Mycobacterium Tuberculosis-Associated Uveitis: Infection and Autoimmunity

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Mycobacterium Tuberculosis-Associated Uveitis: Infection and Autoimmunity

Mycobacterium tuberculosis-geassocieerde uveïtis: infectie en auto-immuniteit

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So verily, with every difficulty, comes ease. (the Quran 94:6)

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Chapter 1

General Introduction

Tuberculosis and its immunopathogenesis
Uveitis
Uveitis in the spectrum of tuberculosis

TUBERCULOSIS AND ITS IMMUNOPATHOGENESIS

Tuberculosis (TB) is an airborne infectious disease caused by microorganisms of the *Mycobacterium tuberculosis (Mtb)* complex, and mainly affects the lungs. Although primarily a pulmonary pathogen, *Mtb* can affect almost all organs. The World Health Organization (WHO) estimates that one-third of the world's population is infected with *Mtb*, but only ten percent of infected persons develop clinical manifestations of TB. Of this 10%, 16%-27% have extrapulmonary TB involvement including the eye (*Mtb*-associated uveitis: 1.4% in the United States (US) before the 1990s and 18% in Spain recently).[1, 2] Risk factors for extrapulmonary TB are: age over forty, female gender, and human immunodeficiency virus (HIV) infection.[3] In many low-income and middle-income countries, TB continues to be a major cause of morbidity and mortality, and drug-resistant TB is a significant concern in many settings.

Clinical manifestations and diagnosis

Mtb mainly affects the lungs, therefore, an active pulmonary TB is characterized by a chronic cough, fever, hemoptysis and general symptoms such as appetite loss, night sweats and sustained weight loss.[4] Clinical manifestations of active TB infection in other organs will depend on the location of the infection.

The gold standard for diagnosing active TB infection is sputum smear microscopy and culture in liquid medium with subsequent drug-susceptibility testing. Sputum smear microscopy has many limitations, but it continues to be widely used in limited facility settings. Sputum smear microscopy will detect active TB from the first sputum smear test in only 75% in HIV-negative individuals and even in lower percentage in HIV-positive individuals (57%).[5] Thus, the WHO recommended a PCR based test: Xpert *MTB*/RIF (Cepheid Inc., Sunnyvale, California, USA) which has better accuracy than sputum smear microscopy with a sensitivity of 90% in HIV-negative individuals and up to 77% in HIV-positive individuals.[6]

Radiography imaging of the chest (chest X-ray/CXR) is useful in diagnosing pulmonary TB in clinically suspected cases that could not be confirmed with microbiological testing or as a screening tool, especially in HIV positive individuals. As a screening tool, CXR has a higher sensitivity for pulmonary TB than screening for TB symptoms. However, CXR lacks specificity thus, the pulmonary TB diagnosis based on CXR needs to be followed up clinically and by microbiological tests.[7]

Exposure to *Mtb* will lead to a memory immune response by T lymphocytes (T cells). If a patient has memory T cells that have been exposed to *Mtb* previously, he/she will have an *in vivo* response to Mycobacterium antigen subcutaneously; the tuberculin skin test (TST) and *in vitro* from blood; as in QuantiERON-Gold TB tests (QFTs). However,

QFT or TST is not a good measurable correlate of disease activity or immunity for TB.[8] Positive QFT or TST does not necessarily indicate that the host has a clinical manifestation of active TB infection and *Mtb* active replication and thus can transmit the disease. On the other hand, latent TB is not necessarily representing protection against developing of active TB. It is assumed that *Mtb* has resided within different microenvironmental conditions driving the micro-organism into a state of non-replication or slow replication.[9] There is no immunological biomarker that correlates with the risk whether *Mtb* has been resuscitated or *Mtb* has already been cleared. The lack of clinical correlation between host immunity, host disease manifestation, and *Mtb* bacterial persistence is referred as TB heterogeneity **(Table 1).**[10,11] To understand TB heterogeneity, we need to understand the immunopathogenesis of TB disease, especially how the host initially responded to *Mtb* exposure. This may result in an equilibrium between the host and *Mtb*,

TABLE 1. Heterogeneity in Tuberculosis

Clinical Term	Bacterial Term	Immunological term	Risk of reactivation
Active or reactivation of TB Visible symptoms Contagious (high *Mtb* transmission)	Resuscitation Bacterial phenotype: active replication, active metabolism (Positive in culture)	Poor host immune control	--
Subclinical low active TB disease Non-visible symptoms Contagious sporadically (low *Mtb* transmission)	Bacterial persistence (Positive in culture)	Poor host immune control	Yes
Latent TB Non-visible symptoms Not contagious (no *Mtb* transmission)	Bacterial persistence Bacterial phenotype: low levels of replication, minimal metabolism (Negative in culture)	Poor host immune control	Yes
Latent TB Non-visible symptoms Not contagious (no *Mtb* transmission)	Bacterial persistence Bacterial phenotype: low levels of replication, minimal metabolism (Negative in culture)	Active host immune control	No/ Low risk
Latent TB Cleared infection Non-visible symptoms Not contagious (no *Mtb* transmission)	No *Mtb*	Detectable host T cell response to *Mtb*	No risk
No infection	No *Mtb*	No detectable host T cell response to *Mtb*	No risk

to clinical disease, or towards *Mtb* clearance. Knowledge about the *Mtb*-host relation will lead to a better understanding of *Mtb*-associated uveitis in the heterogeneity of TB and will clarify how to improve the approach of *Mtb*-associated uveitis.

First exposure to Mtb

Mtb is primarily an airborne infectious pathogen that enters the host via the respiratory tract in aerosolized droplets and subsequently gains entry into host cells within the alveoli. *Mtb* will be ingested by professional phagocytic cells in the lungs such as alveolar macrophages[12,13], neutrophils[14], and dendritic cells.[15] Alveolar epithelial cells might have a role in recognizing *Mtb* and the modulation of the immune response.[16]

Phagocytosis of *Mtb* could be mediated through opsonic and non-opsonic mechanisms. Several C-type leptin receptors (CLRs) such as macrophage mannose receptors (MMR)[17] and dendritic cells (DC)-specific intracellular adhesion molecule 3 – grabbing nonintegrin (DC-SIGN)[18] recognize the repeating molecular pattern on mycobacterial cell wall lipoglycan, mannose-capped liparabinomannan (ManLAM) as non-opsonic phagocytosis. Opsonic phagocytosis mediated by scavenger receptors (SRs), complement receptors (CRs), and Fcγ receptors (FcγR) can target host iC3b and IgG on *Mtb*.[19] The receptor-ligand interaction used by *Mtb* is important to determine the intracellular fate of *Mtb*. Uptake through MMR is associated with a more virulent *Mtb* infection because it can avoid phagosome maturation intracellularly, while ingestion by DC-SIGN will result in lysosomal delivery to the *Mtb*.[20] In addition, the receptor-ligand interaction influences the innate cytokine signaling in response to *Mtb*.[19,21,22]

Mtb fate in the phagocytic cells

After *Mtb* is phagocytosed there are four possible routes of fate: 1). Bacterial death, 2). bacterial survival or growth, 3). restricted growth, and 4). bacterial survival and growth with transmission (**Figure 1** adapted from ref[19]). The aim of phagocytosis is to eliminate the infection through several mechanisms, but *Mtb* can benefit from those mechanisms by manipulating the host response in order to provide itself with a cellular niche for further expansion.

Mtb will be internalized into endosomes and phagosomes which will further undergo a maturing process. The final destination in this process is fusion with a lysosome for extensive degradation of *Mtb*. The phagolysosome fusion has anti-microbial properties such as a very low PH, hydrolytic enzymes defensins and other bactericidal peptides as well as the ability to generate toxic oxidative radicals. *Mtb* could prevent this toxic maturation of the phagosomes by inhibiting “phosphatidylinositol 3-phosphate” (PI3P) and “GTP-bound Rab 7” generation. Because of this inhibition, *Mtb* promotes maintenance within early Rab5 endosomes where it still could grow by acquiring nutrients while

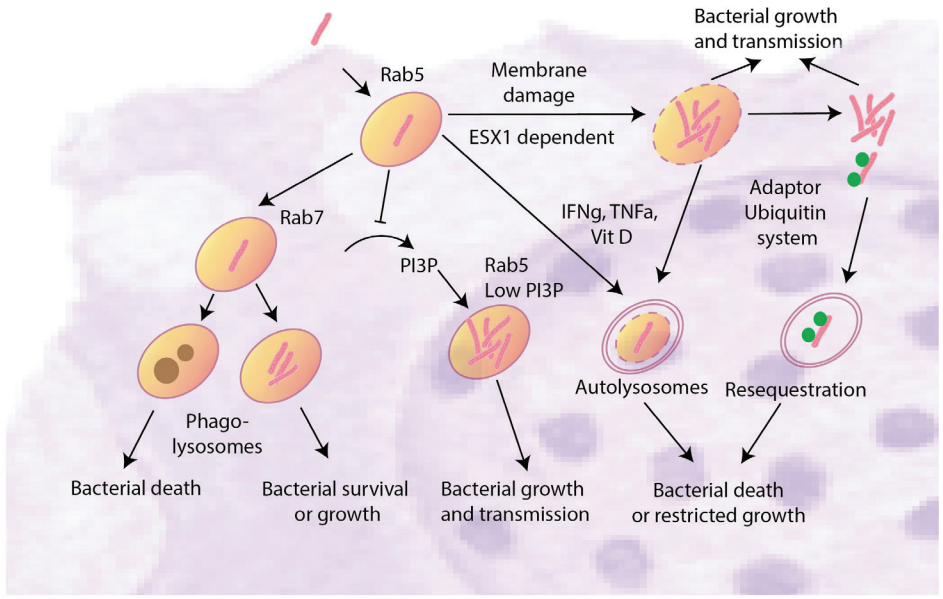

Figure 1. Mtb fates in phagocytic cells.
Ideally *Mtb* within Rab5 early endosome will mature to Rab7 phagolysosome and experience bacterial death. Several *Mtb* may survive and are able to grow within the phagolysosome. *Mtb* is able to prevent phagosome maturation by inhibiting phosphatidylinositol 3-phosphate (PI3P) generation in the phagosome and impairing recruitment of active GTP-bound Rab7. They will stay in Rab5 low PI3P and able to grow and further transmit *Mtb*. IFNγ, TNFα and vitamin D will enhance the killing activity within the cell and further induce bacterial death or restricted growth. Through *Mtb* virulence factor ESX1, *Mtb* could damage the membrane and access the cytosol. *Mtb* could escape and further grow and transmit or be resequestered by the ubiquitin system (adapted from ref[19] with permission).

avoiding the acidic degradative environment.[23] PI3P generation is also needed in autophagy, and its disruption will impair the clearance of *Mtb* by autophagy as well. However, appropriate phagolysosome formation will not always result in impaired bacterial replication. *Mtb* can respond, resist, and even persist in the moderately acid environment of the phagosome or phagolysosome.[24]

Although *Mtb* could survive inside of macrophages, *Mtb* infected macrophages can also be activated to increase their killing capacity by specific cytokines such as IFNγ, tumor necrosing factor (TNF)α[25], and other factors such as vitamin D.[26-32] However, *Mtb* virulence factor 6 kDa early secretory antigenic target (ESAT-6) secretion system 1 (ESX1) enables escape from the phagosome and subsequent replication in the cytosol inducing cell death via necrosis.[33-37] Cell necrosis will aid cell-to-cell spread of *Mtb*. Furthermore, *Mtb* infected macrophages can express DC markers and may actively exit the site of infection leading to hematogenous dissemination from the primary infection site in the early infection state.[38, 39]

Innate immune response to Mtb

Mtb is recognized by multiple pattern-recognition-receptors (PRRs), among them are Toll-like receptors (TLRs) and CLRs on the cell membrane and intra-cellular and, the nucleotide-binding oligomerization domain (NOD)-like receptors/NLRs and retinoic acid-inducible gene (RIG)-I-like receptors (RLRs) in the cytoplasm.[40] These initial steps to phagocytosis are of evident importance, based on studies with toll-like receptor (TLR)2 and TLR9 single-knockout mice or TLR2/TLR9 double-knock out mice. The double-knock out mice are more susceptible to *Mtb* than the single knock-out mice.[41] Moreover, in knock-out mice, second messengers that could coordinate upstream stimulation of TLRs, such as myeloid differentiation primary response 88 (MyD88), make the mice even more susceptible.[42, 43]

Downstream PRR activation will regulate innate immune responses and also initiates the adaptive immune response. PRR activation results in production of pro-inflammatory cytokines, including TNFα and interleukin (IL)-1β through activation of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB). This process increases macrophage effector functions including intracellular killing, and local/systemic immune cell mobilization and activation.[44] *Mtb* has evolved multiple mechanisms to evade phagocyte killing and eventually subvert and delay the initiation of the adaptive immune response (as discussed below).

Type 1 Interferons

Type 1 interferons (IFNs) are proteins that may "interfere" with intracellular infections. Their expression is primarily induced by cytoplasmic PRRs, and endosomal TLRs and can further activate certain interferon regulatory factors (IRFs) resulting in interferon-inducible gene expression.[45] The role of type I IFN in *Mtb* infection has recently emerged by the findings of type 1 IFN inducible gene expression in active pulmonary TB patients, whose differential gene expression could differentiate them from healthy controls and other diseases such as sarcoidosis and pneumonia.[46-49] This type 1 IFN-inducible gene expression (also referred to as Type 1 IFN signature) normalizes in response to successful anti-tuberculosis treatment (ATT).[46, 47] Type 1 IFN signature also correlates with the risk of progression from latent TB infection to active TB infection.[50]

Despite mounting evidence about the role of type 1 IFNs in active TB, the exact mechanism in the pathogenesis of TB is not fully understood. *Mtb* stimulated cytosolic PRRs induce type 1 IFNs, which lead to IFN-β production in an IRF3-dependent way. This is turn, induces CCL2/CCR2-dependent migration of *Mtb* permissive inflammatory macrophages (iM) and inflammatory dendritic cells (iDC) to the lung.[51] Within these cells, type 1 IFNs will change the arachidonic acid metabolism which renders the cells more susceptible to necrotic death.[52] Additionally, type 1 IFNs limits IL-1β mediated neutrophil

activation to reduce excessive inflammation and associated damage. This type 1 IFN mediated inhibition acts through IL-10, which does however require that permissive cells have been primed by IFNγ.[53, 54]

Granuloma formation

Histopathologically, *Mtb* is known to induce granuloma formation, these granulomas are organized immune cell aggregates developing in response to persistent stimuli.[55] It was previously thought that granuloma formation correlated with *Mtb* sequestration and elimination by host immune cells. Granuloma formation is the pathological basis for latent *Mtb* infection.[56]

A granuloma starts as a collection of mature macrophages in response to the persistent stimulus of *Mtb*. Mature macrophages are assumed to be more phagocytic and microbicidal with their increased cytoplasmic size, larger numbers of organelles, and ruffled cell membranes. These mature macrophages also underwent fusion into multinucleated giant cells. Some of the mature macrophages can transform into epithelioid cells which have tightly interdigitated cell membranes in zipper-like arrays that link adjacent cells. Within this cellular context are areas of necrosis due to cell death, including macrophages. In gross pathology studies, this is known as caseum. Other cells involved in granuloma formation are neutrophils, DCs, B cells, T cells, natural killer cells (NK-cells), fibroblasts, and epithelial cells.[56] With these characteristics, it was long believed that granulomas play an important role in controlling the infection thereby protecting the host. But, studies in animal models show that in the early phase of granuloma formation *Mtb* growth is still rapid and reaches a plateau until adaptive immunity develops, generating mature granulomas.[57, 58] Moreover, in developing zebra fish with still a morphologically and functionally immature adaptive immunity, granuloma formation coincided with accelerated *Mtb* proliferation.[59, 60] In a live imaging study in zebra fish, macrophage reaction depends on *Mtb* virulent factor ESX-1 secretion (encoded by region of difference (RD) 1 virulence locus). RD1 deficient *Mtb* will have fewer macrophages attracted to the granuloma, their morphology is more rounded, and they move more slowly. In consequence, this will coincide with poor granuloma formation, and limited *Mtb* proliferation and dissemination within the host. In contrast, in more RD1 positive virulent *Mtb*, there is rapid and continuous migration of the macrophages to the granuloma site. Besides the signal from RD1 positive *Mtb*, there will be a second signal generated by infected yet dying macrophages to attract additional macrophages to phagocytose the contents of dying infected macrophages. As a result higher numbers of infected cells will be found.[61] Moreover, ESAT6 peptide secreted by ESX-1 positive *Mtb*, could induce macrophage-independent MMP9 secretion from epithelial cells surrounding the granuloma to act as a chemoattractant for macrophages. MMP9-knockout mice have decreased macrophage

recruitment to the lung and poor granuloma formation upon *Mtb* infection, and consequently, it is associated with decreased bacterial loads.[62] The host forms granulomas to concentrate the host defenses in an organized immune structure but *Mtb* alters it (facilitates) as a safe niche for its survival and growth and delay adaptive immunity.

Adaptive immune responses to Mtb and the "Immune Equilibrium"

Adaptive immune responses require the transport of live bacteria from lungs to the draining lymph nodes by dendritic cells (DCs) which takes 8-10 days after *Mtb* infection (in comparison, only 20 hours for influenza virus).[15, 63] Besides the delay of presenting *Mtb* to T cells, adaptive immune responses are also hindered due to delayed arrival of effector T cells by regulatory T cells (Tregs) [64] and delayed activation of effector T cells[65, 66] Therefore, it is possible that early granuloma formation is used by *Mtb* to evade the host immune system.[56]

The adaptive immune response is dependent on IL-12 (p40/p35) which is mainly secreted by *Mtb*-activated DCs through a TLR-dependent mechanism. In the lymph node, migrated DCs will drive naïve T cells to differentiate towards a T helper cells (Th) 1 phenotype. Protective antigen-specific Th_1 cells will migrate back to the lung and produce IFNγ a major cytokine in granuloma formation. IFNγ leads to activation, cytokine production, the induction of anti-microbial factors and bacterial control. This protective immune response is mainly mediated by CD4+ Th_1 cells, but other cells also play a role in producing IFNγ; such as CD8+, NK cells, γδ T cells, and CD1-restricted cells.[21]

The adaptive immune response will arrest the progressive growth of the bacterial population but its ability to eliminate *Mtb* is limited. Immunocompetent mice which have been infected with virulent strains of *Mtb* will maintain a plateau population of bacteria with accumulation of effector CD4+ and CD8+ T cells in the lungs until the mice die.[67] This phenomenon, together with the fact that CD4+ T cell-deficient mice or CD4+ T cell lymphopenic HIV patients are highly susceptible to TB, illustrates the crucial role of an appropriate immune response to maintain the immune equilibrium against *Mtb* activation.[22]

Reactivation of latent TB from immune equilibrium.

As mentioned above, the best known mechanism for TB reactivation was observed in the profound CD4+ T cell depletion in HIV/ acquired immune deficiency syndrome (AIDS) patients.[68] Similar findings were found in simian immunodeficiency virus (SIV) infection in non-human primates and CD4+ T cells depleted-mice during the chronic stage of *Mtb* infection.[65, 69] Recent knowledge was also obtained from the phenomenon of TB reactivation in patients treated with TNF neutralizing biologics. TNF-blocking treatment inhibits the induction of pro-inflammatory cytokines by blocking

TNF-mediated NFκB activation and further diminishes anti-mycobacterial activity of macrophages.[25, 70, 71] Medical conditions such as diabetes mellitus and treatment with corticosteroids are also associated with *Mtb* reactivation.[72,73] Other possible mechanisms that might cause reactivation are 1) T cell exhaustion[74-76], 2) altered antigen expression by *Mtb* [77, 78] , 3) decreased migratory capacity of specific myeloid and lymphoid cells [79], and 4) resuscitation-promoting factors from the *Mtb* itself [10].

UVEITIS

Uveitis is an inflammatory disease involving the uvea, the highly vascularized and pigmented middle layer of the eye located between the retina and sclera. The uvea is composed of the iris, ciliary body, and choroid **(Figure 2).** All the tissues surrounding the uveal tract, including the optic nerve, retina, vitreous, and sclera can also be involved in the inflammatory process, either secondary or primary, and therefore, the name uveitis is usually used to indicate any type of intraocular inflammation.

Uveitis is considered as an uncommon disease with an incidence of approximately 52 persons per year per 100.000 individuals and prevalence of 115/100.000 person in developed countries.[80] The incidence and prevalence of uveitis are probably much higher in developing countries, however there are no precise numbers available.

Uveitis is an important cause of visual impairment and blindness In the western world; uveitis results in visual impairment in approximately 35% and blindness in 5%-10% of cases.[81] In developing countries the blindness due to uveitis reaches up to 25%.[82]

Uveitis can be classified in several ways as summarized in **Table 2**. Since the causal classification is not always feasible, especially in the initial phase of the inflammation, the anatomical uveitis classification gained most popularity. Anatomical classification of uveitis differentiates into anterior uveitis, intermediate uveitis and posterior uveitis. Furthermore, when uveitis comprises all parts of the uvea it is designated as panuveitis (Figure 2). Uveitis classification is important for diagnostic and therapeutic purposes as well as for the prognosis. Table 3 summarizes the relation of anatomical classification of uveitis with infectious and systemic non-infectious diseases and ocular syndromes (adapted from ref [83-85]).

Worldwide, infectious uveitis is probably the most common uveitis entity. Infections are most frequent in the developing countries, with the prevalence of infectious uveitis between 30% and 50%.[81] In contrast, prevalence of infectious uveitis in the developed countries is substantially lower, namely between 11% and 21%.[81] Identification of the causative micro-organism is critical because the treatment with appropriate

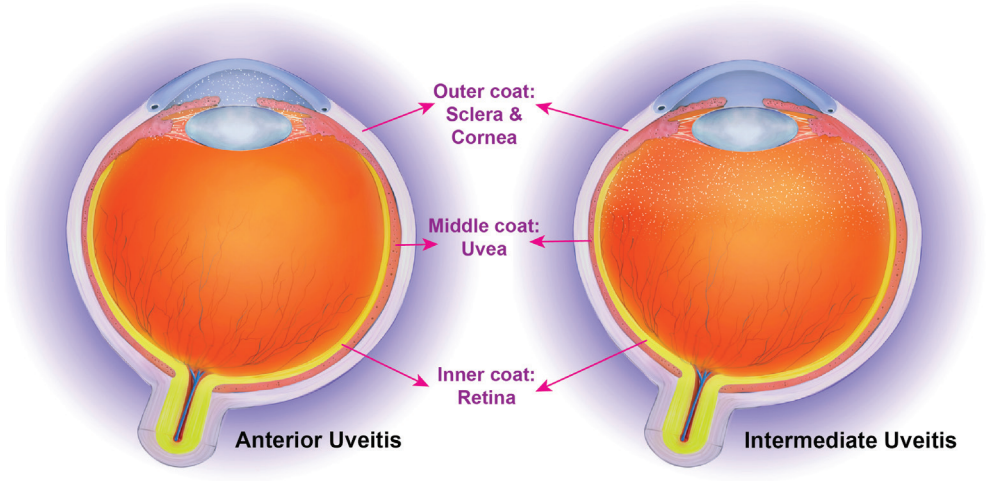

Figure 2. Anatomical location of uveitis.
The eye consists of three coats; outer (sclera and cornea), middle (uvea), and inner coat (retina). Inflammatory cells and localization Inflammation within the eye determine the "anatomic classification" diagnosis of uveitis. Anterior uveitis is predominantly affecting anterior chamber, intermediate uveitis is affecting predominantly vitreous and peripheral retina, and posterior uveitis is predominantly affecting retina and choroid. Panuveitis has no predominance and affect all part of the uvea. The structure adjacent to uvea; cornea, sclera, and optic nerve, are usually also affected either primarily or secondarily.

antimicrobial agents might decrease inflammatory activity and even cures. In developing countries, the most common intraocular infections include tuberculosis and toxoplasmosis. Other less common infections causing uveitis are leprosy, leptospirosis, onchocerciasis, cysticercosis, and trypanosomiasis.[81] In developed countries, viral infections

TABLE 2. General uveitis classification

Characteristic	Classification
Anatomical location	Anterior uveitis, intermediate uveitis, posterior uveitis, panuveitis
Course	Acute, chronic, recurrent
Laterality	Unilateral, bilateral (asynchronous or simultaneous)
Morphology	Retinitis vs choroiditis; paucifocal vs multifocal; granulomatous vs non-granulomatous
Cause	Infectious vs non-infectious (associated with systemic disease or ocular syndrome without systemic disease), masquerade (malignant, non-malignant), trauma.
Others	Child vs adult, immunocompromised vs immunocompetent

and toxoplasmosis are most common. In the last decades, HIV epidemic caused the increased prevalence of cytomegalovirus (CMV) retinitis in both, developed and developing countries.[86]

It is extremely important to distinguish early between infectious and non-infectious causes, as they require completely different treatment strategies. The diagnosis of infectious uveitis cannot be performed on the clinical features only, and the ancillary diagnostic tests are necessary. Serologic tests are easily available, however, they are not always informative about the cause of inflammation within the eye. Therefore, local diagnostic test based on the analysis of the intraocular fluid is required. Intraocular fluid can be assessed by polymerase chain reaction (PCR) and/or Goldmann-Witmer coefficient (GWC), which indicates the intraocular production of specific antibodies. However, PCR and GWC tests adapted for intraocular fluid are not easily accessible in developing countries where the infectious causes are most prevalent.

UVEITIS IN THE SPECTRUM OF TUBERCULOSIS HETEROGENEITY

Uveitis caused by *Mtb* is clinically hard to recognize because it can involve all anatomical locations (see **Table 3**) and it has protean ocular clinical manifestations (see **Table 4,** adapted from ref [3, 87]). Obtaining *Mtb* from the eye to confirm the diagnosis is extremely difficult, therefore worldwide there is a lack of consensus on how to classify and diagnose *Mtb*-associated uveitis.[88, 89]

Recently a new classification was proposed about the involvement of *Mtb* in uveitis **(Table 5).** Definitive diagnosis of ocular TB is performed by confirmation of the presence of *Mtb* in ocular fluid or tissue by microbiological tests. However, most of the diagnoses are presumptive. A probable *Mtb*-associated uveitis diagnosis is made in cases

TABLE 3. Etiology and disease-associations with infectious and non-infectious uveitis

Anatomic Location	Primary site of inflammation	Infectious	Non-infectious	
			Associated with systemic disease	Ocular syndromes with no systemic disease
Anterior uveitis	Iris, ciliary body	CMV, HSV, VZV, Rubella virus, HIV, Syphillis, TB, Ebola	HLA-B27 associated uveitis, Behcet, JIA, Sarcoidosis,TINU, IBD	FUS
Intermediate uveitis	Vitreous, pars plana of ciliary body and peripheral retina	Syphilis, Lyme, TB, HTLV-1	MS, Sarcoidosis	Pars planitis
Posterior uveitis	Retina, choroid	Toxoplasmosis, ARN, PORN, DUSN, CMV retinitis, Bartonella, Lyme, TB, Toxocariasis, Candida, Aspergillus, Onchocerciasis, Syphilis, TB, Zika virus	Sarcoidosis, SLE, TINU	MEWDS, APMPPE, Amp. Choroiditis, BSCR, Serpiginous choroiditis, MFCPU, PIC
Panuveitis	(all of the above without predominance)	Toxoplasmosis, Syphilis, Lyme, TB	VKH, Behcet, Sarcoidosis	SO

Abbreviations: CMV: cytomegalovirus; HSV: herpes simplex virus; HIV: human immunodeficiency virus; TINU : tubulointerstitial nephritis and uveitis; IBD : inflammatory bowel disease; HTLV-1: human T-lymphotropic virus-1; FUS: Fuchs uveitis syndrome; TB; tuberculosis; JIA: Juvenile idiopathic arthritis-associated uveitis; MS: multiple sclerosis;; ARN: Acute retinal necrosis; PORN: Posterior outer retinal necrosis; DUSN: Diffuse unilateral subacute neuro retinitis; MEWDS: Multiple evanescent white dot syndrome; ORV: Occlusive retinal vasculitis; SLE: Systemic lupus erythematosus; GPA: Granulomatosis with polyangiitis; APMPPE: acute posterior multifocal placoid pigment epitheliopathy; Amp: Ampiginous; BSCR: Birdshot chorioretinitis; MFCPU: Multifocal choroiditis with panuveitis; PIC: Punctate inner choroiditis; VKH; Vogt-Koyanagi-harada; SO: Sympathetic ophthalmia.

TABLE 4. Possible clinical manifestations of uveitis associated with *Mtb*

Primary site of inflammation	Possible presentations
Iris, ciliary body	Granulomatous, non-granulomatous anterior uveitis, iris nodules, iris or angle granuloma, ciliary body tuberculoma
Vitreous, pars plana	Granulomatous, non-granulomatous with organizing exudates in the pars plana/ peripheral uvea
Retina and optic nerve	Retinitis and retinal vasculitis, occlusive retinal vasculitis, macular edema, neuroretinitis, optic neuropathy
Choroid	Choroidal tubercle, choroidal tuberculoma, subretinal abscess, serpiginous-like/ serpiginoid choroiditis
All sites/ no predilection	Panuveitis, endophthalmitis, panophthalmitis

with immunological evidence of *Mtb* infection complemented with a microbiologically proven active *Mtb* infection from another organ or a chest radiographic imaging highly suspected of active TB (reviewed in [4, 90]). A possible *Mtb*-associated uveitis diagnosis is made when uveitis is associated with a host immune response to *Mtb* antigen (QFT or TST positive latent TB infection without other evidence of clinically active TB; **Table 5**, adopted from ref[91]). In addition, possible *Mtb*-associated uveitis can be diagnosed only after other causes of uveitis were excluded. The infection of the eye by *Mtb* is thought to play a role in both definitive and probable TB-associated uveitis. The pathogenesis of possible *Mtb*-associated uveitis (uveitis of unknown cause with positive QFT and no clinical evidence of TB infection) is not yet clear. Depending on the geographical location, the association of IGRA with uveitis might be coincidental or might be either due to infection or immune reaction or both. Sometimes, the clinical response of uveitis to ATT (six until 12 months) is being used as additional criterion for the involvement of *Mtb* in the pathogenesis.[87]

In the clinical practice, a patient that fulfills the criteria of possible *Mtb*-associated uveitis poses a treatment dilemma to the clinician. As the ATT is characterized by its long-term use and might be associated with adverse effects, the decision to treat or not to treat should not be taken lightly. QFT and/or TST positivity indicates that the patient has been ever infected by *Mtb*; however, the level of QFT or induration area of TST are not associated with the probability of active TB disease nor with enhanced immunity against *Mtb*.[8] Positive QFT and/or TST do not indicate that the host has active *Mtb* replication. Unfortunately, so far, there is no marker that indicates the risk whether *Mtb* has been resuscitated or *Mtb* has already been cleared. Availability of such a measure would be of great value to guide optimal treatment for the above indicated problematic uveitis group.

TABLE 5. Classification of confirmed ocular TB and presumed Mtb-associated uveitis

Uveitis classification	Clinical manifestations Mtb-associated uveitis	Microbiological evidence of Mtb from ocular fluids/ tissues	*Radiological evidence consistent of TB active infection (e.g. chest X-ray) OR *microbiological evidence Mtb from extraocular tissues (e.g. sputum etc)	Documented exposure of TB OR Immunological evidence of TB infection (TST or QFT positive)	Other radiological OR Microbiological evidence (but not as characteristic as*)
Confirmed ocular TB	+	+	+/-	+/-	+/-
Probable *Mtb*-associated	+	-	+	+	-
Possible *Mtb*-associated	+	-	-	+/-	+/-

* Reviewed in [4, 90]

Epidemiology

In the western world, TB is an infrequent cause of ocular infection. Data of *Mtb*-associated uveitis vary across the region and across time. Around the 1940s, TB was estimated to account for 80% of granulomatous uveitis cases. This decreased to 22% in the 1960s due to better knowledge of other possible causes of uveitis, including toxoplasmosis and sarcoidosis.[1, 92, 93] The percentage declined further in the decades afterward to around 1.4% to 18%.[2, 81, 94-102] However, recent increase of *Mtb*-associated uveitis was noted and is probably related to HIV infection, immigration from TB endemic areas, new immunosuppressive treatments, inadequate TB control, and increased drug resistance.[87] Epidemiological data differ between TB endemic and non-endemic countries. In non-endemic countries such as the US, Italy, and Japan, the prevalence rates of *Mtb*-associated uveitis range between lower than 1% to 7%. In endemic countries such as in Asia and Africa, the prevalence rate of what ranges between 4% to 10%.[81] In "middle" endemic countries such as Saudi Arabia and Singapore; the prevalence rate is still quite high: 6%-18%.[95, 100, 102, 103] These differences are not only related to differences in disease burden but also due to variations in diagnostic strategies and classifications used.[104] The prevalence of latent TB is high in endemic countries and association of latent TB and uveitis might be due to coincidence. Therefore, it is difficult to confirm whether a latent TB is related to the ocular inflammation and decide whether an individual patient should be treated.

Clinical manifestations of Mtb-associated uveitis

Mtb-associated uveitis is considered a great mimicker of other uveitis entities and may affect all anatomical locations. The typical clinical features involve granuloma formation located on iris or in the irido-corneal angle, ciliary body, choroid, occasionally leading to subretinal abscess formation, endophthalmitis, and panophthalmitis. All these features are assumed to be due to direct infection. However, there are several clinical manifestations that may be related to an immune-mediated mechanism such as the serpiginous or serpiginoid choroiditis, occlusive retinal vasculitis, and anterior uveitis.[87] The described features and/or the most frequently reported clinical manifestations should prompt the clinician to check for TB. Also, an unspecific clinical manifestation with chronic or recurrent course, active despite the immunosuppressive treatment can be related to *Mtb*.

Anterior uveitis accounts for approximately 12%-36% of *Mtb*-associated uveitis.[3] Besides the typical features of iris or angle granuloma and ciliary body tubercles, broad-based posterior synechiae were reported to be associated with *Mtb*-associated uveitis.[105] Other ocular manifestations that have been reported in relation to TB are mutton fat keratic precipitates and less frequently hypopyon and iris atrophy. [3, 106, 107]

Intermediate uveitis accounts for about 11% of *Mtb*-associated uveitis.[3] The clinical manifestations usually comprise a low-grade, smoldering chronic vitritis, snowball opacities, snow banking, peripheral vascular sheathing and peripheral choroidal granuloma.[87]

Posterior uveitis is the most common clinical presentation, comprising 35%-42% cases of *Mtb*-associated uveitis.[3] The clinical features in posterior *Mtb*-associated uveitis manifestations include multifocal choroiditis[108], retinitis (usually concomitantly with choroiditis)[87], serpiginous-like choroiditis[109-112], tubercles[113, 114], tuberculomas[113, 115-120], subretinal abscesses (occur as a result of liquefaction necrosis in caseating granulomas and can develop in patients with disseminated TB),[121] occlusive retinal vasculitis and other vasculopathies.[122-124]

Panuveitis is seen in 11%-20% of *Mtb*-associated uveitis cases.[125] *Mtb*-associated panuveitis might primarily develop and have aspecific features or may progress from a severe form of posterior or intermediate uveitis.[87] Generalized inflammation that has an acute onset and shows rapid progression with the destruction of intraocular tissues can manifest as endophthalmitis and panophthalmitis.[87]

PATHOGENESIS OF MTB-ASSOCIATED UVEITIS

The exact pathogenesis of "*Mtb*-associated uveitis" is so far unclear. Is it caused by bacterial spread to the eye and subsequent replication? And in what degree is the immune system involved? Several pathophysiological models were reported.[126] We postulated four possible pathological mechanisms that may explain the pathogenesis of "*Mtb*-associated uveitis".

