# Cytokine immune response profiles during intestinal helminths and *Mycobacterium tuberculosis* coinfection: An *in vitro* and human *ex vivo* study in KwaZulu-Natal

By

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**Submitted in the fulfilment of the requirements for the degree of Master's in Medical Science in Medical Microbiology, School of Laboratory Medicine and Medical Sciences, College of Health Sciences, University of KwaZulu Natal, Durban**

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**2023**

# DECLARATION

I, **Khethiwe Nomcebo Bhengu,** declare that the dissertation entitled "**Cytokine immune response profiles during intestinal helminths and *Mycobacterium tuberculosis* coinfection: An *in vitro* and human *ex vivo* study in KwaZulu Natal"** is the result of my original work, except where otherwise indicated. I completed this research under the supervision of **Prof Zilungile L. Mkhize-Kwitshana**, **Dr Ravesh Singh** and **Dr Pragalathan Naidoo** from the University of KwaZulu Natal's College of Health Sciences.

I also declare that,

1. This dissertation has not been submitted to UKZN or other tertiary institutions for purposes of obtaining an academic qualification, whether by me or any other party.
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# DEDICATION

To my family: my late husband Khumbulani John Bhengu – **Zohamba izinsizwa kosala izibongo**, my late children Philani Bhengu and Siphiwokuhle Bhengu, my children Nokubonga Luzwakele Bhengu, Mfundiso Malusi Bhengu, Sithembele Zanokuhle Bhengu and Sibonelo Gamelihle Wakhiwe Bhengu.

To my parents: Mandla Bethwell Mlaba and Thembekile Nester Mlaba

To my brothers: Mondli Bertrand Mlaba and Thobelani Ricardo Mlaba

To my late siblings: Zethembe Mlaba and Ntombizomusa Mlaba

I would also like to dedicate this work to the Mangosuthu University of Technology, without whom I would not have had this opportunity.

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**UDUMO NOKUBONGA KUNGOKWAKHE – BABA NYAZI LWEZULU NGIYABONGA KAKHULU NKOSI YAMAKHOSI.**

**AMEN**

# LIST OF PUBLICATIONS

## 1. Publication from the Master's project:

1.1 **Bhengu KN**, Naidoo, P, Singh R, Mpaka-Mbatha MN, Nembe, N, Duma Z, Pillay R, and Mkhize-Kwitshana ZL. 2022. Immunological Interactions between Intestinal Helminth Infections and Tuberculosis. *MDPI Diagnostics*. 12: 2676. doi.org/10.3390/diagnostics12112676. [Review article].

## 2. Research manuscript from the Master's project:

2.1 **Bhengu KN**, Naidoo, P, Singh R, Mpaka-Mbatha MN, Nembe, N, and Mkhize-Kwitshana ZL. 2023. Cytokine responses during *Mycobacterium tuberculosis* H37Rv and *Ascaris lumbricoides* costimulation using human THP-1 and Jurkat cells, and a pilot human tuberculosis and helminth coinfection study. *MDPI Microorganisms*. Provisionally accepted. Manuscript reference number: 2357988.

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3.2 Duma Z, Chuturgoon AA, Ramsuran V, Edward V, Naidoo P, Mpaka-Mbatha MN, **Bhengu KN**, Nembe N, Pillay R, Singh R, Mkhize-Kwitshana ZL (2022). The challenges of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) testing in low-middle 114 income countries and possible cost-effective measures in resource-limited settings. 115 *Globalisation and Health Journal*. 18(1):5. doi: 10.1186/s12992-022-00796-7.

3.3 Naidoo P, Ghazi T, Chuturgoon AA, Naidoo RN, Ramsuran V, Mpaka-Mbatha MN, Bhengu **KN**, Nembe N, Duma Z, Pillay R, Singh R, Mkhize-Kwitshana ZL (2021). SARS-CoV-2 and helminth coinfections, and environmental pollution exposure: An epidemiological and immunological perspective. *Environment International*. 156: 106695. 137 doi.org/10.1016/j.envint.2021.106695

# LIST OF PRESENTATIONS

1. Oral presentation: Helminth coinfection training workshop. Title: Interaction with TB patients in the clinic, 08 February 2023, at University of KwaZulu Natal – Nelson Mandela School of Medicine – by **Bhengu K.N.**

2. Oral presentation: Research article critique at the Helminth research group update: Article title **"Hookworm infection among patients with pulmonary tuberculosis: Impact of coinfection on the therapeutic failure of pulmonary tuberculosis",** Ahmad Farooq Alsayed Hasanain, Ali Abdel-Azeem Hasan Zayed, Reem Ezzat Mahdy, Amany Mohamed Adawi Nafee, Rasha Abdel-Monem Hassan Attia, Asmaa Omar Mohamed, Published: International Journal of Mycobacteriology 4 (2015) 318 – 322 - 20 May 2022 – at the University of KwaZulu Natal – Howard College by **Bhengu K.N**

3. Oral presentation: Inkosi Albert Luthuli Central Hospital (IALCH) /National Health Laboratory Services (NHLS)/UKZN Journal Club Proposal Presentation: Nutritional status and patient immune responses co-infected with *Mycobacterium tuberculosis* and intestinal helminths in Kwa-Zulu Natal, 31 March 2022 at Inkosi Albert Luthuli Central Hospital board room – by **Bhengu K.N.**

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# LIST OF ABBREVIATIONS

| | |
|---|---|
| AIDS | Acquired Immunodeficiency syndrome |
| APCs | Antigen presenting cells |
| BCG | Bacillus Calmette- Guerin |
| CLRs | C type lectins |
| DCs | Dendritic cells |
| DST | Drug Susceptibility Testing |
| DOTS | Direct Observation Treatment Course |
| ES | Excretory/secretory products |
| GCP | Good clinical practice |
| GXP | Gene Xpert |
| IFN-γ | Interferon-gamma |
| IGRA | Interferon-gamma release assay |
| IALCH | Inkosi Albert Luthuli Central Hospital |
| IL | Interleukin |
| HIV | Human Immunodeficiency Virus |
| KZN | KwaZulu Natal |
| LAM | Lipoarabinomannan |
| MDR-TB | Multi Drug Resistant Tuberculosis |
| MTB | Mycobacterium tuberculosis |
| NHLS | National Health Laboratory Services |
| NRAMP | Natural Resistance Associated Macrophage Protein 1 |
| Nb | Nippostrongylus brasiliensis (Nb) |
| NTDs | Neglected Tropical diseases |
| PCR | Polymerase chain reaction |
| PRR | Pathogen Recognition Receptors |
| PPD | Purified Protein Derivative |
| STHs | Soil Transmitted Helminths |
| SDG | Sustainable Development Goals |
| SOP’s | Standard Operating Procedures |
| TAT | Turnaround Time |
| TB | Tuberculosis |

| | |
|---|---|
| TGF-β | Transforming Growth Factor-β |
| Th1 | Type helper type 1 |
| Th2 | Type helper type 1 |
| Th17 | Type helper type 17 |
| TLR | Toll-like Receptors |
| TNF-α | Tumour necrosis factor-α |
| Tregs | Regulatory T cells |
| TSLP | Thymic Stromal Lymphopoetin |
| UCT | University of Cape Town |
| UKZN | University of KwaZulu Natal |
| WHO | World Health Organization |

# ABSTRACT

**Background:** There is a striking geographic overlap between helminths and tuberculosis (TB), particularly in developing countries like Africa. Underprivileged communities are more susceptible to these illnesses due to poverty, poor sanitation, and other environmental factors. Helminth and tuberculosis infections exhibit distinct immune responses, which may be antagonistic in coinfected hosts and lead to poor prognosis. Helminth infections induce anti-inflammatory Th2/Treg responses contrary to the pro-inflammatory Th1 responses triggered by *Mycobacterium tuberculosis* (*Mtb*) infection. Reduced TB protection has been associated with a strong Th2 response. Uncertainty exists on how helminth infection affects the host's resistance to TB. This necessitates further investigation of immune responses in helminths and TB coinfection cases, particularly in KwaZulu-Natal (KZN).

**Aim:** To determine the cytokine response profiles during intestinal helminth and TB coinfection using lymphocytic Jurkat and monocytic THP-1 cell lines for the *in vitro* study and TB and helminth coinfected South African adults for the human *ex vivo* study.

**Methods:** Lymphocytic Jurkat and monocytic THP-1 cell lines were stimulated for 24 and 48 hours with *Mtb* H37Rv and *Ascaris lumbricoides* (*A. lumbricoides*) excretory-secretory protein extracts for the *in vitro* study. A cross-sectional study on consenting adult participants (≥18 years) (n = 414) recruited from primary health care clinics was conducted between March 2020 and August 2021 in Durban, KwaZulu Natal, for the pilot human *ex vivo* study. Blood and stool samples were collected from the recruited participants. The Kato-Katz and Mini-Parasep faecal parasite concentration techniques were used to detect intestinal parasite infections in stool samples. Blood samples were analysed to determine *A. lumbricoides*-specific immunoglobulin E (IgE) and immunoglobulin G4 (IgG4) levels to improve microscopy sensitivity.

In this study, cytokine analysis was undertaken for 164 participants; 96 were HIV infected and had to be excluded, leaving 68 eligible participants. The eligible individuals were subdivided into uninfected controls (no helminth and TB infection) *(n* = 18), helminth only infected (*n* = 35), TB only infected *(n* = 6), and TB and helminth co-infected (*n* = 6) groups. Thereafter, for both the *in vitro* and *ex vivo* study, the gene expression profiles of the T helper type 1(Th1) and transcription factors [*Interferon*-γ (*IFN*-γ), *Tumour necrosis factor*-α (*TNF*-α), *Interleukin-2* (*IL*-

*2*), *Nuclear factor of activated T cells 2 (NFATC2*), *Eomesodermin (eomes),* T helper 2 (Th2) and transcription factors (*Interleukin-4 (IL-4), Interleukin5* (*IL-5, Transforming growth factor-β (TGF-β),* T helper type 17 (Th17) *(Interleukin-17 (IL-17*), immune protein and proteases (*Granzyme B, Perforin),* Regulatory T cells (Tregs) (*Interleukin-10 (IL-10) and Fork head box P3 (FoxP3)*] and the uninfected controls, TB alone, helminth alone and coinfected groups were determined using RT-qPCR.

**Results:** (i) ***In vitro study*:** TB-stimulated Jurkat cells had significantly higher levels of IFN-γ, TNF-α, Granzyme B, and perforin compared to unstimulated controls, LPS, *A. lumbricoides*, and *A. lumbricoides* plus TB costimulated cells ($p<0.0001$). IL-2, IL-17, Eomes, and NFATC2 levels were also higher in TB-stimulated Jurkat cells ($p<0.0001$). TB alone stimulated cells had lower IL-5 and IL-4 levels compared to *A. lumbricoides* alone stimulated and TB plus *A. lumbricoides* costimulated Jurkat and THP-1 cells ($p<0.0001$. *A. lumbricoides* alone stimulated cells had higher IL-4 levels compared to TB plus *A. lumbricoides* costimulated Jurkat and THP-1 cells ($p<0.0001$). TGF-β levels were also lower in TB alone stimulated cells compared to TB plus *A. lumbricoides* costimulated cells. IL-10 levels were lower in TB stimulated Jurkat and THP-1 cells compared to TB plus *A. lumbricoides* costimulated cells ($p<0.0001$. (ii) ***Ex vivo study*:** Similar results were noted for both the *in vitro* and the *ex vivo* study, although the human study had a smaller sample size.

**Conclusion:** Data suggest that helminths induce a predominant anti-inflammatory Th2 and Treg response which may downregulate critical proinflammatory Th1 responses crucial for TB protection.

**Keywords:** *Mycobacterium tuberculosis* H37Rv, *Ascaris lumbricoides* excretory-secretory proteins, Jurkat cells, THP-1 cells, human tuberculosis and helminth coinfection, cytokine gene expression.

# CHAPTER 1: INTRODUCTION

**Background, study rationale, research question, aims, objectives, and dissertation outline.**

## 1.1 Background

Soil-transmitted helminths (STHs) are parasitic worm infections that infect more than 1.5 billion people globally (WHO, 2022a). Most helminth cases occur in sub-Saharan Africa, the Americas, China and East Asia (WHO, 2022a). Forty per cent of the global helminth disease burden affects children (WHO, 2022a). Endemicity in Africa and other developing countries is due to tropical and subtropical climatic conditions, malnutrition, overcrowding, poverty and poor sanitary conditions that favour repeated infections and make effective treatment and eradication of infection challenging (Hotez *et al.*, 2006; Mkhize-Kwitshana *et al.*, 2011).

Humans get infected with helminths after ingesting eggs or larvae from contaminated ‘soil or food or through active skin penetration by infective larval stages (hookworm) found in contaminated soil (Hotez *et al.*, 2004). The most common intestinal helminth species infecting the human population worldwide are roundworms (*A. lumbricoides*), whipworms (*Trichuris trichuria* ), *Schistosoma species* and hookworms (*Necator americanus* and *Ancylostoma duodenale)* (Hotez *et al.*, 2004). Intestinal helminths have been linked to poor physical and cognitive development in children, worker productivity, pregnancy outcomes, and nutritional status (Garisch, 2014). Despite their harmful influence on health, helminths are classified as a neglected tropical disease (NTD) (Garisch, 2014).

Following infection, intestinal parasites cause conditions such as anaemia and malnutrition. For example, hookworms feed directly from the host’s blood, causing chronic intestinal blood loss leading to iron and protein loss, which may result in the development of anaemia (Loukas *et al.*, 2016). Intestinal helminths reduce nutrient absorption and can compete for vitamin A (WHO, 2022a). Furthermore, intestinal helminths cause appetite loss, lowering nutritional intake, and can cause diarrhoea and dysentery, exacerbating malnutrition (WHO, 2022a). Morbidity and death are related to worm burden and illness severity. Low worm burden is generally asymptomatic compared to high-intensity infection (Garisch, 2014).

Tuberculosis (TB) is an infectious disease caused by highly contagious infectious pathogens belonging to the Mycobacterium tuberculosis complex (Dye *et al.*, 2005). This complex consists of seven species, including *Mycobacterium tuberculosis* (*Mtb*), *M. canetti*, *M.*

*africanum, M. pinnipedii, M. microti, M. caprae* and *M. bovis* (Dye *et al.*, 2005). Despite the close genetic similarities of these species and common progenitor, these organisms differ significantly in epidemiology, pathogenicity, and even host spectrum (Brosch *et al.*, 2002). *Mtb* is the most common pathogen causing TB in humans (Dye *et al.*, 2005). Infection occurs through the inhalation of droplets containing viable bacteria, and these droplets travel to the alveoli, where they are ingested by macrophages and dendritic cells (O'Garra *et al.*, 2013). TB is predominantly a disease of the lung, with pulmonary TB accounting for about 70% of the cases (O'Garra *et al.*, 2013). Extra-pulmonary disease sites include lymph nodes, bone, and meninges (O'Garra *et al.*, 2013).

Despite its coexistence with humans since ancient times, TB continues to be a devastating public health problem worldwide (WHO, 2022b). It is the leading cause of death caused by a single infectious agent worldwide, particularly among HIV-infected individuals (Borkow and Bentwich, 2004; WHO, 2022b). HIV infection significantly increases the chance of developing active TB after exposure or having latent disease reactivated (Narasimhan *et al*., 2013). The risk of TB activation from latent to active disease increases as immunosuppression progresses (Narasimhan *et al*., 2013). There is a 10% lifetime chance of developing active TB disease for HIV-uninfected individuals with latent TB and a 10% annual risk for HIV-infected individuals (Narasimhan *et al.*, 2013).

TB infects over 2 billion people (almost a fourth of the world's population) (WHO, 2022b). In 2020, the World Health Organization (WHO) reported an estimated 10 million TB cases and TB has been ranked among the top 10 causes of death globally (WHO, 2022b). In 2020, approximately 1.4 million TB fatalities were reported worldwide, and more than 95% of tuberculosis deaths occur in developing countries such as Africa (WHO, 2022b). Africa accounts for 24% of the estimated 10 million new cases of TB (WHO, 2021). Africa bears 75% of the world's 1.03 million cases of TB and HIV coinfection (WHO, 2021). In addition, TB was identified as one of the leading causes of death in HIV infected patients living in Africa (WHO, 2021).

South Africa (SA) has one of the highest TB burdens and is among the top eight countries, accounting for two-thirds of the 87% of new TB cases (WHO, 2021). Approximately 80% of South Africans are latently infected with TB (Kanabus, 2016). Immunocompromised

individuals such as people living with HIV, malnutrition, severe kidney disease, certain cancers, those on chemotherapeutic drugs, the elderly, diabetics, smokers, and other chronic inflammatory conditions are all risk factors for TB disease progression (Kaufmann *et al*., 2002; Zu *et al*., 2012, Narasimhan *et al*., 2013).In SA, individuals at risk for TB infection are household contacts of TB patients, pregnant women, children under five, former miners, people who work in or live near mines, healthcare professionals, jail inmates, and prison personnel (Stop TB Partnership, 2017).

KwaZulu Natal (KZN) had a TB incidence of 685 per 100 000 in 2015 (Kanabus, 2016). KZN remains one of the top three South African provinces with the highest TB incidence (Kanabus, 2016). The Sustainable Development Goals (SDG) aim to eradicate TB by 2030 (WHO, 2020b). In addition, the WHO End TB Strategy aims to reduce TB deaths by 90% and new infections by 80% by 2035 (WHO, 2020b). South Africa has chosen the 90:90:90 TB strategy to meet the SDGs and WHO End TB Strategy targets. The strategy entails screening 90% of people in critical populations for TB, initiating treatment for 90% of those diagnosed with the disease, and ensuring that 90% of those who begin treatment complete it effectively.

The TB vaccine, Bacillus Calmette-Guerin (BCG), is the only currently used vaccine worldwide; however, its efficacy against pulmonary TB in adults in many high-burden countries is variable (Hawn *et al.*, 2014). A study carried out in Ethiopia revealed that patients with concurrent helminth infections had impaired cellular immune responses to tuberculin pure protein derivative (PPD), which likely indicated decreased resistance to mycobacterial conditions. The study's findings could help explain why BCG is less effective in combating TB in helminth-endemic regions of the world. (Elias *et al.*, 2001). A decrease in eosinophils and IL-10 levels following albendazole treatment compared to the placebo group demonstrated that asymptomatic helminth infection during active TB significantly impacted host immunity. The dewormed patients gained weight, and this finding suggested that their immunity to TB was improving. (Abate *et al.*, 2015).
Additionally, it was also determined that chronic parasitic infection reduced the immunogenicity of BCG in humans and that this was accompanied by higher levels of transforming growth factor beta (TGF-β) (Elias *et al.*, 2008). In light of this evidence and the fact that South Africa has a geographic overlap of TB and helminths, different vaccine

strategies will probably be required since there are gaps in the understanding of protective immunity against TB (Hawn *et al.*, 2014).

TB immune response requires an effective proinflammatory (Th1) cell response. The Th1 response includes the production of *interleukin 1 (IL-1), IL-6, IL-12, interferon-gamma (IFN-γ), tumour necrosis factor alpha (TNF-α*) and Th17 responses *(IL-17* and *IL-23)* (Romero-Adrian, 2015). Immune responses and other factors, such as genetic conditions, play an essential role in the progression of TB infection (Romero-Adrian, 2015). Other factors, such as bacterial virulence and environmental factors, poverty, malnutrition, overcrowding and pathogen exposure have been shown to influence host susceptibility to TB (Romero-Adrian, 2015).

Polymorphisms in genes encoding natural resistance-associated macrophage protein 1 (NRAMP1), IL-1 gene cluster, the vitamin D receptor, and mannose-binding lectin have been linked to susceptibility to TB (Bellamy *et al*., 2000; RJ *et al*., 2000). The essential role of Th1-associated cytokines such as IFN-γ and IL-12 was confirmed by the increased susceptibility to mycobacterial infections in patients with mutations in genes coding for these cytokines (Bellamy *et al.*, 2000; RJ *et al.*, 2000).

The dually infected host mounts an immune response against TB and intestinal helminths; however, the responses to each infection differ. As mentioned earlier, TB infection induces Th1 cell activity (Romero-Adrian *et al.*, 2015; Sia, Georgieva and Rengarajan, 2015). In contrast, intestinal helminth infections induce an anti-inflammatory Th2 and regulatory T cells (Tregs) immune response (Allen and Maizels, 2011; Inclan-Rico and Siracusa, 2018). Therefore, in TB and helminth coinfection, the cytokine immune responses may be skewed, leading to poor host prognosis.

Clinical conditions presumed to be affected by helminths include HIV/AIDS, TB, autoimmune diseases and allergic disorders (Maizels, 2016; Maizels and Mcsorley, 2016). There is an increased T regs activity in the case of TB and helminth coinfections, and these cells have been implicated in impairing Th1 responses to TB (Abate *et al.*, 2015). TB commonly overlaps

geographically with helminths, especially in developing countries (Hartgers and Yazdanbakhsh, 2006; Abate *et al.*, 2012; Babu and Nutman, 2016; Gashaw, 2018). Therefore, in TB and intestinal helminth coinfection, the Th1 and Th2 responses act antagonistically, resulting in dysregulated immune responses to both infections (Babu and Nutman, 2016). Helminths have been reported to have immunomodulatory effects which aid parasite establishment and survival while reducing immune response to allergens, autoimmune disorders, and microbiota determinants. They achieve this by interfering with epithelial cell alarmins, dendritic cell activation, macrophage function, and T-cell responsiveness by promoting an immunoregulatory milieu (Maizels, 2019).

## 1.2 Study Rationale and Significance

STH's are endemic in South Africa, particularly in the tropical and subtropical regions of the country, affecting many people in poor, overcrowded communities with a lack of sanitation, hygiene, insufficient clean water supply and poor public health service delivery (Angie, 2020). A study conducted by Molvik *et al*. on children in rural South Africa showed a 60% prevalence of *Schistosoma haematobium* and helminth prevalence ranging from 20% - 50% (Molvik *et al.*, 2017). Schistosomiasis is also present in the North West, Limpopo, Mpumalanga, KwaZulu Natal and Eastern Cape provinces (Kabuyaya *et al.*, 2017). However, there are few studies that generated data regarding the prevalence of helminths in adults in South Africa (Kwitshana *et al.*, 2008, Mkhize-Kwitshana *et al*, 2011, Adeleke *et al*., 2015).

There is a triple disease burden of helminth infection with HIV/AIDS and TB in South Africa (Mkhize-Kwitshana *et al*, 2014). In addition, SA faces a high burden of intestinal helminths and poverty, both of which exacerbate the HIV/TB epidemic (Adeleke, Yogeswaran and Wright, 2015). The prevalence of intestinal helminths was shown to be high in HIV/TB endemic areas (Borkow and Bentwich, 2004; Adeleke, Yogeswaran and Wright, 2015). This overlap between the two infections poses a significant health risk in the tropical and subtropical regions in developing countries such as SA.

Stats SA released data showing that poverty is rising in South Africa. The "Poverty Trends in South Africa" report showed that poverty levels rose in South Africa, with more than half of the population being poor (Angie, 2020). As of 2022, over 18.2 million South Africans were living in extreme poverty, with the poverty line set at 1.90 US dollars per day. This meant that 123 000 more people fell into poverty between 2021 and 2022 (Galal, 2022). In 2022 the annual consumer inflation reached another 13-year high, increasing to 7,8% in July from 7,4% in June 2022 (StatsSA, 2022), and the unemployment rate of 32,7% further worsened poverty (StatsSA, 2022). Helminths and TB are diseases of low-income populations. Therefore, these poor settings provide a vessel for continuing helminth and TB infections.

KZN is one of the top three provinces in South Africa with the highest TB incidence, with reported rates of 685 per 100,000 (Kanabus, 2021). KZN is significantly plagued by TB and HIV epidemics, with an estimated 70% HIV-TB coinfection incidence (Snyman, 2015). TB is one of the top causes of death in HIV-coinfected patients (WHO, 2022b).

The large extracellular helminth parasites induce a Th2 predominant immune response. On the other hand, the intracellular pathogen *Mtb* activates a Th1 immune response (Hasanain *et al.*, 2015). These two arms of immune responses counter regulate each other; for instance, IL-4 and IL-10 decrease IFN-γ producing CD4 cells (Toulza *et al.*, 2016). The Th2 dominance is thought to attenuate the Th1 immune response to mycobacterial antigens and the BCG vaccination during persistent helminth infection (Elias *et al.*, 2008). Helminth and TB coinfection also poses a risk of developing active TB, reactivating latent TB, and having active TB progression (Monin *et al.*, 2015).

In South Africa, past research on parasites has mainly focused on children, mostly on prevalence surveys which reported high prevalence of these infections , ranging between 5,3% and 64,8% particularly the coastal areas of KwaZulu-Natal, Western Cape and Mpumalanga (Mabaso *et al.*, 2004; Saathoff *et al.*, 2004; Adams *et.al* .,2005; Bhat *et al.*, 2013; Banhela *et al.*, 2017; Zulu *et.al*, 2020) and effects of helminthiasis on malnutrition, stunting (Jinabhai, Taylor and Sullivan, 2003) and cognitive development (Appleton and Kvalsvig, 2017; Banhela *et al.*, 2017). There is paucity of data on adult helminth infection, the impact of coinfection

with intestinal helminths and TB, and immunological responses to helminth and TB coinfections in South Africa, as well as the influence that these infections have on protective immune responses. Furthermore, investigations in other parts of the world investigated different helminth species and have yielded disparate results regarding the impact of TB/ helminth coinfection (Elias *et al.* (2008); Monin *et al.* (2015); Kumar *et al.*, 2020; Bewket *et al.*,2022). This raises the need for more research in local communities.

Considering the different effects that the various helminth species have on the immunity to TB, the current study was conducted in KwaZulu Natal to investigate the impact of TB/helminth regulatory coinfection on cytokine immune responses using *in vitro* and human *ex vivo* experiments. The data presented by this study is expected to provide pertinent information in SA, particularly in the KZN province, which is known for its high TB prevalence, subtropical climate, and poverty suitable for transmission of helminths. Such data may assist in updating the existing mass deworming program, which only targets school-aged children and excludes adults. The information will also contribute to TB management strategies in helminth-endemic areas.

### 1.3 Null Hypothesis

Co-infection with TB and helminths skews the host's immunity to a predominant Th2 and immune response, therefore, a reduced Th1 responses required for TB, leading to a poor TB prognosis.

### 1.4 Research question

Do helminth infections alter the cytokine immune response during intestinal helminth and TB coinfection?

### 1.5 Aim

To determine the cytokine response profiles during intestinal helminth and TB coinfection using lymphocytic Jurkat and monocytic THP-1 cell lines for the *in vitro* study and TB

(GeneXpert positive TB patients) and helminth coinfected South African adults for the human *ex vivo* study.

### 1.6 Objectives

1. To appraise published literature on immune responses during coinfections with intestinal helminth and TB.

2. To investigate the cytokine immune response profiles in lymphocytic Jurkat and monocytic THP-1 cell lines costimulated with H37Rv strain of *Mtb* and *A. lumbricoides* excretory-secretory protein antigens.

3. To investigate the cytokine immune response profiles in adult participants coinfected with intestinal helminths and TB (GeneXpert positive).

### 1.7 Outline of the dissertation

This work is presented as a dissertation by manuscripts, as per the University of KwaZulu Natal's recommendations. Therefore, the chapters include a background/ introduction, literature review in a form of a published review manuscript and a research manuscript which has passed the reviewers and is pending approval by the journal's academic editor. The font of the review manuscript has been formatted in accordance with the journal in which the article was published. The font of the research manuscript that is under review is also formatted according to the journal to which it was submitted.

**Chapter 1: Introduction.** This chapter introduces the literature and study rationale, highlighting the significance and contribution of this study to scientific knowledge. This chapter then addresses the aims and objectives presented as chapters in the dissertation.

**Chapter 2: Literature review.** This chapter addresses objective 1, which was to review existing literature on the immunologic impact of coinfection with intestinal helminths and TB. This manuscript was published in the *MDPI Diagnostics* journal in November 2022: **Immunological Interactions between Intestinal Helminth Infections and Tuberculosis.**

**Bhengu KN**, Naidoo, P, Singh R, Mpaka-Mbatha MN, Nembe, N, Duma Z, Pillay R, and Mkhize-Kwitshana ZL. 2022. Immunological Interactions between Intestinal Helminth Infections and Tuberculosis. *MDPI Diagnostics*. 12: 2676. doi.org/10.3390/diagnostics12112676. [Review article].

**Chapter 3:** This chapter addresses objectives 2 and 3; the actual research that was undertaken to explore the immunological responses during intestinal helminth and TB coinfection, using *in vitro* and human *ex-vivo* experimental work. The research manuscript "**Cytokine responses during Mycobacterium tuberculosis H37Rv and *Ascaris lumbricoides* costimulation using human THP-1 and Jurkat cells, and a pilot human tuberculosis and helminth coinfection study"** was submitted to the *MDPI Microorganisms* journal - manuscript number – **2357988.**

**Bhengu, K.N.;** Singh, R.; Naidoo, P.; Mpaka-Mbatha, M.N.; Nembe-Mafa, N.; Mkhize-Kwitshana, Z.L. Cytokine responses during *Mycobacterium tuberculosis* H37Rv and *A. lumbricoides* coinfection using human THP-1 and Jurkat cells, and a pilot human tuberculosis and helminth coinfection study. was submitted to the *MDPI Microorganisms* journal, has passed the reviewers and is awaiting the academic editor's decision - manuscript number – 2357988.

**Chapter 4:** Synthesis, conclusion, recommendations and list of appendices

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# CHAPTER TWO:

## LITERATURE REVIEW MANUSCRIPT

## Preface to Chapter 2

*Mtb* and intestinal helminth infections continue to be significant public health concerns. In addition to HIV coinfection being a substantial risk factor for higher tuberculosis mortality rates, intestinal helminth co-infection must also be considered. Geographically, intestinal helminthiasis and tuberculosis overlap extensively in tropical and subtropical areas of the world, possibly due to the paradoxical impact of immune responses against them (Babu and Nutman, 2016). Th1 and Th2 cells that fight *Mtb* and intestinal helminths cross-regulate each other via cytokine production, notably IFN-γ and IL4, respectively (Gashaw, 2018).

Furthermore, helminths have been shown to affect the immunogenicity of BCG, the only known vaccine against *Mtb* (Elias *et al.*, 2008). A number of studies have been conducted to study the impact of helminthiasis on the host immunological response to tuberculosis and the efficacy of BCG vaccination, resulting in conflicting and inconclusive findings (Elias, H. Wolday, *et al.*, 2001; Elias *et al.*, 2005, 2008; Biraro *et al.*, 2014). On the other hand, strong evidence suggests that exposure to intestinal helminths reduces the risk of immune-mediated illnesses (McSorley, Hewitson and Maizels, 2013; Wammes *et al.*, 2014; McFarlane *et al.*, 2017) As a result, the immunological interactions of the co-infection with *Mtb* and helminths are explored in this review paper, which also aims to provide insights into the management of these infections in areas where they are co-endemic.

These immunological interactions are discussed in the review below, which was published in the manuscript titled **"Immunological Interactions between Intestinal Helminth Infections and Tuberculosis."**

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**Review**

# Immunological Interactions between Intestinal Helminth Infections and Tuberculosis

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## Abstract

Helminth infections are among the neglected tropical diseases affecting billions of people globally, predominantly in developing countries. Helminths' effects are augmented by coincident tuberculosis disease, which infects a third of the world's population. The role of helminth infections on the pathogenesis and pathology of active tuberculosis (TB) remains controversial. Parasite-induced suppression of the efficacy of Bacille Calmette-Guerin (BCG) has been widely reported in helminth-endemic areas worldwide. TB immune response is predominantly proinflammatory T-helper type 1 (Th1)-dependent. On the other hand, helminth infections induce an opposing anti-inflammatory Th2 and Th3 immune-regulatory response. This review summarizes the literature focusing on host immune response profiles during single-helminth, TB and dual infections. It also aims to necessitate investigations into the complexity of immunity in helminth/TB coinfected patients since the research data are limited and contradictory. Helminths overlap geographically with TB, particularly in Sub-Saharan Africa. Each disease elicits a response which may skew the immune responses. However, these effects are helminth species-dependent, where some parasites have no impact on the immune responses to concurrent TB. The implications for the complex immunological interactions that occur during coinfection are highlighted to inform government treatment policies and encourage the development of high-efficacy TB vaccines in areas where helminths are prevalent.

**Keywords:** *Mycobacterium tuberculosis*; helminths; coinfection; immune response; Bacille Calmette-Guerin; vaccination

## 1. Introduction

Intestinal helminths are parasitic worms infecting over 1.5 billion people globally [1]. Most helminth cases occur in tropical and sub-tropical areas such as Sub-Saharan Africa, the Americas, China and East Asia [1]. Humans are infected with helminth parasites after ingesting eggs or larvae from contaminated water, soil or food or through active skin penetration by infective hookworm larvae in contaminated soil [2]. Climate change, malnutrition, overcrowding, poverty and poor sanitary conditions are risk factors associated with the high helminth prevalence in Africa and other developing countries, making effective treatment and the eradication of infection challenging [1–4]. The most common intestinal helminth species infecting humans are *Schistosoma mansoni, Trichuris trichuria* (whipworm), *A. lumbricoides* (roundworm), *Necator americanus* and *Ancylostoma duodenale (hookworms)* [1, 2].

Tuberculosis (TB) is an infectious bacterial disease caused by different strains of acid-fast bacilli belonging to the *Mycobacterium tuberculosis (Mtb)* complex [5]. The TB bacteria are airborne, and transmission occurs when a TB-infected person coughs, sneezes, or spits, expelling the infected droplets into the air. Inhalation of these aerosols may result in infection of the next host [6]. TB continues to be a public health problem across the world, with the World Health Organization (WHO) reporting over 10 million TB cases in 2020 [7]. Approximately 1.5 million TB-related deaths were reported worldwide in 2020 [7]. Globally, Africa accounts for 50% of cases of TB and human immunodeficiency virus (HIV) coinfection [7]. Furthermore, in Africa, TB is commonly observed in HIV-infected patients, and it is the leading cause of death among them [7].

TB exposure results in the initiation of an immune response to fight the infection. The immune response to TB involves the interaction of innate and adaptive immune responses. It is dependent on the cellular immune response, which is mediated by proinflammatory T-helper type 1 (Th1) and Th17 cells [8–10]. The Th1 cytokines, which are interferon-γ (IFN-γ), interleukin 12 (IL12) and tumour necrosis factor-α (TNF-α) and Th17 cytokines (IL-17, IL-21, IL-22 and IL-23) play a role in combating bacterial and viral infections [8–10]. Helminth exposure, on the other hand, induces an anti-inflammatory Th2 immune response which is

characterized by the production of cytokines such as IL-4, IL-5, IL-9, IL-10 and IL-13, and increased levels of circulating immunoglobulin E (IgE) antibodies, eosinophils, and mast cells, regulatory T cells (Tregs) and transforming growth factor-β (TGF-β) [11, 12].

TB commonly overlaps geographically with soil-transmitted helminths, especially in developing countries [13–16], and this co-endemicity has implications for public health and the afflicted hosts. Helminth infection-induced immune responses could promote the pathogenesis of severe TB infections [16–18]; others report that they can also be beneficial in reducing TB severity [19–22]. However, there is no conclusive evidence to confirm whether helminth-induced immunity modulates TB-specific immune responses or vice-versa, and studies have yielded contradictory results. Therefore, knowledge on the interaction between TB and helminth infections is limited, as are the available data.

Given the current evidence on potential immunologic implications, such as those that could influence TB vaccination, treatment and diagnosis, more research is needed to determine the influence of helminth coinfection on TB control and how to negate any adverse effects. As a result, this review will summarize what is currently known about TB and helminths' immune responses in human and experimental studies, both separately and in the context of coinfection. The review will also elucidate the effects of TB and helminth coinfections on vaccine efficacy and the implications for long-term health care.

## 2. Article Search Strategy for the Current Review

An electronic search of online databases such as Google Scholar, Google, PubMed, Science Direct, online library sources, and Web of Science were utilized to extract research and review articles using phrases and words: helminth, tuberculosis, helminth and tuberculosis coinfection, helminth and tuberculosis vaccine and helminth and tuberculosis diagnosis in humans, animals and *in vitro* studies.

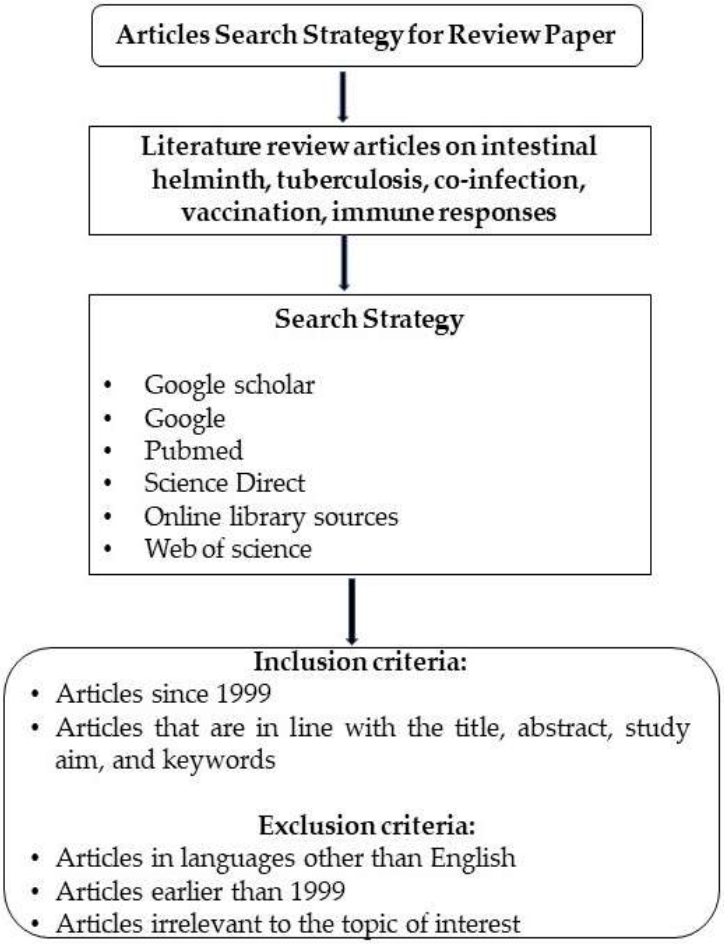


**Figure 1.** PRISMA flow diagram of the search strategy and the research design process.

## 3. The Host Immune Response to Helminths

Helminths are parasitic and multicellular organisms that coevolve with their hosts [23]. These parasitic infections are often asymptomatic, but there are cases of heavy worm burden. These have been linked to persistent health conditions such as anaemia, fatigue, growth stunting and poor cognitive development [24]. Helminths are the driving force behind how immunity is initiated and maintained [25]. They typically create long-term infections in their hosts. They can influence physiological and immunological homeostasis to ensure their continuing existence [25].

Helminths mature within the infected subject and lay eggs for transfer to another host, exposing them to multiple stages of parasite development, each of which elicits a unique immune response [26]. Helminths have evolved to exploit a range of host immunoregulatory

mechanisms and activate generic suppressive pathways that can suppress bystander responses to other antigens, allergens, and self-antigens [12]. Helminths have been dubbed "masters of immunoregulation" because of their capability to control immunity to escape being eliminated by the host [25, 27]. Helminths enter the body through the skin or intestinal epithelium's barrier surface, where they block the transcription of numerous molecules that keep the epithelium intact [28].

