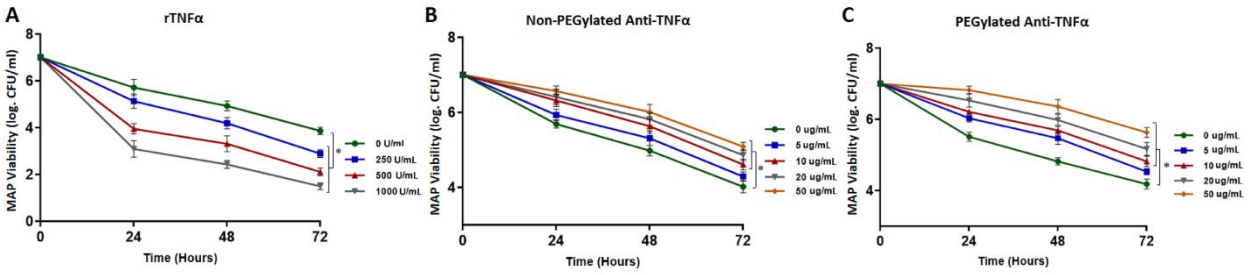

Figure 9: MAP viability in monocyte-derived infected macrophages pulsed with rTNFα (A), non-PEGylated anti-TNFα (B) and PEGylated anti-TNFα (C) therapeutics, following 24, 48 and 72 hours of infection.

***P<0.05**

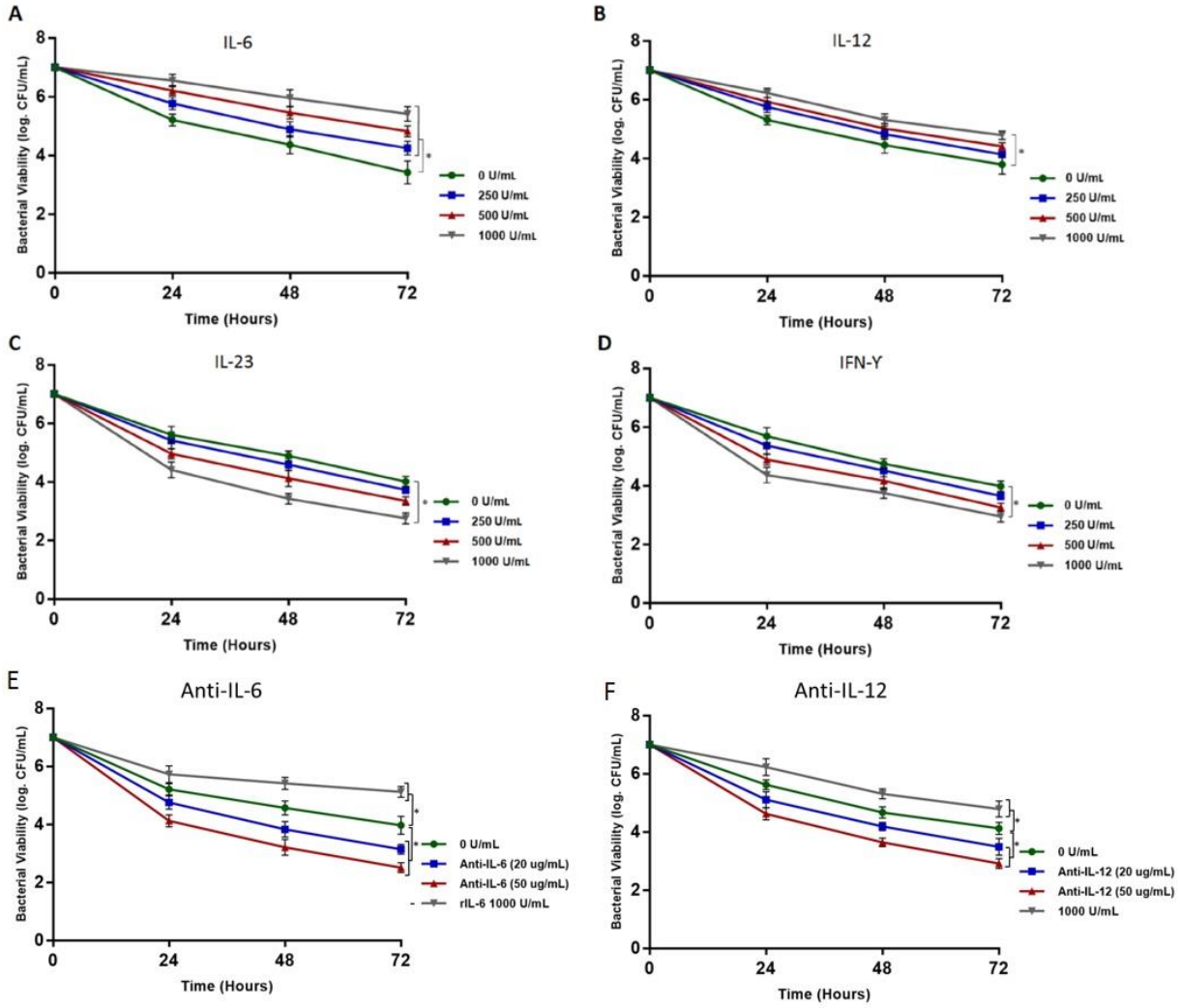

Figure 10: MAP viability in monocyte-derived infected macrophages pulsed with IL-6 (A), IL-12 (B), IL-23 (C), IFN-Y (D), anti-IL-6 and anti-IL12 following 24, 48 and 72 hours of infection.

***P<0.05**

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CHAPTER THREE: GENETIC POLYMORPHISMS IN TUMOR NECROSIS FACTOR RECEPTORS (TNFRSF1A/1B) ILLUSTRATE DIFFERENTIAL TREATMENT RESPONSE TO TNFα INHIBITORS IN PATIENTS WITH CROHN'S DISEASE

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Introduction

Tumor necrosis factor-α (TNFα) is a pro-inflammatory cytokine that has been found to be dysregulated in numerous inflammatory disorders including rheumatoid arthritis (RA), psoriasis and Crohn's disease (CD) [1, 2]. It is secreted primarily by macrophages, but it can also be produced by lymphocytes, natural killer cells and mast cells [3]. In order to exert its biological activity, TNFα binds to two different cell-surface receptors; tumor necrosis factor-α receptor superfamily 1A (*TNFRSF1A*), which is expressed in most tissues, and tumor necrosis factor-α receptor superfamily 1B (*TNFRSF1B*), which is typically found in immune cells [4].

Targeting TNFα by monoclonal antibodies (Anti-TNFα) such as adalimumab (Humira®) and infliximab (Remicade®) has shown that blocking this cytokine signaling pathway may control the symptoms of hyperactive immune response in CD initially [5]. However, there is a significant variable response to anti-TNFα therapeutics among patients receiving this treatment [6]. Additionally, clinical observations have reported that 10-30% of inflammatory bowel disease (IBD) patients, have no initial response to anti-TNFα treatment, and over 50% of the initial responders have lost response to treatment over time [7].