1. Direct *Mtb* infection of the retina
2. *Mtb* induces a specific anti-retinal immune process due to antigenic mimicry
3. Latent *Mtb* immuno-surveillance induces a general higher level of immune cell (monocytic and CD4+ T cell) activation that results in uveitis in predisposed individuals
4. Combination of the above postulated mechanisms

Pathogenesis of *Mtb*-associated uveitis is probably related to pathogenesis of heterogeneous manifestations of TB infection as indicated above **(Table 1)**. In extreme cases of TB infection such as in lymphohematogenous dissemination of *Mtb* (miliary TB)[127], there are reports of granulomas in choroid that responded to ATT like in other infected organs.[128] There is a possibility that macrophages from *Mtb*-laden granuloma in foci in the lungs exit and spread and directly infected the eye.[129] Guinea pigs infected with aerosolized *Mtb* developed uveal granulomatous lesions containing acid-fast staining

positive organisms.[130] The risk of ocular involvement is also increased in HIV-positive patients [2], and their clinical manifestations are granulomatous and are more likely to be caused by a direct infection with *Mtb* because as there was an evidence of infection in other organs as well.[131]

In latent TB however, the presence and/or activity of *Mtb* in the body might vary according to bacterial and immunological functions **(Table 1).** A histopathological study from eyes with proven presence of *Mtb*, revealed that systemic TB infection is not always found.[132, 133] The authors reported that *Mtb* is distributed mainly in RPE cells and only one or two bacilli were found associated with a near giant cell or an area of necrosis.[132] These lesions generated vascular endothelial growth factor (VEGF) expression in the neuroretina and retinal pigment epithelium (RPE).[134] These studies showed RPE cells have an important role in *Mtb*-associated uveitis. An *in vitro* study showed that RPE cells were able to phagocytose *Mtb* with comparable efficacy as a monocytic cell line (THP-1) but with higher *Mtb* survival rates.[135] This suggests that RPE cells could be a niche for *Mtb* and may be a locus where reactivation or reinfection could begin. RPE cells might have a role in sequestering *Mtb* as in granuloma formation in the lung or lymph node. RPE cells might also have a role in regulating the immune equilibrium thus controlling the inflammation. This hypothesis is supported by a study in which *Mtb* genome was found in sub-retinal fluids of rhegmatogenous retinal detachment patients that were without any signs of uveitis in either eye.[136]

Due to the difficulty to obtain *Mtb* from ocular tissues in presumed *Mtb*-associated uveitis cases, it has been hypothesized that *Mtb*-associated uveitis might be caused by autoimmune reactions rather than by an infection itself. *Mtb* has nearly 500 experimentally verified human T cell epitopes which may stimulate the immune system.[137] There are numerous studies on autoimmune uveitis in animal models.[138] The first experimental autoimmune uveitis (EAU), which could be consistently induced, was obtained by subcutaneous inoculation of inter-photoreceptor retinal binding protein (IRBP) emulsified in oil and complete Freund's adjuvant (CFA).[139, 140] CFA consist of dried extract *Mtb* strain H37Ra which will stimulate the PRRs and activate innate immune responses and drives differentiation of retina-specific T cells (by inter-photoreceptor retinoid binding protein, IRBP) in a pro-inflammatory type.[141] This type of activated autoreactive T cells was found in peripheral blood, but these cells need to pass blood-retinal barrier to induce uveitis. In an experiment of adoptive transfer of naïve "hen egg lysozyme" (HEL)-specific CD4+ T-cells into transgenic mice expressing HEL, under a retina-specific promoter, showed proliferation of those specific T cells in the draining eye lymph nodes, without inducing any uveitis. However, if the same mice model was infected systemically with murine cytomegalovirus (MCMV) engineered to express HEL, there was proliferation of transferred naïve CD4+ T cells and induction of uveoretinitis, while

this was not observed when wild-type MCMV was used. Therefore, ocular autoantigens likely will not induce uveitis if not presented in the context of inflammation, which may be induced by infection.[142]

Autoreactive T cells toward retinal crude extract (RCE) were observed in vitreous samples of *Mtb*-associated uveitis, these cells were resistant to activation-induced cell death which might contribute to the inflammation. Even though the T-cells were poly-functional after stimulation with *Mtb* antigen ESAT6 and RCE (which was not found in non-*Mtb*-associated uveitis models), the population of retinal auto-reactive T-cells was different from the population of *Mtb* reactive T cells in terms of their sensitivity to activation-induced cell death.[143]

Another study in a Bacille-Calmette-Guerin (BCG)-induced bilateral granulomatous anterior uveitis, peripheral blood T-cell proliferation upon exposure to retinal antigens and purified protein derivative (PPD) was found.[144] The authors reported there was amino acid sequence homology between proteins from *Mtb*, BCG and retinal antigens, hence antigenic mimicry as the cause of uveitis may be possible.[144]

As a more general mechanism; it can be speculated that pulmonary TB or latent TB in an adult immunocompetent patient causes a state of a hyperactive anti-mycobacterial immune response. This may increase anti-retinal immunity in patients with a pre-existent subclinical immune response against retina and may hypothetically induce uveitis. Increased delayed-type hypersensitivity to TB antigens was associated with a more likelihood to suffer from active disease or to develop it.[145, 146] Increased IFNγ production was also found in the peripheral blood, lung tissue, bronchoalveolar lavage (BAL) fluid, pleural effusion and lymph nodes of active TB patients, which decreased upon treatment.[147, 148] Moreover, several gene expression studies also implicate an increase in type I IFN activity and type 1 IFN regulated genes in active pulmonary TB patients or latent TB cases that eventually develop active pulmonary TB.[46, 47, 149, 150] Therefore, in combination with antigenic mimicry, the hyperactive anti-mycobacterial immune response may also play a role.

"Immune recovery uveitis" occurs in HIV-positive patients with TB who develop immune reconstitution after starting antiretroviral therapy.[151] The findings in "immune recovery uveitis" can vary widely and include anterior uveitis, hypopyon, vitritis, papillitis, panuveitis, retinal or optic disc neovascularization, retinal detachment, cystoid macular edema, epiretinal membrane formation, vitreomacular traction, and macular hole.[152] These findings are aspecific and can be observed in diverse uveitis entities.

Animal EAU demonstrates that uveitis is driven by Th_{17} or Th_1 dominated immune response, the predominant pathway will be determined by the antigen exposure. CFA drives more Th_{17} response while in vitro antigen-pulsed DCs drives Th_1 response.[141] B cells contribute to the adaptive immune response towards TB and are abundant in

tertiary lymphoid structure (TLS) formed during TB infection. TLS formation is further supported by IL23, and IL-17.[153] B cell(-/-) mice display more severe immunopathology toward *Mtb* infection as evidenced by elevated recruitment of neutrophils, higher production of IL-10, and higher bacterial burden in the lung, which all can be inverted by adoptive transfer of B cells.[154] Autoreactive B-cells might develop from TLS under the influence of type 1 IFN and B-cell activating factor (BAFF) in Th_{17} driven autoimmune disease.[155] Although there were findings of intra-retinal TLS formation in EAU animal model[156], generally little is known about B cell involvement in uveitis.[154]

Challenges in diagnosis and management of Mtb-associated uveitis

The diagnostic procedures for *Mtb*-associated uveitis are frequently not conclusive, which implies the need to rule out other causes of uveitis. The identification of *Mtb* in ocular fluids represents a challenge; most techniques are unfortunately either unreliable or not sensitive enough.

- ***Ocular Mtb staining and culture***

Obtaining *Mtb* directly from the eye as the gold standard for the diagnosis of *Mtb*-associated uveitis is often not feasible because of the sensitivity limits of the staining and culture procedures. For positive microbiological conformation a certain minimal number of bacilli is required that is at least 1 x 10^4. bacilli/ml for acid-fast staining of sputum and 1 x 10^2 bacilli/ml for bacterial culture.[157] These are the requirements for the regular diagnostic tools for sputum in patients with active pulmonary TB. Yet, this amount is difficult to obtain in ocular TB due to the small volume and likely minimal bacterial load in intraocular fluids.

- ***Diagnostic PCR***

Although the latest nucleic acid amplification test through PCR can detect as minimal as ten femtogram (fg) of DNA which equals to 2-3 tubercle bacilli, this test reveals variable results in intraocular fluid and is not commonly performed in diagnosing of ocular TB.[157-159] The reasons for this unreliable PCR results in intraocular fluid include the non-uniform distribution of *Mtb* inside the eye and the paucibacillary nature of *Mtb* infection. Histopathological studies found that only 1-2 *Mtb* bacilli could be found in the retinal pigment epithelium layer in the posterior part of the eye and already cause severe panuveitis.[133]

There are several other caveats in using PCR as diagnosis tool for *Mtb*-associated uveitis. First, as already stated, the location of tissue sampling may also affect the results of PCR. A nested PCR approach for the gene target *MPB-64* showed 78% sensitivity from epiretinal membranes as obtained by vitrectomy, while this decreased to 21% when the

test was performed from a vitreous biopsy.[160, 161] PCR in anterior chamber fluid from possible and probable ocular TB cases obviously also showed a low yield of positivity.[162] Second, different *Mtb* gene targets will give variable results. Some *Mtb* strains have zero or low copy numbers of certain gene targets (e.g. *IS6110*), therefore, a multi-target gene (*IS6110* + *MPB-64* or *IS6110* + 38 kDa + *MPB-64*) PCR approach will improve the sensitivity.[158, 163] Lastly, PCR does not differentiate between the viable and nonviable microorganisms.

- ***Immunological testing***

Although active pulmonary *Mtb* infection is supposed to be easier to diagnose, there is still a problem in sputum smear-negative TB with and without positive radiological findings. At least, exposure to *Mtb* can be tested by measuring the T-cell immunological response to *Mtb* through QFT or TST. Unfortunately, these tests cannot differentiate between an active and a latent *Mtb* infection. They have a low positive predictive value (PPV) and high negative predictive value (NPV) in sputum-negative, culture-proven active pulmonary TB.[164-166] The low PPV and high NPV were also found in *Mtb*-associated uveitis cases (PPV 71% and NPV 95%).[167] These *Mtb*-associated uveitis cases were based on criteria by Gupta et al., which include a 4-6 weeks ATT treatment observation.[87] The low PPV of QFT does not solve the problem how to treat QFT-positive uveitis of unknown cause. There is a need for additional tests and biomarkers to further stratify that group of patients to optimize treatment choice.

Recent reports demonstrate a differential type-1 IFN signature in patients with active pulmonary *Mtb* infection and patients with latent infection.[149] Both, the activation of the type-1 IFN cascade and its downstream genes present a characteristic pattern in immune cells. Until now, no studies are available that linked type-1 IFN-regulated gene expression in peripheral blood of patients with uveitis and *Mtb* infection.

Increasing number of studies reporting on the association between positive TST and/or QFT and uveitis were reported.[168-176] Moreover, TST and/or QFT-positive uveitis patients may benefit from complete ATT.[177] The reason for this is not entirely clear, but might be related to minimal numbers of *Mtb* bacilli within the retina that cause retinitis.[87] Alternatively, the immunological response to mycobacteria may generate an immune response that is autoreactive to retinal antigens.

As mentioned above, there is still a diagnostic problem in sputum smear-negative TB with and without positive radiological findings. A T-cell immunological response to *Mtb* peptides as measured by QFT or TST cannot differentiate active from latent TB infection. Moreover, the tests can be positive in a certain proportion of healthy individuals; e.g., India (23%)[178], Thailand (17%)[179], USA (5%)[180], Denmark (4.5%)[181], and Tanzania (41%)[182]. Due to these ambiguities, the diagnosis and management of *Mtb*-associated

uveitis may differ between different parts in the world. The choice of first-line diagnostic tools also differs: TST is preferable over QFT in low-income countries. Chest CT is often ordered in high-income countries to detect subtle changes and to detect other common non-infectious diseases such as sarcoidosis.

In conclusion, the evaluation of systemic and ocular signs and symptoms in patients suspected from *Mtb*-associated uveitis represents a first important step in diagnosing *Mtb*-associated uveitis.[159] In contrast to the classical findings of granulomatous inflammation, in latent TB, a broad spectrum of novel ocular manifestations was recently recognized, which includes broad-based synechiae, serpiginous-like retinitis, and retinal occlusive vasculitis, features previously not linked to TB infection.[105, 162, 174] There is an urgent need for an early and more conclusive diagnostic procedure for *Mtb*-associated uveitis.

MANAGEMENT

ATT is the mainstay treatment for *Mtb*-associated uveitis.[87, 183, 184] However, there are no guidelines for the commencement and duration of ATT in patients with uveitis in the setting of positive QFT.[88, 89] The present guidelines mainly suggest that confirmed ocular TB warrants ATT treatment. On the other hand, the diagnosis of *Mtb*-associated uveitis is presumptive in the majority of patients due to the lack of microbiological proof. In *Mtb*-associated uveitis, ATT administration is even a part of diagnosis criteria; a decrease of inflammation after 4-6 weeks of ATT was considered to support the diagnosis.[87] Beside subsiding inflammation, reduced recurrences after the treatment will also facilitate the diagnosis. This management approach, is desirable for patients at risk of losing the vision as the diagnostic uncertainty and/or delay may lead to permanent vision loss.[185]

Recommended regimens for drug-susceptible pulmonary TB from the World Health Organization (WHO) is 6-month rifampicin-based regimen (2 months of isoniazid (H), rifampicin (R), pyrazinamide (Z) and ethambutol (E), and four months of H and R). Daily dosing is still recommended for uveitis patients in initial and continuation phase.[186] However, there is a wide variation in the type of drugs, regimen, and duration of treatment for ocular TB or *Mtb*-associated uveitis. Initial RHZE regimens varied from a minimum of 2 months up to 3-4 months and subsequent HR regimens varied from 4 months up to 15 months.[183] The problems with the multi-drug regimen and the long duration of ATT are the toxicity, poor compliance of the patient and possible drug-resistant *Mtb* induction and in some cases also the costs.[187] There are many side effects reported from ATT, the most concerning one is toxic optic neuropathy associated with ethambutol.[188]

It is a rare but vision-threatening condition, which is ironic because the drug is used for regaining visual acuity from inflammation due to *Mtb*.

Variations also exist in the use of systemic corticosteroids and other immunosuppressive treatments. The use of oral corticosteroids in low-dose during the first 4-6 weeks along with ATT may be beneficial to limit tissue destruction. However, a systematic literature review revealed no differences in clinical outcome of patients with and without use of corticosteroids [183] The use of corticosteroids alone, without ATT, was inconsistently reported as being both detrimental and beneficial.[189, 190] Probably, the initial concomitant use of corticosteroids or other immunosuppressives could bias the true benefit of ATT as well. Some cases, such as for example serpiginoid choroiditis attributed to TB, were difficult to manage by ATT solely, without addition of corticosteroids or immunosuppressive drugs.[111] Even after the full ATT was finished, 15/136 (11%) of the patients needed additional corticosteroid therapy.[191] It was also shown that some patients suspected from *Mtb*-associated uveitis reached remission without ATT, and used only corticosteroids.[190] The decision to use corticosteroids or corticosteroids-sparing agents must be made very carefully and monitored in well-equipped expert centers.

The beneficial effect of ATT is expected from its anti-microbial properties, which will eliminate *Mtb* as the inciting agent of uveitis. In a systematic review of 28 studies including 1917 patients suspected from *Mtb*-associated uveitis, it was concluded that the use of ATT benefits *Mtb*-associated uveitis with or without concomitant systemic corticosteroids. However, there was no control group included, no standardized recruitment and treatment protocol.[183] Additionally, there might be a publication bias toward the good results of ATT.

There is a possibility that ATT might have direct anti-inflammatory properties. In a study of experimental autoimmune encephalomyelitis (EAE), rifampicin treatment associated with suppression of inflammation acts on demyelination in spinal cords of EAE mice, through inhibition of Th_{17} cell differentiation.[192] In another study with mice suffering from autoinflammatory atopic dermatitis induced by 1-chloro 2,4-dinitrobenzene (DNCB), rifampicin treatment tapered the dermatitis symptoms. Rifampicin was associated with the suppression of β-hexosaminidase and histamine from human mast cell (HMC)-1 cells, and therefore inhibited the secretion of inflammatory mediators by mast cells (TNF-α and PGD_2). Though, there is no data yet how strong the anti-inflammatory properties of ATT are uveitis cases.

There are still many questions left about the immunopathogenesis of *Mtb*-associated uveitis. In the future, better knowledge on the pathophysiology will help the clinician to diagnose and to treat *Mtb*-associated uveitis patients in the most safe and effective way.

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Chapter 2

Aims and scope of the thesis

AIMS AND SCOPE OF THE THESIS

Mtb-associated uveitis has various clinical manifestations and represents a sight-threatening condition. At present, it is unknown whether "*Mtb*-associated uveitis" is an infection, an autoimmune disorder or a combination of both. In consequence, the correct treatment of this disorder is elusive.

The overall goal of this thesis is to increase our knowledge on the pathophysiology and clinical manifestations of *Mtb*-associated uveitis in order to improve its diagnosis and treatment.

- To achieve this, we formulated the following aims;
- To examine ocular manifestations of TB and point out difficulties in the diagnosis and management of *Mtb*-associated uveitis.
- To examine clinical manifestations and treatment outcomes of uveitis associated with QFT-positive outcomes in regions endemic and non-endemic for TB.
- To examine whether RPE can be infected with *Mtb* and how RPE cells respond to this infection.
- To examine occurrence of anti-retinal and anti-nuclear antibodies in patients with *Mtb*-associated uveitis.
- To determine whether a peripheral blood type 1 IFN signature is associated with *Mtb*-associated uveitis and may serve as biomarker for *Mtb*-associated uveitis risk stratification in OFT positive patients with uveitis of unknown cause.

In the discussion, we summarize and interpret the results of our investigations, and speculate about further research directions based on the findings in this thesis.

Chapter 3

Uveitis related to *Mycobcaterium tuberculosis* in Indonesia and the Netherlands

Chapter 3.1

Clinical manifestations of patients with intraocular inflammation and positive QuantiFERON–TB Gold In-Tube Test in a country nonendemic for tuberculosis

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ABSTRACT

Purpose: To evaluate clinical manifestations of patients with uveitis and scleritis of unknown origin and positive QuantiFERON–TB Gold In-Tube test (QFT) in a country not endemic for tuberculosis.

Design: Multicenter retrospective cohort study.

Methods: Retrospective review of the clinical, laboratory, and imaging data of 77 patients. Main outcome measures consisted of ocular and systemic features as well as results of laboratory examinations.

Results: Out of all, 60 of 71 (85%) were living for at least 6 months in tuberculosis-endemic regions. Location of uveitis was variable; posterior uveitis (29/77; 38%) was the most frequent. Two clinical entities were commonly noted: retinal occlusive vasculitis (21/77; 27%) and serpiginoid choroiditis (11/77; 14%). Antituberculosis treatment was completed in 32 patients; 29 of them (91%) achieved complete remission. Mean QFT level was 7.5 U/ mL; 71% had values above 2 U/mL and 41% above 10 U/ mL. We observed no associations between QFT levels and clinical and/or imaging features. Previous tuberculosis infection was diagnosed in 5 of 77 patients (6.5%), while hilar/mediastinal lymphadenopathy was found in 25 of 76 patients (33%). Of these, 12 were consistent with the diagnosis of sarcoidosis, 9 were typical for (prior) tuberculosis, and 4 were compatible with both diagnoses.

Conclusions: Ocular features of patients with idiopathic uveitis and positive QFT were diverse, but retinal occlusive vasculitis and serpiginoid choroiditis were common. The QFT levels were usually highly elevated and 33% of patients exhibited lymphadenopathy, suggesting frequently the diagnosis of sarcoidosis. Ocular inflammation reacted favorably to antituberculosis treatment, although only a small minority had documented (prior) tuberculosis.

INTRODUCTION

Infection with Mycobacterium tuberculosis may result in active disease, characterized by the active multiplication of mycobacteria with formation of granulomas and tissue destruction of the infected organs, preferentially the lungs. Ocular involvement during active tuberculosis (TB) can consist of formation of granulomas in various parts of the eye or orbit.[1, 2] However, the majority of infected people will not advance to active disease but live with a latent TB infection.[3, 4] During the last decade, interferon gamma release assay tests have been developed, which allow identification of patients with latent TB infection with better specificity than the tuberculin skin test and differentiate between infection and prior vaccination.[5] It has become apparent that a portion of the patients with uveitis of so far unidentified origins in fact suffer from TB infection–related uveitis.[6-8] The presumed pathogenesis of uveitis related to latent TB infection consists of inflammation caused by presence of low numbers of bacteria within the eye with or without superimposed immune reaction to mycobacterial or ocular antigens.[3, 9] So far, limited data are available on the ocular manifestations in latent TB infection and most information is based on populations from TB-endemic areas. Various ocular manifestations have been reported, including features distinct from characteristic granulomatous inflammation.[7, 10-15] Herein we evaluate the clinical manifestations of patients with uveitis of unknown origin and positive QuantiFERON–TB Gold In-Tube test (QFT; Cellestis Limited, Carnegie, Victoria, Australia) living in an area not endemic for TB.

METHODS

We identified 96 consecutive human immunodeficiency virus–negative patients with uveitis and scleritis and positive QFT results from 3 tertiary centers (Erasmus Medical Center Rotterdam, University Medical Center Utrecht, and Rotterdam Eye Hospital). In the Netherlands TB is not endemic and Bacillus Calmette Guérin (BCG) vaccination is not routinely performed.

The clinical, laboratory, and imaging data of these patients were reviewed. This study was performed in accordance with the Declaration of Helsinki and with the approval for retrospective review of patients' data by the Institutional Review Boards of Erasmus Medical Center Rotterdam, University Medical Center Utrecht, and Rotterdam Eye Hospital.

In this study, the participating centers previously included a QFT test as part of their screening for all new patients with uveitis and scleritis. In addition, several patients with unexplained chronic uveitis who underwent the screening before the interferon gamma

release assay era were also tested. All our new uveitis patients received the QFT and tuberculin skin tests simultaneously during the screening; in several patients the tuberculin skin test was performed previously, but in all cases more than 6 months before the QFT tests were performed. The QFT cutoff value was 0.35 U/mL.

A specific cause of uveitis and/or associated systemic disorder was determined in 19 patients (ocular toxoplasmosis, n = 4; Behçet disease, n = 2; human leukocyte antigen B27–associated acute anterior uveitis, n = 2; viral anterior uveitis, n = 5; masquerade syndromes, n = 3; Vogt-Koyanagi-Harada disease, n = 1; Wegener disease n = 1; and presumed ocular histoplasmosis syndrome, n = 1). Uveitis in these patients was considered unrelated to latent TB infection and these cases were excluded from the final analysis. Four patients with biopsy-proven sarcoidosis and positive QFT results were included in our series because both diseases might share common pathogenic pathways and the differential diagnosis of sarcoidosis and TB can be difficult.[16] All 77 included patients had been subjected to uveitis screening that included erythrocyte sedimentation rate, blood counts, serum angiotensin converting enzyme level, serology for syphilis, and, in those with anterior uveitis, also human leukocyte antigen B27 testing. Chest radiograph examination was performed in 69 patients; out of the remaining 8 patients, 7 had a computerized tomography (CT) scan primarily performed and in 1 patient the imaging data could not be retrieved.

The classification of uveitis was performed according to current criteria.[17] In this study, the term intraocular inflammation included uveitis and scleritis. The diagnosis of serpiginoid chorioretinitis was performed in patients with multiple serpiginous-like chorioretinal lesions that exhibited multiple areas of activity and were not located adjacent to the optic disc.[18, 19] Immunosuppressive treatment was defined as systemic treatment with corticosteroids, sometimes in combination with methotrexate, azathioprine, or cyclosporine A. An anti-TB treatment was defined as a course of at least 3 antibiotics—isoniazid (INH), rifampin, pyrazinamide—in the initial 2-month phase of treatment followed by a 4-month consolidation phase using rifampin and INH (in total, 6 months).[20] Three patients from one of the centers (Utrecht) received a longer consolidation phase (7 months, encompassing a total treatment of 9 months). In the absence of signs of pulmonary and/or systemic TB in the majority of patients, the decision to treat the patients with anti-TB drugs was based on the severity of the ocular disease and the patient's preference in case of non-sight-threatening uveitis. The presence of macular edema was confirmed by optical coherence tomography in all clinically suspected patients.

Data were entered and analyzed using SPSS software version 20.0 (IBM Inc, Chicago, Illinois, USA). For all calculations with visual acuity (VA) data, Snellen VA was converted to the logarithm of the minimal angle of resolution (logMAR). For easier understanding,

the logMAR results were converted back to Snellen VA and are presented as such in this report. In patients with unilateral involvement, visual acuity of the affected eye was included; to prevent the bias of bilateral disease, only the right eye was included in bilateral cases.

RESULTS

The general characteristics of the 77 patients (44 male and 33 female) are given in Table 1. The mean age at onset was 46 years and the median follow-up time was 3 years. Out of all patients, 24 of 71 (32%) were born in the Netherlands, of whom 13 stayed longer than 6 months in a country endemic for TB; 47 of 71 (66%) came from and stayed longer than 6 months in a country endemic for TB (demographic data could not be traced for 6 patients).

The anatomic location of intraocular inflammation was diverse, but posterior uveitis was most frequent (Table 2). Anterior uveitis without posterior segment involvement was observed in 19 of 77 patients (25%). Anatomic location of uveitis did not differ between the patients born in the Netherlands and those born in an endemic country or between the patients who never visited an endemic country and those who were born in or stayed longer than 6 months in an endemic country.

The clinical manifestations of inflammation varied widely; however, 2 explicit clinical posterior segment entities were commonly noted, specifically occlusive retinal vasculitis (21/77; 27%) and serpiginoid choroiditis (11/77, 14%). Occlusive retinal vasculitis manifested preferentially in male subjects ($P < 0.001$) and was associated with younger age ($P = 0.04$, Table 1). Intraocular granulomas were not observed. Corneal opacities (other than band keratopathy) were noted in 7 of 77 patients (9%). Granulomatous keratic precipitates were present in 10 of 77 patients (13%; 10/19 with anterior uveitis, of whom 4 were bilateral and 6 unilateral). Vitreous inflammation was present in 46 of 77 patients (60%) and vitreous hemorrhage in 6 of 77 (8%), and 2 patients exhibited retinal fibro- vascular tumors. Vasculitis (arterial, venous, or both) was noted in 34 of 77 patients (44%) and 21 of the 34 (62%) exhibited vascular occlusions and retinal ischemic areas. The most common ocular complications included macular edema, glaucoma, and cataract (Table 3). Vitreous hemorrhage and retinal neovascularizations were most common in retinal occlusive vasculitis compared to other types of uveitis ($P = 0.001$, Table 3). The number of patients with 1 or more complications was 55 of 77 (71%). Out of 77 patients, 35 (45%) had to undergo 1 or more intraocular surgical procedures (n = 29) and/or laser treatment (n = 8).

TABLE 1. General Characteristics of QuantiFERON–TB Gold Test–Positive Patients with Uveitis and Scleritis

	Total	Anterior Uveitis	Occlusive Retinal Vasculitis	Serpiginoid Chorioretinitis	Lived in an Endemic Country >6 Months[a]	Did Not Stay in Endemic Country >6 Months[a]
	N = 77	N = 19	N = 21	N = 11	N = 60	N = 11
Mean age at onset of uveitis (y)	46	49	40c	42	45.5	44.4
Male-to-female ratio	1.3:1	6:13[b]	20:1[c]	5:06	7:05	5:06
Living in an endemic country >6 months	60/71 (85%)	15/17 (88%)	19/21 (91%)	8/11 (73%)	N.A.	N.A.
Contact with TB patient	20/77 (26%)	4/19 (21%)	5/21 (24%)	3/11 (27%)	10/60 (17%)	7/11 (64%)
Positive TST	51/55 (93%)	14/15 (93%)	13/14 (93%)	5/6 (83%)	38/41 (93%)	8/9 (89%)
Proven (previous) TB infection	5/77 (7%)	0/19 (0%)	3/21 (14%)	0/11 (0%)	3/60 (5%)	2/11 (18%)
Hilar and/or mediastinal adenopathy on radiologic and/or CT examination	25/76 (33%)d	5/19 (26%)	7/21 (33%)	4/11 (36%)	21/60 (35%)	4/11 (36%)
Mean quantiferon levels in U/mL (median)	7.49 (5.75)	7.89 (4.9)	6.29 (2.2)	5.55 (2.1)	7.6 (7.1)	5.5 (2.6)
Quantiferon levels >10 U/mL	30/73 (41%)	7/18 (39%)	7/19 (37%)	4/11 (36%)	24/56 (43%)	3/11 (27%)

CT = computed tomography; N.A. = not applicable; TB = tuberculosis; TST = tuberculin skin test.
[a]Data were not available for 6 patients.
[b]Anterior uveitis occurred more frequently in female subjects (P = 0.01).
[c]Occlusive retinal vasculitis occurred more frequently in male subjects (P = 0.001). Patients with occlusive retinal vasculitis were younger (P = 0.04) compared to the remainder.
[d]Of these 25 patients, 12 were considered compatible with the diagnosis of sarcoidosis (symmetrical configuration of lymph nodes and absence of old calcifications), 9 with tuberculosis (asymmetrical presentation, calcification, and old scars), and 4 were consistent with both diagnoses.

TABLE 2. Ocular Characteristics of QuantiFERON-TB Gold Test-Positive Patients With Uveitis and Scleritis

Characteristic	Total	Unilateral	Bilateral
	N = 77	N = 26	N = 51
Unilateral-to bilateral ratio	1:2	Not applicable	Not applicable
Location of inflammation			
Anterior uveitis	19 (25%)	11 (42%)	8 (16%)
Intermediate uveitis	9 (12%)	1 (4%)	8 (16%)
Posterior uveitis	29 (38%)	10 (39%)	19 (37%)
Panuveitis	16 (21%)	2 (8%)	14 (28%)
Scleritis	4 (5%)	2 (8%)	2 (4%)
Serpiginoid chorioretinitis	11/77 (14%)	4/26 (15%)	7/51 (14%)
Retinal vasculitis	34/77 (44%)	10/26 (39%)	24/51 (47%)
Occlusive retinal vasculitis	21/77 (27%)	6/26 (23%)	15/51 (29%)
Papillitis[a]	34/73 (47%)	8/26 (31%)	26/51 (51%)
Fibrovascular retinal lesions	2/77 (3%)	2/26 (8%)	0 (0%)
Corneal opacities	7/77 (9%)	2/26 (8%)	5/51 (10%)
Optic disc atrophy	7/77 (9%)	3/26 (12%)	4/51 (8%)

[a]Fluorescein angiography and/or appropriate clinical information were not available for 4 patients.

Mean QFT value for the whole series was 7.49 U/ mL (median 5.75 U/mL, Table 1); 52 of 73 patients (71%) had levels above 2 U/mL and 30 (41%) above 10 U/mL. No associations were found between the levels of QFT and various clinical manifestations, specifically when the following groups of patients were compared: patients with and without anterior uveitis (mean 7.9 U/mL vs 7.4 U/ mL, P = 0.757), with and without occlusive retinal vasculitis (mean 6.3 U/mL vs 7.9 U/mL, P = 0.329), with and without serpiginoid choroiditis (mean 5.6 U/mL vs 7.8 U/mL, P = 0.261), and with and without hilar and/or mediastinal lymphadenopathy (mean 8.3 U/mL vs 7.1 U/mL, P= 0.437). An elevated serum angiotensin converting enzyme level was noted in 16 of 68 patients (24%). A positive association was noted between elevated serum angiotensin converting enzyme and QFT levels (P= 0.04). A positive tuberculin skin test was present in 51 of 55 patients (93%), out of which 15 (15/51; 29%) had a test result exceeding 20 mm. An association between highly positive tuberculin skin test result and clinical and/or laboratory characteristics was not observed. There were 4 patients with negative tuberculin skin test (despite positive QFT results). Two patients were previously diagnosed with active TB, of whom 1 was still on anti-TB treatment, and 3 were diagnosed with TB during the assessment for uveitis. There was no difference in the levels of QFT for patients born in the Netherlands vs not born in the Netherlands (P = 0.78), nor was there an association with age noted (P = 0.095). Contact with a TB patient was recalled more

TABLE 3. Ocular Complications of QuantiFERON–TB Gold Test–Positive Patients with Uveitis and Scleritis

	Total	Anterior Uveitis	Occlusive Retinal Vasculitis	Serpiginoid Chorioretinitis	Lived in an Endemic Country >6 Months[a]	Did Not Stay in Endemic Country >6 Months[a]
	N = 77	N = 19	N = 21	N = 11	N = 60	N = 11
Cystoid macular edema	34 (45%)	4 (21%)	8 (38%)	7 (64%)	27 (45%)	6 (55%)
Glaucoma	18 (24%)	5 (26%)	7 (33%)	2 (18%)	14 (23%)	4 (22%)
Cataract	12 (16%)	1 (5%)	3 (14%)	3 (27%)	10 (17%)	2 (17%)
Vitreous hemorrhage	6 (8%)	0 (0%)	6 (28%)	0 (0%)	6 (10%)	1 (9%)
Subretinal neovascularization	8 (10%)	0 (0%)	7 (33%)	1 (9%)	6 (10%)	1 (9%)
Other complications[b]	12 (15%)	1 (5%)	7 (33%)	3 (27%)	13 (22%)	0 (0%)

[a]Data were not available for 6 patients.
[b]Includes hypotony, band keratopathy, hyphema, macular atrophy, macular pucker, tractional retinal detachment, and subretinal fibrosis.

often in the group who never visited an endemic country (7/11; 64% vs 10/60; 17%, P = 0.003).

Hilar and/or mediastinal adenopathy was diagnosed in 25 of 76 patients (33%) (10 by chest radiograph, 13 by CT scan and 2 by both imaging methods; Table 1) and was characterized as compatible with the diagnosis of sarcoidosis in 12 of 25 (48%); in 9 of 25 (36%) the adenopathy was compatible with the diagnosis of TB (asymmetrical configuration, calcifications, and/or presence of lung parenchyma lesions suggestive of TB) and in 4 (16%) was compatible with both diagnoses. Out of 12 patients with bilateral adenopathy on chest radiograph, 4 also had CT scans done, confirming the chest findings in 2; in 2 patients, however, the CT scan revealed no abnormalities. Out of 15 patients with hilar adenopathy on CT scan, 9 had concurrent chest radiographs available, of which 2 were concordant, but in 7 no abnormalities were seen on chest radiograph. No correlation was observed between the presence of hilar/mediastinal adenopathy and general characteristics such as sex, age, BCG status, QFT levels, diameter of tuberculin skin test induration, serum angiotensin converting enzyme level, location of uveitis, and presence of specific ocular characteristics. Biopsy of enlarged chest lymph nodes was performed in 12 of 25 patients (48%) with hilar and/or mediastinal adenopathy. Microscopic evaluation revealed only 1 patient with granuloma and associated necrosis; 2 showed nonspecific inflammatory infiltrates and the remainder exhibited granulomas without necrosis consistent with the diagnosis of sarcoidosis. The patient with necrotic granuloma in the lung showed negative results in culture, polymerase chain reaction

TABLE 4. Visual Acuity and Treatment Regimens of QuantiFERON–TB Gold Test–Positive Patients With Uveitis and Scleritis

	Visual Acuity Before Treatment	Visual Acuity 1 Year After Treatment	P Value (Related Samples Wilcoxon Test)
ATT full course, total (n = 32)	Median 20/40 Mean 20/91	Median 20/25 Mean 20/45	0.004
No ATT (n = 38)	Median 20/33 Mean 20/51	Median 20/25 Mean 20/37	<0.001

ATT = antituberculosis treatment (for definitions see Methods section).
Visual acuity was calculated using the logarithm of the minimal angle of resolution (logMAR). For easier understanding the logMAR results were converted back to Snellen visual acuity and presented here. In bilateral cases, only the right eye is included to prevent a bias of similar bilateral presentation. In patients without ATT, visual acuity before and 1 year after the onset of any kind of treatment was included.

(PCR), and Ziehl- Neelsen stain, but 3 (out of 12) other biopsies were positive for M tuberculosis (2 by culture, of which 1 was also positive by PCR and 1 by Ziehl-Neelsen stain only); these 3 patients had occlusive retinal vasculitis. Two out of these 3 patients were previously treated with immunosuppressive agents compared to 1 out of 9 with negative biopsies. Four additional patients underwent bronchoalveolar lavage, but none tested positive for M tuberculosis by staining, PCR, or culture. In addition, 4 patients had no thoracic lymph-adenopathy but had enlarged lymph nodes elsewhere (in the pancreas, liver, spleen, and neck).