Tissue injury activates the production of "alarmins" (IL-33 and thymic stromal lymphopoietin (TSLP)) and the identification of invaders by pattern recognition receptors (PPRs) in the host [28]. The Th1 proinflammatory cytokine production is driven by pattern recognition receptors (PRRs) such as toll-like receptors (TLRs) or C-type lectin receptors (CLRs), whereas IL-33 and TSLP initiate a Th2 anti-inflammatory response [28]. Helminths stimulate increased mucin synthesis, smooth muscle contractility and epithelial cell turnover as a host defense to eliminate the infection. There is also increased IgE and IgG1 production in mice and IgE and IgG4 production in humans [12, 28]. All these processes work together to drive worm expulsion and wound-healing responses, which control worm-induced tissue damage [28].

The Th2 immune response induced by helminths includes interleukins (IL-4, IL-5, IL-9, IL-10, and IL-13), broad or localized eosinophilia and hyperplasia of goblet and mucosal mast cells [12, 28]. The CD4-positive Th2 cells were initially identified as an essential source of IL-4, IL-5, IL-9, IL-10 and IL-13 cytokines [29]. Eosinophils, basophils and innate lymphoid cells (ILCs) can also produce some of these cytokines in response to helminth infections [29]. Although the Th2 immune response induced by helminth parasites is stereotypical, the initiation, progression and culmination of this response require interaction with different cell types, most notably: epithelial or stromal cells, ILCs, antigen-presenting cells, dendritic cells, macrophages, T cells, B cells, eosinophils, mast cells and basophils [12].

Tregs maintain the Th2 dominance, IL-10 and TGF-β, which mediate the suppression of competing Th1 and Th17 cell populations [30]. Tregs modulate the immune system to prevent tissue damage induced by proinflammatory responses, maintain tolerance to self-antigens and abrogate autoimmune disease [31]. These cells can be divided into two subsets: natural Tregs

that develop in the thymus and induced Tregs that arise from conventional CD4-positive T cells in the periphery, which are promoted by chronic antigen exposure [32]. The forkhead/winged-helix transcription factor (Foxp3) is a crucial marker for identifying these subsets, but it may be expressed on activated CD4-positive T cells [32]. Helminth-induced suppression of immunopathology also involves CD4+ Tregs (Foxp3+ or Foxp3), CD8+ Tregs, regulatory B cells (Bregs), IL-4-responsive cells, TGF-β, and IL-10 [33]. Since an increased Th2 response can potentially induce disease, a regulated response must be generated. This is referred to as the modified Th2 cell response and is characterized by the downregulation of Th2 cytokines [12].

According to the hygiene hypothesis, in developed countries where sanitation is good, and helminths have been eliminated, there is an increase in allergic diseases such as asthma and allergic rhinitis, and autoimmune diseases such as Crohn's disease [34]. This hypothesis has led to many human and animal studies conducted using live helminth parasites to determine whether helminths do nullify the effect of allergies and autoimmune disorders. Human studies conducted in underdeveloped countries where helminths are still prevalent showed fewer allergies and autoimmune diseases [34–36]. Others have reported evidence of decreased allergies in developing countries [37].

Helminths induce various immune and physiologic modifications to survive the hostile immune response directed against them and their general survival. These survival mechanisms include this modified Th2 response [27]. These parasites also promote angiogenesis, which changes tissue vascularity and thus provides a good niche for their survival [38]. The overall immune modulation of helminths invokes immunosuppression, immunologic and physiological tolerance and a modified Th2 response [27]. These can lead to a reduced immune response, thus amplifying susceptibility to infection with other pathogens, reduced anti-tumor immunity and reduced vaccine efficacy.

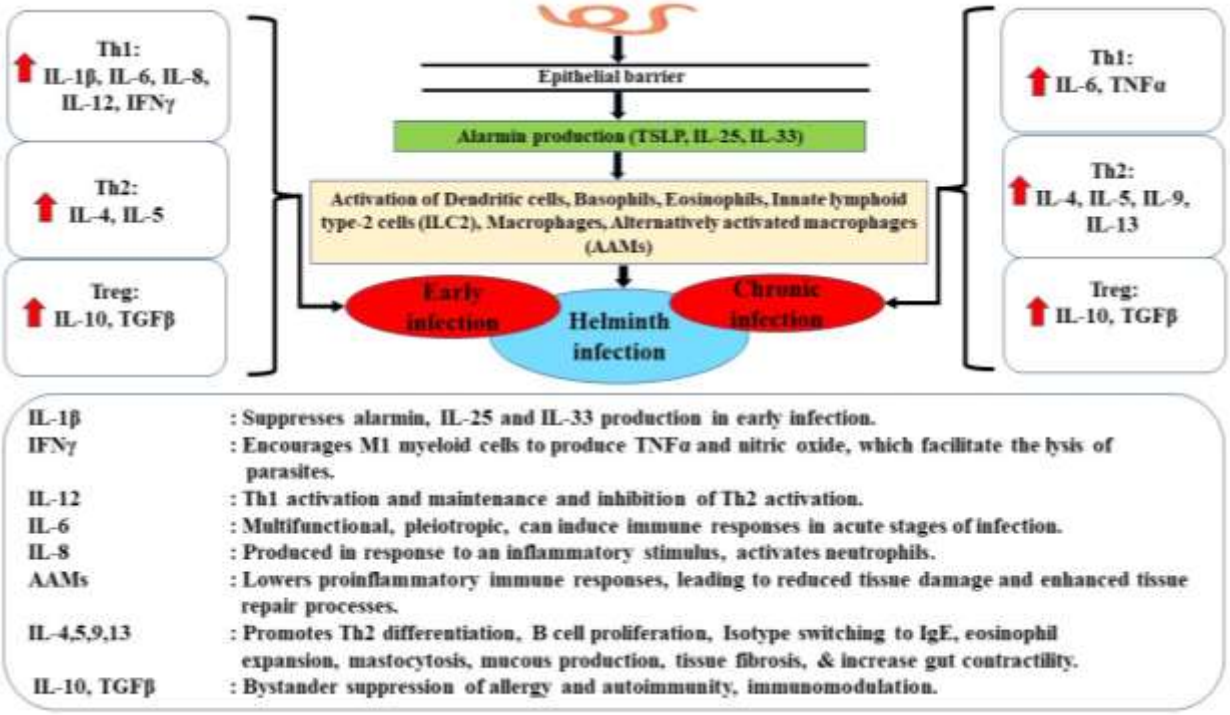


**Figure 2.** Immune response profiles during helminth infection. Migration of helminths damages epithelial barrier cells and tissues, triggering an immune response. Helminths produce damage and pathogen-associated molecular patterns (DAMPS and PAMPS). DAMPS and PAMPS activate various cells, such as epithelial, which release alarmins such as Thymic stromal lymphopoietin (TSLP), IL-25 and IL-33. Alarmins stimulate innate lymphoid cells (ILCs), aiding collagen deposition and tissue repair, and are a source of IL-5 required for eosinophil activation. Eosinophils enter tissues during helminth infection-induced inflammation. Eosinophilia is a crucial feature of the host response to helminth infection. Alarmins promote B cell activation and induction of alternatively activated macrophages (AAMs). AAMs stimulate IL-10 and TGF-β, which reduce the host's immune response to pathogens to avoid damaging the host and maintain normal tissue homeostasis. Classically activated macrophages, stimulated by IFN-γ produce proinflammatory cytokines (IL-1β, IL-6, IL-8, IL-12 and TNF-α).

Figure 1 Footnotes: IL: interleukin; IFN-γ: interferon-gamma; TGFβ: transforming growth factor beta; TNFα: tumor necrosis factor-alpha; ILCs: innate lymphoid cells; TSLP: Thymic stromal lymphopoietin; AAMs: alternatively activated macrophages; DAMPS: damage-associated molecular patterns; PAMPS: pathogen-associated molecular patterns. Red arrow pointing up indicates cytokines that are upregulated/increased during the early stages of helminth infection and those that are upregulated during the chronic stages.

## 4. The Host Immune Response to TB

TB enters the body via inhaled droplets to the alveoli. It interacts with the alveolar macrophages, infecting and multiplying inside them, thus making these cells the first line of defence against infection [6]. In immunocompetent individuals, macrophages are activated, and they phagocytose and remove TB.

In some cases, the disease is controlled and kept inactive or latent in distinct foci known as granulomas bacteria [9, 15, 39, 40]. However, some bacteria can escape this fate, multiply and eventually cause an active infection. This may be due to the intrinsic capacity of the macrophage, the immune status of the host or the virulence of the infecting bacteria [9, 15, 39, 40]. *Mtb* is, therefore, a pathogen that can cause both latent and active disease [41].

### **4.1.** *Innate Responses to TB*

The initial stages of TB infection include phagocytosis of the bacteria by macrophages [6]. Receptors that recognize a broad spectrum of mycobacterial ligands cause phagocytosis [9]. Pathogen recognition receptors, TLRs, complement receptors (C.R.), Nucleotide Oligomerization Domain (NOD)-like receptors and C-type lectins have all been implicated in the recognition of mycobacteria and the initiation of the cytokine response [8].

When phagocytic cells encounter TB, they get activated and generate cytokines, including proinflammatory cytokines such as TNF-, IL-1, IL-6, IL-12 and IFN-γ [8]. Increased susceptibility to TB was reported to be linked to genetic abnormalities in IFN-γ production [15, 42]. IFN-γ is involved in activating macrophages that fight mycobacteria through intracellular killing and antigen presentation to T lymphocytes [43]. Vitamin D is also involved in killing *Mtb*, which is aided by the creation of the peptide cathelicidin [44]. The presentation of TB antigens by dendritic cells in lymph nodes, possibly aided by neutrophils, initiates a local immune response that culminates in pathogen killing by reactive oxygen species (ROS) and antimicrobial peptides [8].

Cells required in the host's defence against *Mtb* include monocytes, macrophages, neutrophils, natural killer (NK), and dendritic cells. Together, these cells form a primary granuloma, which may allow *Mtb* growth while containing the infection until T cells are recruited to the infection site, a response process that takes weeks [8]. Phagolysosomal fusion, reactive oxygen and nitrogen intermediates, and antimicrobial peptides such as cathelicidin induced by vitamin D are innate mechanisms against Mtb [44].

NK cells may eliminate intracellular *Mtb* through the activation of perforin, where the antimycobacterial factor granulysin binds to the bacterial cell surface and disrupts the membrane, resulting in bacterial osmotic lysis [45]. Apoptosis is a critical mechanism for the infected host cell to limit *Mtb* replication to a minimum. Phagocytic cell apoptosis may prevent the spread of disease, diminish the viability of intracellular mycobacteria and reduce the risk of infection [46].

### *4.2. Adaptive Immune Responses to TB*

Adaptive immunity develops after exposure to mycobacterial antigens or vaccination with BCG. This part of the immune system is triggered when the innate immune response is insufficient to suppress TB infection. The control of TB requires Th1 immune responses (IFN-γ, IL-12 and TNF-α) and Th17 responses (IL-17 and IL-23). Th1 responses are proinflammatory and develop a cell-mediated reaction [39]. Th1 cells produce IFN-γ through the T-box transcription factor (TBX21). Both IL-12 and IFN-γ are the leading cytokines in Th1 responses, where IL-12 is secreted by antigen-presenting cells [40, 47]. The IL-12 receptor, which is expressed on the surface of T cells, interacts with IL-12. The increased T-bet (encoded by TBX21) boosts the signal transducer and activator of transcription 4 (STAT4), a regulator of Th1 cells [47]. T-bet binds to and affects the expression of Th1-specific genes and Th1 and Th17 cell expression [47]. This is important since the control of TB requires Th1 responses. STAT4 and T-bet work together to ensure optimal IFN-γ levels, and their depletion eliminates IFN-γ production [47].

TB immunity involves many cells, such as T cells, B cells and natural killer (NK) cells, with CD4+ T cells being the primary cell type in TB control [48]. The CD4+ Th1 cells are central to the control of TB; these cells secrete IFN-γ and TNF-α, which are both critical in the management of TB [39]. IL-12 regulates the induction of IFN-γ, and mutations in the genes coding for IL-12, IL-12R, IFN-γR or STAT1 or depletion of CD4+ T cells (as seen in HIV infection) all promote susceptibility to disseminated TB [39]. IFN-γ stimulates phagocytosis, phagosome maturation, the production of reactive oxygen intermediates (ROS) and antigen presentation in macrophages.

IFN-γ is regarded as the primary cytokine that regulates TB infection and eradication. It works by activating the infected macrophage, resulting in the production of reactive oxygen and nitrogen species, which have a microbicidal role [49]. In terms of memory immune responses, CD4+ Th17 cells and Th1 cells have been identified as enhancing the host's resistance to TB [50]. Th17 cells are a lineage of CD4+ T helper cells that produce the cytokine IL-17, IL-17F and IL-22, and they play a role in developing an optimal Th1 response [51].

Th17 was first described as a distinct population of the T helper cells controlled by the transcription factor RAR-related orphan receptor gamma (RORyt) [52]. They develop independently of T-bet, STAT4, GATA-3 and STAT6 transcription factors critical for the development of Th1 and Th2 development, respectively [52]. The central effector cytokines of Th17 are IL-17; other cytokines are IL-22 and IL-26 [53]. The immune response to TB infection is directed mainly by a Th1 response, with contributions from Th17 and other cells. A strong proinflammatory milieu also characterizes TB infection. On the other hand, human innate immune responses to *Mtb* infection are still poorly understood, owing to the limitations in examining pulmonary-specific immunity.

Therefore, understanding the interaction of innate and adaptive immune cells in human TB is crucial for identifying new immunomodulatory targets and clarifying protective immunity processes.

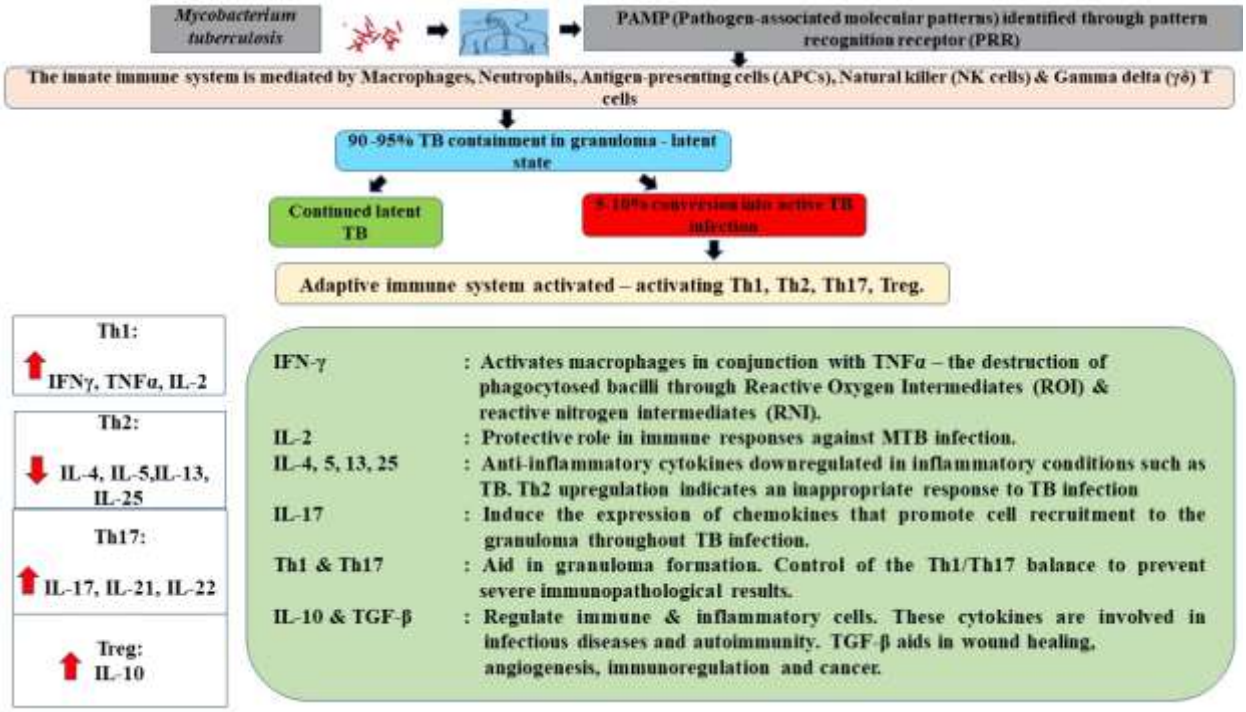


**Figure 3.** Immune response profiles during tuberculosis infection. Mycobacteria encounter alveolar macrophages where they are phagocytosed, kept inside phagosomes and exposed to antimicrobial peptides and degrading lysosomal enzymes (lysozyme). However, pathogenic mycobacteria have developed strategies to subvert the host's defences. Th1-cell activity (IFN-γ, IL-12 and TNF-α) is required for *Mycobacterium tuberculosis* immunity. IFN-γ activation of macrophages promotes bacterial killing by forming toxic reactive oxygen intermediates (ROI) and reactive nitrogen intermediates (RNI). An array of cytokines and chemokines, including tumour necrosis factor (TNF-α), induces a proinflammatory response and directs immune cells to the infection site. Dendritic cells migrate to draining lymph nodes, where they encounter many immature T cells. In the presence of proinflammatory cytokines such as IFN-γ and IL12, T cells become activated, multiply and differentiate into T helper (Th)1 cell. IFN-γ stimulates macrophages and triggers the potent antimicrobial activities of the primed Th1 cells. Innate and Th1-dominant adaptive immune responses interact to produce granulomas. Innate and adaptive immune responses are critical for microorganism eradication.

Figure 2 footnotes: IL: interleukin; IFN-γ: interferon-gamma; TNF-α: tumor necrosis factor-α; ROI: reactive oxygen intermediates; RNI: reactive nitrogen intermediates. Red arrow pointing up indicates cytokines that are upregulated/increased during TB infection. Red arrow pointing down indicates cytokines that are downregulated during TB infection.

## 5. Host Immune Response during Helminth Coinfection with TB

The geographic distributions of helminths and TB overlap substantially, particularly in underdeveloped countries, resulting in an increased likelihood of coinfection with both pathogens [15, 16]. This coexistence has also led to the hypothesis that helminths can worsen the effects of TB There have been suggestions that the anti-inflammatory response induced by helminths in cases of coinfection might dampen protective and immunopathological responses to TB [15, 16].

An Ethiopian study investigated the association between intestinal helminths and active TB and found that helminth infection increases the likelihood of developing active TB [54]. This and other studies also suggested that patients with coinfection may have antagonistic effector cell responses in responding to and regulating these diseases [30, 55]. This can also imply that the efficacy of the vaccines may be reduced.

One school of thought suggests that helminths create an environment that weakens the host's defenses against TB By activating the IL-4 receptor pathway, a preexisting helminth infection inhibits an innate pulmonary anti-TB defense [56]. In coinfected mice models, helminth-induced lung alterations increased susceptibility to TB [56]. Macrophages can be classically or alternatively activated. Classically activated macrophages (CAMs) increase the activity of nitric oxide synthase (iNOS), which converts L-arginine to nitric oxide and citrulline. Nitric oxide promotes intracellular *Mtb* killing. On the other hand, alternatively activated macrophages (AAMs) induce arginase, which competes with iNOS for L-arginine, thereby reducing nitric oxide production for the intracellular killing of Mtb [49]. *Mtb* resistance in helminth-infected mice is promoted by AAMs. This major cellular pathway compromises the helminth-infected host's ability to limit *Mtb* growth [56].

A review in support of this proposed role of the Th2-dominant phenotype on *Mtb* control illustrated that AAMs might inhibit the macrophage killing of *Mtb* [49]. Conversely, a murine study in South Africa using *Nippostrongylus brasiliensis* (*Nb*) revealed that *Mtb* colonies were

reduced in the lungs of *Nb*-infected mice. The stimulation of pulmonary CD4+ T cells and Th1 and Th2 cytokines, neutrophils and alveolar macrophages was elevated. This suggests that *Nb* infection triggers a macrophage response, which protects the host throughout the early phases of mycobacterial disease and subsequent illness [19].

Both helminths and TB have independent mechanisms for initiating the host immune response, with significant consequences for the immunology of each infection [15, 16]. The coexistence of helminth infection and active tuberculosis has been demonstrated in epidemiological, cross-sectional and case-control studies that looked at the prevalence and correlation of the two diseases. Pulmonary TB patients were found to have a significant rate of intestinal nematode infection, indicating that helminth immunomodulation may affect the control of TB [54], [57].

In Ethiopia, some studies reported an increase in the prevalence of helminth coinfection in TB patients, where one study found a higher risk of parasites among active TB patients than in healthy community controls [17, 58, 59]. Likewise, in Iran, a higher prevalence of intestinal helminths was found in tuberculosis patients compared to the uninfected subjects (Taghipour et al., 2019). Taghipour and colleagues also determined that immunocompromised TB patients are more vulnerable to parasitic gastrointestinal infections [60]. It was reported that Blastocystis subtype 1 was the most common subtype found in TB patients; however, a phylogenetic analysis revealed no distinction between Blastocystis isolates from TB patients and those from the uninfected [61].

*S. mansoni* was also a risk factor for TB infection, and it altered the clinical presentation and pathogenesis of TB in Tanzania [62]. The authors recommended treatment of this parasite using praziquantel in TB infection management [62]. A systematic review suggested that health education be implemented to help prevent intestinal helminth infection. It further added that screening for helminths should be possibly included in the treatment strategies for tuberculosis patients [63]. Another review suggested an association between *Toxoplasma gondii (T.gondii*) seropositivity and having tuberculosis, with *T. gondii* seropositivity, which indicates chronic infection, being relatively common among tuberculosis patients [63].

*Strongyloides stercoralis* coinfection with pulmonary TB was implicated in the cause of the skewed immune response to mycobacterial disease [64]. The proinflammatory Th1 cytokines were reduced, whereas the anti-inflammatory Th2 and Th3 cytokines were elevated, thus leading to a conclusion that helminth coinfection may modulate protective immune responses in latent TB [64]. A study of immunological correlates in TB coinfection with *S. mansoni* in Kenya, on the other hand, discovered that the expression of TB-specific Th1 cytokines was maintained. Individuals with latent tuberculosis and *S. mansoni* infection had more CD4+ Th1 cells than those who were only latently TB-infected [22]. There were similar results in a Brazilian study, whose findings revealed that A. *lumbricoides* infection had no impact on Th1, Th2 and Th17 responses or the T cell populations [21].

A Th1 immune response observed during persistent filarial infection was characterized by a reduction in Purified Protein Derivative (PPD)-specific IFN-γ and IL17 responses [65]. The study suggested that filaria infection reduced the PPD-specific IFN-γ and IL17 responses. In addition, it was observed that onchocerciasis patients' peripheral T cells had a weak response to *Mtb* antigens [66]. Elias and colleagues illustrated that compared to dewormed patients, helminth-infected individuals displayed low Th1 immune response and IFN-γ production in response to mycobacteria infection [67]. Lastly, it has been suggested that a robust Th1 response characterizes cell mediated protection against TB infection, and coinfection with helminths could modulate these immune responses by driving Th2 and Treg cells [17, 68].

Furthermore, enhanced Treg function is associated with helminth infection and may suppress Th1 responses against unrelated antigens [12, 68]. This finding was supported by studies which showed that intestinal helminth coinfection was associated with a reduced Th1 response in active TB [16], [69]. Type I immunity and its proinflammatory cytokines, such as IFN-γ, IL-12 and TNF-α, have a protective role against *Mtb*. By contrast, the induction of type 2 immunity, e.g., Th2 and Treg cells (as seen in helminth infections) and their anti-inflammatory cytokines, were reported to suppress the efficient immune response against TB [39].

A mouse model study of Schistosoma mansoni showed a reduced protective efficacy of BCG vaccination against Mtb [67]. Another study demonstrated that concomitant helminth

infections significantly impair the immunogenicity of BCG vaccines, an impairment associated with increased TGF-β production [30]. During active TB, asymptomatic helminth infection has been shown to have a considerable impact on host immunity in a double-blind, randomized clinical study [17]. In comparison to the placebo group, eosinophils and IL-10 levels decreased after albendazole treatment [17]. Another albendazole treatment study was conducted to determine the immunological effects of deworming on proinflammatory cytokine responses to plasmodial antigens. The study demonstrated improvements in immune hypo responsiveness, where anthelmintic treatment significantly increased proinflammatory cytokine responses to Plasmodium falciparum-infected red blood cells [70].

In Egypt, it was determined that hookworm infection was one of the risk factors for the failure of TB therapy [71]. However, a human study in the United Kingdom (U.K.), where the authors studied migrants from Nepal, found that hookworm infection reduced TB growth and may reduce the risk of infection [20]. According to the evidence presented above, some studies demonstrated that helminthiasis has a negative impact on TB diseases, while others showed a beneficial effect. Table 1 summarizes some of the studies investigating helminth and TB coinfections.

Although HIV is not covered in this review, there is evidence of a concurrent distribution of triple disease burden involving tuberculosis, helminths and HIV, particularly in Sub-Saharan Africa. This necessitates a greater focus on disease management strategies by various policymakers.

**Table 1.** Summary of experimental and human studies focusing on helminth and tuberculosis coinfections.

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| References | Study Type, Location and Helminth(s) | Study Aim | Major Findings |
|---|---|---|---|
| [73] | Human study in Kenya.<br>*Wuchereria bancrofti* and *Schistosoma haematobium* | To investigate whether prenatal immunity to helminths persists in childhood and if it alters the immune response to BCG | Compared to patients with prenatal sensitization 10–14 months after BCG immunization, T cell IFN-γ production was 26-fold higher in infants not sensitized to filariae or schistosomes in utero. |
| [74] | Human study in South Africa.<br>*Ascaris lumbricoides* and *Trichuris trichiura* | To determine total serum IgE before and after tuberculosis therapy | TB therapy resulted in reduced serum *Ascaris*-specific IgE levels. Tuberculin induration was found to be inversely related to IgE in patients but not in controls. |
| [66] | Human study in West Cameroon.<br>*Onchocerca volvulus* | To determine total serum IgE before and after tuberculosis therapy | TB therapy resulted in reduced serum *Ascaris*-specific IgE levels. Tuberculin induration was found to be inversely related to IgE in patients but not in controls. |
| [65] | Human study in East Ethiopia.<br>*Ascaris lumbricoides, hookworms, Trichuris trichiura, Strongyloides stercoralis, Hymenolepis nana* and *Taenia spp*. | To investigate the effect of intestinal helminths on the immune response to PPD in naturally immunized or BCG-vaccinated individuals | Individuals who received BCG vaccination and were infected with helminths had reduced T cell and PPD skin test responses. Increased T cell proliferation and IFN were associated with improved BCG efficacy following anthelmintic therapy. |
| [67] | An experimental study in Ethiopia.<br>*Schistosoma mansoni* | To investigate whether chronic helminth-infected individuals have reduced efficacy of BCG vaccine compared to uninfected persons | Possibly through attenuation of protective immune responses to mycobacterial antigens and/or by polarizing the general immune responses to the Th2 profile, *S. mansoni* infection reduced the |

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| | | | protective efficacy of BCG vaccination against *Mtb*. |
| [54] | Human study in Ethiopia.<br>*Ascaris lumbricoides, Hookworm, Strongloides stercoralis, Trichuris trichiura, S. mansoni* and *Enterobius vermicularis* | To study the prevalence of intestinal helminth infections and their association with active TB in TB patients and healthy household contacts | In addition to HIV infection, intestinal helminth infection may be a risk factor for the development of active pulmonary TB This discovery could have significant consequences for the control of tuberculosis in helminth-endemic areas around the world. |
| [30] | Human study in Ethiopia.<br>*Trichuris trichiura, Ascaris lumbricoides, hookworms, Taenia spp, Hymenolepis nana* and *Enterobius vermicularis* | This study tested anti-helminthic medication before BCG vaccination to determine if it could improve BCG vaccination immunogenicity in helminth-infected patients | Chronic worm infection reduced BCG immunogenicity in humans. This was linked to increased TGF-β production but not a better Th2 immune response. |
| [75] | Human study in South Africa.<br>*Ascaris lumbricoides* and *Trichuris trichiura* | To investigate whether helminth infection could affect a child's ability to generate a proper Th1 immune response, which was defined by a positive tuberculin skin test (TST) | Helminth infection/exposure may reduce the immune response to *Mtb* infection. In younger children, being Ascaris IgE-positive significantly reduced the likelihood of being TST-positive, but this effect faded as they grew older. |
| [76] | Human study in Venezuela.<br>*Ascaris lumbricoides* and *Trichuris trichiura* | To investigate the effects of parasite infections, malnutrition and plasma cytokine profiles on tuberculin skin test (TST) positivity | TST positivity was associated with low plasma Th1 cytokine levels in indigenous Venezuelan children with TB contacts and helminth infections. |
| [19] | Animal study in South Africa.<br>*Nippostrongylus brasiliensis (Nb)* | To investigate the impact of acute *Nb*-induced lung damage and long-term parasite lung conditioning on the host's ability | The findings show that early stage *Nb* infection induces a macrophage response that protects against subsequent mycobacterial infection. |

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|---|---|---|---|
| | | to control mycobacterial infection | |
| [77] | Human study in Ethiopia.<br>*Giardia lamblia, Ascaris lumbricoides, Hookworm spp., Strongyloides stercoralis, Trichuris trichuria, Enterobius vermicularis, Taenia spp., Hymenolepis nana, Schistosoma mansoni* or trophozoite stage of *Entamoeba histolytica.* | To diagnose latent *Mtb* infection using the tuberculin skin test (TST) and the IFN-γ release assays in helminth infected school children | The tuberculin skin test should be used with caution in areas where parasitic intestinal infections are common. |
| [78] | Human study in Uganda.<br>*Hookworm, Trichuris trichiura, Hymenolepis nana, Schistosoma mansoni, Ascaris lumbricoides, Hymenolepis nana* and *Schistosoma mansoni* | To determine whether coinfections such as helminths, malaria and HIV modulate the immune system and increase susceptibility to latent tuberculosis infection (LTBI), leading to the persistence of the tuberculosis epidemic | Concurrent helminth, malaria and HIV infections did not affect cytokine responses profile in individuals with LTBI. |
| [79] | Human study in Ethiopia.<br>*Schistosoma mansoni* | To investigate whether maternal helminth infection affects maternal and neonatal immunological function and TB immunity | The combination of early secretory antigenic target 6 (ESAT-6) and culture filtrate protein 10 (CFP-10) elicited a significantly lower IFN-γ response in helminth-positive than in helminth-negative participants. Cord blood mononuclear cells' (CBMCs) IFN-γ response, total IgE and cross-placental transfer of TB-specific IgG were all negatively correlated with maternal helminth infection. |

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|---|---|---|---|
| [17] | Human study in Ethiopia.<br>*Ascaris lumbricoides*<br>*Hookworm spp*<br>*Strongyloides stercoralis*<br>*Trichuris trichiura*<br>*Hymenolepis nana*<br>*Taenia spp.* | To examine the clinical and immunological effects of helminth infection on TB | Asymptomatic helminth infection had a profound influence on the immunological profile of individuals with TB This favored Th2 immune responses such as increased regulatory T cells and IL-5 and IL-10 secreting cells. |
| [80] | Human study in Ethiopia.<br>*Ascaris lumbricoides* | To investigate the clinical and immunological outcomes of patients coinfected with helminths and TB after albendazole treatment | The decrease in eosinophil counts and IL-10 demonstrated that asymptomatic helminth infection considerably impacts host immunity during tuberculosis and can be efficiently reversed with albendazole treatment. Helminth infection has clinical effects on chronic infectious diseases such as tuberculosis, and these effects should be further explored. |
| [81] | An animal study in the USA.<br>*Schistosoma mansoni* | To investigate whether *Mtb*-specific T cell responses can be reversibly impaired by treatment of *S. mansoni* coinfection, without impacting arginase-1-expressing macrophage-mediated TB control | Anthelminthic treatment improved Mtb-specific T cell responses. In TB-infected mice, arginase-1-expressing macrophages in the lung formed granulomas and exacerbated inflammation. |
| [82] | An experimental animal study in USA.<br>*Heligmosomoides polygyrus* | To investigate whether Mtb infection would be modulated in mice with chronic *H. polygyrus* infection | Despite a systemic increase in FoxP3+ T regulatory cells, neither primary nor memory immunity conferred by Mycobacterium bovis BCG vaccination were affected in mice with chronic enteric helminth infection. |

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| [83] | Human study in India.<br><br>*Strongyloides stercoralis* | To investigate whether helminth modulation of cytokine responses in latent TB coinfection is reversible after anthelminthic therapy | In *Strongyloides stercoralis*-latent TB coinfection, anthelmintic therapy reversed the modulation of systematic and TB antigen-stimulated cytokine responses. |

## 6. Effect of Helminth Infection on TB Vaccine

BCG is currently the only TB vaccine available; it celebrated its 100th anniversary in 2021. Alternative vaccines are being developed [84]. The BCG vaccine is still the only option for protection against human TB, and it is inexpensive, safe and widely available. BCG effectiveness against TB, however, varies in the high helminth-burden areas of the world [84]. Children are typically given the BCG vaccine. A review reported that BCG could provide protection against severe forms of TB, including meningitis and miliary [85].

The BCG vaccine is administered to more than 80% of all newborns and babies in countries where it is included in the national childhood immunization program; however, it does not prevent the development of latent tuberculosis or the reactivation of pulmonary disease in adults[86]. BCG has been reported to be less effective in TB-coinfected individuals living in helminth-endemic areas [65]. However, another study reported no difference in BCG vaccination status and tuberculin skin testing (TST) responses in patients with or without TB and helminth coinfection [68].

An Ethiopian study found that helminth infection influenced BCG vaccination outcomes, and PPD-specific cellular immune responses improved in helminth-treated individuals compared to untreated controls (Elias et al., 2001) [65]. Deworming was shown to boost the efficacy of BCG immunization in this randomized experiment [65]. In addition, it was found that the BCG vaccination of PPD-negative individuals in a helminth-infected population in Ethiopia had poor immunogenicity, and they concluded that this was due to a high Th2 bias in immunological responses caused by chronic helminth infection [65].

Furthermore, in another study, *S. mansoni* was found to reduce the protective efficacy of BCG vaccination against *Mtb*, possibly by attenuating protective immune responses to mycobacterial antigens and polarizing general immune responses to a Th2 profile [67]. Th2-like IL-10 responses elicited by intestinal helminths may interfere with Th1-like IFN responses induced by BCG, altering the protective immune response to BCG vaccination [87]. The impact of helminth infection is due to the antigen-specific modification of cell-mediated immunity, and the diminished efficacy could be owing to impaired immune responses to recall antigens [88].

Furthermore, helminth infection during pregnancy has been shown to persist into childhood and shift immunity away from Th1 responses, which are required in TB infection and vaccination [73]. Chronic helminth infections increase susceptibility to TB infections requiring Th1 responses and also lead to impaired efficacy of the BCG vaccine [30, 89]. While there is mounting evidence that helminth prophylaxis could have a role in combating the HIV/AIDS and TB pandemics [90], observational research and randomized controlled trials have not revealed a uniform clinical picture. Deworming programs may help to enhance community-based health measures such as proper sanitation, access to clean water and adequate education [91]. More intervention research is required to demonstrate the impact of deworming on tuberculosis disease progression.

## 7. Helminth and TB Coinfection-Immune Mediated Pathology

The typical immune response to helminths, characterized by decreased IFN-γ, reduced T cell proliferation and IL-2 as a result of increased Th2/Treg cytokines, attenuates a potent anti-tuberculosis IFN-γ immune response and therefore uncontrolled TB pathology [15]. Furthermore, the helminth-induced expansion of AAMs and nitric oxide synthase suppression could also contribute to the impaired intracellular killing of TB in macrophages, thereby enhancing TB disease process [15]. In addition, the helminth-induced anergy of cognate and bystander T cells and increased apoptosis further impair TB responses and increase the pathogenesis [89].

## 8. Effect of Deworming during TB-Helminth Coinfection

The effects of deworming can be used to determine the impact of helminth infections. It was shown that the use of anthelminthic drugs to treat patients with helminths resulted in increased T cell proliferation and IFN-γ production of PBMC stimulated with PPD. The study showed that T cell responses to PPD were improved in filarial-infected patients treated with diethylcarbamazine [56,66]. The treatment of helminth-infected patients with albendazole during BCG vaccination increased proliferative and IFN-γ responses to PPD, suggesting that persistent helminth infection during BCG vaccination may contribute to a decreased T cell response to mycobacterial antigens. This meant that removing helminths via anthelminthic treatment would reduce Th2 cell and cytokine inhibitory effects on Th1 responses [92].

Toulza *et al*. found that anthelminthic therapy altered antimycobacterial immune responses in U.K. migrants. Patients with helminth infection had a higher frequency of CD4 + Fox P3 + T cells (Tregs) and a lower frequency of CD4 + IFN-γ + T cells, but these effects were reversed after treatment [69]. Another study in Gabon found that anti-helminth treatment with praziquantel against *Schistosoma* infection resulted in a significant decrease in CD4 + Fox P3 + T cells after treatment [93]. Since helminth infections cause widespread immunological alterations that revert to normal after the helminth infection is eradicated, their role in the interaction between their host and other pathogens could be substantial [94].

From the above, it is apparent that concurrent helminth and TB infections have demonstrated various effects on the host. These reactions could be due to different helminth species, their location in the body, different life cycles, variable (excretory/secretory) E/S products and *Mtb* infection. The virulence and infection route of the mycobacterial strain may also contribute. Some in vitro studies have been reported to have shown that helminth infection affects *Mtb* infection in terms of immune response and disease severity, but the clinical and treatment outcome is unknown, possibly due to underpowered studies, the type or intensity of the infecting helminth and the various methodologies used to detect helminth infection [15].

## 9. Concluding Remarks

Concurrent helminth infection and TB both produce antagonistic immune responses. Helminths have the potential to impair the host's ability to respond to bystander infections such as TB Helminth and TB's spatial overlap may impair the host's ability to respond to mycobacterial conditions. Th1 responses are required for TB immunity, whereas helminths mount an opposing Th2 response, which tends to dominate and thus skew the immune response. Furthermore, chronic helminth infections impair innate and adaptive immune responses to TB and induce immunoregulatory responses, lowering TB immunity even further. However, whether these opposing immune responses in helminth and TB coinfection affect pathological outcomes is unclear.

In helminth-endemic areas, it is suggested that chronic helminth infections reduce the efficacy of BCG, the currently available TB vaccine. There is conflicting evidence regarding the effectiveness of regular anti-helminth medication in the treatment of TB, and this requires further investigation. Clarification of the effect of deworming in concurrent helminth-TB infections may aid in the development of government treatment policies. Since vaccines can prevent TB infection, the co-occurrence of helminths and TB must be considered when developing new vaccines and conducting research on them. Finally, more research is needed to understand better the effects of multicellular coinfecting pathogens on immune responses.