Clinical studies have shown that anti-TNFα therapeutics have many deleterious side effects, such as malignancy, neurologic disorders, heart failure and more importantly multiple infections [8]. Since TNFα plays a critical role in the immune defense against infections, it was unsurprising to notice that TNFα-deficient animal models are more susceptible to develop mycobacterial infections compared to wild-type controls [9]. On the other hand, patients receiving anti-TNFα treatment are at higher risk for meningitis,
sepsis, histoplasmosis, and pneumonia [10, 11, 12]. Moreover, the risk for tuberculosis development has substantially increased in patients receiving anti-TNFα, which might raise a question about their effect on *Mycobacterium avium* subspecies *paratuberculosis* (MAP) infection in a subset of CD patients [13, 14, 15,16]. Thus, prescribing anti-TNFα to CD patients without considering MAP infection could worsen disease condition eventually.
Genetic polymorphisms are not only associated with the overall risk of developing an inflammatory disorder, but they also play a role in the treatment outcome. For instance, polymorphisms in *TNFRSF1A* and *TNFRSF1B* have been shown to affect anti-TNFα treatment response significantly among CD patients [17, 18, 19]. However, the mechanism of those effects remains unknown. Predicting the efficacy of anti-TNFα treatment based on patient's genetics and MAP infection status would be more effective and beneficial to patients by reducing the risk for adverse effects and treatment cost.
In this study, we investigated the frequency of single nucleotide polymorphisms (SNPs) in *TNFα, TNFRSF1A* and *TNFRSF1B* among CD patients in comparison to healthy controls, in addition to their effect on gene expression and susceptibility to MAP intracellular infection. Finally, we further linked the effect of SNPs in these genes and presence of MAP to anti-TNFα treatment response in CD patients.

Materials and Methods

Clinical Samples:

Peripheral blood from a total of 104 subjects (54 CD patients and 50 healthy controls) were included in his study. All participants provided written informed consent prior to enrollment, and the study was approved by the University of Central Florida Institutional Review Board #IRB00001138.

Each subject provided two 4.0-mL K_2-EDTA coded blood tubes, where one tube was processed for detection of MAP infection, and the second tube was processed for gene expression analysis and genotyping of nine SNPs in *TNFα, TNFRSF1A* and *TNFRSF1B.* There was no significant difference in average age or gender between the two groups (CD vs healthy controls). All subject demographics are listed in Table 3.

Extraction of DNA and Detection of MAP by IS900 Nested PCR:

Purified white blood cells separated from blood samples were cultured in BD Bactec™ MGIT™

Para-TB medium for 6 months of incubation at 37 °C. Mycobacterial growth was measured initially using the UV illuminator and 1.0 mL was used for DNA extraction and nested PCR (nPCR) analysis as described earlier [15, 20]. Briefly, MAP infection was detected using *MAP-specific IS900* derived oligonucleotide primers. Round one of amplification was performed using P90 (GTTCGGGGCCGTCGCTTAGG) and P91 (GAGGTCGATCGCCCACGTGA) primers following these conditions conditions: 95 °C for 5 min, then 34 cycles of 95 °C for 1 min, 58 °C

for 1.5 min, 72 ℃ for 1.5 min. Final extension of 10 min at 72 °C. The amplified product from this round was 398 bp.

A second round of amplification involved using AV1 (ATGTGGTTGCTGTGTTGGATGG) and AV2 (CCGCCGCAATCAACTCCAG) primers, which was performed following these conditions: 95 °C for 5 min, then 34 cycles of 95 °C for 1 min, 58 °C for 1.5 min, 72 °C for 1.5 min. Final extension of 10 min at 72 °C. The final product following this round was 298 bp, which was identified on 2% agarose gel.

Identification of SNPs in TNFα, TNFRSF1A and TNFRSF1B Genes:

Genomic DNA was purified from peripheral blood leukocytes using QIAamp® DNA Blood Mini Kit (Qiagen™) following manufacturer's instructions. TaqMan™ genotyping assays (Applied Biosystems™) for *TNFα* (rs1800629, rs1799964, and rs1799724), *TNFRSF1A* (rs4149584, rs767455, and rs4149570) and *TNFRSF1B* (rs1061624, rs1061622, and rs3397) were performed on isolated DNA samples following manufacturer protocol at the University of Florida Pharmacotherapy and Translational Research Department (Gainesville, FL). Briefly, the reaction mixture had 2x TaqMan™ master mix and 20x assay working stock (primers with VIC and FAM fluorophore attachment). Isolated DNA samples along with reaction mixture and controls were added into a 384-well microtiter plate, following RT-PCR assay (one cycle at 95°C for 10 minutes, 92°C for 15 seconds and 50 cycles at 58°C for 1 minute) using Applied Biosystems™ QuantStudio™ RT-PCR System. The plate was analyzed for VIC and FAM fluorophores for at 551 nm and 517 nm, respectively. Fluorescence of VIC or FAM alone determined that the sample had allele 1 or allele 2, while VIC and FAM together determined that the sample was

heterozygous for each allele. Gene mutation, location and phenotype for SNPs used in this study are summarized in Table 4 [21-27].

Gene Expression of TNFα, TNFRSF1A, and TNFRSF1B by RT-PCR:

RNA was isolated from 1.0 mL of whole blood samples, and used for cDNA synthesis in order to analyze gene expression of *TNFα, TNFRSF1A,* and *TNFRSF1B* via RT-PCR. Briefly, 1.0 mL of blood was transferred into 2.0 mL RNA-ase free microcentrifuge tube, and then centrifuged at 1000 rpm for 5 minutes. Pellets containing leukocytes were collected and suspended in 1.0 mL of TRIzol® reagent (Invitrogen) for 15 minutes of gentle shaking. A total volume of 0.2 mL of chloroform was added to each tube, and the mixture was then incubated at room temperature for 3 min. All tubes were then centrifuged at 11,400 RPMs for 15 minutes at 4°C. The upper aqueous colorless phase containing RNA was transferred into a new 2.0 mL RNA-ase free microcentrifuge tubes. A total volume of 0.5 mL of 100% isopropanol was then added following 10 minutes of incubation at room temperature. Each tube was then centrifuged at 11,400 RPMs for 10 minutes at 4°C. A total volume of 1.0 mL of 75% ethanol was used to wash RNA pellets and then centrifuged at 8,700 RPMs for 5 minutes at 4°C. RNA pellets were air-dried for 15-30 min, suspended in 20 ul of RNase free H_2O, and finally heated at 60°C for 10 minutes.

For cDNA synthesis, 600 ng of RNA from each sample was added to 0.25 mL tubes containing 0.2 mL of PCR reaction, 4 uL of iScript™ Reverse Transcription (Bio-Rad®), and up to 20 uL RNase free H_2O. All tubes were then transferred to thermal cycler (MyGene™ Series Pelteir Thermal Cycler) and ran for 5 minutes at 25°C, 20 min at 46°C and 1 min at 95°C. The final concentration of cDNA synthesized for each sample was 30 ng/uL. A total volume of 1 uL of cDNA was mixed with 10 uL of Fast SYBR Green Mastermix (Thermofisher Scientific®), 1 uL

of either *TNFa, TNFRSF1A,* or *TNFRSF1B* PrimePCR SYBER Green Assay mix (Bio-Rad®) and 8 uL of molecular biological grade sterile H_2O in a 96-well microamp RT-PCR reaction plate. Controls of 18s RNA gene oligonucleotide primers (forward primer: 5'-GTA ACC CGT TGA ACC CCA TT-3'; reverse primer: 5'-CCA TCC AAT CGG TAG TAG CG-3') were used in order to obtain baseline CT readings. The 7500 Fast Real-Time PCR System (Applied Biosystems®) was used to perform the RT-PCR reaction. Relative mRNA expression levels were calculated using the equation ($2^{\wedge(-\Delta CT)} \times 1000$), where ΔCT = Sample RT-PCR CT reading - 18s CT baseline.