Anti-TB treatment was initiated in 42 patients. Eight patients stopped the medication prematurely (2 patients because of liver toxicity, 1 because of general malaise, and 5 for nonmedical reasons); 2 were still on treatment at the time of data collection (Table 4). Thirty-two patients finished a full course of anti-TB treatment (10 in combination with corticosteroids, an additional 12 patients after a course with corticosteroids and/or other immunosuppressive drugs) and had an average follow-up after anti-TB treatment of 2.1 years (standard deviation: 3.2 months). Out of 32 patients who received a full anti-TB treatment course, 29 (91%) exhibited gradual decrease of inflammatory activity with complete disappearance of inflammation even after withdrawing all other (local and/or systemic) treatment. Two showed persisting intraocular inflammation; in 1 patient intraocular inflammation recurred 3 years after completion of anti-TB treatment. Development of VA during the various treatment regimens is shown in Table 4. Mean VA at 1-year follow-up (after the initiation of anti- TB treatment) was improved in patients who finished a full course of anti-TB treatment (whether or not in combination with immunosuppressive medication) (P = 0.004;Table 4). Patients who did not receive anti-TB treatment had a better visual acuity at onset compared to those with anti-TB

treatment, but also this group showed an improved VA at 1-year follow-up (P < 0.001, Table 4).

Eighteen patients were initially treated with systemic immunosuppressive medications (4 of whom received INH prophylaxis); 11 of 18 had shown ongoing activity and/or recurrences of uveitis, of whom 1 patient developed active TB disease; 6 reached a quiet stage and 1 was lost to follow-up. Out of these 18 patients, 9 received a subsequent full anti-TB treatment. Out of 4 patients who received prophylaxis with INH, none developed ongoing activity and/or recurrences of uveitis.

Nineteen patients with a positive QFT test but with other identified causes of their uveitis were treated with appropriate therapies, and INH prophylaxis was given in 3 out of 4 patients who needed systemic immunosuppressive agents or prednisone. Patients did not receive a full TB treatment since they had no chest abnormalities and their uveitis was considered not related to their latent TB. None developed manifest tuberculosis and/or ocular signs indicative of ocular TB.

DISCUSSION

Our study shows that QFT-positive, but otherwise unexplained, intraocular inflammation in the Netherlands occurred preferentially in patients who stayed longer than 6 months in TB-endemic countries and was characterized by a wide spectrum of ocular features with 2 distinct posterior segment entities: occlusive retinal vasculitis and serpiginoid choroiditis. Further, one third of these patients exhibited hilar and/or mediastinal adenopathy on radiologic and/or CT scan examinations, which was considered consistent with either sarcoidosis or tuberculosis, or could be compatible with both. Anti-TB treatment was associated with decrease of inflammatory activity, few recurrences, and increase of VA, although only a small minority had documented (prior) M tuberculosis disease.

Our findings of a high prevalence of posterior segment involvement with frequent occurrence of occlusive retinal vasculitis and serpiginoid choroiditis are consistent with previously reported results.[7, 8, 21] In our study, vasculitis was a common sign observed in 44%, which is similar to findings from the United Kingdom and India.[7, 8] Our results are in concordance with a Singaporean study that found predominant posterior segment inflammation in uveitis associated with latent TB infection and observed a high prevalence of retinal phlebitis (61%).[21] In contrast, 2 studies (1 from Germany and 1 from India) reported on a higher prevalence of serpiginoid choroiditis compared to retinal vasculitis in interferon gamma release assay–positive patients with uveitis (55% and 45%, respectively).[14, 22] Anterior uveitis was observed in 25% of our patients, which is

similar to the 29% reported from Singapore and India, while only 5%-11% was reported from Germany and the United Kingdom.[7, 8, 21] The above differences may have been influenced by inclusion bias as most studies included only patients with clinical manifestations suspicious of TB disease. We could not confirm the finding of broad-based posterior synechiae as a frequent sign of latent and/or active TB infection–associated uveitis, which might have been in part caused by incomplete documentation in our retrospective study.[21] We observed occlusive retinal vasculitis predominantly in young male subjects, which is consistent with male predominance of Eales disease.[23, 24]

Latent TB infection is defined as a dormant state of TB infection, associated with only a small number of viable, but not multiplying, mycobacteria and satisfactory immune protection to prevent progression to active TB. The standard test for TB infection for many decades has been the tuberculin skin test; but both sensitivity and specificity of this test are suboptimal. Distinct from tuberculin skin test, interferon gamma release assay tests enable the differentiation between the vaccinated and truly infected individuals, enabling the diagnosis of latent TB infection with superior specificity compared to the tuberculin skin test. A moderate concordance of 83% has been shown between positive outcomes of QFT and tuberculin skin test, which is entirely consistent with our results.[25]

The baseline QFT levels in adult patients with active TB vary for different populations; levels between 2 and 4 IU/mL were reported for adult patients with active TB infection.[26-31] The number of T cells secreting interferon gamma in response to M tuberculosis antigens provides a quantitative measurement of specific T-cell immunity that is not correlated with protection from disease.[32] The interferon gamma release assay tests measure an immunologic response of the patient dependent on the patients' immunologic capacity and many other factors.[33, 34] Our finding of extremely high QFT levels (mean 7.49 U/mL) in patients with uveitis is surprising and might indicate an abundant immune reaction to M tuberculosis antigens. We did not find any association between the level of QFT and specific clinical features. Neither have we observed an association between QFT levels and indurations of tuberculin skin test, but the different antigens used by the different tests might explain this discrepancy. The absence of intraocular granulomas and the presence of vasculitis in the majority of patients with latent TB infection and uveitis was previously noted and might also be explained by an enhanced immune response rather than by the direct invasion of intraocular tissues by M tuberculosis.[35] The identification of M tuberculosis in intraocular tissues of patients is usually not feasible and the scarce presence of M tuberculosis in ocular tissues in proven ocular tuberculosis cases was reported.[36]

One third of our patients exhibited mediastinal/hilar adenopathy, of whom a majority of the adenopathy was consistent with the diagnosis of sarcoidosis. The histologic

characteristics as well as the negative results of staining, cultures, and PCR for M tuberculosis in 9 of 12 lymph node biopsies were considered consistent with the diagnosis of sarcoidosis. One could speculate either that our diagnostic tests were not sensitive enough to reveal mycobacteria or that this was attributable to a sampling error since the presence of mycobacteria in lymph node tissue might be scarce.[36] In contrast, our findings might also indicate that a specific type of sarcoid reaction could occur triggered by TB infection. Our findings emphasize the possible relation- ship between infection with M tuberculosis and development of enlarged hilar and/or mediastinal lymph nodes consistent with the diagnosis of sarcoidosis. The association of tuberculosis and sarcoidosis has been repeatedly reported and various studies suggest that mycobacterial antigens might represent the inciting agent in a proportion of patients with sarcoidosis.[37-39] DNA of M tuberculosis was observed in granulomas of patients with sarcoidosis and specific antigens (catalase-peroxidase and the 70-kDa mycobacterial heat shock protein) were pointed out as possible inciting agents.[38, 39] QFT results also seem to be reliable in patients with sarcoidosis, in contrast to their anergy to tuberculin skin test.[40, 41]

So far, systematic assessments of radiologic and histologic findings in a large number of patients with uveitis and positive QFT test results are not available. Mediastinal lymphadenopathy was also observed in 4 of 21 cases (19%) with presumed intraocular TB in the United Kingdom.[8] The symmetrical radiologic appearance and histologic findings as well as negative results of various tests for TB in the biopsies of our 15 patients might also indicate that mycobacteria are not (anymore) present and that a sarcoid reaction has developed, possibly triggered by mycobacterial antigens. It is not yet clear whether uveitis in our QFT-positive population can be considered as an active ocular infection (probably with slowly multiplying mycobacteria) or just as a purely immunologic reaction to previous infection or a combination of both phenomena. The positive reaction to anti-TB treatment in our series favors the presence of infection, and it might be feasible that anti-TB treatment eliminates M tuberculosis either in the eye or elsewhere in the body and stops a perpetual immune response that is leading to uveitis.

In the absence of a diagnostic test for ocular TB, a therapeutic trial was considered justified in patients with severe sight-threatening intraocular inflammation and latent TB infection.[42] The indication for anti-TB treatment in our study was biased by the severity of ocular disease; predominantly, patients with active uveitis and poor VA were treated. Our study documents a very good overall effect of anti-TB treatment, as 29 of 32 patients (90%) with completed anti-TB treatment exhibited a disappearance of intraocular inflammation in contrast to patients who did not receive anti-TB treatment. The improvement of VA in patients who did not receive anti-TB treatment can be explained by a natural course of their uveitis as well as initial favorable effect of immunosuppressive therapy. Interestingly, 11 out of 19 patients who were initially

treated with immunosuppressive drugs showed ongoing activity and, in the long-term follow-up, decrease of VA, and 9 of these patients showed a satisfactory effect on their inflammatory activity after subsequent anti-TB treatment.

The drawbacks of our study include the limitations of retrospective studies; there was no systematic data collection, diagnostic procedures differed, and no systematic treatment criteria were applied. However, even in this limited series the beneficial effect of anti-TB treatment was clear.

In conclusion, we show a favorable outcome of anti-TB treatment in QFT-positive patients with a uveitis of undetermined cause. Our results show the absence of intraocular granulomas and confirm the presence of occlusive retinal vasculitis and serpiginoid choroiditis, as previously observed in patients positive for interferon gamma release assay tests in endemic populations. Further, we report on highly elevated QFT levels in patients with intraocular inflammation and point out the limited number of patients with documented (prior) TB disease. We identify the association of QFT-positive uveitis with hilar and/or mediastinal lymphadenopathy suggestive of the diagnosis of sarcoidosis. Future studies are required to elucidate the exact pathogenesis of QFT-associated intraocular inflammation and lymphadenopathy, and to show whether viable mycobacteria are present in affected tissues or whether the inflammation represents an immune reaction or a combination of both processes. In the view of the multitude of infected individuals in the world and the associated visual morbidity, intensive research on this topic is needed.

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Chapter 3.2

Tuberculosis and other causes of uveitis in Indonesia

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ABSTRACT

Purpose: To assess the causes of uveitis in Indonesia and determine the importance of tuberculosis (TB) as a cause o uveitis.

Methods: Prospective cohort study examining 146 consecutive new HIV-negative patients with active uveitis between June 2014 and May 2015. We assessed the anatomic locations and specific causes of uveitis as well as associations with infectious and non-infectious systemic diseases. We determined the prevalence of positive Quantiferon-G test (QFT) results in Indonesian patients with uveitis and calculated the number of patients with active systemic TB.

Results: Posterior and panuveitis were the most common anatomic entities (38% each). Infections represented the most frequent cause of uveitis (33%); the most prevalent were toxoplasmosis (19%) and active systemic TB (8%). The majority of patients were QFT-positive (61%). A specific diagnosis could not be established in 45% of the patients. At first presentation to the ophthalmologist, the majority of patients (66%) had a visual acuity of less than finger counting at 3 m and already exhibited various complications of uveitis. When classifying the QFT-positive patients with unexplained uveitis into a TB-related group, the percentage of "TB-associated" uveitis cases increased from 8% to 48%. Highly elevated QFT levels were observed in patients with uveitis of unknown cause and no signs of active systemic TB.

Conclusion: In Indonesia, infectious uveitis was the most common type of uveitis, and the leading causes consisted of toxoplasmosis and TB. The association observed between highly elevated QFT results and uveitis of otherwise unexplained origins indicates that a link exists between the latent TB infection and the development of uveitis.

Keywords*:* uveitis in Indonesia, tuberculosis-related uveitis

INTRODUCTION

Uveitis is an ocular disorder that is associated with multiple infectious and non-infectious causes and a major cause of blindness worldwide. Determining the cause of uveitis in each patient is essential for disease management.[1] Tuberculosis (TB) has historically been considered a major cause of uveitis, but the importance of TB as a primary cause of uveitis has gradually decreased in the last decades, although it has recently re-emerged.[2-4]

TB is an infectious disease caused by *Mycobacterium tuberculosis (M. tuberculosis)*. One-third of the world's population is thought to be infected with *M. tuberculosis*, and in 2015,10.4 million new infections occured.[5] TB is typically viewed as a poverty-related disease, and immunodeficiency, including infection with human immunodeficiency virus (HIV), is an important risk factor. The typical risk factors for extrapulmonary TB including eye involvement are HIV infection, young age, female gender and non-white race.[6]

Ocular involvement has prevalence between 1.4% and 18% in pulmonary TB patients.[7, 8] The diagnosis of ocular TB is difficult to achieve because access to intraocular tissues is limited, and few mycobacteria invade the intraocular environment. Previously, if a patient had unexplained uveitis, evidence of active systemic TB elsewhere in the body justified treatment for ocular TB.[9] In recent years, the introduction of Interferon Gamma Release Assay (IGRA) tests has made it possible to identify patients with latent TB infections. Identifying uveitis with active and/or latent TB is important because it can lead to substantial visual loss or even blindness if left untreated.[10, 11] Previous reports have emphasized the presence of granulomas as a pathognomonic feature of ocular TB, whereas recent publications have shown that serpiginoid choroiditis and occlusive retinal vasculitis are ocular manifestations attributable to latent TB infection.[12]

There are no precise data regarding ocular TB and other potential causes of uveitis in Indonesia, a country with a high prevalence of all three TB categories (i.e., TB, TB/HIV and multi-drug-resistant TB) and that will carry a high disease burden for the years 2016-2020 according to the World Health Organization (WHO).[5] In 2016, the prevalence rate of TB was estimated at 395 per 100,000.[5] In the current prospective study, we determined the anatomical location and causes of uveitis in Indonesia and evaluated the prevalence of active and latent TB infections in the uveitis population using standard screening examinations including radiologic chest imaging and concurrent tuberculin skin tests (TSTs) and QuantiFERON-Tb Gold tests (QFTs).

SUBJECTS AND METHODS

We performed a prospective cohort study that included all 247 consecutive new patients with uveitis who were referred to the Infection and Immunology Division of the Ophthalmology Department of the Medical Faculty Universitas Indonesia/Cipto Mangunkusumo Hospital, Jakarta Indonesia. Our study occurred from June 2014 until May 2015 and was approved by the local medical ethics committee. All included patients provided informed consent. In all, 146 of the 247 enrolled patients were included in the final analysis. We excluded patients unwilling to participate and/or patients who did not complete the screening work-up and those who did not attend follow-up examinations (n=72). Additional 29 HIV-positive patients with uveitis were seen in the same time-frame; they are reported separately.

A medical history was obtained, and a full ophthalmological examination, including slit-lamp biomicroscopy and indirect ophthalmoscopy, was performed for all included patients. Color photographs were obtained, and fluorescein angiography, optical coherence tomography (OCT) and visual field tests were performed according to standard clinical practices. Uveitis cases were classified and graded according to the Standardization of Uveitis Nomenclature (SUN) system.

All patients were submitted to standard screening protocols for uveitis to obtain the erythrocyte sedimentation rate and red and white blood cell counts. Additionally, serological testing for HIV, syphilis and toxoplasmosis, urine analysis and radiological chest examination were performed. TSTs (RT23 SSI-2 T.U/0.1 mL Statens Serum Institute, Copenhagen, Denmark) and QFTs (Cellestis Inc., Carnegie, Australia) were also performed. In all cases, the QFT was performed before the TST. Because of a temporary lack of tuberculin supply, the TST was performed in only 115/146 (79%) of the patients. In Indonesia, a TST result with an induration larger than 10 mm in diameter is considered positive.[13] The QFT cut-off value was 0.35 IU/mL.[14] In HIV positive patients, however, QFT and TST tests were not systematically applied because we had very limited supply of tests and the outcomes of laboratory tests in HIV-infected patients are not reliable. In addition, some HIV-positive patients (13/29, 45%) were previously diagnosed with systemic TB and treated, and their QFT and TST outcomes would not be contributory.

A radiological examination; standard postero-anterior chest X ray (CXR) was performed in all patients. When needed, additional imaging examinations, including apico-lordotic CXR (n=5) and chest computed tomography (CT) scans (n=16), were conducted. Cases involving iris and/or choroidal granulomas, sub retinal abscesses, serpiginoid choroiditis and retinal vasculitis of unknown cause (n=16) underwent chest CT scanning because this modality can be useful for diagnosing granulomatous uveitis.[15]

Human leucocyte antigen (HLA) B27 testing was not available at our institution at the time of this study.

Microbiological examinations (i.e., sputum culture (n=1), Ziehl *Nielsen* (ZN) staining of sputum (n=21), and polymerase chain reaction (PCR) for *M. tuberculosis* in sputum (N=10)) were performed in selected cases. Additional PCRs of intraocular fluids was performed in 44 patients with a high suspicion of infectious uveitis etiology and no response to standard treatments with corticosteroids including herpes simplex virus (HSV, n=10), varicella zoster virus (VZV, n=3), cytomegalovirus (CMV, n=18), Toxoplasma (n=5), and *M. tuberculosis* (n=37). PCR for *M. tuberculosis* was performed on samples isolated using a QIAamp DNA and Blood Mini Kit and primers specific for IS 1660 in a thermal cycler. The products were further analyzed using gel electrophoresis.

Diagnoses of pulmonary TB were based on the Indonesian Society of Respirology (ISR)TB guidelines (see the algorithm illustrated in supplemental Figure 1).[16, 17] Briefly, diagnoses of active pulmonary TB were based on a general clinical examination and microbiological and/or radiological findings. A review of chest X-ray and CT scan data was performed by two pulmonologists who are specialists in TB (one from Indonesia and one from the Netherlands), and cases were classified as active TB, prior but inactive TB and abnormalities other than TB.

Clinical diagnoses, such as Behcet's disease, Vogt Koyanagi Harada (VKH) syndrome, and Fuchs uveitis syndrome (FUS), were diagnosed according to current diagnostic criteria. All diagnoses of sarcoidosis were achieved according to current diagnostic criteria for patients with negative TB tests. Patients showing no evidence of active systemic TB but who had *Mtb*-PCR-positive intraocular fluids were not classified as having active systemic TB and were separately indicated. Diagnoses of ocular toxoplasmosis were based on clinical manifestations including focal active chorioretinitis located adjacent to a chorioretinal scar in combination with positive serological results and a good response to antiparasitic drugs. We registered all demographic data and the results of all diagnostic and ophthalmological examinations.

All statistical analyses were performed using chi-squared tests and one-way ANOVA tests. Statistical significance was defined as a p value less than 0.05. We performed Bonferroni post-hoc analyses, and all p values shown were adjusted. Multiple regression analysis was used to compare all clinical and laboratory characteristics related to QFT values. For all calculations with visual acuity data, we used the logarithm of the Minimum Angle of Resolution (logMAR). In bilateral cases, we used the average logMAR visual acuity; in unilateral cases, the visual acuity of the affected eye was used.

RESULTS

The general characteristics of the included patients, causes of TB and its associations with systemic diseases are presented in Tables 1 and 2. The mean age at onset of uveitis was 41 years old, and no difference was observed between genders. At first clinical presentation, a duration of uveitis of longer than 1 year was observed in approximately 25% of patients, and the majority of patients (97/146, 66%) had a visual acuity (VA) of less than finger counting at 3 m. The majority of cases in our series were unilateral (100/146, 68%), and anterior uveitis was observed in 29/146 (20%) of patients. Posterior and pan-uveitis were the most common presentations (38% for each, Table 2).

Ocular infections were diagnosed in 48/146 (33%) of the patients. Toxoplasmosis was the most common (30/48, 63%), followed by active TB-associated uveitis (12/48, 25%). Toxoplasmosis was the major cause of posterior uveitis (27/56,48%). An association with a non-infectious systemic disease was observed in 11/146 (7.5%) cases. The most frequently observed presentations were Behcet's disease and VKH syndrome (4/146, 3% each). Ocular clinical syndromes were diagnosed in 21/146 (14%) patients, among which masquerade syndromes (8/146, 5.5%) and FUS (4/146, 3%) were the most common. A specific diagnosis was not established in 66/146 (45%) cases.

Using ISR TB guidelines, active pulmonary TB was diagnosed in 12/146 (8%) patients, and evidence of previous pulmonary TB was observed in 23/146 (16%; Table 3) patients. Patients with active pulmonary TB-associated uveitis showed no differences compared with the other groups in gender, laterality, anatomical location of uveitis, age of onset, VA at first visit, duration of uveitis, or complications. A borderline association was found for the development of hypotonic and/or phthisical eye (p=0.055, chi-squared test). No specific clinical manifestations were prevalent in patients with active pulmonary TB-associated uveitis except for choroidal granulomas (2/12, 17% vs 0/134, 0% in other groups, p<0.000). Of the two patients with serpiginoid choroiditis, one had active systemic TB, and the other had previous pulmonary TB.

The majority of uveitis patients were QFT-positive (89/146, 61%; Table 2). The prevalence of QFT-positive tests was highest for the patients with active pulmonary TB-associated uveitis (10/12, 83%) and previous TB (18/23, 78%). One (1/12, 8%) patient with active pulmonary TB who was diagnosed using ISR TB guidelines had a negative QFT test. The QFT was more frequently positive in female patients (52/89, 58% vs 21/53, 40% in males; p=0.038), but there were no differences in age, laterality, VA at first visit, duration, clinical manifestations or complication rates of uveitis. Although unilateral cases were more prevalent in this series, QFT-positive patients were more likely to exhibit bilateral involvement than QFT-negative patients (44/89, 50% *vs* 13/53, 25%; p=0.005, multivariate binary logistic regression analysis; odds ratio, 3).

TABLE 1. General characteristics and QuantiFERON–TB Gold outcomes in patients with uveitis in Indonesia

General characteristics	Total	QFT Positive N = 89				QFT negative N= 53		
		Total	Active pulmonary TB[b]	Established cause (other than active systemic TB)	Cause not established	Total	Established cause	Cause not established
	N=146[a]	N = 89/146 (61%)	N= 10	N= 21	N= 58	N = 53/146 (36%)	N = 26	N = 27
Age at onset of uveitis (mean ± SD)	41 ± 16	**42.5 ± 15**	40 ± 18	37 ± 13	45 ± 14	**38** ± 17	38 ± 17	38 ± 17
Male–to-female ratio	71: 75	**37 : 52**	7 : 3	14 : 7	16 : 42	**32 : 21**	16 : 11	16 : 10
Positive TST [3] 15 mm (N=115)	45/115 (39%)	**42/74 (57%)**	2/9 (22%)	7/16 (44%)	33/49 (68%)	**2/38 (5%)**	1/ 19 (5%)	1/ 19 (5%)
Positive TST [3] 10 mm (N=115)	66/115 (57%)	**57/74 (77%)**	9/9 (100%)	9/16 (56%)	39/49 (80%)	**8/38 (21%)**	5/19 (27%)	3/19 (16%)
QFT levels (IU/ml) (median; IQR)	1.0; (0.04 – 5)	**3.6; (1.4 – 8.6)**	1,7; (1.1 – 5,6)	1.6; (0.7 – 4.8)	5.0; (2.1 – 10.4)	**0.01; (-0.005 – 0.12)**	0.03; (-0.004 – 0.14)	0.007;(-0.01 - 0.06)
QFT levels ≥ 5 IU/ml	36/146 (25%)	**36/89 (40%)**	2/10 (20%)	5/21 (24%)	29/58 (50%)	**0**	0	0

QFT: QuantiFERON–TB Gold, IQR: Interquartile range, SD: Standard deviation, IU: international units, TB: tuberculosis, TST: tuberculin skin test
[a] 4 patients had indeterminate QFT-G test outcomes (data are not shown)
[b] Two additional patients were diagnosed with active pulmonary tuberculosis according to Indonesian guidelines (one was QFT negative and one had indeterminate QFT result)

TABLE 2. Ocular characteristics, tuberculosis infection and QuantiFERON–TB Gold outcomes in patients with uveitis in Indonesia

Characteristics	Total	QFT Positive N = 89				QFT Negative N=53		
		Total	Active pulmonary TB[b]	Established cause (other than active systemic TB)	Cause not established	Total	Established cause	Cause not established
	N=146[a]	N= 89	N = 10	N= 21	N= 58	N= 53	N = 26	N = 27
Unilateral-to-bilateral ratio	100 : 46	45 : 44	6 : 4	14 : 7	25 : 33	40 : 13	19 : 7	21 : 6
Location of inflammation								
Anterior uveitis	29/146 (20%)	21/89 (23.5%)	1/10 (10%)	2/21 (9.5%)	18/58 (31%)	7/53 (13%)	2/26 (8%)	5/27 (18.5%)
Intermediate uveitis	4/146 (3%)	3/89 (3.5%)	0	0	3/58 (5%)	1/53 (2%)	0	1/27 (4%)
Posterior uveitis	56/146 (38%)	27/89 (30.5%)	3/10 (30%)	13/21 (62%)	11/58 (19%)	27/53 (51%)	20/26 (77%)	7/27 (26%)
Panuveitis	55/146 (38%)	36/89 (40.5%)	6/10 (60%)	6/21 (28.5%)	24/58(41%)	18/53 (34%)	4/26 (15%)	14/27(52%)
Scleritis	2/146 (1%)	2/89 (2%)	0	0	2/58 (4%)	0	0	0
Etiology of uveitis								
Infections, total	**48/146 (33%)**	**25/89 (28%)**	**10/10 (100%)**	**15/21 (71%)**	**0/58**	**21/53 (40%)**	**21/26(81%)**	**0/27**
Toxoplasmosis	30/48 (63%)	12/25 (48%)	0	12/15 (87%)	0	17/21 (81%)	17/21 (81%)	0
Pulmonary TB	12/48 (25%)	10/25 (40%)	10/10 (100%)	0	0	1/21(5%)	1/21 (5%)	0
Others	6/48 (12%)	3/25 (12%)	0	3/15 (13%)	0	3/21 (14%)	3/21 (14%)	0
Associated with non-infectious systemic diseases, total	**11/146 (7.5%)**	**6/89 (7%)**	**0/10**	**6/21 (29%)**	**0/58**	**5/53 (9%)**	**5/26 (19%)**	**0/27**
Behcet disease	4/11 (36.5%)	2/6 (33%)	0	2/6 (33%)	0	2/5 (40%)	2/5 (40%)	0
VKH	4/11 (36.5%)	3/6 (50%)	0	3/6 (50%)	0	1/5 (20%)	1/5 (20%)	0
Others	3 (27%)	1/6 (17%)	0	1/6 (17%)	0	2/5(40%)	2/5 (40%)	0
Ocular clinical syndromes, total	**21/146 (14%)**	**9/89 (10%)**	**0/10**	**0/21**	**9/58 (15.5%)[c]**	**12/53 (23%)**	**0**	**12/27 (44%)**
Masquerade	8/21 (38%)	3/9 (33%)	0	0	3/9 (33%)	5/12 (42%)	0	5/12 (42%)
FUS	4/21 (19%)	1/9(11%)	0	0	1/9 (11%)	3/12 (25%)	0	3/12 (25%)
Others	9/21 (43%)	5/9 (56%)	0	0	5/9 (56%)	4/12 (33%)	0	4/12 (33%)
Unknown cause	**66/146 (45%)**	**49/89 (55%)**	**0/10**	**0/21**	**58/58 (100%)**	**15/53 (28%)**	**0**	**27/27 (100%)**

QFT: QuantiFERON–TB Gold, TB: tuberculosis, VKH: Vogt Koyanagi Harada, FUS: Fuchs uveitis syndrome
[a] 4 patients had indeterminate QFT-G test outcomes.
[b] Two additional patients were diagnosed with active pulmonary tuberculosis according to Indonesian guidelines (one was QFT negative and one had indeterminate QFT result).
[c] Ocular clinical syndromes were considered as uveitis without established cause.

The QFT was more frequently positive in patients with uveitis of unknown cause than in those for whom there was an established cause other than active pulmonary TB (58/87, 67% vs 21/47, 45% in patients with an established cause; p=0.054, multivariate binary logistic regression analysis; odds ratio, 2). When patients with a positive QFT result and otherwise unexplained uveitis were classified as TB-related, the percentage of "TB-associated" patients increased from 8% to 48% (70/146) (66% or 70/106 of patients with infectious uveitis). Focal chorioretinal scars were more frequently observed in QFT-negative patients (22/53, 41.5% *vs* 17/89, 19%, p=0.01; odds ratio, 2.7). The specific QFT level observed in each group is indicated in Table 3. In QFT-positive patients, the QFT levels in patients with active pulmonary TB and those without pulmonary TB did not differ (median value, 1.5 IU vs 0.5 IU, p=0.570, Mann-Whitney test). The median QFT value was higher in patients with uveitis of unknown cause than in those with an established cause (2.2 IU vs 0.3 IU, p=0.008). Moreover, QFT values higher than 5 IU/ml were more frequently observed in patients in which uveitis had an unknown cause than in patients with an established cause (29/85; 34% vs 5/46; 11%, p=0.019; odds ratio, 3.5).

The TST result was ≥10 mm in 65/112 (58%) and ≥15 mm in 44/112 (40%) of the tested patients. The prevalence of a TST result of ≥15 mm was highest in patients with previous TB (16/20, 80%), while in active pulmonary TB-associated uveitis, only 2/10 (20%) had a TST result of ≥15 mm (p=0.000). There was no association between TST positivity and gender, laterality, location of uveitis, ocular complications or clinical characteristics (except for focal chorioretinal scars, which were more commonly found in

TABLE 3. Pulmonary tuberculosis in uveitis patients according to the Indonesian Society of Respirology Tuberculosis guidelines

Classification	Total[a]	Active pulmonary tuberculosis	Previous pulmonary tuberculosis	No pulmonary tuberculosis
	N=146	N=12/146 (8%)	N=23/146 (16%)	N=101/146 (69%)
QFT positive patients	89/146 (61%)	10/ 12 (83%)	18/ 23 (78%)	54/101 (53.5%)
QFT (median; IQR)[b]	1; 0.05 – 5.3	1.5; 1 – 4.7	6.2; 1.2 – 11	0.5; 0.01 – 3.6
QFT ≥ 5 IU	36/146 (25%)	2/12 (17%)	11/23 (48%)	22/101 (69%)
TST ≥15 mm	45/115 (39%)	2/ 10 (20%)	16/21 (76%)	26/ 78 (33%)
TST ≥ 10mm	66/115 (57%)	10/10 (100%)	18/21 (86%)	35/78 (46%)

QFT: QuantiFERON–TB Gold, TST; Tuberculin skin test; IU; international unit, IQR: Interquartile range
[a] 10 patients who could not be correctly classified according to Indonesian guidelines are not shown.
[b] 4 patients with indeterminate QFT results were not included in the median QFT calculation.

TST-negative patients: 23/68 or 34% of patients had negative TST results vs 5/44 or 11% of patients with positive TST results; p=0.008). All patients with active pulmonary TB had positive TST results (≥10 mm). In accordance with the QFT results, a TST value of ≥15 mm was more frequently observed in patients with uveitis of unknown origin than in those with an established diagnosis (34/68, 50% vs 8/34, 23.5%; p=0.011).

The QFT and TST results were highly correlated (p<0.000), and this relationship was even stronger in patients with QFT levels ≥5 IU/ml (TST ≥15 mm, p=0.000; TST ≥10 mm, p=0.001). Out of the 74 QFT-positive patients, 42/74 (57%) had a TST result ≥15 mm, and 57/74 (77%) had a TST result ≥10 mm.

Out of 37 PCR runs to assess the presence of *M. tuberculosis* in intraocular fluid, 4 yielded a positive result. These four patients were all QFT-positive, and one was classified as having active pulmonary TB (positive sputum for *M. tuberculosis* determined by PCR). Two patients had anterior uveitis, and two had pan-uveitis. Small nodules were observed on the iris in two of these patients, but no other similarities in clinical manifestations were observed.

TABLE 4. Characteristics in Human Immunodeficiency Virus (HIV)-positive patients with uveitis

Characteristic	HIV positive patients N- 29
Age (mean ± SD), years	37 ± 9
Male–to-female ratio	24 : 5
Unilateral-to-bilateral uveitis ratio	14 : 15
Tuberculin skin test >10 mm	1/7 (14%)
Positive QuantiFERON–TB Gold test	1/2 (50%)
Location of inflammation	
Anterior uveitis	3/29 (10%)
Intermediate uveitis	2/29 (7%)
Posterior uveitis	12/29 (41.5%)
Panuveitis	12/29 (41.5%)
Aetiology of uveitis	
Infections, total	**18/29 (62%)**
Toxoplasmosis	4/18 (22%)
Tuberculosis	3/18 (17%)
Cytomegalovirus	9/18 (50%)
Syphilis	2/18 (11%)
Associated with non- infectious systemic diseases, total	**0/29 (0%)**
Ocular clinical syndromes, total	**0/29 (0%)**
Undetermined	**11/29 (38%)**

TST: Tuberculin skin test QFT: QuantiFERON–TB Gold, TB: tuberculosis, VKH: Vogt Koyanagi Harada, FUS: Fuchs uveitis syndrome

The characteristics of 29 HIV-positive uveitis patients are provided in Table 4. The majority of the HIV-associated uveitis cases was of infectious origin (18/29, 62%), with CMV retinitis being most common (9/18, 50%) followed by toxoplasmosis (4/18, 22%). Association of active systemic TB infection with uveitis at the time of presentation was found in 3/29 (10%) cases.

DISCUSSION

This prospective study demonstrates that the known etiology of uveitis is most commonly infectious in Indonesia. Furthermore, we show that the majority of patients consulted an ophthalmologist when uveitis had entered the late stage and they already had poor VA and complications. This late presentation limits the visual prognosis and precludes the recognition of typical clinical manifestations, which impairs the correct diagnosis of uveitis. We demonstrate that active pulmonary TB remains a frequent cause of uveitis in Indonesia (8% of all uveitis cases) and ranks second after toxoplasmosis among infectious causes of uveitis. We also found that a majority of uveitis patients were QFT-positive (61%) and observed an excess of highly positive QFT results in patients with uveitis of unknown cause and without any signs and symptoms of active systemic TB. This finding suggests that QFT-positive uveitis might be associated with previous or latent TB, as has been previously suggested.[7, 18, 19] Interestingly, QFT levels were much higher in patients with uveitis associated with latent and previous TB than in patients with active systemic TB-associated uveitis. The majority of patients with uveitis had a positive QFT result, illustrating the limited diagnostic value of this test in endemic populations such as ours.

In western countries, infections account for approximately 16% of all uveitis cases. This percentage is higher in developing countries (range, 20% to 30%).[3, 20] Because only proven infections are classified as infectious cases, it is likely that in reality these percentages are much higher. In our series, infectious causes accounted for 33% of all cases. The majority were caused by toxoplasmosis, followed by active systemic TB-associated uveitis. Our findings are similar to those described in reports from South America, Thailand and Nepal. In contrast, reports have shown that in other countries in Asia (including India, Singapore and Myanmar), uveitis is more often TB-associated than caused by toxoplasmosis.[21-23] However, these reports classified patients with uveitis and positive QFT and/or TST results (without additional evidence of systemic TB) as having TB. While this may explain the higher prevalence of TB-associated cases, it also prevents comparisons with our series. If the QFT-positive and unexplained cases of uveitis in our series were all classified as TB-associated, the percentage of "TB-associated" cases of

uveitis would increase from 8% to 48%, which illustrates the importance of classification methods. Similar disparities in the prevalence of (presumed) ocular TB can also be found in studies performed in western and middle-eastern countries (range, from under 1% to 18%).[3, 24, 25]These large variations might be caused by genuine geographical differences or potentially by a lack of appropriate classification criteria for TB-associated uveitis.