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# CHAPTER THREE: RESEARCH MANUSCRIPT under review – pending *MDPI Microorganisms* - academic editor decision

**"Cytokine responses during Mycobacterium tuberculosis H37Rv and Ascaris lumbricoides co-infection using human THP-1 and Jurkat cells, and a pilot human tuberculosis and helminth co-infection study."**

## Preface to Chapter 3

TB has become the most important infectious disease to resurface globally and has one of the highest mortality rates due to a single infectious agent (Borkow and Bentwich, 2004; WHO, 2022b). TB coinfection with HIV has increased mortality rates (WHO, 2021). Almost a quarter of the global population is infected with TB (WHO, 2022b). In 2020 there were 10 million reported TB cases, with a mortality rate of approximately 1,5 million worldwide (WHO, 2022b). Most TB deaths are said to have occurred in Sub-Saharan Africa (WHO, 2022b). Helminths are also highly prevalent in tropical and subtropical countries, causing chronic infections that can persist for up to twenty years or more, depending on the infecting species (WHO, 2022a). There is a striking overlap between TB and helminth infections, requiring serious attention and interventions (Babu and Nutman, 2016; Kumar *et al.*, 2020).

Chronic helminth infection has been shown to bias the immune response toward Th2, which may subvert the Th1 response to TB and other intracellular pathogens (Resende Co *et al.*, 2007; Verhagen *et al.*, 2012; Chatterjee and Nutman, 2015). Numerous publications have suggested the mechanisms by which persistent human helminth infections modify immune responses to other unrelated viral, bacterial, allergic and autoimmune diseases (Borkow *et al.*, 2000; Maizels *et al.*, 2004). Even though there is a sizable body of research examining the relationships between intestinal parasites and TB, many of these studies are observational and cross-sectional studies with very few longitudinal studies involving large populations, which may boost our understanding of the interaction between helminths and TB (latent or active).

Some studies have shown that the type 2 immune responses that predominate during helminth infection are detrimental to TB immunological responses, vaccine and treatment to vaccine (Elias *et al.*, 2005, 2008; Resende Co and Hirsch, 2006; Abate *et al.*, 2015; Hasanain *et al.*, 2015; Kumar *et al.*, 2020). Other studies found helminths to have no effect on clinical effects on the development and presentation of pulmonary (George *et al.*, 2014; Santos *et al.*, 2019). There are opposing views on whether helminths are beneficial or harmful to their hosts. Some believe it is to protect the host against re-infection, while others suggest it is a parasite-driven mechanism of resisting inflammatory Th1 onslaught or a mix of host-parasite mediated survival strategies (Maizels *et al.*, 2004).

This chapter sought to determine whether there is cytokine dysregulation during coinfections with TB and helminths. The current study aimed to determine the cytokine immune responses to tuberculosis and helminths using *in vitro* and human *ex vivo* analyses. The study hypothesis was that coinfection with tuberculosis and helminths resulted in a reduction of Th1 immune responses to TB and increased Th2 and Treg immune responses in dually infected hosts as compared to single or uninfected individuals. The study was divided into *in vitro* and *ex* vivo arms. The *in vitro* studies used the human monocytic THP-1 and lymphocytic Jurkat cells. These cells were stimulated with *Mtb* (H37Rv) and *Ascaris lumbricoides* excretory-secretory proteins. The human *ex vivo* pilot study included participants with TB and TB plus helminth coinfection.

The resultant research paper was submitted to *MDPI Microorganisms* – manuscript number – **2357988** – pending academic editor decision and titled "**Cytokine responses during Mycobacterium tuberculosis H37Rv and Ascaris lumbricoides coinfection using human THP-1 and Jurkat cells, and a pilot human tuberculosis and helminth coinfection study.**

**Original article**

# Cytokine responses during *Mycobacterium tuberculosis* H37Rv and *Ascaris lumbricoides* costimulation using human THP-1 and Jurkat cells, and a pilot human tuberculosis and helminth coinfection study.

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**Abstract: Background:** Helminth infections are widespread in tuberculosis-endemic areas and are associated with an increased risk of active tuberculosis. In contrast to the pro-inflammatory Th1 responses elicited by *Mycobacterium tuberculosis* (*Mtb*) infection, helminth infections induce anti-inflammatory Th2/Treg responses. A robust Th2 response has been linked to reduced tuberculosis protection. There are several studies that show the effect of helminth infection on BCG vaccination and TB, but the mechanisms remain unclear. **Aim:** To determine the cytokine response profiles during tuberculosis and intestinal helminth coinfection. **Methods:** For the in vitro study, lymphocytic Jurkat and monocytic THP-1 cell lines were stimulated with Mtb H37Rv and *Ascaris lumbricoides* (*A. lumbricoides*) excretory-secretory protein extracts for 24 hrs. and 48 hrs. The pilot human ex vivo study consisted of participants infected with Mtb, helminths, or coinfected with both Mtb and helminths. Thereafter, the gene expression profiles of IFN-γ, TNF-α, Granzyme B, Perforin, IL-2, IL-17, NFATC2, Eomesodermin, IL-4, IL-5, IL-10, TGF-β and FoxP3 in the uninfected controls, TB alone, helminth alone and coinfected groups were determined using RT-qPCR. **Results:** TB-stimulated Jurkat cells had significantly higher levels of IFN-γ, TNF-α, Granzyme B, and perforin compared to unstimulated controls, LPS, *A. lumbricoides*, and plus *A. lumbricoides* TB costimulated cells. IL-2, IL-17, Eomes, and NFATC2 levels were also higher in TB-

stimulated Jurkat cells. TB alone stimulated cells had lower IL-5 and IL-4 levels compared to *A. lumbricoides* alone stimulated and TB plus *A. lumbricoides* costimulated Jurkat and THP-1 cells. Only *A. lumbricoides* stimulated cells had higher IL-4 levels compared to TB plus *A. lumbricoides* costimulated Jurkat and THP-1 cells. TGF-β levels were also lower in TB alone stimulated cells compared to TB plus *A. lumbricoides* costimulated cells. IL-10 levels were lower in TB stimulated Jurkat and THP-1 cells compared to TB plus *A. lumbricoides* costimulated cells. Similar results were noted for the human ex vivo study, albeit with a smaller sample size.

Keywords: *Mycobacterium tuberculosis* H37Rv; *Ascaris lumbricoides* excretory-secretory proteins; Jurkat cells; THP-1 cells; human tuberculosis and helminth co-infection; cytokine gene expression

## 1. Introduction

Tuberculosis (TB) infection is caused by *Mycobacterium tuberculosis (Mtb),* a significant global health challenge and one of the deadliest diseases caused by a single infectious agent [1]. Ten million TB cases and 1,4 million fatalities were reported globally in 2020 [1]. A quarter of the global population is latently infected with TB [1]. A competent immune system contains the TB infection in an asymptomatic/latent state. However, there are underlying factors in 5-10% of hosts that may lead to the development of active TB from latent infection [1, 2].

Helminths infect 1.5 billion people worldwide, and *A. lumbricoides,* the most prevalent helminth, infects an estimated 807 million–1.2 billion people worldwide [3]. There is a significant geographic overlap between TB and helminth infection, particularly in low and middle-income countries (LMICs), with 20-35 per cent of people being co-infected [4]. The impact of helminths on cell-mediated immunity has been the subject of numerous investigations [5–9]. However, it is still unclear if parasite infection is associated with TB activation from a dormant condition to active disease development [5].

An efficient T-helper type 1 (Th1)/ pro-inflammatory response is required to control intracellular Mtb [7, 10]. The Th1/ pro-inflammatory response is characterised by the production of interferon-gamma (IFN-γ), tumour necrosis factor-alpha (TNF-α), and interleukins (IL-1, IL-6 and IL-12) [11]. In contrast, helminths skew the immunity towards a predominant T-helper type 2 (Th2)/ anti-inflammatory and Regulatory (Treg) response, leading to the release of IL-4, IL-5, IL-9, IL-10, IL-13, and transforming growth factor-beta (TGF-β) [10, 12]. These two arms of immune responses counter-regulate each other. Subsequently, helminths have been shown to reduce Bacille Calmette-Guerin (BCG) immunogenicity [13, 14], weaken Mtb-specific Th1 responses, downregulate co-stimulatory molecules [15], induce anergy [16], and reduce treatment response, particularly in pulmonary TB [17, 18].

However, in some studies, helminths were demonstrated to have no impact on human tuberculin skin tests [19] and Mtb infection [20] or the improvement of TB disease management [20]. Therefore, reports on TB immune responses in cases of helminth coinfection are variable and dependent on the infecting parasite and the type of study [8, 9, 21]. Studies involving *Nippostrongylus brasiliensis* (*Nb*) and mycobacterial

mice coinfection yielded divergent findings on Mtb growth control. One study determined that mycobacterial clearance in the lungs of tuberculosis and *Nb*-coinfected mice was not delayed and that the helminth-induced Th2 responses do not exacerbate tuberculosis infection [22]. It was also reported that early-stage *Nb* infection increased macrophage production, which confers protection against subsequent stages of the mycobacterial disease [23]. Conversely, another study reported that mycobacterial burden was higher in tuberculosis and *Nb*-coinfected mice and that these animals had reduced resistance to TB infection [24]. In human studies, *A. lumbricoides* infection was associated with negative tuberculin skin tests in children, suggestive of poor tuberculosis immune response [25, 26].

Therefore, the effect of different helminth species and their antigens on immunity, particularly the macrophages, the primary effector cells in tuberculosis infection, remains unclear. Hence the present study compared the cytokine immune responses in human THP-1, and Jurkat cells stimulated with and without coincident tuberculosis and antigen to *A. lumbricoides* simulate coinfection. The study was also extended to humans to determine the cytokine immune responses in *ex vivo* data. The detailed abbreviations and definitions used in the paper are listed in Table 1.

Table 1. List of abbreviation and acronyms used in the paper.

| **Abbreviation** | **Definition** |
|---|---|
| ATCC | American Type Culture Collection |
| BCG | Bacille Calmette-Guerin |
| Eomes | Eomesodermin |
| ESP | Excretory-secretory protein |
| FoxP3 | Fork head box P3 |
| GAPDH | Glyceraldehyde 3 diphosphate dehydrogenase |
| IFN-γ | Interferon gamma |
| IL | Interleukin |
| LPS | Lipopolysaccharide |
| MHC | Major Histocompatibility Complex |
| *Mtb* | *Mycobacterium tuberculosis* |
| *Nb* | *Nippostrongylus brasiliensis* |
| NFATC2 | Nuclear factor of activated T-cells |
| OADC | Oleic acid albumin dextrose catalase enrichment |
| RT-qPCR | Real time quantitative polymerase chain reaction |
| SA | South Africa |
| TGF-β | Transforming growth factor beta |
| Th1 | T-helper type 1 |
| Th2 | T-helper type 2 |
| TNF-α | Tumour necrosis factor-alpha |
| Treg | Regulatory T cells |

## 2. Materials and Methods

### *2.1. Part 1: In vitro studies:*

#### 2.1.1. Bacterial cultures

The H37Rv strain of *Mtb* (bacterial strain number 25618) was purchased from the American Type Culture Collection (ATCC) through Thistle QA Laboratory Services Cc in Johannesburg, South Africa (SA). H37Rv was cultured to log phase at 37°C in 5% $CO_2$ in Middlebrook 7H9 broth with 0.05% Tween-80 and 10% oleic acid albumin dextrose catalase enrichment (OADC) (Becton Dickinson). Colony-forming units were counted by serial dilutions on Middlebrook agar plates. The H37RV was heat inactivated, therefore there was no MOI of infection. The protein concentration of the heat inactivated H37Rv was determined using the Bradford assay [27] and an optimal concentration of 5μg/ml was used for cell stimulation. Cells were preserved in 1 ml aliquots at -80°C until further use.

#### 2.1.2. Helminth (*A. lumbricoides*) excretory-secretory protein extracts

Whole worm excretory-secretory protein (ESP) extracts of *A. lumbricoides,* kindly donated by Prof William Horsnell, were prepared and supplied by the Division of Immunology, Department of Pathology from the Faculty of Health Sciences at the University of Cape Town, SA. Adult *A. lumbricoides* were obtained from patients from the Red Cross War Memorial Children's Hospital (Cape Town, South Africa), and were used to acquire excretory proteins. The *A. lumbricoides* excretory proteins were obtained by keeping the worms alive at 37°C in Dulbecco modified essential medium with 1% Pen-strep (Thermofisher Scientific, Waltman, Mass), and 1% glucose (wt./vol). The media was collected three times a day. Using Amicon ultra concentrator, extract proteins were concentrated and resuspended in 5ml of phosphate-buffered saline (Merck). All antigens were measured for protein content with a BCA protein estimation kit (Thermofisher Scientific) or by using the Bradford assay previously described [27] and stored at -80°C at a standard concentration of 500 μg/ml until further use.

#### 2.1.3. Cell culture and treatment

Human monocytic THP-1 (lot number: TIB-202) and lymphocytic Jurkat (lot number TIB-152) cells were purchased from the ATCC by Thistle QA Laboratory Services Cc in Johannesburg, SA. The cells were maintained in 25 $cm^3$ cell culture flasks containing Roswell Park Memorial Institute (RPMI) supplemented with 2 mM L-glutamine, 5% HEPES, 100 U/ml penicillin, 100 μg/ml streptomycin, and 10% foetal bovine serum (FBS) at 37°C in a humidified atmosphere of 5% $CO_2$.

Thereafter, the Jurkat and THP-1 cells were aliquoted into the 24 well multi-well plates in 1ml aliquots (>$1x10^6$) and unstimulated or stimulated with either lipopolysaccharide (LPS) (Thermofisher – catalogue number 00-4976-93) , *Mtb* H37Rv or *A. lumbricoides* ESP extracts. The unstimulated cells served as the control group, the LPS stimulated group received 1 mg/ml LPS and served as a positive control, the , *A. lumbricoides* alone group were stimulated with 5μ/ml of *A. lumbricoides* ESP extracts only, the TB alone group were stimulated with 5μg/ml of *Mtb* H37Rv only, and

lastly, the costimulated group were co-stimulated simultaneously ( to emulate real-life situation) with both 5µg/ml of *A. lumbricoides* ESP extracts and 5µg/ml of Mtb H37Rv. Two independent experiments were set up in triplicate. Thereafter, the Jurkat and THP-1 unstimulated/ stimulated cells were incubated for 24-hrs or 48-hrs at 37°C. At the end of the incubation period, the viability of the Jurkat and THP-1 cells was tested, and it was more than 90%. The cells were collected, stored in Trizol® (Invitrogen; Thermo Fisher Scientific, Inc. catalogue 15596026) and stored in the -80$^0$C freezer for RNA extraction and gene expression studies using Quantitative PCR.

2.1.4. Real-Time - Quantitative PCR (RT-qPCR)

RNA was extracted from unstimulated/ stimulated Jurkat, and THP-1 cell lines using the Trizol® reagent (Invitrogen; Thermo Fisher Scientific, Inc. catalogue 15596026) and the Pure Link™ RNA Mini Kit (Thermofisher Scientific, catalogue number 12183018A). The total RNA had to be DNA- free, therefore Pure link® DNase treatment at 80µL per sample was done. The DNase treatment included 88µL10X DNase buffer, 110 µL resuspended DNase and 620µL RNase free water. The prepared DNase mixture was added directly onto the surface of the spin cartridge membrane, incubated at 15 minutes, washed with buffer and then ethanol was added, the cartridge was spun. RNase-free water was added to the spin cartridge and incubated for 1 minute. The spin cartridge was spun with the recovery tube. The RNA preparation was added to a Nanodrop 2000 spectrophotometer (Thermofisher Scientific) to check for purity and concentration. Thereafter, the isolated RNA was reverse transcribed to cDNA using the High-Capacity cDNA Reverse Transcription Kit with RNase Inhibitor (Thermofisher Scientific, catalogue number 4374966), as per the manufacturer's instructions and reaction protocol. The Nanodrop 2000 spectrophotometer (Thermofisher Scientific) quantified the total cDNA. The cDNA samples with an optical density at 260/280 nm ($OD_{260/280}$) >1.8 was used for RT-qPCR.

The Applied Biosystems Quant Studio 5 PCR instrument and software (Thermofisher Scientific, Waltham, MA) were used to determine the expression of the cytokine genes of interest listed in Table 1 in the unstimulated (control cells), tuberculosis stimulated, *A. lumbricoides* stimulated, LPS stimulated, and *A. lumbricoides* and tuberculosis co-stimulated (coinfected) cells.

The PCR master mix was prepared by adding 5 µl PCR-grade water (Thermofisher Scientific, catalogue number 10977023), 0,50 µl FAM-labelled cytokine probe mix (Thermofisher Scientific) (Table 1), 2,50 µl Fast Start 4x probe master mix (Thermofisher, catalogue number A15300) and 2 µl cDNA to make a total of 10 µl per sample. Glyceraldehyde 3-diphosphate dehydrogenase (GAPDH) was used as a housekeeping gene. PCR-grade water (Thermofisher Scientific, catalogue number 10977023), instead of cDNA, was used as a negative control.

**Table 2.** FAM-labelled cytokine probe mix purchased from Thermofisher Scientific and their corresponding catalogue number.

| Cytokine gene | Thermofisher Catalogue number |
|---|---|
| Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (housekeeping gene) | Hs99999905_m1 |
| Interferon-gamma (INF-γ) | Hs00989291_m1 |

| | |
|---|---|
| Tumour necrosis factor-alpha (TNF-α) | Hs00174128_m1 |
| Granzyme B | Hs00188051_m1 |
| Perforin | Hs00169473_m1 |
| Interleukin - 2 (IL-2) | Hs00174114_m1 |
| Interleukin - 17 (IL-17) | Hs01056316_m1 |
| Nuclear factor of activated T-cells 2 (NFATC2) | Hs00905451_m1 |
| Eomesodermin (Eomes) | Hs00172872_m1 |
| Interleukin - 4 (IL-4) | Hs00174122_m1 |
| Interleukin - 5 (IL-5) | Hs99999031_m1 |
| Interleukin -10 (IL-10) | Hs00961622_m1 |
| Transforming growth factor beta (TGF-β) | Hs00234244_ml |
| Fork head box P3 (FoxP3) | Hs01085834_m1 |

The PCR was performed at 95°C for 1 min, followed by 45 cycles comprising denaturation at 95°C for 30 seconds, annealing at 60°C for 30 seconds and extension at 72°C for 30 seconds. All PCR reactions were run in duplicate. Data were collected using the Applied Biosystems Quant Studio 5 V.2.3 software (Thermofisher Scientific, Waltham, MA).

Serial dilutions of pooled cDNA synthesised from the total RNA were performed for each target gene and GAPDH, which served as standard curves for quantitative analysis, ranging from 1ng/μL to 1000ng/μL. Gene expression results were depicted as the expression of the gene of interest divided by the expression of GAPDH.

*2.2. Part B: Human ex-vivo experiment*

Sample size determination:

$n = Z^2(pq)/e^2$

$= 1,96(29,2)(100-p)/25$

$= 1,96(29,2)(70,8)/25$

$= 1,96(2067,36)/25$

$= 318$

The Th-1, Th-17 and Treg cytokine gene and transcription factor's transcription levels study were also piloted for human ex-vivo experiments to compare the human and in vitro cytokine profile results. The current analysis is a sub-study of a previously described cohort of 414 individuals recruited from 6 primary healthcare clinics in a peri-urban, poor settlement in the eThekwini district of KwaZulu-Natal [28]. In this study, cytokine analysis was undertaken for 164 participants; of those, 96 were HIV infected and had to be excluded (because the focus of the study was TB only and not HIV. Another study on HIV on the same cohort was conducted separately and data had already been published), leaving 68 eligible participants. Thereafter, the eligible individuals were subdivided into uninfected controls [no helminth and TB (GeneXpert negative)] (n = 18), helminth only infected (n = 35), TB (GeneXpert positive, recently diagnosed, first episode infection and not on treatment) only infected (n = 6), and TB and helminth co-infected (n = 6) groups. Intestinal helmith species identified included *A. lumbricoides* which

caused the majority of parasite infections, *Schistosoma spp, Taenia spp, Strongyloides spp, Trichuris trichiuria, Enterobius vermicularis* and protozoa such as *Entamoeba coli and Hymenolepis spp* [29].

Stool samples were collected for microscopical detection of helminth eggs /larvae using the Kato-Katz and Mini Parasep methods. Microscopy results can be inaccurate if the sample contains few egg or because of variation in egg excretion e.g. if the host is infected by male parasites. Furthermore because the excretion of eggs depend on the individual's immune response, genetic and environmental factors. Blood samples were also collected for parasite serology (Ascaris-specific IgE and IgG4) to improve the sensitivity and specificity of parasite detection and to indicate any exposure (past or present) to parasite infection (Mpaka-Mbatha et al., 2023). TB diagnosis and confirmatory results were obtained from the district hospital laboratory that services the clinics where participants were recruited. The sputum was analysed using the GeneXpert Infinity 48s (Catalog number: Infinity-48).

Blood samples collected from the recruited participants were also stored in Trizol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.) at -80$^0$C for RNA extraction and RT-qPCR-based gene expression studies as described for the *in vitro* experiments above.

### *2.3. Statistical analysis*

A standard curve method was used to calculate gene expression, whereby the the value of the target gene was divided by the value of the housekeeping gene (GAPDH). GAPDH was validated as the most suitable reference gene due to PCR efficiency, and based on literature. Values were expressed as medians. All cytokine gene expression data were analysed using GraphPad Prism 5 (GraphPad Software, Inc., San Diego, CA, USA) statistical software package. For the *in vitro* and human *ex vivo* studies, analysis of variance (ANOVA) or the Kruskal-Wallis test with Tukey or Dunn's Multiple Comparison was used to assess for statistical significance in cytokine gene expression profiles between the different groups (uninfected/ unstimulated controls, helmith alone, TB alone infected/ stimulated and coinfected/ costimulated groups). Thereafter, the Mann-Whitney or Student's t-test was used to calculate the p-value between the two groups. All data presented in Figure 1 – 9 are expressed as the median and interquartile range. A $p < 0.05$ was considered statistically significant.

## 3. Results

### *3.1. Part 1: In vitro study*

Profiling of cytokine and transcription factor gene transcription levels was performed using THP-1 and Jurkat cells to investigate whether TB stimulation would upregulate pro-inflammatory and Th1 cytokines and whether *A. lumbricoides* coinfection would downregulate these. Furthermore, it was aimed to determine whether *A. lumbricoides* would upregulate Th2 and regulatory cytokines.

#### 3.1.1. Th1/pro-inflammatory immune responses.

Cytokine gene transcription levels levels in unstimulated and stimulated human cell lines are summarised in the figures below, showing a significant increase of Th1/proinflammatory cytokine genes after TB stimulation.

IFN-$\gamma$ and TNF-$\alpha$ (at both 24 and 48 h stimulation time points), granzyme B (24 h stimulation only) and perforin (48 h stimulation only) levels were significantly higher in the TB-alone stimulated Jurkat cells compared to the unstimulated controls, LPS- and *A. lumbricoides*-alone stimulated Jurkat cells, and *A. lumbricoides* plus TB-costimulated Jurkat cells ($p < 0.0001$) (Figure 1). Similar results were noted for the THP-1 stimulated cells, apart from perforin, where similar findings were noted at 24 h and 48 h (Figure 2). IL-2, IL-17, Eomes, and NFATC2 (at both 24 and 48 h stimulation) were significantly higher in TB-alone stimulated Jurkat cells compared to the unstimulated controls, LPS and *A. lumbricoides*-alone stimulated Jurkat cells, and TB plus *A. lumbricoides*-costimulated Jurkat cells ($p < 0.0001$) (Figure 3). Similar findings resulted from tests on THP-1 cells ($p < 0.0001$) (Figure 4).

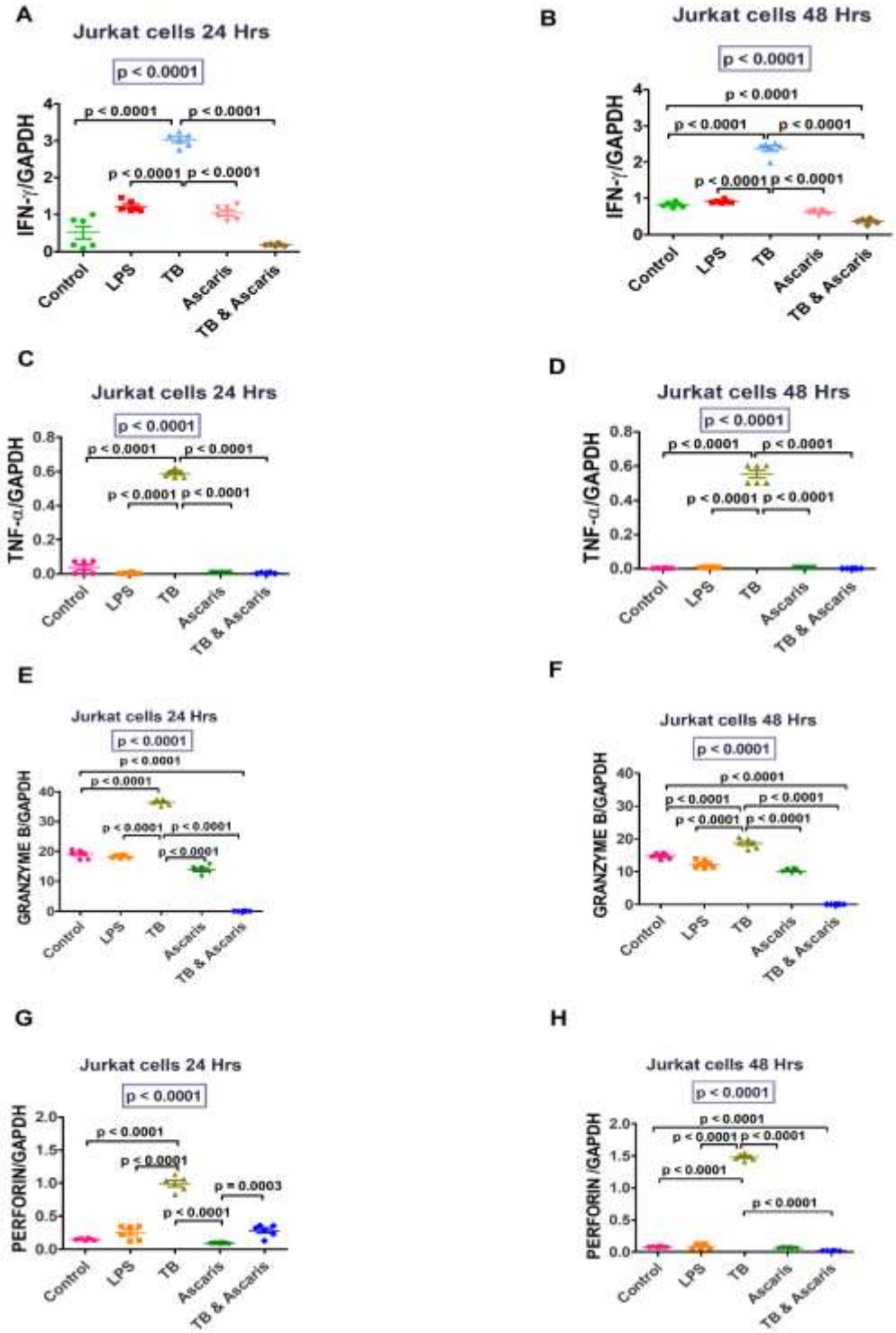



**Figure 1.** Transcription levels data for IFN-γ, TNF-α, granzyme B, and perforin are shown for Jurkat cells at 24 and 48 h time points. The unstimulated control cells and LPS-stimulated cells were negative and positive controls, respectively. **(A)** IFN-γ levels for Jurkat cells stimulated for 24 h, **(B)** IFN-γ levels for Jurkat cells stimulated for 48 h, **(C)** TNF-α levels for Jurkat cells stimulated for 24 h, **(D)** TNF-α levels for Jurkat cells stimulated for 48 h, **(E)** granzyme B levels for Jurkat cells stimulated for 24 h, **(F)** granzyme B levels for Jurkat cells stimulated for 48 h, **(G)** perforin levels for Jurkat cells stimulated for 24 h, and **(H)** perforin levels for Jurkat cells stimulated for 48 h. The p value in box denotes the overall

p value for all the results in that particular figure. The p value on the significance lines between the groups denotes the value among groups.

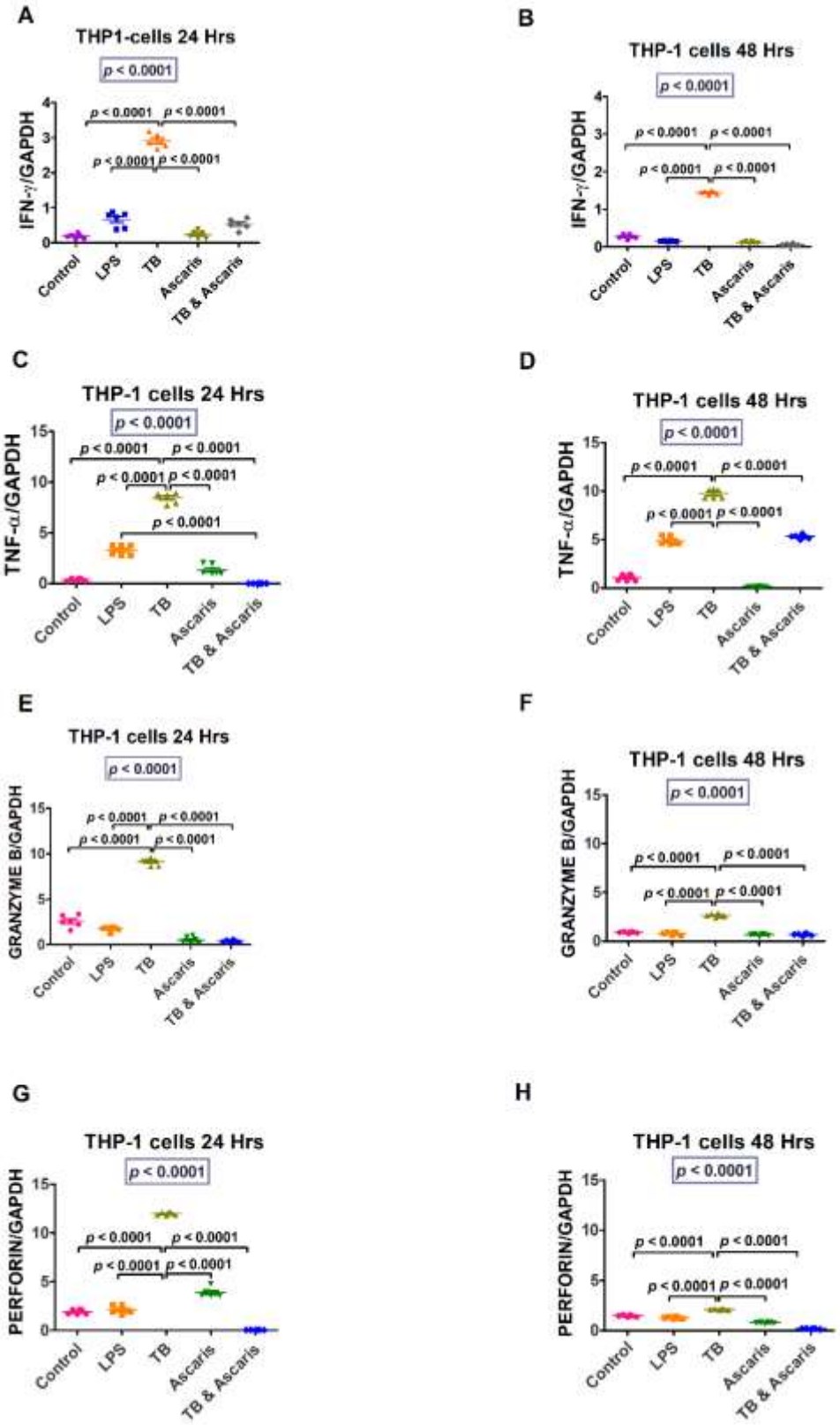


**Figure 2:** Transcription level data for IFN-γ, TNF-α, granzyme B, and perforin are shown for THP-1 cells at 24 and 48 h time points. The unstimulated control cells and LPS-stimulated cells were negative and positive controls, respectively. **(A)** IFN-γ levels for THP-1 cells stimulated for 24 h, **(B)** IFN-γ levels for THP-1 cells stimulated for 48 h**, (C)** TNF-α levels for THP-1 cells stimulated for 24 h, **(D)** TNF-α levels for THP-1 cells stimulated for 48 h, **(E)** granzyme B levels for THP-1 cells stimulated for 24 h, **(F**) granzyme B levels for THP-1 cells stimulated for 48 h, **(G)** perforin levels for THP-1 cells stimulated for 24 h, and **(H**) perforin levels for THP-1 cells stimulated for 48 h. The p value in box denotes the overall p value for all the results in that particular figure. The p value on the significance lines between the groups denotes the value among groups.

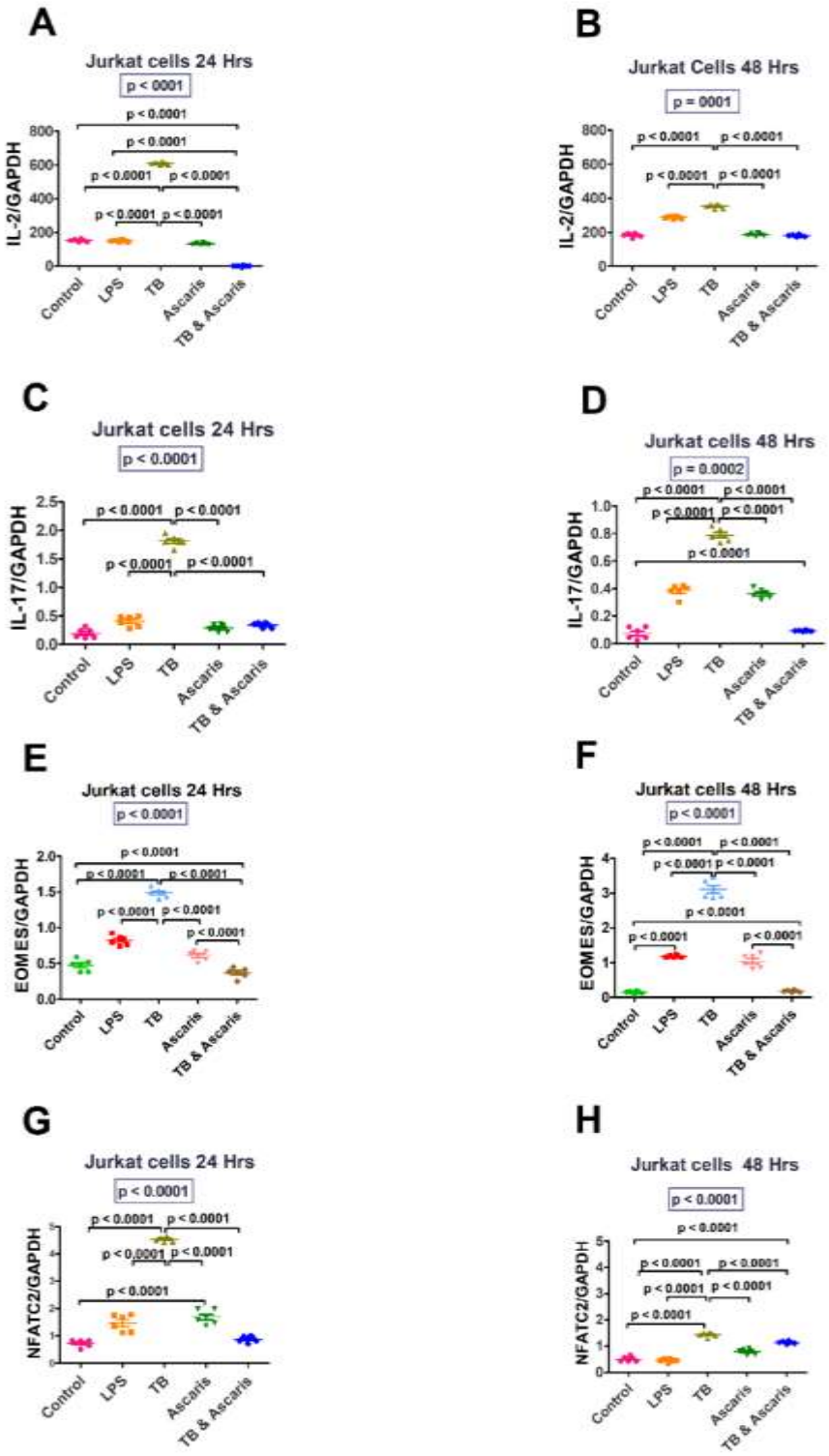



**Figure 3.** Jurkat cell line responses for Th1/pro-inflammatory (IL-2 and IL-17) and transcription factors (Eomes and NFATC2) at 24 and 48 h time points. The unstimulated control cells and LPS stimulated cells were negative and positive controls, respectively. **(A)** IL-2 levels for Jurkat cells stimulated for 24 h, **(B)** IL-2 levels for Jurkat cells stimulated for 48 h, **(C)** IL-17 levels for Jurkat cells stimulated for 24 h, **(D)** IL-17 levels for Jurkat cells stimulated for 48 h, **(E)** EOMES

levels for Jurkat cells stimulated for 24 h, **(F)** EOMES—transcription levels for Jurkat cells stimulated for 48 h, **(G)** NFATC2 levels for Jurkat cells stimulated for 24 h, and **(H)** NFATC2 levels for Jurkat cells stimulated for 48 h. The p value in box denotes the overall p value for all the results in that particular figure. The p value on the significance lines between the groups denotes the value among groups.

