The Eicosanoid system – effects on pathogenesis and potential as targets for Host Directed Therapy in Tuberculosis

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Abbreviations

AA – Arachidonic acid
AIDS – Acquired immunodeficiency virus
ART – Antiretroviral therapy
ALOX5- Arachidonate 5-lipoxygenase gene
APC – Antigen presenting cell
BCG – Bacille Calmette Guérin
cAMP – Cyclic adenosin monophosphate
CD – Cluster of differentiation
CFU – Colony-forming unit
COX – Cyclooxygenase
COX-I – Cyclooxygenase inhibitor
CREB – cAMP response element binding protein
DC – Dendritic cell
DOT – Direct observed therapy
ELISA – Enzyme-linked immunosorbent assay
EP1-4 – Prostaglandin E2 receptor 1-4
EPTB – Extrapulmonary TB
Erk 1/2 - Extracellular signal kinase 1/2
ESAT – Early secretory antigenic target
FcR – Fc Receptor
FoxP3 – Forkhead box P3
GM-CSF – Granulocyte macrophage – colony stimulating factor
HDT – Host directed therapy
HIV – Human immunodeficiency virus
HLA-DR – Human leukocyte antigen DR isotype
IRIS – Immune reconstitution inflammatory syndrome
IFN – Interferon
IGRA – Interferon y release assay
IL- Interleukin
iNOS- Inducible nitric oxide synthase
IRF – Interferon regulatory factor
LC-MS – Liquid chromatography-mass spectrometry
LOX – Lipoxygenase
LPS - Lipopolysaccharide
LTA4H – Leukotriene A4 hydrolase
LTB4 – Leukotriene B4
LTBI – Latent tuberculosis
LXA4 – Lipoxin A4
Mav – Mycobacterium avium
MCP – Monocyte chemoattractant protein
MDM – Monocyte-derived macrophage
MDR-TB – Multidrug-resistant TB
MDSC – Myeloid derived suppressor cell
MHC – Major histocompatibility complex
MIP – Macrophage inflammatory protein
MMP – Matrix metalloproteinase
mRNA – Messenger RNA
Mtb – Mycobacterium tuberculosis
MTBC – Mycobacterium Tuberculosis Complex
NAAT – Nucleic acid amplification test
NFkB – Nuclear factor kB
NO – Nitric oxide
NSAID – Non-steroidal anti-inflammatory drug
NTM – Nontuberculous mycobacteria
P38 MAPK – p38 Mitogen activated protein kinase
PAMP – Pathogen associated molecular pattern
PBMC – Peripheral blood mononuclear cell
PD-1 – Programmed death receptor 1
PD-L1 – Programmed death ligand 1
PGE2 – Prostaglandin E2
PGES – Prostaglandin synthase
PKA – Protein kinase A
POC – Point of care

POCT – Point-of-care-testing
PPD – Purified protein derivative
PRR -Pattern recognition receptor
RCT – Randomized clinical trial
RNA – Ribonucleic acid
ROI – Reactive oxygen intermediate
ROS – Reactive oxygen species
TB – Tuberculosis
Tbet – T-box transcription factor
TCR – T cell receptor
TGF – Tumor growth factor
Th – T helper cell
TLR – Toll like receptor
TNF – Tumor necrosis factor
Treg – T regulatory cell
TST – Tuberculin skin test
VEGF – Vascular endothelial growth factor
WHO – World Health Organization
XDR-TB – Extensively drug-resistant TB

List of publications

Paper 1 **Elevated Levels of Anti-inflammatory Eicosanoids and Monocyte Heterogeneity in Tuberculosis**

Kristin Grotle Nore, Marthe Jøntvedt Jørgensen, Anne Ma Dyrhol-Riise, Synne Jenum, Kristian Tonby.
Front. Immunol. 2020. 11:579849.

Paper 2 **Plasma LOX-products and monocyte signaling is reduced by adjunctive cyclooxygenase-2 inhibitors in a phase I clinical trial of patients with tuberculosis**

Marthe Jøntvedt Jørgensen, Kristin Grotle Nore, Synne Jenum, Hans Christian D. Aass, Kjetil Taskén, Dag Kvale, Emilie Layre, Jérôme Nigou, Rasmus Mortensen, Kristian Tonby, Anne Ma Dyrhol-Riise.
Front. Cell Infect Microbiol. 2021. 11:669623.

Paper 3 **The Cyclooxygenase 2 inhibitor etoricoxib as adjunctive therapy in Tuberculosis impairs macrophage control of mycobacterial growth**

Kristin Grotle Nore, Claire Louet, Marit Bugge, Alexandre Gidon, Marthe Jøntvedt Jørgensen, Synne Jenum, Anne Ma Dyrhol-Riise, Kristian Tonby, Trude Helen Flo
In manuscript, to be submitted fall 2022

Summary 1 (English)

Worldwide, Tuberculosis (TB) is one of the top 10 causes of death and poses a great challenge to global health. Current TB treatment strategies consist of long-lasting multiple drug regimens with risk of serious side effects, and reduced compliance to treatment contributes to the development of multi-drug resistant TB (MDR-TB). Thus, new knowledge of immunopathogenesis in TB and discovery of novel treatment modalities are warranted. Host-Directed Therapy (HDT) in TB is adjuvant treatment aiming to modulate host inflammatory pathways by 1) reducing excessive and harmful inflammation and 2) improving immune effector mechanisms for more effective pathogen control.

Previous studies, and in particular preclinical animal studies, indicate that lipid mediators such as eicosanoids, are of great importance in modulating the host response during infection with *Mycobacterium tuberculosis (Mtb)*. In humans, there are few studies exploring the eicosanoid pathways in *Mtb* infection. Thus, the overall aim of this thesis has been to explore the effects of eicosanoids on immune pathogenesis in *Mtb* infection and investigate the potential of selective targeting of eicosanoid pathways with Cyclooxygenase-2 inhibitors (COX-2is) as HDT adjunctive to standard TB treatment.

In paper 1, we report that anti-inflammatory eicosanoids are associated with severity of TB disease. Levels of the anti-inflammatory mediator Lipoxin A4 (LXA4) at diagnosis were elevated in active disease compared to patients with latent infection, while there were no differences in levels of Prostaglandin E2 (PGE2) and Leukotriene B4 (LTB4) between different clinical states of *Mtb* infection. LTB4 was the only mediator to be reduced upon treatment, along with a reduction of the LTB4/LXA4 ratio, emphasizing that the balance between eicosanoids may be of importance during *Mtb* infection. Further, we showed that both innate and adaptive immune cells seemingly make important contributions to eicosanoid mediator production.

In paper 2, we explored monocyte intracellular signaling and levels of eicosanoids in samples from a phase 1/2 clinical trial (TBCOX2 study), analyzing effects of a COX-2i as adjunctive therapy. We found that specific intracellular signaling pathways in monocytes, as well as plasma levels of pro-inflammatory cytokines, were reduced in the COX-2i group compared to

controls. Consequently, COX-2i modulates the phosphorylation patterns in monocytes by reducing the responsiveness upon stimulation. Lipoxygenase (LOX)-derived products and pro-inflammatory cytokines were associated with TB disease severity, and were reduced during TB therapy, possibly accelerated by adjunctive COX-2i.

In paper 3, monocyte-derived macrophages from patients receiving adjunctive COX-2i treatment in the TBCOX2 study had lower capacity to control *in vitro* mycobacterial infection compared to macrophages from the control group. Macrophages from patients in the COX-2i group also had lower basal expression of inflammatory genes and lowered pro-inflammatory cytokine secretion compared to controls. Our data implies that the effect of adjunctive COX-2i treatment may be imprinted in circulating immune cells. Thus, COX-2i impair macrophage potential to control mycobacterial growth, an effect possibly caused by an overall lowered pro-inflammatory function.

Taken together, our studies provide important knowledge about dynamics of eicosanoids in different stages of *Mtb* infection. Importantly, our data show potential unfavorable effects of COX-2i treatment in *Mtb* infection and may not support COX-2i as HDT in TB.

Summary 2 (Norwegian)

Tuberkulose (TB) er en av topp 10 ledende dødsårsaker på verdensbasis med påfølgende store konsekvenser for global helse. Verdens Helseorganisasjon (WHO) anslår at 10 millioner personer får TB hvert år og at 1,3 millioner dør som følge av sykdommen. Nåværende behandlingsstrategier for tuberkulose består av langvarige regimer med stor risiko for bivirkninger og dermed redusert etterlevelse. Dette utgjør en stor fare for resistensutvikling, som er et enormt og økende problem globalt. Utvikling av nye behandlingsstrategier prioriteres høyt internasjonalt med spesiell oppmerksomhet på såkalt Host-Directed Therapy (HDT), immunmodulerende behandling som retter seg mot vertens inflammatoriske respons. Målet er å fremme gunstige vertsresponser samt begrense replikasjon og spredning av mykobakterier.

Lipid mediatorer, kjent som Eikosanoider, er vist å være av stor betydning for immumodulering av vertsresponsen under infeksjon og har vist seg å ha stor innflytelse på utfallet av TB i prekliniske modeller. Tradisjonelt antas de COX-avledede prostaglandinene, hvor Prostaglandin E2 (PGE2) er sentral, å ha pro-inflammatoriske egenskaper, mens 5-LOX-deriverte leukotriener og lipoxiner har henholdsvis pro- og antiinflammatoriske effekter. Balansen av disse mediatorene blir sett på som en avgjørende komponent for det medfødte antimykobakterielle forsvaret, hvor antagonistiske effekter av eikosanoider ser ut til å være av stor relevans i TB patogenesen. Studier av immunceller og deres forhold til eikosanoid-biosyntese kan avsløre viktig informasjon om vertsresponsen under *Mtb*-infeksjon og muligens gi nye mål for immunterapeutisk intervensjon.

PhD-prosjektet **"The Eicosanoid system – effects on pathogenesis and potential as targets for Host Directed Therapy in Tuberculosis"** utforsker effektene av eikosanoider på immunpatogenesen ved TB, med videre studier av forholdet mellom eikosanoider og andre komponenter i immunsystemet. Vårt overordnede mål var å få en bedre forståelse av immunresponser ved *Mtb* infeksjon, samt utforske effekter ved adjuvant behandling (tilleggsbehandling) ved tuberkulose sykdom. I dette prosjektet har vi kartlagt immunresponser hos pasienter inkludert i den kliniske studien «TBCOX2: An open label phase I clinical trial of the therapeutic TB H56:IC31 vaccine and cyclooxygenase-inhibitors» (NCT02503839). I denne studien ble en betennelsesdempende medisin (COX-hemmer

etoricoxib) gitt i tillegg til standard TB behandling med mål om en forbedret immunrespons i TB. Hovedfokus i våre studier var å undersøke eikosanoiders potensiale som mål for HDT, der vi i Artikkel 1 utforsket nivået av eikosanoider og betydningen av monocyttheterogenitet i forskjellige stadier av *Mtb*-infeksjon. Artikkel 2 utforsket intracellulær signalisering i medfødte immunceller (monocytter) og evaluerte nivåer av eikosanoider i pasientprøver fra TBCOX2-studien. Artikkel 3 utforsket ytterligere immunresponser i pasientprøver fra TBCOX2-studien, inkludert studier av makrofagers mykobakterielle drapseffekt etter etoricoxib-behandling. Vårt overordnede mål var å undersøke immunresponser med fokus på eikosanoidnettverket i TB, samt studere hvordan disse nettverkene påvirkes av selektiv hemming av signalveier. Oppsummert gir vårt arbeid ny kunnskap om eikosanoid-nettverket ved human *Mtb*-infeksjon og TB-sykdom, der våre resultater bekrefter de mulige skadelige effektene av antiinflammatoriske eikosanoider. Videre viser våre resultater potentielt ugunstige effekter ved bruk av COX-hemmere som HDT ved *Mtb*-infeksjon. Samlet gir våre studier ny innsikt i de potensielle effektene av HDT rettet mot eikosanoid-biosyntese ved TB.

1 Introduction: Tuberculosis

1.1 Epidemiology and global health

Tuberculosis (TB) is a communicable disease with a huge impact on global health. In 2021, the World Health Organization (WHO) reported 10.0 million (8.9-11.0) new cases of TB, causing 1.2 million (1.1-1.3) deaths worldwide [1]. It is estimated that one-fourth of the planet's population is latently infected with *Mycobacterium tuberculosis* (*Mtb*), the causative agent of TB, thus representing a considerable reservoir for possible reactivation of TB disease [2].

Interlinked with poverty, most of the TB burden is carried by underdeveloped countries, wherein 90 % of new TB cases worldwide arise in 30 countries. These high TB burden countries include India, Indonesia, China, Pakistan, Nigeria, Bangladesh, and South Africa[3]. Factors such as crowding and poor living conditions, comorbidity, undernutrition, air pollution and lack of access to proper health care are important determinants that drive the transmission of disease [4].

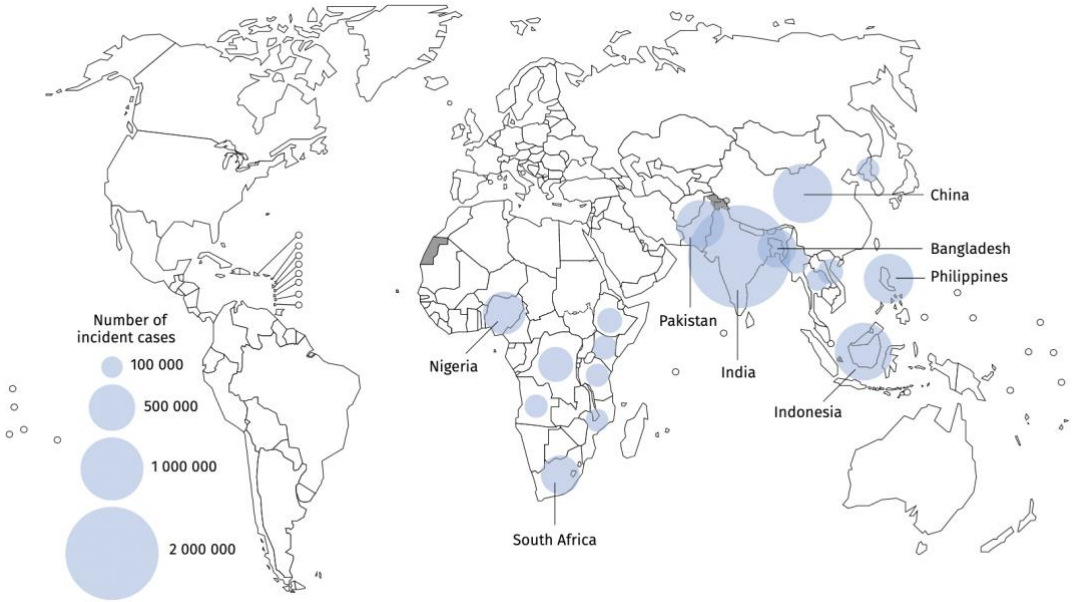


Figure 1 Estimated TB incidence in 2020, for countries with at least 100 000 incident cases. Global tuberculosis report 2021. Geneva: World Health Organization; 2021. License: CC BY-NC-SA 3.0 IGO.

The emergence of the HIV epidemic fueled the incidence of new TB cases [5]. TB remains the leading cause of death among people living with HIV, and in 2021, 8.2 % of confirmed TB cases were HIV positive [6]. HIV/TB co-infection leads to a higher risk of reactivation and progression of TB disease and increases the risk of severe or fatal forms of TB [7]. With the emergence of drug resistant *Mtb* strains, these are two major challenges to treatment. In 2019, an estimated 3.3% of newly infected cases and 17% of previously treated cases were reported as resistant to one or more of the first-line TB drugs, making drug resistance one of the most critical obstacles to ending the TB epidemic [8]. Drug resistance requires increasingly toxic, costly, and lengthy treatment regimens, and challenges with patient compliance and lack of access to diagnostics such as molecular methods of resistant testing, propose a risk of resistance transmission [9]. Thus, the continuing spread of multi-drug resistant (MDR)-TB is one of the most urgent and difficult challenges facing global control, making it one of the focus points to consider in the strategy to eradicate TB [2].

The WHO's End TB Strategy from 2014 aims to reduce deaths and limit new cases by 90 % within 20 years [10]. Although incidence rates have steadily decreased, as of 2020, the milestone to accomplish a 20 % reduction in new cases was not reached [11]. Moreover, the Covid-19 pandemic has been devastating for the efforts to control the TB epidemic, with reversal of progress in reaching the global End TB targets[12]. Notably, the Covid-19 pandemic makes access to proper health care services challenging, and it is estimated that 1.4 million fewer people received care in 2020 [13]. In countries with high TB burden, scarce resources have been allocated to manage the Covid-19 response, which has undermined TB testing and treatment programs, including drug delivery and follow-up[12]. Thus, the 21% reduction of new cases in 2020 is not due to a sudden success in TB programs worldwide but a sign of reduced access to proper health care. Overwhelmed health-care services and the economic impact of Covid-19 further diminish global control efforts. Consequently, it is estimated a 0,5 million excess TB deaths due to the pandemic, and in the wake of Covid-19, for many people worldwide, it once again faces status as a neglected disease[14].

1.2 Transmission, Pathogenesis, and immunology

1.2.1 Mycobacterium Tuberculosis and the Mtb complex

TB is caused by the acid-fast bacteria *Mtb*, which is part of the *Mycobacterium tuberculosis* complex (MTBC) consisting of genetically related mycobacteria *(Mtb, M. africanum, M. bovis and M, canetii)[15].* It has been shown that strain variations within MTBC may lead to differences in TB transmission, clinical manifestations of disease, and host immune responses [16]. The global distribution and genetic diversity of MTBC phylogenetic lineages are shown in Figure 2 [17].

The interaction between *Mtb* genotype and host genetic factors result in variable immune responses when it comes to effector functions of innate immune cells, bacterial clearance, and phagocytosis as well as initiation of adaptive responses [16]. Further, variation in strains might affect diagnostics, vaccine development and treatment. Thus, lineage diversity may constitute a major factor to consider when developing host-directed therapy in TB [16].