Statistical Analysis:

All statistical tests were performed using GraphPad Prism® 7.02. MAP infection incidence was used to estimate the power of this study. Since CD patients have MAP infection incidence proportion of nearly 40%, while in healthy controls MAP infection incidence proportion is about 10% [13, 14], at 90% power and alpha (Type I error) of 0.05, our calculated sample size was 84 samples (42 CD and 42 healthy controls). We also aimed for a similar number of Matsukura's et al. anti-TNFα treatment response study which included 81 participants [17]. For genotype frequency, we used two-tailed Z test and odd ratio analysis to compare between the presence of SNPs in CD patients vs. healthy controls. At each locus examined, SNP genotypes were subcategorized into 4 groups (major, heterozygous, homozygous and both heterozygous + homozygous), then tested for significance within each subcategory at $P<0.05$ and a 95% confidence interval (CI). For haplotype analysis, we used Fisher's exact test since we had a smaller number of samples. CD Patients were subcategorized into 4 haplotype groups and then tested for significance in between MAP infection groups. P-value of less than 0.05 was

considered significant. For gene expression analysis, first, we compared the average gene expression in CD vs. healthy control for each gene regardless of their genotype using unpaired two-tailed t-test at $P<0.05$ and a 95% confidence interval (CI), then we compared individuals who carried two major alleles with others for each SNP tested by using one-way ANOVA, where Newman-Keuls post test was selected for multiple comparisons. For MAP infection susceptibility, we compared infection proportions between SNP genotypes and major alleles in CD group and healthy controls separately using two-tailed Z test at $P<0.05$. Age and gender were not included as covariates as for all data sets no age or gender effects were observed.

Results

Frequency of SNPs in TNFα, TNFRSF1A, and TNFRSF1B among CD patients

We have assessed 104 subjects (54 CD patients and 50 healthy controls) for three SNPs in *TNFα* (rs1800629, rs1799964, and rs1799724), three SNPs in *TNFRSF1A* (rs4149584, rs767455, and rs4149570) and three SNPs in *TNFRSF1B* (rs1061624, rs1061622, and rs3397). Genotype distribution of these SNPs conveyed the Hardy-Weinberg equilibrium.

We identified one SNP in *TNFα* (*TNFα:rs1800629*) with a significant difference in frequency in CD patients compared to healthy controls ($P<0.05$). The *TNFα:rs1800629* GA genotype was found in 20/54 CD patients (37%) compared to 7/50 healthy controls (14%) [OR = 3.6, 95% CI: 1.37-9.54]. As shown in Table 5, the other two SNPs (*TNFα:rs1799964* and *TNFα:rs1799724*) have shown no significance among CD patients compared to healthy controls ($P>0.05$). Similarly, there was a significant difference in frequency of one SNP in *TNFRSF1A* (*TNFRSF1A:rs767455*) among CD patients compared to healthy controls ($P<0.05$). The

TNFRSF1A:rs767455 GG genotype was found in 15/54 CD patients (28%), while it was only found in 2/50 healthy controls (4%) [OR = 9.2, 95% CI: 1.98-42.83]. The other two SNPs (*TNFRSF1A:rs4149584* and *TNFRSF1A:rs4149570*) have shown no significance (P>0.05).

Additionally, two SNPs in *TNFRSF1B* (*TNFRSF1B:rs1061624* and *TNFRSF1B:rs3397*) were found to be significant in CD patients compared to healthy controls (P<0.05). The *TNFRSF1B:rs1061624* AG genotype was found in 27/54 CD patients (50%) compared to 15/50 healthy controls (30%) [OR = 2.3, 95% CI: 1.04-5.22]. The *TNFRSF1B:rs3397* CT genotype was found in 31/54 CD patients (57%) compared to (15/50) healthy controls (30%) [OR = 3.1, 95% CI: 1.40-7.07]. Besides, the *TNFRSF1B:rs3397* TT genotype was also significantly found in 15/54 CD patients (28%) compared to (4/50) healthy controls (8%) [OR = 4.4, 95% CI: 1.36-14.14]. However, *TNFRSF1B:rs1061622* was not found to be significant among CD patients in comparison to healthy controls (P>0.05). A complete list of SNPs and genotyping frequency is shown in Table 5.

SNPs Downregulates TNFRSF1A and TNFRSF1B Gene Expression in CD

We quantified relative gene expression level of *TNFα, TNFRSF1A,* and *TNFRSF1B* in all 104 study participants. Then, we further analyzed the data according to each SNP present, in order to find out if SNPs are associated with gene expression level. In general, the relative expression of *TNFα* was more than three times higher in CD patients (2.44 ± 0.30), in comparison to healthy subjects (0.72 ± 0.20), regardless of SNPs involved (P<0.5). However, none of *TNFα* SNPs (rs1800629, rs1799964, and rs1799724) was associated with gene expression level significantly (P >0.05) [Figure 11A, 10B, and 11C].

Overall, the expression level of *TNFRSF1A* and *TNFRSF1B* was significantly downregulated in CD patients (0.38 ± 0.15; P<0.5 and 0.34 ± 0.13; P<0.05, respectively), compared to healthy subjects (0.79 ± 0.24 and 0.66 ± 0.17, respectively) [Figure 11D - 10I]. Nevertheless, CD patients with the SNP *TNFRSF1A:rs767455* who had GG genotype, had a significantly lower relative gene expression level compared to patients without SNP who had AA genotype (0.26 ± 0.09 vs 0.49 ± 0.12; P<0.5) [Figure 11E].

Similarly, the expression level of *TNFRSF1B* was significantly lower in CD patients with the SNP *TNFRSF1B:rs3397* who had either CT (0.32 ± 0.11) or TT (0.2 ± 0.09) genotype, compared to patients without SNP (0.59 ± 0.10) who had CC genotype (P<0.5) [Figure 11I].

SNPs in TNFRSF1A and TNFRSF1B Induce Susceptibility to MAP Infection

We evaluated MAP infection status in all 104 study participants by *IS900* nPCR analysis, which is very sensitive and specific for MAP. Overall, 31/54 CD patients were infected with MAP compared to only 4/50 healthy control [OR = 15.5, 95% CI: 4.88-49.22, P<0.05].

We further looked for any association between a specific genotype in all SNPs we tested and the presence of MAP infection. Interestingly, CD patients with the SNP *TNFRSF1A:rs767455* who had either AG or GG genotype were more susceptible to MAP infection (63% and 66.6%, respectively), while CD patients without SNP (AA genotype) were 41% infected with MAP. However, this result was not statistically significant (P>0.05) [Figure 12A]. On the other hand, CD patients with the SNP *TNFRSF1B:rs3397* who had either CT or TT genotype were significantly susceptible to MAP infection (58% and 70.5%, respectively), compared to CD patients without SNP (CC genotype), who were only 17% infected with MAP (P<0.05) [Figure

12B]. None of the additional SNPs we tested (rs1800629, rs1799964, and rs1799724 of *TNFα,* rs4149584 and rs4149570 of *TNFRSF1A,* rs1061624 and rs1061624 of *TNFRSF1B*) were found to be significantly associated with higher susceptibility to MAP infection.

SNP Haplotypes Distribution and MAP Infection Susceptibility

After we have identified *TNFRSF1A:rs767455* and *TNFRSF1B:rs3397* as SNPs associated with lower gene expression and higher MAP infection susceptibility in CD patients, we further performed haplotype analysis of these two SNPs. The haplotype frequencies among CD patients and healthy controls showed a significant difference for the G – T haplotype ($P<0.05$), which was found in 25/54 CD patients (46%), while in healthy controls it was 7/50 (14%) [Figure 13]. Consequently, MAP infection was significantly higher among CD patients who had the G – T haplotype (17/25), which contributes to 31% of CD patients overall, while none of the healthy controls had the G – T haplotype and MAP infection at the same time. As described in Table 6, none of the other SNP haplotypes inferred from *TNFRSF1A* and *TNFRSF1B* (A – C, G – C and A – T) were found to be associated with susceptibility to MAP infection significantly ($P>0.05$).