It is currently unknown whether uveitis associated with a positive IGRA test in the absence of signs of active TB represents a real ocular infection with *M. tuberculosis*, an immune reaction, or a combination of both. The presence of *M. tuberculosis* in ocular tissues has occasionally been demonstrated in active systemic TB-associated uveitis, but a histopathological study demonstrated that very few bacilli are required to cause uveitis.[26, 27] In India, patients with uveitis and positive IGRA outcomes had positive PCR results in the absence of systemic signs of TB infection.[28] These findings show that a low-grade intraocular infection with *M. tuberculosis* is possible even in patients showing no other systemic signs of TB. The diagnostic role of performing PCR on intraocular fluids to identify TB is not yet clear, and the results vary according to the selected gene targets.[12, 18, 29] Because patients can have negative PCR findings in ocular fluid while showing uveitis and active systemic TB or positive PCR findings while showing no evidence of active systemic TB,[29-32] dividing patients into those with either active or latent TB infection is inadequate. These results indicate that there is a broad spectrum of disease severity.

Leprosy is a mycobacterial disease that is endemic in Indonesia, and the possibility of cross-reactivity in the QFT must be taken into account. The peptides ESAT6 and CFP10, which are included in the QFT, were reported to show positive results after infection *M. leprae*.[33, 34] One study conducted in the USA, a non-endemic country, reported that 5/10 (50%) of patients with tuberculoid leprosy patients had positive QFT results, whereas none with lepromatous leprosy (n=40) were QFT-positive.[35] As the leprosy is still prevalent in Indonesia (yearly incidence in 2015 was 7 leprosy cases per 100.000 population versus 395 TB cases per 100.000 population), we cannot exclude that some QFT positivity might be caused by infection with *M. leprae*.[5, 36] However, isolated uveitis without any systemic signs of leprosy is exceedingly rare. In addition, our study included only one patient with a history of treated leprosy, and the patient's QFT result was negative.

In our series, patients with uveitis and signs of previous pulmonary TB had very high QFT levels, as did QFT-positive patients with idiopathic uveitis but no signs of systemic TB. These results are consistent with those described in previous reports.[37-39] High QFT levels might reflect the induction of additional immune reactions by *M. tuberculosis* antigens in (genetically) susceptible patients.[38]The link between uveitis and a positive IGRA test is not entirely clear and the specific pathogenesis of uveitis accompanied by

a positive IGRA test remains unknown. In this study, the lack of associations between ocular features and manifestations of TB may in part be have resulted from the large number patients with end-stage uveitis; it was not feasible to recognize specific clinical features in these patients.

Although this study had a prospective design, it has several limitations. First, our center provides tertiary care, and it is possible that our population of patients was biased towards more severe and chronic cases. The prevalence of patients with anterior uveitis (20%) was, however, very similar to that in other series performed at tertiary centers worldwide. In Indonesia, peripheral ophthalmologists commonly lack the appropriate methods to diagnose patients with uveitis, and the socioeconomic status of patients frequently precludes a timely medical consultation. For example, most of the patients in our study presented with complications and already had very limited VA. This creates a complex and challenging situation for organizations in the ophthalmology health system in Indonesia. Ophthalmologists in Indonesia should be aware of the high prevalence TB-and other infection-associated uveitis. Close collaboration between infectious diseases specialists and pulmonologists is needed to distinguish between infectious and non-infectious uveitis and to thereby enable the selection of an appropriate treatment. In addition, we did not evaluate the QFT value in HIV-positive patients due funding limitations and the unreliability of these tests in the HIV-infected population. Indeed, QFT in HIV-positive patients is reported to have low sensitivity and to frequently exhibit indeterminate results.[40] Therefore, the prevalence of positive QFT outcomes in HIV-positive patients could not be compared to HIV-negative patients; nonetheless, the proportion of uveitis associated with active TB infection was similar for HIV-positive and HIV-negative patients with uveitis (3/29, 10% vs 12/146, 8%, respectively)

In conclusion, infections are the leading cause of uveitis in Indonesia, and most patients consult with an ophthalmologist when their VA is already severely compromised, and their clinical complications are apparent. Infectious uveitis is potentially treatable, and blindness is therefore preventable in these patients. Our findings will hopefully increase the early recognition of uveitis etiologies and play a role in preventing unnecessary blindness.

SUMMARY

What was previously known:

- Active ocular TB has historically been an important cause of uveitis. Recently, in western countries, active ocular TB has been found to primarily occur in immunosuppressed patients.

- The introduction of Interferon Gamma Release Assay/Quantiferon-G tests (QFTs) made it possible to identify patients with latent TB infections, and it seems probable that some types of uveitis are associated with latent TB infection.
- Data related to the causes of uveitis in Indonesia, a country in which TB is endemic, are entirely lacking.

Novel findings of this study:

- Infectious uveitis was identified as the most common type of uveitis in Indonesia (33% of all cases). The leading causes were toxoplasmosis and uveitis associated with active pulmonary TB.
- Latent TB was present in 40% of patients with uveitis of unknown origin, and when we classified cases of uveitis of unknown cause and latent TB as TB-associated uveitis, the percentage of TB-associated uveitis increased from 8% to 48% of all uveitis cases.
- Extremely high QFT levels were found in patients with uveitis of otherwise unexplained origin, indicating a clear link between uveitis and latent TB. These data suggest the involvement of an associated (auto)immune reaction to TB antigens.
- The proportion of cases associated with active systemic TB was similar in HIV-positive and HIV-negative patients with uveitis.

FINANCIAL DISCLOSURE

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(R.L.D.N., A.R., M.B., G.S.); preparation (R.L.D.N., A.R.), review (R.L.D.N., A.R., M.B., M.P.v.H., R.S., M.S., L.E., S.S., G.S.), and approval of the manuscript (R.L.D.N., A.R., M.B., M.P.v.H., R.S., M.S., L.E., S.S., G.S.).

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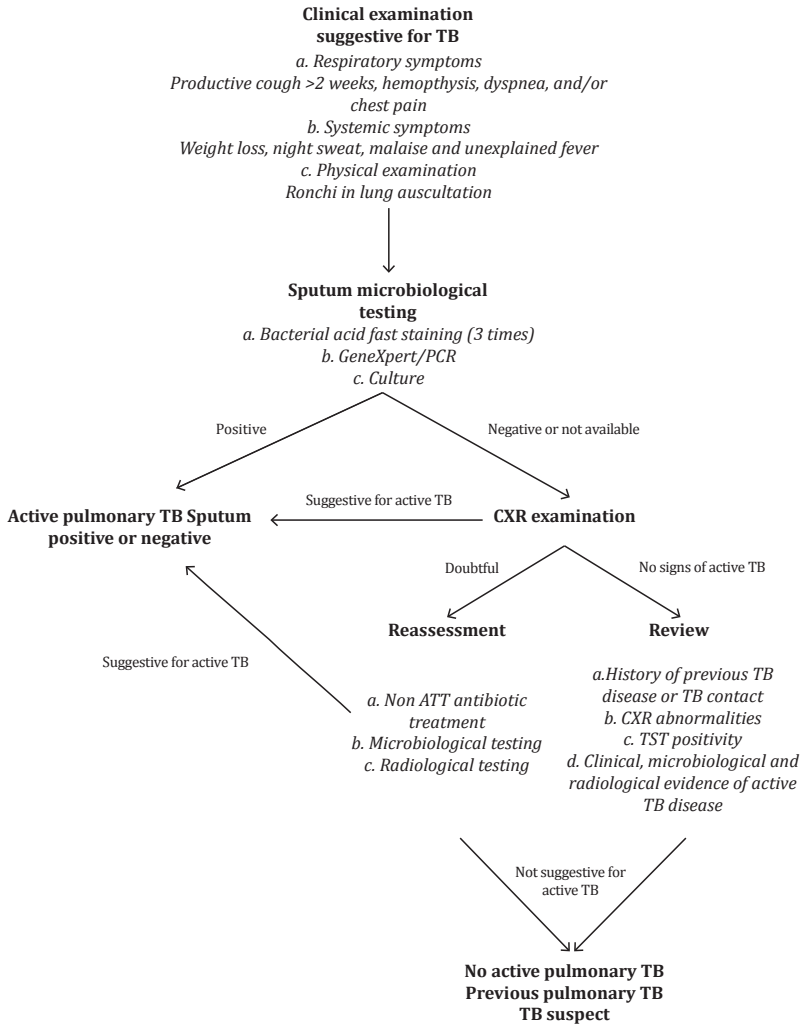
SUPPLEMENTAL DATA



Supplemental Figure 1. Indonesian Society of Respirology (ISR) TB diagnostic guideline.
Scheme depicting the TB diagnostic algorithm in Indonesia. The guideline starts with evaluation of clinical signs suggestive for tuberculosis (TB). In patients with signs suggestive of pulmonary TB infection, an acid-fast staining examination from the sputum is required and a positive result leads to diagnosis of active pulmonary TB. If the culture examinations from the sputum are negative, radiologic imaging is reviewed and classified When imaging is considered to exhibit signs of active pulmonary TB, the patient is diagnosed with active pulmonary TB. Therefore, active pulmonary TB will be either acid-fast bacillus (AFB) positive or AFB negative. The diagnosis of AFB-positive active pulmonary TB is based on positive culture or positive GeneXpert outcomes, and AFB-negative TB is based on radiologic signs of active pulmonary TB and clinical improvement after anti-tuberculosis antibiotic treatment.
TB: tuberculosis, AFB: acid-fast bacillus, GeneXpert: a molecular test that detects bacterial DNA from *Mycobacterium tuberculosis*, PCR: polymerase chain reaction, CXR: chest X-ray, TST: tuberculin skin tes

Chapter 4

Pathogenesis of Uveitis related to *Mycobacterium tuberculosis*: infection or autoimmunity?

Chapter 4.1

Retinal pigment epithelial cells control early *Mycobacterium tuberculosis* infection via interferon signaling

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ABSTRACT

Purpose: Mycobacterium tuberculosis (*Mtb*) bacilli have been found in retinal pigment epithelial (RPE) cells from uveitis patients without signs of systemic tuberculosis (TB) infection. RPE cells are important for the ocular immune privilege and uveitis development.

Methods:To address a potential role for *Mtb*-infected RPE cells in the development of uveitis, we delineated the response to *Mtb* infection in human RPE cells and primary human macrophages, the main target cell of *Mtb*. Primary human RPE cells, the human RPE cell line ARPE-19 and monocyte-derived pro-inflammatory M1 and anti-inflammatory M2 macrophages were infected with DsRed-expressing *Mtb* strain H37Rv. Infection rates and clearance were addressed along with RNA sequencing analysis, a confirmation analysis by dual-color reverse-transcriptase multiplex ligation-dependent probe amplification (dcRT-MLPA) and cytokine secretion.

Results: RPE cells robustly controlled intracellular outgrowth of *Mtb* early after infection. The response in RPE cells to control *Mtb* survival was dominated by interferon (IFN)-signaling and further characterized by prominent regulation of cell death/survival-associated genes and low-level production of Th1-associated cytokines. In contrast, macrophages engaged a plethora of responses including IFN signaling and communication between innate and adaptive immune cells to induce granuloma formation.

Conclusions: Together, our data demonstrate that RPE cells display a strong response to *Mtb* infection that appears however incomplete in comparison to the macrophage response to *Mtb*. The RPE response might reflect a balance between mechanisms aimed at *Mtb* eradication and mechanisms that limit retinal inflammation.

Keywords: Tuberculosis, Retinal Pigment Epithelial (RPE) cells, Macrophages, IFN signaling, Immune profiling.

INTRODUCTION

Tuberculosis (TB) is a global health issue with one-fourth of the world population being latently infected. It is the world's leading cause of death from infectious diseases as 1.8 million people died from TB in 2015 alone.[1] TB primarily infects (alveolar) macrophages in the lungs, and its causative agent *Mycobacterium tuberculosis* (*Mtb*) spreads via airborne droplets from active pulmonary TB patients to other susceptible individuals.[2] The infected host first initiates an innate immune response to limit *Mtb* infection which is followed by an adaptive immune response and formation of caseating granulomas.[3, 4] However, *Mtb* exhibits immune evasion strategies to survive and replicate within macrophages and disseminate from the primary focus throughout the body via blood and lymphatic system.[5]

Mtb can also infect other cell types, including epithelial cells, endothelial cells, fibroblasts, adipocytes and neuronal cells.[5, 6] Persistence of *Mtb* in such cell types distant from the lungs may constitute reservoirs that can reactivate TB disease resulting in extrapulmonary tuberculosis.[5] Notably, several histopathological studies have described uveitis cases that, due to a lack of systemic symptoms, were not suspected to be active TB cases but obviously did contain *Mtb* in the retinal pigment epithelium (RPE).[7, 8] Although very few *Mtb* bacilli were detected in RPE cells, necrosis or severe granulomatous inflammation around the RPE cells was observed, suggesting a role for *Mtb*-infected RPE cells in driving these responses. Therefore, knowledge of the host-pathogen interaction between *Mtb* and RPE cells and the immune responses induced by *Mtb*-infected RPE cells will provide critical pathophysiological insights into how *Mtb* can cause uveitis. Importantly, RPE cells have been described to phagocytose *Mtb,* resulting in increased expression of Toll-like receptor (TLR)2 and TLR4, which are major pattern recognition receptors (PRRs) for sensing *Mtb*.[9, 10] Moreover, chemical inhibition of TLR2 and TLR4 on RPE cells reduced the number of intracellular *Mtb* bacilli, suggesting an essential role for these receptors in phagocytosis of *Mtb* by RPE cells[9]. Yet, limited data are available on the response of RPE cells to *Mtb* infection and how this response compares to the response elicited in human macrophages, the predominant target cell of *Mtb* in the lungs.

In this study, we infected primary human RPE cells (OZR1), the human RPE cell line ARPE-19, and primary human monocyte-derived pro-inflammatory (M1) and anti-inflammatory (M2) macrophages with *Mtb* to investigate the host-pathogen interaction between these cells and *Mtb* as well as the host response induced in these cells to infection with *Mtb*. Transcriptomics and cytokine secretion data were analyzed to discover crucial signaling pathways/networks and biological effector functions regulated in *Mtb*-infected RPE cells and macrophage subtypes. Our findings suggest that while M2 cells have a plethora of responses, including IFN signaling, to control *Mtb* infection,

RPE cells primarily depend on IFN signaling to control the early phases of *Mtb* infection successfully.

MATERIALS AND METHODS

Cell culture

Primary human RPE cells (OZR1)[11] cells and the human RPE cell line ARPE-19 (CRL-2302; American Type Culture Collection ATCC) were maintained in Iscove's Modified Dulbecco's Medium (IMDM) supplemented with 10% fetal bovine serum (FBS; Greiner Bio-One, Alphen aan den Rijn, Netherlands). For experiments, OZR1 cells were used between 6th-10th and ARPE-19 cells between 25th-39th passage.

Pro-inflammatory M1 and anti-inflammatory M2 macrophages were generated from monocytes isolated from whole blood of healthy donors by FICOLL gradient separation and CD14 MACS sorting (Miltenyi Biotec, Teterow, Germany) followed by differentiation for 6 days in the presence of either 5 ng/ml recombinant granulocyte macrophage-colony stimulating factor (GM-CSF; Life Technologies, Bleiswijk, The Netherlands) or 50 ng/ml recombinant macrophage-colony stimulating factor (M-CSF; R&D Systems, Abingdon, United Kingdom), respectively, as previously reported.[12] Macrophages were maintained in Gibco Roswell Park Memorial Institute (RPMI) 1640 medium (Life Technologies) supplemented with 10% FBS (Greiner Bio-One), 100 U/ml penicillin and 100 mg/ml streptomycin (Life Technologies).

Mycobacterial culture

DsRed-expressing *Mtb* strain H37Rv was cultured in Difco Middlebrook 7H9 broth (Becton Dickinson, Breda, The Netherlands) supplemented with 10% albumin dextrose catalase (ADC; Becton Dickinson), 0.5% Tween-80 (Sigma-Aldrich Chemie B.V., Zwijndrecht, The Netherlands) and 50 µg/ml Hygromycin B (Life Technologies). DsRed was expressed from expression plasmid pSMT3 and expression was under the control of the mycobacterial heat shock promoter Phsp60.[13]

Mycobacterial infection

Mycobacterial cultures were diluted to pre-log phase density with appropriate antibiotics one day before infection (optical density at 600 nm (OD600) of 0.4). Immediately before infection, bacterial density was determined, and the bacterial suspension was diluted to 30x10^6 bacteria/ml in cell culture medium without antibiotics (Multiplicity of infection (MOI) = 10). The accuracy of bacterial density was verified by colony forming unit (CFU) assay. RPE cells, seeded 24h prior to infection at 30x10^4 cells/24-well, were

inoculated with 100 µl of the bacterial suspension, centrifuged (3 minutes, 129g) and incubated (60 minutes: 37°C, 5% CO_2). Plates were then washed with culture medium containing 30 µg/ml gentamicin sulfate (Lonza BioWhittaker, Basel, Switzerland) and incubated at 37°C and 5% CO_2 in medium containing 5 µg/ml gentamicin until readout by flow cytometry, confocal microscopy or colony forming unit (CFU) assay as described previously.[14]

Confocal microscopy

27x10^4 cells were grown overnight on 35mm glass bottom microwell dishes (MatTek corporation, Ashland, Maryland, USA) and infected as described above. Samples were stained with Phalloidin-Alexa Fluor® 488 (Life Technologies) in PBS to visualize F-actin. Samples were subsequently fixed for 30 minutes at RT with 4% paraformaldehyde (PFA), embedded in VectaShield with 4'6-diamidino-3-phenylindole (DAPI, Brunschwig Chemie, Amsterdam, The Netherlands). Microscopy slides were examined on a Leica TCS SP5 confocal microscope (Leica Microsystems B.V., Endhoven, The Netherlands).

Phagocytosis

To quantify the phagocytic capacity of cells, fluorescent polysterene particles of 2 µm in diameter with carboxylate coating (Fluoresbrite®YGcarboxylate microspheres, Polyscience, Eppelheim, Germany) were used as described.[15] For details see supplementary information.

RNA isolation and Dual-color Reverse-Transcriptase Multiplex Ligation-dependent Probe Amplification (dcRT-MLPA)

RNA isolation and dcRT-MLPA were performed as described elsewhere.[16] For details see supplementary information. Trace data were analyzed using GeneMapper software 5 package (Applied Biosystems, Warrington, UK). The areas of each assigned peak (in arbitrary units) were exported for further analysis in Microsoft Excel spreadsheet software. Data were normalized to *GAPDH* and signals below the threshold value for noise cutoff in GeneMapper (log2 transformed peak area 7.64) were assigned the threshold value for noise cutoff. Finally, the normalized data were log2 transformed for statistical analysis.

RNA-sequencing and pathway and network analysis

RNA-Sequencing was performed by ZF-Screens B.V (Leiden, The Netherlands). For detail see supplementary information. Differential gene expression results were analyzed using Partek (Partek® Genomics Suite® software, version 6.6 Copyright ©; 2014 Partek Inc., St. Louis, MO, USA.), Ingenuity Pathway Analysis (IPA; QIAGEN Redwood City,

CA, USA), DAVID (open access Database for Annotation, Visualization and Integrated Discovery DAVID Bioinformatics Resources, Frederick, MD, USA) and Instem/OmniViz Treescape (version 6.1.13.0; Instem scientific, Staffordshire, UK) software tools.

Cytokine, chemokine, and growth factor assay analysis

Culture supernatants were analyzed using the Bio-Plex Pro Human Cytokine, Chemokine, and Growth Factor Assay (BioRad, Hercules, CA, USA) for: IL-1β, IL-1Ra, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8/CXCL8, IL-9, IL-10, IL-12 (p70), IL-13, IL-15, IL-17, bFGF, eotaxin, granulocyte colony-stimulating factor (G-CSF), GM-CSF, IFNg, interferon gamma-induced protein (IP)-10/CXCL10, monocyte chemotactic protein (MCP)-1/CCL2, macrophage inflammatory protein (MIP)-1α/CCL3, MIP-1β/CCL4, PDGF-BB, regulated on activation, normal T cell expressed and secreted (RANTES/CCL5), TNFα and VEGFA. The assay was performed according to the manufacturer's instructions.

Statistical analysis

Mtb bacterial load, cytokine/chemokine/growth factors, and dcRT-MLPA data were analyzed for statistical significance using a two-tailed unpaired Student's t-test. A p-value ≤0.05 was considered significant. For the RNA-Sequencing data set, p-values were adjusted for multiple testing using the Benjamini-Hochberg procedure, which controls the false discovery rate (FDR). The statistical computation was performed using GraphPad 7 (La Jolla, California, USA) and SPSS 22 software (IBM Corp, Armonk, NY, US).

RESULTS

Macrophages and RPE cells infected with Mtb control intracellular bacterial growth

To validate previous findings that *Mtb* can be phagocytosed by RPE cells,[9] ARPE-19 and OZR1 cells were infected with DsRed-expressing *Mtb* strain H37Rv. Confocal microscopy and Z-scan analyses clearly revealed intracellular localization of *Mtb* bacilli, confirming that *Mtb* can infect RPE cells (Figure 1A). The percentage of *Mtb*-infected cells was markedly higher in pro-inflammatory M1 and anti-inflammatory M2 macrophages than in the RPE cells ARPE-19 and OZR1 (Figure 1B). *Mtb* requires active phagocytosis by host cells and the differences in infection rate between macrophage subsets and RPE cells indeed correlated with their phagocytic capacity (Figure 1C) and expression of phagocytosis-associated cell surface markers (Figure 1D). To address whether RPE cells, like macrophages, control *Mtb* infection, intracellular bacterial loads were determined immediately after infection with *Mtb* (t=1h) and compared to intracellular bacterial load

24h after infection (Figure 1E). Comparable with M1 and M2 macrophages, a significant reduction in CFUs was observed in ARPE-19 and OZR1 cells over time, indicating that RPE cells are able to reduce the intracellular survival of *Mtb* and control the mycobacterial infection. However, OZR1 cells showed more *Mtb* load to begin with (t =1h) than ARPE-19 cells, which is in line with the higher proportion of phagocytic cells observed for OZR1 in comparison to ARPE-19 (Figure 1C).

Characterization of the host response at the transcriptomic level in RPE cells and macrophages infected with Mtb

Next, we characterized the host response induced in RPE cells and macrophages upon *Mtb* infection to (1) identify crucial host signaling pathways and networks regulating intracellular bacterial survival and (2) address whether similar regulatory networks are operational in RPE cells and macrophages to control outgrowth of *Mtb*. Global transcriptional changes induced in primary human RPE cells OZR1 and human anti-inflammatory M2 macrophages following *Mtb* infection were determined in triplicate samples by RNA-sequencing. M2 macrophages were selected for RNA-sequencing since they share many characteristics with alveolar macrophages, the predominant target of *Mtb in vivo* in the human lung. Principal component analysis (PCA) analysis indicated profound differences between the overall gene expression patterns of *Mtb*-infected cells and their uninfected counterparts, with the largest changes found between infected and uninfected M2 macrophages (Figure 2A). This could either reflect a stronger regulation of commonly altered genes in M2 compared to OZR1 cells and/or regulation of a larger and more diverse gene repertoire in macrophages compared to RPE cells.

To first identify individual genes that were differentially expressed following *Mtb* infection, cut-off values for fold-change >1.5 and p-value ≤0.01 were applied to the data set. 390 and 1638 genes were differentially expressed in *Mtb*-infected OZR1 and M2 cells compared to their uninfected controls, respectively, of which 130 genes were commonly regulated by OZR1 and M2 cells in response to *Mtb* infection (Figure 2B and Supplementary Table 1). Notably, not only does the host response to *Mtb* infection in M2 macrophages involve the regulation of an extended set of genes compared to OZR1 cells, but the magnitude of the response also appears superior in M2 cells compared to OZR1 as indicated by a larger overall fold induction of regulated genes (Figure 2C).

Identification of signaling pathways and networks regulated in RPE cells and macrophages upon infection with Mtb

Next, genes that were found to be differentially expressed in OZR1 and M2 cells following *Mtb* infection were fed into Ingenuity Pathway Analysis to identify critical signaling pathways and networks involved in the host response of RPE cells and macrophages

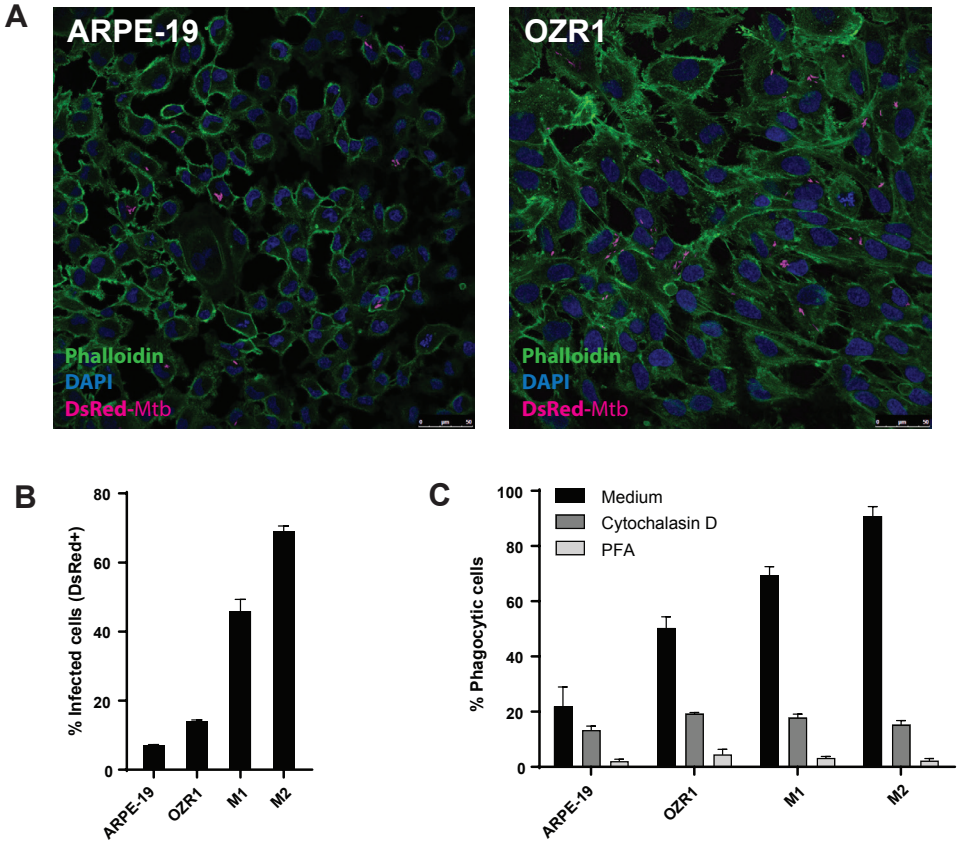
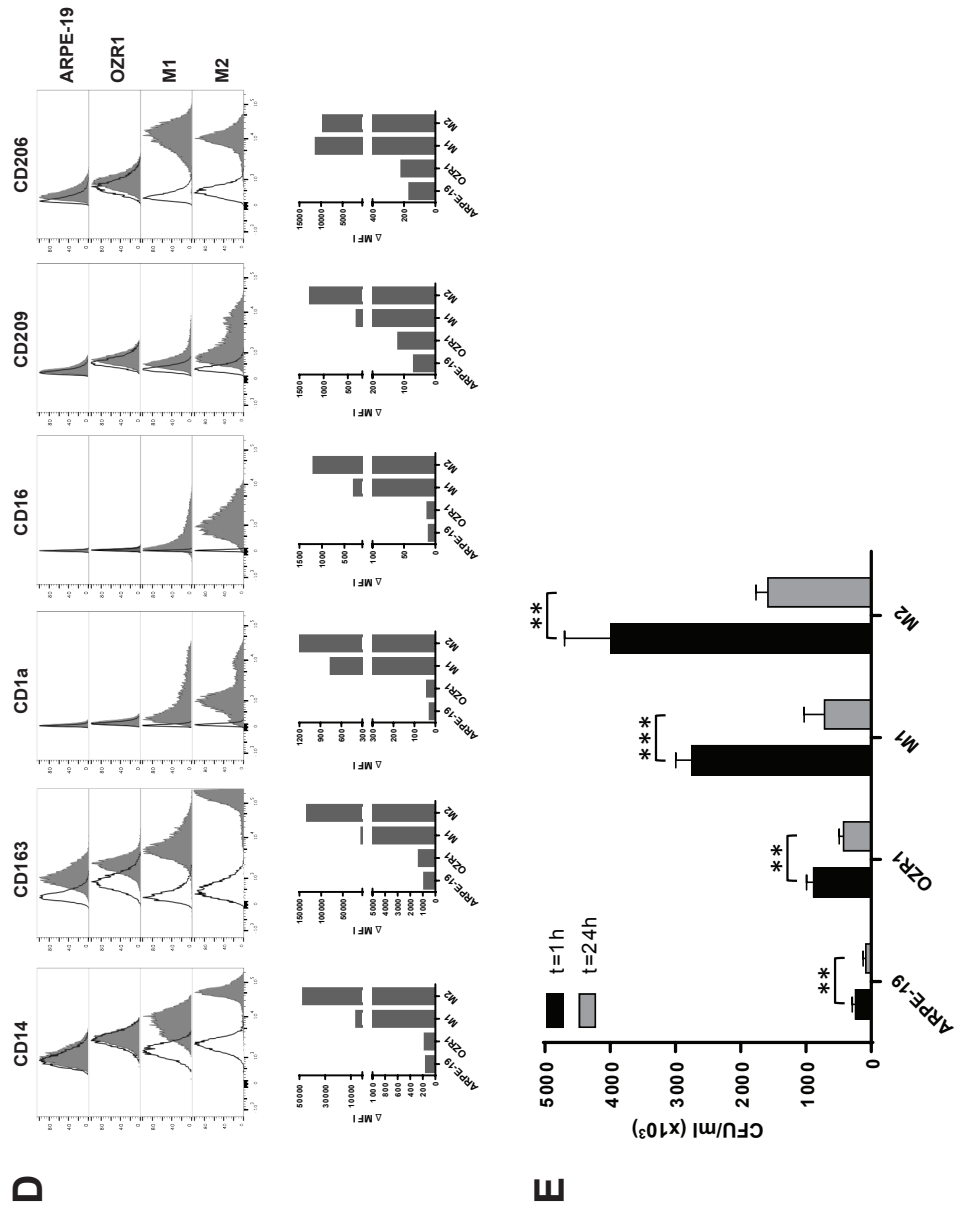


Figure 1. Both RPE and macrophage subsets infected with *Mtb* can control intracellular bacterial survival.
(**A**) Confocal microscopy of RPE cells 24 hours after infection with DsRed-expressing *Mtb*. (**B**) Flow cytometric analysis of RPE cells (ARPE-19 and OZR1) and macrophage subsets (M1 and M2) 24 hours after infection with DsRed-expressing *Mtb*. Percentages of DsRed positive events are indicated. A representative result of 3 experiments is shown. Bars display mean of triplicates ± standard deviation. (**C**) Percentage of phagocytic cells after trypan blue quenching was determined by confocal microscopy. Percentages of phagocytic cells were determined in each cell type, without (black bars) or with pre-incubation of phagocytosis inhibitors; 5 μM Cytochalasin D (dark gray bars) or 4% paraformaldehyde (PFA) (light gray bars). (**D**) Expression of cell surface markers associated with phagocytosis was determined by flow cytometry. D Mean Fluorescence Intensity (MFI) (bottom panel) was calculated between antigen-specific staining (grey histograms) and isotype control staining (white histograms) (top panel). (**E**) Colony forming unit assays were performed at t=1h (end of the infection period) and t=24h after infection with *Mtb*. A representative result of 3 experiments is shown. Bars display mean of triplicates ± standard deviation. Statistical significance was tested using a Student's t-test. ** = p<0.01, *** = p<0.001.



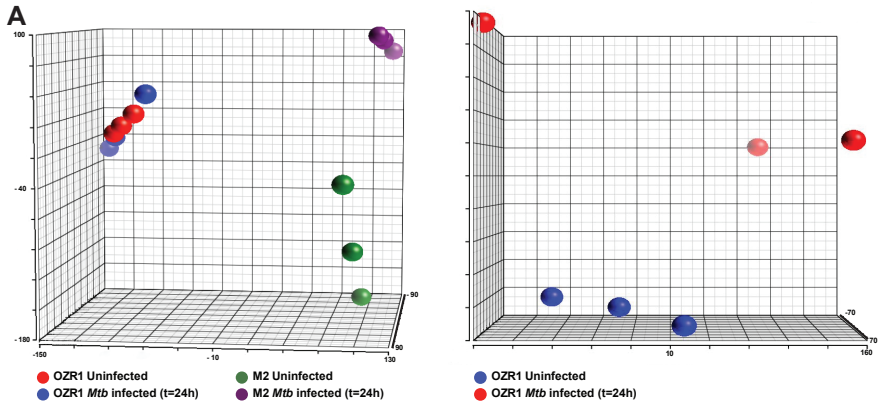


Figure 2. Characterization of the host response at the transcriptomic level in RPE cells and macrophages infected with *Mtb*.
(**A**) PCA analysis of the overall gene expression profiles of uninfected and *Mtb*-infected (t=24h) OZR1 and M2 macrophages. Blue and red spheres represent triplicate samples of uninfected and *Mtb*-infected OZR1 cells, whereas green and purple spheres represent triplicate samples of uninfected and *Mtb*-infected M2 macrophages, respectively. (**B**) Venn diagram of the number of differentially expressed genes (fold change >1.5; p-value ≤0.01) in *Mtb*-infected OZR1 and M2 macrophages compared to their uninfected controls. (**C**) OmniViz Treescape is displaying hierarchical clustering of the differentially expressed transcripts identified in uninfected versus *Mtb*-infected M2 (left panel) and uninfected versus *Mtb*-infected OZR1 (right panel). Red indicates increased gene expression levels compared to the geometric mean; Blue indicates decreased gene expression levels compared to the geometric mean. The colour intensity correlates with the magnitude of the calculated fold change.

to control intracellular survival of *Mtb*. Hierarchical clustering of the top 25 canonical pathways indicated that the host response of human macrophages upon *Mtb* infection involved a broader spectrum of signaling pathways than primary human RPE cells, as the host response in RPE cells was primarily dominated by interferon (IFN) signaling (Figure 3). A detailed analysis of the signaling pathways commonly or exclusively regulated by M2 and OZR1 cells is shown in Supplementary Table 2 and Supplementary Figure 1. A key canonical pathway regulated by both M2 and OZR1 cells in response to infection was IFN signaling (Figure 3 and Supplementary Figure 1). Of the differentially expressed genes annotated within this canonical pathway, ten genes were shared between M2 and OZR1, while RPE cells differentially regulated eight additional IFN-inducible genes and M2 cells regulated two additional IFN-inducible genes (Figure 4). More importantly, the host response of both M2 macrophages and OZR1 cells involved type I as well as type II IFN signaling genes. Network analysis corroborated a central role for IFN signaling in the host response of both OZR1 and M2 cells (Figure 5). In addition, OZR1 cells selectively induced a cluster of *TRAIL* and *PARP* genes, belonging to a family of proteins mainly involved in DNA repair and programmed cell death, while M2

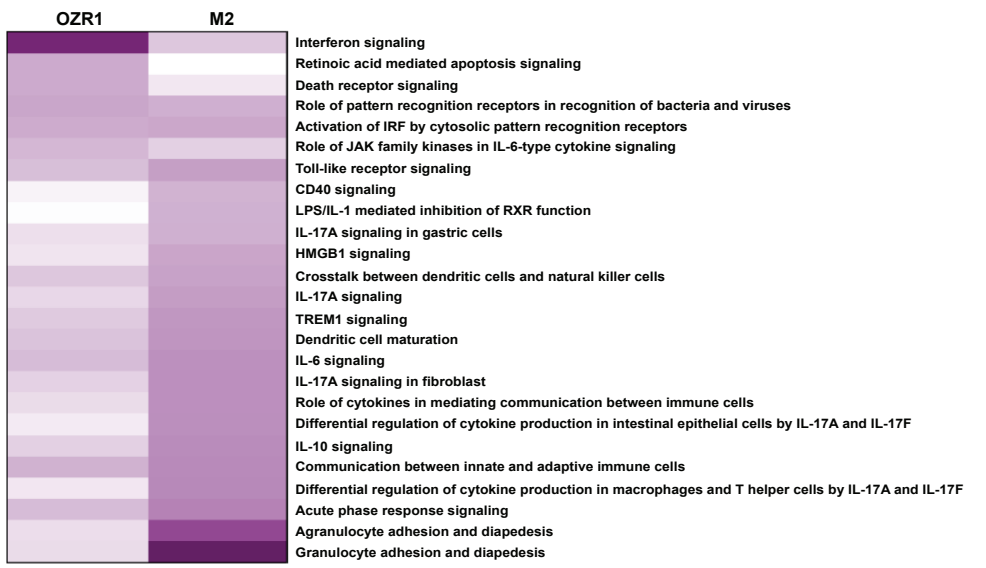


Figure 3. Identification of signaling pathways and networks regulated in RPE cells and macrophages upon infection with *Mtb*.
Hierarchical clustering of the top 25 canonical pathways that were regulated with statistical significance in OZR1 and M2 cells following infection with *Mtb* (t=24h). The colour coding of the map corresponds to the –log(p-value) of each canonical pathway.