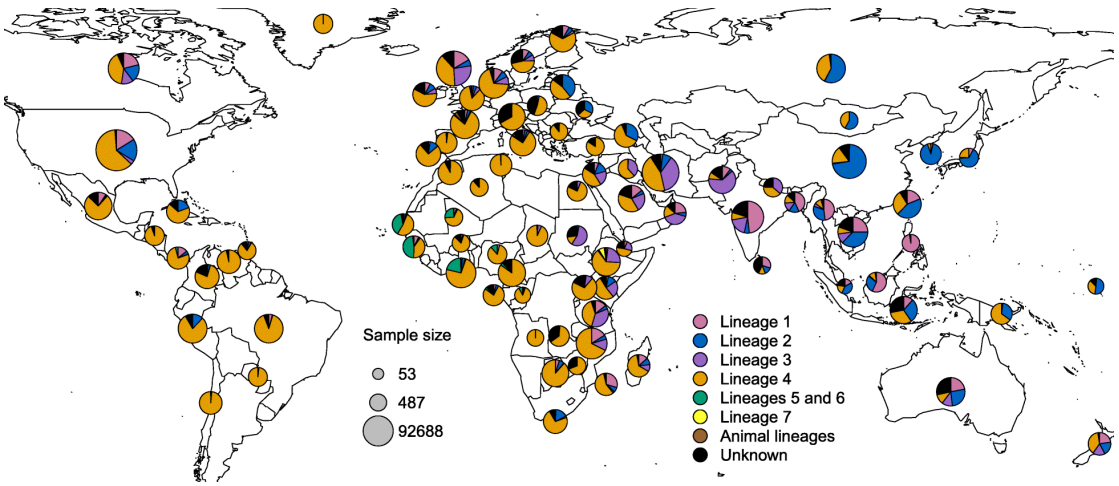


Figure 2 The global distribution and genetic diversity and of MTBC phylogenetic lineages. Wiens, K.E., Woyczynski, L.P., Ledesma, J.R. et al. Global variation in bacterial strains that cause tuberculosis disease: a systematic review and meta-analysis. BMC Med 16, 196 (2018). https://doi.org/10.1186/s12916-018-1180-x.[17] Reprinted by permission.

1.2.2 Transmission of Mtb

Individuals with pulmonary TB may transmit airborne bacilli in droplets or aerosols by talking, sneezing, or coughing, where the risk of transmission is dependent on several factors, such as exposure duration and intensity[18].

When transmission of bacilli occurs, inhaled droplets containing bacteria reach the lungs of the exposed individual and may result in infection and disease, depending on bacterial and host factors[19]. The transmitted bacilli infect resident innate immune cells in the small airways and alveoli of the host that generate an inflammatory response, which in most cases lead to bacterial containment[19, 20]. However, if the host cannot generate a sufficient immune response towards *Mtb*, active disease may develop[19].

1.2.3 The innate immune response

The early events of *Mtb* infection largely affect the establishment of infection and the risk of disease[20]. Once *Mtb* has reached the lung alveoli, it is detected by pathogen recognition receptors (PRRs) on host immune cells, effectively creating an innate immune response[20]. Resident alveolar macrophages rapidly engulf invading bacteria, which are then internalized and contained in endocytic vacuoles called phagosomes[21]. This process is a well-known strategy that innate immune cells utilize to rid the host of invading pathogens, as the phagosome maturation cycle known as the phagosome-lysosome fusion creates a microenvironment that is hazardous and detrimental for many pathogens[22]. Low acid pH inside the phagolysosome, and the production of reactive oxygen intermediates (ROI), lysosomal enzymes, and toxic peptides, are only some of the mechanisms utilized to eliminate microorganisms[23]. However, *Mtb* may survive inside phagosomes by inhibiting phagosome-lysosome fusion, thus avoiding discovery by adaptive immune cells [23, 24].

Once the innate immune response recognizes pathogens, in parallel to engaging bacterial killing mechanisms, it also produces mediators that alarm and attract other immune cells to infection site [20]. Production of pro-inflammatory cytokines IL-6, IL-12, IL-1β by macrophages, neutrophils, and other immune cells initiates a cascade of events leading to local and systemic inflammatory effects that are critical to combat invading mycobacteria but

may cause harmful tissue injury when produced in a dysregulated fashion [24]. Production of pro-resolving cytokines like IL-10, TGFb, IL-1RA and VEGF-A act in an antagonistic manner, limiting tissue injury and promotes tissue repair [25].

Processed mycobacterial antigens carried by antigen-presenting cells (APCs) such as dendritic cells (DCs) and monocytes reach the lymph nodes and are presented to cells of the adaptive immune system by major histocompatibility complex (MHC) class II molecules [26]. By expressing co-stimulatory signals such as APC-secreted Interleukin-12 (IL-12), innate cells efficiently initiate a proper adaptive immune response and promote development of CD4+ T cells of the TH1 subtype, necessary for bacterial clearance [20]. IFN-γ-dependent activation of macrophages by these CD4+ T cells is imperative to successful immunity against intracellular bacilli [23, 27].

To contain the spread of bacteria, an organized inflammatory focus composed of macrophage-derived giant cells with surrounding lymphocytes begins to form. This is called a granuloma and is one of the hallmarks of TB [27]. Infected macrophages in the center of the granuloma undergo apoptosis and necrosis (i.e., programmed, and non-programmed cell death), leading to a caseous center of dead cells and debris surrounded by a cellular zone of lymphocytes, monocytes, and fibroblasts[27]. Here, the body's immune system can eliminate or contain the bacteria in a dormant state. However, if the host response is weakened or waned, bacteria may replicate, escape, and spread within the lung, resulting in active disease[27, 28]. Figure 3 shows characteristics of granulomas in different stages with different levels of immune control and bacterial burden [29].

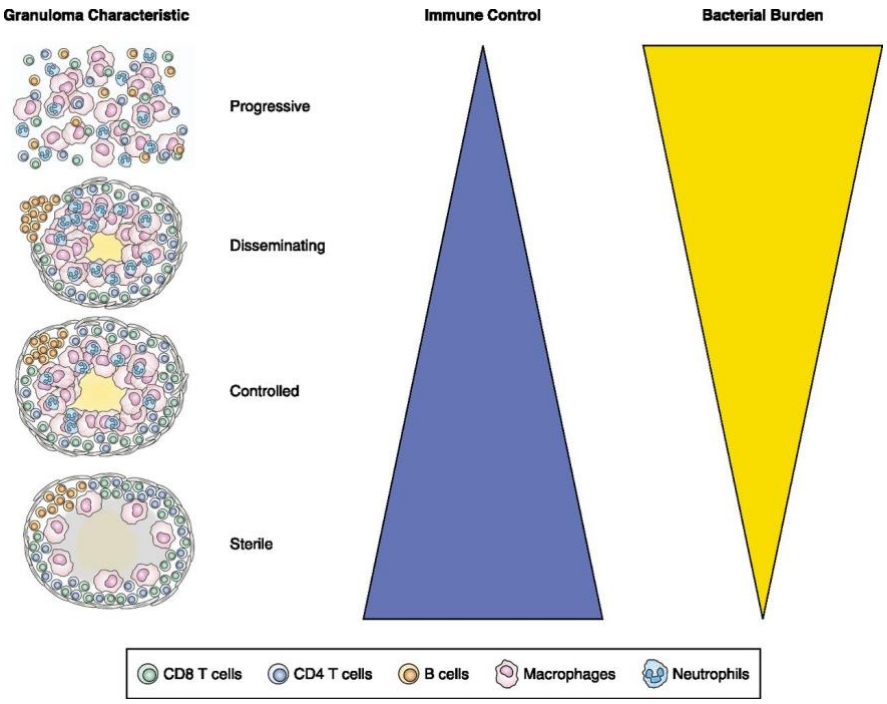


Figure 3 Spectrum of granuloma types in *Mtb* infection. The End of the Binary Era: Revisiting the Spectrum of Tuberculosis, Philana Ling Lin, JoAnne L. Flynn, The Journal of Immunology November 1, 2018, 201 (9) 2541-2548; DOI: 10.4049/jimmunol.1800993[29]. Reprinted with permission.

The immunologic microenvironment within granulomas is exceedingly complex and represents a spectrum of pathology, where developing therapeutic strategies are designed to shift the balance of TB granuloma formation toward protective rather than destructive processes [21, 23, 27, 28]. With the establishment of *Mtb* infection, and progression of disease, granulomas enlarge and undergo alterations in their morphological feature, becoming increasingly necrotic. As such, because of exhausted or dysfunctional immunity, the granuloma loses its protective functions, beneficial for *Mtb* [21, 27, 28]. These processes increase the potential for bacilli to spread from the primary site of infection, the lung, to other tissues and organs [30]. Some argue that the local host–pathogen response in each individual granuloma determines the overall control of the infection, where disruption of a single granuloma may lead to dissemination [20, 30].

Although macrophages are considered the dominant innate immune cell engaged in protective responses against mycobacteria, other innate immune cells are highly involved [20]. Monocytes are bone marrow-derived leukocytes that circulate in the blood and differentiate into monocyte-derived macrophages and monocyte-derived dendritic cells that govern innate and adaptive immunity [31]. They are thought to be critical in the antimycobacterial defense

as they are recruited to the lung during infection and participate in phagocytosis, innate activation, and promote the triggering of adaptive immune mechanisms [31, 32]. In humans, three circulating monocyte subsets are classified based on their relative expression levels of CD14 and CD16 surface proteins, namely classical, intermediate, and non-classical subsets, where subsets have been shown to harbor both phenotypically and functionally different characteristics [33]. Researchers propose that these inherent functional differences in monocyte subsets contribute to the overall outcome of infection following potential skewing of one subset over the others [20, 34-36]. Here, an expansion of the intermediate subset has been linked to severity of TB disease, though contrary reports exist [31, 37].

Early events following infection is critical in determining outcome, and for development of effective HDT, a clear understanding of early host responses against *Mtb* is necessary [38]. Importantly, the interaction between *Mtb* and the cells of the innate immune system have considerable consequences on the subsequent adaptive immune response, and thus, the overall bacterial control [20].

1.2.4 The adaptive immune response

The adaptive immune system is antigen-specific and can recognize previously encountered pathogens[39, 40]. Although it is uncertain what constitutes a protective immunity in *Mtb* infection, cell-mediated immunity and CD4 T cells play a crucial role, and clearance of bacteria depends on the success of the macrophage-T cell interaction [41].

A characteristic of adaptive immunity in TB is that it is delayed for several weeks following infection, which has been proposed to be due to both immune-evasive mechanism by mycobacteria as well as host-factors [39, 42]. Dendritic cells (DCs) are professional antigen-presenting cells (APCs) that are critical in bridging innate and adaptive immunity by capturing, processing, and presenting antigens [43]. Here, DC traffic to the lymph nodes where they prime naïve T cells and induce proliferation of effector cells. Almost 3 weeks after infection, these effector T cells arrive in the lung, which gives *Mtb* an opportunity to replicate and modulate inflammation at local sites of infections [20, 39]. The reason for the delayed onset of adaptive immunity is incompletely understood, although several mechanisms have been suggested. *Mtb* may interfere and inhibit maturation of DCs that limit the trafficking,

while another possible explanation is that *Mtb* inhibits apoptosis of macrophages and neutrophils, thereby delaying antigen-presentation [40].

Production of IFN-γ by CD4 T cells of the TH1 subtype is critical to immune control in *Mtb* infection as it leads to activation of the macrophage and enhances bacterial killing mechanisms [44, 45]. This is demonstrated by the increased mycobacterial susceptibility in hosts that lack IFN-γ [45]. However, while IFN-γ production is necessary for mycobacterial control, it is not sufficient alone to provide protection. Other mediators that activate macrophages include tumor necrosis factor α (TNF-α), also necessary for granuloma formation and bacterial containment [46]. Further, Granulocyte-macrophage colony-stimulating factor(GM-CSF) and IL-1β also promote macrophage activation, though the latter is mainly produced by macrophages to inhibit intracellular growth of mycobacteria and induce the inflammatory response [45]. As host immunity works synergistically, the production of IL-12, IL-18, and IL-15 by APCs after phagocytosis of bacilli initiate TH1 polarization and IFN-γ production [41].

Although CD4 T cells of the TH1 subtype is considered the most critical for the antimycobacterial defense, also other adaptive cells are involved [47]. CD8 T cells contribute to bacterial clearance by cytokine production and promoting cell lysis [48]. Regulatory T cells (Tregs) limit excessive inflammation by producing anti-inflammatory mediators such as IL-10 and transforming growth factor β (TGFβ) [49, 50], and has been shown to be both beneficial and detrimental [27, 51, 52]. T cells of the TH2 subtype produce IL-4, IL-5, IL-10 and IL-13, and is shown to have antagonistic effects to the TH1 subtype, thus thought to confer unfavorable effects in *Mtb* infection [53]. The role of the third subset of helper T cells, TH1-IL-17 producing CD4 T cells (Th17 cells) in TB, is not yet clear [54-57]. Understanding the roles of these different cell types during infection might provide opportunities to discover new protective effector functions and develop methods to augment their function as part of new vaccination or treatment strategies [58].

1.2.5 Host inflammatory networks

Macrophages initiate the acute phase response by production of IL-1 and IL-6 while initiating IFN-γ producing T cells by generating IL-12, IL-15, and IL-18 [58, 59]. IL-12, the main TH1 inducing cytokine, is essential to establish a successful immune response against

mycobacteria [60]. Production of IL-8, MCP-1, and MIP-1a is important for chemotaxis [61], while for initiation of adaptive immunity, production of CD1, CD80, IL-12, IL-18, TNF-α and IL-1β is critical [41]. In parallel to production of pro-inflammatory mediators, other soluble molecules to induce resolution and promote tissue repair, are induced. Important mediators of this class include IL-1Ra, IL-4, IL-10, and transforming growth factor beta (TGFβ) [62].

Chemotactic cytokines (Chemokines) are a family of small mediators that facilitate immune cell recruitment to site of infection[63]. CXCL1, CXCL2 and CXCL8 drive leukocyte migration, while CCL5, CXCL9, CXCL10 and CXCL11 are crucial for T cell recruitment to infection site [64]. In TB, chemokines have been associated with numerous key processes that lead to *Mtb* containment, from recruitment of myeloid cells into the lung to activation of adaptive immunity, formation of protective granulomas and vaccine recall responses [63]. However, imbalances in several key chemokine mediators can shift the delicate balance of cytokines and cellular responses, thus promoting tissue destruction and spread of *Mtb* [63].

1.2.6 Immune evading mechanisms by Mtb

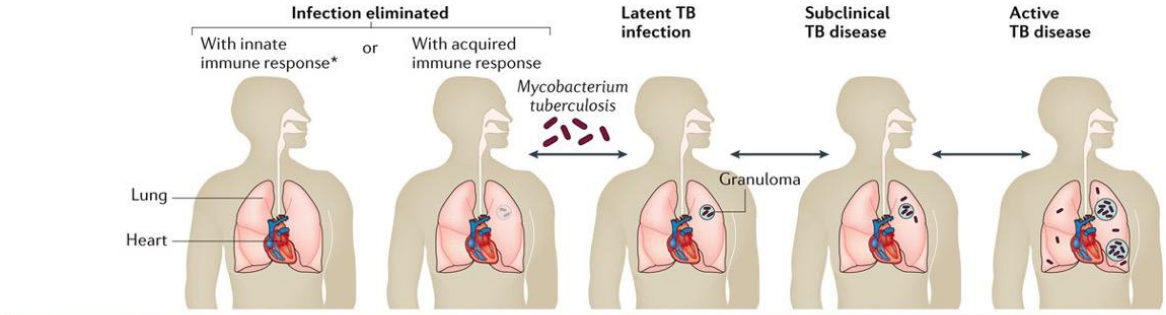
Mtb employs extensive immune-evasive mechanisms to evade the bactericidal activity of macrophages, thus successfully establishing a proliferative niche [65] and allowing bacteria to replicate [66]. First, *Mtb* actively blocks phagosome-lysosome fusion, apoptosis, and autophagy, effectively creating a microenvironment that favors bacteria survival [67, 68]. Second, bacterial manipulation of host cytokine networks disrupts innate responses, such as induction of excessive Type 1 Interferons or TNF-α production [59, 69]. Further, delaying adaptive immune cell responses in early stages by hindering DC migration and priming, interfering with activation of antigen-specific T cells, or inducing Tregs that dampen adaptive immune cells, blocks effective protective immunity [67]. In late stages, bacteria limit antigen presentation, inhibit T cell activation and promotes induction of T cell exhaustion to disrupt host immune responses, which poses a great challenge to vaccine development [67, 70, 71].

1.3 Clinical perspectives: Spectrum of Mtb infection, diagnostics, and treatment

1.3.1 The spectrum of Mtb infection

After transmission, there are mainly three possible outcomes of infection and disease, determined by host, bacterial and environmental factors[18]. About 5-10% of cases develop active disease following infection [5]. However, in about 1/3 of cases, the innate or adaptive immune system eliminate the invading pathogen without leaving a trace of infection [72]. If not fully eliminated, bacteria can persist in the host, though contained by the immunological response, without allowing further spread of bacilli [29]. Thus, mycobacteria are forced into a state of dormancy, referred to as latent tuberculosis infection (LTBI). The host develops a persistent immunological response without clinical evidence of active disease or risk of spread of infection[18]. Latently infected individuals carry a 5-15% risk of reactivation, which mostly occurs within the first two years following exposure[5]. Factors that increase the risk of developing active disease include comorbidities that impair the host immune system, including HIV, cancer, diabetes, organ transplantation, undernutrition, and aging [5].

Traditionally, TB is commonly conceptualized as a simple binary distinction between active TB disease and latent infection (TB infection). However, researchers now argue this to be an outdated concept [29]. Classically, latent TB is defined as evidence of immunological sensitization by mycobacterial proteins in the absence of clinical signs and symptoms of active TB disease, proved by molecular tests such as the tuberculin skin test (TST) and Interferon-Gamma Release Assay (IGRA) [73]. Now, however, the LTBI concept is expanded and is no longer regarded as a static condition but ranges from presumably cleared infection to subclinical disease without overt symptoms [20, 29]. Figure 4 denotes the complexity of staging in *Mtb* infection, from elimination of *Mtb* upon infection to “classical” latent TB, subclinical TB disease and overt TB disease. As shown in Figure 4, progression from *Mtb* infection to clinical disease appears via several continuous infection states without clinical signs of disease, ranging from TST-and/or IGRA negative to cases to subclinical disease, often described as having intermittently detectable *Mtb* in sputum, but with a normal chest x-ray and no symptoms of TB [29].



TST	Negative	Positive	Positive	Positive	Usually positive
IGRA	Negative	Positive	Positive	Positive	Usually positive
Culture	Negative	Negative	Negative	Intermittently positive	Positive
Sputum smear	Negative	Negative	Negative	Usually negative	Positive or negative
Infectious	No	No	No	Sporadically	Yes
Symptoms	None	None	None	Mild or none	Mild to severe
Preferred treatment	None	None	Preventive therapy	Multidrug therapy	Multidrug therapy

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Figure 4 The spectrum of TB. Pai, M. *et al.* (2016) Tuberculosis *Nat. Rev. Dis. Primers* doi:10.1038/nrdp.2016.76[18]. Reprinted with permission.

In TB disease the individual has developed clinical symptoms of *Mtb* infection. Active disease has a myriad of clinical outcomes ranging from mild to moderate to severe disease, affecting lungs (pulmonary TB) or other organs (extrapulmonary TB, EPTB)[18]. If the individual develops TB disease directly following exposure, it is referred to as primary TB, while if disease occurs because of reactivation, it is regarded as post-primary TB [18]. These two states are distinguished as they frequently carry unique clinical features, including radiological findings [74]. Further, although TB is predominantly regarded as a pulmonary pathogen, it can affect almost any other part of the human body[75]. EPTB more frequently occurs in immunocompromised patients, where factors such as granuloma disruption can lead to loss of containment of bacilli and lymphohematogenous dissemination [29]. Although the mechanism of *Mtb* dissemination from the pulmonary site to other organs is not fully elucidated, researchers believe that both genetic and host immune factors, especially at local sites of infection, are important [76-78]. In immunocompromised hosts, such as in patients with HIV co-infection or individuals using immunosuppressive medication, infection with *Mtb* can result in a severe, disseminated form of TB characterized by massive lymphohematogenous spread of bacilli, which is often fatal if left untreated[77].