Discussion

Increased level of TNFα has been found in the inflamed intestinal mucosa of CD patients, which is considered an essential mediator of immunologic response for this inflammatory disease [28]. Consequently, TNFα plays a role in increasing intestinal permeability, granuloma formation and coagulation pathways [3]. Most recently, we elucidated the relationship between MAP infection and the upregulation of TNFα expression *in vitro*, which has shown that both MAP and *M. tuberculosis* induce gene expression significantly in comparison to other mycobacteria and non-

mycobacteria strains [29]. Although inhibition of TNFα has shown a positive clinical outcome in some CD patients, poor response was also found in others, which leaves those patients with severe side effects and higher susceptibility to infections [13]. Therefore, several pharmacogenetic studies have evaluated the variation of anti-TNFα treatment response among CD patients in order to predict treatment response ultimately [17, 18, 19].

Genetic polymorphisms in *TNFRSF1A* and *TNFRSF1B* were found to be correlated with anti-TNFα treatment response in CD patients. Specifically, the *TNFRSF1A:rs767455* AG and GG genotype has a significant difference in frequency among non-responders to anti-TNFα treatment compared to the majority of drug responders who had the AA genotype [16]. Similarly, the *TNFRSF1B:rs3397* CT and TT also have a significant difference in frequency among anti-TNFα non-responders in comparison to CD patients who were classified as drug responders (CC genotype) [18, 30]. However, these observations were limited to drug response only, while the molecular effects of these SNPs were not discussed.

Since MAP is a suspected microbial causative agent of CD [14, 15, 31, 32], it was worthy to find out if genetic polymorphisms are inducing susceptibility to MAP infection in CD patients, which will ultimately influence anti-TNFα treatment outcome. Therefore, we tested 104 subjects (54 CD patients and 50 healthy controls) for selective SNPs in *TNFα* (rs1800629, rs1799964, and rs1799724), *TNFRSF1A* (rs4149584, rs767455, and rs4149570) and *TNFRSF1B* (rs1061624, rs1061622, and rs3397). First, four out of these nine SNPs were found to have a significant difference in frequency among CD patients compared to healthy controls (*TNFα:rs1800629, TNFRSF1A:rs767455, TNFRSF1B:rs1061624 and TNFRSF1B:rs3397*), which also correlates with previous reports [33, 34, 35]. Secondly, we quantified gene expression level of *TNFα,*

TNFRSF1A and *TNFRSF1B* in all study participants. We found that *TNF*α relative expression level is about 3.4 folds higher in CD patients compared to healthy controls overall. However, none of *TNFα*-associated SNPs had a significant association with *TNF*α gene expression level. The expression of *TNFRSF1A* and *TNFRSF1B* was significantly downregulated (~ 2 folds) in CD patients compared to healthy controls regardless of SNPs involved. Furthermore, the SNPs *TNFRSF1A:rs767455* and *TNFRSF1B:rs3397* were both found to be associated with a significant lower gene expression. Additionally, MAP infection was predominantly found among CD patients in comparison to healthy controls (57% vs 8%, respectively). Interestingly, MAP infection was also associated with the SNPs *TNFRSF1A:rs767455* and *TNFRSF1B:rs3397*. Furthermore, our SNP haplotype analysis of *TNFRSF1A:rs767455* and *TNFRSF1B:rs3397* indicates that the G – T haplotype has a significant difference in frequency among CD patients (46%) and MAP infection susceptibility is also associated with this specific haplotype (31%).

It is relevant to compare between MAP and *M. tuberculosis* infection since mycobacterial protein tyrosine phosphatase (PtpA) in MAP shares 90% homology to PtpA in M. tuberculosis [36]. This protein inhibits phagosome–lysosome fusion in the macrophage by dephosphorylating the host vacuolar protein sorting 33B (VPS33B). Consequently, the pathogen will be able to avoid containment eradication and it establishes a successful intracellular infection by preventing fusion of lysosome and phagosome into the phagolysosomal complex [37, 38]. Therefore, the primary mechanism for MAP eradication is by inducing apoptosis of the infected macrophage through TNFα dependent mechanism [39, 40]. This is a critical point when we consider anti-TNFα treatment for CD patients who are infected with MAP. In addition to genetic testing of selective SNPs we discussed, MAP infection screening method should be implemented before initiation of therapy. Finally, in figure 14 we suggest following a treatment algorithm for CD,

based on patient's haplotype inferred from *TNFRSF1A* rs767455 *(A/G)* and *TNFRSF1B* rs3397 *(C/T)*.

Figures

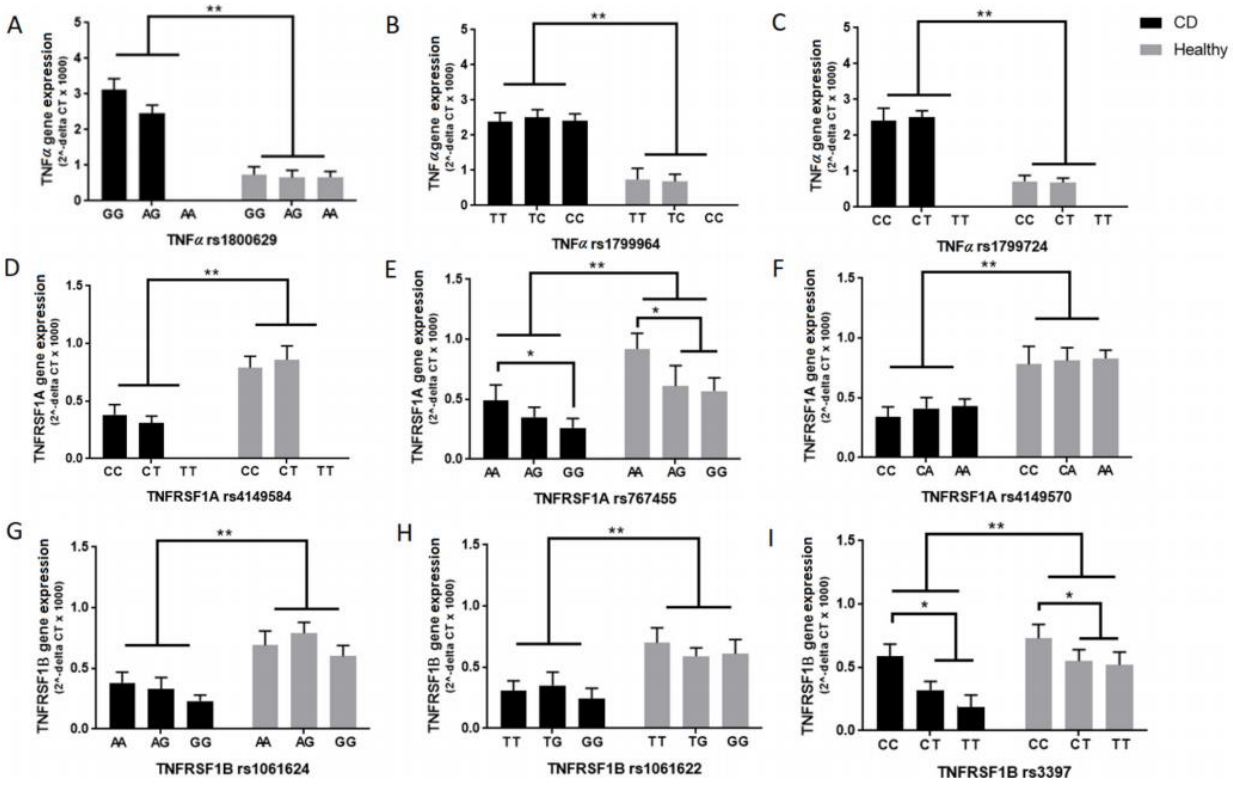

Figure 11: Gene expression level of *TNFα, TNFRSF1A* and *TNFRSF1B* according to each allele type in selected SNPs (A – I) among CD patients (*N = 54)* and healthy controls (*N = 50)*. Unpaired two-tailed t-test at P<0.05 and a 95% confidence interval (CI) was used to test gene expression significance in CD patients vs healthy controls, then one-way ANOVA, where Newman-Keuls post test was selected for multiple comparisons was used to test individuals who carried two major alleles with others for each SNP.