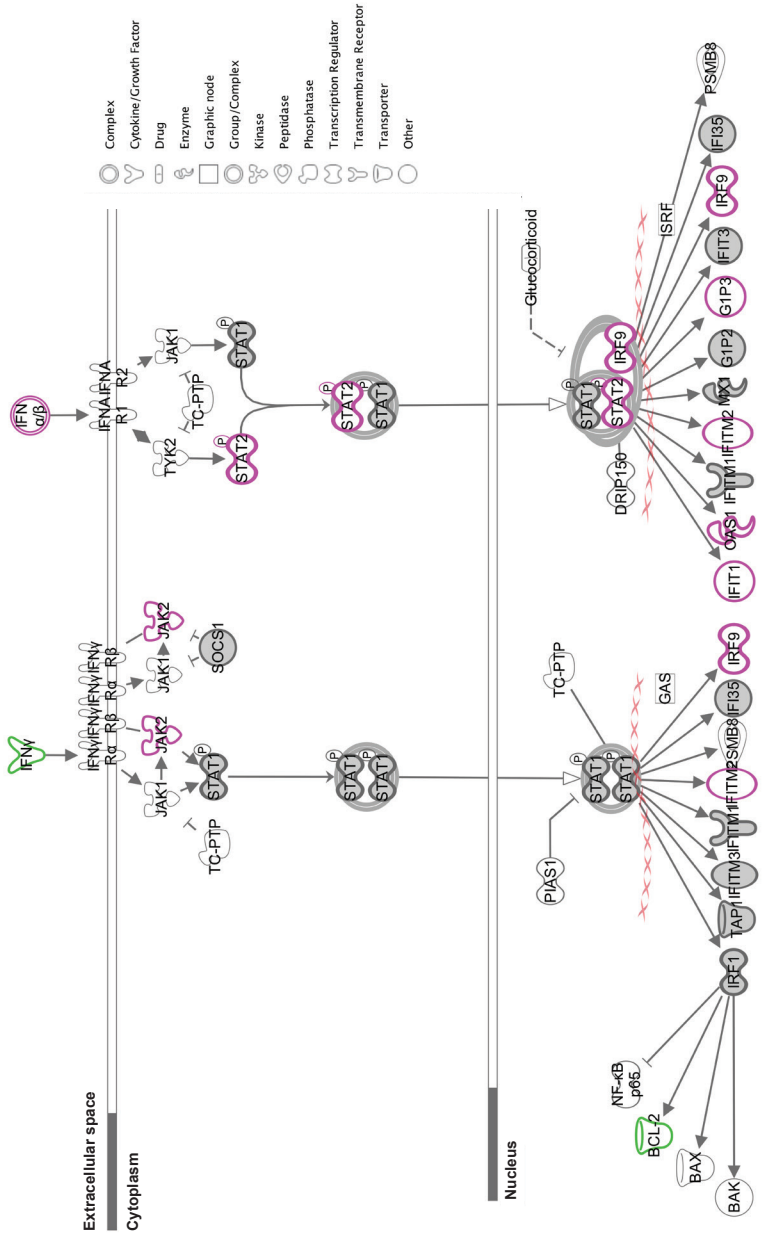


Figure 4. Both type I and II IFN signaling pathways are involved in the host response of RPE cells and marcrophages against *Mtb*.
Interferon signaling canonical pathway (Ingenuity Pathway Analysis). Symbols represent gene function. Indicated are genes that were differentially expressed in both OZR1 and M2 (grey), or solely regulated by OZR1 (pink) or M2 (green) in response to infection with *Mtb* (t=24h).

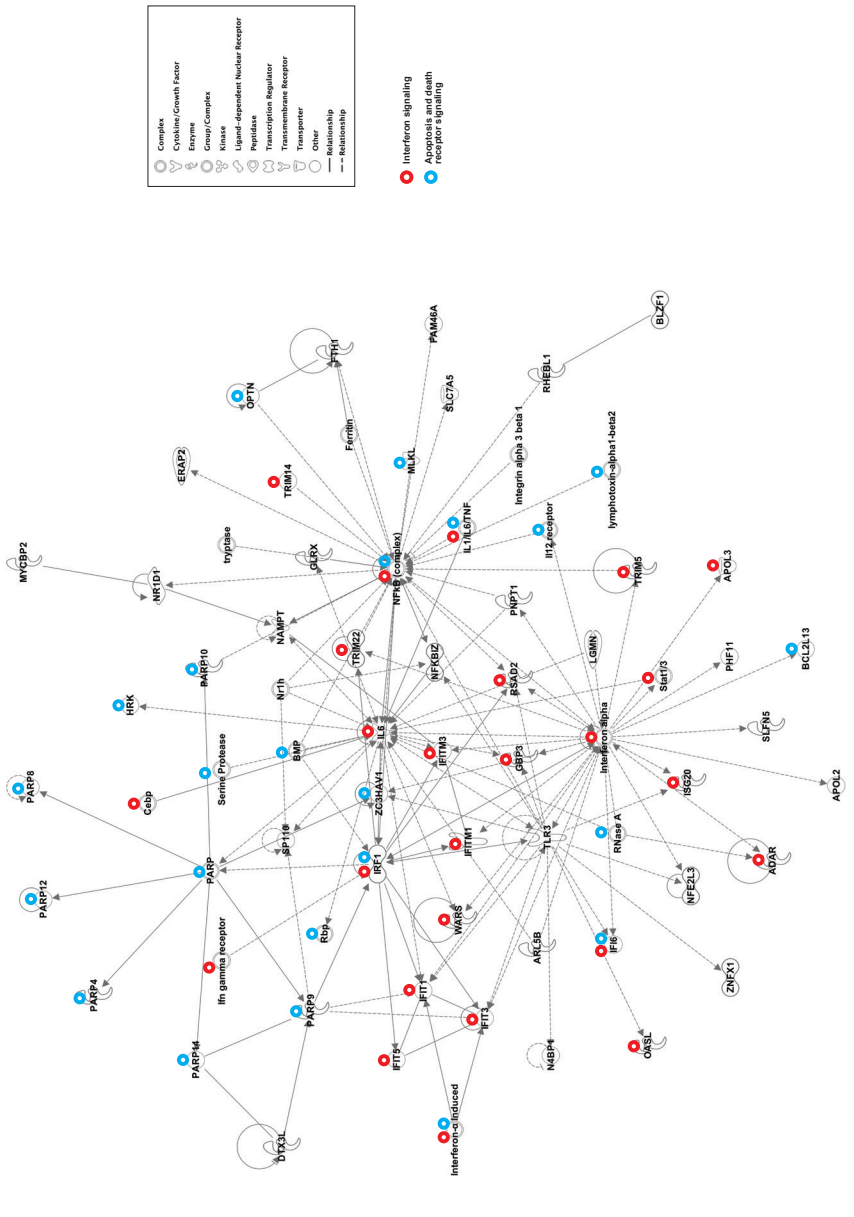
macrophages specifically regulated a cluster of ubiquitin specific peptidases (*USP*) and a cluster of inflammasome-associated genes (Figure 5 and Supplementary Figure 2).

Validation of the Mtb-induced IFN response signature using a focused gene expression profiling platform

To (1) validate independently the identified IFN response signature in OZR1 cells and M2 macrophages following *Mtb* infection and (2) investigate whether the identification of the IFN response signature can be confirmed in ARPE-19 cells and M1 macrophages upon *Mtb* infection, dcRT-MLPA was performed. This approach confirmed the IFN response signature in OZR1 and M2 cells in independent infection experiments (Supplementary Figure 3). More importantly, the *Mtb*-induced IFN response signature was also confirmed in ARPE-19 cells, suggesting that the observed host response in OZR1 cells dominated by IFN signaling is a more general phenomenon in RPE cells. Interestingly, although macrophages displayed higher basal levels of IFN-inducible genes and a larger induction of expression of these transcripts following *Mtb* infection than RPE cells, the response kinetics to infection was significantly faster in RPE cells. Many IFN-inducible genes already reached maximum expression levels in RPE cells 1h after *Mtb* infection, while in macrophages the expression levels of most IFN-inducible genes were still comparable to baseline levels 1h after infection and were only found elevated 24h after infection (Supplementary Figure 3).

Characterization of the host response at the protein secretion level in RPE cells and macrophages infected with Mtb

Similar to the transcriptomic response, macrophages also secreted quantitatively and qualitatively more cytokines/chemokines/growth factors than RPE cells after *Mtb* infection (Figure 6). Both OZR1 and ARPE-19 cells displayed a significantly enhanced secretion of IL-6, IL-8, and MCP-1 upon *Mtb* infection while OZR1 also displayed enhanced secretion of IFNγ, VEGFA, IL-12, IP10, TNFα and RANTES. In both M1 and M2 macrophages MCP-1 secretion significantly decreased upon *Mtb* infection, while secretion of the other measured cytokines/chemokines/growth factors increased, except IL-5 and IL-13 (undetectable before and after infection). Interestingly, the protein secretion profiles of M1 and M2 were highly similar and both macrophage subsets strongly induced secretion of MIP-1α, MIP-1 β and RANTES to absolute levels that profoundly surpassed the levels observed in RPE cells (Supplementary Table 3). Together, these data suggest that while RPE cells focus their host response on eradicating *Mtb* through a response dominated by IFN signaling, macrophages upregulate in addition to IFN response-associated transcripts specifically the production of chemokines and cytokines involved in



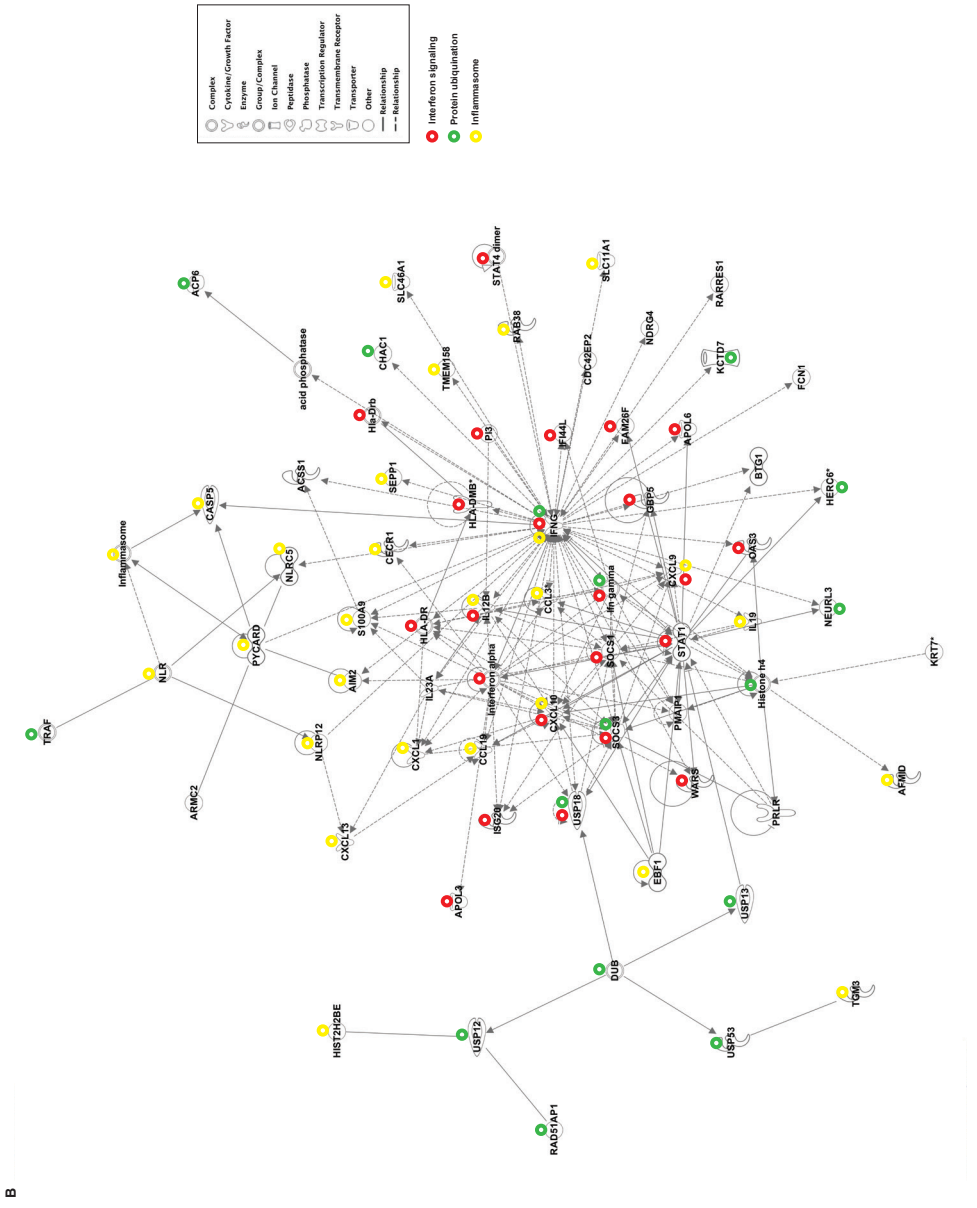


Figure 5. Network analysis of the host response in RPE cells and macrophages following *Mtb* infection. Ingenuity based pathway analysis of genes that were differentially expressed between (A) uninfected and *Mtb*-infected OZR1 cells and (B) uninfected and *Mtb*-infected M2 macrophages (t=24h). Shown are the top networks identified in both cell types. Type I and II IFN-inducible genes are highlighted in red, apoptosis and death receptor signaling genes are highlighted in blue, protein ubiquitination-associated genes are indicated in green, and inflammasome-associated genes are shown in yellow.

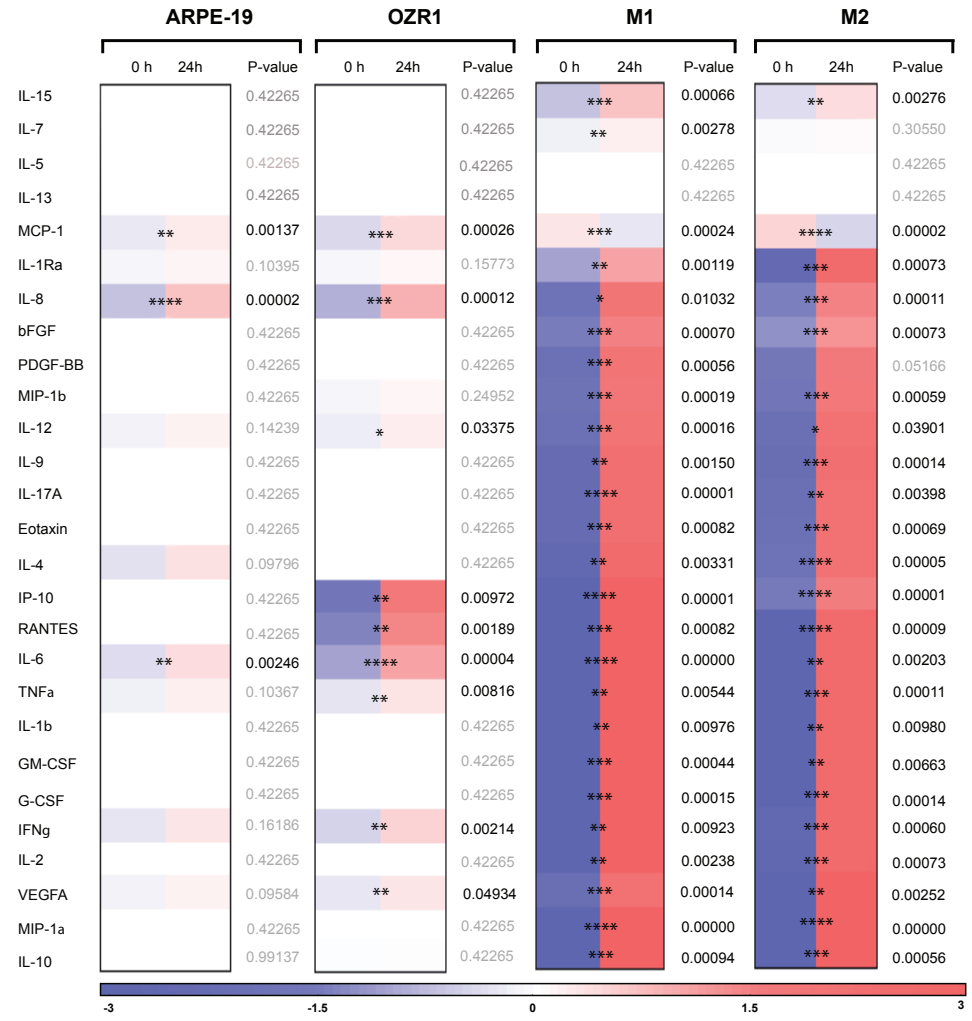


Figure 6. Characterization of the host response at the protein secretion level in RPE cells and macrophages infected with *Mtb*.
The heat map is representing changes in cytokine/chemokine/growth factor secretion before and after *Mtb* infection based on the average of triplicate samples. Primary RPE cells OZR1, RPE cell line ARPE-19, pro-inflammatory M1 and anti-inflammatory M2 macrophages were infected with *Mtb* for 1h. Culture supernatants were harvested at 24h and analysed. Red indicates increased protein expression levels compared to the geometric mean; Blue indicates decreased protein expression levels compared to the geometric mean. The colour intensity correlates with the magnitude of the calculated fold change. Statistical significance was tested using a Student's t-test. * = p<0.05, ** = p<0.01, *** = p<0.001, **** = p<0.0001.

the granulomatous response by recruiting other macrophages and immune cells to the site of infection.

DISCUSSION

We demonstrated that *Mtb* could infect human RPE cells, although with a lower efficiency than human primary macrophages, and that RPE cells, like macrophages, control the intracellular survival of *Mtb* bacilli. Furthermore, we uncovered that the regulatory host response to *Mtb* in both pro-inflammatory M1 and anti-inflammatory M2 macrophages involved a more diverse spectrum of genes and secreted proteins compared with RPE cells. Moreover, the extent of the transcriptomic and secreted cytokine response was superior in macrophages compared to RPE cells. On the other hand, RPE cells expressed particular apoptosis and death receptor genes which might reflect their immunoregulatory role in deviating cellular immune responses which is important for maintenance of immune privilege of the eye. Thus, our findings suggest that while macrophages appear to engage a plethora of responses, including IFN signaling to control *Mtb* infection and in communication between innate and adaptive immune cells to induce granuloma formation, RPE cells initiate a strong yet incomplete anti-*Mtb* response that primarily depends on IFN signaling to successfully control the early phases of *Mtb* infection in the retina.

The canonical IFN signaling pathway appeared to be the mutual pathway activated in both RPE cells and macrophages, along with IL-6 signaling and the pattern recognition receptor (PRR) pathway involved in bacterial and viral recognition (Supplementary Figure 1). However, IFN signaling was most strongly activated in *Mtb*-infected RPE cells, with type I IFN (IFNα/β) signaling being dominant over type II (IFNg) signaling (Figures 3 and 4). This is in line with observations in *Mtb*-infected human pulmonary alveolar epithelial cells.[17-19] Activation of genes associated with IFN signaling in RPE is, however, not specific for *Mtb* infection as this was also observed upon infection with West Nile Virus, vesicular stomatitis virus (VSV, also a single-stranded RNA virus) or upon stimulation with a synthetic analogue (poly I:C) for viral double-stranded RNA.[20-22] Therefore, the observed activation of IFN-inducible genes in *Mtb*-infected RPE cells most likely reflects a general immune response to (intracellular) infection rather than being *Mtb* specific.

IFNs exert both protective and detrimental effects on the host during intracellular bacterial infection. IFNγ is known to induce T-helper (Th)1 responses, and to activate and enhance the expression of anti-bactericidal molecules in phagocytes to kill intracellular bacteria.[21, 23] In RPE cells, both type I and type II IFN are known to inhibit cytomegalovirus (CMV) and Toxoplasma gondii replication, which involves indoleamine 2,3 dioxygenase (IDO) induced tryptophan depletion.[24, 25] IDO-1 was also one of the IFN induced genes we found in *Mtb* infected RPE. Possibly IDO induced tryptophan depletion inhibits *Mtb* replication in RPE, but this would require further study.[26] In contrast, excessive IFNα/β signaling has been linked to high *Mtb* disease activity in patients with clinical disease.[18, 19] Suppression of cytokines crucial for host defense against *Mtb*, including

IL-1α, IL-1β, IL-12 and TNFα, as well as repression of innate cell responsiveness to IFNg have been proposed as important mechanisms of IFNα/β-mediated immunosuppression during *Mtb* infection.[4, 20, 21, 27-31] Moreover, RPE-derived IFNβ can down-regulate CXCL9 and intercellular adhesion molecule 1 (ICAM-1) expression by RPE in an autocrine manner. This potentially limits retinal T-lymphocyte/ natular killer (NK) cell recruitment and has been proposed as an immuno-suppressive mechanism that protects the retina from excessive inflammation, for instance in case of RPE infection.[20] Our data suggest that in RPE cells, at least during the early stages of *Mtb* infection, IFN signaling may have a beneficial effect by inhibiting the outgrowth of intracellular *Mtb*. However, it could be speculated that the (prolonged) high induction of IFNα/β could potentially be detrimental at later stages of infection and thereby contributes to *Mtb*-mediated uveitis due to chronic infection or *Mtb*-latency in the RPE cells. Clearly, unraveling the exact role or balance of IFN signaling and Th1 induction in RPE in *Mtb*-mediated uveitis requires further investigation.

Molecules related to protein ubiquitination and inflammasome pathways were amongst the genes more specifically identified in the M2 regulatory network (Figure 5B). These findings are in line with other *in vitro* studies demonstrating that *Mtb*-infected macrophages exhibit innate immune functions (including inflammasome activation and ubiquitin-mediated autophagy) to kill intracellular mycobacteria but also to induce adaptive immunity.[3, 32] A canonical pathway more prominently regulated in RPE upon *Mtb* infection compared to M2 macrophages was related to cell death and survival (Figures 3 and 5A). One of the apoptosis-related genes that increased in *Mtb*-infected RPE (TNFSF10/TRAIL/APO2L; Supplementary Figure 2) encodes the pro-apoptotic membrane expressed molecule TNF-related apoptosis-inducing ligand (TRAIL). TRAIL is expressed by many ocular tissues, including RPE, and contributes to ocular immune privilege, most likely by inducing apoptosis of infiltrating inflammatory cells that express TRAIL-receptor 2 (TRAIL-R2/DR5/TNFRSF10B).[33] Type I IFNs are known to enhance TRAIL expression, for instance on dendritic cells, which enables these cells to induce T-lymphocyte apoptosis.[34] Induction of TRAIL on RPE may thus represent a way to deviate the cellular immune response in order to protect the retina from excessive inflammation and to limit the immunopathologic damage. On the other hand, human RPE cells express TRAIL-R2 (this study and[33]) and necrotic *Mtb*-positive RPE cells have been observed in human eyes,[8] suggesting that TRAIL-TRAIL-R2 interactions between RPE cells may induce death of RPE cells and possibly contribute to inflammation.

RPE cells are well-known producers of mediators that attract and activate different types of leukocytes and as such are considered important initiators/regulators of retinal inflammation.[35, 36] Yet *Mtb*-infected RPE secreted substantial lower amounts of cytokines than *Mtb*-infected macrophages did. This might reflect the local ocular function of RPE

where only short distance inter-cellular communication is required as opposed to that of macrophages that need to instruct other (immune) cells over longer distances. *Mtb* infection was associated with increased IL-12 production by RPE cells, although it was upregulated to a lesser extent than seen in macrophages. It is tempting to hypothesize that IL-12 derived from *Mtb*-infected RPE cells is involved in local Th1-lymphocyte activation and uveitis-like disease. Also, production of RANTES and IP-10 by *Mtb*-infected RPE cells may further amplify local T-lymphocyte recruitment and activation. This is further supported by observations in a primed mycobacterial uveitis model in rats that was associated with increased production of IP-10.[37] Besides local Th1-lymphocyte activation, it is possible that RPE function in maintaining immune privilege is compromised after a certain degree of infection. *Mtb*-infected RPE cells produced IL-6 that antagonizes TGF-β which has an important regulatory role in ocular immune privilege.[38] Further insight into the involved pathways may contribute to a better immunopathologic understanding of *Mtb*-associated uveitis, which may also be of benefit for finding new treatment modalities.

In conclusion, we demonstrated that *Mtb* can infect RPE cells and that RPE cells can control the intracellular survival of *Mtb* bacilli similar to macrophages. However, RPE cells phagocytosed *Mtb* less effectively than macrophages. RPE cells displayed a clear yet restricted anti-*Mtb* response that primarily used IFN signaling to control early phases of *Mtb* infection while macrophages engage a more diverse repertoire of responses including IFN signaling and production of cytokines/chemokines to facilitate communication between innate and adaptive immune cells to induce granuloma formation. RPE cells more strongly activated death receptor and retinoic acid-mediated apoptosis signaling pathways which are postulated to represent an immuno-suppressive mechanism to protect the retina from excessive inflammation and damage.

CONFLICT OF INTEREST

The authors declare that they have no conflicting and competing financial interests.

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AUTHOR CONTRIBUTIONS

P.M.v.H. W.A.D., M.v.V., T.H.M.O., and M.C.H. conceptualized and designed the study. R.L.D.N., W.A.D., S.M.A.S., P.J.vd.S., and M.C.H. analyzed the transcriptomic and secreted protein data and wrote the manuscript. K.V.W. designed and performed the *Mtb* infection experiments and analyzed the data. E.Q. and R.L.D.N. executed the dcRT-MLPA experiments. M.C.H. had primary responsibility for the final content of the manuscript; and all authors have read and approved the final manuscript.

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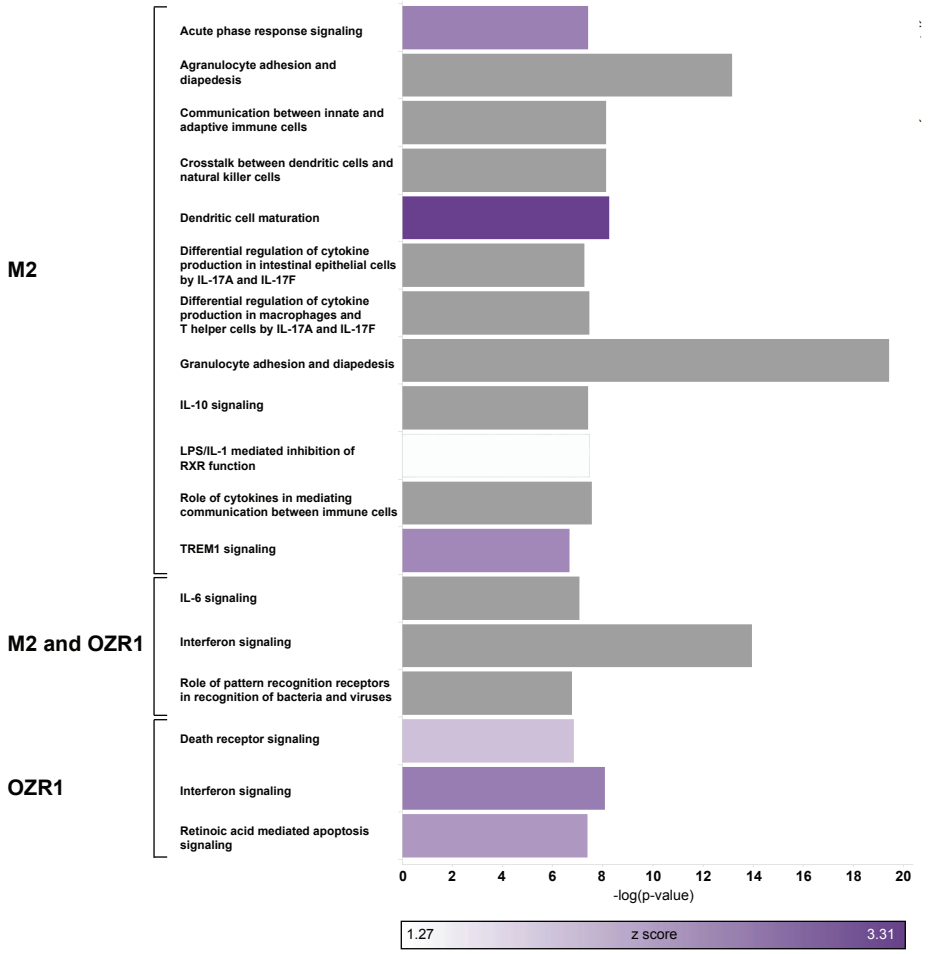
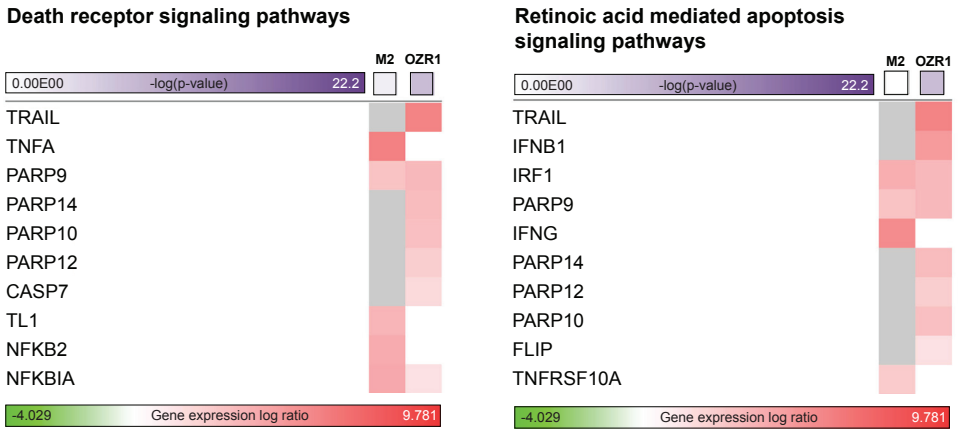
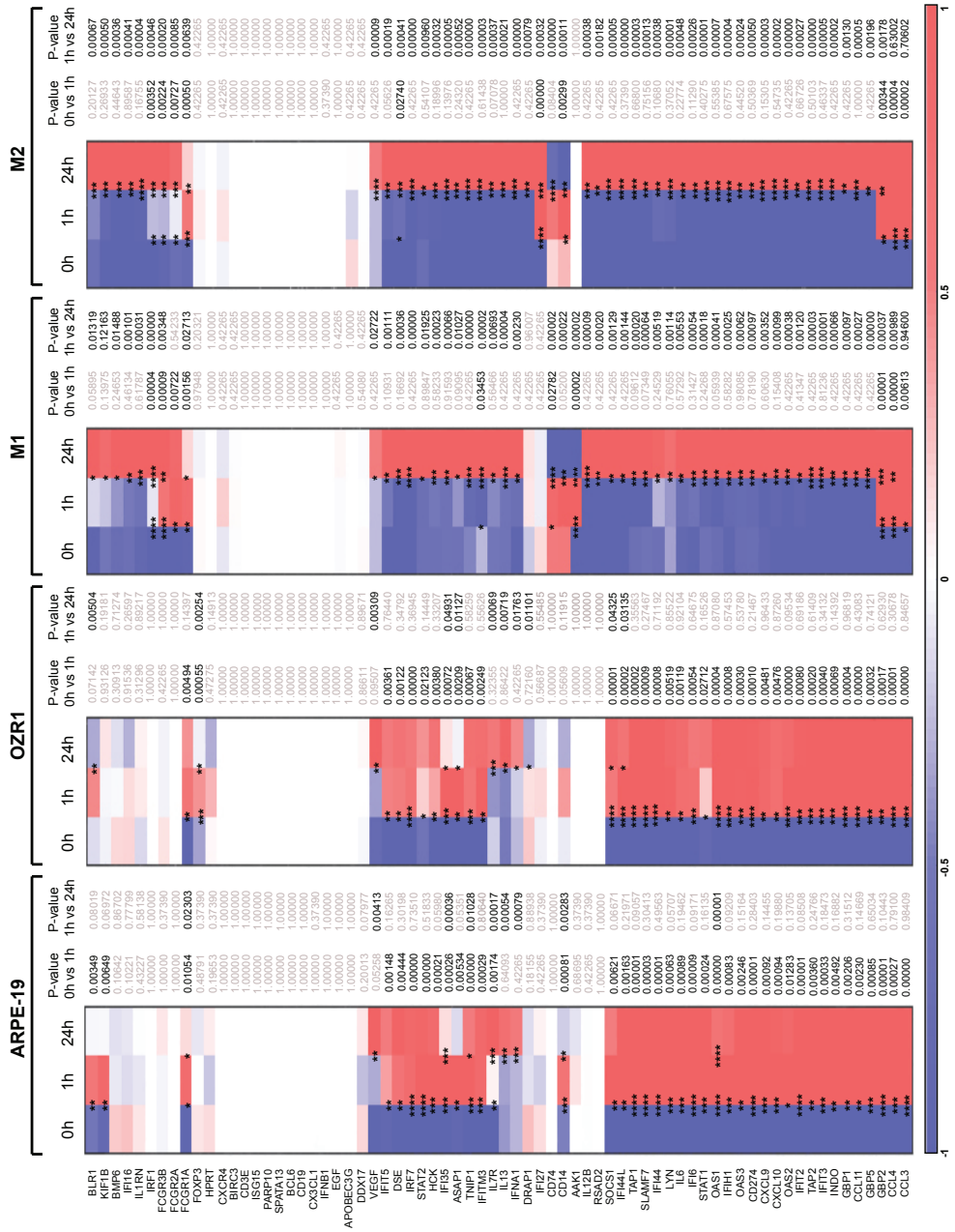
SUPPLEMENTAL DATA



Supplementary Figure 1. Molecular signatures of Mtb infection in OZR1 and M2 cells identified by Ingenuity Pathway Analysis differ in intensity and quality.
Bar charts represent canonical pathways that were common (middle panel) or exclusively regulated by either M2 (top panel) or OZR1 cells (lower panel) upon infection with *Mtb*. The color coding of the bars corresponds to the z-score of each canonical pathway.



Supplementary Figure 2. Heatmaps of genes belonging to the death receptor and retinoic acid-mediated apoptosis signaling pathways that were differentially expressed upon *Mtb* infection.
Shown are the –log(p-values) of the death receptor and retinoic acid-mediated apoptosis signaling pathways in M2 and OZR1 cells, indicating both pathways were regulated more profoundly in OZR1 than in M2 following infection with *Mtb*. The log ratio of the top 10 differentially expressed genes within each pathway is also indicated. Green defines lower gene expression levels, and red defines higher gene expression levels compared to the uninfected control.



Supplementary Figure 3. Validation of the host response against infection with *Mtb* in RPE cells and macrophage subsets using dcRT-MLPA.
Relative expression levels are depicted of selected genes in uninfected ARPE-19, OZR1, M1 and M2 cells or cells infected with *Mtb* for 1h (end of the infection period) or evaluated 24h after infection. Red indicates increased gene expression levels compared to the geometric mean; Blue indicates decreased gene expression levels compared to the geometric mean. The colour intensity with the magnitude of the calculated fold change. Statistical significance between uninfected and 1h infected cells, and between 1 h and 24h infected cells was tested using a Student's t-test. * = p<0.05, ** = p<0.01, *** = p<0.001, **** = p<0.0001.