Recent advances in technology, including imaging such as PET-CT, has been important in challenging the dogma of TB pathogenesis [29]. Here, identification of metabolically active

granulomas (e.g., characteristics of patients with subclinical or active disease) in patients with LTBI, has been important to increase the understanding of the complexities of *Mtb* infection and disease [29]. Yet, the TB disease spectrum remains a challenge to diagnostics where the traditional binomial approach to manage TB falls short when selecting treatment strategy as TB heterogeneity complicates patient selection for preventive treatment, and carries a risk of overtreatment, undertreatment, loss of follow up and invasive investigations[79]. Further, it's difficult to predict which patients are at risk for developing progression to TB disease[79, 80]. *Mtb* infection does not prevent reinfection with new *Mtb* strains, and reinfection might occur later in life, even in patients that have undergone treatment [29]. Thus, the clinical distinction of reactivation from reinfection may be a challenge in clinical settings, particularly in TB endemic settings where re-infection occurs frequently [18, 29].

1.3.2 Signs and symptoms

In pulmonary TB, the most common symptoms include persistent cough, chest pain, low-level fever, night sweats, weight loss, enlarged lymph glands, and fatigue lasting more than fourteen days[81]. In EPTB, symptoms will vary depending on the organ affected[76]. Signs associated with TB are usually non-specific, and TB should be suspected in individuals with persistent cough with sputum production and/or hemoptysis for two weeks or more, especially if accompanied by weight loss, malaise, night sweats or fever[82, 83]. In addition, known previous exposure or stays in regions with a high prevalence of TB increase the likelihood of *Mtb* to be the causative agent, and diagnostic tests are warranted [83].

1.3.3 Diagnostics of Mtb infection

The diagnostic algorithm for TB determination includes microscopy, molecular tests, culture-based methods, and imaging techniques (chest x-rays, CT or PET-CT) [84]. However, the test of choice depends on the purpose of testing, i.e., if testing for TB infection or TB disease.

Diagnosis of *Mtb* infection is indirect and based on detecting cellular immune responses, demonstrated by a positive Tuberculin skin test (TST) or Interferon-Gamma Release Assay (IGRA) [85]. The TST measures a delayed-type hypersensitivity reaction to intradermal injection of mycobacterial antigens, while the IGRA blood test (QuantiFERON®) rely on measuring Interferon-γ (IFN-γ) production by T cells following bacterial encounter [2]. TST

is regarded as an outdated method in high-income low-endemic settings as it may yield a false positive result from cross-reactivity with nontuberculous *Mycobacterium* species (NTMs) and be confounded by previous BCG vaccination [85, 86]. Yet, it is a method still frequently used in low-income high-endemic settings. TB-IGRA tests are more reliable, and in contrast to the TST, not affected by previous BCG vaccination [20]. However, also this test may be prone to cross-reactivity to some NTMs and has reduced sensitivity in immunocompromised hosts[9]. Although an improvement over TST, IGRA remains an imperfect test, and requires specific laboratory equipment, and use in low-income regions is thus limited [9]. In high-income settings, it is widely used as a screening tool for *Mtb* infection, especially in patients with a history of exposure. A positive test result usually warrants further examination and/or monitoring and evaluation of preventive treatment. Importantly, neither IGRA nor TST differentiate infection (e.g., latent infection) from disease [29, 82, 87].

To diagnose TB disease, WHO recommends a biomolecular test as the initial diagnostic test [9]. Nucleic acid amplification tests (NAAT), such as the Gene Xpert MTB/RIF, have been used as diagnostic tools for pulmonary TB since 2013 [9]. The Xpert MTB/RIF is highly sensitive, has higher accuracy compared to microscopy in detecting pulmonary TB disease and can detect drug resistance to Rifampicin, a first-line TB drug, simultaneously [88]. However, this technology is expensive and requires access to laboratory facilities, and thus may not be an option in resource-poor settings [9, 88]. Culture-based techniques are gold standard for the diagnostic confirmation of active TB and important for detecting resistant strains to establish effective treatment [9]. Though highly specific, this method is resource-demanding and time-consuming, owing to the slow growth rate of *Mtb* which may be up to 6-8 weeks [89]. Smear microscopy for acid fast bacilli by Ziehl-Neelsen or fluorescent stain is often used in tandem with culture, especially in resource poor settings [18]. A primary tool for the diagnosis of TB, the results of microscopy may largely determine further handling of the patients in clinical settings. However, efficacy drops significantly in children and immunocompromised hosts due to lower levels of *Mtb* or reduced ability to produce sputum [90]. Chest x-rays is an important tool for triaging and screening for pulmonary TB and is useful to aid diagnosis when pulmonary TB cannot be confirmed bacteriologically[9]. For diagnostics of extrapulmonary TB, advanced radiological procedures with CT scans and/or invasive procedures for tissue sampling, may be necessary[9].

Although there are advances in diagnostic tests of TB, including rapid tests such as molecular-based methods, simpler point-of-care -tests (POCTs) tests are needed for low-income settings [85]. POCTs facilitate early detection, immediate treatment, and reduced transmission of TB disease as pointed out in a recent review by Hong et al. [91-93]. Further, a major disadvantage to diagnostics in TB is that no test can reliably predict which individuals with LTBI are at the highest risk of progression to active disease, and thus this complicates the selection of patients that will benefit preventive treatment [83]. However, efforts of exploring biomarkers for identification of those at high risk of disease progression are ongoing [29].

1.3.4 Treatment and prevention

Treatment of presumably drug-sensitive TB consists of a prolonged treatment with a combination of several antimicrobial drugs, where a 6-month regimen is considered standard duration [94]. According to WHO guidelines, preferred treatment consists of a 2-month long intensive phase with Rifampicin, Isoniazide, Ethambutol and Pyrazinamide with a continuation phase with Isoniazide and Rifampicin for four months [95]. Monitoring treatment efficacy with repeated sputum smears, cultures, and imaging is usually necessary [96]. As low compliance due to drug toxicity and prolonged duration remains a challenge, to enhance optimal adherence, a monitoring approach by Directly observed therapy (DOT) was introduced in 1994 [97]. This method requires that health professionals directly supervise treatment administration, and although such treatment monitoring is widely used, there is a lack of clinical trial data supporting effectiveness on compliancy by this approach [98].

To treat drug-resistant TB, an individually tailored treatment regimen based on drug susceptibility profile is necessary [96]. MDR-TB is defined as resistance to rifampicin and isoniazide, where treatment duration can span from 9 to 20 months, with a global success rate of 57% [99]. The backbone of MDR-TB treatment consists of a combination of drugs from different groups: Group A (fluoroquionolons, bedaquiline, linezolid), Group B (clofazimine, cycloserine) and Group C (ethambutol, delamanid, pyrazinamide, carbapenems, amikacin, ethionamide, PAS) [100]. Prolonged MDR-TB treatment is encumbered by a variety of side effects posing a risk for non-compliance and unsuccessful treatment [96]. Recently, WHO published a rapid communication with recommendation of 6 months treatment of MDR-TB in

selected cases [101]. This constitutes a game changer in the treatment of MDR-TB worldwide. Although the important obstacle for compliance and treatment success is the duration of treatment, the considerable concerns of drug toxicity and sequelae is not solved [9]. As follows, the last decade, reports of both increasing numbers of XDR-TB (MDR-TB also resistant to fluoroquinolones and one injectable) and total drug-resistant strains threatens treatment effectiveness, emphasizing the need for new TB drugs and new TB treatment strategies[102]. Further, in TB endemic areas, an additional risk of TB treatment is the prevalent co-infection with HIV [5], and although cure rates of TB treatment have been reported to be similar in HIV-positive versus HIV- negative patients, co-infected patients have greater risk of adverse events during treatment [103].

Timely and effective diagnosis and treatment for combatting and eradicating TB is at the heart of preventive measures according to WHO guidelines [9]. Further, management of latent infection to reduce the risk of developing TB disease remains a main prevention measure, although the WHO recommends preventive treatment to be limited to people with high risk of exposure or high risk of severe disease [83][104]. Pre-covid-19, the Bacille Calmette-Guérin (BCG) vaccine was the most widely used vaccine in the world and has been included in the standard childhood immunization program in 153 countries [105, 106]. Yet, the BCG vaccine has only been documented to confer protection from severe forms of TB in children, though a recent proof-of-concept study revealed that revaccination can prevent sustained infection in young adults [107]. The variability of BCG in protection and immunogenicity may be due to several factors as prior exposure to environmental mycobacteria other than *Mtb* and possible other co-infections such as HIV, leishmaniasis and cytomegalovirus infections [105, 108]. Thus, some countries have removed BCG from standard immunization programs and recommend vaccination of high-risk individuals only[9]. Identifying protective mechanisms necessary for an effective TB vaccine, or even correlates of protective immunity, has proved difficult, although efforts to achieve this goal are ongoing. In fact, there are several TB vaccine candidates being pursued in clinical trials [45, 109].

For a next generation vaccine to be effective, it must achieve prevention of infection or prevention of disease or prevention of recurrence or relapse [38]. More than a dozen vaccines have entered clinical trial assessments, with more in preclinical development. The main strategies in development of new TB vaccines the last decades have been to 1) replace the BCG vaccine with an improved whole-organism vaccine such as a recombinant BCG or an

attenuated strain of BCG or 2) developing a subunit vaccine to enhance protection by BCG [108]. Further, new TB vaccine strategies are also investigating new vaccination routes such as inhalation, aerosol, and mucosal immunization[38]. In addition, more focus has also been to other aspects of immunogenicity with special interest in trained innate immunity, tissue resident memory T cells and *Mtb* surface antibodies [109, 110]. Lastly, in the wake of the successful mRNA vaccines against Covid-19, there has been several calls for developing mRNA vaccines against TB, although none are in the pipeline yet [38, 111]. For future work, to develop effective vaccines and novel drug regimens, knowledge of the host response and immunological defenses in TB are essential [21].

1.4 Eicosanoids in TB

Though their complete effects during *Mtb* infection remain elusive, findings indicate that Arachidonic acid-derived signaling molecules, such as eicosanoids, are of great importance in modulating the host response [112]. Eicosanoids regulate a variety of physiological responses, including hemostasis, platelet aggregation, protection of gastric mucosa, inflammation, tissue remodeling, parturition, nociception and pyrexia [113]. They have been shown to exert major influence on the outcome of experimental TB by regulating both innate and adaptive immunity [114, 115]. Produced by the enzymatic breakdown of arachidonic acid and released from membrane phospholipids by phospholipases [116], lipid mediators are generated through three main pathways: (1) the cyclooxygenase pathway (COX-1 and COX-2) that produces prostaglandins and thromboxanes, (2) the lipoxygenase pathway (5-LOX, 12-LOX, and 15-LOX), that generates the production of leukotrienes and lipoxins, and (3) the cytochrome p40 pathway, which generates hydroxyeicosatetraenoic and epoxieicosatrienoic acids [113]. Downstream of the main enzymes, there are multiple other enzymatic reactions, leading to a production of a multitude of mediators with vast and multifaceted effects [117-119]. Thus, the network is exceedingly complex and not fully understood[113]. An overview of the main pathways and metabolites are shown in Figure 5.

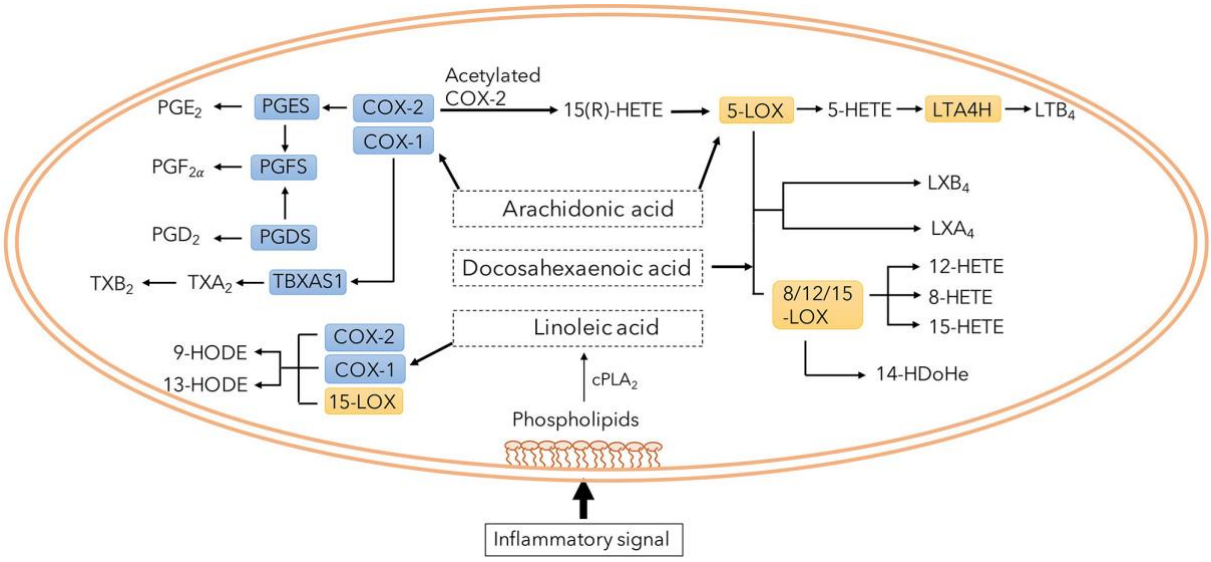

Figure 5 Overview of eicosanoid pathway and its metabolites. Jøntvedt Jørgensen M et al, Plasma LOX-Products and Monocyte Signaling Is Reduced by Adjunctive Cyclooxygenase-2 Inhibitor in a Phase I Clinical Trial of Tuberculosis Patients. *Front. Cell. Infect. Microbiol.* 11:669623. Doi: 10.3389/fcimb.2021.669623[120]. Reprinted with permission.

COX-derived prostaglandins, of which Prostaglandin E2 (PGE2) is central, mainly exert pro-inflammatory effects, whereas the LOX-pathway produces both pro-inflammatory leukotrienes and anti-inflammatory lipoxins, where Leukotriene B4 (LTB4) and Lipoxin A4 (LXA4) are important mediators in the context of TB[112] Here, *Mtb* specifically modulates the production of certain eicosanoids, including PGE2, LTB4 and LXA4, a mechanism dependent on bacterial virulence [121]. Opposing functional roles of different eicosanoids in TB pathophysiology seems to be of great relevance in the pathogenic process [122, 123]. Further, innate immune cells are major producers of eicosanoids and may exert immunostimulatory and/or immunosuppressive effects through eicosanoid-dependent mechanisms [124].

Essentially, biological active derivatives that exert effects in an autocrine and paracrine fashion, eicosanoids interact with G-protein-coupled receptors on the cell surface or in the nuclear envelope [113, 125]. PGE2 exerts its effects by binding to one of four different receptors, Prostaglandin E2 receptor 1-4 (EP1-4), where EP2 and EP4 are the most studied [126, 127]. Traditionally regarded as a strict pro-inflammatory mediator, PGE2 has also been proposed to harbor many immune-regulatory actions [126]. Effects on innate immunity include regulating IFN-γ, nitric oxide, and Il-12 production and modulating phagocytosis and antimicrobial functions of innate immune cells [114, 128, 129]. Further, PGE2 is an important

regulator of host cytokine networks, where a potential beneficial effect is conferred by the inhibition of Type 1 Interferons and the induction of TNF-α [130, 131]. In preclinical evidence, PGE2 has been shown to lead to several, in part contradictive effects on TB host responses; it induces apoptosis of macrophages, down-modulates Th1 cytokines, limits T cell proliferation and expand T regs and TH2 cells[52, 132-134]. Although thought beneficial early in infection, PGE2 has been shown to harbor suppressive effects on adaptive immunity during chronic stages [126, 129, 135, 136].

Production of leukotrienes and lipoxins is governed by a range of different isoenzymes of LOX [135]. This pathway drives the oxidation of arachidonic acid into a diverse group of intermediate products, ultimately leading to the production of pro- or anti-inflammatory mediators [137]. The intermediate enzyme leukotriene A4 hydrolase (LTA4H), represents an important crossroad enzyme, where activity determines production of either pro- or anti-inflammatory products [138-140]. Like PGE2, LTB4 is a classical pro-inflammatory mediator that induces TNF-α, IL-12, NO, and IFN-γ production, recruits inflammatory cells such as neutrophils, monocytes, and macrophages, enhances phagocytosis and bactericidal activity and recruits adaptive immune cells [129]. By contrast, LXA4 is dominantly an anti-inflammatory mediator, which has been proposed to carry unfavorable effects if excessively produced during *Mtb* infection [121]. LXA4 has been shown to inhibit TNF-α production, reduce recruitment of neutrophils, and induce production of IL-10 [121, 128]. Importantly, its role in driving macrophages to necrosis has been highlighted as a detrimental effect in TB [121, 141]. For proposed and shown functions of PGE2, LTB4 and LXA4 in TB, see Table 1.

Table 1 | Functions of eicosanoids in TB

Lipid mediators	Pathway	Functions	
Prostaglandin E2 (PGE2)	Cyclooxygenase-2 (COX-2)	*Biomarker potential*	
			↑↓ PGE2 in active tuberculosis ([69, 142-147] ↓ PGE2/LXA4 in severe disease [69, 143] ↓ PGE2 in severe disease [146]
		Cytokine networks	Regulates IFN-γ-production [129, 146, 148, 149]
			Inhibits Type 1 Interferons [69, 142, 150, 151]
			Induce TNF-α production [152, 153]
		Genotype-polymorphism	Prostaglandin 2 (EP2) receptor modulates susceptibility [142, 154]
		Innate immunity	Induces macrophage apoptosis [114, 155-157]
			Modulates phagocytosis and antimicrobial functions [129, 148]
			Regulates production of nitric oxide[148, 152]
			Inhibits neutrophil migration [158] Suppresses NK-cells, downregulates MHC 2 and inhibits NADPH oxidase [159]
			Promotes macrophage efferocytosis [158]
			Enhances autophagy in monocytes and macrophages [146, 160]
		Adaptive immunity	Modulates expansion of T regulatory (Treg) cells [52, 127, 136, 161, 162]
			Induce TH2/TH17 polarization [112, 142]
			induce myeloid-derived suppressor cells (MDCS) [142]
			induce IL-10, IL-23, IL-17 [163]
			Modulates production of Il-12 [152, 163]
			Enhance T cell priming [114]
			Modulates TH1 cytokines [129]
		Granuloma formation	Enriched in solid granulomas [164]
			Promotes lipid droplet formation [165]
Lipoxin A4 (LXA4)	5-lipoxygenase (5-LOX)	*Biomarker potential*	↑↓LXA4 in active tuberculosis [143-145]
			↑ LXA4/LTB4 in severe disease [145]
		Cytokine networks	Inhibits TNF-α production[137, 148]
		Genotype-polymorphism	Leukotriene hydrolase A4 (LTA4H) modulates susceptibility[137]
		Innate immunity	Induces macrophage necrosis [114, 137]
			Inhibits recruitment of neutrophils [152, 158]
			Promotes macrophage efferocytosis [152, 158]
			Induces production of Il-10 [158]
			Reduces TNF-α production [137, 148]
			Modulates PGE2 production [69, 148]
		Adaptive immunity	Downregulates TH1-derived cytokines [123]
			Inhibits CD4+ and CD8+ responses [114, 166]
		Granuloma formation	Generates poorly formed, non-necrotizing granulomas [138, 164]

Leukotriene B4 (LTB4)	5-lipoxygenase (5-LOX)	*Biomarker potential*	↑↓ LTB4 in active tuberculosis [145, 147]
		Cytokine networks	Induces TNF-α production [137]
		Genotype-polymorphism	Leukotriene hydrolase A4 (LTA4H) modulates susceptibility [137]
		Innate immunity	Induces macrophage necrosis [137]
			Recruits neutrophils, monocytes and macrophages [152, 167]
			Induces IFN-γ-production [168]
			Enhances phagocytosis and bactericidal activity [158, 168]
			Stops the neutrophil response [169]
		Adaptive immunity	Recruits TH1, TH2 and TH17 cells [152]
			induces IL-12 and nitric oxide [168]
		Granuloma formation	Causes caseous granuloma formation and necrosis in colocalization with TNF-α [138, 164]

In TB, the eicosanoids influence on macrophage cell death is of great consequence to spread of bacilli [170]. Further, the relationship between lipid mediators and essential cytokines in TB pathogenesis remain a topic of great research interest [27]. Recent research has focused on their contribution to granuloma formation [164], while their effects on the adaptive immune system remain a controversial topic, as do their role as potential biomarkers [171]. Thus, to successfully target eicosanoids in HDT, there is a need for a more refined understanding of eicosanoids involvement during *Mtb* infection [172].