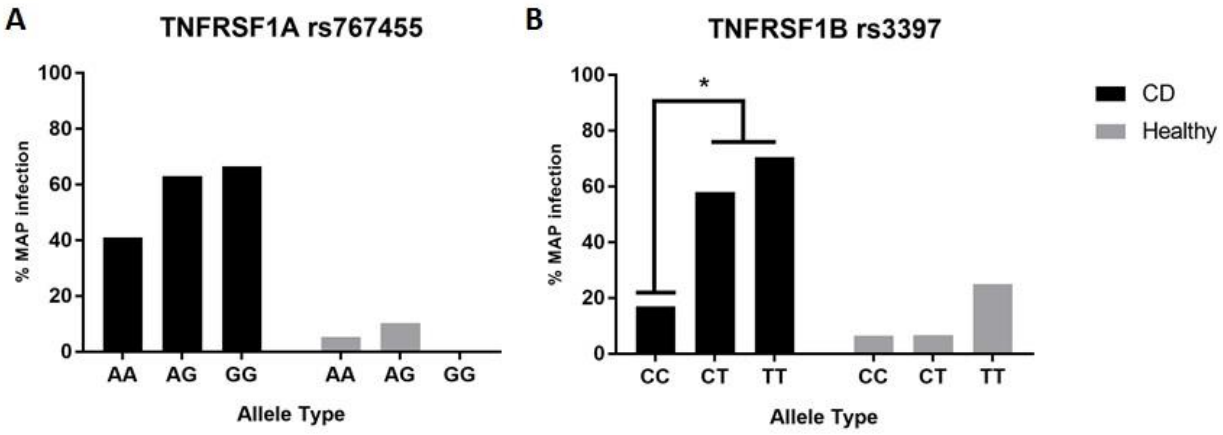

Figure 12: Influence of *TNFRSF1A (*rs767455) and *TNFRSF1B* (rs3397) SNPs on MAP infection susceptibility in CD patients (*N* = 54) and healthy subjects (*N=50*). Infection proportions were compared between SNP genotypes and major alleles in CD patients and healthy controls separately using two-tailed Z test at P<0.05.

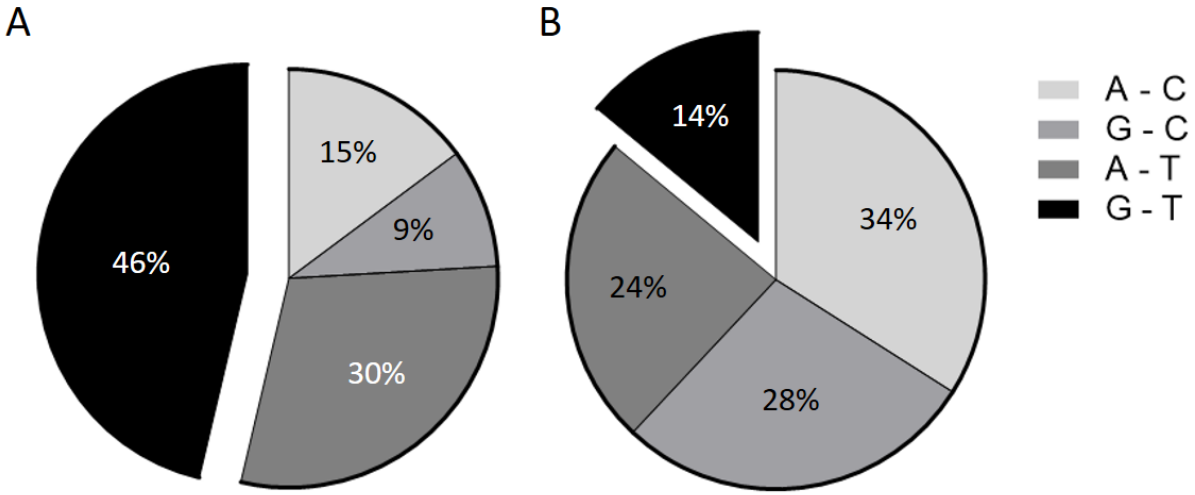

Figure 13: Haplotypes inferred from rs767455 (A/G) and rs3397 (C/T), and their distributions in CD patients (A) and healthy subjects (B).

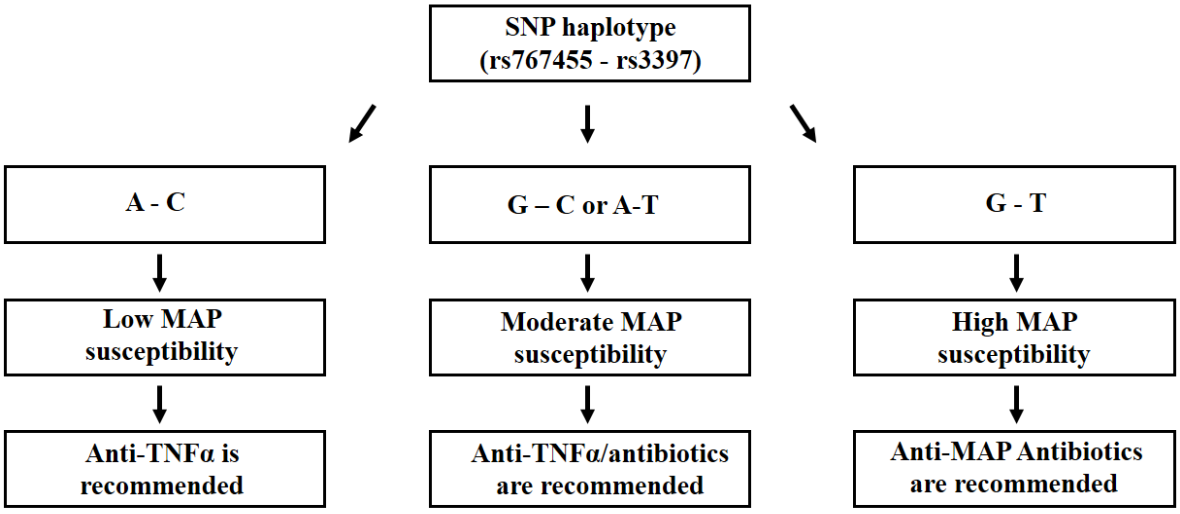

Figure 14: Recommended CD treatment algorithm based on haplotypes inferred from *TNFRSF1A* rs767455 *(A/G)* - *TNFRSF1B* rs3397 *(C/T)*.

Tables

Table 3: Demographics of Study Participants.

Diagnosis	**Number**	**Age Range**	**Average Age**	**Gender Ratio (Male : Female %)**
All Subjects	104	20 - 66	35	53:47
Crohn's Disease	54	21 - 66	39	48:52
Healthy Controls	50	20 - 63	31	58:42

Table 4: Gene mutations, locations and mutation phenotypes of SNPs selected for this study.