Supplementary Table 1. List of differentially expressed genes in OZR1 and M2 cells following infection with Mtb.

Supplementary Table 2. List of all canonical pathways differentially regulated in OZR1 and M2 cells upon Mtb infection.

Supplementary Table 3. Expression levels of secreted proteins in the supernatant of OZR1 and M2 cells following infection with Mtb.

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Chapter 4.2

Type 1 interferon-inducible gene expression in QuantiFERON Gold TB-positive uveitis: a tool to stratify a high versus low risk of active tuberculosis?

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Submitted

ABSTRACT

QuantiFERON-Gold TB (QFT)-positive patients with undetermined uveitis are problematic in terms of whether to diagnose and treat them for tuberculosis (TB). Here, we investigated whether peripheral blood expression of type 1 interferon (IFN)-inducible genes may be of use to stratify QFT-positive patients with uveitis into groups of high versus low risk of having active TB-associated uveitis. We recruited all new uveitis patients in Cipto Mangunkusumo Hospital, Jakarta, Indonesia for one year. We included 12 patients with uveitis and clinically diagnosed active pulmonary TB, 58 QFT-positive patients with uveitis of unknown cause, 10 sputum positive active pulmonary TB patients without uveitis and 23 QFT-negative healthy controls. Expression of 35 type 1 IFN-inducible genes was measured in peripheral blood cells from active pulmonary TB patients without uveitis and healthy controls. Differentially expressed genes were identified and used for further clustering analyses of the uveitis groups. A type-1 IFN gene signature score was calculated and the optimal cut-off value for this score to differentiate between active pulmonary from healthy controls was determined and applied to QFT-positive patients with uveitis of unknown cause. Ten type 1 IFN-inducible genes were differentially expressed between active pulmonary TB and healthy controls. Expression of these 10 genes in QFT-positive patients with uveitis of unknown cause revealed three groups: 1). patients resembling active pulmonary TB, 2). patients resembling healthy controls, and 3). patients displaying an in-between gene expression pattern. A type 1 IFN gene signature score ≥5.61 displayed high sensitivity (100%) and specificity (91%) for identification of active TB. Application of this score to QFT-positive patients with uveitis of unknown yielded two groups with expected different likelihood (high vs. low) of having active-TB uveitis, and therefore may be useful to stratify into groups of high or low likelihood of being active TB uveitis.

INTRODUCTION

Tuberculosis (TB) is one of the major health problems worldwide. TB associated uveitis represents a major cause of infectious uveitis in Indonesia and in other countries endemic for TB.[1-5] The diagnosis of TB uveitis is mainly based on microbiological proof of active TB infection in the eye or a positive culture in other organs, most frequently the lungs. However, the diagnosis of TB uveitis is challenging in the absence of clinically apparent pulmonary disease because ocular tissue examinations are not readily available and are troublesome to perform.[6]

The advent of the IFN-γ release assay (IGRA) or QuantiFERON Gold TB (QFT) test enabled the identification of individuals with a prior *Mycobacterium tuberculosis* (*Mtb)* infection. However, although the QFT test provides evidence of an immune response to *Mtb,* it lacks the specificity to distinguish between active and latent TB.[7] Indeed, whether QFT-positive uveitis is the result of a direct infection of the retina, an anti-retinal immune response in QFT-positive individuals or a combination of both is unclear.[8] Therefore, using QFT as a diagnostic test for active TB is not feasible in cases suspect of active TB uveitis.

Histopathological studies in patients with uveitis, but without pathological lung findings, documented a *Mtb* infection of the retinal pigment epithelium (RPE). This finding shows that ocular infection may be present even in patients without signs of active systemic TB.[9, 10] Anti-tuberculous therapy (ATT) treatment can be very successful in QFT-positive uveitis of unknown cause patients, but the results are not conclusive.[11, 12] Thus, whether a patient has uveitis due to active TB infection, retinal autoimmunity or a combination of both is unclear. Discrimination of active TB infection from latent TB infection-associated uveitis is thus an important goal for adjusting therapy and optimizing visual outcomes in patients with QFT-positive uveitis.

Recent reports have indicated that active TB is associated with a specific activation pattern of the type 1 IFN signaling cascade.[13-16] Active pulmonary TB can be discriminated from latent TB by the expression of type 1 IFN-inducible genes in immune cells.[13, 15] In this study, we explore whether the expression of type 1 IFN-inducible genes in peripheral blood cells from patients with QFT-positive uveitis can serve as a tool to stratify these patients into groups with a high or low likelihood of having active TB uveitis.

PATIENTS AND METHODS

Patients

Patients with uveitis were selected from a prospective uveitis cohort study (June 2014 to May 2015) in which we consecutively recruited 247 patients (after receiving informed consent) with new uveitis referred to the Infection and Immunology Division of the Ophthalmology Department of the Medical Faculty Universitas Indonesia/Cipto Mangunkusumo Hospital, Jakarta, Indonesia. The whole clinical study is recently in press.[1] For the present study, we excluded 177 of these 247 uveitis patients for the following reasons: 72 had an incomplete screening workup, 29 were human immunodeficiency virus (HIV) positive, 53 were QuantiFERON-Gold TB (QFT) negative, 2 had an indeterminate QFT value without evidence of clinically active TB, and 21 QFT-positive patients had another established cause of uveitis. In total, we included 12 cases with uveitis with clinically diagnosed active pulmonary TB (of whom two were *Mtb* sputum-positive and 10 were *Mtb* sputum-negative) and 58 patients with uveitis who were QFT positive without any signs of active TB and in whom no alternative cause for uveitis could be established.

As a positive control group, we additionally included 10 newly diagnosed *Mtb* sputum-positive active pulmonary TB patients (HIV negative) without uveitis or a history of ATT from the outpatient clinic of Persahabatan Hospital, East Jakarta, Indonesia. We also included 23 Indonesian healthy controls who were QFT negative, had no history of uveitis and did not use any medication at the time of the study.

All included patients underwent a full ophthalmic examination. The uveitis classification and grading were performed according to standardized uveitis nomenclature (SUN).[17] The diagnosis and workup were performed as described previously.[1] QFT was performed for all included uveitis patients with the QuantiFERON-Tb Gold (QFT; Cellestis Inc., Carnegie, Australia) test using a positive cut-off value of >0.35 U/mL.[1] The tuberculin skin test (TST) (RT23 SSI-2 T.U/0.1 mL, Statens Serum Institute, Copenhagen, Denmark) was performed in 59/70 uveitis patients due to a temporary unavailability of tuberculin. The TST was considered positive in patients with an induration larger than 10 mm in diameter.[18] The QFT was always performed before the TST, and the TST was not performed in the healthy controls or the active pulmonary TB patients without uveitis.

Diagnosis of pulmonary TB

Pulmonary TB was diagnosed according to the Tuberculosis Guidelines from the Indonesian Society of Respirology (the algorithm is depicted in S1 Fig).[19, 20] Briefly, the diagnosis of active pulmonary TB was based on a clinical examination with the support of

microbiological and radiological findings. The review of the chest X-rays and computed tomography (CT) scans was performed by two independent pulmonologists specialized in TB (one from Indonesia (GS) and one from the Netherlands (MB)). The results were classified as being compatible with 1) active TB, 2) prior TB or 3) abnormalities other than TB. The study was approved by the Faculty of Medicine University of Indonesia (FMUI) medical ethics committee, and written informed consent was obtained.

Whole blood collection

Blood was collected in PAXgene tubes (PreAnalytix, Hombrechtikon, Switzerland) according to the manufacturer's protocol. The collection was performed at the patient's first visit to our clinic along with a routine blood draw for the standard uveitis work-up. The collected blood was stored at -80 °C for further processing.

Real-time quantitative PCR

Total RNA was extracted from the whole blood samples using the Blood RNA Extraction Kit (PreAnalytix) and subsequently reverse-transcribed into complementary DNA (cDNA) according to the manufacturer's instructions. The expression levels of 35 type 1 IFN-inducible genes selected based on previous reports[13-15, 21, 22] (*CCL2*, *CCL7*, *CX3CR1*, *CXCL10*, *DDX58*, *FCGR1B*, *GBP1*, *GBP4*, *IFI16*, *IFI27*, *IFI44*, *IFI44L*, *IFIH1*, *IFIT1*, *IFIT2*, *IFIT3*, *IFITM1*, *IFNA1*, *IFNB1*, *IL15RA*, *IL1B*, *IRF7*, *ISG15*, *LY6E*, *MyD88*, *MxA*, *OAS1*, *OAS2*, *RSAD2*, *SERPING1*, *STAT1*, *TBK1*, *TLR8*, *UBE2L6*, and *USP18*) were determined. To calculate the relative expression levels, all the samples were normalized to the expression of the housekeeping gene *ABL* (ΔC_t).[23]

Statistical analysis

Descriptive statistics (mean and standard deviation (SD) or median and interquartile range (IQR)) were used to summarize demographic factors, age, gender, and the TB-related test results (QFT, sputum smear, and TST). Then, the groups were compared using the non-parametric Kruskal-Wallis test or chi-square test. Post hoc analysis was performed with the Bonferroni test.

To identify which of the 35 type 1 IFN-inducible genes differed significantly between the patients with active pulmonary TB without uveitis and the healthy controls, the Mann-Whitney U test was performed. Due to significant age and gender differences between the groups, the analysis was adjusted for age and gender using binary logistic regression.

All statistical analyses were performed in SPSS, and a *P* value <0.05 indicated statistical significance. Unsupervised hierarchical clustering analysis of the identified genes was performed using OmniViz version 6.1.1.13.0 (Instem scientific, Inc.)

Type 1 IFN signature score

Individual type 1 IFN-inducible genes were assigned a score ($\Delta\Delta C_t$ value) by deducting the ΔC_t value of each gene in each subject from the average ΔC_t value of that particular gene within the healthy control group and dividing by the standard deviation (SD) of the ΔC_t value of that particular gene in the healthy control group. Subsequently, a type 1 IFN signature score was assigned to the total gene set by calculating the sum of the individual gene scores ($\Delta\Delta C_t$ values).[22-24]

Receiver operating characteristic (ROC) curve analysis was used to identify the optimal cut-off value of the type 1 IFN signature score associated with active TB. The maximum Youden index (sensitivity + specificity − 1) was used to obtain the optimal cut-off point to discriminate between the active pulmonary TB patients and the healthy controls.

RESULTS

Patient groups

The characteristics of the patients and controls are summarized in Table 1. A significant difference was found in age and gender between the study groups. The post hoc analysis revealed that the healthy controls were significantly younger than the QFT-positive group with uveitis of unknown origin (p<0.0001). The post hoc analysis of the gender distributions did not reveal any significant differences between the groups.

Among the 12 uveitis patients clinically diagnosed with active pulmonary TB, only 2 had positive *Mtb* staining and PCR (GeneXpert) results in their sputum samples. QFT testing was positive in 10/12 (83%) of these patients. Of the 10 patients with active pulmonary TB but without uveitis, 7/10 (70%) were QFT positive.

Type 1 IFN-inducible gene expression

Among the 35 type 1 IFN-inducible genes, 18 genes differed significantly in their expression levels between the patients with active pulmonary TB without uveitis and the healthy control group (S2 Fig.). After adjustment for age and gender, the expression of 10 genes remained significantly different between these two groups. These 10 genes (*UBE2L6* (p=0.004), *FCGR1B* (p=0.012), *GBP1* (p=0.013), *IL1B* (p=0.015), *MYD88* (p=0.018), *TLR8* (p=0.02), *IRF7* (p=0.028), *STAT1* (p=0.032), *SERPING1* (p=0.036), and *IFIT2* (p=0.037)) were included in the subsequent analyses.

TABLE 1 General characteristic of the patients

	Total (N=103)	Active Pulmonary TB Without uveitis (N=10)	Uveitis with clinically diagnosed active pulmonary TB (N=12)	QFT (+) uveitis of unknown cause (N=58)	Healthy controls (N=23)	P value
Gender						
Male	38/103 (37%)	7/10 (70%)	8/12 (67%)	16/58 (28%)	7/23 (30%)	0.008
Female	65/104 (63%)	3/10 (30%)	4/12 (33%)	42/58 (72%)	16/23 (70%)	
Age (mean ± SD)	40 ± 15	41 ±16	42 ± 17	46 ± 13	31 ± 9	0.000
Microbiology evidence of Mtb positive	12/104 (11.5%)	10/10 (100%)	2/12 (17%)	0/58 (0%)	0/23 (0%)	0.058
QFT-G value * (median;IQR)	2.8; 0.02 – 3.7	2.2; 0.2 – 5.5	1.4; 1 – 4.2	5.0; 2.1 – 10.3	0.01; 0.00 – 0.07	0.000
QFT-G positive*	75/104 (72%)	7/10 (70%)	10/12 (83%)	58/58 (100%)	0/23 (0%)	0.000
QFT-G negative*	28/104 (27%)	3/10 (30%)	1/12 (8%)	0/58 (0%)	23/23 (100%)	
TST > 10 mm**	49/59 (83%)	NA	10/10 (100%)	39/49 (84%)	NA	0.293

*QFT-G indeterminate in 1/103 (1%) in suspected tuberculous uveitis with sputum AFB positive
SD: Standard Deviation, IQR: Interquartile Range, QFT: QuantiFERON Gold test, TB: Tuberculosis, *Mtb*: Mycobacterium tuberculosis
** The TST was always performed after the IGRA test, and the TST was not performed in the healthy controls or the active pulmonary TB patients without uveitis.

Cluster analysis of the patient groups and the healthy controls

Unsupervised hierarchical clustering of the 10 active pulmonary TB patients without uveitis and 23 healthy controls based on the 10 genes yielded two main clusters. Cluster 1 contained the majority of the healthy controls (19/23; 83%); in contrast, cluster 2 contained the majority of the active pulmonary TB patients without uveitis (9/10; 90%) (S3 Fig.). Subsequently, the two *Mtb* sputum-positive uveitis patients with clinically active pulmonary TB were included in this analysis, and both patients were grouped in the cluster that contained 9/10 of the active pulmonary TB patients without uveitis (cluster 2, Fig 1).

Due to the ability of this 10-gene expression pattern to distinguish the majority of the *Mtb* sputum stain-positive active pulmonary TB patients (both with and without uveitis) from the healthy controls, the same analysis was performed after including the 10 uveitis cases with clinically diagnosed active pulmonary TB who were *Mtb* sputum stain negative. This analysis clustered 7/10 cases (70%) with the majority of the healthy controls (cluster 1), whereas 3 cases (30%) were clustered with the active pulmonary TB cases without uveitis (cluster 2; Fig 2).

Next, the analysis was performed for the 58 QFT-positive patients with uveitis of unknown cause. This analysis revealed that 34/58 patients (59%) were clustered in the vicinity of the majority of the active pulmonary TB patients without uveitis (clusters 2 and 1B, Fig 3), whereas 23/58 (37%) were clustered close to the majority of the healthy controls (cluster 1A, Fig 3). Additionally, 1/58 patients (4%) was grouped together with 7 healthy controls and 1 active TB without uveitis case (cluster 1C, Fig 3). However, the gene expression pattern in cluster 1B was more similar to that of cluster 2, which contained the majority of the active pulmonary TB cases.

Type 1 IFN gene signature score

Based on the 10-gene expression patterns, a type 1 IFN signature score was calculated for each individual included in the study groups. This calculation revealed a significant difference between the active pulmonary TB patients without uveitis and the healthy controls ($p<0.0001$; Fig 4). The type 1 IFN signature score of QFT-positive patients with uveitis of unknown cause had significantly lower scores than the active pulmonary TB patients without uveitis (*P* value 0.002) but significantly higher scores than the healthy controls (*P* value 0.01; Fig 4). In the patients with uveitis and clinically diagnosed active pulmonary TB, the two sputum-smear-positive TB uveitis patients and three other cases revealed type 1 IFN signature scores that were comparable to those seen in the active pulmonary TB without uveitis group. Furthermore, in the QFT-positive patients with uveitis of unknown cause, the type 1 IFN signature scores varied, with a range from scores comparable to those seen in the active pulmonary TB (without uveitis) group to

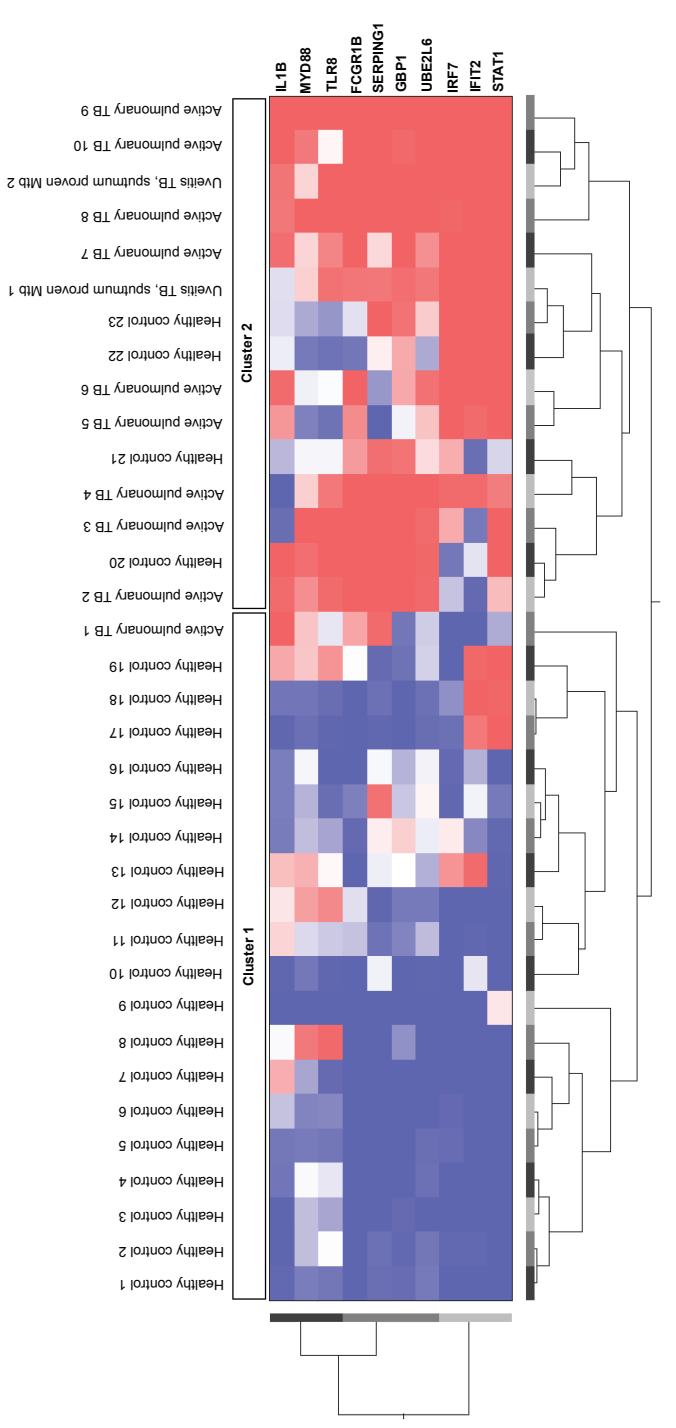

Figure 1. Cluster analysis of patients with uveitis with clinically active, *Mtb* sputum positive, pulmonary TB and patients with active pulmonary TB without uveitis.
Unsupervised hierarchical clustering analysis based on 10 type 1 IFN-inducible genes (indicated on the right). Active pulmonary TB subjects indicate the active pulmonary TB patients without uveitis. Uveitis TB subjects indicate uveitis patients with clinically diagnosed active pulmonary TB. Cluster 1 contains the majority of the healthy controls (19/23; 83%) and one active pulmonary TB case without uveitis (1/10; 10%). Cluster 2 contains the majority of the active pulmonary TB cases (9/10; 90%), two uveitis with clinically diagnosed active pulmonary TB with sputum-positive *Mtb* (2/2; 100%) and 4 healthy controls (4/23; 17%). Red indicates increased gene expression levels compared to the geometric mean, and blue indicates decreased gene expression levels compared to the geometric mean. Color intensity correlates with the magnitude of the calculated fold change.

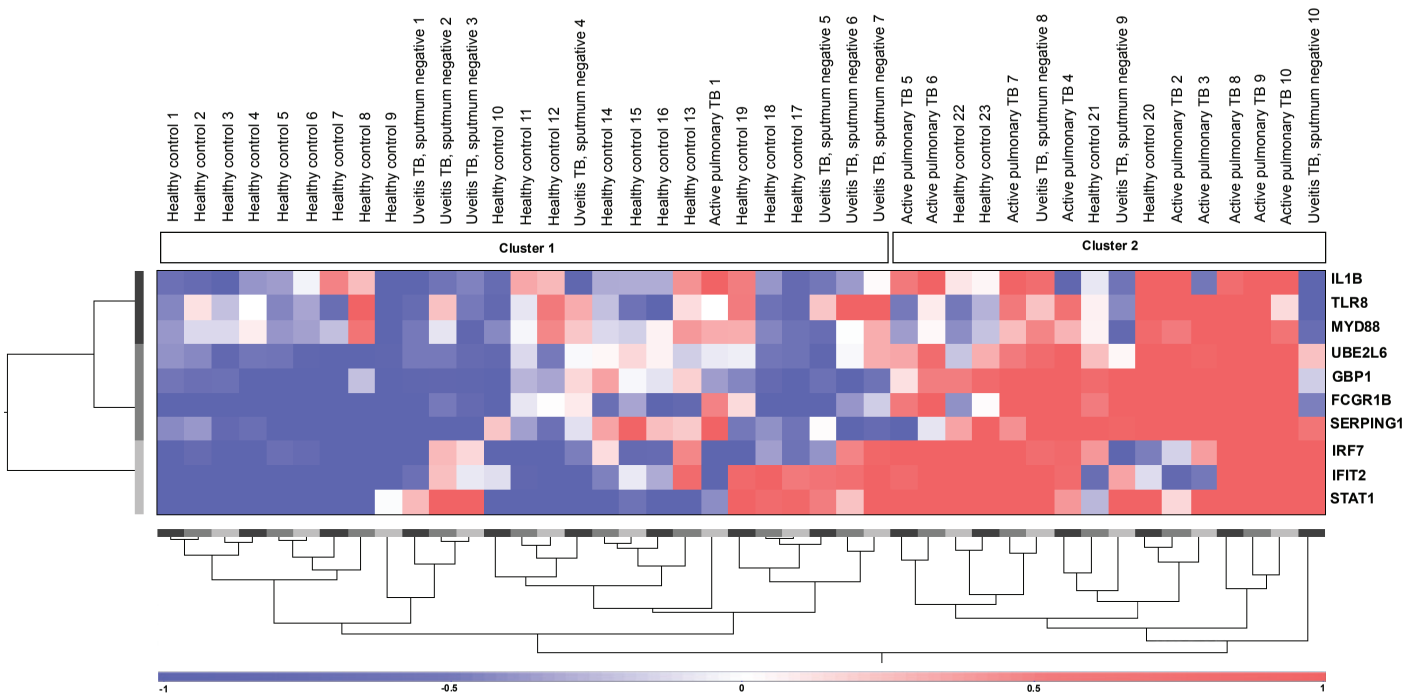

Figure 2. Cluster analysis of patients with uveitis who were sputum *Mtb* negative but were diagnosed with active TB infection on clinical grounds, patients with active pulmonary TB without uveitis and healthy controls.
Unsupervised hierarchical clustering analysis based on 10 type 1 IFN-inducible genes (indicated on the right). Active pulmonary TB subjects indicate the active pulmonary TB patients without uveitis. Uveitis TB subjects indicate uveitis patients with clinically diagnosed active pulmonary TB. Cluster 1 contains the majority of the healthy controls (19/23; 83%) and one active pulmonary TB case without uveitis (1/10; 10%). Cluster 2 contains the majority of the active pulmonary TB cases (9/10; 90%) and 4 healthy controls (4/23; 17%). Ten uveitis patients had clinically diagnosed active pulmonary TB infections but negative *Mtb* sputum tests. Only 3 patients (3/10; 30%) were grouped in cluster 2, which primarily contained clustered active pulmonary TB patients. Red indicates increased gene expression levels compared to the geometric mean, and blue indicates decreased gene expression levels compared to the geometric mean. Color intensity correlates with the magnitude of the calculated fold change.

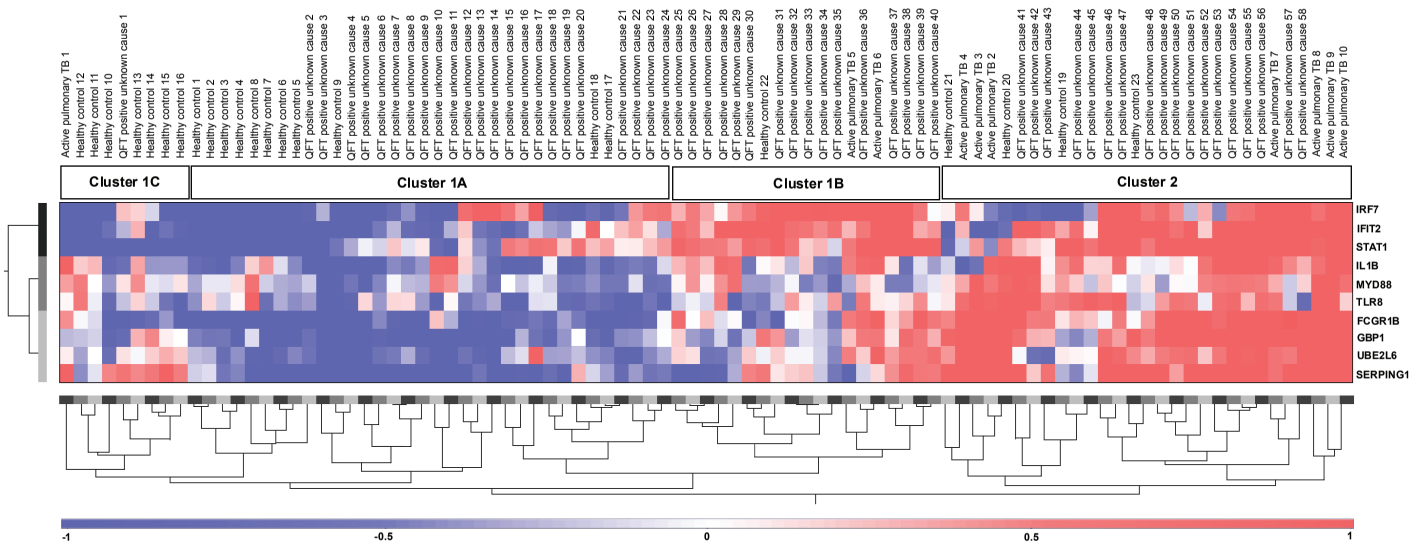

Figure 3. Cluster analysis of QFT-positive patients with uveitis of unknown cause, patients with active pulmonary TB without uveitis and healthy controls.
Unsupervised hierarchical clustering analysis based on 10 type 1 IFN-inducible genes (indicated on the right). Active pulmonary TB subjects indicate the active pulmonary TB patients without uveitis. Uveitis TB subjects indicate uveitis patients with clinically diagnosed active pulmonary TB Cluster 1 (A+B+C) contains the majority of the healthy controls (19/23; 83%), cluster 1A contains 11 healthy controls (11/23; 48%) and 23 patients with uveitis of unknown cause were QFT positive (23/58; 40%), cluster 1B contains 1 healthy control (1/23; 4%), 2 patients with active pulmonary TB without uveitis (2/20; 20%) and 16 patients with uveitis of unknown cause were QFT positive (16/58; 28%) and cluster 1C contains 7 healthy controls (7/23; 30%), 1 patient with active pulmonary TB without uveitis (1/10; 10%) and 1patient with uveitis of unknown cause were QFT positive (1/58; 2%). Cluster 2 contains the majority of the active pulmonary TB cases without uveitis (9/10; 90%), 4 healthy controls (4/23; 17%) and 18 patients with uveitis of unknown cause were QFT positive (18/58; 31%). Red indicates increased gene expression levels when compared to the geometric mean, and blue indicates decreased gene expression levels when compared to the geometric mean. Color intensity correlates with the magnitude of the calculated fold change.

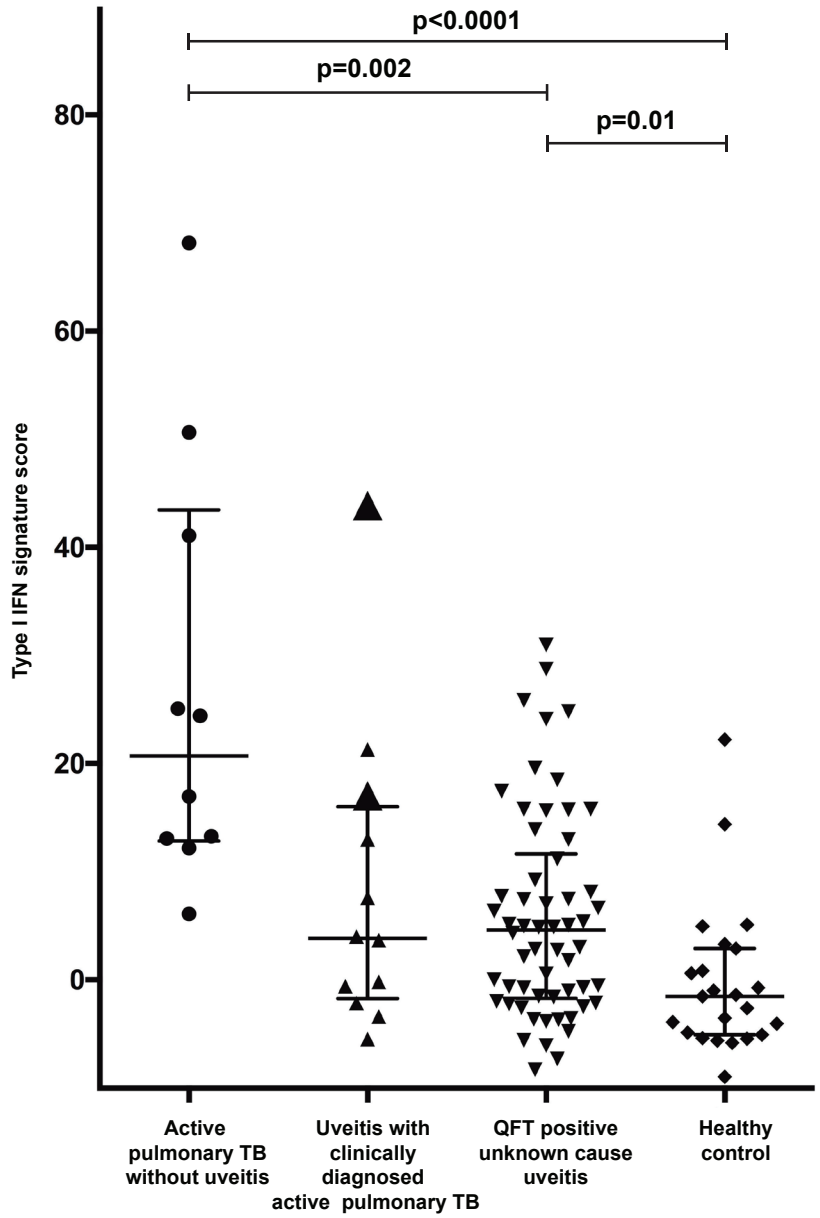

Figure 4. Scatter plot of type 1 IFN signature scores for the four study groups.
A type 1 IFN gene signature score was calculated for every individual patient sample based on the expression of the 10-gene set. Each point represents one individual. Horizontal bars indicate the median value within a group. Enlarged triangles within the TB uveitis group represent individuals within that group with a sputum stain positive for *Mtb*.
Statistical significance was assessed using the Kruskal-Wallis test, and subgroup analyses were performed with Dunn's multiple comparison test.

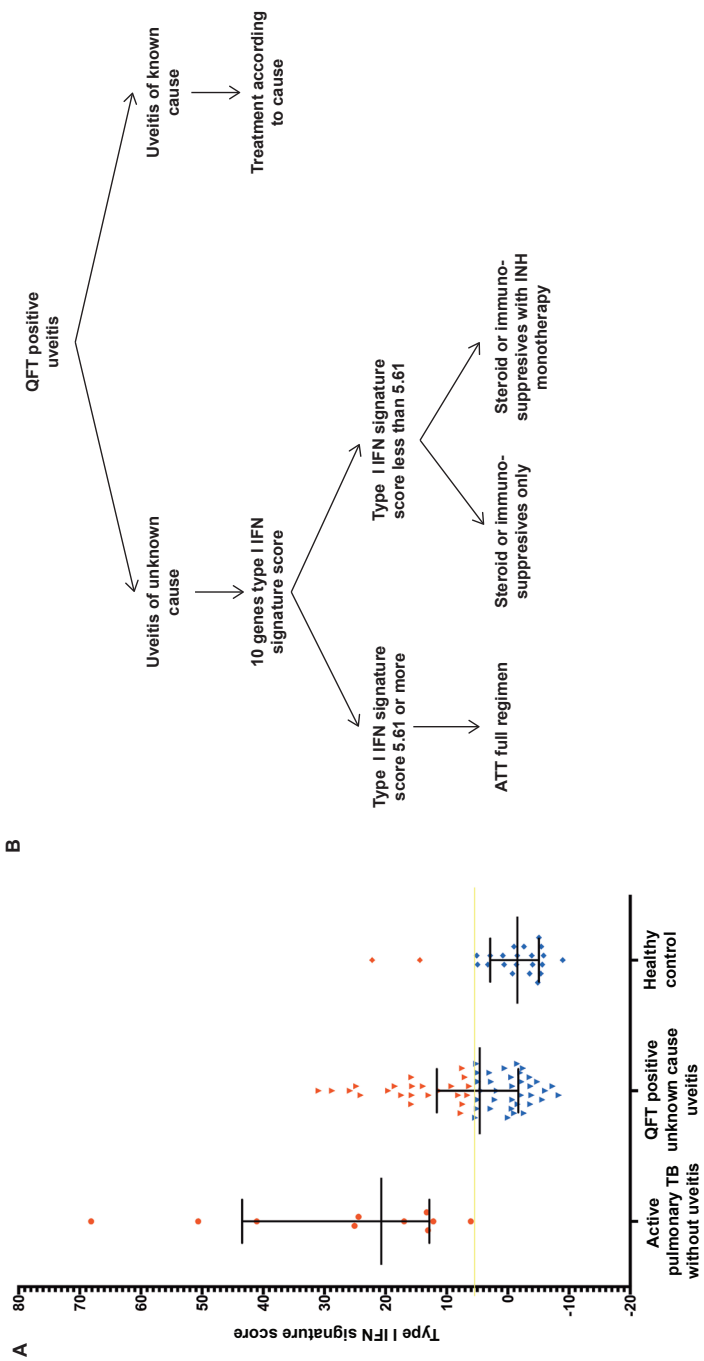

Figure 5. Proposed algorithm for the diagnostic work-up of QFT-positive uveitis.
A. Scatter plot of type 1 IFN signature scores for QFT-positive patients with uveitis of unknown cause, patients with active pulmonary TB without uveitis and healthy controls.
Each point represents one individual. Horizontal bars indicate the median value within a group. Yellow horizontal bar marks the cut off value of 5.61 for a positive versus a negative type 1 IFN gene signature score. Therefore, symbols indicated in red represent type1 IFN gene signature score positive individuals and blue symbols indicate type1 IFN gene signature score negative individuals.
B. Algorithm for the management of QFT-positive uveitis patients.
QFT-positive patients with uveitis of known causes should be treated appropriately for that cause. In QFT-positive patients with uveitis of unknown causes, the 10-gene type 1 IFN score should be calculated. When the score is 5.61 or more, the patient should receive a full ATT regimen and the necessary anti-inflammatory treatment regimen. When the score is less than 5.61, the patient can defer ATT and can undergo anti-inflammatory treatment with or without an Isoniazid (INH) prophylaxis regimen with strict observation.

scores overlapping those seen in the healthy control group (Fig 4). Further, we determined a type 1 IFN signature score cut-off value using ROC curve analysis that yielded an area under the curve (AUC) of 0.961 with a *P* value <0.00001 (S4 A Fig.); a cut-off value ≥5.61 displayed the highest sensitivity (100%), specificity (91%) and Youden index (0.913) for the identification of active TB (S4 B Fig. and Fig 5 A).