1.5 Host-Directed Therapy in TB

Standard TB drug treatments target the pathogen, while Host-Directed Therapy (HDT) targets host immune responses [173]. HDT aims to modulate the immunological microenvironment surrounding the bacteria to limit replication and spread by augmenting favorable host-responses or inhibiting dysfunctional mechanisms [69, 174, 175]. Thus, HDT in TB is adjunctive treatment aiming to improve treatment outcomes, shorten treatment duration and preserve normal lung architecture after *Mtb* infection [176]. The development of HDTs is focused on two general approaches: 1) modulate the host inflammatory pathways to reduce excessive inflammation and tissue destruction 2) augment the host's innate and adaptive immune effector mechanisms for more effective pathogen control [173]. If proven useful, HDT could be widely implemented, probably at low cost, having a considerable impact on TB management and outcomes [173].

Eicosanoids can potentially be targeted therapeutically to enable the immune system to clear *Mtb* and/or reduce pathogenicity [177]. Drug re-purposing is a prospective strategy for TB treatment, and anti-inflammatory drugs that target the COX-axis are already in use as adjunctive therapy: Corticosteroids reduce morbidity and mortality in TB meningitis while non-steroidal anti-inflammatory drugs (NSAIDS), such as COX-is relieve symptoms in non-severe cases of paradoxical TB immune reactions (TB-IRIS) [178-181]. These drugs represent an option with potential beneficial effects on excessive inflammation during *Mtb* infection [182, 183]. By directly and indirectly targeting the COX-axis, corticosteroids adjunctive to standard TB treatment in specific clinical contexts such as TB meningitis and IRIS have proven useful in several studies, including in the HIV/TB co-infection framework [184]. Supporting evidence demonstrates improved lung radiological lesions, earlier symptomatic improvement, and reduced morbidity in severe disease [173, 178, 185]. In pulmonary TB, although there is some evidence for improvement in selected clinical parameters with corticosteroid therapy, there is no high-quality evidence showing decreased mortality or sustained improved microbiological or clinical outcomes when comparing adjunctive corticosteroid treatment to placebo in pulmonary TB [186]. Although none are listed for pulmonary TB, pending trials are well underway to illuminate the possible beneficial effects of corticosteroids as adjuvant therapy in HIV-associated (NCT03092817) and non-HIV associated TB meningitis (NCT03100786), as well as in TB-IRIS (NCT01924286) [187]. Potential drugs that target other pathways of the eicosanoid system include Zileuton,

Montelukast and other LOX-inhibitors as well as supplementation of eicosanoid agonists [188]. Moreover, indirect targets for eicosanoids as HDT include manipulation of host-mediator networks [189], with drugs targeting the crosstalk between eicosanoids, IL-1β, Type 1 interferons, TNF-α, and IFN-γ, which is a subject under intense investigation [69].

Potential HDTs in TB treatment have been extensively discussed in recent reviews [184, 186, 190, 191], including repurposed compounds such as metformin, verapamil, statins, everolimus and nutritional supplements [184, 190, 192, 193]. Other strategies that aim to improve treatment outcome involve cytokine immunotherapies as well as adjunctive vaccines [38, 175, 194-199]. Studies targeting eicosanoids as HDT in *in vitro* and preclinical studies are shown in Table 2, whilst Table 3 gives an overview of ongoing or completed clinical trials targeting eicosanoids in *Mtb* infection.

Table 2 | *In vitro* studies and preclinical experiments using drugs targeting eicosanoids

	Drug	Effect	Model	Challenge	Time	Reference
In vitro cultures	Valdecoxib	Increased bacterial loads	Culture (BMDM)	H37RV	-	[69]
	NS398 (COX-2 inhibitor)	Suppressed T-reg expansion	Culture (DC)	BCG	-	[136]
	Indomethacin	Downregulated *Mtb*-specific FOXP3-regulatory cells, cytokine responses and T cell proliferation	Culture (PBMC)	ESAT-6, Ag85	36h	[52]
	Celecoxib	No difference in bacterial load (CFU)	Whole-blood bactericidal activity (WBA) model	H37RV	7 days	[200]
	PGE2 supplementation	Suppresses *Mtb*-growth in dose-dependent manner, increased secretion of IL-6/IL-8, decreased cell viability	Culture (PBMC)	H37RV	24 days	[201]
	LXA4 supplementation	Reduced TNF-α, increased PGE2 and LXA4	Culture (BMDM)	H37RV	24 hours	[148]
Preclinical	Celecoxib	Enhanced 60-day survival. Increased LTB4, IL-12 and IFN-γ, decreased Il-10 and PGE2	Balb/c mice	H37RV	30/60 days	[168]

Celecoxib	Reduction in bacterial load, enhanced IFN-γ and NO release, increased phagocytosis	SV129 mice	H37RV	15/30 days	[168]
Celecoxib	Reduced bacterial load, decreased TNF-α, increased IFN-γ , increased NO, increased monocytes and neutrophils, increased phagocytosis,	SV129 mice	H37RV	30	[129]
Low dose aspirin	Increased survival, reduced lung pathology and decreased bacillary load in late stages of disease, anti-inflammatory effects	C3HeB/FeJ mice	H37RV	28 days	[202]
Low dose aspirin	Increased survival, lower bacillary load, lower levels of pro-inflammatory mediators	C3HeB/FeJ	H37RV	28 days	[203]
LTB4 supplementation	Increased susceptibility, worsened lung inflammation and tissue damage	Alox -/- mice	H37RV	30	[129]
MK886	Increased bacterial burden, impaired influx of leukocytes	Balb/c mice	BCG	30	[167]
MK886	Reduce 60-day survival, increased bacterial burden. Reduced LTB4, IL-12, IFN-γ and NO	Balb/c mice	H37RV	30/60 days	[168]
PGE2, Zileuton	Bacterial load and lung pathology reduced	Il1a/Il1b -/- mice	H37RV	21 days	[69]
ASA	Reduced bacterial load	LTA4H-high genotype zebrafish	M.marinum	3 days	[174]
LTB4 antagonist	Reduced bacterial load	LTA4H-high genotype zebrafish	M.marinum	3 days	[174]
PD146176 (15-LOX inhibitor)	Increased TNF-α, decreased bacterial burden	Zebrafish	M.marinum	-	[153]

Table 3 | Clinical trials using drugs targeting eicosanoids

No	Drug	Phase	Conditions	Status	Location
NCT02060006)	Meloxicam (NSAID)	3	Tuberculosis	Unknown	University of Stellenbosch, South Africa
NCT02781909	Ibuprofen (NSAID)	2	Tuberculosis	Completed	National Center for Tuberculosis and Lung Disease, Tbilisi, Georgia
NCT02503839	Eterocoxib (NSAID)	1	Tuberculosis	Completed	Oslo University Hospital, Norway
NCT02602509	Celecoxib (NSAID)	1	Tuberculosis	Completed	National University Hospital Singapore, Singapore
NCT04145258	Aspirin (NSAID)	3	Tuberculous meningitis	Recruiting	Cocody University Hospital, Abidjan, Côte D'Ivoire
NCT02237365	Aspirin (NSAID)	2	Tuberculous meningitis	Completed	Hospital for Tropical Diseases, Ho Chi Minh City, Vietnam
NCT04575519	Aspirin, Ibuprofen (NSAID)	2	Tuberculosis	Recruiting	National Center for Tuberculosis and Lung Disease, Tbilisi, Georgia, Perinatal HIV Unit (PHRU)- Chris Hani Baragwanath Hospital and PHRU- Matlosana, Tshepong Hospital MDR Unit, South Africa
NCT03927313	Aspirin (NSAID)	2	Tuberculous meningitis	Recruiting	Livingstone Hospital Port Elizabeth, Eastern Cape, South Africa

2 Aims of study

In this thesis, we have conducted three studies testing different hypotheses concerning the effects of eicosanoids on immunopathogenesis in TB and exploring the potential effects of COX-2is as HDT. The specific aims were:

Paper 1: Investigate the levels of the key lipid mediators (PGE2, LXA4 and LTB4) and the phenotype of monocyte and T cells subsets in different clinical stages of *Mtb* infection and after 6 months of standard TB treatment.

Paper 2: Investigate plasma levels of eicosanoid derivatives following adjunctive COX-2i treatment in the TBCOX2 study, and study systemic profile of lipid mediators in active TB at day 14 and 56 after treatment with standard TB treatment with/without adjunctive COX-2i. Investigate phosphorylation of specific intracellular pathways related to eicosanoid and cytokine production, and study changes following selective inhibition pathways with COX-2i.

Paper 3: Compare mycobacterial growth inhibition capacity, cytokine production and host inflammatory gene profile of macrophages before and after *in vitro* mycobacterial challenge in samples obtained from TB patients after 56 days of standard TB therapy with/without COX-2i.

3 Summary of results

In Paper 1, we showed that pulmonary TB patients had elevated levels of the anti-inflammatory mediator LXA4 at diagnosis compared to LTBI, while levels of PGE2 and LTB4 were comparable between different clinical states of *Mtb* infection. LTB4 was the only mediator to be reduced upon treatment, along with a reduction of the LTB4/LXA4 ratio. Pulmonary TB patients had higher levels of total monocytes at diagnosis compared to end-of-treatment and LTBI. Further, TB patients showed a relative increase in the classical monocyte subset. All monocyte subsets had low basal expression of COX-2 and 5-LOX, which were markedly increased upon stimulation with PPD. By contrast, the expression of EP2 was reduced following stimulation. CD4 T cells expressed low basal COX-2 activity that showed a modest induction upon stimulation, whereas their basal expression of 5-LOX was considerable. We concluded that the levels of eicosanoids in plasma seem to vary between clinical states of *Mtb* infection, where both innate and adaptive immune cells contribute to eicosanoid production.

In Paper 2, LXA4 and other lipoxygenase-derived metabolites, but not PGE2, were associated with disease severity in TB, and showed a reduction upon treatment that was possibly accelerated by COX-2is. *In vivo* COX-2i inhibition reduced the LPS-induced phosphorylation potential in monocytes and affected downward signaling cascades involved in cytokine production. Phosphorylation of p38 MAPK, NFkB, Erk1/2, and Akt in monocytes as well as plasma levels of MIG/CXCL9 and procalcitonin were reduced in the COX-2i group compared to controls. Thus, we concluded that COX-2i may affect immune regulation in TB also by affecting the LOX-pathway in addition to modulating intracellular pathways in innate cells.

In Paper 3, after 56 days of adjunctive etoricoxib treatment in patients from the TBCOX2 trial, macrophage capacity to control *in vitro* mycobacterial infection was lowered compared to controls. Uninfected macrophages harvested from patients on COX-2is had significantly lower expression of genes important for controlling *Mtb* such as TNF, IL-1β, CCL4, CXCL9 and CXCL10. Further, levels of IFN-β, CD40L and S100A8/A9 were significantly lowered in the COX-2i group and a trend of lowered levels of IL-2, IL-4, IFN-a, IP-10, IL12/IL23p40 and IL-1RA was observed compared to controls. We concluded that *in vivo* COX-2i affects *ex vivo* macrophage functioning and cytokine production, but where the complete mechanism for the demonstrated reduced microbicidal activity, remains incompletely understood. Further,

our results may imply that the effect of adjunctive COX-2i treatment may be imprinted in circulating innate immune cells with potential of changing their antibacterial capacity.

4 Methodological considerations in Paper 1-3

The studies included in this thesis were exploratory in essence, meant to generate hypotheses regarding the role of eicosanoids in TB and the possible effects of COX-2i as host-directed therapy. However, they contain three major limitations; 1) First, the small sample size reduces power of statistical calculations; thus, subtle differences may be overlooked 2) changes and dynamics in peripheral blood do not accurately reflect immune responses at local sites of *Mtb* infection and 3) the study design limits our possibility to conclude on causative factors.

4.1 Study design, patient selection and ethical considerations

Figure 6 gives an overview of the patients and materials included in the different papers with the corresponding approvals from the regional ethics committee. In paper 1 and 2, material from patients recruited in a prospective observational cohort (called "observational cohort"), were selected. This cohort constituted pulmonary and extrapulmonary TB and LTBI patients admitted to the Department of Infectious Diseases and the Department of Pulmonary Medicine at Oslo University Hospital (OUS). The diagnosis was funded on the presence of clinical symptoms, microbiological specimen (culture/PCR), and radiologic findings. EPTB was defined as non-pulmonary TB. Plasma and peripheral blood mononuclear cells (PBMC) were collected and stored in the department's ongoing biobank in 2013-2020. For PTB and EPTB, patients were included for the study if they had 1) confirmed active disease with drug-sensitive *Mtb* isolates, 2) available plasma and PBMC samples at diagnosis and after a 6-month follow-up, 3) negative HIV status, and 4) no known immunocompromising disease, including diabetes. Eligibility criteria for LTBI were defined as 1) QuantiFERON®-TB-positive, 2) no clinical signs of active disease, 3) negative HIV status, and 4) no known immunocompromising disease.

For Paper 2 and 3, samples were collected from a phase I/II/clinical trial at OUS, Norway in the period 2015-2019 (TBCOX2, *NCT02503839*) designed to study the safety and immunogenicity of the COX-2i etoricoxib (Arcoxia, MSD) as an adjunctive to standard TB treatment in drug-sensitive active TB [204]. Patients aged ≥18 years with confirmed drug-sensitive pulmonary or extrapulmonary TB (GeneXpert MTB/RIF®) without comorbidities (including a negative HIV test), willing to participate (written informed consent), were

included. A clinical history with a clinical examination, lab work-up, and chest X-ray were performed at baseline. 18 patients received adjunctive COX-2i treatment (etoricoxib) for 140 days in addition to standard TB treatment and 21 patients received only standard TB treatment. All participants experienced clinical improvement and culture conversion after two months of treatment. For Paper 2, peripheral blood was included at baseline, day 14, and day 56. For this study, an additional five patients (age 18-70) with pulmonary TB were included in a pilot study with blood sampling before TB treatment initiation. For Paper 3, peripheral blood mononuclear cells (PBMCs) were collected at day 56 after start of treatment.

Patients in Paper 1 were included in "Research Biobank Infectious Diseases ("Forskningsbiobank Infeksjonssykdommer" (REK 1.2006.181-S-0885, SHDNR. 09/513). The phase I/II/clinical trial at Oslo University Hospital, Norway, in the period 2015-2019 (TBCOX2, *NCT02503839*), was approved by the Regional Ethics Committee (TBCOX2, REK SØ 2015/692) and The Norwegian Medicines Agency (EudraCT Number 2014-004986-26).

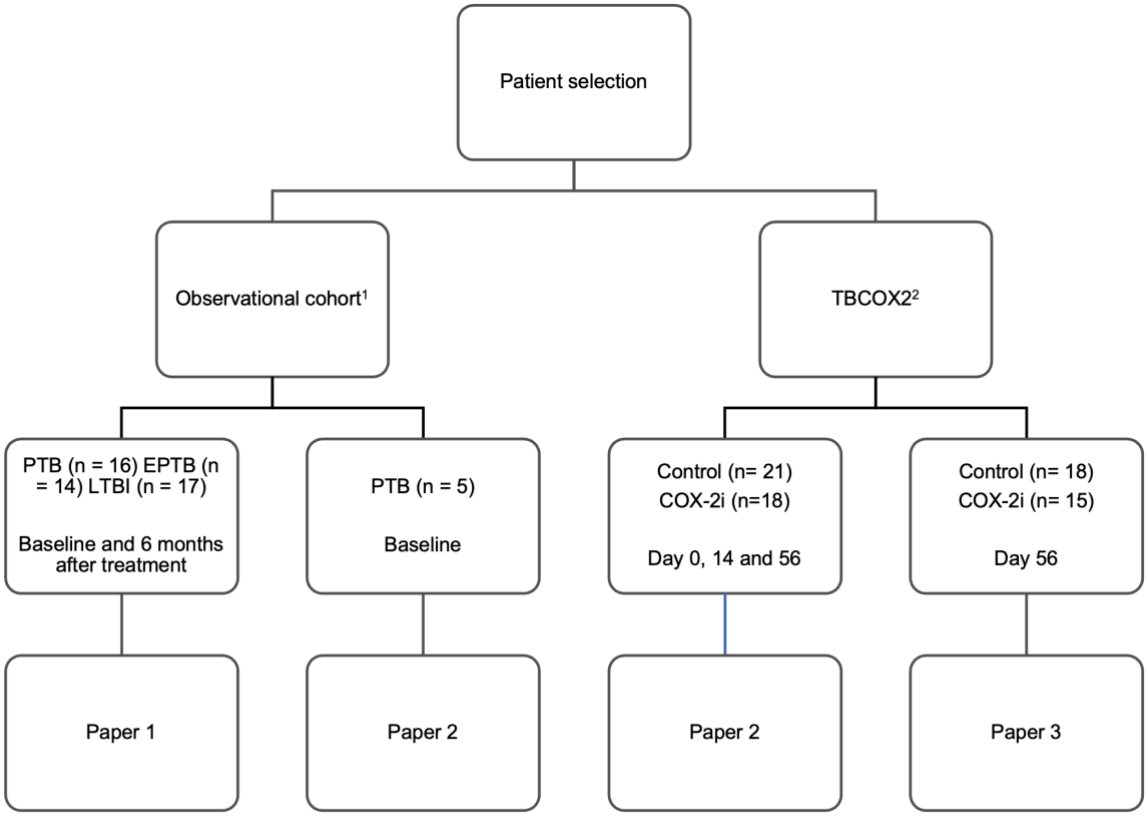

Figure 6 Overview of patient cohorts in Paper 1-3 with corresponding approvals**. 1)** «Prognostic Immunological markers», approved by the Regional Committees for Ethics in Medical Research (REK-Sør-Øst 2016/2123). Biobank samples from "Research Biobank Infectious Diseases"

("Forskningsbiobank Infeksjonssykdommer" (REK 1.2006.181-S-0885, SHDNR. 09/513), Department of Infectious Diseases, OUS, Ullevål **2)** The clinical study «TBCOX2», NCT02503839, approved by the Regional Ethics Committee (TBCOX2, REK SØ 2015/692) and The Norwegian Medicines Agency (EudraCT Number 2014-004986-26).