Gene	Reference SNP	Gene Mutation*	Location and AA Change*	Mutation Phenotype	Reference
TNF	rs1800629	G→A	Promoter	Higher susceptibility to CD	22
	rs1799964	T→C	Promoter	Associated with IBD in general	23
	rs1799724	C→T	Promoter	Linked to ankylosing spondylitis	24
TNFRSF1A	rs4149584	C→T	Exon 4 (R→Q)	Higher susceptibility to MS	25
	rs767455	A→G	Exon 1: No AA change	Used to predict anti-TNFα response in CD	17
	rs4149570	G→T	320bp upstream of gene	Used to predict anti-TNFα response in RA	26
TNFRSF1B	rs1061622	T→G	Exon 6 (M→R)	Higher susceptibility to IBD	27
	rs1061624	G→A	Exon 10	Higher susceptibility to IBD	27
	rs3397	C→T	Exon 10	Used to predict anti-TNFα response in CD	18

* Gene mutation and location data were obtained from the National Center for Biotechnology Information (NCBI) [21]

Table 5: Genotype frequencies of selected SNPs for CD patients and healthy controls.

Genotype	**CD Patients (*N = 54)***	**Healthy Controls *(N = 50)***	**P-value***	**OR**	**95% CI**
TNFα rs1800629					
GG (Reference allele)	34 (64%)	41 (82%)	0.03		
GA	20 (37%)	7 (14%)	0.01	3.6	1.37-9.54
AA	0 (0%)	2 (4%)	0.13	0.2	0.01-5.20
GA + AA	20 (37%)	9 (18%)	0.03	2.7	1.08-6.64
TNFα rs1799964					
TT (Reference allele)	33 (61%)	34 (68%)	0.75		
TC	18 (33%)	16 (32%)	0.89	1.2	0.51-2.64
CC	3 (6%)	0 (0%)	0.09	7.2	0.35-144.9
TC + CC	21 (39%)	16 (32%)	0.73	1.4	0.60-3.00
TNFα rs1799724					
CC (Reference allele)	45 (83%)	43 (86%)	0.70		
CT	9 (16%)	7 (14%)	0.38	1.2	0.42-3.59
TT	0 (0%)	0 (0%)	N/A	N/A	N/A
CT + TT	9 (16%)	7 (14%)	0.70	1.2	0.42-3.59

Genotype	**CD Patients (*N = 54)***	**Healthy Controls *(N = 50)***	**P-value***	**OR**	**95% CI**
TNFRSF1A rs4149584					
CC (Reference allele)	52 (96%)	49 (98%)	0.60		
CT	2 (4%)	1 (2%)	0.60	1.9	0.17-21.40
TT	0 (0%)	0 (0%)	N/A	N/A	N/A
CT + TT	2 (4%)	1 (0%)	0.60	1.9	0.17-21.40
TNFRSF1A rs767455					
AA (Reference allele)	17 (31%)	29 (58%)	0.01		
AG	22 (41%)	19 (38%)	0.29	2.0	0.83-4.65
GG	15 (28%)	2 (4%)	0.01	9.2	1.98-42.83
AG + GG	37 (68%)	21 (42%)	0.01	3.0	1.34-6.71
TNFRSF1A rs4149570					
GG (Reference allele)	25 (46%)	26 (52%)	0.56		
GT	13 (24%)	18 (36%)	0.18	0.75	0.31-1.84
TT	16 (30%)	7 (14%)	0.06	2.4	0.83-6.75
GT + TT	29(54%)	25(50%)	0.70	1.2	0.56-2.59

Genotype	**CD Patients (*N = 54)***	**Healthy Controls *(N = 50)***	**P-value***	**OR**	**95% CI**
TNFRSF1B rs1061624					
AA (Reference allele)	13 (24%)	27 (54%)	0.01		
AG	27 (50%)	15 (30%)	0.04	2.3	1.04-5.22
GG	14 (26%)	8 (16%)	0.02	3.6	1.21-10.83
AG + GG	41 (76%)	23 (46%)	0.01	3.7	1.61-8.53
TNFRSF1B rs1061622					
TT (Reference allele)	24 (44%)	31 (62%)	0.07		
TG	22 (41%)	16 (32%)	0.36	1.8	0.77-4.1
GG	8 (15%)	3 (6%)	0.14	3.44	0.82-14.4
TG + GG	30 (%)	19 (38%)	0.07	2.03	0.93-4.47
TNFRSF1B rs3397					
CC (Reference allele)	12 (22%)	31 (62%)	0.01		
CT	31 (57%)	15 (30%)	0.01	3.1	1.4-7.07
TT	15 (28%)	4 (8%)	0.01	4.4	1.36-14.14
CT + TT	46 (85%)	19 (38%)	0.01	6.25	2.66-14.69

Two-tailed Z test and odd ratio analysis were used to compare between the presence of SNPs in CD patients vs. healthy controls

*P-value of <0.05 was considered as significance threshold

Table 6: Haplotypes inferred from TNFRSF1A rs767455 (A/G) - TNFRSF1B rs3397 (C/T) and MAP infection distributions among CD patients.

Haplotype* **(rs767455 - rs3397)**	**CD MAP (+)** ***(N = 31)***	**CD MAP (-)** ***(N = 23)***	**Overall**	**P-Value**
A - C	2 (4%)	6 (11%)	8 (15%)	0.14
G - C	3 (5%)	2 (4%)	5 (9%)	0.64
A - T	9 (17%)	7 (13%)	16 (30%)	0.59
G - T	**17 (31%)**	**8 (15%)**	**25 (46%)**	**0.01**

Fisher's exact test was used to test group significance at $P<0.05$.

CD: Crohn's Disease

MAP: *Mycobacterium avium* subspecies *paratuberculosis*

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CHAPTER FOUR: HIGHER POTENCY AND BROADER SPECTRUM OF RHB-104 ANTI-MICROBIAL ACTIVITY IS OBSERVED IN ONE CAPSULE FORMULATION FOR EFFECTIVE TREATMENT OF CROHN'S DISEASE

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Introduction

The current treatment guidelines of Crohn's disease (CD) include immunosuppressants, anti-inflammatory drugs, nutritional therapy and antibiotics [1]. MAP was isolated from intestinal tissues, milk and blood samples from CD patients at a higher frequency than controls [2]. Other microorganisms have been associated with CD such as *L. monocytogenes, S. aureus, K. pneumoniae* and invasive *E.coli* [4]. CD patients treated with prolonged combination of macrolide-based antimycobacterial regimens in randomized clinical trials have achieved reversal of CD symptoms [5]. We have recently evaluated the effects of an investigational triple antibiotics regimen known as RHB-104 (by RedHill Biopharma) against several clinical MAP strains and other non-MAP mycobacterial strains *in vitro* [6]. Using BACTEC™ MGIT Para-TB medium, based on fluorescence quenching technology, our data demonstrated that lower concentrations of the triple combination of RHB-104 active ingredients provided synergistic anti-MAP growth activity compared to individual or dual combinations of the drugs. RHB-104 active ingredients are composed of 63.3% Clarithromycin (CLA), 6.7% Clofazimine (CLO) and 30% Rifabutin (RIF). However, in our earlier study we were unable to dissolve the capsule formulation in one compatible solution. Therefore, RHB-104 active ingredients solution was

prepared at a final concentration of 1 mg/mL by combining the 3 individually dissolved drugs at their respective percent composition in RHB-104.