DISCUSSION

In this study, we demonstrate that a blood transcriptional signature of 10 type 1 IFN-inducible genes differentiates QFT-positive uveitis patients into distinct different groups.

Type 1 IFN (IFNα/β)-inducible gene expression signatures in peripheral blood cells have consistently been reported as a potential biomarker for active pulmonary TB compared with signatures in healthy controls or patients with other diseases.[13] Berry *et al.* first reported the association between active pulmonary TB and the expression of numerous type 1 IFN-inducible genes in peripheral blood cells in regions with both an intermediate TB burden (London, UK) and a high TB burden (South Africa).[13] Importantly, these type 1 IFN-inducible gene transcripts differed from those observed in systemic autoimmune diseases, including systemic lupus erythematosus (SLE), which is among a group of systemic autoimmune diseases that contains subgroups of patients displaying a type 1 IFN-inducible gene expression pattern.[13, 21, 25, 26] Several other studies have corroborated the use of type 1 IFN-inducible gene expression in the peripheral blood to distinguish active from latent TB infection.[14-16, 27, 28] Moreover, a rapid normalization of the type 1 IFN gene expression pattern upon successful ATT was reported.[13, 15] Together, these observations underscore the role of type 1 IFN signaling in the pathogenesis of TB and the potential usefulness of the peripheral blood type 1 IFN transcriptome as a biomarker and monitoring tool in TB diagnostics and management. However, no studies of type 1 IFN-inducible blood transcriptome have been conducted in uveitis cases in relation to TB infection.

In our study, we identified a peripheral blood cell transcriptome consisting of 10 type 1 IFN-inducible genes that were strongly associated with active pulmonary TB without uveitis. When a type 1 IFN signature score was applied to this 10-gene set, a score ≥5.61 displayed the optimal sensitivity and specificity for distinguishing active pulmonary (*Mtb* sputum smear-positive) TB patients without uveitis from healthy controls. In line with this result, the two TB uveitis cases diagnosed with active pulmonary TB and having positive *Mtb* sputum smear displayed a type 1 IFN signature score >5.61. This finding indicates that microbiologically proven active pulmonary TB with or without uveitis is associated with high expression of type 1 IFN-inducible genes. However,

several additional uveitis cases, who were QFT-positive, displayed a positive type 1 IFN signature despite being *Mtb* sputum negative.

Over-diagnosis of uveitis TB due to the lack of a gold standard diagnostic test (i.e., laboratory and radiological investigations) is a recognized problem that may result in overzealous treatment of uveitis with TB drugs.[29] Within the uveitis group diagnosed with pulmonary TB on clinical grounds solely, we found 7 cases with a type 1 IFN signature score <5.61. In these cases, the uveitis was probably not related to active TB. Importantly, the type 1 IFN signature scores revealed two subgroups within the QFT-positive patients with uveitis of unknown cause. We propose that QFT-positive patients with uveitis of unknown cause and a type 1 IFN signature score <5.61 are unlikely to have uveitis related to active TB infection. This conclusion is supported by the high concordance within the set of 10 type 1 IFN-inducible genes identified in our study and the type-1 IFN inducible genes identified in other studies that did compare active with latent TB. Furthermore, this finding is in agreement with other studies that compared patients with active and latent TB and reported a rapid decline in peripheral blood cell expression of type 1 IFN-inducible genes in active TB patients treated with a full ATT regimen.[13, 15] Consequently, we expect that patients within the QFT-positive group with uveitis of unknown cause who display a positive type 1 IFN signature score (≥5.61) will have a higher likelihood of having uveitis due to active TB infection. Our series, however, lacks the results of type 1 IFN signature in QFT-positive patients without uveitis. Nevertheless, we propose that measurement of a peripheral blood type 1 IFN gene signature score can aid to the diagnostic workup and choice of treatment in patients with QFT positive uveitis, as is indicated in Fig 5 B.

Elevated expression of type 1 IFN-inducible genes in peripheral blood cells is not specific to active TB disease. Type 1 IFN gene signatures are also associated with systemic autoimmune diseases, such as in Sjögren's syndrome cases (~55%), systemic lupus erythematosus (~50%), and systemic sclerosis (~30%), and are also present in healthy control subjects (~5%).[21, 25, 26] However, differential expression of type 1 IFN-inducible genes between TB and systemic autoimmune diseases is clear. *MxA* is a type 1 IFN-inducible gene that encodes an important mediator of the early innate immune defense against viruses.[22] Elevated *MxA* expression is part of the peripheral blood type 1 IFN signature in systemic autoimmune disease patients.[22] Yet in our study elevated *MxA* gene expression was not found in peripheral blood from patients with active pulmonary TB compared to healthy control subjects (S2 Fig.). Therefore, our data support differential involvement of type 1 IFN-inducible genes among different diseases associated with type 1 IFN activity.[13]

In conclusion, we identified a blood transcriptional signature of 10 type 1 IFN-inducible genes highly expressed in active pulmonary TB patients. Although further

studies are required, our data suggest that this peripheral blood type 1 IFN gene signature has the potential to stratify patients with suspected active TB infection-associated uveitis into groups with either a low or high risk of having uveitis due to TB. We expect that this stratification forms a constructive basis for future diagnostic and treatment studies in QFT/ TST positive uveitis patients.

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4. The authors declare that they have no conflicting and competing financial interests.

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AUTHOR CONTRIBUTIONS

R.L.D.N , P.M.v.H. and W.A.D., conceptualized and designed the study. R.L.D.N, R.S, M.S, L.E, S.S, G.S, and RR.D.H collected the patient data. R.L.D.N did the RT PCR experiments. M.A.V, supervised and analyzed the *MxA* EIA experiment. R.L.D.N, S.M.A.S and P.J.v.d.S. analyzed the RT PCR data. R.L.D.N and A.R analyzed the clinical data. R.L.D.N and W.A.D wrote the manuscript. W.A.D. had primary responsibility for the final content of the manuscript; and all authors have read and approved the final manuscript.

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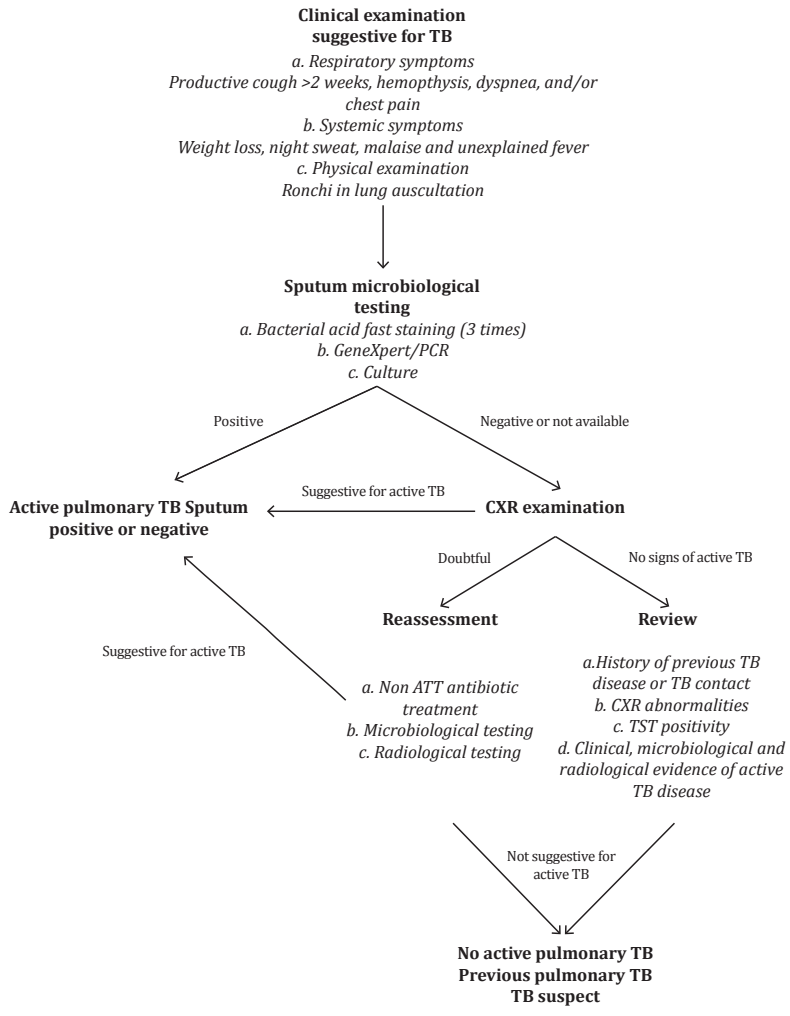
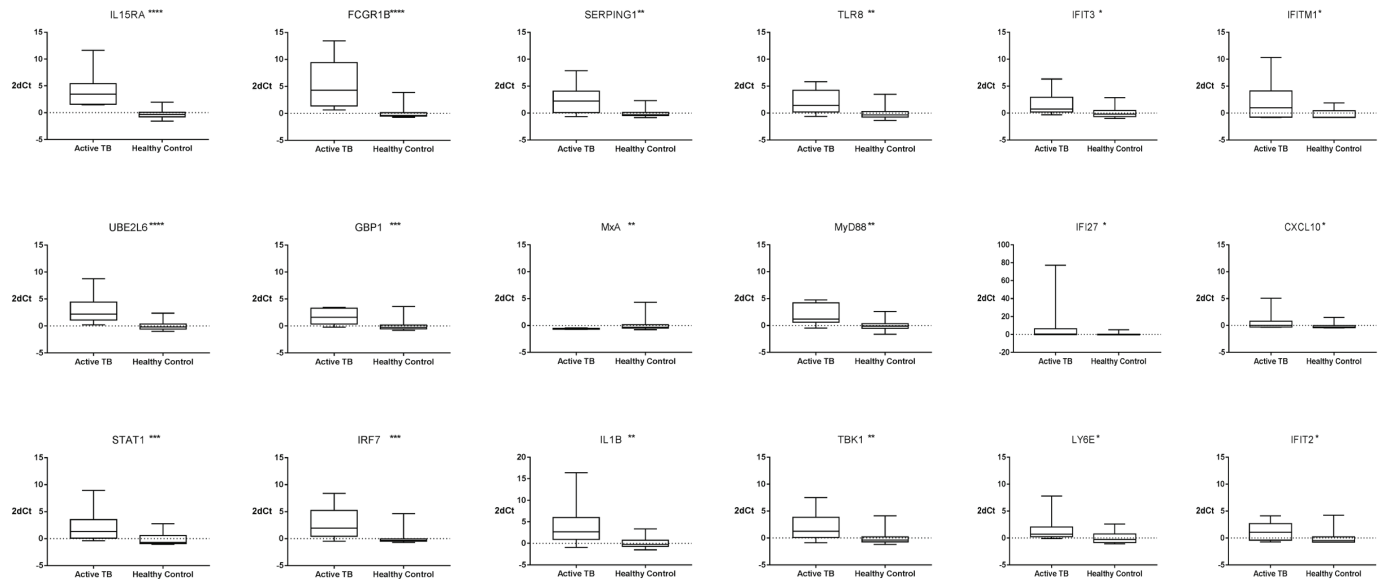
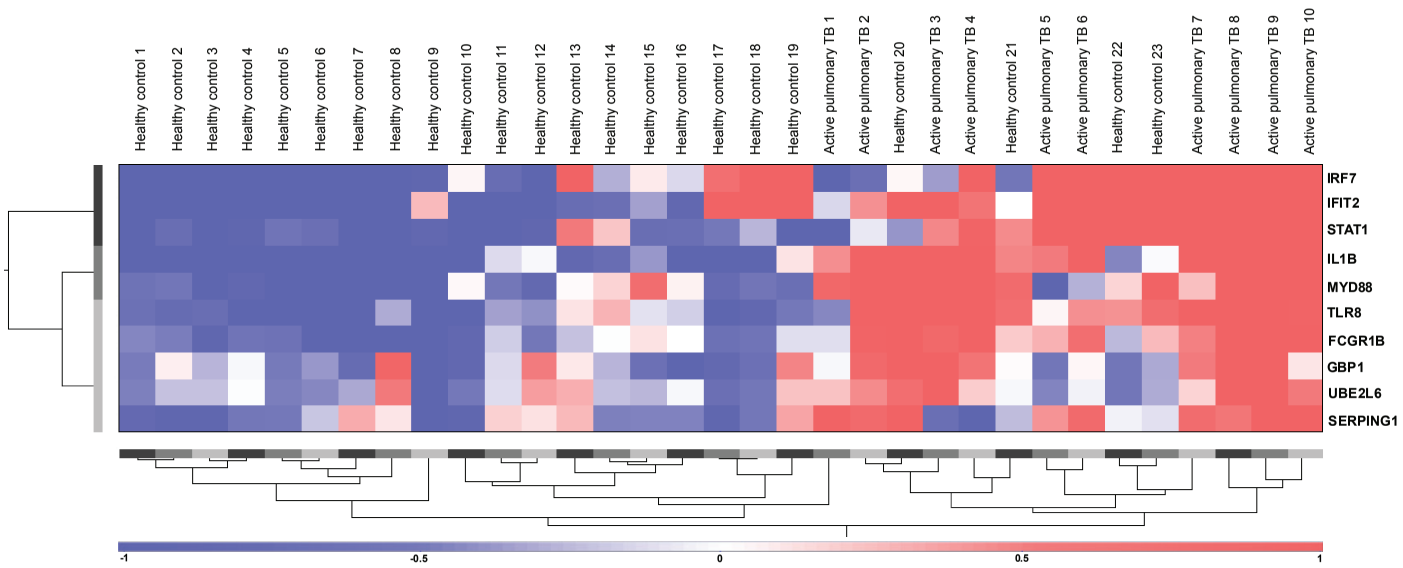
SUPPLEMENTAL DATA



Supplemental Figure 1. Indonesian Society of Respirology (ISR) TB diagnostic guideline.
Scheme depicting the TB diagnostic algorithm in Indonesia. The guideline starts with evaluation of clinical signs suggestive for tuberculosis (TB). In patients with signs suggestive of pulmonary TB infection, an acid-fast staining examination from the sputum is required and a positive result leads to diagnosis of active pulmonary TB. If the culture examinations from the sputum are negative, radiologic imaging is reviewed and classified When imaging is considered to exhibit signs of active pulmonary TB, the patient is diagnosed with active pulmonary TB. Therefore, active pulmonary TB will be either acid-fast bacillus (AFB) positive or AFB negative. The diagnosis of AFB-positive active pulmonary TB is based on positive culture or positive GeneXpert outcomes, and AFB-negative TB is based on radiologic signs of active pulmonary TB and clinical improvement after anti-tuberculosis antibiotic treatment.
TB: tuberculosis, AFB: acid-fast bacillus, GeneXpert: a molecular test that detects bacterial DNA from *Mycobacterium tuberculosis*, PCR: polymerase chain reaction, CXR: chest X-ray, TST: tuberculin skin test

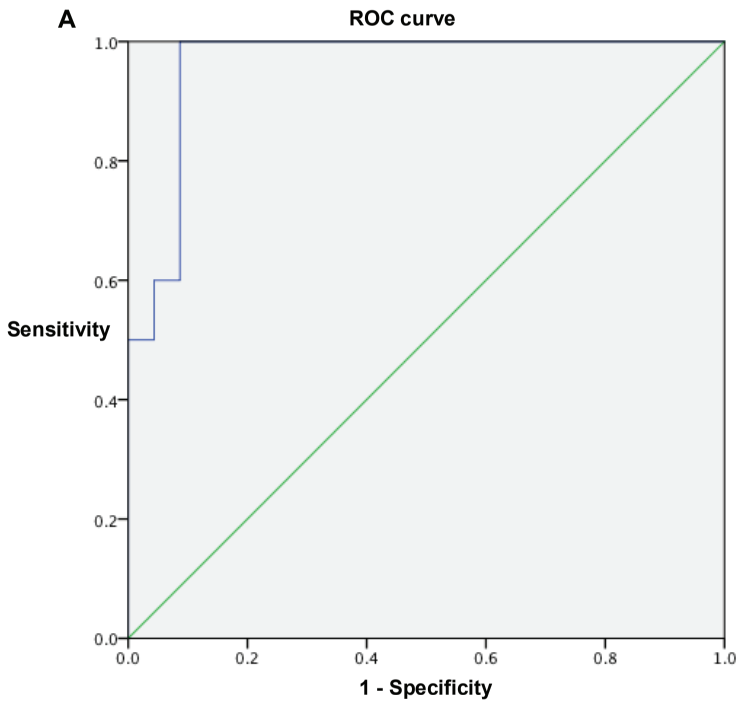


Supplemental Figure 2. Type 1 IFN-inducible genes with significant differences in expression between active pulmonary TB patients without uveitis and healthy controls.
Of a total of 35 type 1 IFN-inducible genes measured in the peripheral blood, 18 genes differed significantly in their expression between the active pulmonary TB without uveitis patients and the healthy controls without correction for age differences between the groups. Statistical significance was assessed using the Mann-Whitney U test.
**** *P* value <0.0001, *** *P* value <0.001, ** *P* value <0.01, * *P* value <0.05



Supplemental Figure 3. Cluster analysis of active pulmonary TB patients without uveitis and healthy controls based on type 1 IFN-inducible gene expression patterns.
Unsupervised hierarchical clustering analysis based on 10 type 1 IFN-inducible genes (indicated on the right). Active pulmonary TB subjects indicate the active pulmonary TB patients without uveitis. Cluster 1 contains the majority of the healthy controls (19/23; 83%) and one active pulmonary TB case without uveitis (1/10; 10%). Cluster 2 contains the majority of the active pulmonary TB cases without uveitis (9/10; 90%) and 4 healthy controls (4/23; 17%). Red indicates increased gene expression levels when compared to the geometric mean, and blue indicates decreased gene expression levels when compared to the geometric mean. Color intensity correlates with the magnitude of the calculated fold change.

A



Area Under the Curve

Area	Std. Error	Asymptotic Sig	Asymptotic 95% Confidence Interval	
			Lower Bound	Upper Bound
0.961	0.031	0.000033	0.9	1

B

Coordinates of the Curve				Youden index (J)
Type-1 IFN score positive if Greater Than or Equal To	Sensitivity	1 - Specificity	Specificity	
-9.96	1.000	1.000	0.000	0.000
-7.39	1.000	.957	0.043	0.043
-5.71	1.000	.913	0.087	0.087
-5.52	1.000	.870	0.130	0.130
-5.40	1.000	.826	0.174	0.174
-5.22	1.000	.783	0.217	0.217
-4.97	1.000	.739	0.261	0.261
-4.45	1.000	.696	0.304	0.304
-3.96	1.000	.652	0.348	0.348
-3.71	1.000	.609	0.391	0.391
-3.07	1.000	.565	0.435	0.435
-2.06	1.000	.522	0.478	0.478
-1.46	1.000	.478	0.522	0.522
-1.19	1.000	.435	0.565	0.565
-0.86	1.000	.391	0.609	0.609
-0.05	1.000	.348	0.652	0.652
0.74	1.000	.304	0.696	0.696
1.87	1.000	.261	0.739	0.739
3.10	1.000	.217	0.783	0.783
4.13	1.000	.174	0.826	0.826
5.04	1.000	.130	0.870	0.870
5.61	1.000	.087	0.913	0.913
9.15	.900	.087	0.913	0.813
12.64	.800	.087	0.913	0.713
13.18	.700	.087	0.913	0.613
13.84	.600	.087	0.913	0.513
15.69	.600	.043	0.957	0.557
19.60	.500	.043	0.957	0.457
23.33	.500	0.000	1.000	0.500
24.76	.400	0.000	1.000	0.400
33.08	.300	0.000	1.000	0.300
45.87	.200	0.000	1.000	0.200
59.41	.100	0.000	1.000	0.100
69.17	0.000	0.000	1.000	0.000

Supplemental Figure 4. ROC curve analysis and Youden Index calculation for determining the optimal cut-off value of type 1 IFN signature score. **A.** The ROC curve analysis and area under the curve (AUC) **B.** Youden index analyses indicate that a type 1 IFN signature score cut-off value ≥5.61 represents the optimal cut-off value for distinguishing active pulmonary TB patients without uveitis from healthy controls with 100% sensitivity, 91% specificity and a Youden index of 0.913.

Chapter 4.3

Antinuclear and antiretinal antibodies in uveitis associated with active and latent tuberculosis

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Editor,

The pathogenesis of uveitis in the setting of active and latent tuberculosis (TB) is not entirely clarified. Next to genuine infection [1], an important part of pathogenesis was attributed to (auto)immune reactions initiated by *Mycobacterium tuberculosis (Mtb)*. Infection with *Mtb* can be associated with the production of diverse serum autoantibodies.[2] Herein, we investigate the influence of (latent) TB on the presence of serum antinuclear and antiretinal autoantibodies (ANA and ARA) in Indonesian patients with uveitis.

Blood samples from patients with uveitis associated with active (not yet treated) pulmonary TB (N=10) and uveitis of unknown cause (N=85) were collected from June 2014 until May 2015. Classification of patients was performed according to SUN classification, and specific diagnoses were determined after the basic work-up for uveitis as indicated in our previous publication.[3] The diagnosis of active pulmonary TB was based on clinical and/or microbiological and radiological findings.[3]. This study was performed with the approval of the local medical ethical committee.

All patients underwent QuantiFERON-Tb Gold (QFT) (Cellestis Inc., Carnegie, Australia). Screening for the presence of ANA using HEp-2 cells (Inova, San Diego, California) and the presence of ARA using primate retinal tissue (Euroimmun, Lubeck, Germany) was performed by indirect immunofluorescence as described before.[4] Logistic regressions with correction for age and gender were performed using SPSS to analyse differences in the presence of ANA and ARA between the diagnosis groups.

Patients with uveitis of unknown cause were was divided according to their QFT results in 58 patients with latent TB (QFT positive) and 27 patients without evidence of prior TB exposure (QFT negative). All QFT positive patients were assessed by the pulmonologist and examined for the possible presence of pulmonary and extrapulmonary TB, but no cases of extrapulmonary involvement were found. The group with an unknown cause of uveitis and latent TB consisted of more female patients compared to the other groups (72% vs 30% in uveitis in the setting of active pulmonary TB group and 41% in the uveitis of unknown cause and QFT negative group, p-value 0.044) and older age patients (mean age 46 years vs 40 years in uveitis in the setting of active pulmonary TB group and 39 years in uveitis of unknown cause and QFT negative group, p-value 0.003). The median QFT value in patients with uveitis of unknown cause and latent TB was 5.0 IU/ml, and in patients with known tuberculosis induced uveitis 1.7 IU/ml.

Patients' serum ANA and ARA results are shown in Table 1. Patients with uveitis and either active or latent TB were characterized by high prevalence of systemic autoreactivity (ANA positive). In contrast, a higher proportion of organ-specific autoreactivity (ARA positive) was found in uveitis patients without evidence of any previous contact with *Mtb*. Induction of ANA, reported previously for active TB [5], apparently also occurs

in latent TB. Interestingly, organ-specific autoreactivity (ARA) appears to be suppressed in both active and latent TB, however, the local production of ARA in ocular fluid samples was not investigated in this series. The presence of serum autoantibodies, directed against endothelial cells, and their decrease following treatment was reported in age-related macular degeneration.[6] Unfortunately, we have no samples of our patients after they completed the treatment. Further studies are warranted to dissect the pathogenesis of this selective systemic induction of autoreactivity in uveitis patients as a result of *Mtb* infection and its implication on disease course.

TABLE 1. Prevalence of antinuclear and antiretinal antibodies in tuberculosis induced uveitis and uveitis of unknown cause with positive or negative QuantiFERON–TB Gold outcomes

	ANA positive	ARA positive
Total	18/95 (19%)	39/87 (45%)
1. Uveitis in the setting of active pulmonary TB	5/10 (50%)	4/9 (44%)
2. Uveitis of unknown cause, QFT positive	12/58 (21%)	19/52 (37%)
3. Uveitis of unknown cause, QFT negative	1/27 (4%)	16/26 (62%)
P-value: 1 vs. 2 vs. 3	0.023	0.049
P-value: 1 vs. 2	>0.05	>0.05
P-value: 2 vs. 3	>0.05	0.014
P-value: 1+2 vs. 3	0.03	0.021

Legend: Serum ANA were more prevalent in patients with TB-induced uveitis (50%) than in patients with uveitis of unknown cause with latent TB (21%) or without latent TB (4%; p=0.023; Table 2). The prevalence of ARA was higher in QFT negative patients with unknown uveitis cause (62%) than in QFT positive uveitis cases (p=0.049). Prevalence of ARA or ANA did not differ between TB-induced uveitis and QFT positive uveitis of unknown cause. Abbreviations: ANA= antinuclear antibodies, ARA = antiretinal antibodies, OR = odds ratio, QFT = QuantiFERON–TB Gold test

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COMPETING INTERESTS

The authors declare that they have no competing interests.

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Chapter 5

Summary and discussion

SUMMARY

The pathophysiology of Mycobacterium tuberculosis (*Mtb)*-associated uveitis is not entirely understood, and in consequence, the diagnosis and the treatment of *Mtb*-associated uveitis are challenging. The studies included in this thesis aim to increase our knowledge of this debilitating ocular disorder and aim to develop specific biomarkers for its diagnosis as defined in chapter 2.

Chapter 1 provides a general introduction to tuberculosis (TB) disease and its immunology and *Mtb*-associated uveitis and summarizes our current knowledge of this topic. Uveitis is a potentially sight-threatening disease, and therefore, early recognition of its cause is of great importance as the correct treatment might prevent unnecessary deterioration of visual acuity. Especially in patients with infectious uveitis, the disease may rapidly progress and lead to a permanent visual loss. The definitive diagnosis of "*Mtb*-associated uveitis" is frequently not feasible and the diagnosis is in the majority of cases presumptive ("probable or possible *Mtb*-associated uveitis*")*.

Chapter 3.1 reports a retrospective study conducted on QuantiFERON–TB Gold In-Tube test (QFT)-positive patients with an otherwise undetermined cause of uveitis in the Netherlands. In this study, the ocular features of patients with uveitis of undetermined cause, but positive QFT were diverse, yet two distinct clinical entities could be specifically identified: retinal occlusive vasculitis occurring predominantly in young males, and serpiginoid choroiditis. The QFT levels of patients with occlusive vasculitis were highly elevated. Further, 25/76 (33%) of the patients exhibited lymphadenopathy, suggestive for the diagnosis sarcoidosis. Moreover, in 10 out of 12 lymph node biopsies, the presence of granulomas was found, but *Mtb* culture was positive in only 3 of them. The role of *Mtb* in sarcoidosis is an ever-recurring subject, as *Mtb* antigens were previously found in sarcoid biopsies.[1] Ocular inflammation reacted favorably to anti-tuberculosis treatment (ATT), but in 3 cases corticosteroid therapy was started because of relapse of disease after ATT was completed.

In **Chapter 3.2** the results of a prospective study of consecutive patients with uveitis in Indonesia showed that infectious uveitis was the most common type of uveitis. The leading causes of infectious uveitis included toxoplasmosis and TB. At the first presentation to the ophthalmologist, the majority of patients had a visual acuity of less than finger counting and already exhibited various complications of uveitis. When classifying the "QFT-positive patients with unexplained uveitis" into a TB-related group, the percentage of 'TB-associated' uveitis cases increased from 8 to 48%. Further, an excess of patients with "uveitis of undetermined cause" was found within the group of patients positive for QFT, which suggests a link between latent *Mtb* infection and the development of uveitis. The QFT levels in otherwise healthy patients with "uveitis of undetermined

origin" were higher than QFT levels of patients suffering from active pulmonary TB. This suggests that these patients have a "high immune response" as measured by IFNγ production, however we cannot rule out T- lymphocyte exhaustion in the active TB group. Meanwhile, the study in the Netherlands, a non-endemic TB country, showed that QFT positive patients exhibited an association with certain ocular features in contrast to patients in Indonesia. This might be in part related to late presentation of patients to the ophthalmologist in Indonesia.

Mtb bacilli have been found in retinal pigment epithelium (RPE) cells from uveitis patients without signs of systemic TB infection.[2, 3] RPE cells are essential for maintenance of the retina and are involved in both the ocular immune privilege and uveitis development. In **Chapter 4.1** the cellular response of RPE cells after *Mtb* infection was studied and compared to that of macrophages (the main target cell of *Mtb*). RPE cells were found to robustly control intracellular outgrowth of *Mtb* early after infection. This control of *Mtb* was associated with prominent activation of interferon (IFN)-signaling pathways as well as altered expression of cell death/survival-associated genes and low-level production of T helper (Th)1-associated cytokines. The RPE response upon *Mtb* infection differed from that in macrophages, with the latter displaying a plethora of responses including IFN signaling and communication between innate and adaptive immune cells to induce granuloma formation. These data demonstrate that RPE cells display a strong response to *Mtb* infection that appears however incomplete in comparison to the macrophage response. The RPE response might reflect a balance between mechanisms aimed at *Mtb* eradication and mechanisms that limit retinal inflammation.

As type 1 IFN-inducible genes are prominent expressed in active systemic TB and even locally in *Mtb* infected RPE cells the possibility whether a type 1 IFN gene signature could stratify the problematic group "QFT positive uveitis of unknown cause" was studied in **Chapter 4.2**. This revealed ten type 1 IFN-inducible genes that were differentially expressed between active pulmonary TB and healthy controls; *UBE2L6, FCGR1B, GBP1, IL1B, MYD88, TLR8, IRF7, STAT1, SERPING1*, and *IFIT2*. Exploration of the peripheral blood expression pattern of these ten type 1 IFN-inducible genes (type 1 IFN gene signature) in QFT-positive patients with uveitis of unknown cause revealed three groups: 1). patients with a type 1 IFN gene signature resembling that of patients with active pulmonary TB (without and with uveitis), 2). patients with a type 1 IFN gene signature resembling that of healthy controls, and 3). patients displaying a type 1 IFN gene signature in-between the other two patterns indicated above. Subsequently, a type 1 IFN gene signature score was calculated as previously described in the context of systemic autoimmune diseases.[4, 5]

A type 1 IFN gene signature score ≥ 5.61 displayed high sensitivity (100%) and specificity (91%) for identification of patients with active TB. It is proposed that application

of this score to QFT-positive patients with "uveitis of unknown cause" may help to stratify patients within this group into those with high versus low likelihood of having uveitis due to active TB.

High score type 1 IFN gene signature in peripheral blood and the ability for *Mtb* to infect RPE suggest that an infectious origin is probably the most important factor in the pathogenesis of *Mtb*-associated uveitis. However, it has been shown that *Mtb*-associated uveitis cases sometimes require an immunosuppressive treatment even in cases which have been treated with a long-term ATT.[6-8] These findings support the possibility of an autoimmune component in the pathogenesis. In **Chapter 4.3,** a cohort study was performed to investigate whether humoral autoimmunity plays a role in *Mtb*-associated uveitis. The presence of antinuclear antibody (ANA) and antiretinal antibody (ARA) were determined in QFT positive and negative patients. It was shown that patients with

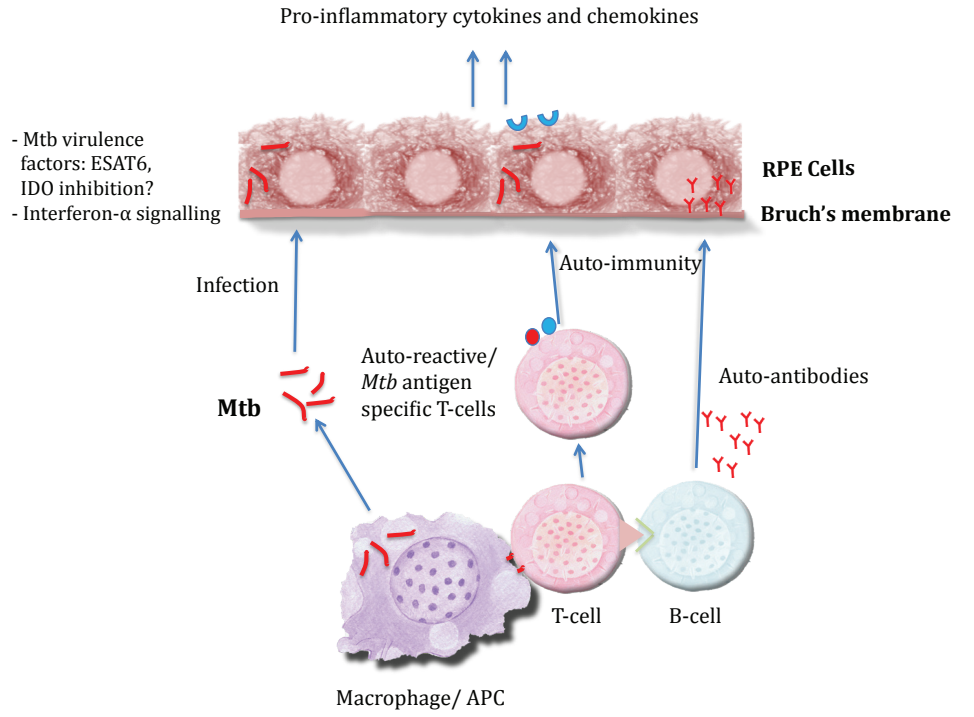

Figure 1. Hypothetical simplified concept of the pathogenesis of *Mtb*-associated uveitis.
RPE cells represent central players in the pathogenesis of *Mtb*-associated uveitis as they can be infected by *Mtb* and induce a local inflammatory response. It is speculated that *Mtb* induces T-cell depended retinal autoimmunity that may result or contribute to uveitis. Our studies showed that anti-retinal antibodies are not a common finding in *Mtb*-associated uveitis and thus not mandatory in the pathogenesis.

uveitis and either active or latent systemic TB were characterized by a high prevalence of ANA indicating systemic auto-reactivity. In contrast, a higher proportion of ARA, suggesting organ-specific autoreactivity, was observed in patients with uveitis who had no evidence of any previous contact with *Mtb*.

Induction of ANA was previously reported in active TB, but apparently also occurs in latent TB. Interestingly, organ-specific autoreactivity determined by presence of ARA appears to be suppressed in both, active and latent TB. It seems that autoimmunity through a humoral immune response to retinal antigens does not play an important role in *Mtb*-associated uveitis. A simplified hypothetical concept of the pathogenesis of *Mtb*-associated uveitis is presented in Figure 1.

DISCUSSION

TB is an airborne disease, but is heterogeneous in disease manifestations, in *Mtb* replication state, and in host immunological state. Active TB can start from latent infection at the moment the immune system cannot prevent *Mtb* replication anymore, the host develops clinical manifestations. From its primary focus in the lung, *Mtb* might disseminate haematogenously into many organs including the eye. Therefore, *Mtb*-associated uveitis can potentially be caused by direct invasion of *Mtb* into the retina. Apart from macrophages, *Mtb* is able to infect epithelial cells, and RPE cells are thus potential targets. Moreover, RPE has biologic similarities with macrophages, i.e., phagocytosis, pro-inflammatory cytokine production and expression of HLA class 2 molecules.[9] Macrophages may destruct or hide *Mtb*. In that sense, it is intriguing if *Mtb* is able to hide in RPE, facilitated by the production of molecules that hamper the cellular defense of the RPE. *Mtb* derived molecules such as 6 kDa early secretory antigenic target (ESAT6) are able to manipulate the host cell defense.[10] On the other hand, *Mtb* may induce in a certain stage of development a "RPE inflammatory state" by inducing pro-inflammatory cytokine/chemokine release and subsequent T-cell infiltration, a process that will result in local tissue damage which by itself accelerates local inflammation.

We need more insights into the contribution of RPE cells within the spectrum of *Mtb* infection, whether and how RPE cells eliminate *Mtb* and how they are involved in hiding *Mtb*. Previous studies reported *Mtb* within RPE cells, it is however unknown whether if *Mtb* is sequestered in RPE due to a supposed role as immune privilege site or is it in fact facilitated *Mtb* evasion. RPE differential gene expression after *Mtb* infection supports the possibility of both, therefore we need further studies to elaborate the mechanism and kinetics.