The prospective observational cohort used for Paper 1 allows for the inclusion of many study subjects and simultaneous analysis of longitudinal samples by different laboratory techniques. Its major limitation is that such study designs fail to evaluate causality. Thus, it can be useful to explore associations, but cannot prove the effect of an intervention, which would require intervention RCTs. This was applied for Paper 2 and 3, though limited by the small sample size.

For ethical considerations, all studies were conducted in in compliance with the Declaration of Helsinki principles, and for the TBCOX2 study, in accordance with the International Conference on Harmonisation's Good Clinical Practices guidelines and registered in ClinicalTrials.gov (TBCOX2, NCT02503839). Registration and storage of patient data was conducted in accordance with national personal data laws as well as regulations for clinical research. The patient perspective in the study was safeguarded by informed consent. The study protocol for TBCOX2 was submitted to the National Regulator Authority (Norwegian Medicines Agency), and not started before approval was received. Before enrollment, the study protocol was approved by the regional ethics committee. Serious or unexpected adverse events were reported to the National Regulatory Authority. Written informed consent included information of aims, methods, anticipated benefits, and potential hazards, and it was emphasized that participation was voluntary. It was ensured that the information was understood by the patients before enrollment, to secure autonomy, rights, safety, and well-being of the patients included. This was done to ascertain voluntarism, information disclosure and decision-making capacity regardless of cultural background, education, social class or language barriers.

4.2 Sample material

For Paper 1-3, PBMCs were isolated in cell preparation tubes using a density gradient, cryopreserved in freezing media and stored at -150°C to secure structurally intact living cells [205]. This method is a major advantage that allows for longitudinally collected samples to be analyzed consecutively in batches, facilitating comparability of data. Immediate thawing at 37°C in water-bath and drop-wise adding of pre-heated cell medium was performed for all PBMCs used in Paper 1-3. For plasma samples used in Paper 1 and 2, these were collected in tubes containing Ethylenediaminetetraacetic acid (EDTA) to prevent coagulation, and red blood cells were removed by centrifugation. Finally, tubes were snap-frozen (< 30min) and stored a -80 °C until further analysis. While this method might induce changes or even degradation of cytokines and other soluble molecules it simplifies the logistics and allows for batch analysis of samples [206, 207].

Much of our current knowledge of immunological processes involving eicosanoids comes from animal models and *in vitro* systems. However, these approaches have intrinsic limitations, especially when studying the complex host-pathogen relationship during human *Mtb* infection[208, 209]. TB is a heterogeneous disease, and animal models fail to replicate the immunopathology seen in human TB, although some argue that macaque models used for preclinical testing of treatment and vaccines reflect the full spectrum of infection and disease [210]. Contrasting reports may result from varying models of infection [186, 203, 209], differences in experimental protocols [22, 137, 170] and disparities between *in vitro* findings and studies conducted on experimental animal models *in vivo* [171, 211]. Further, the same inconsistencies mentioned in animal models are present in *in vitro* studies, where cell cultures, although a useful tool, often are limited to studying a few biological processes [208]. In addition, these cells differ from the tissue-resident ones, and when used in *in vitro* cultures, they lack the environmental stimuli that ultimately shape cellular responses to infection[212-214].

Although the genetic uniformity and precisely defined experimental conditions available in an animal model are advantageous for basic research, the clinical relevance of studying human biological samples is indisputable. However, by using PBMCs and plasma, we examine systemic responses and mechanisms that may represent secondary effects of the primary

response in the lung tissue [145, 215, 216]. Thus, these designs cannot accurately reflect the mechanisms taking place on-site of infection [217]. Further, cells isolated from peripheral blood have a different biological composition, immune phenotype, and activation status than cells found at sites of infection. To correctly investigate such cells, bronchoalveolar lavage fluid (BAL) collected from lungs can be applied [217]. However, this technique is expensive, invasive and presents a potential risk for complications [217].

4.3 Plasma quantification – LC-MS, ELISA, and Multiplex

For Paper 1 and 2, Enzyme-linked Immunosorbent assay (ELISA) and Liquid chromatography-mass spectrometry (LC-MS) were used to quantify lipid mediators. ELISA is an assay technique designed to detect and quantify soluble substances in biological sample material such as plasma, serum, urine, or tissue [218]. The technique uses capture antibodies covalently bound to enzymes that, when bound to a specific molecule of interest, changes fluorescence intensity that can be measured by colorimetric reading using an internal standard [219]. It is relatively cheap, sensitive, robust, and available for a wide range of soluble molecules, although limited in that it can only measure one target at a time. For quantification of PGE2, LTB4 (only in Paper 1), and LXA4, competitive ELISA assays were used (Cayman Chemical and Oxford Biochemical), where the target of interest competes with another molecule for attachment to the binding site of the target-specific antibody (Figure 7). Thus, the absorbance is inversely proportional to the amount of analyte in the sample and measured by a four-parameter logistic fit. To ensure validity, samples were run in duplicate, and inter and intra-assay controls were used to evaluate precision and reliability (%CV). For all assays, inter and intra-assay variation was less than 10%.

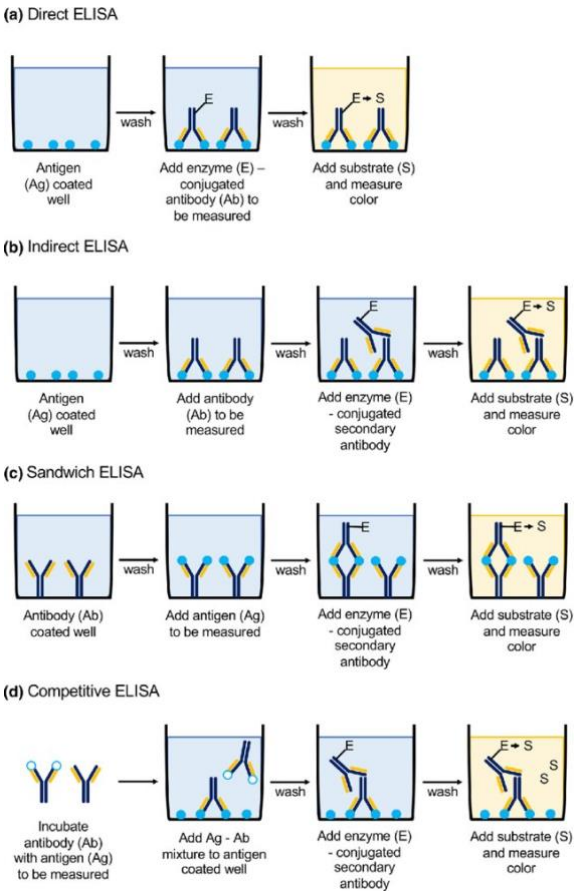

Figure 7 Schematic presentation of basic types of ELISA: a) direct, b) indirect, c) sandwich, d) competettive; *Ag* antigen, *Ab* antibody, *E* enzyme, *S* substrate. Adapted from Boguszewska, K., Szewczuk, M., Urbaniak, S. *et al.* Review: immunoassays in DNA damage and instability detection. *Cell. Mol. Life Sci.* **76**, 4689–4704 (2019).[220]. Available by license (http://creativecommons.org/licenses/by/4.0/).

LC-MS is a technique that involves the physical separation of target analytes followed by mass-based detection[221]. This allows for high specificity while quantifying a wide range of molecules simultaneously[221]. While liquid chromatography separates mixtures with multiple components, mass spectrometry provides spectral information that may help to identify each separated component [222, 223]. For Paper 2, quantification was performed as previously described [222] at the MetaToul Lipidomic Core Facility (I2MC, Inserm 1048, Toulouse, France, MetaboHUBANR-11-INSB-0010). Metabolites that were not detectable in more than 30% of the samples were excluded for further analysis.

For quantification of cytokines in Paper 2 and 3, a microsphere-based multiplex bioassay technique was used. Like ELISA, this technique is based on capture antibodies, that by using color-coded magnetic beads (microspheres), enable the simultaneous quantification of a wide range of different analytes [224]. The sample was added to a mixture containing color-coded

beads pre-coated with analyte-specific capture antibodies for analysis. Subsequently, detection antibodies conjugated to biotin were bound to the analytes of interest. Phycoerythrin (PE) – conjugated streptavidin was then added, which binds to the biotinylated detection antibody. Fluorescence was then measured on a Luminex instrument. As for ELISA, quantification is performed by extrapolation from an internal standard. In Paper 3, cytokines in supernatants from human macrophages before and after challenge with *Mav* were quantified using a custom panel from Procartaplex (Affymetrix, eBioscience) and analyzed with a Luminex 200 instrument (Invitrogen). Out-of-range values in the upper region were set to the highest measurable concentration, and values below blank were set as zero. Values that were out of the standard range but stipulated from the standard curve were included.

Methods such as ELISA, Luminex and LC-MS are potent tools in characterizing individual soluble molecules in complex biological material [218]. Although these methods show several advantages, they do have certain limitations. ELISA is limited by its potential of high background which can reduce the sensitivity of the method [218]. Compared to LC-MS, ELISA often reports higher analyte concentration due to non-specific binding, a process that might be amplified in assays that do not include steps to stop the color reaction, as the method often show short signal stability [221]. Further, as the method requires numerous washing steps to reduce variation and unspecific binding, it may not detect weak interactions [222]. Its advantages are that it's relatively simple and easy to perform, is generally considered to have high sensitivity and specificity, and that the method is cost effective and cheap [218].

The major advantage of LC-MS is that it is a robust analytical technique that provides high sensitivity and selectivity when detecting molecules in sample mixtures, and where the combination of two distinctive analytical techniques improves accuracy [222]. Its major limitation is that its costly and requires trained personnel to operate. Further, although high sensitivity LC-MS can quantify a wide range of molecules simultaneously, it can be limited by using a pooled standard reagent [221]. In Paper 2, we experienced difficulties in that several lipid mediators fell below the limit of detection. This was partly due to a compromise in the optimal titration of the sample and the set standard curve, as well as the fact that some eicosanoids are prone to extensive *in vitro* metabolism. Thus, methods such as ELISA that quantify single metabolites may be more reliable in certain instances[218]. For both techniques, when quantifying eicosanoids, purification steps are needed to remove contaminants that interfere with the analysis. Purification of the sample with alcohol to

precipitate proteins and isolation of lipids using a C18 column technique was applied for both. However, even with such precautions, there is no certainty that the sample does not contain molecules that cross-react. For this reason, analyzing lipids is challenging, and more reliable methods are hopefully available in the future.

While the Luminex method has several advantages over ELISA, this method also has similar challenges [224]. Cross-reactivity between detection antibodies and non–specific analytes may interfere with validity. Plasma contains proteases that degrade cytokines while freeze-thaw cycles may affect the levels of cytokines. Another challenge is that assay panels often contain targets that are present at radically different concentrations, and thus, covering the range of individual analytes remains a problem and targets may fall below limit of detection, which result in loss of information [224]. Its advantages are that it's able to quantify a wide range of targets simultaneously at a reduced cost and generates high throughput with less sample handling [224].

4.4 Immune cell phenotyping – Flow Cytometry and phospho-flow cytometry

Flow cytometry is a well-known technique used to characterize the simultaneous expression of a wide range of cell surface or intracellular molecules, allowing rapid defining of cell types in a heterogeneous cell population [225]. This is done by linking fluorochromes to monoclonal antibodies that bind to different molecules on the cell surface or intracellularly [226]. Cells pass through a laser beam, where the light scattered is characteristic, and light is absorbed and emitted in a band of wavelengths. Here, the flow cytometer measures fluorescence intensity produced by the fluorescent-labeled antibodies [227]. Thus, multi-color flow cytometry allows for the analysis of multiple parameters and cell characteristics simultaneously, making it able to rapidly provide a vast amount of data [228].

For Paper 1, flow cytometry was used to phenotype monocytes and T cells. PBMCs were thawed, washed, and counted, where only samples with >80% viability were included for further analysis, with most above 90%. Cells were either unstimulated or stimulated with Purified protein derivative (PPD, SSI, Denmark) and stained for surface markers.

Cells were then stained for intracellular markers with conjugated antibodies. See Table 4 for antibodies used for phenotyping monocytes and T cells, respectively. For 5-LOX staining, an indirect technique was applied: an unconjugated antibody for 5-LOX was used for initial stain, followed by two wash steps with PBS and an additional incubation with a secondary antibody. To control for unspecific binding, fluorescence minus one (FMO) was used for gating of COX-2, EP2, and 5-LOX, and for the monocyte panel, FcR receptor block was added prior to staining. In Paper 2, intracellular phosphorylation was assessed by phospho-flow, a phospho-specific approach that enables analysis of phosphorylation events by using phospho-epitope specific antibodies. The analysis was performed as previously described[229]. Briefly, prior to permeabilization, the different stimuli conditions were barcoded with different combinations of Pacific Blue and Pacific Orange at room temperature. After barcoding, cells were washed and stained.

Table 4 Markers used in flow cytometry analysis in Paper 1, 2 and 3

Marker	Function
5-LOX	Member of the lipoxygenase family of enzymes. Catalyze the first step in production of leukotriene and lipoxins[230]
CD3	Protein complex and T cell co-receptor, responsible for activating both cytotoxic and helper T cells[231]
CD4	T cell co-receptor, binds to antigens presented by APCs. Marker for T helper cells. [231]
CD8	T cell co-receptor, binds to antigens presented by APCs. Marker for cytotoxic T cells[231]
CD14	Pattern recognition receptor, binds to LPS. Associated with TLR4. [232]
CD16	FC receptor. Mediating antibody-dependent cellular cytotoxicity and phagocytosis. [232]
COX-2	Rate-limiting enzyme in the production of prostaglandins, induced by inflammation.[233]
EP2	Prostaglandin E2 receptor 2[234]
HLA-DR	MHC class II Receptor that binds endocytosed peptides and presents antigens to T cells[232]

Flow cytometric analysis can be effectively applied to the interrogation of a multitude of cell populations from a variety of tissues and is distinguished by its high throughput that generates large amounts of data [225]. However, the technique requires trained personnel to operate and perform analysis, and the method is relatively costly [227]. The biggest challenge and the source of unreliable data in flow cytometry is spectral overlap, that decreases validity [227]. Further, it may give rise to interpretation issues, as gating of immune cells are highly

subjective. This may lead to variations in data [228]. In flow cytometry, the number of recorded events and the number of cells in the population of interest should be sufficient to reduce the % coefficient of variation (CV) [228]. Observed dispersion of data may result from slight differences in fluorochrome staining and flow cytometry settings rather than relevant biological differences in the samples [228]. To avoid this, in Paper 1-3, patient samples with low viability or low cell numbers that resulted in few recorded events were excluded from further analysis.

We encountered several challenges when using flow cytometry in the papers included. In Paper 1, indirect staining was performed for 5-LOX due to the lack of commercially available direct-conjugated antibodies. Controls (FMO) of both the primary and secondary antibody used was included to evaluate background and unspecific binding of each antibody. However, we cannot be sure that the signal generated was not due to at least some unspecific binding, which limits the validity of the finding. We initially included an antibody specific for Leukotriene A4 Hydrolase to investigate the activity of this enzyme during *Mtb* infection but had to exclude the antibody due to extensive unspecific binding. Further, monocytes have a high abundance of Fc-receptors on their cell surface, which may cause inherent unspecific binding. Thus, in Paper 1, a commercially available FcR-blocking reagent was applied to correct for a potential erroneous signal.

Another point to consider is the differentiation potential of monocytes in culture. In Paper 1, monocytes underwent considerable phenotypical changes of CD14 and CD16 expression during the 18h stimulation step. A potential explanation for this transformation is that monocytes spontaneously differentiate to monocyte-derived macrophages (MDMs) in culture [235]. Furthermore, as more monocytes differentiate to MDMs, they quickly adhere to culture plates and are thus increasingly difficult to resuspend. Attached monocytes can thus be lost in the following steps before flow cytometry analysis. For this reason, culture plates containing polypropylene rather than polystyrene was used to reduce plate adherence. To avoid contamination of non-monocytes from PBMCs, a lineage antibody cocktail containing CD3 (T cells), CD19 (B cells), CD20 (B cells) and CD56 (NK cells) was used in both in Paper 1 and 2. However, for Paper 2, due to incompatibility between the permeabilization buffer and antibodies used, only HLA-DR and CD14 was used to obtain a crude monocyte population. It is therefore possible that samples were contaminated by other cell subsets such as activated T

cells (HLA-DR+) and CD14-expressing myeloid cells (macrophages, neutrophils, and dendritic cells).

4.5 M. avium culture, infection and CFU analysis

Growth inhibition assays are functional assays that include most aspects of the complex host immune response after infection with mycobacteria. It can be used to reveal differences in the control of mycobacterial growth after *in vivo* or *in vitro* intervention [236, 237]. For Paper 3, this technique was used to evaluate the control of mycobacterial growth of macrophages from patients included in the TBCOX2 study.

PBMCs (See flow chart in Figure 6 for cohort) were seeded in triplicate into plates and monocyte-derived macrophages (MDMs) were generated by plastic adherence. Cells were then washed to remove non-macrophages and assessed by light microscopy. MDMs were stimulated with M-CSF to induce macrophage differentiation for an additional six days, after which they were subjected to *M. avium* (*Mav)* infection. Macrophages seeded at 50,000 cells/well were infected in two separate plates with the *Mav* clinical isolates at an MOI of 10. One plate was infected for 2 hours and the other for 6 days. Wells were lysed and the contents plated in serial dilutions on 7H10 Middlebrook plates in triplicate to enumerate uptake by counting the number of CFU (time zero). On day 6, supernatant and RNA were harvested from both uninfected and infected wells. Then, the remaining wells were lysed, and serial dilutions were plated on 7H10 Middlebrook plates in triplicate. Total CFUs from both plates were counted after 21 days of incubation at 37°C.