Materials and Methods

RHB-104 active ingredients is composed of 63.3% Clarithromycin (CLA), 6.7% Clofazimine (CLO) and 30% Rifabutin (RIF). In our earlier study [1], we were unable to dissolve the drug formula in one compatible solution and therefore, RHB-104 active ingredients solution was prepared at a final concentration of 1 mg/mL by combining the 3 individually dissolved drugs at their respective percent composition in RHB-104. In this study, we successfully dissolved RHB-104 formulation in one solution (due to propriety ownership, RHB-104 solvent information can be requested directly from RedHill Biopharma). RHB-104 formulation solution was then evaluated against MAP and several other microorganisms. Using same methodology, Minimum inhibitory concentration (MIC) was determined for RHB-104 formulation and RHB-104 active ingredients solution [1].

Results

In this study, we successfully dissolved RHB-104 formulation in one solution (due to propriety ownership, RHB-104 solvent information can be requested directly from RedHill Biopharma). RHB-104 formulation solution was then evaluated against MAP and several other microorganisms. Using same methodology, Minimum inhibitory concentration (MIC) was determined for RHB-104 formulation and RHB-104 active ingredients solution The dissolving solution was tested against mycobacterial and other non-mycobacterial strains and it has shown no inhibitory effect on their growth at concentration as high as 12.5% (V/V).

The synergistic effects of RHB-104 capsule formula dissolved in one solution was more efficient against mycobacterial strains than adding the three active ingredients individually to the culture (Figure 15). This higher potency was observed against MAP strains and non-MAP mycobacteria strains. RHB-104 capsule formula has shown bactericidal effect at 2 ug/ml MIC level against MAP strain *MS 137* isolated from intestinal tissues of CD patients, compared to 4ug/ml of active ingredients added individually. Similarly, RHB-104 capsule formulation was bactericidal at lower MIC level than combining individual active ingredients against *M. avium JF7* isolated from the blood of HIV patient, *M. fortuitum* subspecies *fortuitum, M. smegmatis* (ATCC 27199) and *M. tuberculosis* strain HR237. The activity RHB-104 capsule formula against mycobacterial strains in comparison to its individual active ingredients in details is shown in Table 7. Additionally, we were interested to test the effect of RHB-104 against the other microbial causative agents of CD. The drug was effective against *S. aureus* (ATCC 25923) and *L. monocytogenes* (ATCC 19112) at MIC level of 0.125 and 0.25 ug/ml, respectively. Since CLA composes about 63% of RHB-104 we tested it individually against those bacterial strains. The MIC of CLA alone was 1.5 ug/ml for both *S. aureus* and *L. monocytogenes*. However, RHB-104 had no bactericidal effects against *E. coli* (ATCC 8739) and *K. pneumonia* (ATCC 13883) at concentrations as high as 40ug/ml.

Conclusion

These additional data support our original study and confirms that RHB-104 should be effective as a first-line treatment for CD associated with MAP, *L. monocytogenes* or *S. aureus.* This new

regimen is given in one capsule formulation with lower concentration of 3 drugs instead of giving them individually which increases patient's compliance and minimizes side effects.

Figures

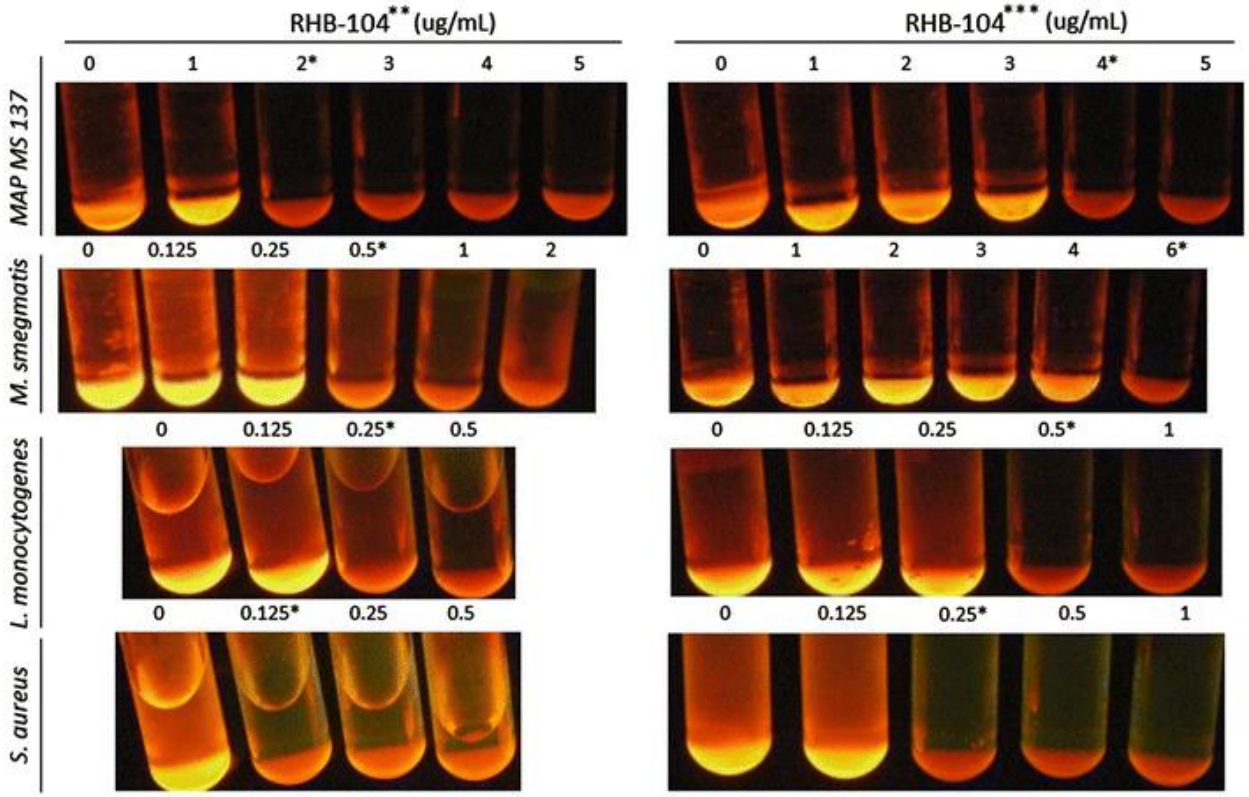

Figure 15: Comparing the bactericidal activity of RHB-104 capsule formulation to RHB-104 active ingredients added individually against: *MAP MS 137, M. smegmatis, L. monocytogenes* and *S. aureus* at MIC level.

***MIC level**
****RHB-104 capsule formulation dissolved in one compatible solution (1mg/ml).**
*****RHB-104 active ingredients added into the culture individually; CLA was dissolved in a solution made of sodium acetate in water (pH 5.0) at 1mg/ml, CLO (1mg/ml) was prepared by using hydrochloric acid and sodium dodecyl sulfate in water, RIF (1mg/ml) was prepared by using absolute ethanol.**

Tables

Table 7: MIC of the proprietary RHB-104 capsule formulation and RHB-104 analog

Microorganism	**Minimum inhibitory concentration (µg/mL)**	
	CLA-CLO-RIF (RHB-104 analog)[a]	**Proprietary RHB-104 capsule formulation[b]**
MAP MS 137	4.0	2.0
M. avium—JF7	4.0	2.0
M. fortuitum ss fortuitum	15	10
M. tuberculosis HR237	10	5.0
M. smegmatis ATCC 27199	6.0	0.125
S. aureus ATCC 25923	0.25	0.125
L. monocytogenes ATCC 19112	0.5	0.25
K. pneumonia ATCC 13883	>40	>40
Recombinant *E. coli*	>40	>40

All cultures were performed in duplicates and have been repeated three times.

[a]RHB-104 analog where the 3 drugs dissolved individually were combined in one solution at their proprietary RHB-104 capsule formulation composition percentage.