With the dominance of IFN signaling in RPE cells after early *Mtb* infection, the levels of IFNγ, IFNα, and IFNβ intraocularly are of interest. In CMV infected RPE cells, IFNγ and IFNβ are known to inhibit cytomegalovirus (CMV) and Toxoplasma gondii replication. [11,12] This inhibition acts by inducing the indoleamine 2,3 dioxygenase (IDO) pathway which would convert tryptophan to kynurenine. Tryptophan deprivation will inhibit CMV replication and will be reversed by tryptophan addition in this model. It is not known yet whether *Mtb* will also be inhibited by IDO-1 induced tryptophan depletion as observed in CMV and Toxoplasma gondii. On the other hand, IDO-1 exerts an immune-dampening effect by suppressing the response of macrophages and effector T cells, IDO-1 expression by RPE cells may in part explain RPE-mediated immune suppressive effects.[13] Whether the IDO pathway is involved in either *Mtb* tolerance or destruction is subject for further studies. Previously, a clinical study reported the beneficial effect of IFN alpha-2a in presumed intraocular TB. In this study, patients finished complete ATT regimen and experienced recurrence of inflammation despite the ATT and corticosteroid treatment.[14]

In our *Mtb* infected RPE cultures, we found production of interleukins, T cell chemokines, and adhesion molecules, potentially enabling the infected RPE cells to facilitate a uveitis condition. Upregulation of *TRAIL* gene expression was observed, and this might have an important role in uveitis pathophysiology as it can be important in ocular immune privilege. The role of TRAIL needs further elaboration in *Mtb* infected RPE.

The RPE infection studies were performed after 24 hours culture. There is a need to investigate the survival of *Mtb* in RPE and the cellular response of RPE in long-term cultures. Drug sensitivity of intracellular *Mtb* is another important subject which has to be studied in long-term cultures, sensitivity of RPE phagocytosed *Mtb* to ATT is unknown. These items are subjects for future research, for which these long term *Mtb*-infected RPE cultures are warranted. It is not known whether the RPE response depends on a particular *Mtb* strain. RPE infection with various strains may identify strains that have a more pro-inflammatory characteristic while other *Mtb* strains that hide may produce facilitating factors. At this moment the role of the type of *Mtb* strain in *Mtb* associated uveitis needs further exploration.

Another issue that needs attention: can other retinal cells (such as glial cells, i.e., Müller cells, microglia, and astroglia) be infected by *Mtb*? Microglia cells are the most interesting candidates: these are phagocytic cells required for neuronal homeostasis and innate immune defense. The immunological potential of microglia is comparable with blood monocytes and macrophages,[15] so these cells may destroy phagocytosed *Mtb* or even may hide *Mtb*..

There are no studies yet showing whether RPE cells can contribute to choroid granuloma formation comparable to alveolar epithelial cells in the lung.[16] Interestingly,

increased MMP9 gene expression was observed in *Mtb* infected RPE which was not found in the macrophages (data is not shown). MMP9 is an important factor for homing of macrophages and is produced by alveolar epithelial cells and supports granuloma formation. *Mtb* infected RPE cells have the capability to produce this factor, it is tempting to hypothesize that MMP9 contributes to *Mtb* induced choroidal granuloma formation as previously reported.[17, 18]

Important granuloma inducing or stimulating cytokines, i.e., IFNγ and TNFα are produced by *Mtb* infected RPE cells. The role of IFNγ is well established in tuberculosis and granuloma formation, on the contrary, no *Mtb*-associated uveitis has been described yet in patients with neutralizing anti– IFNγ autoantibodies, these patients develop recurrent non-tuberculous mycobacterial infections or disseminated TB.[19] In *Mtb* infections, several questions can be addressed according to the host factors or who develops *Mtb*-associated uveitis? From many host factors, it can be speculated that they contribute to susceptibility to develop disease. More understanding came from genetic studies in severe TB. In particular, Mendelian susceptibility to mycobacterial diseases (MSMDs) studies, which usually involves weak virulent mycobacterial species, such as BCG and environmental mycobacteria.[20] Several mutations were described including germline mutations in seven autosomal (*IFNGR1*, *IFNGR2*, *IL12B*, *IL12RB1*, *STAT1*, *IRF8, ISG15*) and two X-linked (*NEMO*, *CYBB*) genes. These genes are related to functionality of IFN γ, cellular responses or intracellular microbe degradation such as in chronic granulomatous disease.[21]

GWAS studies identified an association between four polymorphisms in the *TLR8* gene and pulmonary TB.[22] Moreover, previous candidate gene studies identified variants in purinergic receptor P2X7,[23] vitamin D receptor,[24] IL-1β,[25] LTA,[26] IL-10,[27] IFNγ,[27-30] and TLR2 in extrapulmonary disease.[31] This supports the hypothesis of a genetic predisposition for extrapulmonary tuberculosis. Up to now no genetic variants were described concerning *Mtb* associated uveitis. Genetic studies may help to identify genetic variants in patients who are at risk to develop *Mtb* associated uveitis.

The association between uveitis and positive TST or QFT in otherwise healthy patients are frequently reported. As described above, these patients may benefit from complete ATT as it is assumed that minimum numbers of bacteria reach the retina and cause the inflammation. However, a substantial number of patients (~21%) developed recurrent disease after full ATT therapy, without proof of reinfection or multi-resistant *Mtb*. These patients recover with immunosuppression without a systemic outbreak of systemic TB.[7]

Immunotherapy with Bacille-Calmette-Guerin (BCG) instillation in patients with bladder cancer can be complicated by uveitis as well.[32-34] It is unclear if BCG can infect the retina, but an autoimmune reaction to the retina is expected because no signs of a

peripheral BCGitis was described. Reactive polyarthritis (Poncet's disease) occurring in TB patients manifests in the absence of detectable mycobacteria in the joint spaces[35] Indicating an autoimmune reaction in non-articular TB. Moreover, complete Freund's adjuvants (CFA) induce autoimmunity in animal models including uveitis, even without co-administration of retinal antigens.[36] Retinal autoantibodies are seemingly not playing a role in *Mtb* associated uveitis as described in this thesis. CD4+ T lymphocytes are supposed to be the major players in autoimmune uveitis, and obviously also in TB, as human immunodeficiency virus (HIV) resulted in an epidemic increase in patients with TB. The contribution of cellular autoimmunity in *Mtb* associated uveitis, in particular, autoreactive T-lymphocytes needs further investigation. Interestingly, autoreactive T cells directed against retinal crude extract (RCE) were observed in vitreous samples of *Mtb*-associated uveitis cases, and these were not found in other uveitis cases. The phenotypes include both T-central memory lymphocytes and T-effector memory T lymphocytes[37] ; they found T-effector memory cell preponderance while the cytokine response was significantly stronger in T-central memory lymphocytes. The mechanisms underlying these phenomena are not known yet, especially whether the T cells were activated in the periphery or locally in the eye. If a specific type autoreactive T cell can be identified in peripheral blood or the eye through *Mtb* epitopes, it may contribute to the diagnosis and monitoring of *Mtb*-associated uveitis [36] Single nucleotide polymorphisms (SNPs) that associate with TB severity also associate with autoimmune disease, for example, *CTLA-4* autoimmunity associated genotype contributes to TB severity.[38] An in-depth T-lymphocyte analysis in patients with *Mtb* associated uveitis may give more insights in the immune process and may deliver additional monitoring tools.

An immunological response to mycobacteria may also generate an immune response to retinal antigens; both responses might play a role in the development of uveitis. It is further of interest to measure the type 1 IFN gene signature in these "autoimmune" patients (without known active TB disease) to stratify them in the group latent TB or active TB to adjust the most effective therapy. Comparison with the type 1 IFN gene signature of other autoimmune diseases such as M. Sjögren's or lupus erythematosus is of interest to identify gene expressions typical for autoimmunity or other infection besides *Mtb* infection (CMV or Toxoplasma gondii). Another hypothesis for the ATT success and recurrent inflammation in high QFT and high type-1 IFN gene signature is whether patients with latent TB have a higher state of immune surveillance; this may stimulate the development of uveitis in predisposed individuals. A small, short-term preliminary follow-up (in the Indonesian cohort) retrospective study suggests an association of high QFT value with ATT treatment success and an association of high type-1 IFN gene signature score (≥5.61) to persistent uveitis inflammation or recurrence in non-ATT treated patients (unpublished data).

In the near future, there is a need to perform a randomized controlled trial of ATT treatment in patients with "QFT-positive unknown cause uveitis" based on the type 1 IFN gene signature score with long follow up of the clinical manifestations and monitoring of the type 1 IFN scores during the course of treatment in order to draw further conclusions.

CONCLUSIONS

Infectious uveitis was the most common type of uveitis in Indonesia and uveitis in the setting of systemic TB was one of its major causes. The studies performed in this thesis further indicate that *Mtb* infection plays a crucial role in a significant proportion of patients "with uveitis of undetermined cause" but positive QFT reaction. Two specific clinical manifestations were recognized in patients with uveitis of undetermined cause and positive QFT in the Netherlands, a country not endemic to *Mtb*. QFT positive patients with uveitis might benefit from ATT, despite the absence of systemic signs of active TB. Further, *Mtb* infected RPE cells dominantly showed interferon signaling canonical pathway and network. In the peripheral blood, type 1 IFN gene signature score stratified *Mtb*-associated uveitis in three types. These findings might form the basis for a prospective trial with ATT and/or corticosteroids. Lastly, we found no evidence that antibodies against retinal antigens are involved in the pathogenesis in *Mtb*-associated uveitis, and we assume that -if autoimmune processes are involved- a cellular response is more likely.

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Appendix

Abbreviations

Samenvatting

Acknowledgments

Curriculum Vitae

PhD Portfolio

Publications

ABBREVIATIONS

AFB	Acid-fast bacillus
AIDS	Acquired immune deficiency syndrome
Amp	Ampiginous
APMPPE	Acute posterior multifocal placoid pigment epitheliopathy
ARN	Acute retinal necrosis
ATT	Anti-tuberculosis treatment
AUC	Area under the curve
BAFF	B-cell activating factor
BCG	Bacille-Calmette-Guerin
BSCR	Birdshot chorioretinitis
CD	Cluster of differentiation
CFA	Complete Freund's adjuvant
CLR	C-type leptin receptors
CMV	Cytomegalovirus
CR	Complement receptors
CT	Computerized/ computed tomography
CXR	Chest X-Ray
DC	Dendritic cells
DC-SIGN	Dendritic-cells specific intracellular adhesion molecule 3 – grabbing nonintegrin
DNA	Deoxyribonucleic acid
DNCB	1-chloro 2,4-dinitrobenzene
DUSN	Diffuse unilateral subacute neuro retinitis
E	Ethambutol
EAU	Experimental autoimmune uveitis
ESAT-6	6 kDa early secretory antigenic target
ESX1	ESAT-6 secretion system 1
FcγR	Fcγ receptors
FMUI	Faculty of Medicine University of Indonesia
FUS	Fuchs uveitis syndrome
GPA	Granulomatosis with polyangiitis
GWC	Goldmann-Witmer coefficient
H (or INH)	Isoniazid
HEL	Hen egg lysozyme
HIV	Human immunodeficiency virus
HMC	Human mast cells

HSV	Herpes simplex virus
HTLV	Human T-lymmphotropic virus
IBD	Inflammatory bowel disease
iDC	Inflammatory dendritic cells
IFN	Interferon
IGRA	Interferon-γ release assay
IL	Interleukin
iM	Inflammatory macrophages
INH (or H)	Isoniazid
IQR	Interquartile range
IRBP	Inter-photoreceptor retinoid binding protein
IRF	Interferon regulatory factor
ISR	Indonesian Society of Respirology
IU	International units
JIA	Juvenile idiopathic arthritis
kDa	kilo Dalton
logMAR	Logarithm of the minimum angle of resolution
M. leprae	Mycobacterium leprae
ManLAM	Mannose-capped liparabinomannan
MEWDS	Multiple evanescent white dot syndrome
MFCPU	Multifocal choroiditis with panuveitis
MMR	Macrophage mannose receptors
MS	Multiple sclerosis
Mtb	*Mycobacterium tuberculosis*
MyD88	Myeloid differentiation primary response 88
NFκB	nuclear factor kappa-light-chain-enhancer of activated B cells
NK-cells	Natural killer cells
NLR	NOD-like receptors
NOD	Nucleotide-binding oligomerization domain
NPV	Negative predictive value
OCT	Optical coherence tomography
ORV	Occlusive retinal vasculitis
PCR	Polymerase chain reaction
PGD	Prostaglandin
PI3P	Phosphatidylinositol 3-phosphate
PIC	Punctate inner choroiditis
PORN	Posterior outer retinal necrosis
PPD	Purified protein derivative
PPV	Positive predictive value
PRR	Pattern-recognition-receptor
QFT	QuantiFERON-Gold TB
R	Rifampicin
RCE	Retinal crude extract
RD	Region of difference
RHZE	Rifampicin, Isoniazid, Pyrazinamide, Ethambutol
RIG	Retinoic acid-inducible gene
RLR	RIG-I-like receptors
RNA	Ribonucleic acid
ROC	Receiver operating characteristic
RPE	Retinal pigment epithelium
SD	Standard of deviation
SIV	Simian immunodeficiency virus
SLE	Systemic lupus erythematosus
SO	Sympathetic ophthalmia
SR	Scavenger receptor
SUN	Standardization of uveitis nomenclature
T cells	T lymphocytes
TB	Tuberculosis
Th	T helper cells
TINU	Tubulointerstitial nephritis and uveitis
TLR	Toll-like receptor
TLS	Tertiary lymphoid structure
TNF	Tumor necrosis factor
Tregs	Regulatory T cells
TST	Tuberculin skin test
US	United States of America
VA	Visual acuity
VEGF	Vascular endothelial growth factor
VKH	Vogt Koyanagi Harada
VZV	Varicella zoster virus
WHO	World Health Organization
Z	Pyrazinamide
ZN	Ziehl Nielsen

SAMENVATTING

De pathogenese van uveitis, geassocieerd met een infectie door Mycobacterium tuberculosis (*Mtb*), de zogenaamde *Mtb*-geassocieerde uveïtis, wordt niet volledig begrepen. Als gevolg hiervan zijn de diagnose en de behandeling van *Mtb*-geassocieerde uveïtis een uitdaging. Mtb-geassocieerde uveitis kan het gezichtsvermogen verminderen en tot blindheid leiden. De studies opgenomen in dit proefschrift hebben tot doel de kennis over Mtb-geassocieerde uveïtis te vergroten en zijn vooral gericht op de ontwikkeling van specifieke biomarkers voor diagnose en therapie, zoals gedefinieerd in de "Aims" in hoofdstuk 2.

Hoofdstuk 1 geeft een algemene inleiding over tuberculose (TB) en de immunologie hiervan. Daarnaast beschrijft dit hoofdstuk de indeling en oorzaken van uveitis en de huidige kennis over *Mtb*-geassocieerde uveitis. Uveïtis is een potentieel gezichtsvermogen bedreigde ziekte. Vroegtijdige herkenning van uveitis alsmede de identificatie van de oorzaak is daarom van groot belang omdat de juiste behandeling onnodige (irreversibele) verslechtering van het gezichtsvermogen kan voorkomen. Vooral bij patiënten met een infectieuze uveïtis, kan de ziekte snel leiden tot ernstig verlies van gezichtsvermogen. De definitieve diagnose "*Mtb*-geassocieerde uveitis" is vaak moeilijk te stellen daarom krijgt de diagnose in de meeste gevallen het predicaat "vermoedelijk" (dit wordt weer onderverdeeld in "waarschijnlijke" of "mogelijke" *Mtb*-geassocieerde uveïtis).

In hoofdstuk 3.1 wordt een retrospectieve studie gepresenteerd die werd uitgevoerd bij QuantiFERON-TB test (QFT)–positieve patiënten met een onverklaarde uveïtis in Nederland. In deze studie waren de oogsymptomen divers, maar toch konden vooral twee verschillende klinische entiteiten herkend worden: retinale occlusieve vasculitis (voornamelijk bij jonge mannen) en serpigineuze choroïditis. De QFT-niveaus van patiënten met occlusieve vasculitis waren opvallend hoog. Verder hadden 25/76 (33%) van de patiënten lymfadenopathie, klinisch suggestief voor de diagnose sarcoïdose. Bovendien werd in 10 van de 12 lymfeklierbiopten de aanwezigheid van granulomen gevonden, maar de *Mtb*-kweek was slechts positief in 3 lymfeklieren. De rol van *Mtb* bij sarcoïdose is overigens een steeds terugkerend onderwerp, aangezien *Mtb*–componenten werden aangetroffen in sarcoïd granulomen. De uveïtis reageerde gunstig op behandeling met tuberculostatica (ATT), maar in 3 gevallen werd behandeling met corticosteroïden gestart vanwege nieuwe ziekte-activiteit nadat de ATT behandeling was voltooid.

In **hoofdstuk 3.2** worden de resultaten gepresenteerd van een prospectieve studie van patiënten met uveitis die de oogheelkunde kliniek van de "University of Indonesia" in Jakarta consulteerden. Er werd aangetoond dat aldaar, infectieuze uveïtis het meest frequent voorkomt. De belangrijkste oorzaken van infectieuze uveïtis waren;

toxoplasmose en TB. Bij de eerste presentatie aan de oogarts had de meerderheid van de patiënten een gezichtsscherpte van minder dan “aantal vingers tellen” en er bestonden ook al verschillende complicaties van de uveïtis. Bij het classificeren van de QFT-positieve patiënten met onverklaarde uveïtis in de TB-gerelateerde groep, nam het percentage TB-geassocieerde uveïtis gevallen toe van 8 tot 48 %. Er werd een oververtegenwoordiging gevonden van “onverklaarde uveïtis” binnen de QFT positieve patiënten, dit suggereert een verband tussen latente *Mtb*–infectie en de ontwikkeling van uveïtis.

Mtb-bacilli zijn gevonden in retinale pigmentepitheelcellen (RPE) van uveïtispatiënten die verder geen tekenen van systemische Mtb-infectie vertoonden. RPE-cellen zijn essentieel voor het behoud van het netvlies en zijn betrokken bij zowel de oculaire immuniteitsprivilege als bij de ontwikkeling van uveïtis. In **hoofdstuk 4.1** worden de resultaten van de cellulaire respons van RPE-cellen na *Mtb*-infectie gepresenteerd. Deze respons werd vergeleken met die van macrofagen (de belangrijkste doelcel van *Mtb*). RPE-cellen bleken in staat om de intracellulaire groei van *Mtb* te beïnvloeden. Intracellulair *Mtb* was geassocieerd met een prominente activering van de interferon (IFN)–signaleringsroutes en verder veranderde expressie van celdood/overleving-geassocieerde genen en productie van, weliswaar in geringe mate, Th1-geassocieerde cytokines. De RPE-respons als reactie op *Mtb*-infectie verschilde met die van macrofagen, waarbij de laatsten overmatige activatie-responsen lieten zien waaronder IFN-signalering en interactie tussen de innate en adaptieve immuniteit, die een rol speelt bij granuloomvorming. Deze bevindingen tonen aan dat RPE-cellen een sterke reactie vertonen op een *Mtb*-infectie maar dat deze reactie onvolledig is in vergelijking met de reactie van macrofagen. De cellulaire RPE-reactie kan een weerspiegeling zijn van de balans tussen mechanismen die gericht zijn op *Mtb*-degradatie en de mechanismen die netvlies ontsteking beperken.

Aangezien type 1 IFN-induceerbare genen significant tot expressie komen in actieve systemische TB en ook lokaal in *Mtb* geïnfecteerde RPE-cellen, werd de mogelijkheid bestudeerd of type 1 IFN-genexpressie (IFN-handtekening) in bloedcellen de moeilijk definieerbare groep “onverklaarde QFT-positieve uveïtis “ zou kunnen stratificeren. De resultaten worden in **hoofdstuk 4.2** gepresenteerd. Er werden 10 type 1 IFN-induceerbare genen geïdentificeerd, die voornamelijk tot expressie werden gebracht bij actieve pulmonale tuberculose; UBE2L6, FCGR1B, GBP1, IL1B, MYD88, TLR8, IRF7, STAT1, SERPING1 en IFIT2. Onderzoek van deze type 1 IFN-handtekening in QFT-positieve patiënten met onverklaarde uveïtis leverde 3 groepen op: 1). patiënten met een type 1 IFN-handtekening die lijkt op die van patiënten met actieve pulmonale tuberculose (onafhankelijk van het voorkomen van uveïtis), 2). patiënten met een type 1 IFN-handtekening die lijkt op die van gezonde controles en 3). patiënten die een type

1 IFN-handtekening vertonen met kenmerken van zowel groep 1 als groep 2. Vervolgens werd een type 1 IFN-handtekening score gemeten zoals eerder beschreven in de context van systemische auto-immuunziekten. Een type 1 IFN-handtekening score van ≥ 5,61 toonde een hoge sensitiviteit (100%) en specificiteit (91%) voor patiënten met actieve TB. Toepassing van deze score op QFT-positieve patiënten met “onverklaarde uveïtis “ leidde tot stratificatie van uveïtispatiënten met een hoge of lage overeenkomst met actieve TB. Deze resultaten vormen een basis voor verder prospectief therapeutisch onderzoek.

Een hoge type 1 IFN-handtekening score in perifeer bloed en het vermogen van *Mtb* om RPE te infecteren suggereert dat een infectie met *Mtb* waarschijnlijk de belangrijkste rol speelt in de pathogenese van *Mtb*-geassocieerde uveïtis. Er is echter met regelmaat gerapporteerd dat patiënten met *Mtb*-geassocieerde uveïtis soms een immunosuppressieve behandeling nodig hebben, zelfs patiënten die langdurig zijn behandeld met ATT. Deze bevindingen ondersteunen de mogelijkheid dat tevens een auto-immuuncomponent bijdraagt aan de pathogenese. In **hoofdstuk 4.3** wordt een cohortonderzoek gepresenteerd waarin onderzocht werd of humorale auto-immuniteit een rol speelt bij *Mtb*-geassocieerde uveïtis. De aanwezigheid van antinucleaire antistoffen (ANA) en anti-retinale antistoffen (ARA) werd bepaald bij QFT-positieve en -negatieve patiënten met en zonder uveïtis. Er werd gevonden dat patiënten met uveïtis en actieve of latente systemische TB werden gekenmerkt door een hoge prevalentie van ANAs wat systemische autoreactiviteit suggereert. Inductie van ANAs werd eerder gerapporteerd bij actieve TB, maar komt blijkbaar ook voor bij latente TB. Daarentegen werd een hogere frequentie ARAs, wat orgaanspecifieke autoreactiviteit representeert, waargenomen bij patiënten met uveïtis die geen bewijs hadden van enig eerder contact met *Mtb.* Interessant in deze is, dat orgaanspecifieke autoreactiviteit, weergegeven door de aanwezigheid van ARAs, lijkt te zijn onderdrukt in het geval van zowel actieve als latente TB. De resultaten suggereren dat een humorale auto-immuunrespons tegen retinale antigenen geen dominante rol speelt bij *Mtb*-geassocieerde uveïtis.

CONCLUSIES

Infectieuze uveïtis is de meest voorkomende vorm van uveïtis in Indonesië, waarbij uveïtis ten gevolge van systemische tuberculose behoort tot één van de belangrijkste oorzaken. De studies in dit proefschrift geven verder aan dat een *Mtb*-infectie een cruciale rol speelt bij een aanzienlijk deel van de patiënten “met onverklaarde uveïtis”. Twee specifieke klinische manifestaties werden vooral gezien bij QFT-positieve patiënten met “uveïtis van onbekende origine” in Nederland: retinale occlusieve

vasculitis en serpigineuze choroïditis. Bij deze twee vormen van uveïtis moet men dus alert zijn op de mogelijkheid van een *Mtb*-geassocieerde uveïtis. QFT-positieve patiënten met uveïtis kunnen baat hebben van behandeling met ATT, ondanks de afwezigheid van systemische kenmerken van actieve TB. *Mtb* geïnfecteerde RPE-cellen reageerden met een sterke interferon-signalering en in het perifere bloed werd een specifieke type 1 IFN-handtekening score gemeten die geassocieerd is met actieve TB (met en zonder uveitis). Op basis van deze perifeer bloed type 1 IFN-handtekening kan bij QFT-positieve patiënten met een onverklaarde uveïtis, een patiënt-stratificatie plaatsvinden. Deze laatste bevinding kan de basis vormen voor een prospectieve therapeutische studie. Ten slotte werd er geen evident bewijs gevonden dat auto-antistoffen tegen retinale antigenen betrokken zijn in de pathogenese van *Mtb*-geassocieerde uveïtis. Indien auto-immuunprocessen een rol spelen in *Mtb*-geassocieerde uveïtis lijkt een cellulaire T-cel respons waarschijnlijker.

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Home is where your heart is and the first long-term apartment I rent together with dr. Yulia Rosa Saharman (Lili) was entirely a home for me in Rotterdam. Thank you dr. Lili, you have been a friend and even a sister during our PhD journey. We had fun in that apartment, and we were lucky to have great landlords, Stephan Hoogwerf and Monique. We felt welcome and in one way or another, it provided a significant support in our PhD process. You can find the picture of our lovely apartment on the cover of this book. The photo was taken when I was on one of my afternoon run and saw a beautiful view of the sunset from across the water. I rediscovered my love for running during my stay in Rotterdam. I even joined Rotterdam ladies run (my first 5K run) with my neighbor Siskha and Dowty. Looking back, my early PhD years in that apartment left such fond memories.

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CURRICULUM VITAE

Rina La Distia Nora was born on the 7th November 1979 in Banda Aceh, Indonesia. She grew up in Jakarta and graduated from high school at 8 Senior High School, Jakarta in 1998. She entered Faculty of Medicine Universitas Indonesia in 1998, and she obtained her medical degree in 2004. She was accepted for ophthalmology residency in Faculty of Medicine Universitas Indonesia/ Cipto Mangunkusumo Hospital (FKUI/RSCM), Jakarta, Indonesia in 2005 and graduated as an ophthalmologist in 2009. She was recruited to become a medical staff in Infection and Immunology division in Ophthalmology Department FKUI/RSCM. Consequently, she underwent an internal fellowship program in that division for two years and officially started as medical staff in February 2011. In December 2013, she officially began her Ph.D. study described in this thesis under the supervision of Prof. dr. P. Martin van Hagen (Clinical Immunology, Internal Medicine) and Prof. dr. A. Rothova (Ophthalmology).

PHD PORTFOLIO

Name PhD student:	Rina La Distia Nora
Erasmus MC Department:	Immunology
PhD period:	December 2013 – June 2018
Promotors:	Prof. dr. P. Martin van Hagen, Prof. dr. A. Rothova

Courses and workshops

2014	Basic Flow Cytometry & Advanced Flow Cytometry, Immunology Department, Erasmus MC, Rotterdam
2014	Photoshop and Illustrator CS6 course for PhD-students and other researcher, Postgraduate School Molecular Medicine, Erasmus MC, Rotterdam
2014	Molecular Diagnostic IX, Postgraduate School Molecular Medicine, Erasmus MC, Rotterdam
2014	Next Generation Sequencing (NGS) in DNA Diagnostic Course, The Postgraduate School Molecular Medicine, Erasmus MC, Rotterdam
2014	Biomedical Research Techniques XII, Postgraduate School Molecular Medicine, Erasmus MC, Rotterdam
2014	Research Management for PhD-students, Postgraduate School Molecular Medicine, Erasmus MC, Rotterdam
2014	Molecular Genetics and Therapeutics of Ophthalmic Disorders Seminar and Practical workshop on DNA Isolation, PCR, and RT-PCR in Ophthalmology Department FKUI/RSCM, Jakarta, Indonesia, in collaboration with Radboud University Nijmegen, the Netherlands.
2015	Partek Course on Microarray and Next Generation Sequencing, Postgraduate School Molecular Medicine, Erasmus MC, Rotterdam
2015	English Biomedical Writing and Communication, Erasmus MC, Rotterdam
2015	Good Clinical Practice Course & Workshop, by Clinical Study Unit, Faculty of Medicine, Universitas Indonesia
2015	Advanced Immunology Course, by Immunology Department and the Postgraduate School Molecular Medicine, Erasmus MC, The Netherlands
2015	Genetic for Dummies, by the Postgraduate School Molecular Medicine, Erasmus MC, The Netherlands
2015	11th Vienna Ophthalmic Wetlab Course, Vienna, Austria

2015	SNP Course XII: SNPs and Human Disease, The Postgraduate School Molecular Medicine, Erasmus MC, The Netherlands
2016	Workshop Ingenuity Pathway Analysis (IPA), The Postgraduate School Molecular Medicine, Erasmus MC, The Netherlands
2016	Course On R, by the Postgraduate School Molecular Medicine, Erasmus MC, The Netherlands
2016	Research Integrity, Department Medical Ethics and Philosophy of Medicine, Erasmus MC, The Netherlands
2017	CPO Course 2017 Patient Oriented Research: Design, Conduct, and Analysis, Erasmus MC, The Netherlands
2017	Panelist in Global Ocular Inflammation Workshop (GOIW), Asia Pacific Intraocular Inflammation Study Group (APIISG), Indonesian Infection and Immunology Society Meeting (INOIIS), Bali, Indonesia
2017	Leadership for Effective Team Management, Medical Faculty Universitas Indonesia (FKUI), Jakarta
2017	Training of trainers, held by the Royal College of Ophthalmologist and Kolegium Oftalmologi Indonesia (KOI), Bandung, Indonesia.
2017	Bayesians statistics and JASP, the Postgraduate School Molecular Medicine, Erasmus MC, The Netherlands
2018	Microsoft access basic, the Postgraduate School Molecular Medicine, Erasmus MC, The Netherlands

Presentations

2013	2nd Dutch Ophthalmology PhD Students' (DOPS) conference meeting, Nijmegen, the Netherlands. Title: QuantiFERON Positive Uveitis in the Netherlands?
2014	Meet the expert in Kursus Penyegar dan Penambah Ilmu Kedokteran (KPPIK) FKUI 2013, Jakarta, Indonesia. Title: The Red Eye, Current Concept for Primary Care Decision: Microbial conjunctivitis, to treat or not to treat?
2014	Indonesian Radio/ Radio Republik Indonesia (RRI) about the publication: Clinical characteristic and therapy results of presumed ocular tuberculosis and their relation to HIV status. (Med J Indones. 2012;21:214-9)

2014	12th International Ocular Inflammation Society Congress, Valencia, Spain. Title: 1. Clinical Manifestations of Patients with Intraocular Inflammation and Positive QuantiFERON-TB Gold in-Tube Test in Country Non-endemic for Tuberculosis 2. Intracameral Levofloxacin for Intra Operative Prophylaxis of Acute Endophthalmitis Post Cataract Surgery: Safety Profile 3. E-poster: A rare case of Blau Syndrome from Indonesia 4. E-poster: Intracameral levofloxacin for intra operative prophylaxis of acute endophthalmitis post cataract surgery: efficacy profile
2015	40th Annual Scientific Meeting of Indonesian Ophthalmologist, Bandung, Indonesia. Title: Tuberculosis Related Uveitis in Indonesia
2015	13th International Ocular Inflammation Society Congress, in San Fransisco, California, the United States. Title: Tuberculosis Related Uveitis in Indonesia
2016	41st Annual Scientific Meeting of Indonesian Ophthalmologist, Jakarta, Indonesia. Title: Tuberculous Uveitis
2017	Symposium Bandung Eye Center. Title: Ocular surface problem Related to systemic diseases
2017	41st Annual Scientific Meeting of Indonesian Ophthalmologist, Malang, Indonesia. Title: Ocular manifestation of skin diseases
2017	Symposium Childhood Eye Disorder, Departemen Medik Mata RSCM Kirana. Title: Pediatric uveitis
2017	ASEAN Ophthalmology Society Congress 2017, Jakarta, Indonesia. Title: Challenges in microbiological proven etiology from intraocular fluid in uveitis patients
2017	14th Congress of The International Ocular Inflammation Society (IOIS) and The 4th International Assembly Ocular Inflammation Societies, the Topic was Prognostic Clinical Features of Mycobacterium Tuberculosis Intraocular Inflammation, Lausanne, Switzerland
2018	Poster in Molecular medicine day, the Postgraduate School Molecular Medicine, Erasmus MC, The Netherlands.

(Inter)national conferences

2013	2nd Dutch Ophthalmology PhD Students' (DOPS) conference meeting, Nijmegen, the Netherlands
2014	ARVO-Ned, Utrecht, The Netherlands
2015	5th Dutch Ophthalmology PhD Students' (DOPS) conference meeting, Nijmegen, the Netherlands
2015	European Society of Ophthalmology Congress, Vienna, Austria
2015	40th Annual Scientific Meeting of Indonesian Ophthalmologist, Bandung, Indonesia
2015	13th International Ocular Inflammation Society Congress, in San Fransisco, California, the United States
2016	8th WID Expert Meeting, Santpoort, the Netherlands
2016	9th International Symposium of Uveitis, International Uveitis Study Group (IUSG), Dublin, Ireland
2016	'Eilanddagen Oogheelkunde' Congress, Schiermonnikoog, The Netherlands
2016	41st Annual Scientific Meeting of Indonesian Ophthalmologist, Jakarta, Indonesia
2017	Indonesian Primary Immunodeficiency Clinical Care Meeting, Jakarta, Indonesia
2017	42nd Annual Scientific Meeting of Indonesian Ophthalmologist, Malang, Indonesia
2017	14th Congress of The International Ocular Inflammation Society (IOIS) and The 4th International Assembly Ocular Inflammation Societies, Lausanne, Switzerland

Teaching activities

2013	Supervising research master 4th year medical student of Erasmus MC to do retrospective descriptive study on Uveitis and scleritis in Indonesia.
2011- current	Lecturer in Ophthalmology Department Universitas Indonesia for medical students and ophthalmology residents.

Other academic activities

April- September 2016	Weekly clinical work at uveitis outpatient department of ophthalmology, Erasmus MC
April- September 2016	Weekly clinical ophthalmology-immunology seminars, Erasmus MC

PUBLICATIONS

1. Sitompul R, **La Distia Nora R**. Glaucoma and dry eye disease: the role of preservatives in glaucoma medications. Med J Indones 2011;20:302-305.

2. **La Distia Nora R**, Sitompul R, Susiyanti M, Edwar L, Sjamsoe S. Clinical characteristic and therapy results of presumed ocular tuberculosis and their relation to HIV status. Med J Indonesia 2012;21:214-219.

3. **La Distia Nora R**, Van Velthoven MEJ, Ten Dam-Van Loon NH, et al. Clinical manifestations of patients with intraocular inflammation and positive QuantiFERON-TB gold in-tube test in a country nonendemic for tuberculosis. Am J Ophthalmol 2014;157:754-761.

4. Asroruddin M, **La Distia Nora R**, Edwar L, Sjamsoe S, Susiyanti M. Various factors affecting the bacterial corneal ulcer healing: A 4-years study in referral tertiary eye hospital in Indonesia. Med J Indonesia 2015;24:150-155.

5. Cut Putri S, **La Distia Nora R**, Susiyanti M. Response of plasminogen deficiency associated ligneous conjunctivitis to topical fresh frozen plasma with heparin. Journal of Case Reports 2015;5:132-136.

6. Ratnasari M, **La Distia Nora R**, Susiyanti M. Folliculitis extending into preseptal cellulitis and abscess in children. Journal of Case Reports 2015;5:45-52.

7. Rhendy R, **La Distia Nora R**, Susiyanti S. Challenging diagnosis of atypical toxoplasmic neuroretinitis in children: a case series. Journal of Case Reports 2015;5:564-571.

8. **La Distia Nora R**, Sitompul R, Bakker M, et al. Tuberculosis and other causes of uveitis in Indonesia. Eye (Lond) 2017.

9. **La Distia Nora R**, Walburg KV, van Hagen PM, et al. Retinal Pigment Epithelial Cells Control Early Mycobacterium tuberculosis Infection via Interferon Signaling. Invest Ophthalmol Vis Sci 2018;59:1384-1395.