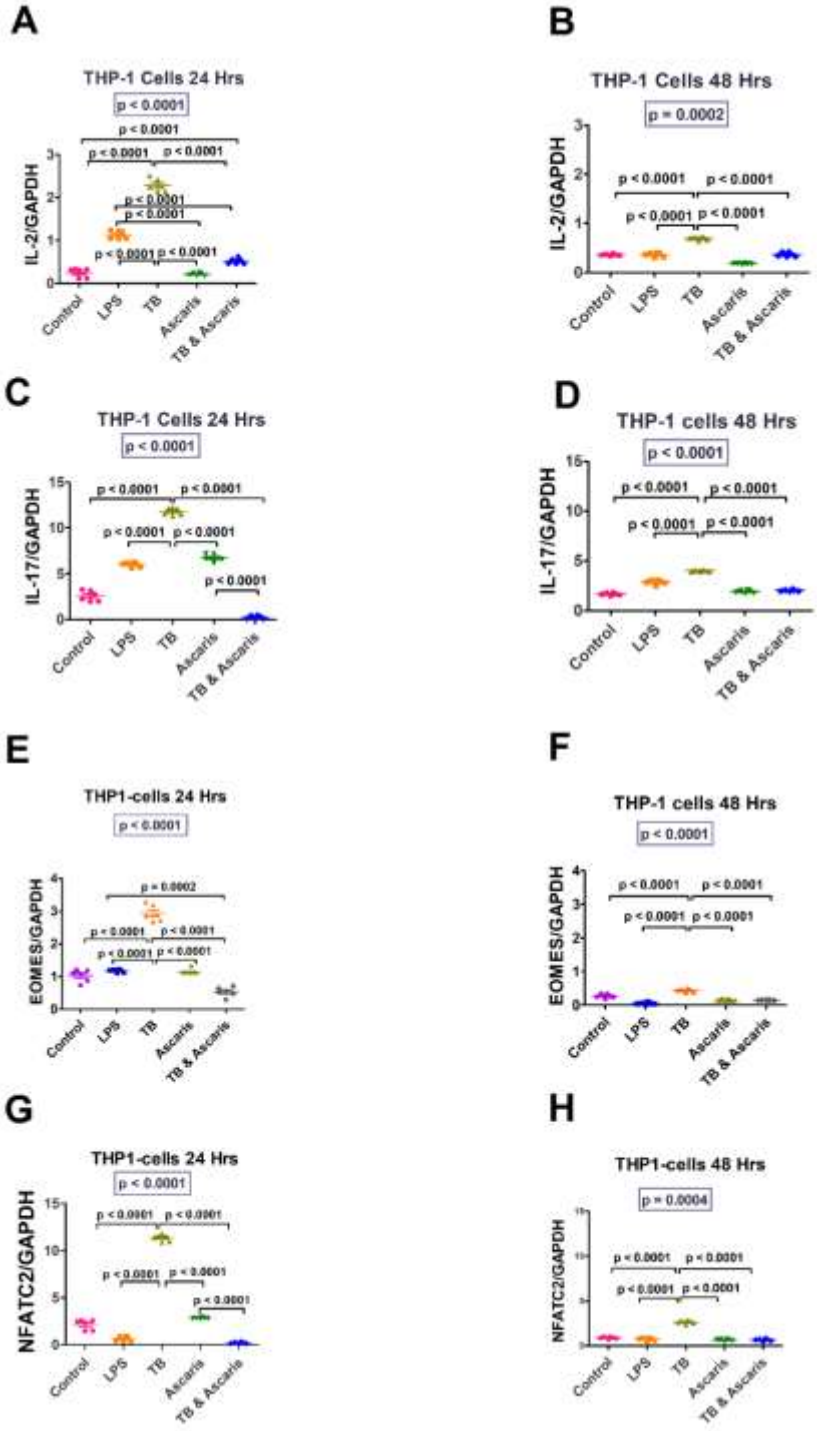
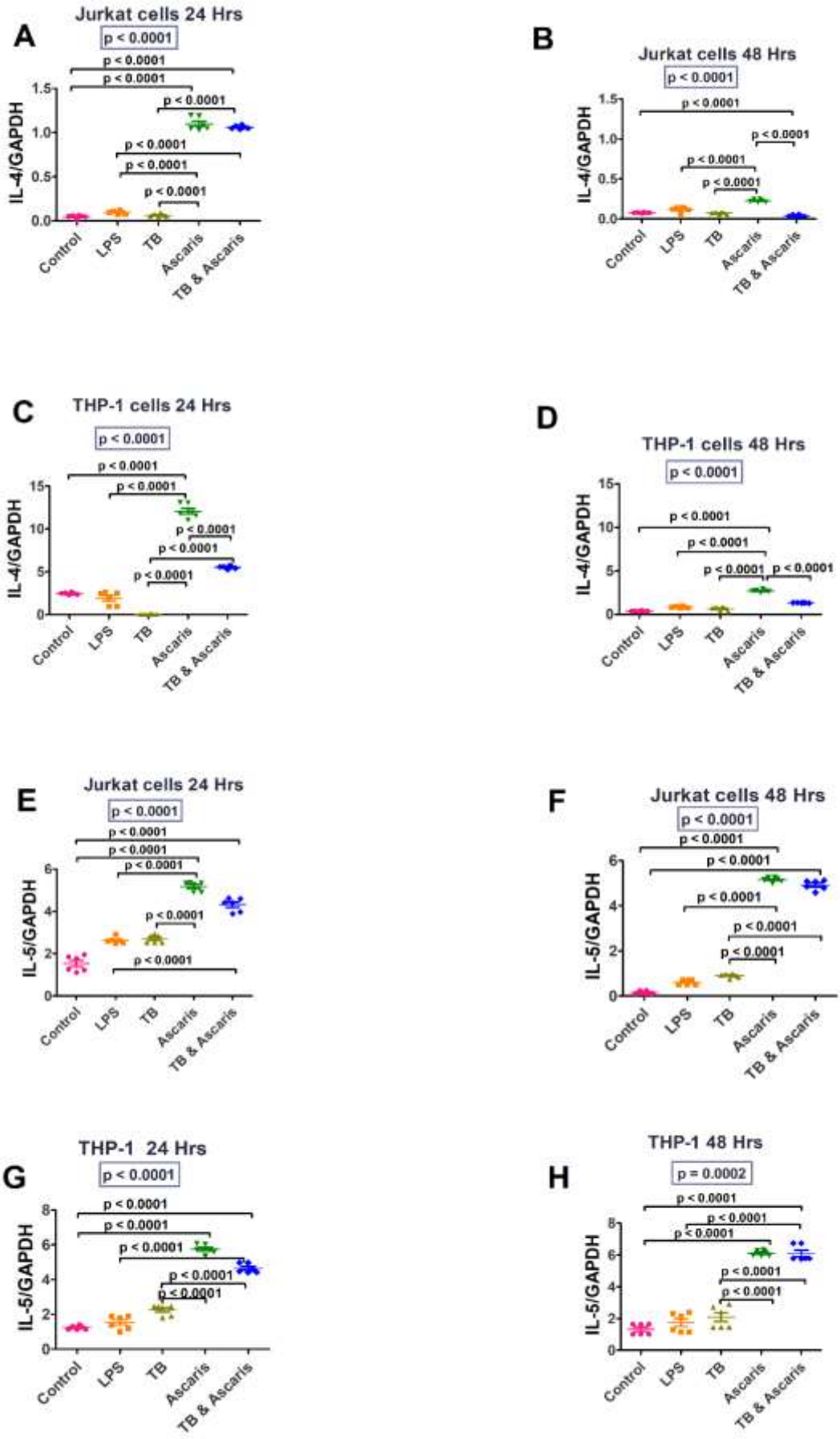


**Figure 4.** THP-1 cell line responses for Th1/pro-inflammatory (IL-2 and IL-17) and transcription factors (Eomes and NFATC2) at 24 and 48 h time points. The unstimulated control cells and LPSstimulated cells were negative and positive controls, respectively. **(A)** IL-2 levels for THP-1 cells stimulated for 24 h, **(B)** IL-2 levels for THP-1 cells stimulated for 48 h, **(C)** IL-17 levels for THP-1 cells stimulated for 24 h, **(D)** IL-17—transcription levels for THP-1 cells stimulated for 48 h, **(E)** EOMES levels for THP-1 cells stimulated for 24 h, **(F)** EOMES levels for THP-1 cells stimulated for 48 h, **(G)** NFATC2 levels for THP-1 cells stimulated for 24 h, and **(H)** NFATC2 levels for THP-1 cells stimulated for 48 h. The p value in box denotes the overall p value for all the results in that particular figure. The p value on the significance lines between the groups denotes the value among groups.

### 3.1.2. Th2/Anti-Inflammatory, Immune Responses

Type 2 cytokine responses after stimulation of cell lines in Figure 5 show that both IL-4 and IL-5 were increased after *A. lumbricoides*-antigen stimulation of both cell lines. IL-5 (24 and 48 h stimulation) levels were significantly lower in the TB-alone stimulated cells compared to the *A. lumbricoides*-alone stimulated and TB plus *A. lumbricoides* costimulated Jurkat and THP-1 cells, which had similar transcription levels ($p < 0.0001$). Similar findings were noted for IL-4; however, the *A. lumbricoides*-alone stimulated cells had significantly higher IL-4 levels compared to the TB plus *A. lumbricoides*-costimulated Jurkat (48 h stimulation) and THP-1 cells (24 and 48 h stimulation) ($p < 0.0001$).


A
Jurkat cells 24 Hrs
p < 0.0001
IL-4/GAPDH
Control
LPS
TB
Ascaris
TB & Ascaris
B
Jurkat cells 48 Hrs
C
THP-1 cells 24 Hrs
D
THP-1 cells 48 Hrs
E
Jurkat cells 24 Hrs
IL-5/GAPDH
F
Jurkat cells 48 Hrs
G
THP-1 24 Hrs
H
THP-1 48 Hrs
p = 0.0002

2372

**Figure 5.** IL-4 and IL-5 responses in TB, *A. lumbricoides*, and dually stimulated Jurkat and THP-1 cells at 24 and 48 h time points. **(A)** IL-4 levels for Jurkat cells stimulated for 24 h, **(B)** IL-4 levels for Jurkat cells stimulated for 48 h, **(C)** IL-4—levels for THP-1 cells stimulated for 24 h, **(D)** IL-4 levels for THP-1 cells stimulated for 48 h, **(E)** IL-5 levels for Jurkat cells stimulated for 24 h, **(F)** IL-5 levels for Jurkat cells stimulated for 48 h, **(G)** IL-5 levels for THP-1 cells stimulated for 24 h, and **(H)** IL-5—transcription levels for THP-1 cells stimulated for 48 h.

### 3.1.3. Regulatory cytokines

Figure 6 illustrates regulatory cytokine transcription levels in the cell lines. In both Jurkat and THP-1cells, both *A. lumbricoides* and *A. lumbricoides* plus TB stimulation significantly increased TGFβ and IL-10 transcription levels at 24 h and remained high at 48 h in *A. lumbricoides*-stimulated cells. FoxP3 was increased in both *A. lumbricoides* and TB plus *A. lumbricoides* stimulation in both cell lines and at both time points (24 and 48 h). TGF-β levels were significantly lower in the TB-alone stimulated Jurkat cells (24 h stimulation) compared to the TB plus A. lumbricoides-costimulated cells, however, the opposite trend was observed for THP-1 cells (24 h stimulation) ($p < 0.0001$). In contrast, no significant differences were noted in Jurkat and THP-1 cells (48 h stimulation) between the TB- alone stimulated and TB plus *A. lumbricoides*-costimulated cells. IL-10 levels were significantly lower in the TB-stimulated Jurkat (24 and 48 h stimulation) and THP-1 (24 h stimulation) cells compared to the TB plus *A. lumbricoides*-costimulated cells ($p < 0.0001$). FoxP3 levels were also significantly lower in the TB-alone stimulated Jurkat and THP-1 cells (24 and 48 h stimulation) in comparison to the TB plus *A. lumbricoides*-costimulated cells ($p < 0.0001$). (Figure 6). The p value in box denotes the overall p value for all the results in that particular figure. The p value on the significance lines between the groups denotes the value among groups.

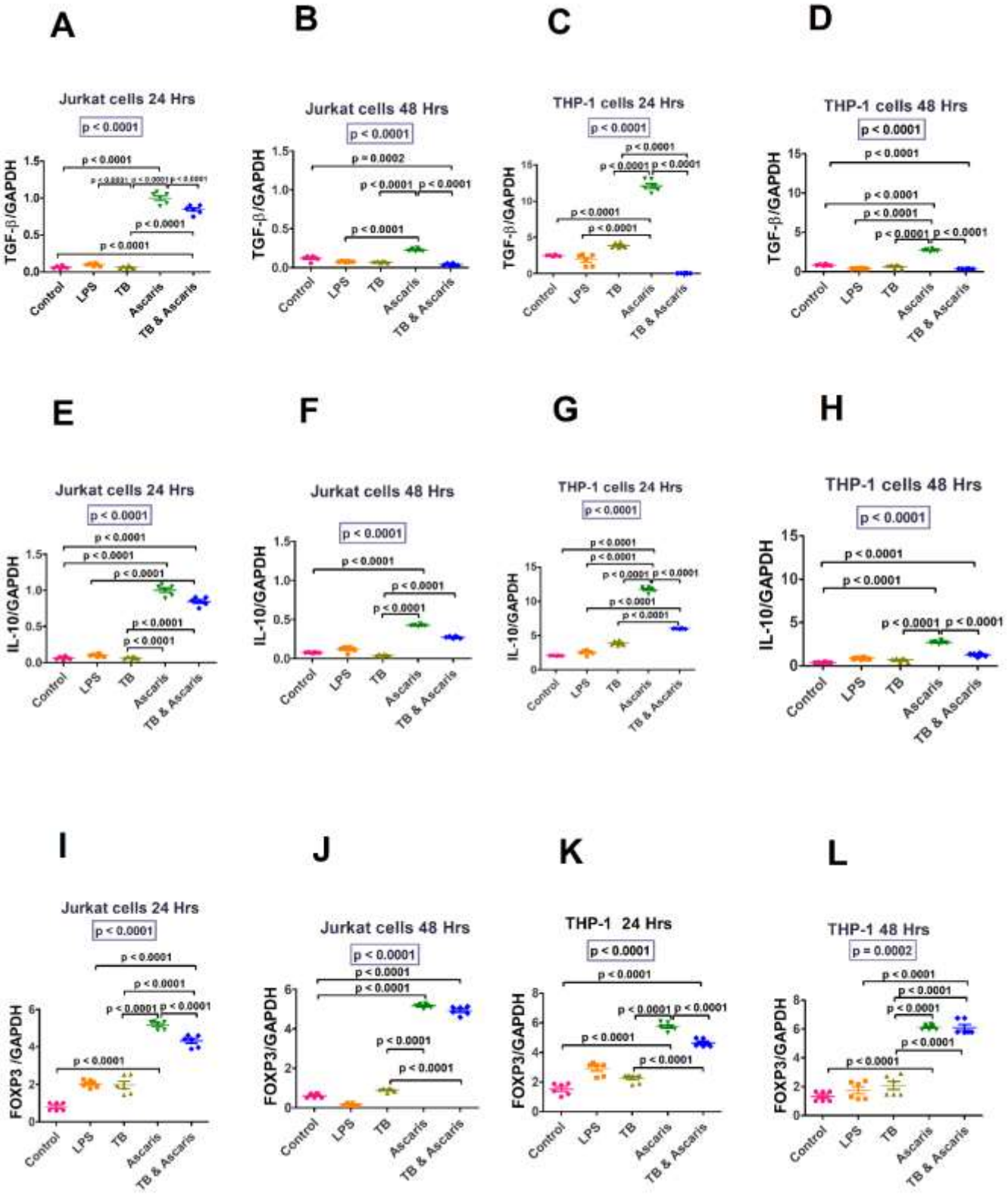



**Figure 6**. Jurkat and THP1 cell responses for regulatory cytokines (TGF-β, IL-10, and FoxP3) at 24 and 48 h time points. The unstimulated control and LPS-stimulated cells were used as negative and positive controls, respectively. **(A)** TGF-β levels for Jurkat cells stimulated for 24 h, **(B)** TGF-β levels for Jurkat cells stimulated for 48 h, **(C)** TGF-β levels

for THP-1 cells stimulated for 24 h, **(D)** TGF-β levels for THP-1 cells stimulated for 48 h, **(E)** IL-10 levels for Jurkat cells stimulated for 24 h, **(F)** IL-10 levels for Jurkat cells stimulated for 48 h, **(G)** IL-10 levels for THP-1 cells stimulated for 24 h, **(H)** IL-10 levels for THP-1 cells stimulated for 48 h, **(I)** FoxP3 levels for Jurkat cells stimulated for 24 h, **(J)** FoxP3 levels for Jurkat cells stimulated for 48 h, **(K)** FoxP3 levels for THP-1 cells stimulated for 24 h, **(L)** FoxP3 levels for THP-1 cells stimulated for 48 h. The p value in box denotes the overall p value for all the results in that particular figure. The p value on the significance lines between the groups denotes the value among groups.

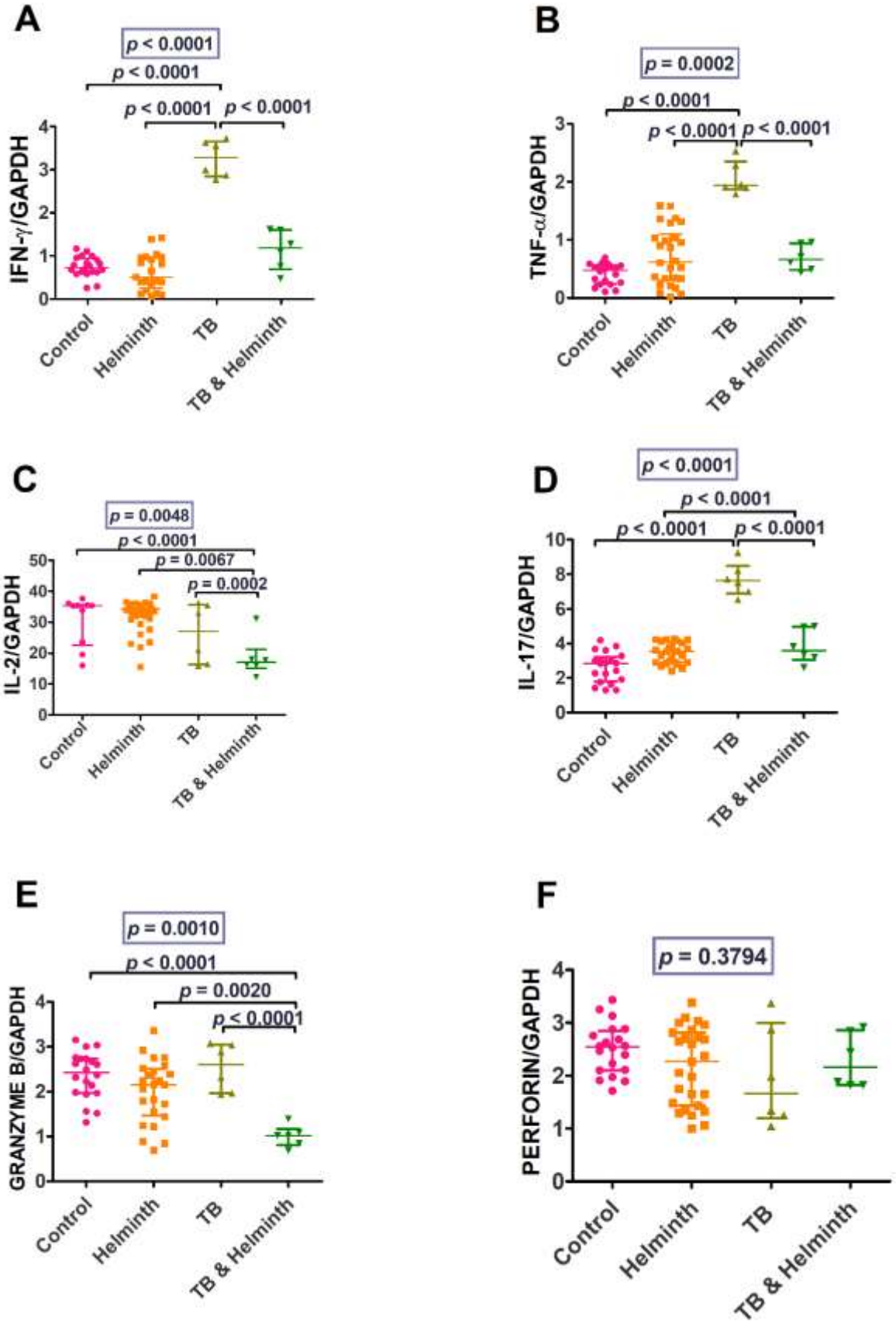
### *3.2. Part 2: Human ex-vivo experiment results*

A total of 414 participants were recruited in the main study [29]; of those, a subpopulation of 164 were eligible for cytokine gene transcription level analysis, based on blood sample availability. However, 96 were HIV-infected and were excluded, leaving 68 eligible participants. Of the eligible participants, 18 were uninfected and were used as controls; 35 were helminth-infected (24 were infected with *A. lumbricoide*s, 3 Trichuris trichiura, 3 Taenia spp., 3 Schistosoma spp., and 2 had Stronglyloides spp.), 6 had TB, and another 6 had TB and helminth (3 had *A. lumbricoides*, 1 Schistosoma spp., 1 Trichuris trichiura and 1 with Taenia spp.) coinfection.

Regardless of the small sample sizes for these two groups, the Th1/pro-inflammatory cytokines (IFN-γ, TNF-α, granzyme B, IL-2, and IL-17), critical cytokines for TB, were significantly higher among the TB-alone infected individuals compared to the uninfected controls and helminth-infected groups (Figure 5). In the presence of helminth and TB coinfection, these cytokines were decreased, although there was no significant difference noted between the coinfected group and the TB- alone infected group, except for granzyme B, where the TB and helminth-coinfected group had lower levels compared to the TB-alone infected group (Figure 7).

Eomes and NFATC2 were significantly higher in the control group compared to the coinfected group. The coinfected group also had lower Eomes and NFATC2 levels compared to the TB-alone infected and helminth-alone infected groups (Figure 8).

IL-4 and IL-10 responses were variably increased in the helminth-infected individuals. TGF-β levels were variably increased in the controls and decreased in TB-alone infected and the helminth and TB-coinfected individuals. FoxP3 levels also differed between the controls and the TB-alone infected groups and between the helminth-alone infected and TB-alone groups. The low number of TB-infected individuals resulted in even lower numbers of the coinfected groups, thus making statistically valid analytical comparisons difficult (Figure 9).


A
p < 0.0001
p < 0.0001
p < 0.0001
p < 0.0001
IFN-γ/GAPDH
Control
Helminth
TB
TB & Helminth
B
p = 0.0002
p < 0.0001
p < 0.0001
p < 0.0001
TNF-α/GAPDH
C
p = 0.0048
p < 0.0001
p = 0.0067
p = 0.0002
IL-2/GAPDH
D
p < 0.0001
p < 0.0001
p < 0.0001
p < 0.0001
IL-17/GAPDH
E
p = 0.0010
p < 0.0001
p = 0.0020
p < 0.0001
GRANZYME B/GAPDH
F
p = 0.3794
PERFORIN/GAPDH

2466
2467

**Figure 7.** I Figure 7. IFN-γ, TNF-α, IL2, IL-17, perforin, and granzyme B ex vivo gene transcription levels data. **(A)** IFN-γ levels, **(B)** TNF-α levels, **(C)** IL-2 levels, **(D)** IL-17 levels, **(E)** granzyme B levels, and **(F)** perforin levels.

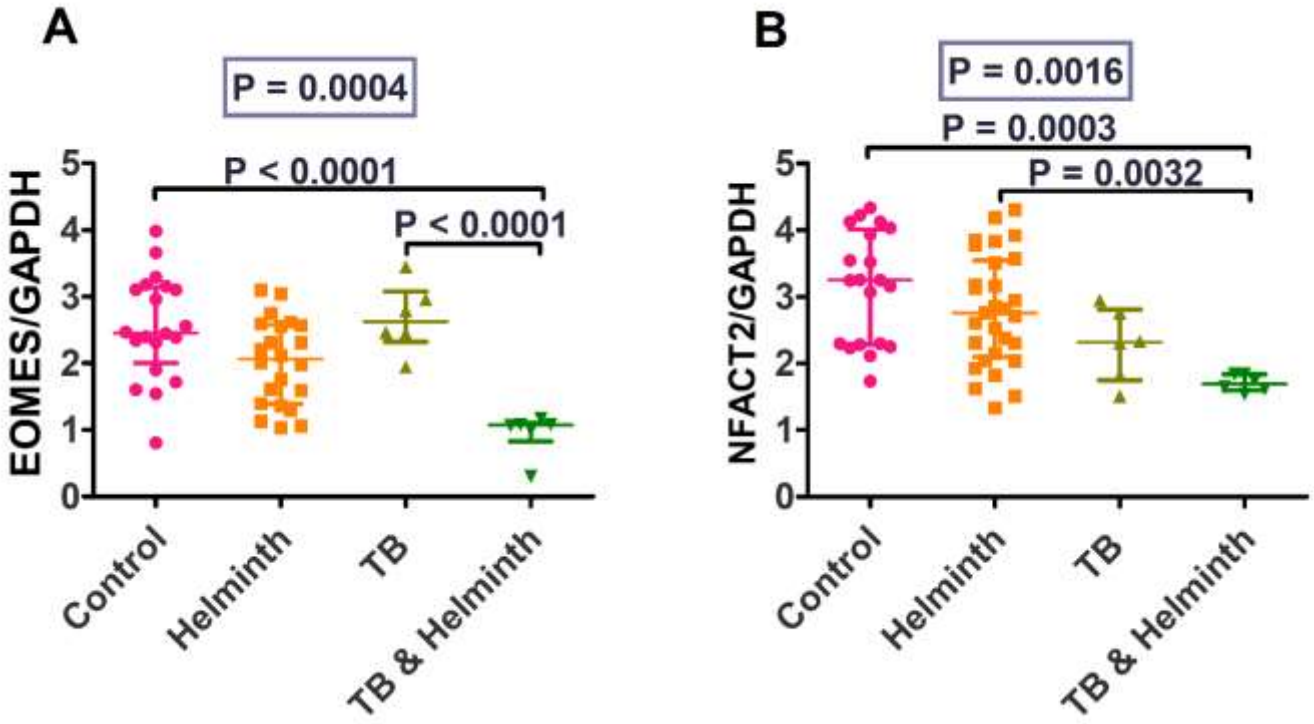



**Figure 8.** Eomes and NFATC2 gene transcription levels values**. (A)** Eomes levels, and **(B)** NFATC2 levels. The p value in box denotes the overall p value for all the results in that particular figure. The p value on the significance lines between the groups denotes the value among groups.

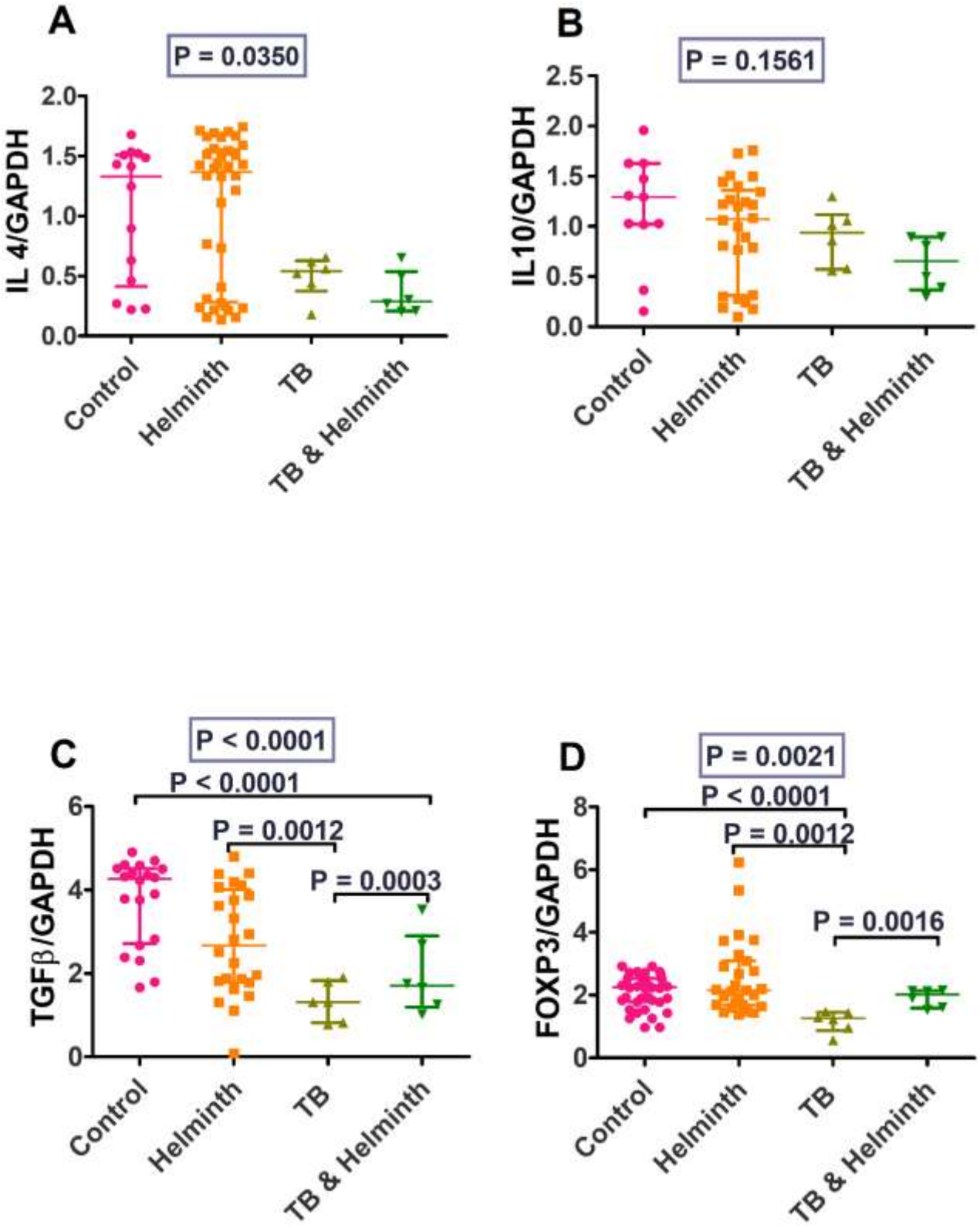



**Figure 9.** IL-4, IL-10, TGF-β and FoxP3 gene transcription levels values**. (A)** IL-1 levels, **(B)** IL-10 levels, **(C)** TGF-β levels, and **(D)** FoxP3 levels. The p value in

box denotes the overall p value for all the results in that particular figure. The p value on the significance lines between the groups denotes the value among groups.

IFN-γ, TNF-α, and IL-17 were highest in TB-infected group (albeit, there were only six individuals). Perforin was similar across all groups, while granzyme B levels differed between the control and coinfected groups ($p < 0.0001$), between the helminthinfected and coinfected groups ($p = 0.0020$), and between TB-infected and coinfected groups ($p < 0.0001$). IL-2 levels differed between the control and the TB plus helminthcoinfection group ($p < 0.0001$) and also between the helminth-infected and coinfected group ($p = 0.0067$).

Eomes levels were higher in the controls than in the TB/helminth co-infected ($p < 0.0001$) and higher in the TB-infected compared to the coinfected individuals ($p < 0.0001$). NFATC2 levels were significantly higher among the controls compared to the coinfected individuals ($p = 0.0003$) and higher in the helminth-infected than in the TB/helminth-coinfected individuals ($p = 0.0032$).

IL-4, IL-10, and TGF-β were higher among uninfected controls and helminth-infected individuals (albeit there was a wide distribution in values) compared to the TB-alone infected and coinfected groups (albeit there was a small sample size). TGF-β was lower in the TB-alone infected group and the coinfected group, compared to the controls ($p = 0.0012$ and $p < 0.0001$, respectively). FoxP3 was significantly lower among the TB-infected compared to both the control ($p < 0.0001$) and helminth-infected groups ($p = 0.0012$).

## 4. Discussion

The present study aimed to determine the profile of cytokines after stimulation of monocytic and lymphoid cells with *A. lumbricoides* and TB antigens to assess whether *A. lumbricoides* infection would decrease the Th1/pro-inflammatory cytokines essential for TB control and increase the Th2/ anti-inflammatory and regulatory cytokines. The human *ex vivo* data was also used to determine the cytokine responses during helminth, TB and in cases of helminth/TB coinfection. The Th1 cytokines were increased in TB-stimulated cells/ infected individuals and reduced during coinfection. The Th2 and regulatory cytokines were variably increased in dual infection.

The Th1/pro-inflammatory cytokines, IFN-γ and TNF-α, were upregulated for TB compared to the *A. lumbricoides* and coinfection stimulation. This finding suggests that Th1/pro-inflammatory cytokines are upregulated by TB and reduced in helminth coinfection. These cytokines are produced more in pro-inflammatory conditions such as TB [7]. The cytokine IFN-γ is essential for protective defence against intracellular infections. IFN-γ is a key modulator of macrophage activation in *Mycobacterium tuberculosis* (*Mtb*) infection [29,30].

TNF-α plays a pivotal role in granuloma formation, which is one of the host's defence mechanisms against TB [31]. According to some studies, TNF-α levels are frequently high in individuals with active TB infection [32, 33]. Our analysis also demonstrated a similar pattern.

The molecules involved in the cell-mediated killing of intracellular pathogens in the pro-inflammatory response included granzyme B and perforin. Granzyme is a serine protease present in the granules of cytotoxic lymphocytes. Perforin and granzyme work together to kill infected or target cells by perforating the cell walls leading to disintegration [34]. Natural Killer (NK) and CD8-positive cells primarily produce Granzyme B and perforin [34, 35]. They attack malignant or infected cells and cause them to undergo apoptosis [35]. Granzyme B and Perforin were both increased at 24 hours in the TB-stimulated cells and reduced at 48 hours, suggesting that they induce apoptosis in infected cells during the early stages of infection. However, for granzyme B and perforin it is crucial to note that more experiments such as tunnel assays or flow cytometry should be performed to validate these results.

The increased levels of IL-2, IL-17, eomes, and NFATC2 for both the *in vitro* and *ex vivo* analysis are in keeping with the pro-inflammatory response. IL-2 is produced by Th1 cells, and it stimulates T-cell proliferation, among other functions. In turn, Th1 cells produce IL-2, which has been found to stimulate cytotoxic T lymphocytes and Th1 cells during intracellular pathogen invasion [36, 37]. Compared to uninfected individuals, patients with active TB have been shown to have high IL-2 cytokine levels suggesting that this cytokine plays a protective role [38].

IL-17, an inflammatory cytokine released during the early stages of TB infection, is suggested to increase the synthesis of chemokines that aid in the recruitment of cells essential for granuloma formation [40]. Limiting *Mtb* growth and immunopathology caused by increased IL-17 production requires a balance between Th1 and Th17 immune responses [39]. Overproduction of IL-17 can increase neutrophil recruitment, which can cause tissue damage [39]. A Th1/Th17 balance is required for anti-mycobacterial immunity and immunological disease prevention [39]. Therefore the current study determined that *in vitro* and *ex vivo,* the Th1/ pro-inflammatory responses are higher in TB infection and reduced in helminth and TB/helminth coinfection cases.

Eomes in our study was increased in both the *in vitro* and *ex vivo* experiments in the TB stimulated/ infected group.Eomes was increased since it plays a role in the differentiation of cytotoxic T cells [40] because the cytotoxic T cells promote the killing of infected cells through the release of granzyme B and perforin [40]. Hence, in our study, eomes was increased in both the *in vitro* and *ex vivo* experiments in the TB stimulated /infected group. NFATC2 overexpression aids cell defence against oxidative stress and electrophilic offences by stimulating antioxidative and detoxifying enzyme synthesis [41]. As expected, pro-inflammatory/ Th1 responses were all increased by TB antigen stimulation and decreased during helminth coinfection in our study. The current study suggests that the *A. lumbricoides* effect of lowering the pro-inflammatory/ Th1 cytokine responses to TB could be detrimental to TB control during TB and helminth coinfection.

In the present study, the Th2/ anti-inflammatory cytokines, IL-4 and IL-5, were higher in the helminths and coinfection *in vitro* stimulations compared to TB single stimulation. This is in keeping with the Th2 predominant immune response produced by the extracellular helminths. IL-4 was increased at 24 hours for both cell lines in the *A. lumbricoides* and coinfection stimulation; however, this was not sustained at 48 hours. The upregulation of IL-4 was shown by the significant differences between *A. lumbricoides* stimulated and *A. lumbricoides*/ TB co-stimulation. High IL-

4 downregulates IFN-γ, which may be deleterious for TB control [42]. IL-5- was elevated in the helminth and coinfection group versus the control group. High levels of IL-5 are commonly observed in intestinal helminth and protozoa-infected hosts, and it also induces eosinophilia, another common manifestation of parasite infection [43]. The current study confirmed the association between *A. lumbricoides* and Th2 cytokine responses.

The regulatory cytokines, IL10 and transcription factor FoxP3, were upregulated in the helminths and coinfected cells as opposed to the TB group. The increase is expected in *A. lumbricoides* -treated cells since helminths polarise immunity towards a Th2 and regulatory immune response [14, 44]. Transcription factor , FoxP3, was also high for the current study's *in vitro* and *ex vivo* experiments. This upregulation of FoxP3 in helminths and the coinfected stimulated cells concurs with the study that determined that helminths increase the secretion of TGFβ, which upregulates FoxP3 and promote differentiation of regulatory cells [45]. Regulatory cytokines, such as IL-10, play a suppressive role in regulating immune homeostasis. Hence IL-10 levels are higher in helminth infection since these parasites have mechanisms of evading the immune system to ensure long-term survival within the host [46]. IL-10 and FoxP3 were increased in the *A. lumbricoides* and coinfection group, suggesting that IL-10 and FoxP3 are upregulated by *A. lumbricoides*. Dual infection stimulation was done to elucidate coinfection scenarios and to determine if there is an effect in the up or downregulation of Th1, Th2, and regulatory cytokines. Regulatory cytokines are high during *A. lumbricoides* infection and also in cases of TB/helminth coinfection compared to TB. This may be due to the downmodulation of the immune response to TB.

The present study demonstrated a typical TB response characterized by an increase in inflammatory cytokines such as IFN-γ, TNF-α, IL-2 and IL-17. However, we did not use costimulatory molecules such as anti-CD-28 or anti CD-49 to enhance the stimulation of the Jurkat cells, since they do not possess antigen presenting properties. Therefore, the Jurkat cell response may be suboptimal, due to the exclusion of immuno costimulatory molecules, which is a limitation for this study.

## 5. Study Limitations

The current study was limited by human studies which were compromised by low sample sizes for TB and TB plus helminth coinfected participants. However, the limited analysis mirrored what was found in, *in vitro* experiments, which showed higher pro-inflammatory / Th1 in TB and lower in the coinfected group. The immune responses to helminths were not differentiated to species level since this was a pilot study and some numbers were very small e.g., *Trichuris trichiura* (n=1) and *Taenia spp* (n=1) therefore would not be possible to control for each individual species differences.

A spurious finding was that some levels of cytokine gene expression levels were highest among the uninfected controls, such as perforin, NFATC-2, TGF-β, and IL-10. This may be due to the fact that the uninfected controls were only screened for helminths and TB in the laboratory and could possibly be exposed to other bacterial, viral or other

immune activating factors that could not be detected during the questionnaire administration that was done for the main study. The demographic profile of the participants may attest to the possibility of other immune-activating environmental factors [29]. In addition, as alluded to above, for the *in vitro* studies, the cell culture experiments did not include co-stimulatory molecules to properly represent the *in vivo* antigen presentation and processing.Therefore these results were suboptimal, despite the fact that the main responses typically depicted TB (Th1/inflammatory) and helminth (Th2/Treg) profiles.

As noted above, there are additional tests, such as flow cytometry and tunnel assays that could be performed to validate the increase in granzyme B and perforin. The gene transcription levels of cytokines in stimulated and unstimulated Jurkat and THP-1 cell lines are not directly correlated with its production. Therefore, to validate the gene transcription levels results at protein level, further tests such as ELISA needed to be performed. Flow cytometry for phenotyping the CD4+/CD8 profile for the Jurkat cells line - TIB 152 could unfortunately not be done owing to the limited quantity of the cells because other analyses had to be done for the main study, since the current study was a pilot. Flow cytometry not being done is another limitation for this study.

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## 6. Conclusion

The *in vitro* findings suggest that pro-inflammatory Th1 responses are increased in TB infection and reduced in cases of coinfection. The study also determined that anti-inflammatory Th2 and regulatory cytokines are increased during single helminth infection and TB and helminth coinfection. The *ex vivo* data, although limited by the sample size, also supported the hypothesis that TB increases Th1 immune responses and those helminths have strong Th2 and regulatory cytokines.