[b]Proprietary RHB-104 capsule formulation was dissolved in one compatible solvent.

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CHAPTER FIVE: CONCLUSION/FUTURE DIRECTIONS

Numerous factors are involved in CD pathogenesis including microbial infection and genetic susceptibility. We support the hypothesis that dysregulated immune response against MAP in genetically susceptible individuals lead to disease development. However, current treatment guidelines do not initially consider antimycobacterial therapy. Usually, therapy consists of anti-inflammatories, immunomodulators and biologics, which may significantly impair macrophages and formation of granulomas, which are essential for isolating and restricting microbial infection. Therefore, an alternative combined therapy should be considered for long-term remission and possibly a cure from this deleterious disease. Combining antibiotics in addition to targeted immunotherapy is a better approach to block source and signs of inflammation.

Here, we identified the detrimental activity of recombinant TNFα, IL-6, IL-12, IL-23 and IFN-Ɣ on MAP infection *in vitro.* Our data shows that anti-TNFα therapeutics do not have any direct bactericidal effects against MAP or other non-mycobacterial strains at supratherapeutic concentrations. However, these medications increased MAP survival in infected macrophages in a concentration-dependent manner, indicating that CD patients receiving such treatment are at a higher risk for MAP infection. On the other hand, MAP viability declined in infected macrophages pulsed with exogenous rTNFα in a concentration-dependent manner, which shows that TNFα plays a vital role in protection against MAP infection.

Furthermore, we found that gene expression level of *TNFα*, *IL-6* and *IL-12* are also increased significantly in MAP or *M. tuberculosis* infected macrophages, which indicates that a high level of these cytokines in CD patients could be a result of MAP infection. Interestingly, MAP survival was increased significantly when exogenous rIL-6 was added to infected macrophages

in a concentration-dependent manner. However, rIL-23 and IFN-Y had a similar effect to rTNFα, where they reduced MAP viability significantly with higher concentrations.

There is a significant variable response among CD patients receiving anti-TNFα, where about 10-30% of IBD patients have no initial response to anti-TNFα treatment. However, Over 50% of the anti-TNFα initial responders lose response to treatment over time. Additionally, genetic polymorphisms in *TNFRSF1A* and *TNFRSF1B* have been shown to affect anti-TNFα treatment response significantly among CD patients. Moreover, the risk for tuberculosis infection has substantially increased in patients receiving anti-TNFα.

The SNPs *TNFRSF1A:rs767455* and *TNFRSF1B:rs3397* were both found to be associated with decreased expression of their corresponding genes, while MAP infection susceptibility was correlated with the SNPs *TNFRSF1A:rs767455* and *TNFRSF1B:rs3397*. Our SNP haplotype analysis of *TNFRSF1A:rs767455* and *TNFRSF1B:rs3397* points out that the G – T haplotype has a significant difference in frequency among CD patients, and MAP infection is correlated with this specific haplotype. Genetic testing for *TNFRSF1A:rs767455* and *TNFRSF1B:rs3397*, in addition to MAP infection screening should be implemented before anti-TNFα treatment initiation in CD for more effective clinical outcome.

In the future, we would like to investigate the relationship between *IL-6, Notch-1, STAT-3* and MAP infection in CD. Cytokines are essential mediators linking inflammatory response in various disease conditions. Since IL-6 was highly upregulated in MAP infected macrophages, it could serve as a potential therapeutic target as well as a prognostic factor. Mainly, IL-6 plays a significant role in inducing acute phase proteins such as C-reactive protein (CRP) [1]. Recently, IL-6 was found to activate Th17 cells and Transforming Growth Factor (TGF-β) [2]. At the same

time IL-6 was also found to be inhibiting T regulatory cells [3]. Thus, IL-6 promotes pro-inflammatory environment.

In CD, IL-6 induces T-cell resistance in the intestinal mucosa and it also induces endothelial chemokines, which helps in recruiting more leukocytes [4]. The circulating levels of IL-6 in the intestinal mucosa is a major risk factor for colon cancer development [5]. Patients with advanced stage colorectal cancer have shown higher serum levels of IL-6 [6]. Although several studies have identified that IL-6 can modulate inflammation, the underlying mechanism remains unclear [7]. We are willing to focus on those mechanisms in our future studies and we would also like to test the role of TNFα inhibitors on *Notch-1*, *IL-6*, apoptosis and MAP infection *in vitro*, in addition to CD clinical samples.

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APPENDIX: CONSENTS FOR PUBLICATION

We, the authors, give our permission to include data and materials described in Qasem et. al. 2017 (below) in the dissertation contents of Mr. Ahmad Qasem for Doctor of Philosophy in Biomedical Sciences at the University of Central Florida.

Article Title: The alternate effects of anti-TNFα therapeutics and their role in mycobacterial granulomatous infection in Crohn's disease.

Authors: Ahmad Qasem, Abed Elrahman Naser and Saleh A. Naser

Journal: *Expert review of anti-infective therapy*, *15*(7), 637-643.

DOI: https://doi.org/10.1080/14787210.2017.1328276

Published: May 17th, 2017

We, the authors, give our permission to include data and materials described in Cao & Qasem et. al. 2018 (below) in the dissertation contents of Mr. Ahmad Qasem for Doctor of Philosophy in Biomedical Sciences at the University of Central Florida.

Article Title: Systematic review and meta-analysis on the association of tuberculosis in Crohn's disease patients treated with tumor necrosis factor-α inhibitors (Anti-TNFα).

Authors: Brent L Cao*, Ahmad Qasem*, Robert C Sharp, Latifa S Abdelli and Saleh A Naser

Journal: *World journal of gastroenterology*, *24*(25), 2764-2775.

DOI: 10.3748/wjg.v24.i25.2764

Published: July 7th, 2018

We, the authors, give our permission to include data and materials described in Qasem et. al. 2018 (below) in the dissertation contents of Mr. Ahmad Qasem for Doctor of Philosophy in Biomedical Sciences at the University of Central Florida.

Article Title: TNFα inhibitors exacerbate Mycobacterium paratuberculosis infection in tissue culture: a rationale for poor response of patients with Crohn's disease to current approved therapy.

Authors: Ahmad Qasem and Saleh A Naser

Journal: *BMJ open gastroenterology*, *5*(1), e000216.

DOI: 10.1136/bmjgast-2018-000216

Published: July 15th, 2018

We, the authors, give our permission to include data and materials described in Qasem et. al. 2019 (below) in the dissertation contents of Mr. Ahmad Qasem for Doctor of Philosophy in Biomedical Sciences at the University of Central Florida.

Article Title: Genetic polymorphisms in tumour necrosis factor receptors (TNFRSF1A/1B) illustrate differential treatment response to TNFα inhibitors in patients with Crohn's disease.

Authors: Ahmad Qasem, Seela Ramesh and Saleh A Naser

Journal: *BMJ Open Gastroenterology*, *6*(1), e000246.

DOI: 10.1136/bmjgast-2018-000246

Published: February 1st, 2019

We, the authors, give our permission to include data and materials described in Qasem et. al. 2016 (below) in the dissertation contents of Mr. Ahmad Qasem for Doctor of Philosophy in Biomedical Sciences at the University of Central Florida.

Article Title: A single capsule formulation of RHB-104 demonstrates higher anti-microbial growth potency for effective treatment of Crohn's disease associated with Mycobacterium avium subspecies paratuberculosis.

Authors: Ahmad Qasem, Mitra Safavikhasraghi and Saleh A Naser

Journal: *Gut pathogens*, *8*(1), 45.

DOI: https://doi.org/10.1186/s13099-016-0127-z

Published: September 29th, 2016