Growth inhibition assays has been used for the evaluation of the cellular and humoral immune response against *Mtb*, and due to its technical simplicity, it is considered suitable for use in clinical studies to evaluate vaccine candidates [237]. The major limitation in our study is the use of nontuberculous mycobacteria (NTM), and not *Mtb*. This was due to logistic reasons, as NTM handling does not require a biosafety level-3 containment. *Mav* is primarily an intracellular pathogen where cellular immunity plays a major role in protection. It has been shown in studies that different mycobacterial strains can have distinctive effects on eicosanoid metabolism[123, 156]. For example, the avirulent mycobacterial strain H37RA stimulates PGE2 generation, while studies report that the virulent strain H37RV induces LXA4

production [121]. Another example is the presence of ESAT-6, an antigen characteristic of virulent mycobacteria (present in *Mtb* and *Mycobacterium bovis*, but not in attenuated strains), which trigger COX-2 expression both *in vitro* and *in vivo* [238]. Thus, whether effects of *Mav* are comparable to *Mtb* on eicosanoid biosynthesis, remains unknown and pose a limitation in our study when evaluating effects of COX-2i as HDT.

4.6 Gene expression analysis

For Paper 3, gene expression in immune cells was investigated by quantification of RNA, achieved using nCounterTechnology. By single-molecule detection with no amplification, this method employs fluorescent barcodes that enable simultaneous detection of a wide range of different target molecules [239]. Here, RNA is directly tagged by a capture probe linked to biotin, and a reporter probe, where once the target complex is hybridized (allowing two complementary single-stranded RNA molecules to form a single double-stranded molecule through base pairing), the complexes are captured on a streptavidin-coated surface [239]. The reporter probe contains a unique fluorescent barcode, and an automated fluorescent microscope then scans the sample. Thus, the amount of RNA for each target can be directly quantified [240].

For the study, total RNA was extracted from cell culture supernatants, and samples were eluted and measured by spectrophotometry (NanoDrop). Genes of interest were quantified by using the nCounter Human Inflammation v2, which consisted of 249 inflammation-related gene and 6 internal reference genes. Negative and positive controls included in probe sets were used for background thresholding. The raw data were normalized to six genes within each tissue type with the lowest coefficient of variation using the nSolver software following the manufacturer's instructions (NanoString Technologies).

By using two hybridization probes where probe pairs are generated for each unique gene [240], the reporter probe carries the signal while the capture probe binds the complex, immobilizing it so that a digital analyzer can detect the hybridized reporter probes. A digital analyzer identifies, scans, and counts the molecular barcode per sample [241]. Thus, a reverse transcription step is not required to generate cDNA. RNA was extracted from cell culture beforehand [240]. This method has several advantages. First, high-quality RNA is not

required, and smaller amounts of starting material is needed compared to other methods an where detection of low-expression RNA is possible [241]. Secondly, there is minimal background signal, which increases validity [241]. Further, data analysis software is freely provided by NanoString Technologies, with readouts that are user friendly and doesn't require bioinformatics [241]. The method is limited in that it is not suitable for biomarker discovery. Further, only a limited number of probes are available to measure isoform or gene expression, and thus, custom probe design is often needed [241].

4.7 Statistical analysis

As all datasets used in this thesis were biological data of relatively small sample size, the data were generally not normally distributed, and non–parametric statistical tests were applied. One exception was the Luminex data in Paper 2, in which a mixed–model ANOVA was applied. Non- parametric tests are "distribution free" and not based solely on parametrized families of probability distributions [242]. Yet, there are some disadvantages with this approach. They can be less sensitive and fail to detect differences between groups that exist and in statistical terms, a type II error failing to reject the false null hypothesis. Furthermore, the multiple comparison problem when running multiple hypothesis tests (i.e increasing the probability of observing significant results due to chance) is a major limitation for ELISA, Luminex and gene expression data. This was corrected for by post-hoc tests for multiple comparison.

Wilcoxon matched pair test was applied when comparing two dependent variables, while Mann-Whitney U test was used to compare two independent variables. Friedman test was used in Paper 2 to compare three or more dependent variables. To compare three or more independent groups, Kruskal Wallis test was applied. All correlation analysis was calculated using Spearman's rank coefficient. For gene expression data, the raw Nanostring signatures were normalized using negative controls, positive controls, and housekeeping genes via nSolver version 4.0 software (Nanostring) and the Advanced Analysis 2.0 plugin (Nanostring) where default settings automatically set normalization probes by using the geNorm algorithm (Vandesompele, 2002). Analysis was performed with the use of XQuartz 2.8.1 Normalization for run-to-run and sample-to-sample variability was done by dividing counts within a lane by the geometric mean of the reference/normalizer probes from the same lane. To calculate differential expression of genes, default settings using the optimal method in the advanced analysis 2.0 plugin (Nanostring) were used. Briefly, a negative binomial mixture model was

applied for low expression probes and a simplified negative binomial model for high expression probes. In situations where the algorithm was not converging, the linear regression method was used instead. Given the number of simultaneous tests and an expected increase in type 1 error, false positives were controlled for using the Benjamini–Yekutieli false discovery rate (FDR).

The major statistical limitation of the studies included in this thesis was the small sample size for each group. Therefore, the studies were based on immunological proxies with an exploratory design and without formal hypothesis testing. Group comparisons resulting in p-values below the decision rule (<0.05) were instead interpreted as hypothesis supporting rather than confirmatory. Also, the heterogeneity of patients called for a careful interpretation of data. Translational research and a priori power calculations were not possible due to the exploratory nature of the studies included. While the low sample size risks type II statistical errors, another consideration is potential type I errors if data is not corrected for multiple testing. However, if several findings across different markers point towards the same biological process, the likelihood of type I error is low. Due to the study's exploratory nature, and to avoid conclusions that could be caused to type I error, correction of multiple comparisons was performed for Luminex, LC-MS, and Nanostring analysis in Paper 2 and 3. In Paper 1, a non-parametric Kruskal-Wallis test was performed when comparing the three different patient groups (active TB, LTBI, and EPTB), correction of multiple comparisons were performed using Dunn's post-hoc correction.

5 Discussion

Current TB treatment regimens have several shortcomings, such as lengthy treatment duration and adverse drug effects, that complicate patient adherence [173]. Research efforts are being directed towards establishing more individualized treatment options for TB, where HDT is a novel way of manipulating targeted host responses and/or the host-pathogen interaction and presents a promising strategy[177, 217]. However, TB patients are notoriously heterogeneous, and it would be naïve to expect that "one size fits all". The host inflammatory response is affected by multiple factors, including pre-existing host immunity, the burden of infection, and the individual inflammatory rebuttal upon antigen exposure [173]. Thus, evaluation of patient heterogeneity with regard to potential beneficial effects of HDT, remains pivotal. Seemingly, an individually-tailored treatment strategy, optimally based on clinical characteristics, biomarkers, disease spectrum and genetic profile would be the goal [173].

The overall aim of this thesis has been to explore the effects of eicosanoids on immune pathogenesis in *Mtb* infection and investigate the potential of COX2-i as HDT adjunctive to standard TB treatment. First, in **Paper 1**, we explored levels of eicosanoids and monocyte heterogeneity in different stages of *Mtb* infection. In **Paper 2**, we investigated monocyte intracellular signaling and levels of eicosanoids in samples from the phase 1/2 clinical trial TBCOX2 study and analyzed the effects of the COX2-i etoricoxib given as adjunctive TB treatment. Finally, in **Paper 3**, further exploratory analyses were performed on samples from the TBCOX2 study, including investigations of macrophage mycobacterial killing capacity, macrophage inflammatory gene profiles and cytokine production.

The following sections will provide an integrated discussion and reflections upon important aspects of eicosanoid pathways as potential for HDT in TB, whilst a detailed discussions of the separate studies are given in **Papers 1-3**.

5.1 Eicosanoid pathways as potential targets for Host-directed therapy in Tuberculosis

Figure 8 gives a simplified overview of relevant eicosanoid pathways. The different pathways of the arachidonic acid cascade are novel avenues to discover potential targets for HDT, with the COX and LOX axis serving as main opposing pharmaceutical targets for drug repurposing [138].

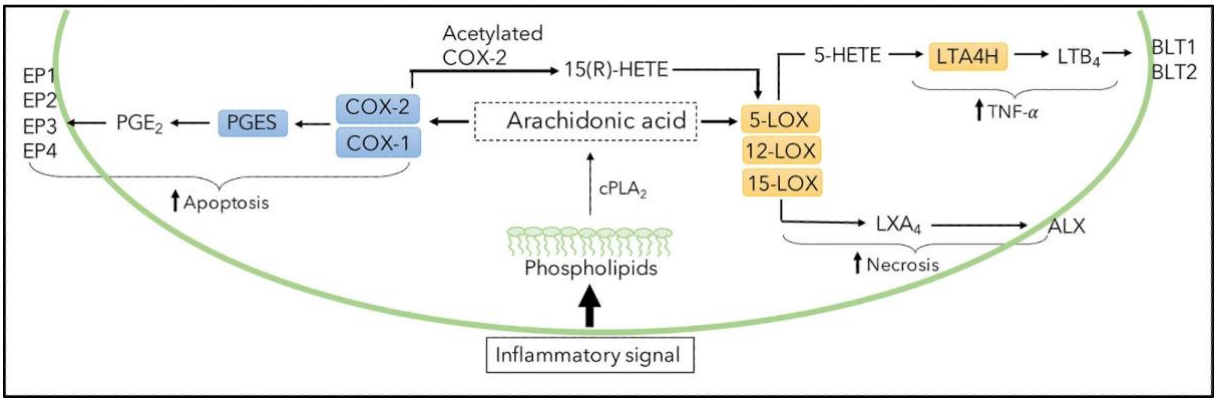

Figure 8 Illustration of eicosanoid biosynthesis. Formation of PGE2, LTB4, and LXA4 through breakdown of arachidonic acid (AA) by Cyclooxygenase (COX) and lipoxygenase (LOX) pathways. COX enzymes and downstream PGES generates PGE2 which binds to receptors EP1-EP4. 5-LOX-derived LTB4 is produced via the precursor 5-HETE, converted by enzyme LTA4H and exerts effects through receptors BLT1 and BLT2. LXA4 is derived from LOX enzymes 5,12 and 15-LOX and binds to the ALX receptor. Nore et al. [243]. Reprinted with permission.

5.1.1 The COX-pathway

COX-2 inhibition, which reduces levels of PGE2, has been proposed to lead to several advantageous effects when used adjunctive to standard TB treatment[186]. Key aspects include increased efflux pump inhibition, leading to elevated TB drug levels inside host cells, potentiated activity of standard treatment, and overall reduced inflammation with limited tissue damage [200]. The theory that COX-2is may be favorable for the host is largely based on PGE2's proposed immunosuppressive effects [146]. Still, evidence investigating the role of PGE2 in *Mtb* infection is conflicting [69, 146].

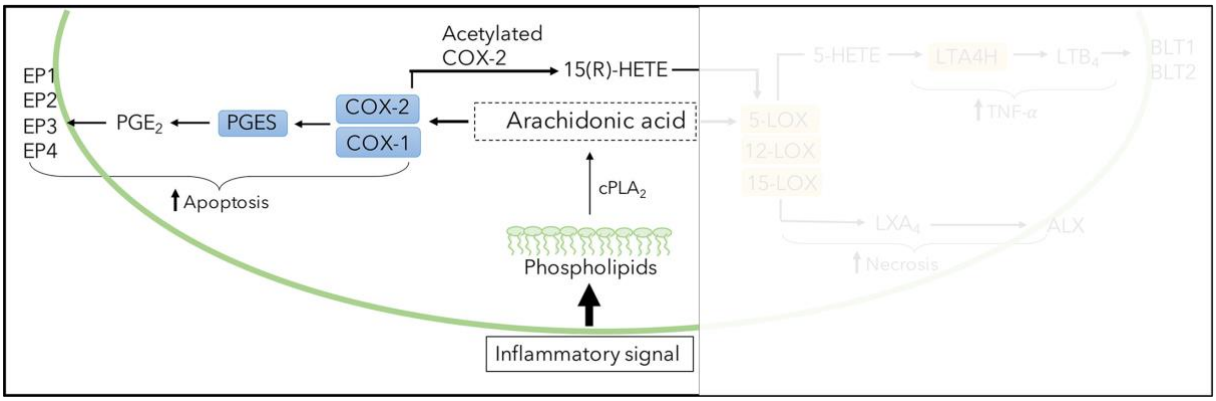

Figure 9 The COX-pathway. *COX enzymes and downstream PGES generates PGE2 which binds to receptors EP1-EP4.* Adapted from Nore et al. [243]. Reprinted with permission.

We hypothesized that levels of PGE2 would differ depending on the clinical state of *Mtb* infection. However, our results from **Paper 1** and **Paper 2** showed no association between PGE2 and clinical state of infection, nor with disease severity. Thus, our results may implicate that PGE2 plays a less important role in *Mtb* infection and extent of TB disease than initially hypothesized. Others have reported increased levels of PGE2 in TB patients compared to healthy donors, possibly reflecting the inflammatory state of TB patients [145, 146]. While both studies compared PGE2 in active TB versus healthy QuantiFERON® (QFT) negative controls, our study participants were QFT positive. Further, differences in patient cohorts must be taken into consideration. In our studies, while the time of infection is difficult to establish, they were all recruited in a high-income low-endemic setting, where they were most likely experiencing late progression of primary TB or reactivation of latent TB and were thus probably in a chronic phase. In comparison, the studies that showed elevated concentrations of systemic PGE2 in active TB were all conducted in high-endemic settings, and one of them even reported a significant negative correlation between PGE2 levels and disease duration[146]. Further, in Pellegrini et al's study, they found lower levels of PGE2 in severe TB disease compared to patients with moderate disease [146]. These findings supports the evidence that PGE2s effect on host immunity is dependent on several factors: 1) the duration of *Mtb* infection 2) the clinical status of each patient 3) the local concentrations of PGE2 at site of infection and 4) both the cell type and the receptors on which the mediator is acting upon [146].

A limitation in our studies is that we did not investigate concentration of PGE2 at site of infection. In late stages of *Mtb* infection, characterized by progressive pneumonia, PGE2

levels have been shown to be elevated in the lungs [134]. These findings seem plausible, considering the proposed effects of PGE2 in chronic phases; inhibited phagocytosis, limited bacterial killing and dampened T cell responses[126, 127, 244]. By contrast, during early phases, PGE2 concentrations have been shown to be relatively low and stable [134]. Animals in this early phase thus experienced exacerbated inflammation and increased bacterial loads following COX-2i treatment [134]. Hence, low and stable PGE2 concentrations may permit temporal control of bacillus growth in early phases of *Mtb* infection, while high PGE2 concentrations during chronic stages confer immunosuppressive effects, permitting disease progression [134, 135, 146].

The proposed beneficial effects of PGE2 during early phases is supported by PGE2s involvement in host mediator networks, as shown by Mayer-Barber et al. [69]. In this study, they highlighted the cross-regulatory network between eicosanoids and cytokines, where IL-1 and PGE2 collectively cooperate to reduce expression of detrimental Type 1 Interferons [69]. Thus, they postulate that IL-1 and Type I IFNs are two major counter-regulatory classes of inflammatory cytokines, functionally linked by PGE2, which may have significant impact on the outcome of *Mtb* infection[69]. In the study, IL-1 deficient mice ($IL1r1^{-/-}$) displayed excess amounts of type I IFNs, reduced levels of PGE2, increased levels of LTB4 and LXA4 and consequently, advanced TB disease and death [69]. HDT targeting eicosanoids with PGE2 supplementation was thus hypothesized to be more effective in severe disease where high type I IFN conditions dominates[69, 245]. In sum, the study by Mayer-Barber et al. contradicts recommendations for use of COX-2is as HDT in TB. However, as documented in the study, the effects of COX-2i inhibition were condition-dependent, with both host and bacterial factors affecting disease outcome [69].

While there is substantial evidence for potential phase-dependent dynamics of PGE2 in *Mtb* infection, there is less knowledge of consequences of inhibiting the COX2/PGE2-axis [126, 134, 186]. In **Paper 2** and **Paper 3,** we analyzed the effects of COX-i adjunctive to standard TB treatment. In **Paper 2**, there were no differences in PGE2 levels after 14 days of treatment in the control group, and surprisingly, neither in the COX-2i group. The latter may be explained by compensating biosynthetic pathways, with a possible increased COX-1 or Microsomal PGES-1 (mPGES-1) activity, as reported by others [69]. In **paper 3**, we reported reduced mycobacterial growth control of macrophages after 56 days of adjunctive COX-2i

treatment. To our knowledge, there are no other studies evaluating the *in vivo* effects of COX-is on *ex vivo* macrophage function in TB.

Other preclinical studies show that use of COX-is in established infection decreases inflammation and bacillary loads, thus increasing survival in murine models[134, 168, 246-248]. In an C3HeB/FeJ model where mice were *Mtb* infected intravenously and treated with an unselective COX-inhibitor, Vilaplana et al. found that Ibuprofen significantly reduced lung lesions and increased survival following treatment[246]. In support of this, another study showed that diclofenac (an unselective COX inhibitor) significantly reduced bacterial counts in lungs and spleen and increased survival in mice, an effect enhanced by combination therapy with diclofenac and streptomycin [247].

While confirming the reports by Villaplana et al. with iv infection, Mortensen et al. showed that aerosol infected mice had increased bacterial burdens following COX-2i treatment and hypothesized that this effect was caused by a diminished protective capacity of CD4 T cells[182]. Byrne et al. showed that Aspirin or Ibuprofen alone did not reduce CFU counts in murine models, but reduced bacterial loads only in combination with isoniazid or pyrazinamide, compared to anti-TB drugs only [249]. Moreover, in a whole-blood bactericidal activity model from healthy volunteers receiving celecoxib adjunctive to standard TB treatment, one study reported that celecoxib had no effect alone or in combination with adjunctive therapy on bactericidal activity[200]. Importantly, the recently published phase I/II randomized TBCOX2 trial of the adjunctive COX-2i etoricoxib given adjunctive to standard TB treatment, did not improve cellular or humoral immunogenicity, although it was well tolerated [204].

In short, PGE2s proposed beneficial effects in early phases of TB disease, suggest that timing of treatment initiation is critical when evaluating COX-2is as HDT [134, 146]. In our studies, we did not show any association between PGE2 and disease severity, nor were there were any measurable effects on PGE2 levels after 14 days of adjunctive COX-2i treatment. The latter supports reports of compensatory mechanisms in PGE2 biosynthesis [69]. Finally, both our reports and the reports of others questions the use of COX-2is in TB[69, 182, 204], and before evaluating the potential risk-to-benefit ratio, other targets of the eicosanoid system, needs to be evaluated.

5.1.2 The LOX-pathway

There are several potential targets other than the COX-pathway in the eicosanoid system. PGE2s immunological counterpart, the anti-inflammatory mediator LXA4, is widely considered to augment TB disease[250]. *Mtb* is shown to redirect the arachidonic acid cascade towards the production of 5-LOX derived metabolites, such as LXA4 and LTB4, and in murine TB, the administration of LOX-inhibitors restricts tissue damage and improve disease outcome [69, 121].