**Authorship contribution statement: K**NB: Patient recruitment, development of study procedures, data collection, specimen processing, analysis of data, conceptualisation, visualisation, methodology, writing - original draft preparation. ZLMK: Project development, data collection and analysis, conceptualisation, visualisation, supervision, funding acquisition, writing - reviewing /and editing. RS, PN: data analysis, writing, reviewing and editing MNMM, NN: Specimen processing, data collection, data analysis, writing, reviewing and editing**.**

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**Institutional Review Board Statement: T**he University of KwaZulu Natal Biomedical Research Ethics Committee (BREC) (BREC/00001983/2020) granted ethical approval. This project was a sub-study of a larger project that had already received BREC approval

(BE351/19). The Provincial (eThekwini) and District (KwaZulu Natal) Health authorities approved this study. Throughout the investigation, ethical rules and values such as confidentiality, dignity, respect, autonomy, fairness, nonmaleficence, and beneficence were followed.

**Informed Consent Statement:** Informed consent was obtained from all participants of the study.

**Data Availability Statement: T**he data collected for the current study are available on request from the corresponding author.

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**Declaration of Competing Interest:** The authors declare that they have no known financial or personal affiliations that could have seemed to affect the work reported in this study.

**Disclaimer: T**he writers' views and opinions expressed in this article are their own. They do not necessarily reflect the official stance or position of any authors' affiliated organisations/institutions.

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# CHAPTER FOUR:

## SYNTHESIS AND CONCLUSION

## 4.1 Preamble

In the current chapter, we will summarise the current knowledge of the field of TB and helminth coinfections and then discuss the principal findings of this thesis, identify gaps, highlight limitations and make recommendations.

This project was conducted in South Africa, and it is general knowledge that Africa is plagued by many challenges, including poverty, lack of clean water supply, poor hygiene and sanitation which aggravate a multitude of infectious diseases and inadequate healthcare systems (Maphumulo *et al.;*2019; Water Crisis in South Africa - Greenpeace Africa, 2022). Unfortunately, these issues are exacerbated by an overlapping burden of helminth and TB coinfection, which has far-reaching public health implications yet is currently receiving little attention (Borkow *et al.*,2004). Hence, helminths are classified as one of the NTDs, the most prevalent human infections in Sub-Saharan Africa (Hotez *et al.*,2009). Evidence suggests that the most common NTDs (soil-transmitted helminths and schistosomiasis) have a high degree of regional overlap with TB, and coinfection is common.

The relationship between helminth infections and immunosuppression is complicated. Numerous variables may influence whether helminth infection suppresses, (Elias *et al.*, 2005, 2008; Resende Co and Hirsch, 2006; Abate *et al.*, 2015a; Hasanain *et al.*, 2015; Kumar *et al.*, 2020) , or has no effect (George *et al.*, 2014; Santos *et al.*, 2019) on immunological responses against TB. These determining factors comprise the type of helminths present, the number of infecting parasites, and whether the disease in the human host is past infection or ongoing. These concerns must be investigated further to address the complicated obstacles of helminth-TB coinfection in tuberculosis diagnosis, treatment, and immunisation regimens. The widespread helminth coinfection in areas of high TB incidence in Africa remains an essential element that will decide the immunomodulation generated by the familiar but diverse helminth infections towards host immunity to TB, diagnostic testing, and the efficiency of preventative TB vaccinations.

Helminths are potent stimulators of anti-inflammatory Th2 and Treg immune responses, while intracellular bacterial infections such as TB induce a proinflammatory Th1 response (Maizels *et al.*,2004). Studies on the immunological profile of helminth-TB coinfection yielded

inconclusive results. Some studies suggest that helminths negatively affect the progression of TB (Elias *et al*., 2006; Bates, Marais, and Zumla, 2015; Babu & Nutman, 2016). Most agreed that intestinal helminth infections induce the anti- inflammatory Th2 immune response development, which down-regulates proinflammatory Th1 cells, which play an essential role in the fight against intracellular infections like TB (Monin *et al.*, 2015; Anuradha *et al.*, 2017; Kathamuthu *et al.*, 2018; Bewket *et al.*, 2022). The immunological profile of TB patients has been demonstrated to be significantly impacted by asymptomatic helminth infection, with a substantial bias towards the Th2 type of immune response, including increased regulatory T cells and cells that secrete IL-5 and IL-10 (Abate et al., 2015). Furthermore, cytokines produced by anti-inflammatory Th2 cells, particularly IL-4, inhibit proinflammatory Th1 cytokine formation (Elias *et al.*, 2005; Gillan and Devaney, 2005) whereas pro-inflammatory cytokines, particularly IFN-γ, released by Th1 due to TB infection disrupt anti-inflammatory Th2 production (Romero-Adrian, 2015; Gashaw, 2018). Furthermore, helminths impair innate and adaptive immunity, making individuals more susceptible to various diseases (Chatterjee and Nutman, 2015; Weatherhead *et al.*, 2020). Such immunological profile cross-regulation may also exacerbate TB in helminth-endemic regions. These findings highlight the impact of the high prevalence NTDs on the health outcomes of TB and offer a new opportunity to develop innovative public health interventions and strategies for these diseases. However, other studies have reported contradictory findings on type effects of helminths on TB disease. For example, (Du Plessis *et al.* 2012) illustrated an improvement in TB pathogenesis, shown by increase in lung macrophages of mice coinfected with the helminth-*Nippostrongylus brasiliensis* (*Nb).* The findings showed that early stage *Nb* infection induces a macrophage response that protects against subsequent mycobacterial infection (du Plessis *et al*.,2012). Furthermore, active TB was found to be associated with lower rates of sputum smear positivity in Ethiopian patients with asymptomatic helminth infection, implying that helminth infection had a favourable effect on TB bacterial loads (Abate *et al.*,2015).

Reports on BCG vaccination indicate that helminth-specific immune responses developed during pregnancy remained throughout childhood (Malhotra *et al.*, 1999). As a result, the prenatal sensitisation brought on by helminths steers T cell immunity away from the Th1 IFN-γ responses linked to defence against mycobacterial infection (Malhotra *et al.*, 1999). Another study suggested that in helminth-infected individuals, the poor immunogenicity of BCG immunisation is coupled with increased TGF-β production (Elias *et al.*, 2008). This is crucial

to note for public health in regions of the world, especially in Africa, where there is a high prevalence of TB and inadequate diagnostic resources.

In areas where TB and HIV are endemic, mass deworming may also be a cost-effective strategy for lowering morbidity from helminths and coinfection. Further research is needed because there is insufficient convincing evidence to link negative effects of helminth/TB coinfections on the host's immunity. Additional rigorous investigations are required to provide a comprehensive understanding of the immunological profiles generated by helminth/TB coinfections in endemic areas.

## 4.2 Summary findings of the current study

In South Africa, underprivileged groups that reside in underserved, highly populated areas are more likely to continue to face significant challenges due to coinfection with intestinal parasites and TB. Both tuberculosis and helminths are poverty-related diseases particularly prevalent in impoverished communities. The current study aimed to explore the host immune responses during coexistent helminth and TB infections. However, it should be noted that properly understanding the helminth/TB immune interaction will require a long follow-up, randomised study with a large cohort, with an extensive control for all confounders.

The study had two arms, an *in vitro* and *ex vivo* component. For the human *ex vivo* analysis, the study population was consenting adults (18 years and above) recruited from clinics in a peri-urban area of KwaZulu Natal, South Africa. The objectives for this study were to (i) To appraise published literature on immune responses during coinfections with intestinal helminth and TB, (ii) To investigate the cytokine immune response profiles in lymphocytic Jurkat and monocytic THP-1 cell lines coinfected with H37Rv strain of *Mtb* and *Ascaris lumbricoides* excretory-secretory protein antigens, and (iii) To investigate the cytokine immune response profiles in patients coinfected with intestinal helminths and TB (newly diagnosed GeneXpert positive and not on treatment).

The study hypothesised that participants with a dual infection TB and helminth infection would fare worse in terms of immune response to TB than TB alone, helminth alone or uninfected participants. The current research project is a sub-study of a previously described cohort of 414 individuals recruited from 6 primary healthcare clinics in a peri-urban, disadvantaged settlement in KwaZulu-Natal's eThekwini district (Mpaka-Mbatha *et al.*, 2023). In this study, cytokine analysis was performed on 164 subjects, 96 of whom were HIV positive and had to be excluded, leaving 68 eligible participants. Thereafter, the eligible patients were split into four groups: control group (no helminth and TB infection) (n = 18), helminth and TB co-infected group (n = 6), helminth infected group (n = 6) and TB (recently diagnosed and not on treatment) infected group (n = 6). Despite the few eligible human participants, this arm of the study was supplementing the *in vitro* experiments which was conducted on lymphocytic Jurkat and monocytic THP-1 cells unstimulated, TB of helminth alone stimulated or co-stimulated with H37Rv strain of *Mtb* and *Ascaris lumbricoides* excretory-secretory products.

In the present study, for both the *in vitro* and human *ex vivo* experiments, proinflammatory Th1 cytokines were significantly elevated in the TB-stimulated Jurkat and THP-1 cells and TB infected participants and decreased during TB/ helminth coinfection. Dual infection, on the other hand, significantly increased anti-inflammatory Th2 and regulatory cytokines. This is in agreement with some studies examining the interaction between helminth infection and TB and demonstrated that helminth parasites could reduce immunity against mycobacterial infection (Abate *et al.*, 2015; Simon, 2016; Gashaw, 2018). The study's overall the result indicated that helminths could impair the immune responses to TB and these effects are deleterious to participants coinfected with both helminths and TB. These observations of reduced proinflammatory Th1 and elevated anti-inflammatory Th2 and Treg cytokines among individuals coinfected provided suggestive evidence of a detrimental effect of both infections on the infected host.

The current study demonstrated that although helminths are commonly associated with children in South Africa, they are also prevalent in adults. These findings further emphasise that helminths, which are part of the NTDs, despite the reports of their high prevalence in SA are indeed neglected and that they have adverse effects on TB immune responses. Much attention has been directed on the bidirectional relationship between TB and HIV, their management and

treatment strategies and less on TB/helminth regimes in adults. Deworming programmes in South Africa have mainly focused on children and not on adults despite the evidence of helminths being common in both adults and children living in impoverished conditions. The prevalence of helminths in adults in KZN has been repeatedly shown to be above 30%, which is significant (Kwitshana *et al.*, 2008; Mkhize *et al.*, 2017; Mpaka-Mbatha *et al.*, 2023) and necessitates paying urgent attention to these findings and develop management, preventive, and treatment strategies for helminth and TB coinfection among adults.

## 4.3 Conclusion

Helminths being part of the NTDs are continually being underrated as a serious public health problem, particularly in SA, even though their prevalence has been reported to be above 30% in adults**.** The response to our current study question, "Do helminth infections alter the cytokine immune response during intestinal helminth and TB coinfection?" suggests that helminths do affect the cytokine profile during coinfection. This is based on our finding that stimulation of Jurkat and THP-1 cells with TB H37Rv only, and individuals with TB only, result in increased Th1/proinflammatory cytokines, while dual stimulation with TB H37Rv and Ascaris antigens, as well as dual infection with helminths and TB result in reduction of these cytokines accompanied by an increase in Th2/Treg cytokines. This has not been reported in the KwaZulu-Natal province of South Africa where both TB and helminthiasis are highly prevalent.

## 4.4 Limitations

Firstly, the human *ex vivo* aspect of the study was limited by the small number of eligible participants with TB single infection and TB plus helminth coinfection. Recruitment of TB infected patients proved to be a challenge. Despite the fact that there were many such patients in the recruitment sites, the majority were not willing to participate. This could be linked to the stigma still largely associated with TB (and indirectly with HIV). Secondly, the overall sample size particularly for the human arm of the study is not representative of the population therefore results not generalizable.

Thirdly, we did not assess whether the participants were in the acute or the chronic stages of both TB and helminth infections. Each of these will induce a different type of response during the acute versus chronic stages. For example, helminths induce an inflammatory response during the early acute phase, however during chronic helminth infection the Th2 cytokine, including IL-10, are upregulated (Caldas *et al*., 2008). As a result, it was impossible to determine if the immune responses corresponded to the stage of the disease. Lastly, the *in vitro* study utilised only *Ascaris lumbricoides* excretory-secretory proteins because they were the only species available for analysis. This made it impossible to compare the cell line's immune responses to other helminth species.

## 4.5 Recommendations

This study revealed several areas of inquiry that need more research, including the epidemiological and immunological interaction between TB and intestinal helminth coinfection in susceptible populations. Such data will impact deworming programmes in coinfected individuals. Future research will need to determine whether prior deworming to TB immunisation is the best practice for attaining optimal vaccine response and for which helminth diseases and human population groups this would be advantageous. The scientific understanding of the health advantages of NTD treatment for HIV and TB patients still needs additional investigation, despite the expansion of NTD treatment programmes and the rising body of evidence supporting the positive health impacts of worm treatment. More research will be required to address the social and logistical elements in implementation and the operational challenges that arise from integrating these therapy programmes.

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# List of appendices

# Appendix A

## Ethical clearance letter from Biomedical Research Ethics Committee (BREC) at UKZN

UNIVERSITY OF KWAZULU-NATAL
INYUVESI YAKWAZULU-NATALI

**03 February 2021**

**Mrs Khethiwe Nomcebo Bhengu (218087035)**
**School of Lab Med & Medical Sc**
**Medical School**

**Dear Mrs Bhengu,**

**Protocol reference number: BREC/00001983/2020**
**Project title: Nutritional status and immune responses in patients co-infected with Mycobacterium tuberculosis and intestinal helminths in Kwa-Zulu Natal**
**Degree Purposes: Masters**

**EXPEDITED APPLICATION: APPROVAL LETTER**

A sub-committee of the Biomedical Research Ethics Committee has considered and noted your application.

The conditions have been met and the study is given full ethics approval and may begin as from 03 February 2021. Please ensure that outstanding site permissions are obtained and forwarded to BREC for approval before commencing research at a site.

This approval is subject to national and UKZN lockdown regulations, see (http://research.ukzn.ac.za/Libraries/BREC/BREC_Lockdown_Level_1_Guidelines.sflb.ashx). Based on feedback from some sites, we urge PIs to show sensitivity and exercise appropriate consideration at sites where personnel and service users appear stressed or overloaded.

This approval is valid for one year from 03 February 2021. To ensure uninterrupted approval of this study beyond the approval expiry date, an application for recertification must be submitted to BREC on the appropriate BREC form 2-3 months before the expiry date.

Any amendments to this study, unless urgently required to ensure safety of participants, must be approved by BREC prior to implementation.

Your acceptance of this approval denotes your compliance with South African National Research Ethics Guidelines (2015), South African National Good Clinical Practice Guidelines (2006) (if applicable) and with UKZN BREC ethics requirements as contained in the UKZN BREC Terms of Reference and Standard Operating Procedures, all available at http://research.ukzn.ac.za/Research-Ethics/Biomedical-Research-Ethics.aspx.

BREC is registered with the South African National Health Research Ethics Council (REC-290408-009). BREC has US Office for Human Research Protections (OHRP) Federal-wide Assurance (FWA 678).

The sub-committee's decision will be noted by a full Committee at its next meeting taking place on 09 March 2021.

Yours sincerely,

Prof D Wassenaar
Chair: Biomedical Research Ethics Committee

**Biomedical Research Ethics Committee**
**Chair: Professor D R Wassenaar**
**UKZN Research Ethics Office Westville Campus, Govan Mbeki Building**
**Postal Address:** Private Bag X54001, Durban 4000
**Email:** BREC@ukzn.ac.za

# Appendix B

## Informed consent form for participant recruitment


**UKZN BIOMEDICAL RESEARCH ETHICS COMMITTEE**

**APPLICATION FOR ETHICS APPROVAL**
**For research with human participants (Biomedical)**

**A pilot study on helminthiasis and microbes interactions: macrobiotic control of microbiota and the effects on Human Immunodeficiency Virus and Mycobacterium tuberculosis diseases, immune responses and nutritional status: Human and *in vitro* studies**

**INFORMATION TO PARTICIPANTS AND INFORMED CONSENT**

**Information Sheet and Consent to Participate in Research**

Date:

Hello:

My name is……
from the School of laboratory medicine and Medical Sciences and my contact details are as follows: cell number is……., office number is ……..and email address is or ……

You are being invited to consider participating in a study that involves research on the consequences of being infected by intestinal worms and tuberculosis or the human immunodeficiency virus at the same time. The aim and purpose of this research is to find out if intestinal worm infection interferes with the ability of the immune system to fight the HIV virus or the TB germs, and also to find out if the worm infections also interfere with the body of an infected person to respond appropriately to treatment for tuberculosis and HIV. We will also check if worm infections do not disturb the good germs that are found in your gut for good health. The study is expected to screen approximately seven hundred individuals for worms and enroll three hundred and fifty participants in total, those with and those without intestinal worms plus tuberculosis or HIV. The potential participants will be identified from and recruited from their clinics in Umlazi area. You are therefore invited to participate t-in the study, if you agree, because you are attending this clinic.

During your consultation in the Clinic for your ailments and the reasons for your coming to the Clinic, some tests will be done by the Clinic, as part of your treatment plans. These tests will include, testing for TB using a machine called the Genexpert, the other test may be a chest XRay. The Clinic will also do the HIV test and may also do CD4 counts. If you agree to participate in this research, we are asking you give us permission to get the results of these tests so that we can use them for the research purposes.

If you agree to participate in this research, you will first be given further details of the study, and then if the researchers are satisfied that you fully understand the study, they will ask you to sign this permission letter with which you will be confirming that you agree to participate and you understand what will be expected of you for the study. The researchers will then ask you questions about yourself, your household income, your health status and sources of water for your household and other questions relating to your health. To assess the nutrition status, you will be weighed, and your height taken,

thereafter will be asked about what you had eaten in the past 24 hours. You will then be asked to donate approximately four teaspoons of blood. These blood samples will be taken to the laboratories to test for the (i) levels of nutrients, (ii) your immune status, (iii) the state of your blood (to see if you do not have weak blood- a test called full blood count). You will then be asked to donate 2 stool samples- you will be given 2 stool jars. You will be requested to return the first stool the following day, and the next one when you return to the Clinic to collect your results of the tests that will be requested through the Clinic for your treatment plan. The stool specimens will be taken to the laboratory to check if they do not have worm eggs (meaning that there are worms in the gut that are producing these eggs into the stool). In the laboratory, the researchers will also test what types of the good germs (microbial or normal flora) are in your gut.

Although you will have been tested for HIV in the HIV Counselling and Testing program of the Clinic, some of your donated blood will be tested for HIV again in the laboratory in order to confirm the results for the research, and to be able to assign you to a group of either those infected or not infected. The researchers will allow you to get your results if you choose to. This will be done through the Clinic's Counselling programme. You will be asked if you would like to get these results back, and if you want them, the researchers will arrange with the HCT staff so that you receive further post-test counselling, and your results will then be disclosed to you following the same procedures for confidentiality.

The duration of your participation if you choose to enroll and remain in the study is expected to be for at most 3 months. However, you will donate the blood once and stools in two days, then the results of the screening for worms will be made available to you within that period. In addition, if there is a need to contact you for any other research related results, the researchers will contact you within that three-month period". If there are no further results you may participate for less than 3 months as soon as all your results are completed.

The study may involve the following risks and/or discomforts. During blood collection, discomfort in the form of mild pain and possible bruising may be experienced. If it occurs, the bruising disappears and heals after a few days by itself. Also, you will be asked personal questions such as income level which may make you feel uncomfortable. You are free to decide not to answer any question that you do not feel comfortable to. We hope that the study will create the following benefits: If you are found to be infected with intestinal worms, you will be provided with a referral letter to your nearest clinic where you will be given deworming treatment. Otherwise, the research will provide no direct benefits to you as a participant. However, the results of the study will help in showing whether worm infections have a bad effect on those who also have TB or HIV infections, and whether the worms make it difficult for such people to get better quickly when they get TB or HIV treatment. The research will also check if those who are infected with worms and HIV or TB have poor nutritional status. This will help the health providers to look after such patients and decide on all the necessary treatment as well as the control of the worm infections in the community. The study will therefore be beneficial in Public Health planning in general which will benefit future generations.

It is expected that the research will not potentially involve any serious risks. However, it may happen that if you have been diagnosed with worm infections and advised to take deworming medication, you may experience some symptoms related to such treatment, some can be mild and short-lived. They may include and not limited to nausea, vomiting, headache. If the symptoms become unbearable, you are advised to consult the health providers.

Because you will be requested to return the stool specimens twice, you will be reimbursed for the return trips for R40, and a small token of R110 for compensation for inconvenience will be provided. You will therefore receive R150.

This study has been ethically reviewed and approved by the UKZN Biomedical research Ethics Committee (provisional approval number_BE351/19). In addition, the study is funded by the South African Medical Research Council.

In the event of any problems or concerns/questions you may contact the researcher (Z.L. Kwitshana) at

School of Laboratory Medicine and Medical Sciences

Ground Floor, Room 25
Durban
4000
KwaZulu-Natal, SOUTH AFRICA
Tel: 27 31 2604769 - Fax: 27 31 2604609
Email: BREC@ukzn.ac.za

Please be aware that participation in this research is voluntary, you may withdraw participation at any point, and in the event of refusal/withdrawal of participation you will not incur penalty or loss of treatment at your clinic or hospital and you will still be entitled to all other benefit to which you are normally entitled. Please note that if you decide to withdraw from the research, you are still expected to continue with your normal scheduled clinic visits. You inform the researchers if you decide to withdraw from the research. If you become very sick, the researcher may terminate your participation from the study and advise you to seek the necessary care from the health provider.

You will be reimbursed for travelling to the clinic as a study participant to bring a second stool sample on the second day after the visit.

Your personal details will be kept in a safe place to make sure that they are protected. Only the researcher will have access to them. We will keep the records in a computer file that can only be accessed by the main researchers. We will assign each participant a study number which will be used to link your personal details in a file which will be kept safely in the computer that will be accessed by the main researcher only. For research purposes, we will only use the study number. Once the research has been completed, your blood samples will be stored for the future research purposes if you agree or will be discarded after the research results have been obtained.

**Name of Researcher providing Information** _____

_____ _____

**Signature of Researcher providing Information** **Date**

**CONSENT**

I (.....) have been informed about the study entitled "A pilot study on helminthiasis and microbes interactions: macrobiotic control of microbiota and the effects on Human Immunodeficiency Virus and Mycobacterium tuberculosis diseases, immune responses and nutritional status: Human and *in vitro* studies
by the researcher).

I understand the purpose and procedures of the study (that I will be expected to donate stool and blood specimens and return the stool samples the following day. I also understand that I will be asked questions about my diet and other personal information. I may refuse to answer questions that I am not comfortable with.

I have been given an opportunity to answer questions about the study and have had answers to my satisfaction.

I declare that my participation in this study is entirely voluntary and that I may withdraw at any time without affecting any treatment or care that I would usually be entitled to.

I have been informed about any available medical advice and care if injury occurs to me as a result of study-related procedures such as bruising after blood donation. If I have any medical issues, for example after taking deworming medication I may seek advice form the health care facility.

If I have any further questions/concerns or queries related to the study I understand that I may contact the researcher, at the following details:

School of Laboratory Medicine and Medical Sciences
Ground Floor, Room 25
George Campbell Building
Howard College Campus
University of KwaZulu-Natal
Tel 031 2601931
Email: kwitshanal@ukzn.ac.za

If I have any questions or concerns about my rights as a study participant, or if I am concerned about an aspect of the study or the researchers then I may contact:

**BIOMEDICAL RESEARCH ETHICS ADMINISTRATION**
Research Office, Westville Campus
Govan Mbeki Building
Private Bag X 54001
Durban
4000
KwaZulu-Natal, SOUTH AFRICA
Tel: 27 31 2604769 - Fax: 27 31 2604609
Email: BREC@ukzn.ac.za

| | |
|---|---|
| **Signature of Participant** | **Date** |
| **Signature of Witness (Where applicable)** | **Date** |
| **Signature of Translator (Where applicable)** | **Date** |

# Appendix C:

## Informed consent form: specimen storage for genetic (DNA) analysis for participant recruitment



**Informed Consent for specimen storage for genetic (DNA) analysis**

**Study Title:**
A pilot study on helminthiasis and microbes' interactions: macrobiotic control of microbiota and the effects on Human Immunodeficiency Virus and Mycobacterium tuberculosis diseases, immune responses and nutritional status: Human and *in vitro* studies.

**Principal Investigator:**
**Dr Zilungile L. Mkhize-Kwitshana**
School of Laboratory Medicine and Medical Science
Ground Floor Room 25
George Campbell Building
Howard College Compass University of KwaZulu -Natal
Tel 031 260 1931
mkhizekwitshanaz@ukzn.ac.za

Hello, my name is……
From: - Laboratory Medicine and Medical Sciences at University of kwaZulu Natal (UKZN)
My contact details are as follows:
Cell number is ……..
Office number is ……
Email address is ……

**Introduction**
You have been invited to voluntarily participate in the study titled "**A pilot study on helminthiasis and microbes' interactions: macrobiotic control of microbiota and the effects on Human Immunodeficiency Virus and Mycobacterium tuberculosis diseases, immune responses and nutritional status: Human and *in vitro* studies**"

During this study blood specimens will be collected from you for the purposes of this study. Specimens that are left over following completion of all testing required for our experiments may be used for future research. You are being asked to consent to the storage of your blood for possible future research that may or may not be related to this study.

**How will you use my stored blood specimens?**
If you agree to participate and donate blood for this study, we also ask for your permission to store some of your blood in the freezers at the University of KwaZulu-Natal, Howard Campus, Medical Microbiology Laboratory Ultra freezers. This blood may be used later to confirm test results and to check the genes (DNA) (which are small substances in our bodies that are responsible for heredity or for inheriting physical qualities or characteristics that we inherit form our parents and ancestors like the colour of our eyes, our complexion, and why some

people get certain diseases while others do not, and why some people respond to certain treatments while others do not).

These stored samples will be used to check further what happens to other genes of the immune system when people are infected with worms and HIV and /or TB at the same time, based on the findings of the present study. These future studies will check whether there can be other substances in the blood of people who have these infections that can be used in future, to tell whether there are changes in the blood if there is infection or not (biomarkers) to assist health care workers to diagnose coinfections better or plan for their management. If you agree to the storage of your blood specimens for possible future research, you will be asked to sign this consent form. A copy of the form will be given to you to keep.

Your blood specimens will not be sold or used in products that make money for the researchers or anyone else. Any studies that use your specimens in future research will be reviewed by the Biomedical Research Ethics Committee of the Nelson R. Mandela School of Medicine, University of KwaZulu-Natal.

We do not plan to contact you with any results from tests done on your stored blood specimens. This is because research tests are often experimental, and we don't think the results will be useful for making decisions about your health. Additionally, these tests will be done in a way that will make it extremely difficult to link the test results to you.

**How long will you keep my blood specimens?**
There is no time limit on how long your blood specimens will be stored.

**Does storage of my blood specimens benefit me?**
You may not benefit directly from this kind of research, but it will help future generations when doctors are able to see early what genes are increased for what diseases, or how they can use these results for treating these diseases. Please note that your personal details will never be disclosed as a source of this research. Also, if and whenever the results of these heredity research are published, they will never be linked to Umlazi.

**What about confidentiality?**
In order to keep your information private, your blood specimens will be labelled with a code. Your personal information, such as name, address, and phone number, will not be placed on the specimens. Only the research clinic where you come for study visits will be able to link the storage code with your personal information.

In the future, when researchers are given your stored specimens to study, they will be given only the code; they will not be given your personal information.

**What are my rights?**
If you do not agree that your blood sample gets stored in the Biobank, it will be discarded after the research has been completed. You are free to agree or disagree to this and still participate in the research.

**What do I do if I have questions?**
If you ever have any questions about the storage of your blood you should contact Dr ZL Mkhize- Kwitshana, Tel: 031 260 1931, e-mail mkhizekwitshanaz@ukzn.ac.za

If you have questions about your rights as a research participant, you should contact the Biomedical Research Ethics Administration, University of KwaZulu-Natal, Research Office,

Westville Campus, Govan Mbeki Building, Private Bag X 54001, Durban, 4000, KwaZulu-Natal, SOUTH AFRICA. Tel: 27 31 2604769 - Fax: 27 31 2604609, Email: BREC@ukzn.ac.za

**Signatures:**

| Participant Name (Print) | Signature of Participant | Date |
|---|---|---|

| Research Staff (Print) | Signature of Research Staff | Date |
|---|---|---|

***The section below is to be completed by the person who administered the informed consent***

Was a copy of the signed copy given to the volunteer: ☐ Yes ☐ No

If no, why not:

# Appendix D:

## Study questionnaire for participant recruitment

**A pilot study on helminthiasis and microbes interactions: macrobiotic control of microbiota and the effects on Human Immunodeficiency Virus and Mycobacterium tuberculosis diseases, immune responses and nutritional status: Human and *in vitro* studies**

### STUDY QUESTIONNAIRE

***Interview Questionnaire.***

**SECTION A**:

Study Identification number .....

Clinic Code : .....

Date of interview

| D | D | / | M | M | / | Y | Y | Y | Y |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | | 2 | 0 | | |

Name of Interviewer: ..... Signature..... Date.....

**SECTION B: Demographic Information**

| Ethnic group | African | | Coloured | | Indian | | White | |
|---|---|---|---|---|---|---|---|---|

| Gender | Male | | Female | |
|---|---|---|---|---|

Marital status

| | |
|---|---|
| Single | |
| Married | |
| Divorced | |
| Widowed | |
| Separated | |
| Living together | |

Area of residence.....

Age of participant .....years

| Date of birth | d | d | m | m | y | y | y | Y |
|---|---|---|---|---|---|---|---|---|
| | | | | | | | | |
| Mobile number | | | | | | | | |
| Phone number (Home) | | | | | | | | |
| Name of Next of Kin: | Cell: | | | | Relation: | | | |

Weight (kg):

Height: (cm):

BMI

**SECTION C**: **Socio-Economic Status**

| | | | | | | |
|---|---|---|---|---|---|---|
| 1. Where do you live? | | | | | | |
| 2. What would you classify the area as? | | Rural | Urban | Peri-urban | | |
| 3. Are you employed? | | Yes | No | | | |
| 4. If no, please specify source of income | | | | | | |
| 5. If yes, what is your income per month? | | <R1000 | R1000–R5000 | R5000–R10 000 | >R10 000 | |
| 6. What is your level of education? | | None | Primary | High school | Tertiary | |
| 7. What is your occupation? | | | | | | |
| 8. How many standard alcoholic drinks, per day, do you have? | None | 1-2 | 3-4 | 5-6 | 7-8 | 10+ |
| 9. How many times, per day, do you take marijuana or any other drug to get high? | Never | 0-1 | 1-2 | 3-4 | 5-6 | 7-8 |

**SECTION D**: **Household Information**

1. What type of house do you live in? .....
2. How many rooms does your house have? .....
3. How many people live in your household?

| | |
|---|---|
| Babies/Pre-school | |
| Primary school | |
| Adults | |
| TOTAL | |

| | |
|---|---|
| River | |
| Own tap- inside the house | |

3250

4. Where does the household usually get drinking water from?

| | |
|---|---|
| Own tap-outside the house | |
| Public tap | |
| Neighbours' tap | |
| Borehole | |
| Other, specify..... | |

5. What kind of water activity do you do?

| | |
|---|---|
| Swim | |
| Wash clothes | |
| Bathe | |
| Fish | |
| Farming | |
| Collect water for household use and cooking | |
| Cross the river | |
| Other, specify..... | |

6. What toilet facilities do the household have?

| | |
|---|---|
| Flush toilet, connected to public pipes | |
| Flush toilet, not connected to public pipes | |
| Pit toilet | |
| None | |
| Other, specify..... | |

7. What is the main source of energy for cooking?

| | |
|---|---|
| Electricity | |
| Wood, open fire outside dwelling | |
| Wood open fire inside dwelling | |
| Gas | |
| Paraffin | |
| Other, specify..... | |

8. From where do you get your food?

| | |
|---|---|
| Local shop(s) | |
| Shops in town | |
| Home garden | |
| Community garden | |
| Own livestock | |
| Food aids/welfare/NGO's | |
| Other, specify..... | |

**SECTION E: Presence of other diseases**

1. Do you presently have any diseases that you are aware of? Yes ☐ No ☐

If yes, please specify

| |
|---|
| |
| |
| |
| |

2. Have you been ill in the past 30 days? Yes ☐ No ☐

3. If yes, please list the disease/s

| |
|---|
| |
| |
| |
| |
| |

4. Have you suffered from any worm infection in the past?

| Yes | | No | |
|---|---|---|---|

5. Has any of your family members suffered from any worm infection?

| Yes | | No | | Don't know | |
|---|---|---|---|---|---|

6. Have you taken any deworming medication in the past 6 months?

| Yes | | No | |
|---|---|---|---|

7. How often is deworming done in your household?

| | |
|---|---|
| Never | |
| Once in 6 months | |
| Once a year | |
| Don't know | |
| Other, specify…… | |

8. Who gets deworming treatment in your household?

| | |
|---|---|
| Children only | |
| Adults only | |
| Everyone in the house | |
| No one in the house | |

9. Have you had an allergic reaction in the past 30 days?

| Yes | | No | |
|---|---|---|---|

10. Do you suffer from any of the following chronic illness?

| | Yes | | No | |
|---|---|---|---|---|
| Diabetes | | | | |
| Arthritis | | | | |
| Heart Disease | | | | |
| Cancer | | | | |
| Asthma | | | | |
| Kidney Disease | | | | |
| Liver disease | | | | |

11. Are you taking any medication?

| Yes | | No | |
|---|---|---|---|

If Yes, please specify ……

**SECTION F: Supplements**

Do you take any supplements? Yes ☐ No ☐

If yes, please name them .....

.....

**SECTION G: Covid-19**

Have you come in close contact with someone who have been diagnosed with COVID-19 in the past 14 days?

Yes ☐ No ☐

Do you have the following: fever, cough, shortness of breath or difficulty breathing, body aches, headache, new loss of taste or smell, sore throat?

Yes ☐ No ☐

Have you been experiencing nausea and/or vomiting?

Yes ☐ No ☐

Have you experienced any recent stomach upset or diarrhoea?

Yes ☐ No ☐

Have you been tested for COVID-19?

Yes ☐ No ☐

If yes, what was the result? .....

Have you ever been diagnosed with COVID-19?

Yes ☐ No ☐

If yes, when? .....

**SECTION H: 24-hour food recall**

1. How often do you eat?
2. Are you constantly hungry?

| | | | per day |
|---|---|---|---|
| Yes | | No | |

*Please indicate everything you ate or drank, including meals, snacks, sweets, beverages, alcohol in the past 24 hours*

| Time of day | What food and drink did you take | How was it prepared | What was added | How much was eaten |
|---|---|---|---|---|
| Waking up to about 9 o'clock (breakfast time) | | | | |
| 9 o'clock to 12 o'clock (mid-morning) | | | | |
| 12 o'clock to 2 o'clock (lunch time) | | | | |
| 2 o'clock to 5 o'clock (afternoon) | | | | |
| 5 o'clock to sunset (supper time) | | | | |
| After supper at bedtime and through the night | | | | |

**Thank you for your participation**

# Appendix E:

# Published review article.

diagnostics

MDPI

*Review*

# Immunological Interactions between Intestinal Helminth Infections and Tuberculosis

**Khethiwe Nomcebo Bhengu [1,2,3,*], Pragalathan Naidoo [1,2], Ravesh Singh [4], Miranda N. Mpaka-Mbatha [1,2,3], Nomzamo Nembe [1,2], Zamathombeni Duma [1,2], Roxanne Pillay [1,2,3] and Zilungile L. Mkhize-Kwitshana [1,2]**

[1] Department of Medical Microbiology, School of Laboratory Medicine and Medical Sciences, College of Health Sciences, Nelson R. Mandela School of Medicine, University of KwaZulu-Natal, Durban 4001, South Africa
[2] Division of Research Capacity Development, South African Medical Research Council (SAMRC), Cape Town 7505, South Africa
[3] Department of Biomedical Sciences, Faculty of Natural Sciences, Mangosuthu University of Technology, Umlazi, Durban 4031, South Africa
[4] Department of Medical Microbiology, School of Laboratory Medicine and Medical Sciences, College of Health Sciences, Howard College, University of KwaZulu-Natal, Durban 4041, South Africa
* Correspondence: bhengukn@mut.ac.za; Tel.: +27-0319077245

**Abstract:** Helminth infections are among the neglected tropical diseases affecting billions of people globally, predominantly in developing countries. Helminths' effects are augmented by coincident tuberculosis disease, which infects a third of the world's population. The role of helminth infections on the pathogenesis and pathology of active tuberculosis (T.B.) remains controversial. Parasite-induced suppression of the efficacy of Bacille Calmette-Guerin (BCG) has been widely reported in helminth-endemic areas worldwide. T.B. immune response is predominantly proinflammatory T-helper type 1 (Th1)-dependent. On the other hand, helminth infections induce an opposing anti-inflammatory Th2 and Th3 immune-regulatory response. This review summarizes the literature focusing on host immune response profiles during single-helminth, T.B. and dual infections. It also aims to necessitate investigations into the complexity of immunity in helminth/T.B. coinfected patients since the research data are limited and contradictory. Helminths overlap geographically with T.B., particularly in Sub-Saharan Africa. Each disease elicits a response which may skew the immune responses. However, these effects are helminth species-dependent, where some parasites have no impact on the immune responses to concurrent T.B. The implications for the complex immunological interactions that occur during coinfection are highlighted to inform government treatment policies and encourage the development of high-efficacy T.B. vaccines in areas where helminths are prevalent.

**Keywords:** *Mycobacterium tuberculosis*; helminths; coinfection; immune response; Bacille Calmette-Guerin; vaccination



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## 1. Introduction

Intestinal helminths are parasitic worms infecting over 1.5 billion people globally [1] Most helminth cases occur in tropical and sub-tropical areas such as Sub-Saharan Africa, the Americas, China and East Asia [1]. Humans are infected with helminth parasites after ingesting eggs or larvae from contaminated water, soil or food or through active skin penetration by infective hookworm larvae in contaminated soil [2]. Climate change, malnutrition, overcrowding, poverty and poor sanitary conditions are risk factors associated with the high helminth prevalence in Africa and other developing countries, making effective treatment and the eradication of infection challenging [1–4]. The most common intestinal helminth species infecting humans are *Schistosoma mansoni*, *Trichuris trichuria* (whipworm), *Ascaris lumbricoides* (roundworm), *Necator americanus* and *Ancylostoma duodenale (hookworms)* [1,2].

Tuberculosis (T.B.) is an infectious bacterial disease caused by different strains of acid-fast bacilli belonging to the *Mycobacterium tuberculosis (Mtb)* complex [5]. The T.B. bacteria are airborne, and transmission occurs when a T.B.-infected person coughs, sneezes or spits, expelling the infected droplets into the air. Inhalation of these aerosols may result in infection of the next host [6]. T.B. continues to be a public health problem across the world, with the World Health Organization (WHO) reporting over 10 million T.B. cases in 2020 [7]. Approximately 1.5 million TB-related deaths were reported worldwide in 2020 [7]. Globally, Africa accounts for 50% of cases of T.B. and human immunodeficiency virus (HIV) coinfection [7]. Furthermore, in Africa, T.B. is commonly observed in HIV-infected patients, and it is the leading cause of death among them [7].