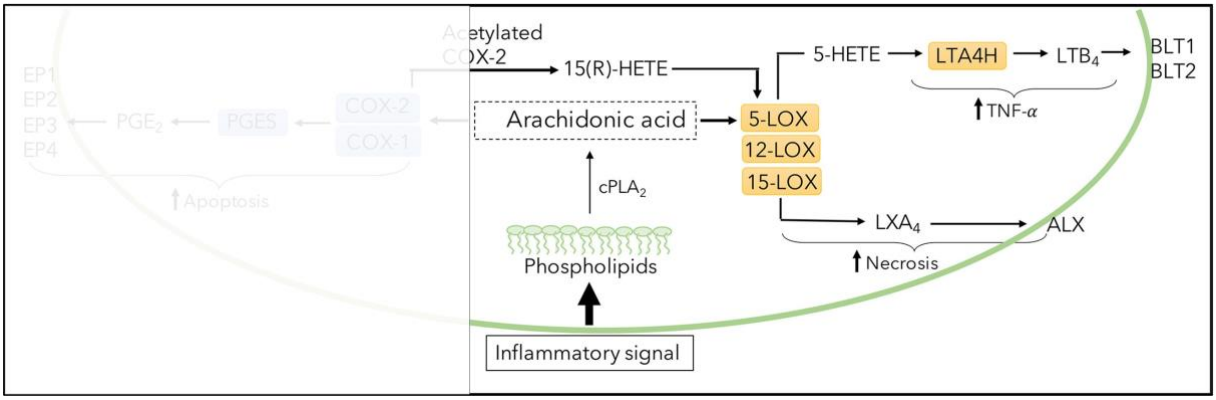

Figure 10 The LOX-pathway. 5-LOX-derived LTB4 is produced via the precursor 5-HETE, converted by enzyme LTA4H and exerts effects through receptors BLT1 and BLT2. LXA4 is derived from LOX enzymes 5,12 and 15-LOX and binds to the ALX receptor. Adapted from Nore et al. [243]. Reprinted with permission.

In **Paper 1**, we found elevated plasma levels of LXA4 in active untreated TB patients compared to LTBI, and in **Paper 2,** we demonstrated that LOX products (LXA4 and 12-HETE) were associated with TB disease severity, with elevated levels in cavitary disease. These findings are supported by others [69, 145, 251]. Kumar et al. showed increased levels of LXA4 in TB individuals with bilateral or cavitary disease, a positive correlation with bacterial burden and subsequent diminished levels of LXA4 after completed TB treatment [145]. Lee et al. suggested that on the basis of their findings with elevated levels of LXA4 in active disease compared to LTBI, which reflects our results, LXA4 may serve as a potential biomarker for TB pathology, differentiating active from latent TB infection in humans[252].

Our results in **Paper 1** and **Paper 2** suggest an important role for LXA4 in TB immunopathogenesis, as shown in preclinical studies [121]. Inherently pro-resolving and anti-

inflammatory, LXA4 leads to reduced chemotaxis and dampened production of pro-inflammatory mediators [253], where in TB, LXA4 has been shown to induce detrimental effects by downregulating critical TH1 cytokines [254], and dampening T cell function[137, 166, 254]. Lack of LXA4 increases apoptosis of macrophages and enhances acquisition of bacterial antigens by DCs, leading to more efficient cross-priming [114, 170, 254]. This is supported by findings in animal models, where bacterial burden is significantly reduced in 5-LOX deficient animals unable to produce lipoxins [69]. Thus, LXA4 makes a possible important mechanistic contribution in delaying adaptive immune response in TB [156, 254].

In **Paper 2**, we found that LOX-derived products, including LXA4, were significantly reduced after 56 days of standard TB treatment, with a surprising possible accelerated effect of COX-2is. These findings contradict other reports [255], where preclinical evidence suggests that the blockade of the COX-1 and COX-2 pathways by administration of NSAIDs, skew the eicosanoid balance with compensatory increased LOX-metabolism [129, 256]. However, it is hypothesized that these pathways do not proceed in a parallel way, but communicate in a dynamic manner, depending on timing and stage of disease [146]. To support this hypothesis, there are studies that demonstrate that since these metabolic pathways utilize arachidonic acid as a common substrate, the potential for bidirectional interaction is present, also without pharmacological intervention[146, 257]. During late stages of infection, eicosanoids reprogram from pro-inflammatory to pro-resolution phenotypes, a term referred to as lipid mediator "class-switching" [158, 245]. During resolution phases, there is a decrease in pro-inflammatory mediators such as PGE2 and LTB4, while increased LXA4 confers effective resolution by inhibiting leukocyte-mediated tissue damage and production of anti-inflammatory mediators[152, 158]. Here, rising PGE2 levels have been proposed to, by itself, act as a "class switch" by shifting the production of PGE2 and LTB4 in favor of LXA4 [131, 158, 258]. Hence, the potential for interaction between these pathways exists, and effects may vary depending on stage of disease [168, 177, 245, 257].

In **Paper 1**, although we did not find any differences in LTB4 levels between the different clinical states of TB, we found that LTB4 and the LTB4/LXA4 ratio decreased after 6 months of standard TB treatment. Previously, some have documented an association between LTB4 and TB disease severity[259, 260], while others have reported contrary findings, actually reporting higher levels of LTB4 in healthy controls compared to TB patients and with no effects of levels after TB treatment [145]. The latter is surprising, as LTB4 is a potent pro-

inflammatory mediator and may be expected to be higher during inflammatory states [113]. In association, the balance between PGE2 and LTB4 has been proposed to be essential in the pathogenesis process of TB, with LTB4 increment related to increased inflammation and propagated bacterial replication [129]. Yet, others have provided conflicting evidence, arguing that LTB4 induces protective response by recruiting innate and adaptive immune cells and increasing production of antimycobacterial cytokines, important for bacterial containment [168, 261].

The reduced ratio of LTB4/LXA4 during TB treatment shown in **Paper 1** contributes to the evidence that the balance between the different eicosanoids seems to be more important than the absolute levels of each individual mediator. Accordingly, the LTB4/LXA4 ratio has been shown to be of great importance during *Mtb* infection, and a relatively new approach to HDT is host-genotype specific therapies aiming to optimize this inflammatory balance [118]. Mutations in the gene encoding for the enzyme Leukotriene A4 Hydrolase (LTA4H) lead to preferential lipoxin or leukotriene (LTB4) accumulation [262]. Marakalala et. al found that LTA4H, which increased activity leads to higher levels of LTB4, was highly abundant in caseous and cavitary granulomas [164] with LTB4 possibly enhancing necrosis by inducing TNF-α production [164, 263]. To further underscore the relevance of the balance of LTB4 and LXA4, Tobin et al, has shown that the genotype with LTA4H-low polymorphisms is skewed toward elevated levels of LXA4 and, subsequently, limited TNF-α production and hypersusceptibility following mycobacterial infection [137]. On the contrary, in LTA4H-high polymorphisms, there are extensive LTB4 and TNF-α production, resulting in hyperinflammation and potent tissue damage [166, 264]. Taken together, high LTA4H activity leads to excessive granuloma formation and abundant necrosis, propagating *Mtb* infection [137]. In the other extreme, an inflammatory response predominated by increased LXA4 exhibits poorly formed granulomas with limited control of bacterial replication. As such, too little or too much of the eicosanoids are seemingly detrimental. Importantly, genetic polymorphisms skewing the local inflammatory milieu may be highly relevant for infection outcome [137-140]. In TB meningitis, the ratio of LTB4 and LXA4 has been shown to be associated with disease severity, possibly due to LTA4H locus polymorphism [137]. This knowledge has been utilized in clinical trials, where locus polymorphism is linked to response of dexamethasone therapy in TB-meningitis [174, 260].

At present, no trials evaluating 5-LOX inhibitors as HDT complementing standard TB treatment are registered on the clinicaltrials.gov resource database. The 5-LOX inhibitor, Zileuton, that decreases levels of both LTB4 and LXA4 is however, approved for treating asthma which could be repurposed as TB HDT and tested to elucidate whether modulation of this pathway improves TB treatment outcomes[173]. Yet, when evaluating drugs such as Zileuton, one must consider the consequences of off-target effects, as Zileuton administration will affect both the lipoxin and leukotriene axis[113].

Ultimately, baseline production of eicosanoids and the effect of these metabolites seem to be dependent on patient characteristics, such as infection stage, severity of disease, and genetic polymorphism, as well as bacterial factors, such as *Mtb* strain [129, 134, 146, 170, 256, 263]. Seemingly, an optimal balance of eicosanoids is required to induce an effective immune response, where the mediators seem to have opposed effects in the acute and chronic stage of infection. To efficiently target this system, there is a need to find the optimal timing for treatment initiation [138]. Further, off-target effects need to be evaluated, as inhibition is likely to influence on other eicosanoids as well [129, 244]. Dampening pro-resolution mediators can have adverse effects in lack of ability to normalize lung architecture after tissue damage, whereas suppressing pro-inflammatory mediators can leave the host vulnerable to other hazardous stimuli [173, 212]. Our evidence that LOX-derived eicosanoids are associated with disease severity support the claim that dampening the lipoxin axis in TB is favorable for *Mtb* clearance, while the repercussions of manipulating leukotriene-levels, remain fully unclear. Considering that many factors potentially impact the efficacy of these treatment options as HDT, and especially for COX-2is as HDT, there is a need to investigate if the proposed beneficial effects outweigh the cost [138, 188, 250]. Importantly, the eicosanoids effects on components of the host response, must be evaluated.

5.2 Monocytes and macrophages in TB and eicosanoid balance

While recruitment of monocytes to sites of infection is crucial in innate infection control and activation and shaping of adaptive immune responses, the role of the different monocyte subsets in TB is still not clearly understood [31, 265]. Studies have found that mononuclear cell populations are highly dynamic during *Mtb* infection, where variations in trafficking and differentiation may contribute to the transition from latent TB infection to active TB disease and/or the severity of disease [266]. Depending on their expression of Fcγ receptor CD16 and

pattern recognition receptor CD14, they are divided into three subsets with different functional properties: Classical monocytes (CD14++CD16-), intermediate monocytes (CD14++CD16) and non-classical monocytes (CD14+CD16 [267, 268]. Relevance of monocytes in TB pathogenesis is supported by findings of altered frequencies of subsets [34, 36] and inflammation markers [35, 37] in TB patients [232, 269, 270]. As such, some report an association between CD16-expressing monocyte expansion (i.e., intermediate, and non-classical monocytes) and disease severity, while others have shown higher levels of CD16-expressing monocytes in patients with subclinical disease and LTBI [32, 265, 269].

In **Paper 1**, we investigated monocyte subset diversity in different stages of *Mtb* infection as well as monocyte contribution to eicosanoid biosynthesis. Contrary to other reports [35, 36, 271], we found increased frequencies of total monocyte population in TB patients, characterized by mostly classical monocytes. Total monocytes were reduced after end-of-treatment while intermediate and non-classical monocytes increased after 6 months successful treatment. While our study was not designed to investigate functional aspects of different monocyte subgroups, others have shown that the classical monocyte subset migrates faster to infection sites during *Mtb* infection, and thus rapidly becomes cellular niches for mycobacteria [272]. Yet, there is also documentation that intermediate and non-classical monocytes contribute to *Mtb* propagation and disease severity by being more permissive to growth and replication, as well as impairing adaptive immunity [272]. In **Paper 1** we explored the differential expression of eicosanoid enzymes and observed higher expression of COX-2 and 5-LOX in the intermediate monocytes, indicating that this subpopulation is more involved in eicosanoid biosynthesis. This finding is plausible, as this subset is known to be highly involved in production of inflammatory mediators[273], and might be a relevant phenotypical characteristic that contributes to the proposed unfavorable effects [32, 265, 269], of this subset in TB.

Mtb rewires host cell signaling to manipulate the early immune responses [274] and several signaling cascades within monocytes/macrophages have been suggested as possible HDT targets to counteract the immune evasion strategies imposed by *Mtb* [35, 275, 276]. To our knowledge, **Paper 2** investigates for the first-time characterization of signaling pathways in monocytes from TB patients and the possible effect on signaling by adjunctive COX-2i treatment. Several overlapping pathways were activated downstream of both endotoxin LPS and mycobacterial antigens (PPD) stimulation, suggestive of activation of similar TLRs in

monocytes. Interestingly, we found no effect on the phosphorylation by *in vitro* COX-2 inhibition in PPD-stimulated monocytes. However, the finding of reduced LPS and PPD induced-phosphorylation after two weeks of COX-2i treatment indicates a lowered phosphorylation potential and possibly reduced ability to transcribe pro-inflammatory cytokines, supported by our observations of macrophage functioning following COX-inhibition in **Paper 3**.

Macrophages are the predominant cell type *Mtb* infects, and a major producer of eicosanoids [20, 22, 277]. Yet, there are few studies examining the impact of COX2-i on macrophage control of mycobacterial growth. In **Paper 3**, we therefore assessed macrophage killing capacity and macrophage inflammatory function following in vivo COX-2i treatment as part of exploratory analysis in the TBCOX2 study. Importantly, we found a reduced ability of macrophages to control mycobacterial growth compared to controls receiving standard treatment alone. This may be linked to the reduced expression of inflammatory genes and productions of cytokines observed in the study. The reduced expression of critical pro-inflammatory genes TNF and IL1β is especially relevant as both are involved in numerous processes such as macrophage activation, granuloma formation, and restriction of intracellular mycobacterial growth [69, 278-281], Thus, lowered levels may leave the host vulnerable to propagated bacterial replication, as shown by others [282]. The finding of reduced expression of genes for chemokines supports this theory, as does the observed reduction in cytokine production. Subsequently, the reduced production of inflammatory cytokines involved in T cell recruitment shown may support the hypothesis that COX-2is impair T cell responses, as shown by Mortensen et al. in mice models[182]. These findings possibly contribute to the lowered microbicidal activity of the macrophages observed, and may indicate that these cytokines are involved in auto-paracrine pathways stimulating anti-microbial defense mechanisms in macrophages, as shown previously for TNF-α and IL-6 in *Mav* infection of macrophages from healthy blood donors [236]. Further, as noted in Paper 3, our findings of reduced mycobacterial control and reduced basal expression of inflammatory gene in the macrophages from the COX-2i treated patients, may imply that the effect of adjunctive COX-2i treatment is imprinted in circulating blood monocytes. Interestingly, this imprint lasts through the *ex-vivo* differentiation of monocytes into macrophages and changes their inflammatory profile and antibacterial capacity to a second, *ex vivo* mycobacterial challenge.

A limitation in **Paper 3** is that we did not investigate other mechanistic explanations for the reduced mycobacterial control observed. This includes cell death modality of macrophages and eicosanoid mediator production, both of which are important immunological processes implicated in macrophage mycobacterial control. Studies indicate that macrophage apoptosis and necrosis is modulated by PGE2 and LXA4, where the balance of these mediators has important impact on infection outcome[114, 156]. As shown in Figure 11, PGE2 may restrict mycobacterial spread by promoting apoptosis, whilst LXA4 induces macrophages necrosis, thus causing propagated bacterial spread (Figure 11) [170]. Hence, a potential mechanism for the reduced bactericidal activity of macrophages following COX-2i inhibition in **Paper 3** may be lower levels of PGE2 and subsequently lower apoptotic induction, a mechanism shown previously [22, 170, 283].

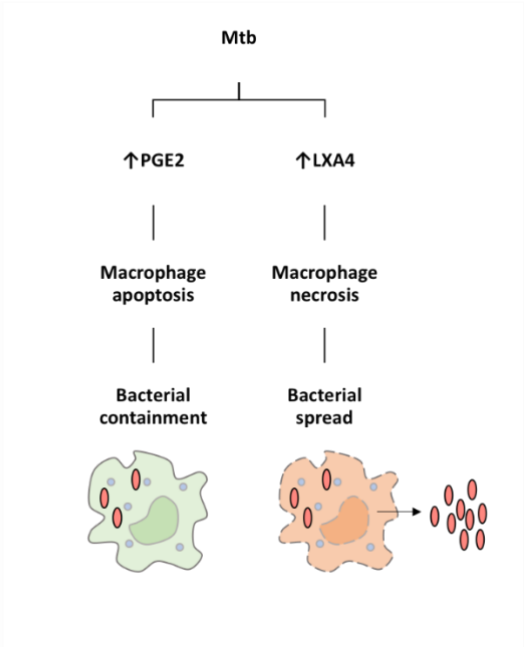

Figure 11 | Modulation of death modality in macrophages by PGE2 and LXA4. Figure by: K.G. Nore.

In **Paper 1-3**, we have found that monocytes and macrophages are important producers of and reciprocally modulated by eicosanoids. Importantly, monocyte and macrophage function are affected by manipulation of eicosanoid levels, where COX-2i inhibition ultimately seems to lower bactericidal activity of macrophages. This warrants further exploration in order to confirm the potential detrimental effects of COX-2is on macrophage function in TB.

5.3 Pros and cons for COX-2is as HDT in TB

The rationale for use of adjunctive anti-inflammatory therapy for TB is to alleviate the excessive and harmful host inflammatory responses that lead to pathological lung lesions [244]. COX-2is are inexpensive and widely available drugs that make them an enticing option as adjunctive therapy in TB [284]. However, their endogenous mechanism of action to *Mtb* and possible off-target effects remain unknown, and thus, alleviated inflammation may come at a cost of reduced protective responses[173].

Ultimately, studies report diverse and opposing effects of COX-is in TB [186]. Possible explanations are variations in drug used, administration form and dosage, experimental model, timing of treatment initiation and duration of therapy [135]. As mentioned in previous chapters, immune responses and eicosanoid production vary between early and chronic infection states and intervention on these targets by HDT is likely to have a similar dependency on timing [146]. Another essential aspect is drug of choice, as selective and unselective COX inhibitors seemingly show different effects when used as HDT[138]. Classical NSAIDs unselectively target both COX isoenzymes, whereas COX-2is are a subgroup that directly target COX-2. Contrary to its counterpart COX-1, a housekeeping enzyme constitutively activated, COX-2 is mainly upregulated during inflammation. Drugs that selectively or unselectively target these enzymes, are widely used for symptom-alleviating treatment in TB [200, 285].

Authors have proposed that inhibition of COX-2 leads to a compensatory upregulation of COX-2 and mPGES-1, as in vivo COX-2i did not affect pulmonary or serum loads of PGE2 after 4-weeks of COX-2i treatment[69]. In **Paper 2**, we observed no effects of COX-2i on PGE2 levels in plasma, which supports the hypothesis of a compensatory mechanism. This might also suggest that there are other off-target effects of COX-2is not yet elucidated. In **Paper 2**, we also reported that etoricoxib surprisingly reduced levels of lipoxygenase-mediators at day 14 and day 56. Others have shown that Ibuprofen influences the production LTB4, and blockade of COX-2 by celecoxib enhanced 5-LOX activity [134, 286]. Here, one might hypothesize that selective and unselective targeting of COX-enzymes lead to differing modulation of other enzymes in the eicosanoid system that might explain variations in susceptibility following treatment. Another possible explanation for the differing results is the

potential for interaction with anti-TB drugs, where murine models show both potentiated and antagonistic effects of COX-2i on standard TB treatment [209, 246, 247, 249, 287, 288].