T.B. exposure results in the initiation of an immune response to fight the infection. The immune response to T.B. involves the interaction of innate and adaptive immune responses. It is dependent on the cellular immune response, which is mediated by proinflammatory T-helper type 1 (Th1) and Th17 cells [8–10]. The Th1 cytokines, which are interferon-γ (IFN-γ), interleukin 12 (IL12) and tumor necrosis factor-α (TNF-α) and Th17 cytokines (IL-17, IL-21, IL-22 and IL-23) play a role in combating bacterial and viral infections [8–10]. Helminth exposure, on the other hand, induces an anti-inflammatory Th2 immune response which is characterized by the production of cytokines such as IL-4, IL-5, IL-9, IL-10 and IL-13, and increased levels of circulating immunoglobulin E (IgE) antibodies, eosinophils, and mast cells, regulatory T cells (Tregs) and transforming growth factor-β (TGF-β) [11,12].

T.B. commonly overlaps geographically with soil-transmitted helminths, especially in developing countries [13–16], and this co-endemicity has implications for public health and the afflicted hosts. Helminth infection-induced immune responses could promote the pathogenesis of severe T.B. infections [16–18]; others report that they can also be beneficial in reducing T.B. severity [19–22]. However, there is no conclusive evidence to confirm whether helminth-induced immunity modulates T.B.-specific immune responses or vice-versa, and studies have yielded contradictory results. Therefore, knowledge on the interaction between T.B. and helminth infections is limited, as are the available data.

Given the current evidence on potential immunologic implications, such as those that could influence T.B. vaccination, treatment and diagnosis, more research is needed to determine the influence of helminth coinfection on T.B. control and how to negate any adverse effects. As a result, this review will summarize what is currently known about T.B. and helminths' immune responses in human and experimental studies, both separately and in the context of coinfection. The review will also elucidate the effects of T.B. and helminth coinfections on vaccine efficacy and the implications for long-term health care.

## 2. Article Search Strategy for the Current Review

An electronic search of online databases such as Google Scholar, Google, PubMed, Science Direct, online library sources, and Web of Science were utilized to extract research and review articles using phrases and words: helminth, tuberculosis, helminth and tuberculosis coinfection, helminth and tuberculosis vaccine and helminth and tuberculosis diagnosis in humans, animals and in vitro studies. A PRISMA flow diagram of the search strategy and research design process for this review is presented in Figure 1.

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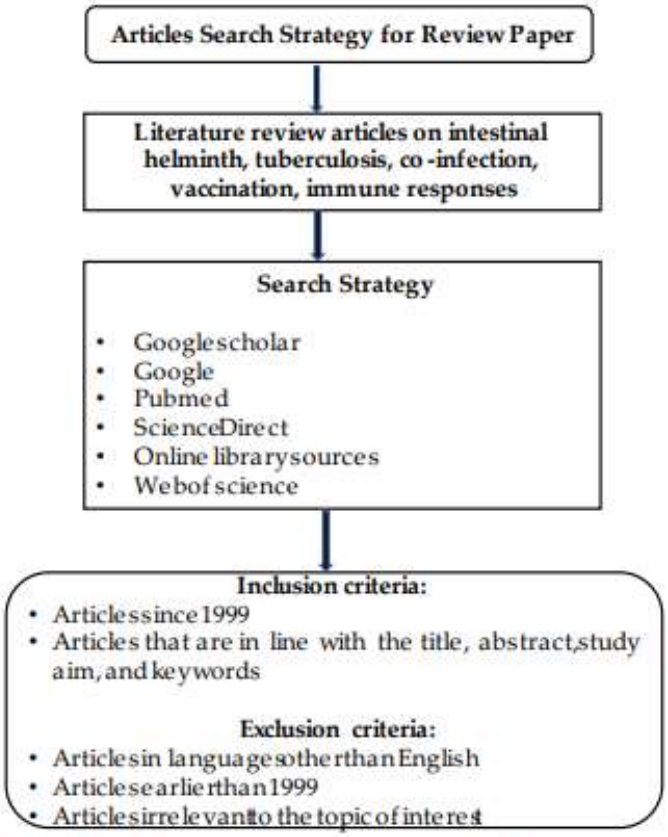


**Figure 1.** PRISMA flow diagram of the search strategy and the research design process.

### 3. The Host Immune Response to Helminths

Helminths are parasitic and multicellular organisms that coevolved with their hosts [23]. These parasitic infections are often asymptomatic, but there are cases of heavy worm burden. These have been linked to persistent health conditions such as anemia, fatigue, growth stunting and poor cognitive development [24]. Helminths are the driving force behind how immunity is initiated and maintained [25]. They typically create long-term infections in their hosts. They have the power to influence physiological and immunological homeostasis to ensure their continuing existence [25].

Helminths mature within the infected subject and lay eggs for transfer to another host, exposing them to multiple stages of parasite development, each of which elicits a unique immune response [26]. Helminths have evolved to exploit a range of host immunoregulatory mechanisms and activate generic suppressive pathways that can suppress bystander responses to other antigens, allergens, and self-antigens [12]. Helminths have been dubbed "masters of immunoregulation" because of their capability to control immunity to escape being eliminated by the host [25,27]. Helminths enter the body through the skin or intestinal epithelium's barrier surface, where they block the transcription of numerous molecules that keep the epithelium intact [28].

Tissue injury activates the production of "alarmins" (IL-33 and thymic stromal lymphopoietin (TSLP)) and the identification of invaders by pattern recognition receptors (PPRs) in the host [28]. The Th1 proinflammatory cytokine production is driven by pattern

recognition receptors (PRRs) such as toll-like receptors (TLRs) or C-type lectin receptors (CLRs), whereas IL-33 and TSLP initiate a Th2 anti-inflammatory response [28].

Helminths stimulate increased mucin synthesis, smooth muscle contractility and epithelial cell turnover as a host defense to eliminate the infection. There is also increased IgE and IgG1 production in mice and IgE and IgG4 production in humans [12,28]. All these processes work together to drive worm expulsion and wound-healing responses, which control worm-induced tissue damage [28].

The Th2 immune response induced by helminths includes interleukins (IL-4, IL-5, IL-9, IL-10, and IL-13), broad or localized eosinophilia and hyperplasia of goblet and mucosal mast cells [12,28]. The CD4-positive Th2 cells were initially identified as an essential source of IL-4, IL-5, IL-9, IL-10 and IL-13 cytokines [29]. Eosinophils, basophils and innate lymphoid cells (ILCs) can also produce some of these cytokines in response to helminth infections [29]. Although the Th2 immune response induced by helminth parasites is stereotypical, the initiation, progression and culmination of this response require interaction with different cell types, most notably: epithelial or stromal cells, ILCs, antigen-presenting cells, dendritic cells, macrophages, T cells, B cells, eosinophils, mast cells and basophils [12].

Tregs maintain the Th2 dominance, IL-10 and TGF-β, which mediate the suppression of competing Th1 and Th17 cell populations [30]. Tregs modulate the immune system to prevent tissue damage induced by proinflammatory responses, maintain tolerance to self-antigens and abrogate autoimmune disease [31]. These cells can be divided into two subsets: natural Tregs that develop in the thymus, and induced Tregs that arise from conventional CD4 positive T cells in the periphery, which are promoted by chronic antigen exposure [32]. The forkhead/winged-helix transcription factor (Foxp3) is a crucial marker for identifying these subsets, but it may be expressed on activated CD4 positive T cells [32].

Helminth-induced suppression of immunopathology also involves CD4+ Tregs (Foxp3+ or Foxp3), CD8+ Tregs, regulatory B cells (Bregs), IL-4-responsive cells, TGF-β, and IL-10 [33]. Since an increased Th2 response can potentially induce disease, a regulated response must be generated. This is referred to as the modified Th2 cell response and is characterized by the downregulation of Th2 cytokines [12].

According to the hygiene hypothesis, in developed countries where sanitation is good, and helminths have been eliminated, there is an increase in allergic diseases such as asthma and allergic rhinitis, and autoimmune diseases such as Crohn's disease [27]. This hypothesis has led to many human and animal studies conducted using live helminth parasites to determine whether helminths do nullify the effect of allergies and autoimmune disorders. Human studies conducted in underdeveloped countries where helminths are still prevalent showed fewer allergies and autoimmune diseases [27,34,35]. Others have reported evidence of decreased allergies in developing countries [36].

Helminths induce various immune and physiologic modifications to survive the hostile immune response directed against them and their general survival. These survival mechanisms include this modified Th2 response [27]. These parasites also promote angiogenesis, which changes tissue vascularity and thus provides a good niche for their survival [37]. The overall immune modulation of helminths invokes immunosuppression, immunologic and physiological tolerance and a modified Th2 response [27]. These can lead to a reduced immune response, thus amplifying susceptibility to infection with other pathogens, reduced anti-tumor immunity and reduced vaccine efficacy. The host immune response profile to helminth infection is presented in Figure 2.

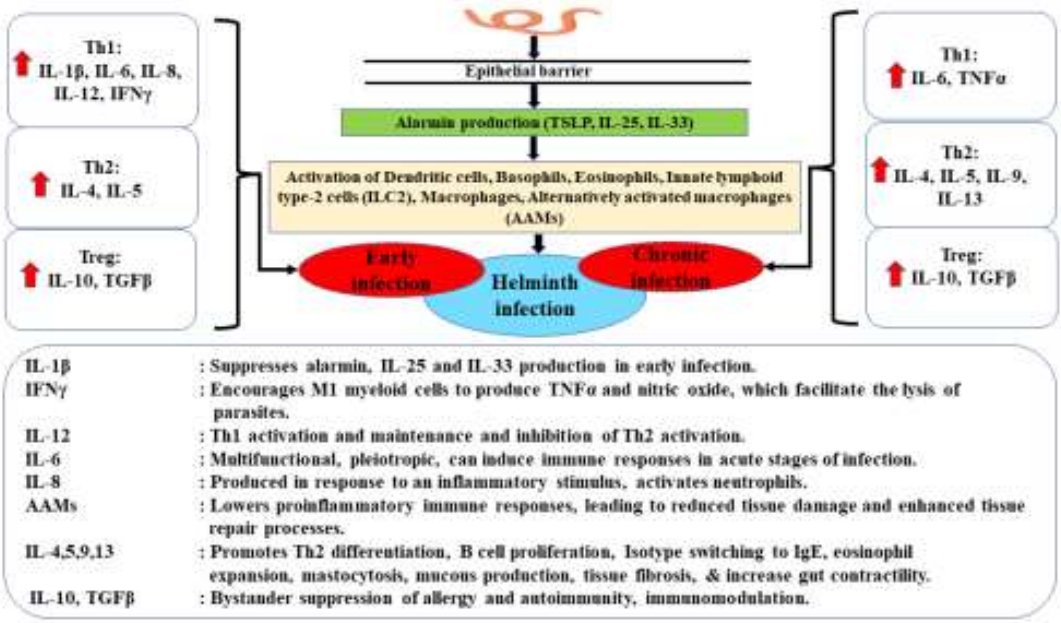


**Figure 2.** Immune response profiles during helminth infection. Migration of helminths damages epithelial barrier cells and tissues, triggering an immune response. Helminths produce damage and pathogen-associated molecular patterns (DAMPS and PAMPS). DAMPS and PAMPS activate various cells, such as epithelial, which release alarmins such as Thymic stromal lymphopoietin (TSLP), IL-25 and IL-33. Alarmins stimulate innate lymphoid cells (ILCs), aiding collagen deposition and tissue repair, and are a source of IL-5 required for eosinophil activation. Eosinophils enter tissues during helminth infection-induced inflammation. Eosinophilia is a crucial feature of the host response to helminth infection. Alarmins promote B cell activation and induction of alternatively activated macrophages (AAMs). AAMs stimulate IL-10 and TGF-β, which reduce the host's immune response to pathogens to avoid damaging the host and maintain normal tissue homeostasis. Classically activated macrophages, stimulated by IFN-γ produce proinflammatory cytokines (IL-1β, IL-6, IL-8, IL-12 and TNF-α).

Figure 2 Footnotes: IL: interleukin; IFN-γ: interferon-gamma; TGFβ: transforming growth factor beta; TNFα: tumor necrosis factor-alpha; ILCs: innate lymphoid cells; TSLP: Thymic stromal lymphopoietin; AAMs: alternatively activated macrophages; DAMPS: damage-associated molecular patterns; PAMPS: pathogen-associated molecular patterns. Red arrow pointing up indicates cytokines that are upregulated/increased during the early stages of helminth infection and those that are upregulated during the chronic stages.

## 4. The Host Immune Response to T.B.

T.B. enters the body via inhaled droplets to the alveoli. It interacts with the alveolar macrophages, infecting and multiplying inside them, thus making these cells the first line of defense against infection [6]. In immunocompetent individuals, macrophages are activated, and they phagocytose and remove T.B.

In some cases, the disease is controlled and kept in an inactive or latent state in distinct foci known as granulomas bacteria [9,15,38,39]. However, some bacteria can escape this fate, multiply and eventually cause an active infection. This may be due to the intrinsic capacity of the macrophage, the immune status of the host or the virulence of the infecting bacteria [9,15,38,39]. *Mtb* is, therefore, a pathogen that can cause both latent and active disease [40].

### 4.1. Innate Responses to T.B.

The initial stages of T.B. infection include phagocytosis of the bacteria by macrophages [6]. Receptors that recognize a broad spectrum of mycobacterial ligands cause phagocytosis [9]. Pathogen recognition receptors, TLRs, complement receptors (C.R.), Nucleotide Oligomerization Domain (NOD)-like receptors and C-type lectins have all been implicated in recognition of mycobacteria and the initiation of the cytokine response [8].

When phagocytic cells encounter T.B., they get activated and generate cytokines, including proinflammatory cytokines such as TNF-, IL-1, IL-6, IL-12 and IFN-γ [8]. Increased susceptibility to T.B. was reported to be linked to genetic abnormalities in IFN-γ production [15,41]. IFN-γ is involved in activating macrophages that fight mycobacteria through intracellular killing and antigen presentation to T lymphocytes [42]. Vitamin D is also involved in killing *Mtb*, which is aided by the creation of peptide cathelicidin [43].

The presentation of T.B. antigens by dendritic cells in lymph nodes, possibly aided by neutrophils, initiates a local immune response that culminates in pathogen killing by reactive oxygen species (ROS) and antimicrobial peptides [8].

Cells required in the host's defense against *Mtb* include monocytes, macrophages, neutrophils, natural killer (NK) cells and dendritic cells. Together, these cells form a primary granuloma, which may allow *Mtb* growth while containing the infection until T cells are recruited to the infection site, a response process that takes weeks [8]. Phagolysosomal fusion, reactive oxygen and nitrogen intermediates, and antimicrobial peptides such as cathelicidin induced by vitamin D are innate mechanisms against *Mtb* [43].

NK cells may eliminate intracellular *Mtb* through the activation of perforin, where the antimycobacterial factor granulysin binds to the bacterial cell surface and disrupts the membrane, resulting in bacterial osmotic lysis [44]. Apoptosis is a critical mechanism for the infected host cell to limit *Mtb* replication to a minimum. Phagocytic cell apoptosis may prevent the spread of disease, diminish the viability of intracellular mycobacteria and reduce the risk of infection [45].

### 4.2. Adaptive Immune Responses to T.B.

Adaptive immunity develops after exposure to mycobacterial antigens or vaccination with BCG. This part of the immune system is triggered when the innate immune response is insufficient to suppress T.B. infection. The control of T.B. requires Th1 immune responses (IFN-γ, IL-12 and TNF-α) and Th17 responses (IL-17 and IL-23). Th1 responses are proinflammatory and develop a cell-mediated reaction [38]. Th1 cells produce IFN-γ through the T-box transcription factor (TBX21). Both IL-12 and IFN-γ are the leading cytokines in Th1 responses, where IL-12 is secreted by antigen-presenting cells [39,46]. The IL-12 receptor, which is expressed on the surface of T cells, interacts with IL-12. The increased T-bet (encoded by TBX21) boosts the signal transducer and activator of transcription 4 (STAT4), a regulator of Th1 cells [46].

T-bet binds to and affects the expression of Th1-specific genes and Th1 and Th17 cell expression [46]. This is important since the control of T.B. requires Th1 responses. STAT4 and T-bet work together to ensure optimal IFN-γ levels, and their depletion eliminates IFN-γ production [46].

T.B. immunity involves many cells, such as T cells, B cells and natural killer (NK) cells, with CD4+ T cells being the primary cell type in T.B. control [47]. The CD4+ Th1 cells are central to the control of T.B.; these cells secrete IFN-γ and TNF-α, which are both critical in the management of T.B. [38]. IL-12 regulates the induction of IFN-γ, and mutations in the genes coding for IL-12, IL-12R, IFN-γR or STAT1 or depletion of CD4+ T cells (as seen in HIV infection) all promote susceptibility to disseminated T.B. [38]. IFN-γ stimulates phagocytosis, phagosome maturation, the production of reactive oxygen intermediates (ROS) and antigen presentation in macrophages.

IFN-γ is regarded as the primary cytokine that regulates T.B. infection and eradication. It works by activating the infected macrophage, resulting in the production of reactive oxygen and nitrogen species, which have a microbicidal role [48]. In terms of memory

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immune responses, CD4+ Th17 cells and Th1 cells have been identified as enhancing the host's resistance to T.B. [49]. Th17 cells are a lineage of CD4+ T helper cells that produce the cytokine IL-17, IL-17F and IL-22, and they play a role in developing an optimal Th1 response [50].

Th17 was first described as a distinct population of the T helper cells controlled by the transcription factor RAR-related orphan receptor gamma (RORyt) [51]. They develop independently of T-bet, STAT4, GATA-3 and STAT6 transcription factors critical for the development of Th1 and Th2 development, respectively [51]. The central effector cytokines of Th17 are IL-17; other cytokines are IL-22 and IL-26 [52]. The immune response to T.B. infection is directed mainly by a Th1 response, with contributions from Th17 and other cells. A strong proinflammatory milieu also characterizes T.B. infection.

On the other hand, human innate immune responses to *Mtb* infection are still poorly understood, owing to the limitations in examining pulmonary-specific immunity.

Therefore, understanding the interaction of innate and adaptive immune cells in human T.B. is crucial for identifying new immunomodulatory targets and clarifying protective immunity processes. The immune response profiles to tuberculosis infection are presented in Figure 3.

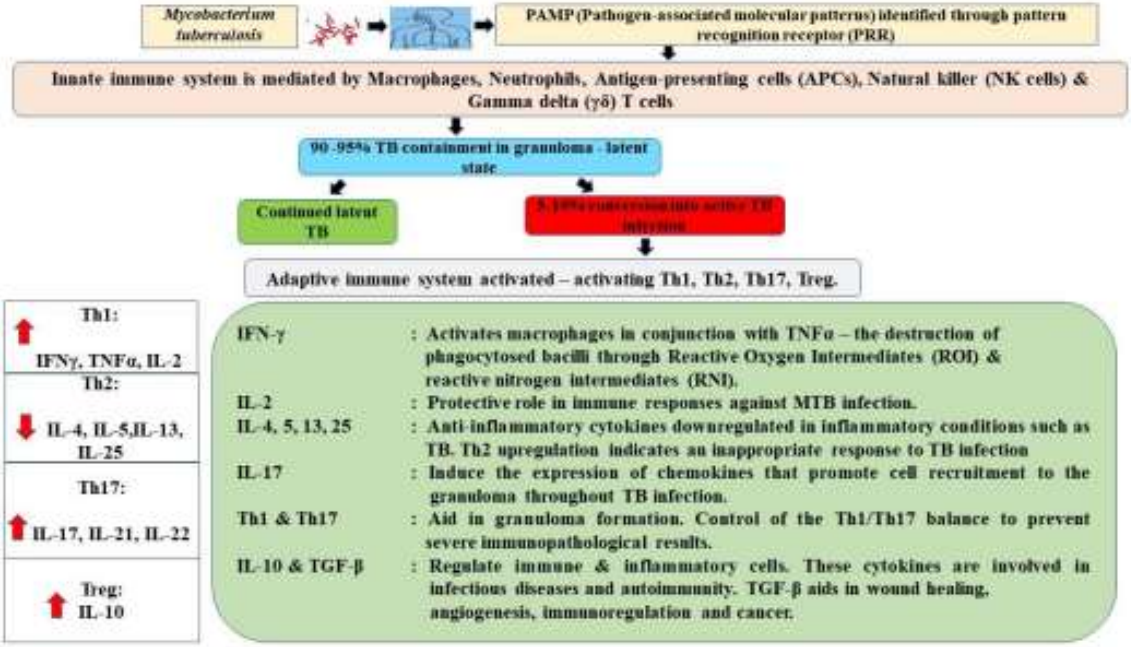


**Figure 3.** Immune response profiles during tuberculosis infection. Mycobacteria encounter alveolar macrophages where they are phagocytosed, kept inside phagosomes and exposed to antimicrobial peptides and degrading lysosomal enzymes (lysozyme). However, pathogenic mycobacteria have developed strategies to subvert the host's defenses. Th1-cell activity (IFN-γ, IL-12 and TNF-α) is required for *Mycobacterium tuberculosis* immunity. IFN-γ activation of macrophages promotes bacterial killing by forming toxic reactive oxygen intermediates (ROI) and reactive nitrogen intermediates (RNI). An array of cytokines and chemokines, including tumor necrosis factor (TNF-α), induces a proinflammatory response and direct immune cells to the infection site. Dendritic cells migrate to draining lymph nodes, where they encounter many immature T cells. In the presence of proinflammatory cytokines such as IFN-γ and IL12, T cells become activated, multiply and differentiate into T helper (Th)1 cells. IFN-γ stimulates macrophages and triggers the potent antimicrobial activities of the primed Th1 cells.

Innate and Th1-dominant adaptive immune responses interact to produce granulomas. Innate and adaptive immune responses are critical for microorganism eradication.

Figure 3 footnotes: IL: interleukin; IFN-γ: interferon-gamma; TNF-α: tumor necrosis factor-α; ROI: reactive oxygen intermediates; RNI: reactive nitrogen intermediates. Red arrow pointing up indicates cytokines that are upregulated/increased during T.B. infection. Red arrow pointing down indicates cytokines that are downregulated during T.B. infection.

## 5. Host Immune Response during Helminth Coinfection with T.B.

The geographic distributions of helminths and T.B. overlap substantially, particularly in underdeveloped countries, resulting in an increased likelihood of coinfection with both pathogens [15,16]. This coexistence has also led to the hypothesis that helminths can worsen the effects of T.B. There have been suggestions that the anti-inflammatory response induced by helminths in cases of coinfection might dampen protective and immunopathological responses to T.B. [15,16].

An Ethiopian study investigated the association between intestinal helminths and active T.B. and found that helminth infection increases the likelihood of developing active T.B. [53]. This and other studies also suggested that patients with coinfection may have antagonistic effector cell responses in responding to and regulating these diseases [30,54]. This can also imply that the efficacy of the vaccines may be reduced.

One school of thought suggests that helminths create an environment that weakens the host's defenses against T.B. By activating the IL-4 receptor pathway, a preexisting helminth infection inhibits an innate pulmonary anti-T.B. defense [55]. In coinfected mice models, helminth-induced lung alterations increased susceptibility to T.B. [55]. Macrophages can be classically or alternatively activated. Classically activated macrophages (CAMs) increase the activity of nitric oxide synthase (iNOS), which converts L-arginine to nitric oxide and citrulline. Nitric oxide promotes intracellular *Mtb* killing.

On the other hand, alternatively activated macrophages (AAMs) induce arginase, which competes with iNOS for L-arginine, thereby reducing nitric oxide production for the intracellular killing of *Mtb* [48]. *Mtb* resistance in helminth-infected mice is promoted by AAMs. This major cellular pathway compromises the helminth-infected host's ability to limit *Mtb* growth [55].

A review in support of this proposed role of the Th2-dominant phenotype on *Mtb* control illustrated that AAMs might inhibit the macrophage killing of *Mtb* [48]. Conversely, a murine study in South Africa using *Nippostrongylus brasiliensis* (*Nb*) revealed that *Mtb* colonies were reduced in the lungs of *Nb*-infected mice. The stimulation of pulmonary CD4+ T cells and Th1 and Th2 cytokines, neutrophils and alveolar macrophages was elevated. This suggests that *Nb* infection triggers a macrophage response, which protects the host throughout the early phases of mycobacterial disease and subsequent illness [19].

Both helminths and T.B. have independent mechanisms for initiating the host immune response, with significant consequences for the immunology of each infection [15,16]. The coexistence of helminth infection and active tuberculosis has been demonstrated in epidemiological, cross-sectional and case-control studies that looked at the prevalence and correlation of the two diseases. Pulmonary T.B. patients were found to have a significant rate of intestinal nematode infection, indicating that helminth immunomodulation may affect the control of T.B. [53,56].

In Ethiopia, some studies reported an increase in the prevalence of helminth coinfection in T.B. patients, where one study found a higher risk of parasites among active T.B. patients than in healthy community controls [17,57,58]. Likewise, in Iran, a higher prevalence of intestinal helminths was found in tuberculosis patients compared to the uninfected subjects [59]. Taghipour and colleagues also determined that immunocompromised T.B. patients are more vulnerable to parasitic gastrointestinal infections [60]. It was reported that Blastocystis subtype 1 was the most common subtype found in T.B. patients; however, a phylogenetic analysis revealed no distinction between Blastocystis isolates from T.B. patients and those from the uninfected [59].

*S. mansoni* was also a risk factor for T.B. infection, and it altered the clinical presentation and pathogenesis of T.B. in Tanzania [61]. The authors recommended treatment of this parasite using praziquantel in T.B. infection management [61].

A systematic review suggested that health education be implemented to help prevent intestinal helminth infection. It further added that screening for helminths should be possibly included in the treatment strategies for tuberculosis patients [59]. Another review suggested an association between *Toxoplasma gondii (T. gondii)* seropositivity and having tuberculosis, with *T. gondii* seropositivity, which indicates chronic infection, being relatively common among tuberculosis patients [62].

*Strongyloides stercoralis* coinfection with pulmonary T.B. was implicated in the cause of the skewed immune response to mycobacterial disease [63]. The proinflammatory Th1 cytokines were reduced, whereas the anti-inflammatory Th2 and Th3 cytokines were elevated, thus leading to a conclusion that helminth coinfection may modulate protective immune responses in latent T.B. [63]. A study of immunological correlates in T.B. coinfection with *S. mansoni* in Kenya, on the other hand, discovered that the expression of T.B.-specific Th1 cytokines was maintained. Individuals with latent tuberculosis and *S. mansoni* infection had more CD4+ Th1 cells than those who were only latently T.B.-infected [22]. There were similar results in a Brazilian study, whose findings revealed that A. *lumbricoides* infection had no impact on Th1, Th2 and Th17 responses or the T cell populations [21].

A Th1 immune response observed during persistent filarial infection was characterized by a reduction in Purified Protein Derivative (PPD)-specific IFN-γ and IL17 responses [64]. The study suggested that filaria infection reduced the PPD-specific IFNγ and IL17 responses. In addition, it was observed that onchocerciasis patients' peripheral T cells had a weak response to *Mtb* antigens [65]. Elias and colleagues illustrated that compared to dewormed patients, helminth-infected individuals displayed low Th1 immune response and IFN-γ production in response to mycobacteria infection [66]. Lastly, it has been suggested that a robust Th1 response characterizes cell mediated protection against T.B. infection, and coinfection with helminths could modulate these immune responses by driving Th2 and Treg cells [17,67].

Furthermore, enhanced Treg function is associated with helminth infection and may suppress Th1 responses against unrelated antigens [12,67]. This finding was supported by studies which showed that intestinal helminth coinfection was associated with a reduced Th1 response in active T.B. [16,68]. Type I immunity and their proinflammatory cytokines such as IFN-γ, IL-12 and TNF-α have a protective role against *Mtb*. By contrast, the induction of type 2 immunity, e.g., Th2 and Treg cells (as seen in helminth infections) and their anti-inflammatory cytokines, were reported to suppress the efficient immune response against T.B. [38].

A mouse model study of Schistosoma mansoni showed a reduced protective efficacy of BCG vaccination against *Mtb* [66]. Another study demonstrated that concomitant helminth infections significantly impair the immunogenicity of BCG vaccines, an impairment associated with increased TGF-β production [30]. During active T.B., asymptomatic helminth infection has been shown to have a considerable impact on host immunity in a double-blind, randomized clinical study [17]. In comparison to the placebo group, eosinophils and IL-10 levels decreased after albendazole treatment [17]. Another albendazole treatment study was conducted to determine the immunological effects of deworming on proinflammatory cytokine responses to plasmodial antigens. The study demonstrated improvements in immune hypo responsiveness, where anthelmintic treatment significantly increased proinflammatory cytokine responses to Plasmodium falciparum-infected red blood cells [69].

In Egypt, it was determined that hookworm infection was one of the risk factors for the failure of T.B. therapy [70]. However, a human study in the United Kingdom (U.K.), where the authors studied migrants from Nepal, found that hookworm infection reduced T.B. growth and may reduce the risk of infection [20]. According to the evidence presented above, some studies demonstrated that helminthiasis has a negative impact on

T.B. diseases, while others showed a beneficial effect. Table 1 summarizes some of the studies investigating helminth and T.B. coinfections.

Although HIV is not covered in this review, there is evidence of a concurrent distribution of triple disease burden involving tuberculosis, helminths and HIV, particularly in Sub-Saharan Africa. This necessitates a greater focus on disease management strategies by various policymakers [71].

**Table 1.** Summary of experimental and human studies focusing on helminth and tuberculosis coinfections.

| References | Study Type, Location and Helminth(s) | Study Aim | Major Findings |
|---|---|---|---|
| [72] | Human study in Kenya. *Wuchereria bancrofti* and *Schistosoma haematobium* | To investigate whether prenatal immunity to helminths persists in childhood and if it alters the immune response to BCG | Compared to patients who had prenatal sensitization 10–14 months after BCG immunization, T cell IFN-γ production was 26-fold higher in infants who were not sensitized to filariae or schistosomes in utero. |
| [73] | Human study in South Africa. *Ascaris lumbricoides* and *Trichuris trichiura* | To determine total serum IgE before and after tuberculosis therapy | T.B. therapy resulted in reduced serum *Ascaris*-specific IgE levels. Tuberculin induration was found to be inversely related to IgE in patients but not in controls. |
| [65] | Human study in West Cameroon. *Onchocerca volvulus* | To determine total serum IgE before and after tuberculosis therapy | T.B. therapy resulted in reduced serum *Ascaris*-specific IgE levels. Tuberculin induration was found to be inversely related to IgE in patients but not in controls. |
| [64] | Human study in East Ethiopia. *Ascaris lumbricoides, hookworms, Trichuris trichiura, Strongyloides stercoralis, Hymenolepis nana* and *Taenia* spp. | To investigate the effect of intestinal helminths on the immune response to PPD in naturally immunized or BCG-vaccinated individuals | Individuals who received BCG vaccination and were infected with helminths had reduced T cell and PPD skin test responses. Increased T cell proliferation and IFN were associated with improved BCG efficacy following anthelmintic therapy. |
| [66] | An experimental study in Ethiopia. *Schistosoma mansoni* | To investigate whether chronic helminth-infected individuals have reduced efficacy of BCG vaccine compared to uninfected persons | Possibly through attenuation of protective immune responses to mycobacterial antigens and/or by polarizing the general immune responses to the Th2 profile, *S. mansoni* infection reduced the protective efficacy of BCG vaccination against *Mtb*. |
| [53] | Human study in Ethiopia. *Ascaris lumbricoides, Hookworm, Strongloides stercoralis, Trichuris trichiura, S. mansoni* and *Enterobius vermicularis* | To study the prevalence of intestinal helminth infections and their association with active T.B. in T.B. patients and healthy household contacts | In addition to HIV infection, intestinal helminth infection may be a risk factor for the development of active pulmonary T.B. This discovery could have significant consequences for the control of tuberculosis in helminth-endemic areas around the world. |
| [30] | Human study in Ethiopia. *Trichuris trichiura, Ascaris lumbricoides, hookworms, Taenia* spp., *Hymenolepis nana* and *Enterobius vermicularis* | This study tested anti-helminthic medication before BCG vaccination to determine if it could improve BCG vaccination immunogenicity in helminth-infected patients | Chronic worm infection reduced BCG immunogenicity in humans. This was linked to increased TGF-β production but not a better Th2 immune response. |

**Table 1.** *Cont.*

| References | Study Type, Location and Helminth(s) | Study Aim | Major Findings |
|---|---|---|---|
| [74] | Human study in South Africa. *Ascaris lumbricoides* and *Trichuris trichiura* | To investigate whether helminth infection could affect a child's ability to generate a proper Th1 immune response, which was defined by a positive tuberculin skin test (TST) | Helminth infection/exposure may reduce the immune response to *Mtb* infection. In younger children, being Ascaris IgE-positive significantly reduced the likelihood of being TST-positive, but this effect faded as they grew older. |
| [75] | Human study in Venezuela. *Ascaris lumbricoides* and *Trichuris trichiura* | To investigate the effects of parasite infections, malnutrition and plasma cytokine profiles on tuberculin skin test (TST) positivity | TST positivity was associated with low plasma Th1 cytokine levels in indigenous Venezuelan children with T.B. contacts and helminth infections. |
| [19] | Animal study in South Africa. *Nippostrongylus brasiliensis (Nb)* | To investigate the impact of acute *Nb*-induced lung damage and long-term parasite lung conditioning on the host's ability to control mycobacterial infection | The findings show that early stage *Nb* infection induces a macrophage response that protects against subsequent mycobacterial infection. |
| [76] | Human study in Ethiopia. *Giardia lamblia, Ascaris lumbricoides, Hookworm* spp., *Strongyloides stercoralis, Trichuris trichuria, Enterobius vermicularis, Taenia* spp., *Hymenolepis nana, Schistosoma mansoni* or trophozoite stage of *Entamoeba histolytica.* | To diagnose latent *Mtb* infection using the tuberculin skin test (TST) and the IFN-γ release assays in helminth infected school children | The tuberculin skin test should be used with caution in areas where parasitic intestinal infections are common. |
| [77] | Human study in Uganda. *Hookworm, Trichuris trichiura, Hymenolepis nana, Schistosoma mansoni, Ascaris lumbricoides, Hymenolepis nana* and *Schistosoma mansoni* | To determine whether coinfections such as helminths, malaria and HIV modulate the immune system and increase susceptibility to latent tuberculosis infection (LTBI), leading to the persistence of the tuberculosis epidemic | Concurrent helminth, malaria and HIV infections did not affect cytokine responses profile in individuals with LTBI. |
| [78] | Human study in Ethiopia. *Schistosoma mansoni* | To investigate whether maternal helminth infection affects maternal and neonatal immunological function and T.B. immunity | The combination of early secretory antigenic target 6 (ESAT-6) and culture filtrate protein 10 (CFP-10) elicited a significantly lower IFN-γ response in helminth-positive than in helminth-negative participants. Cord blood mononuclear cells' (CBMCs) IFN-γ response, total IgE and cross-placental transfer of T.B.-specific IgG were all negatively correlated with maternal helminth infection. |
| [17] | Human study in Ethiopia. *Ascaris lumbricoides Hookworm* spp. *Strongyloides stercoralis Trichuris trichiura Hymenolepis nana Taenia* spp. | To examine the clinical and immunological effects of helminth infection on T.B. | Asymptomatic helminth infection had a profound influence on the immunological profile of individuals with T.B. This favored Th2 immune responses such as increased regulatory T cells and IL-5 and IL-10 secreting cells. |

**Table 1.** *Cont.*

| References | Study Type, Location and Helminth(s) | Study Aim | Major Findings |
|---|---|---|---|
| [79] | Human study in Ethiopia. *Ascaris lumbricoides* | To investigate the clinical and immunological outcomes of patients coinfected with helminths and T.B. after albendazole treatment | The decrease in eosinophil counts and IL-10 demonstrated that asymptomatic helminth infection considerably impacts host immunity during tuberculosis and can be efficiently reversed with albendazole treatment. Helminth infection has clinical effects on chronic infectious diseases such as tuberculosis, and these effects should be further explored. |
| [80] | An animal study in the USA. *Schistosoma mansoni* | To investigate whether *Mtb*-specific T cell responses can be reversibly impaired by treatment of *S. mansoni* coinfection, without impacting arginase-1-expressing macrophage-mediated T.B. control | Anthelminthic treatment improved *Mtb*-specific T cell responses. In T.B.-infected mice, arginase-1-expressing macrophages in the lung formed granulomas and exacerbated inflammation. |
| [81] | An experimental animal study in USA. *Heligmosomoides polygyrus* | To investigate whether *Mtb* infection would be modulated in mice with chronic *H. polygyrus* infection | Despite a systemic increase in FoxP3+ T regulatory cells, neither primary nor memory immunity conferred by Mycobacterium bovis BCG vaccination were affected in mice with chronic enteric helminth infection. |
| [82] | Human study in India. *Strongyloides stercoralis* | To investigate whether helminth modulation of cytokine responses in latent T.B. coinfection is reversible after anthelminthic therapy | In *Strongyloides stercoralis*-latent T.B. coinfection, anthelmintic therapy reversed the modulation of systematic and T.B. antigen-stimulated cytokine responses. |

## 6. Effect of Helminth Infection on T.B. Vaccine

BCG is currently the only T.B. vaccine available; it celebrated its 100th anniversary in 2021. Alternative vaccines are being developed [83]. The BCG vaccine is still the only option for protection against human T.B., and it is inexpensive, safe and widely available. BCG effectiveness against T.B., however, varies in the high helminth-burden areas of the world [83]. Children are typically given the BCG vaccine. A review reported that BCG could provide protection against severe forms of T.B., including meningitis and miliary [84].

The BCG vaccine is administered to more than 80% of all newborns and babies in countries where it is included in the national childhood immunization program; however, it does not prevent the development of latent tuberculosis or the reactivation of pulmonary disease in adults [85]. BCG has been reported to be less effective in T.B.-coinfected individuals living in helminth-endemic areas [64]. However, another study reported no difference in BCG vaccination status and tuberculin skin testing (TST) responses in patients with or without T.B. and helminth coinfection [67].

An Ethiopian study found that helminth infection influenced BCG vaccination outcomes, and PPD-specific cellular immune responses improved in helminth-treated individuals compared to untreated controls [64]. Deworming was shown to boost the efficacy of BCG immunization in this randomized experiment [64]. In addition, it was found that the BCG vaccination of PPD-negative individuals in a helminth-infected population in Ethiopia had poor immunogenicity, and they concluded that this was due to a high Th2 bias in immunological responses caused by chronic helminth infection [64].

Furthermore, in another study, *S. mansoni* was found to reduce the protective efficacy of BCG vaccination against *Mtb*, possibly by attenuating protective immune responses to mycobacterial antigens and polarizing general immune responses to a Th2 profile [66].

Th2-like IL-10 responses elicited by intestinal helminths may interfere with Th1-like IFN responses induced by BCG, altering the protective immune response to BCG vaccination [86]. The impact of helminth infection is due to the antigen-specific modification of cell-mediated immunity, and the diminished efficacy could be owing to impaired immune responses to recall antigens [87].

Furthermore, helminth infection during pregnancy has been shown to persist into childhood and shift immunity away from Th1 responses, which are required in T.B. infection and vaccination [72]. Chronic helminth infections increase susceptibility to T.B. infections requiring Th1 responses and also lead to impaired efficacy of the BCG vaccine [30,88].

While there is mounting evidence that helminth prophylaxis could have a role in combating the HIV/AIDS and T.B. pandemics [89], observational research and randomized controlled trials have not revealed a uniform clinical picture. Deworming programs may help to enhance community-based health measures such as proper sanitation, access to clean water and adequate education [90]. More intervention research is required to demonstrate the impact of deworming on tuberculosis disease progression.

## 7. Helminth and T.B. Coinfection-Immune Mediated Pathology

The typical immune response to helminths, characterized by decreased IFN-γ, reduced T cell proliferation and IL-2 as a result of increased Th2/Treg cytokines, attenuates a potent anti-tuberculosis IFN-γ immune response and therefore uncontrolled T.B. pathology [15]. Furthermore, the helminth-induced expansion of AAMs and nitric oxide synthase suppression could also contribute to the impaired intracellular killing of T.B. in macrophages, thereby enhancing T.B. disease process [15]. In addition, the helminth-induced anergy of cognate and bystander T cells and increased apoptosis further impair T.B. responses and increase the pathogenesis [88].

## 8. Effect of Deworming during T.B.-Helminth Coinfection

The effects of deworming can be used to determine the impact of helminth infections. It was shown that the use of anthelminthic drugs to treat patients with helminths resulted in increased T cell proliferation and IFN-γ production of PBMC stimulated with PPD. The study showed that T cell responses to PPD were improved in filarial-infected patients treated with diethylcarbamazine [55,65].

The treatment of helminth-infected patients with albendazole during BCG vaccination increased proliferative and IFN-γ responses to PPD, suggesting that persistent helminth infection during BCG vaccination may contribute to a decreased T cell response to mycobacterial antigens. This meant that removing helminths via anthelminthic treatment would reduce Th2 cell and cytokine inhibitory effects on Th1 responses [91].

Toulza et al. found that anthelminthic therapy altered antimycobacterial immune responses in U.K. migrants. Patients with helminth infection had a higher frequency of CD4 + Fox P3 + T cells (Tregs) and a lower frequency of CD4 + IFN-γ + T cells, but these effects were reversed after treatment [68].

Another study in Gabon found that anti-helminth treatment with praziquantel against *Schistosoma* infection resulted in a significant decrease in CD4 + Fox P3 + T cells after treatment [92]. Since helminth infections cause widespread immunological alterations that revert to normal after the helminth infection is eradicated, their role in the interaction between their host and other pathogens could be substantial [93].

From the above, it is apparent that concurrent helminth and T.B. infections have demonstrated various effects on the host. These reactions could be due to different helminth species, their location in the body, different life cycles, variable (excretory/secretory) E/S products and *Mtb* infection. The virulence and infection route of the mycobacterial strain may also contribute.

Some in vitro studies have been reported to have shown that helminth infection affects *Mtb* infection in terms of immune response and disease severity, but the clinical and treatment outcome is unknown, possibly due to underpowered studies, the type or intensity of the infecting helminth and the various methodologies used to detect helminth infection [15].

## 9. Concluding Remarks

Concurrent helminth infection and T.B. both produce antagonistic immune responses. Helminths have the potential to impair the host's ability to respond to bystander infections such as T.B. Helminth and T.B.'s spatial overlap may impair the host's ability to respond to mycobacterial conditions. Th1 responses are required for T.B. immunity, whereas helminths mount an opposing Th2 response, which tends to dominate and thus skew the immune response. Furthermore, chronic helminth infections impair innate and adaptive immune responses to T.B. and induce immunoregulatory responses, lowering T.B. immunity even further. However, whether these opposing immune responses in helminth and T.B. coinfection affect pathological outcomes is unclear.

In helminth-endemic areas, it is suggested that chronic helminth infections reduce the efficacy of BCG, the currently available T.B. vaccine. There is conflicting evidence regarding the effectiveness of regular anti-helminth medication in the treatment of T.B., and this requires further investigation. Clarification of the effect of deworming in concurrent helminth-T.B. infections may aid in the development of government treatment policies. Since vaccines can prevent T.B. infection, the co-occurrence of helminths and T.B. must be considered when developing new vaccines and conducting research on them. Finally, more research is needed to understand better the effects of multicellular coinfecting pathogens on immune responses.

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# Appendix F:

# Published research article.

*microorganisms*

MDPI

*Article*

# Cytokine Responses during *Mycobacterium tuberculosis* H37Rv and *Ascaris lumbricoides* Costimulation Using Human THP-1 and Jurkat Cells, and a Pilot Human Tuberculosis and Helminth Coinfection Study

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**Abstract:** Background: Helminth infections are widespread in tuberculosis-endemic areas and are associated with an increased risk of active tuberculosis. In contrast to the pro-inflammatory Th1 responses elicited by *Mycobacterium tuberculosis* (Mtb) infection, helminth infections induce anti-inflammatory Th2/Treg responses. A robust Th2 response has been linked to reduced tuberculosis protection. Several studies show the effect of helminth infection on BCG vaccination and TB, but the mechanisms remain unclear. Aim: To determine the cytokine response profiles during tuberculosis and intestinal helminth coinfection. Methods: For the in vitro study, lymphocytic Jurkat and monocytic THP-1 cell lines were stimulated with Mtb H37Rv and *Ascaris lumbricoides* (*A. lumbricoides*) excretory-secretory protein extracts for 24 and 48 h. The pilot human ex vivo study consisted of participants infected with Mtb, helminths, or coinfected with both Mtb and helminths. Thereafter, the gene transcription levels of IFN-γ, TNF-α, granzyme B, perforin, IL-2, IL-17, NFATC2, Eomesodermin, IL-4, IL-5, IL-10, TGF-β and FoxP3 in the unstimulated/uninfected controls, singly stimulated/infected and costimulated/coinfected groups were determined using RT-qPCR. Results: TB-stimulated Jurkat cells had significantly higher levels of IFN-γ, TNF-α, granzyme B, and perforin compared to unstimulated controls, LPS- and *A. lumbricoides*-stimulated cells, and *A. lumbricoides* plus TB-costimulated cells ($p < 0.0001$). IL-2, IL-17, Eomes, and NFATC2 levels were also higher in TB-stimulated Jurkat cells ($p < 0.0001$). Jurkat and THP-1 cells singly stimulated with TB had lower IL-5 and IL-4 levels compared to those singly stimulated with *A. lumbricoides* and those costimulated with TB plus *A. lumbricoides* ($p < 0.0001$). *A. lumbricoides*-singly stimulated cells had higher IL-4 levels compared to TB plus *A. lumbricoides*-costimulated Jurkat and THP-1 cells ($p < 0.0001$). TGF-β levels were also lower in TB-singly stimulated cells compared to TB plus *A. lumbricoides*-costimulated cells ($p < 0.0001$). IL-10 levels were lower in TB-stimulated Jurkat and THP-1 cells compared to TB plus *A. lumbricoides*-costimulated cells ($p < 0.0001$). Similar results were noted for the human ex vivo study, albeit with a smaller sample size. Conclusions: Data suggest that helminths induce a predominant Th2/Treg response which may downregulate critical Th1 responses that are crucial for tuberculosis protection.

**Keywords:** *Mycobacterium tuberculosis* H37Rv; *Ascaris lumbricoides* excretory-secretory proteins; Jurkat cells; THP-1 cells; human tuberculosis and helminth co-infection; cytokine gene transcription levels

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## 1. Introduction

Tuberculosis (TB) infection is caused by *Mycobacterium tuberculosis* (Mtb), a significant global health challenge and one of the deadliest diseases caused by a single infectious agent [1]. Ten million TB cases and 1.4 million fatalities were reported globally in 2020 [1]. Furthermore, a quarter of the global population is latently infected with TB [1]. A competent immune system contains the TB infection in an asymptomatic/latent state. However, there are underlying factors in 5–10% of hosts that may lead to the development of active TB from latent infection [1,2].

Helminths infect 1.5 billion people worldwide, and *Ascaris lumbricoides* (*A. lumbricoides*), the most prevalent helminth, infects an estimated 807 million–1.2 billion people worldwide [3]. Humans are infected through ingestion of embryonated *A. lumbricoides* eggs containing larvae [3]. The hatched larvae enter the circulation and migrate to the lungs causing pneumonitis and eosinophilia [3]. Larvae mature further in the lungs (10 to 14 days), penetrate the alveolar walls, ascend the bronchial tree to the throat where they are coughed up and swallowed, thereby re-entering the gastrointestinal tract where they mature in the small intestines [3]. There is a significant geographic overlap between TB and helminth infection, particularly in low and middle-income countries (LMICs), with 20–35% of people being co-infected [4]. The impact of helminths on cell-mediated immunity has been the subject of numerous investigations [5–9]. However, it is still unclear if parasite infection is associated with TB activation from a dormant condition to active disease development [5].

An efficient T-helper type 1 (Th1)/pro-inflammatory response is required to control intracellular Mtb [7,10]. The Th1/pro-inflammatory response is characterised by the production of interferon-gamma (IFN-γ), tumour necrosis factor-alpha (TNF-α), and interleukins (IL-1, IL-6 and IL-12) [11]. In contrast, helminths skew the immunity towards a predominant T-helper type 2 (Th2)/anti-inflammatory and Regulatory (Treg) response, leading to the release of IL-4, IL-5, IL-9, IL-10, IL-13, and transforming growth factor-beta (TGF-β) [10,12]. These two arms of immune responses counter-regulate each other. Subsequently, helminths have been shown to reduce Bacille Calmette–Guerin (BCG) immunogenicity [13,14], weaken Mtb-specific Th1 responses, downregulate co-stimulatory molecules [15], induce anergy [16], and reduce treatment response, particularly in pulmonary TB [17,18].

However, in some studies, helminths were demonstrated to have no impact on human tuberculin skin tests [19], Mtb infection [20], or the improvement of TB disease management [20]. Therefore, reports on TB immune responses in cases of helminth coinfection are variable and dependent on the infecting parasite and the type of study [8,9,21]. Studies involving *Nippostrongylus brasiliensis* (Nb) and mycobacterial coinfection in mice yielded divergent findings on Mtb growth control. One study determined that mycobacterial clearance in the lungs of tuberculosis and Nb-coinfected mice was not delayed and that the helminth-induced Th2 responses do not exacerbate tuberculosis infection [22]. It was also reported that early-stage Nb infection increased macrophage production, which confers protection against subsequent stages of mycobacterial disease [23]. Conversely, another study reported that mycobacterial burden was higher in tuberculosis and Nb-coinfected mice and that these animals had reduced resistance to TB infection [24]. In human studies, *A. lumbricoides* infection was associated with negative tuberculin skin tests in children, suggestive of poor tuberculosis immune response [25,26].

Therefore, the effect of different helminth species and their antigens on immunity, particularly on macrophages, the primary effector cells in tuberculosis infection, remains unclear. Hence, the present study compared the cytokine immune responses in human THP-1 and Jurkat cells stimulated with and without coincident tuberculosis and *A. lumbricoides* antigen to simulate coinfection. The study was also extended to humans to determine the cytokine immune responses in ex vivo data. The detailed abbreviations and definitions used in the paper are listed in Table 1.

**Table 1.** List of abbreviations and acronyms used in the paper.

| Abbreviation | Definition |
|---|---|
| ATCC | American Type Culture Collection |
| *A. lumbricoides* | *Ascaris lumbricoides* |
| BCG | Bacille Calmette-Guerin |
| Eomes | Eomesodermin |
| ESP | Excretory-secretory protein |
| FoxP3 | Forkhead box P3 |
| GAPDH | Glyceraldehyde 3 diphosphate dehydrogenase |
| IFN-γ | Interferon-gamma |
| IL | Interleukin |
| LPS | Lipopolysaccharide |
| MHC | Major histocompatibility complex |
| Mtb | *Mycobacterium tuberculosis* |
| Nb | *Nippostrongylus brasiliensis* |
| NFATC2 | Nuclear factor of activated T-cells |
| OADC | Oleic acid albumin dextrose catalase enrichment |
| RT-qPCR | Real-time quantitative polymerase chain reaction |
| SA | South Africa |
| TGF-β | Transforming growth factor-beta |
| Th1 | T-helper type 1 |
| Th2 | T-helper type 2 |
| TNF-α | Tumour necrosis factor-alpha |
| Treg | Regulatory T cells |

## 2. Materials and Methods

### 2.1. Part 1: In Vitro Studies

#### 2.1.1. Bacterial Cultures

The H37Rv strain of Mtb (bacterial strain number 25618) was purchased from the American Type Culture Collection (ATCC) through Thistle QA Laboratory Services Cc in Johannesburg, South Africa (SA). H37Rv was cultured to log phase at 37 °C in 5% $CO_2$ in Middlebrook 7H9 broth with 0.05% Tween-80 and 10% oleic acid albumin dextrose catalase enrichment (OADC) (Becton Dickinson). Colony-forming units were counted by serial dilutions on Middlebrook agar plates. The protein concentration of the H37Rv was determined using the Bradford assay [27] and an optimal concentration of 5 µg/mL was used for cell stimulation. Cells were preserved in 1 mL aliquots at −80 °C until further use.

#### 2.1.2. Helminth (*A. lumbricoides*) Excretory-Secretory Protein Extracts

Whole worm excretory-secretory protein (ESP) extracts of *A. lumbricoides*, kindly donated by Prof William Horsnell, were prepared and supplied by the Division of Immunology, Department of Pathology from the Faculty of Health Sciences at the University of Cape Town, SA. Adult worms were obtained from patients from the Red Cross War Memorial Children's Hospital (Cape Town, South Africa), and were used to acquire *A. lumbricoides* excretory proteins. The *A. lumbricoides* excretory proteins were obtained by keeping the worms alive at 37 °C in Dulbecco modified essential medium with 1% Pen-strep (Thermofisher Scientific, Waltman, MA, USA), and 1% glucose (*wt./vol*). The media was collected three times a day. Using Amicon ultra concentrator, extract proteins were concentrated and resuspended in 5 mL of phosphate-buffered saline (Merck). All antigens were measured for protein content with a BCA protein estimation kit (Thermofisher Scientific) or by using the Bradford assay previously described [27] and stored at −80 °C at a standard concentration of 500 µg/mL until further use.

#### 2.1.3. Cell Culture and Treatment

Human monocytic THP-1 (lot number: TIB-202) and lymphocytic Jurkat (lot number TIB-152) cells were purchased from the ATCC by Thistle QA Laboratory Services Cc in Johannesburg, SA. The cells were maintained in 25 $cm^3$ cell culture flasks containing

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Roswell Park Memorial Institute (RPMI) supplemented with 2 mM L-glutamine, 5% HEPES, 100 U/mL penicillin, 100 µg/mL streptomycin, and 10% foetal bovine serum (FBS) at 37 °C in a humidified atmosphere of 5% $CO_2$.

Thereafter, the Jurkat and THP-1 cells were aliquoted into the 24 well multi-well plates in 1 mL aliquots (>1 × $10^6$) and unstimulated or stimulated with either lipopolysaccharide (LPS) (Thermofisher—catalogue number 00-4976-93), Mtb H37Rv, or *A. lumbricoides* ESP extracts. The unstimulated cells served as the negative control group, the LPS-stimulated group received 1 mg/mL LPS and served as a positive control, the *A. lumbricoides*-singly stimulated group were stimulated with 5 µ/mL of *A. lumbricoides* excretory protein extracts only, the Mtb-singly stimulated group were stimulated with 5 µg/mL of Mtb H37Rv only, and lastly, the costimulated group were co-stimulated with both 5 µg/mL of *A. lumbricoides* excretory protein ESP extracts and 5 µg/mL of Mtb H37Rv. Two independent experiments were set up in triplicate. Thereafter, the unstimulated/stimulated Jurkat and THP-1 cells were incubated for 24 or 48 h at 37 °C. At the end of the incubation period, the cells were collected, stored in Trizol® (Invitrogen; Thermo Fisher Scientific, Inc. catalogue 15596026) and stored in the −80 °C freezer for RNA extraction and gene transcription levels studies using Quantitative PCR.

#### 2.1.4. Real-Time-Quantitative PCR (RT-qPCR)

RNA was extracted from unstimulated/stimulated Jurkat and THP-1 cell lines using the Trizol® reagent (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA, catalogue 15596026) and the Pure Link™ RNA Mini Kit (Thermofisher Scientific, catalogue number 12183018A). The total RNA had to be DNA-free, therefore Pure link® DNase treatment at 80 µL per sample was used. The DNase treatment included 88 µL DNase buffer, 110 µL resuspended DNase, and 620 µL RNase-free water. The prepared DNase mixture was added directly onto the surface of the spin cartridge membrane, incubated at 15 min, washed with buffer, ethanol was added, and the cartridge was spun. RNase-free water was added to the spin cartridge and incubated for 1 min. The spin cartridge was spun with the recovery tube. The RNA preparation was added to a Nanodrop 2000 spectrophotometer (Thermofisher Scientific) to check for purity and concentration. Thereafter, the isolated RNA was reverse transcribed to cDNA using the High-Capacity cDNA Reverse Transcription Kit with RNase Inhibitor (Thermofisher Scientific, catalogue number 4374966), as per the manufacturer's instructions and reaction protocol. The Nanodrop 2000 spectrophotometer (Thermofisher Scientific) was used to quantify the total cDNA. The cDNA samples with an optical density at 260/280 nm ($OD_{260/280}$) >1.8 were used for RT-qPCR.

The Applied Biosystems Quant Studio 5 PCR instrument and software (Thermofisher Scientific, Waltham, MA, USA) were used to determine the transcription levels of the cytokine genes of interest listed in Table 2 in the unstimulated (control cells), tuberculosis-stimulated, *A. lumbricoides*-stimulated, LPS-stimulated, and *A. lumbricoides* and tuberculosis-co-stimulated cells.

The PCR master mix was prepared by adding 5 µL PCR-grade water (Thermofisher Scientific, catalogue number 10977023), 0.50 µL FAM-labelled cytokine probe mix (Thermofisher Scientific) (Table 2), 2.50 µL Fast Start 4× probe master mix (Thermofisher, catalogue number A15300) and 2 µL cDNA to make a total of 10 µL per sample. Glyceraldehyde 3-diphosphate dehydrogenase (GAPDH) was used as a housekeeping gene. PCR-grade water (Thermofisher Scientific, catalogue number 10977023), instead of cDNA, was used as a negative control.

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**Table 2.** FAM-labelled cytokine probe mix purchased from Thermofisher Scientific and their corresponding catalogue number. The cytokines and transcription factors were chosen based on the immune response—[Th-1 and Th-17 cytokines and transcription factors (INF-γ, TNF-α), IL2, IL17, Granzyme B, perforin, NFATC2 and Eomes), Th-2 (IL-4, IL5), Regulatory cytokines and transcription factors (TGF-β, IL-10 and FoxP3)].

| Cytokine Gene | Thermofisher Catalogue Number |
|---|---|
| Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (housekeeping gene) | Hs99999905_m1 |
| Interferon-gamma (INF-γ) | Hs00989291_m1 |
| Tumour necrosis factor-alpha (TNF-α) | Hs00174128_m1 |
| Granzyme B | Hs00188051_m1 |
| Perforin | Hs00169473_m1 |
| Interleukin-2 (IL-2) | Hs00174114_m1 |
| Interleukin-17 (IL-17) | Hs01056316_m1 |
| Nuclear factor of activated T-cells 2 (NFATC2) | Hs00905451_m1 |
| Eomesodermin (Eomes) | Hs00172872_m1 |
| Interleukin-4 (IL-4) | Hs00174122_m1 |
| Interleukin-5 (IL-5) | Hs99999031_m1 |
| Interleukin-10 (IL-10) | Hs00961622_m1 |
| Transforming growth factor beta (TGF-β) | Hs00234244_ml |
| Forkhead box P3 (FoxP3) | Hs01085834_m1 |

The PCR was performed at 95 °C for 1 min, followed by 45 cycles comprising denaturation at 95 °C for 30 s, annealing at 60 °C for 30 s and extension at 72 °C for 30 s. All PCR reactions were run in duplicate. Data were collected using the Applied Biosystems Quant Studio 5 V.2.3 software (Thermofisher Scientific, Waltham, MA, USA).

Serial dilutions of pooled cDNA synthesised from the total RNA were performed for each target gene and GAPDH, which served as standard curves for quantitative analysis, ranging from 1 ng/µL to 1000 ng/µL. Gene transcription levels results were depicted as the transcription levels of the gene of interest divided by the transcription levels of GAPDH.

### 2.2. *Part B: Human Ex-Vivo Experiment*

The Th-1, Th-17 and Treg cytokine gene and transcription factor's transcription levels study were also piloted for human ex-vivo experiments to compare the human and in vitro cytokine profile results. The current analysis is a sub-study of a previously described cohort of 414 individuals recruited from 6 primary healthcare clinics in a peri-urban, poor settlement in the eThekwini district of KwaZulu-Natal [28]. In this study, cytokine analysis was undertaken for 164 participants; of those, 96 were HIV-infected and had to be excluded, leaving 68 eligible participants. Thereafter, the eligible individuals were subdivided into uninfected controls (no helminth or TB) ($n$ = 18), helminth-singly infected only ($n$ = 35), TB-singly infected only ($n$ = 6), and TB and helminth-co-infected ($n$ = 6) groups.

Stool samples were collected for microscopical detection of helminth eggs/larvae using the Kato–Katz and Mini Parasep methods. Blood samples were also collected for parasite serology (*A. lumbricoides*-specific IgE and IgG4) to improve the sensitivity and specificity of parasite detection [29]. TB diagnosis and confirmatory results were obtained from the district hospital laboratory that services the clinics where participants were recruited. The sputum was analysed using the GeneXpert Infinity 48 s (Catalog number: Infinity-48).

Whole blood samples (4 mls) collected from the recruited participants were also stored in Trizol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.) at −80 °C for RNA extraction and RT-qPCR-based gene transcription levels studies as described for the in vitro experiments above.

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### 2.3. Statistical Analysis

A standard curve method was used to calculate gene transcription levels, whereby the transcription levels of the target gene were divided by the transcription levels value of the housekeeping gene (GAPDH). Values were expressed as medians. All cytokine gene transcription levels data were analysed using GraphPad Prism 5 (GraphPad Software, Inc., San Diego, CA, USA) statistical software package. For the in vitro and human ex vivo studies, analysis of variance (ANOVA) or the Kruskal–Wallis test with Tukey or Dunn's Multiple Comparison was used to assess for statistical significance in cytokine gene transcription levels profiles between the different groups (uninfected/unstimulated controls, singly infected/stimulated and coinfected/costimulated groups). Thereafter, the Mann–Whitney or Student's *t*-test was used to calculate the *p*-value between the two groups. All data presented in figures below are expressed as the median and interquartile range. A $p < 0.05$ was considered statistically significant.

## 3. Results

### 3.1. Part 1: In Vitro Study

Profiling of cytokine and transcription factor gene transcription levels was performed using THP-1 and Jurkat cells to investigate whether TB stimulation would upregulate pro-inflammatory and Th1 cytokines and whether *A. lumbricoides* coinfection would down-regulate these. Furthermore, it was aimed to determine whether *A. lumbricoides* would upregulate Th2 and regulatory cytokines.

#### 3.1.1. Th1/Pro-Inflammatory Immune Responses

Cytokine gene transcription levels levels in unstimulated and stimulated human cell lines are summarised in the figures below, showing a significant increase of Th1/pro-inflammatory cytokine genes after TB stimulation.

IFN-γ and TNF-α (at both 24 and 48 h stimulation time points), granzyme B (24 h stimulation only) and perforin (48 h stimulation only) levels were significantly higher in the TB-singly stimulated Jurkat cells compared to the unstimulated controls, LPS- and *A. lumbricoides*-singly stimulated Jurkat cells, and *A. lumbricoides* plus TB-costimulated Jurkat cells ($p < 0.0001$) (Figure 1). Similar results were noted for the THP-1 stimulated cells, apart from perforin, where similar findings were noted at 24 h and 48 h (Figure 2).

IL-2, IL-17, Eomes, and NFATC2 (at both 24 and 48 h stimulation) were significantly higher in TB-singly stimulated Jurkat cells compared to the unstimulated controls, LPS- and *A. lumbricoides*-singly stimulated Jurkat cells, and TB plus *A. lumbricoides*-costimulated Jurkat cells ($p < 0.0001$) (Figure 3). Similar findings resulted from tests on THP-1 cells ($p < 0.0001$) (Figure 4).

#### 3.1.2. Th2/Anti-Inflammatory, Immune Responses

Type 2 cytokine responses after stimulation of cell lines in Figure 5 show that both IL-4 and IL-5 were increased after *A. lumbricoides*-antigen stimulation of both cell lines.

IL-5 (24 and 48 h stimulation) levels were significantly lower in the TB-singly stimulated cells compared to the *A. lumbricoides*-singly stimulated and TB plus *A. lumbricoides*-costimulated Jurkat and THP-1 cells, which had similar transcription levels ($p < 0.0001$). Similar findings were noted for IL-4; however, the *A. lumbricoides*-singly stimulated cells had significantly higher IL-4 levels compared to the TB plus *A. lumbricoides*-costimulated Jurkat (48 h stimulation) and THP-1 cells (24 and 48 h stimulation) ($p < 0.0001$).

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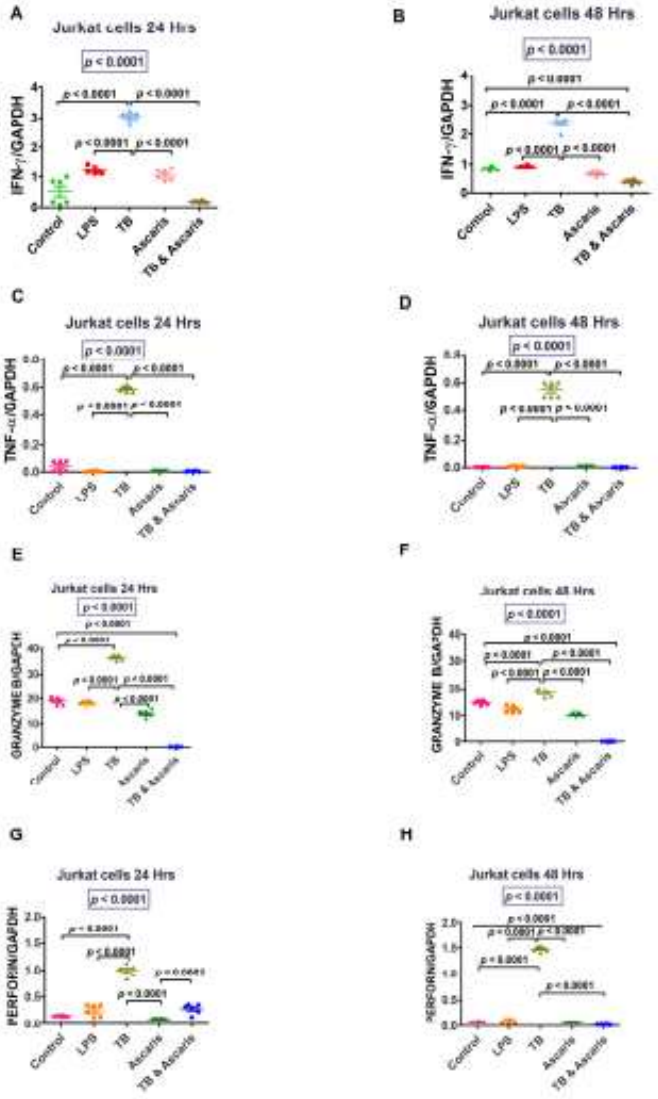


**Figure 1.** Transcription levels data for IFN-γ, TNF-α, granzyme B, and perforin are shown for Jurkat cells at 24 and 48 h time points. The unstimulated control cells and LPS-stimulated cells were negative and positive controls, respectively. (**A**) IFN-γ levels for Jurkat cells stimulated for 24 h, (**B**) IFN-γ levels for Jurkat cells stimulated for 48 h, (**C**) TNF-α levels for Jurkat cells stimulated for 24 h, (**D**) TNF-α levels for Jurkat cells stimulated for 48 h, (**E**) granzyme B levels for Jurkat cells stimulated for 24 h, (**F**) granzyme B levels for Jurkat cells stimulated for 48 h, (**G**) perforin levels for Jurkat cells stimulated for 24 h, and (**H**) perforin levels for Jurkat cells stimulated for 48 h.

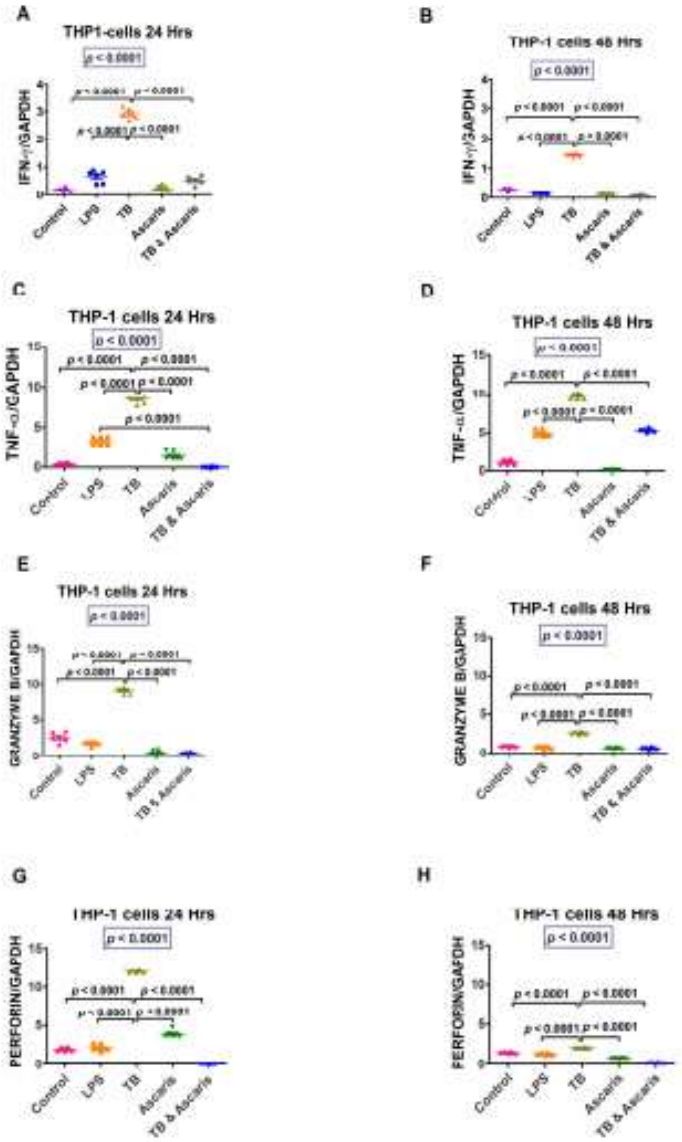


**Figure 2.** Transcription level data for IFN-γ, TNF-α, granzyme B, and perforin are shown for THP-1 cells at 24 and 48 h time points. The unstimulated control cells and LPS-stimulated cells were negative and positive controls, respectively. (**A**) IFN-γ levels for THP-1 cells stimulated for 24 h, (**B**) IFN-γ levels for THP-1 cells stimulated for 48 h, (**C**) TNF-α levels for THP-1 cells stimulated for 24 h, (**D**) TNF-α levels for THP-1 cells stimulated for 48 h, (**E**) granzyme B levels for THP-1 cells stimulated for 24 h, (**F**) granzyme B levels for THP-1 cells stimulated for 48 h, (**G**) perforin levels for THP-1 cells stimulated for 24 h, and (**H**) perforin levels for THP-1 cells stimulated for 48 h.

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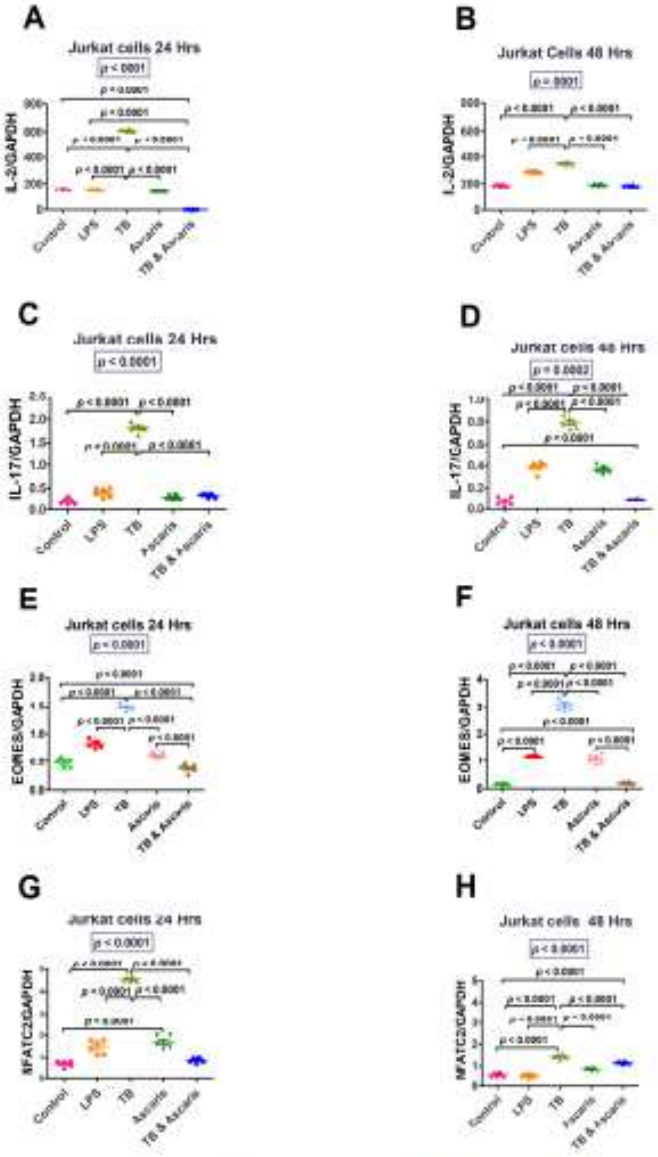


**Figure 3.** Jurkat cell line responses for Th1/pro-inflammatory (IL-2 and IL-17) and transcription factors (Eomes and NFATC2) at 24 and 48 h time points. The unstimulated control cells and LPS-stimulated cells were negative and positive controls, respectively. (**A**) IL-2 levels for Jurkat cells stimulated for 24 h, (**B**) IL-2 levels for Jurkat cells stimulated for 48 h, (**C**) IL-17 levels for Jurkat cells stimulated for 24 h, (**D**) IL-17 levels for Jurkat cells stimulated for 48 h, (**E**) EOMES levels for Jurkat cells stimulated for 24 h, (**F**) EOMES—transcription levels for Jurkat cells stimulated for 48 h, (**G**) NFATC2 levels for Jurkat cells stimulated for 24 h, and (**H**) NFATC2 levels for Jurkat cells stimulated for 48 h.

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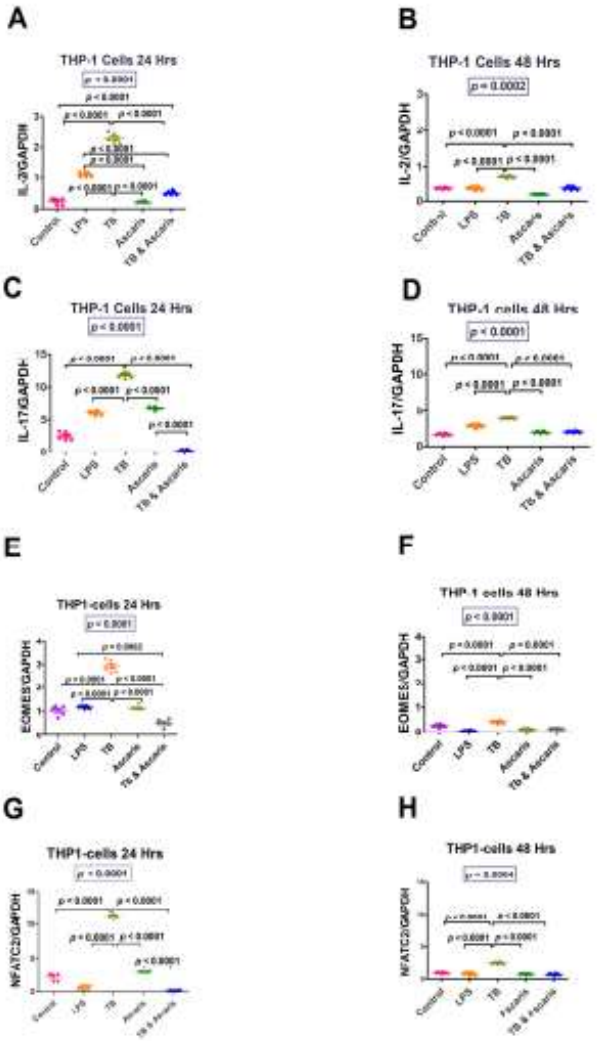


**Figure 4.** THP-1 cell line responses for Th1/pro-inflammatory (IL-2 and IL-17) and transcription factors (Eomes and NFATC2) at 24 and 48 h time points. The unstimulated control cells and LPS-stimulated cells were negative and positive controls, respectively. (**A**) IL-2 levels for THP-1 cells stimulated for 24 h, (**B**) IL-2 levels for THP-1 cells stimulated for 48 h, (**C**) IL-17 levels for THP-1 cells stimulated for 24 h, (**D**) IL-17—transcription levels for THP-1 cells stimulated for 48 h, (**E**) EOMES levels for THP-1 cells stimulated for 24 h, (**F**) EOMES levels for THP-1 cells stimulated for 48 h, (**G**) NFATC2 levels for THP-1 cells stimulated for 24 h, and (**H**) NFATC2 levels for THP-1 cells stimulated for 48 h.

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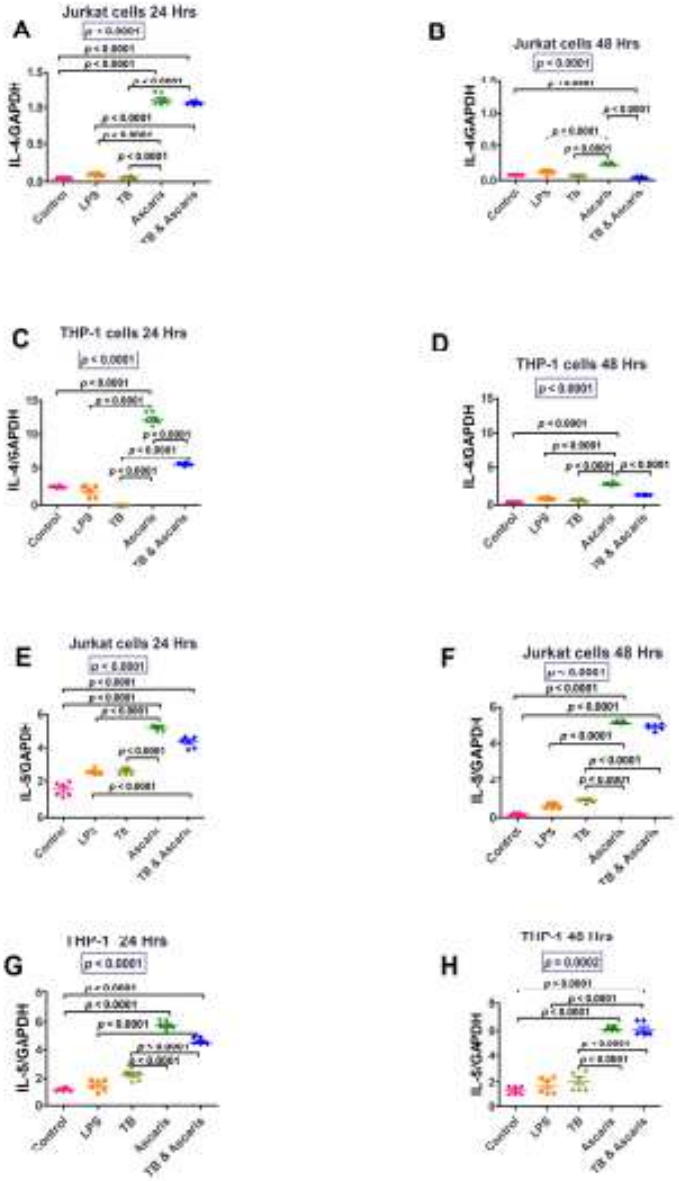


**Figure 5.** IL-4 and IL-5 responses in TB, *A. lumbricoides*, and dually stimulated Jurkat and THP-1 cells at 24 and 48 h time points. (**A**) IL-4 levels for Jurkat cells stimulated for 24 h, (**B**) IL-4 levels for Jurkat cells stimulated for 48 h, (**C**) IL-4—levels for THP-1 cells stimulated for 24 h, (**D**) IL-4 levels for THP-1 cells stimulated for 48 h, (**E**) IL-5 levels for Jurkat cells stimulated for 24 h, (**F**) IL-5 levels for Jurkat cells stimulated for 48 h, (**G**) IL-5 levels for THP-1 cells stimulated for 24 h, and (**H**) IL-5—transcription levels for THP-1 cells stimulated for 48 h.

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#### 3.1.3. Regulatory Cytokines

Figure 6 illustrates regulatory cytokine transcription levels in the cell lines. In both Jurkat and THP-1cells, both *A. lumbricoides* and *A. lumbricoides* plus TB stimulation significantly increased TGFβ and IL-10 transcription levels at 24 h and remained high at 48 h in *A. lumbricoides*-stimulated cells. FoxP3 was increased in both *A. lumbricoides* and TB-*A. lumbricoides* stimulation in both cell lines and at both time points (24 and 48 h).

TGF-β levels were significantly lower in the TB-singly stimulated Jurkat cells (24 h stimulation) compared to the TB plus *A. lumbricoides*-costimulated cells, however, the opposite trend was observed for THP-1 cells (24 h stimulation) ($p < 0.0001$). Conversely, no significant differences were noted in Jurkat and THP-1 cells (48 h stimulation) between the TB-singly stimulated and TB plus *A. lumbricoides*-costimulated cells. IL-10 levels were significantly lower in the TB-stimulated Jurkat (24 and 48 h stimulation) and THP-1 (24 h stimulation) cells compared to the TB plus *A. lumbricoides*-costimulated cells ($p < 0.0001$). FoxP3 levels were also significantly lower in the TB-singly stimulated Jurkat and THP-1 cells (24 and 48 h stimulation) in comparison to the TB plus *A. lumbricoides*-costimulated cells ($p < 0.0001$). (Figure 6).

### *3.2. Part 2: Human Ex-Vivo Experiment Results*

A total of 414 participants were recruited in the main study [29]; of those, a subpopulation of 164 were eligible for cytokine gene transcription level analysis, based on blood sample availability. However, 96 were HIV-infected and were excluded, leaving 68 eligible participants. Of the eligible participants, 18 were uninfected and were used as controls; 35 were helminth-infected (24 were infected with *A. lumbricoides*, 3 *Trichuris trichiura*, 3 *Taenia* spp., 3 *Schistosoma* spp., and 2 had *Strongyloides* spp.), 6 had TB, and another 6 had TB and helminth (3 had *A. lumbricoides*, 1 *Schistosoma* spp., 1 *Trichuris trichiura* and 1 with *Taenia* spp.) coinfection.

Regardless of the small sample sizes for these two groups, the Th1/pro-inflammatory cytokines (IFN-γ, TNF-α, granzyme B, IL-2, and IL-17), critical cytokines for TB, were significantly higher among the TB-singly infected individuals compared to the uninfected controls and helminth-infected groups (Figure 5). In the presence of helminth and TB coinfection, these cytokines were decreased, although there was no significant difference noted betweenthe coinfected group and the TB-singly infected group, except for granzyme B, where the TB and helminth-coinfected group had lower levels compared to the TB-singly infected group (Figure 7).

Eomes and NFATC2 were significantly higher in the control group compared to the coinfected group. The coinfected group also had lower Eomes and NFATC2 levels compared to the TB-singly infected and helminth-singly infected groups (Figure 8). IL-4 and IL-10 responses were variably increased in the helminth-infected individuals. TGF-β levels were variably increased in the controls and decreased in TB-singly infected and the helminth and TB-coinfected individuals. FoxP3 levels also differed between the controls and the TB-singly infected groups and between the helminth-singly infected and TB-singly groups. The low number of TB-infected individuals resulted in even lower numbers of the coinfected groups, thus making statistically valid analytical comparisons difficult (Figure 9).

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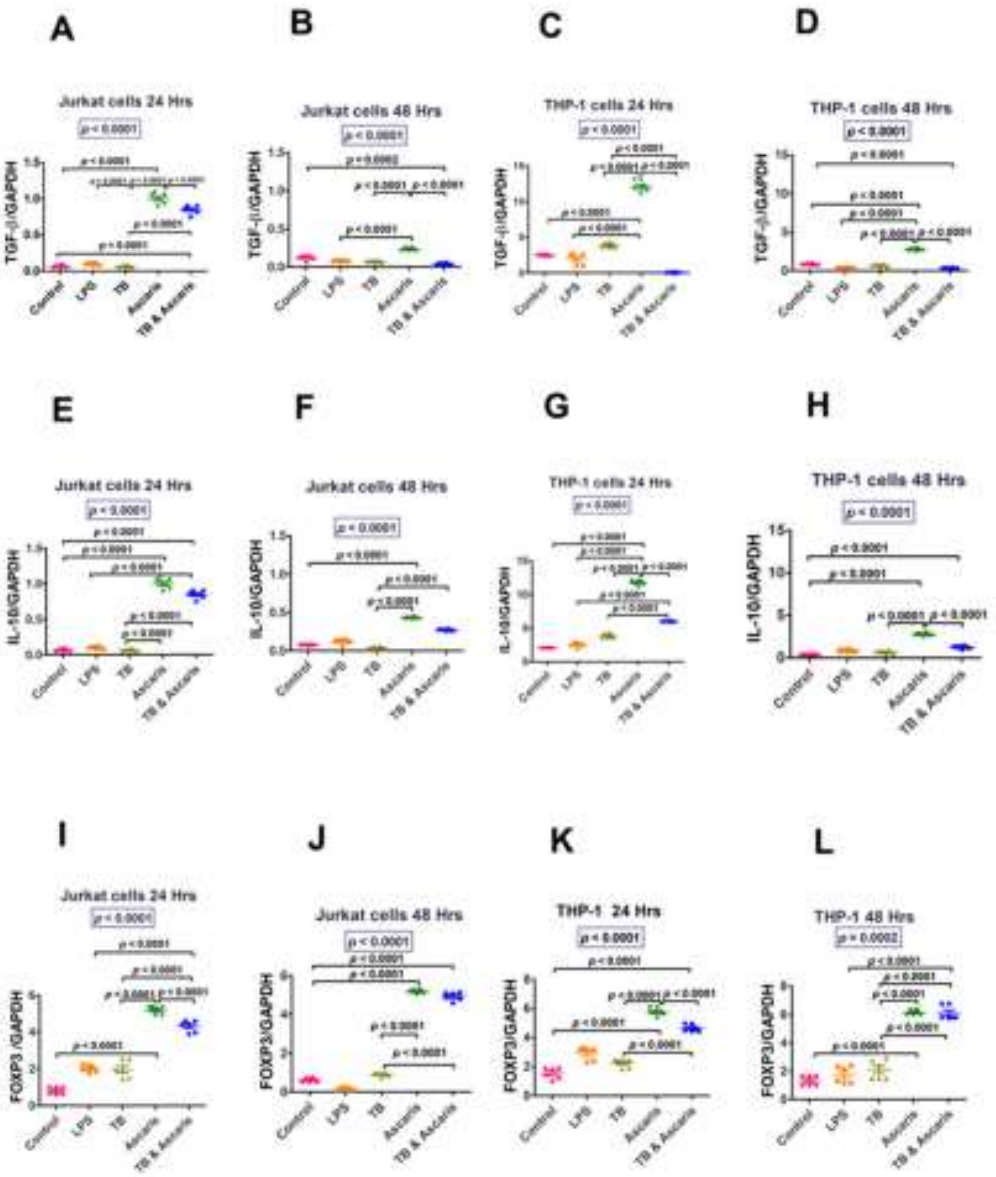


**Figure 6.** Jurkat and THP1 cell responses for regulatory cytokines (TGF-β, IL-10, and FoxP3) at 24 and 48 h time points. The unstimulated control and LPS-stimulated cells were used as negative and positive controls, respectively. (**A**) TGF-β levels for Jurkat cells stimulated for 24 h, (**B**) TGF-β levels for Jurkat cells stimulated for 48 h, (**C**) TGF-β levels for THP-1 cells stimulated for 24 h, (**D**) TGF-β levels for THP-1 cells stimulated for 48 h, (**E**) IL-10 levels for Jurkat cells stimulated for 24 h, (**F**) IL-10 levels for Jurkat cells stimulated for 48 h, (**G**) IL-10 levels for THP-1 cells stimulated for 24 h, (**H**) IL-10 levels for THP-1 cells stimulated for 48 h, (**I**) FoxP3 levels for Jurkat cells stimulated for 24 h, (**J**) FoxP3 levels for Jurkat cells stimulated for 48 h, (**K**) FoxP3 levels for THP-1 cells stimulated for 24 h, (**L**) FoxP3 levels for THP-1 cells stimulated for 48 h.

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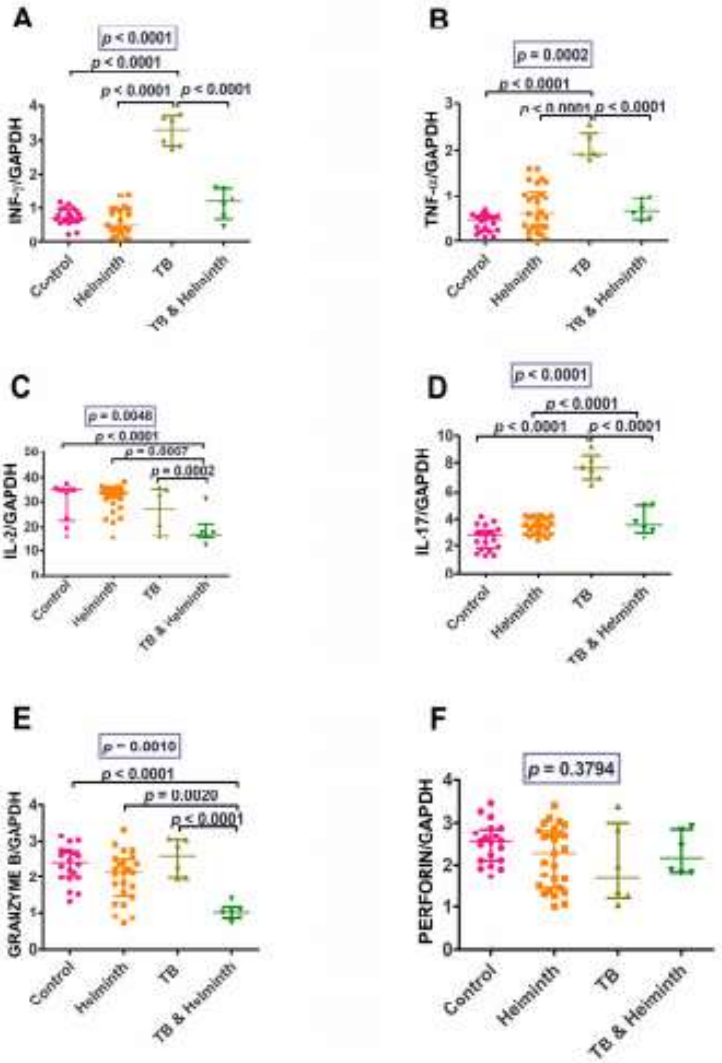


**Figure 7.** IFN-γ, TNF-α, IL2, IL-17, perforin, and granzyme B ex vivo gene transcription levels data. (**A**) IFN-γ levels, (**B**) TNF-α levels, (**C**) IL-2 levels, (**D**) IL-17 levels, (**E**) granzyme B levels, and (**F**) perforin levels.

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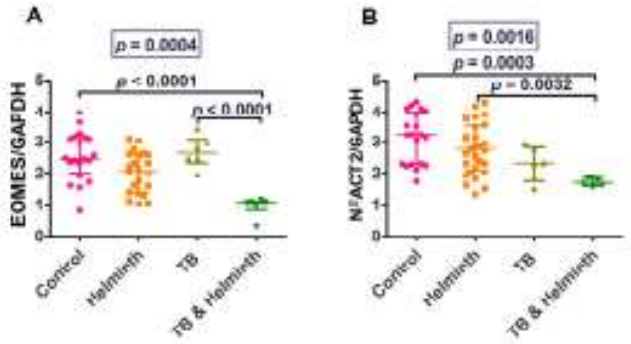


**Figure 8.** Eomes and NFATC2 gene transcription levels values. (**A**) Eomes levels, and (**B**) NFATC2 levels.

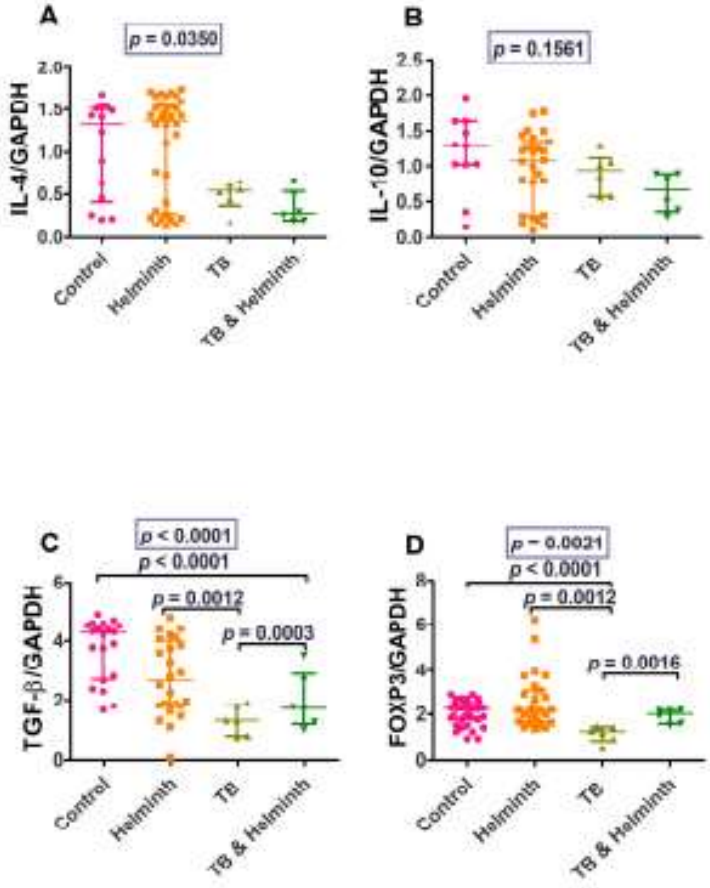


**Figure 9.** IL-4, IL-10, TGF-β, and FoxP3 gene transcription levels values. (**A**) IL-4 levels, (**B**) IL-10 levels, (**C**) TGF-β levels, and (**D**) FoxP3 levels.

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IFN-γ, TNF-α, and IL-17 were highest in TB-infected group (albeit, there were only six individuals). Perforin was similar across all groups, while granzyme B levels differed between the control and coinfected groups ($p < 0.0001$), between the helminth-infected and coinfected groups ($p = 0.0020$), and between TB-infected and coinfected groups ($p < 0.0001$). IL-2 levels differed between the control and the TB plus helminth-coinfection group ($p < 0.0001$) and also between the helminth-infected and coinfected group ($p = 0.0067$).

Eomes levels were higher in the controls than in the TB/helminth co-infected ($p < 0.0001$) and higher in the TB-infected compared to the coinfected individuals ($p < 0.0001$). NFATC2 levels were significantly higher among the controls compared to the coinfected individuals ($p = 0.0003$) and higher in the helminth-infected than in the TB/helminth-coinfected individuals ($p = 0.0032$).

IL-4, IL-10, and TGF-β were higher among uninfected controls and helminth-infected individuals (albeit there was a wide distribution in values) compared to the TB-singly infected and coinfected groups (albeit there was a small sample size). TGF-β was lower in the TB-singly infected group and the coinfected group, compared to the controls ($p = 0.0012$ and $p < 0.0001$, respectively). FoxP3 was significantly lower among the TB-infected compared to both the control ($p < 0.0001$) and helminth-infected groups ($p = 0.0012$).

## 4. Discussion

The present study aimed to determine the profile of cytokines after stimulation of monocytic and lymphoid cells with *A. lumbricoides* and TB antigens to assess whether *A. lumbricoides* infection would decrease the Th1/pro-inflammatory cytokines essential for TB control and increase the Th2/anti-inflammatory and regulatory cytokines. The human ex vivo data were also used to determine the cytokine responses in helminth and TB infection and in cases of helminth/TB coinfection. The Th1 cytokines were increased in TB-stimulated cells/infected individuals and reduced during coinfection. The Th2 and regulatory cytokines were variably increased in dual infection.

The Th1/pro-inflammatory cytokines, IFN-γ and TNF-α, were upregulated in response to TB compared to the *A. lumbricoides* and coinfection stimulation. This finding suggests that Th1/pro-inflammatory cytokines are upregulated by TB and reduced in helminth coinfection. These cytokines are produced more in pro-inflammatory conditions such as TB [7]. The cytokine IFN-γ is essential for protective defence against intracellular infections. IFN-γ is a key modulator of macrophage activation in *Mycobacterium tuberculosis* (*Mtb*) infection [29,30].

TNF-α plays a pivotal role in granuloma formation, which is one of the host's defence mechanisms against TB [31]. According to some studies, TNF-α levels are frequently high in individuals with active TB infection [32,33]. Our analysis also demonstrated a similar pattern.

The molecules involved in the cell-mediated killing of intracellular pathogens in the pro-inflammatory response included granzyme B and perforin. Granzyme is a serine protease present in the granules of cytotoxic lymphocytes. Perforin and granzyme work together to kill infected cells or target cells by perforating the cell walls leading to disintegration [34]. Natural Killer (NK) and CD8-positive cells primarily produce granzyme B and perforin [34,35]. They attack malignant or infected cells and cause them to undergo apoptosis [35]. Granzyme B and perforin were both increased at 24 h in the TB-stimulated cells and reduced at 48 h, suggesting that they induce apoptosis in infected cells during the early stages of infection. However, it is crucial to note that more experiments such as tunnel assays or flow cytometry should be performed to validate these results for granzyme B and perforin.

The increased levels of IL-2, IL-17, Eomes, and NFATC2 for both the in vitro and ex vivo analyses are in keeping with the pro-inflammatory response. IL-2 is produced by Th1 cells, and it stimulates T-cell proliferation, among other functions. In turn, Th1 cells produce IL-2, which has been found to stimulate cytotoxic T lymphocytes and Th1

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cells during intracellular pathogen invasion [36,37]. Compared to uninfected individuals, patients with active TB have been shown to have high IL-2 cytokine levels suggesting that this cytokine plays a protective role [38].

IL-17, an inflammatory cytokine released during the early stages of TB infection, is suggested to increase the synthesis of chemokines that aid in the recruitment of cells essential for granuloma formation [39]. Limiting Mtb growth and immunopathology caused by increased IL-17 production requires a balance between Th1 and Th17 immune responses [40]. Overproduction of IL-17 can increase neutrophil recruitment, which can cause tissue damage [40]. A Th1/Th17 balance is required for anti-mycobacterial immunity and immunological disease prevention [40]. It is notable, then, that the current study determined that, in vitro and ex vivo, the Th1/pro-inflammatory responses are higher in TB infection and reduced in helminth infection and TB/helminth coinfection cases.

Eomes levels were increased in our study, in both the in vitro and ex vivo experiments in the TB stimulated/infected group. Eomes was increased since it plays a role in the differentiation of cytotoxic T cells [39], which promote the killing of infected cells through the release of granzyme B and perforin [39]. Hence, in our study, Eomes was increased in both the in vitro and ex vivo experiments in the TB-stimulated/infected groups. NFATC2 overexpression aids cell defence against oxidative stress and electrophilic offences by stimulating synthesis of antioxidative and detoxifying enzymes [41]. As expected, pro-inflammatory/Th1 responses were all increased by TB-antigen stimulation and decreased during helminth coinfection in our study. Furthermore, the current study suggests that the *A. lumbricoides* effect of lowering the pro-inflammatory/Th1 cytokine responses to TB could be detrimental to TB control during TB and helminth coinfection.

In the present study, the Th2/anti-inflammatory cytokines, IL-4 and IL-5, were higher in the helminth infection and coinfection in vitro stimulations compared to the TB stimulation. This is in keeping with the Th2-predominant immune response produced by the extracellular helminths. IL-4 was increased at 24 h for both cell lines in the *A. lumbricoides* and coinfection stimulations; however, this was not sustained at 48 h. The upregulation of IL-4 was shown by the significant differences between the *A. lumbricoides* stimulation and the *A. lumbricoides*/TB co-stimulation. High IL-4 downregulates IFN-γ, which may be deleterious for TB control [42]. IL-5- was elevated in the helminth and coinfection group versus the control group. High levels of IL-5 are commonly observed in intestinal helminth- and protozoa-infected hosts, and it also induces eosinophilia, another common manifestation of parasite infection [43]. The current study confirmed the association between *A. lumbricoides* and Th2 cytokine responses.

The regulatory cytokines, IL10 and transcription factor FoxP3, were upregulated in the helminth-infected and coinfected cells compared to the TB-infected group. The increase is expected in *A. lumbricoides*-treated cells since helminths polarise immunity towards a Th2 and regulatory immune response [14,44]. Transcription factor, FoxP3, was also high for the current study's in vitro and ex vivo experiments. This upregulation of FoxP3 in helminth- and coinfection-stimulated cells concurs with the study that determined that helminths increase the secretion of TGFβ, which upregulates FoxP3 and promotes differentiation of regulatory cells [45]. Regulatory cytokines, such as IL-10, play a suppressive role in regulating immune homeostasis. Hence, IL-10 levels are higher in helminth infection since these parasites have mechanisms of evading the immune system to ensure long-term survival within the host [46]. IL-10 and FoxP3 were increased in the *A. lumbricoides* infection and coinfection groups, suggesting that IL-10 and FoxP3 are upregulated by *A. lumbricoides*. Dual infection stimulation was done to elucidate coinfection scenarios and to determine if there is an effect in the up or downregulation of Th1, Th2, and regulatory cytokines. Regulatory cytokines are high during *A. lumbricoides* infection and also in cases of TB/helminth coinfection compared to TB infection. This may be due to the downmodulation of the immune response to TB.

The present study demonstrated a typical TB response characterized by an increase in inflammatory cytokines such as IFN-γ, TNF-α, IL-2, and IL-17. However, we did not

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use costimulatory molecules such as anti-CD 28 or anti-Cd49d to enhance the stimulation of the Jurkat cells, since they do not possess antigen-presenting properties. Therefore, the Jurkat cell response may be suboptimal, due to the exclusion of immuno co-stimulatory molecules, which is a limitation of this study.

## 5. Study Limitations

The current study was limited by the small sample sizes in the TB-infected and TB plus helminth-coinfected participant groups. However, the limited analysis mirrored what was found in in vitro experiments, which showed higher pro-inflammatory/Th1 in the TB-infected group and lower in the coinfected group. An unexpected, possibly spurious finding was that some levels of cytokine gene transcription levels levels were highest among the uninfected controls, such as perforin, NFATC-2, TGF-β, and IL-10. This may be because the uninfected controls were only screened for helminths and TB in the laboratory, while they could possibly be exposed to other bacterial, viral, or other immune-activating factors that could not be detected during the questionnaire administration that was used in the main study. The demographic profile of the participants may attest to the possibility of other immune-activating environmental factors [29]. In addition, as alluded to above, the cell culture experiments in the in vitro studies did not include co-stimulatory molecules to properly represent the in vivo antigen presentation and processing.Therefore, these results were suboptimal, despite the fact that the main responses typically depicted TB (Th1/inflammatory) and helminth (Th2/Treg) profiles.

As noted above, there are additional tests, such as flow cytometry and tunnel assays that could be performed to validate the increase in granzyme B and perforin.

The gene transcription levels of cytokines in stimulated and unstimulated Jurkat and THP-1 cell lines are not directly correlated with its production. Therefore, to validate the gene transcription levels results, further tests such as ELISA needed to be performed.

## 6. Conclusions

The in vitro findings suggest that pro-inflammatory Th1 responses are increased in TB infection and reduced in cases of coinfection. The study also determined that anti-inflammatory Th2 and regulatory cytokines are increased during single helminth infection and in TB and helminth coinfection. The ex vivo data, although limited by the sample size, also supported the hypothesis that TB increases Th1 immune responses and responses to helminths involve strong Th2 and regulatory cytokines.

**Author Contributions:** K.N.B.: Patient recruitment, development of study procedures, data collection, specimen processing, analysis of data, conceptualisation, visualisation, methodology, writing—original draft preparation. Z.L.M.-K.: Project development, data collection and analysis, conceptualisation, visualisation, supervision, funding acquisition, writing—reviewing/and editing. R.S. and P.N.: data analysis, writing, reviewing and editing M.N.M.-M. and N.N.-M.: Specimen processing, data collection, data analysis, writing, reviewing and editing. All authors have read and agreed to the published version of the manuscript.

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**Institutional Review Board Statement:** The University of KwaZulu Natal Biomedical Research Ethics Committee (BREC) (BREC/00001983/2020) granted ethical approval. This project was a sub-study of a larger project that had already received BREC approval (BE351/19). The Provincial (eThekwini) and District (KwaZulu Natal) Health authorities approved this study. Throughout the investigation, ethical rules and values such as confidentiality, dignity, respect, autonomy, fairness, nonmaleficence, and beneficence were followed.

**Informed Consent Statement**: Informed consent was obtained from all participants of the study.

**Data Availability Statement**: The data collected for the current study are available on request from the corresponding author.

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**Conflicts of Interest:** The authors declare that they have no known financial or personal affiliations that could have seemed to affect the work reported in this study. The writers' views and opinions expressed in this article are their own. They do not necessarily reflect the official stance or position of any authors' affiliated organisations/institutions.

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# Appendix H:

# GAPDH Raw Data.

**GAPDH Expression values – In vitro study**

| IFN-γ | TNF-α | IL2 | IL-17 - | IL-4 | Il-5 | IL-10 | Eomes | Granzyme B | Perforin | TGF-β | FoxP3 |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 0,6922609 | 4,321772 | 2,242261 | 2,537178 | 4,335555 | 2,960657 | 3,152661 | 3,25318 | 0,8317881 | 4,871032 | 3,788684 | 1,628830778 |
| 0,5549197 | 4,588488 | 1,909364 | 2,237886 | 3,252238 | 3,661663 | 3,00348 | 1,906981 | 0,9539244 | 4,230759 | 4,298737 | 2,989114 |
| 0,2996611 | 4,346796 | 1,619357 | 2,669831 | 3,070656 | 3,981257 | 1,965023 | 2,876768 | 1,166911 | 2,824934 | 1,628830778 | 4,230759 |
| -0,2330997 | 4,51328 | 1,74471 | 1,895694 | 3 543526 | 3,106999 | 2,729767 | 2,553558 | 0,9034979 | 3,65356 | 4,230759 | 2,989114 |
| 0,4977546 | 3,901786 | 3,672405 | 2,226045 | 3,524486 | 3,294524 | 2,318909 | 2,233158 | 0,7700245 | 2,989114 | 2,989114 | 2,627476 |
| -0,2330997 | 1,662275 | 2,987062 | 2,340955 | 4,127658 | 2,343668 | 2,224593 | 2,87356 | 0,9997143 | 4,162461 | 2,989114 | 3,532798 |
| -0,5856284 | 4,900515 | 2,857384 | 0,967743 | 3,159931 | 1,606268 | 2,13924 | 2,56132 | 1,103335 | 3,108827 | 0,513183 | 1,628830778 |
| -0,5295385 | 2,674871 | 3,022936 | 1,925714 | 1,735015 | 1,717568 | 1,561323 | 3,431988 | 1,000331 | 3,93318 | 1,606268 | 0,513183 |
| -0,4597206 | 1,795259 | 1,414609 | 2,324136 | 3,258249 | 1,899205 | 2,369145 | 3,129649 | 0,2557974 | 4,871032 | 1,717568 | 2,486973 |
| 0,5273949 | 4,699629 | 3,268119 | 2,744872 | 2,296954 | 3,157526 | 2,753982 | 2,469137 | 0,6340531 | 4,230759 | 1,899205 | 2,732152 |
| -0,5916582 | 4,502084 | 1,297179 | 2,278145 | 2,299936 | 2,397675 | 2,66854 | 2,101238 | 0,8190182 | 2,824934 | 3,157526 | 1,319866 |
| 0,2641406 | 2,824432 | 3,838855 | 1,428004 | 4,227434 | 2,557481 | 2,486973 | 1,716427 | 0,6814933 | 3,65356 | 2,397675 | 1,940441 |
| 0,5524224 | 3,757952 | 3,032 31 | 1,837248 | 4,036177 | 3,103056 | 2,732152 | 2,754592 | 0,6862319 | 2,989114 | 2,557481 | 3,036252 |
| 0,1213464 | 4,397863 | 2,409717 | 1,259833 | 2,253836 | 1,544569 | 1,319866 | 2,681097 | 0,5775979 | 4,394615 | 3,103056 | 1,519437 |
| -0,4051325 | 2,30721 | 2,273146 | 1,535508 | 4,127534 | 2,447294 | 1,940441 | 2,627476 | 0,2951481 | 4,504033 | 1,544569 | 1,981244 |
| 0,6152731 | 4,224221 | 1,297179 | 2,524384 | 3,935 39 | 0,804874 | 3,036252 | 2,531361 | 0,6187159 | 3,532798 | 2,447294 | 2,606178 |
| 0,5427683 | 2,389303 | 2,862677 | 2,305212 | 2,112916 | 2,465957 | 1,519437 | 1,892317 | 0,6340531 | 4,162461 | 4,397863 | 3,788684 |
| 0,3261259 | 3,788684 | 3,604866 | 2,720523 | 2,279271 | 3,179096 | 1,981244 | 2,390051 | 0,6814933 | 4,989069 | 2,30721 | 4,298737 |
| -0,1640356 | 4,298737 | 2,821346 | 2,913352 | 3,244887 | 2,314863 | 2,606178 | 1,971204 | 0,5775979 | 3,108827 | 4,224221 | 2,279271 |
| -0,1053893 | 4,603799 | 4,177718 | 1,776237 | 2,229232 | 2,387551 | 2,621367 | 2,10706 | 0,7825063 | 3,93318 | 2,389303 | 3,244887 |
| -0,4051325 | 2,30721 | 2,273146 | 1,535508 | 4,127534 | 2,447294 | 1,940441 | 2,627476 | 0,2951481 | 4,504033 | 1,544569 | 1,981244 |

**Gene Expression values – Ex vivo study**

| IFN-γ | TNF-α | IL2 | IL-17 | IL-4 | Il-5 | IL-10 | Eomes | Granzyme B | Perforin | TGF-β | FoxP3 |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 0,5190164 | 2,943441 | 3,538661 | 2,19843 | 3,178767 | 2,569699 | 2,462862 | 3,001704 | 1,421238 | 1,818129 | 0,240723439 | 1,222839 |
| 0,33581 | 1,113195 | 2,965631 | 1,37504 | 1,926758 | 2,119966 | 1,437765 | 1,647541 | 0,061644 | 3,280019 | 1,064294412 | 2,919207 |
| 1,297491 | 4,381172 | 2,905769 | 3,09092 | 4,193833 | 2,612836 | 1,240119 | 2,81215 | 0,39466 | 4,676633 | 0,790887984 | 2,369032 |
| 0,6367406 | 4,060491 | 2,933026 | 2,776843 | 3,851695 | 3,042821 | 2,155234 | 2,960711 | 0,404557 | 2,249749 | 1,34447596 | 2,573783 |
| 1,315363 | 1,30985 | 2,666935 | 3,923942 | 1,826489 | 1,364829 | 2,754981 | 1,322424 | 0,11148 | 3,222649 | 0,276987411 | 3,357564 |
| 1,033749 | 1,881734 | 3,138603 | 1,436769 | 1,512077 | 1,299171 | 1,222839 | 2,269868 | 0,30497 | 2,217039 | 1,083546708 | 0,485645 |
| 0,333913 | 1,460334 | 4,136189 | 2,121936 | 2,311924 | 1,592641 | 2,919207 | 1,34534 | 1,035919 | 4,322372 | 1,200075584 | 4,327118 |
| 0,865759 | 2,517328 | 3,694532 | 2,168298 | 2,532002 | 1,403555 | 2,369032 | 3,381603 | 1,388091 | 4,680031 | 1,757174732 | 0,372239 |
| 0,360723 | 3,321666 | 2,831982 | 2,676748 | 3,511349 | 2,748822 | 2,573783 | 2,373409 | 0,50718 | 3,837501 | 1,497143249 | 4,327118 |
| 0,902673 | 4,798204 | 3,297252 | 1,779467 | 2,708819 | 2,592961 | 3,357564 | 1,00301 | 0,1981 | 3,738384 | 1,507475 | 1,403555 |
| 0,2250222 | 1,966135 | 4,027983 | 2,155854 | 1,627601 | 1,033243 | 2,526359 | 2,684604 | 0,968546 | 2,162665 | 0,892558446 | 2,748822 |
| 0,5256122 | 0,86734 | 3,77832 | 1,993983 | 2,040302 | 2,312579 | 1,824942 | 1,983001 | 0,840963 | 4,6334 | 0,299811376 | 2,592961 |
| 0,1684533 | 4,193946 | 2,555905 | 1,96683 | 3,780639 | 2,309491 | 1,551694 | 1,246989 | 0,976042 | 4,537966 | 0,191065367 | 1,779467 |
| 0,9855033 | 4,119588 | 4,248408 | 2,931244 | 2,859531 | 2,013513 | 0,693730 1 | 2,872575 | 0,09828 | 2,084361 | 1,266654326 | 2,155854 |
| 0,068531 | 3,621221 | 3,541997 | 1,908896 | 2,608577 | 1,985634 | 2,3915 | 2,652 13 | 0,643699 | 4,394615 | 0,999862521 | 1,993983 |
| 1,366936 | 1,622181 | 3,616043 | 1,702161 | 2,951322 | 1,128824 | 0,849627 6 | 1,749542 | 0,12597 | 4,213203 | 1,221891273 | 2,013513 |
| 1,377164 | 2,824432 | 2,39872 | 1,450194 | 2,307015 | 2,207565 | 0,889874 2 | 1,476789 | 0,396515 | 1,641653 | 1,446636 43 | 1,985634 |
| 1,58883 | 3,867269 | 4,222633 | 3,764908 | 2,761006 | 1,759045 | 2,75543 | 1,432016 | 2,965805 | 4,504033 | 1,243631 37 | 1,128824 |
| 0,9575992 | 0,017861 | 4,228666 | 2,319171 | 1,333579 | 2,557481 | 2,237373 | 2,602151 | 0,813492 | 3,393989 | 1,403707778 | 2,207565 |
| 0,3413495 | 1,786398 | 2,906144 | 3,294968 | 2,153944 | 3,103056 | 2,238865 | 3,099903 | 0,419534 | 1,304551 | 1,21512065 | 1,759045 |
| 0,0169081 | 3,757952 | 3,743278 | 1,646529 | 3,132372 | 1,613515 | 2,151155 | 3,031939 | 0,915859 | 3,532798 | 0,807542644 | 2,557481 |
| 1,586365 | 4,397863 | 4,220014 | 1,585312 | 3,92443 | 1,059935 | 2,063699 | 1,058215 | 3,294968 | 2,921379 | 0,175573923 | 3,103056 |
| 0,2083983 | 2,259256 | 3,314526 | 1,47044 | 2,824873 | 1,544569 | 1,725998 | 2,71811 | 1,646529 | 4,364423 | 0,098790 08 | 4,397863 |
| 1,032877 | 0,088 | 2,79329 | 1,688987 | 2,044298 | 2,447294 | 1,792132 | 2,694343 | 1,585312 | 4,989069 | 0,315603678 | 2,259256 |
| 0,602269 | 2,30721 | 4,153199 | 3,74496 | 3,572158 | 0,449383 | 0,915859 | 1,292722 | 1,47044 | 3,544624 | 0,767979358 | 98770,088 |
| 0,083288 | 4,224221 | 3,362108 | 2,126653 | 3,831186 | 1,387793 | 3,294968 | 1,649167 | 1,688987 | 2,174651 | 1,728396214 | 2,30721 |
| 0,496556 | 1,319695 | 2,587809 | 1,522533 | 2,375178 | 2,957363 | 0,807542644 | 2,055555 | 3,74496 | 2,883809 | 1,615156395 | 4,224221 |
| 0,115732 | 2,901474 | 3,805794 | 1,613235 | 4,306144 | 2,465957 | 0,175573923 | 2,442668 | 2,12665 3 | 1,696407 | 1,056665468 | 1,319695 |

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| | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 0,9564 067 | 4,803 145 | 3,851 695 | 1,912 917 | 3,166 459 | 1,87970 5 | 1,43201 6 | 1,821 847 | 2,1539 44 | 1,688 987 | 0,56070 5629 | 2,9014 74 |
| 0,2903 04 | 3,788 684 | 1,826 489 | 2,150 27 | 1,840 26 | 2,08326 5 | 2,60215 1 | 2,839 336 | 3,1323 72 | 3,744 96 | 0,57619 6175 | 4,8031 45 |
| 0,9571 1 | 2,691 775 | 1,512 077 | 2,129 697 | 1,540 133 | 2,31351 9 | 3,09990 3 | 2,919 528 | 3,9244 3 | 2,126 653 | 1,29618 6986 | 3,7886 84 |
| 0,0400 62 | 1,021 831 | 2,311 924 | 1,058 215 | 2,745 588 | 1,00114 4 | 3,85169 5 | 1,817 272 | 1,0582 15 | 2,824 873 | 0,86110 079 | 2,6917 75 |

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