Currently, there are some completed and ongoing clinical trials evaluating effects of NSAIDs as HDT. Importantly, the TBCOX2 trial evaluating effects of the COX-2i etoricoxib as well as the subunit H56:IC31 TB vaccine candidate reported that etoricoxib treatment did not improve cellular or humoral responses to *Mtb*, but rather reduced H56:IC31 specific immunity[204]. Although the researchers did not report any major safety concerns during treatment with etoricoxib, the number of reported adverse events (AE) were higher in the etoricoxib group [204]. Thus, the results did not support adjunctive COX-2 treatment [204]. Now, a pending trial intends to prove the safety and efficacy of adjunctive ibuprofen in extensively drug resistant TB (XDR-TB) (NCT02781909). The results from this study will be valuable in discerning the significance of the many conflicting findings of the pre-clinical research. Other studies pending include the trial NCT04575519, which assesses the efficacy and safety of two repurposed drugs (acetylsalicylic acid and ibuprofen) as adjunctive treatment to standard regimens for drug sensitive and MDR-TB. Another ongoing clinical study evaluates aspirin in combination with corticosteroids in tuberculous meningitis (NCT02237365), while the NCT02060006 trial investigates the efficacy of daily self-administered Meloxicam for prevention of TB-associated Immune Reconstitution Inflammatory Syndrome (TB-IRIS) in HIV-infected adults (NCT02060006).

In short, during the design of new personalized treatments focused on the regulation of the levels of host eicosanoids, various aspects must be considered. The beneficial effect of COX-is based on their ability to limit excessive inflammation and subsequent tissue-injury, as well as dampen the proposed immunosuppressive effects of PGE2 [186, 202]. However, the effects of COX-is are dependent on multiple factors, where phase-dynamics of PGE2 and potential off-target effects are key elements that must be taken into account [134, 186]. Moreover, most of the evidence of COX-2is as adjunctive therapy is limited to preclinical studies, which leaves several open questions that should be addressed in further research. Ultimately, results from clinical trials are long-awaited and may contribute immensely to the ongoing discussion of whether COX-2is should be administered as HDT in TB.

6 Conclusion

Repurposing immunomodulatory drugs such as COX-2is has potential to hold tremendous value for TB research [186]. But despite promising clinical trials in this area, there are many conflicting pieces of evidence[115, 289]. In our studies, we report that lipoxygenase-derived metabolites, but not PGE2, are associated with severity of TB disease and reduced with treatment, possibly accelerated by COX-2is. We show that innate and adaptive immune cells seemingly make important contributions to eicosanoid mediator production. *In vivo* COX-2i inhibition reduced intracellular phosphorylation potential and expression of inflammatory genes in innate immune cells. Finally, we show that the COX-2i etoricoxib reduced macrophage potential to control mycobacterial growth, an effect possibly caused by an overall reduced proinflammatory function. Also, our study may suggest that COX-2i may cause long-lasting effects on innate immune cells. Taken together, our studies provide important knowledge regarding the potential unfavorable effects of COX-2i treatment in *Mtb* infection and disease. We show evidence of the potential detrimental effects of anti-inflammatory eicosanoids, which stress the importance of further investigations of their role in *Mtb* infection in order to illuminate novel targets for HDT in the future.

7 Future perspectives

Despite available treatment, the global TB epidemic has intensified because of HIV co-infection, lack of an effective vaccine, emergence of MDR-bacteria and more acutely, the wake of the Covid-19 pandemic, which has reversed years of progress towards WHO End TB Strategy targets [14]. Alternative HDT strategies could be exploited to improve treatment efficacy and outcome, contain drug-resistant strains and reduce disease severity and mortality [9, 79]. However, the pathogen-host relationship in *Mtb* infection is complicated and considering the spectrum of TB disease and complexity of host immunity, adjunct HDT is unlikely to be efficient as a 'one-size-fits-all' approach [173]. Seemingly, this is also the case for HDT targeting eicosanoid pathways.

To elucidate the role of the eicosanoid system in human *Mtb* infection and disease, and subsequently clarify mediator-specific effects, more studies are warranted. Importantly, in-depth mechanistic studies of the eicosanoid system and longitudinal studies in sufficiently large patient cohorts with rigorous methodology are needed to determine the true potential of eicosanoids as HDT targets. Then, application of unbiased methods such as multi-omics are powerful tools for characterizing the complex eicosanoid network and discover possible biomarker signatures for TB disease. Importantly, the LOX-inhibitor Zileuton, already used in the treatment of asthma, could be repurposed as TB HDT, but intervention RCTs are needed to elucidate potential beneficial effects. Further, to confirm potential unfavorable effects of COX-2is, prospective studies following larger groups in different stages of *Mtb* infection and disease should be conducted. Optimally, such studies should include patients with various ethnicities, disease severity and degree of inflammation. Then, evaluation of timing of administration, treatment-length and route of drug delivery would be critical as there is a need to explore patient-specific characteristics that determine which patients may benefit from such treatment. Importantly, it would be critical to discover which patients are at risk for TB disease progression following COX-2i treatment, as these drugs are widely applied for symptom-alleviating effects in TB.

Ultimately, an individually- tailored treatment strategy based on clinical characteristics, biomarkers, disease spectrum and genetic profile would be the goal to secure effective HDT treatment. Considering that many factors potentially impact the efficacy of these treatment options as HDT, and especially for COX-2is, there is a need to investigate if the proposed

beneficial effects outweigh the cost. As such, the preclinical evidence requires clinical evaluation in larger study cohorts. Such progress is needed to better understand, first, the precise regulation of the eicosanoid system and, second, the effects of pharmaceutical intervention. Such knowledge will potentially allow researchers to uncover mechanisms to harness the immune response for maximum host protection with minimum damage.

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Elevated Levels of Anti-Inflammatory Eicosanoids and Monocyte Heterogeneity in *Mycobacterium tuberculosis* Infection and Disease

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Eicosanoids modulate both innate and adaptive immune responses in *Mycobacterium tuberculosis (Mtb)* infection and have been suggested as possible Host Directed Therapy (HDT) targets, but more knowledge of eicosanoid dynamics in *Mtb* infection is required. We investigated the levels and ratios of eicosanoid mediators and their cellular sources, monocyte subsets and CD4 T cells in Tuberculosis (TB) patients with various clinical states of *Mtb* infection. Patients consenting to prospective enrolment in a TB quality registry and biorepository, 16 with pulmonary TB (before and at-end-of treatment), 14 with extrapulmonary TB and 17 latently infected (LTBI) were included. Plasma levels of Prostaglandin E2 (PGE2), Lipoxin A4 (LXA4), and Leukotriene B4 (LTB4) were measured by enzyme-linked immunosorbent assay. Monocyte subsets and CD4 T cells and their expression of Cyclooxygenase-2 (COX-2), Prostaglandin receptor EP2 (EP2), and 5-Lipoxygenase (5-LOX) were analyzed by flow cytometry with and without Purified Protein Derivate (PPD)-stimulation. Pulmonary TB patients had elevated levels of the anti-inflammatory mediator LXA4 at diagnosis compared to LTBI ($p < 0.01$), while levels of PGE2 and LTB4 showed no difference between clinical states of *Mtb* infection. LTB4 was the only mediator to be reduced upon treatment ($p < 0.05$), along with the ratio LTB4/LXA4 ($p < 0.01$). Pulmonary TB patients had higher levels of total monocytes at diagnosis compared to end-of-treatment and LTBI (both $p < 0.05$), and a relative increase in the classical monocyte subset. All monocyte subsets had low basal expression of COX-2 and 5-LOX, which were markedly increased upon PPD stimulation. By contrast, the expression of EP2 was reduced upon stimulation. CD4 T cells expressed low basal COX-2 activity that increased modestly upon stimulation, whereas their basal expression of 5-LOX was considerable. In conclusion, the level of eicosanoids in plasma seem to vary between clinical states of *Mtb* infection. Mediators in the eicosanoid system are present in

monocytes and CD4 T cells. The expression of eicosanoids in monocytes are responsive to mycobacterial stimulation independent of *Mtb* disease state, but subsets are heterogeneous with regard to eicosanoid-mediator expression. Further exploration of eicosanoid mediators as targets for HDT in TB are warranted.

Keywords: tuberculosis, eicosanoids, prostaglandins, leukotrienes, lipoxins, monocytes, T cells, host-directed therapy

INTRODUCTION

Globally, nearly 10 million new cases of tuberculosis (TB) resulting in 1.3 million deaths, are reported annually despite intensive global strategies to fight the TB epidemic (1, 2). The sustained success of the causative agent, *Mycobacterium Tuberculosis (Mtb)*, is partly explained by complex and multifactorial mechanisms to avoid, evade and subvert host immune responses (3–7).

Host-directed therapy (HDT), aiming to enhance host immune responses and modulate *Mtb*-induced inflammation, is a possible approach to improve treatment outcomes and contribute to shorter treatment regimens (8–10). Lipid mediators of the eicosanoid family are suggested as possible HDT targets (11–14). Eicosanoid biosynthesis consists of several pathways (**Figure 1**). Breakdown of Arachidonic Acid (AA), an integral part of all cell membranes, leads to production of lipid mediators through the gate-keeping enzymes Cyclooxygenase-2 (COX-2) and 5-lipooxygenase (5-LOX). Generated through COX-2, Prostaglandin E2 (PGE2) acts through four distinct receptors, of which Prostaglandin E2 receptor 2 (EP2) seems to play a role in host susceptibility to *Mtb* infection (15, 16). In parallel, 5-LOX generates pro-inflammatory leukotrienes and anti-inflammatory lipoxins, of which Leukotriene B4 (LTB4) and Lipoxin A4 (LXA4) seem to modulate innate and adaptive immune responses by exerting pro-inflammatory or pro-resolution effects (17–19). In *Mtb* infection, the dynamics between eicosanoids may impact on protective host immune responses important for containment or progression of TB disease (6, 14, 20). Disease severity in TB has been reported to be associated with increased ratio of LXA4/LTB4 and/or reduced ratio of PGE2/LXA4, rather than changes in absolute levels of specific metabolites (21, 22). Still, it is unclear whether eicosanoids can act as biomarkers, separating latent from active TB cases or reflecting responses to TB therapy (22).

Macrophages, innate immune cells of critical importance in TB pathogenesis and disease, are major producers of eicosanoids (8, 23). Monocytes are macrophage precursors circulating in peripheral blood and are divided into different subsets; classical monocytes ($CD14^{++}CD16^{-}$), intermediate monocytes ($CD14^{++}$, $CD16^{+}$), and non-classical monocytes ($CD14^{+}CD16^{++}$) (24). These subsets harbor diverging biological roles in antigen presentation, phagocytosis and production of inflammatory mediators during *Mtb* infection (4) suggesting an influence on innate and adaptive host responses (25), of which effector CD4 T-cells exert crucial functions in anti-*Mtb* host defense by facilitating macrophage activation (5, 26, 27).

We hypothesized an imbalance in the levels of eicosanoids secondary to *Mtb*-induced increased COX-2 activity. We therefore explored absolute levels of lipid mediators and their ratios in plasma in different clinical states of *Mtb* infection. Further, we mapped the expression and function of relevant eicosanoid enzymes and receptors in monocyte subsets and T cells in active TB compared to latently infected individuals (LTBI). We also analyzed changes during TB treatment to better understand eicosanoid-mediated immunomodulatory mechanisms in *Mtb* infection and disease.

Abbreviations: AA, Arachidonic acid; CM, Classical monocytes; COX-2, Cyclooxygenase 2; EP2, Prostaglandin E2 receptor 2; HDT, Host-directed therapy; IFN-γ, Interferon-γ; IM, Intermediate monocytes; IL-1, Interleukin 1; LTB4, Leukotriene B4; LXA4, Lipoxin A4; *Mtb*, Mycobacterium tuberculosis; NCM, Non-classical monocytes; PGE2, Prostaglandin E2; PBMC, Peripheral blood mononuclear cells; TB, Tuberculosis; TNF-α, tumor necrosis factor α; 5-LOX, 5-lipoxygenase.

MATERIALS AND METHODS

Study Population

Participants aged 18–70 years diagnosed with pulmonary TB (PTB), extrapulmonary TB (EPTB) or latent TB infection (LTBI) were recruited from the Dep. of Infectious Diseases and Dep. of Pulmonary Medicine at the Oslo University Hospital into a prospective observational cohort study (*Prognostic Immunological markers in Tuberculosis*). The TB diagnosis was based on positive sputum smears for acid fast bacilli, radiological findings, and/or *Mtb* positive cultures. MDR-TB and disseminated TB cases were excluded. All patients were treated with standard TB therapy following WHO guidelines: intensive phase of 2 months with 4 anti-TB drugs (isoniazid (INH), rifampicin (RIF), ethambutol (EMB), pyrazinamide (PZA)) and a continuation phase of 4 months with 2 anti-TB drugs. All patients received and responded to standard TB therapy for 6–12 months. LTBI were defined by positive QuantiFERON®-TB In Tube assay (> 0.70 IU/ml), absence of TB suspected clinical symptoms and, if relevant, negative *Mtb* cultures. Blood samples and clinical information was collected at diagnosis and, for patients with PTB disease, also at the end of anti-TB therapy after 6–12 months. To obtain an immunologically more homogenous patient cohort, exclusion criteria for all participants were HIV infection, diabetes, immunosuppressive diseases, and/or use of immunosuppressive medication.

Eicosanoid Measurements in Plasma

Blood samples collected in EDTA vacutainers were centrifuged immediately for 20 min at 2,000 g, snap-frozen and stored at -80°C

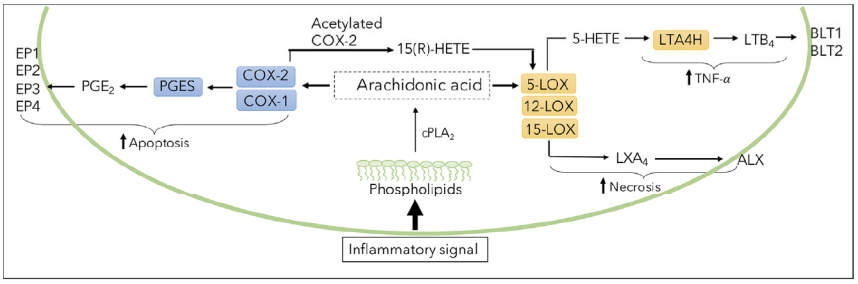

FIGURE 1 | Illustration of eicosanoid biosynthesis. Formation of PGE2, LTB4, and LXA4 through breakdown of arachidonic acid (AA) by cyclooxygenase (COX) and lipoxygenase (LOX) pathways. COX enzymes (constitutive COX-1 or inducible COX-2) and downstream PGES generates PGE2 which binds to various receptors (EP1-EP4). 5-LOX-derived LTB4 is produced *via* the precursor 5-HETE, converted by enzyme LTA4H and exerts effects through receptors BLT1 and BLT2. LXA4 is derived from LOX enzymes 5,12 and 15-LOX and binds to the ALX receptor.

until analysis. Using a competitive parameter immunoassay, human plasma concentrations of LTB4 and PGE2 were quantified using commercial EIA kits (Cayman chemical, Ann Harbour, MI). Plasma concentrations of LXA4 were assessed using enzyme immunoassay (EIA) kits (Oxford Biomedical Research, Oxford, MI). All assays were performed according to manufacturer's instructions. Briefly, samples underwent extraction protocols using C18-SPE Cartridges (Waters inc) or extraction using acetone precipitation, acidification and ethyl acetate extraction prior to analysis. Samples were then run in duplicates and optical density was determined at 450 or 650 nm using a Spectramax Abs plus microplate reader (Molecular devices Corporation). Lipid concentrations were calculated based on a sigmoidal standard curve using a 4-Parameter logistic fit. Inter-assay and intra-assay controls were included in all experiments.

Cell Preparation and Stimulation

Peripheral blood mononuclear cells (PBMC) drawn on cell preparation tubes, CPT (BD Biosciences, San Jose, CA), were isolated by centrifugation and cryopreserved in 20% DMSO/80% fetal calf serum at -150 °C until analysis. Cryopreserved PBMCs were thawed in a water bath at 37°C and washed with pre-warmed RPMI 1640 (Sigma-Aldrich) media supplemented with 1% L-glutamine and 10% fetal calf serum and rested for 2h prior to cell counts and viability check (Tryphan blue microscopy). Only samples with >80% viability were included in analysis. PBMC ($8x10^5$ cells) were either unstimulated or stimulated with 10 ug/ml Purified protein derivative (PPD, SSI, Denmark) and placed in a 37°C degree (with 5% CO_2) incubator overnight (18h). Cells were subsequently stained with either a monocyte or a T cell antibody panel (See **Supplementary Tables 1** and **2**).

Flow Cytometry Analyses

Cells were washed, stained and incubated for 30 min at 4°C. The monocyte panel consisted of Fixable Viability Dye 660, HLA-DR Alexa 700, Lineage cocktail 1 (CD3, CD20, CD56, and CD19) APC, CD14 PerCP, CD16 BV605, and EP2 PE. The T cell panel consisted of Fixable Viability Dye 660, CD3 PerCP, CD4 Alexa 700, EP2 PE. Then, cells were fixed and permeabilized according to the manufacturer's instructions (T cell panel with eBioscience FoxP3 transcriptional factor staining kit and monocyte panel with Cytofix/cytoperm staining kit, BD Bioscience) and subsequently stained for intracellular markers with conjugated antibodies (COX-2 FITC). Unconjugated antibody for 5-LOX were stained for 30 min at 4°C, followed by 2x wash with PBS and an additional incubation with Alexa Fluor® 405-conjugated secondary antibody (Abcam) was performed for another 30 min at 4°C. Flow cytometry (FACS Canto II, BD Biosciences) was performed in a blinded random order to remove any analysis bias. Fluorescence minus one (FMO) were used for gating of COX-2, EP2, 5-LOX (**Supplementary Figure 1C**). Total monocytes (HLA-DR^+ cells gated from the monocyte cloud) were further gated into the monocyte subsets classical monocytes ($CD14^{++}CD16^-$), Intermediate monocytes ($CD14^{++}$, $CD16^+$) and non-classical monocytes ($CD14^+CD16^{++}$) (**Supplementary Figure 1A**) (24). T cells were gated from live CD3 and CD4 positive cells with measurement of COX-2, EP2 and 5-LOX (**Supplementary Figure 1B**). Data are given as frequencies or mean fluorescent intensity (MFI). Flow analysis was performed using FlowJo software (Tree Star Inc.).

Statistical Analysis

All data are expressed with median and interquartile range (IQR). Non-parametrical statistical methods were applied. For ELISA quantification, p-values were calculated using the Kruskal-Wallis test with Dunn's post-hoc for multiple comparisons and Wilcoxon signed rank test was used for paired samples. For flow cytometry data, Mann-Whitney U test was used for groupwise comparison of unpaired data and Wilcoxon for matched pair test by using Graphpad Prism version 8.0 (Graphpad Software, LA Jolla, CA) and SPSS (IBM).

Ethical Considerations

The study was approved by the Regional Committees for Ethics in Medical Research (REK-Sør-Øst 2016/2123). Biobank samples were collected and stored in the "Research Biobank Infectious Diseases" ("Forskningsbiobank Infeksjonssykdommer" (REK 1.2006.181-S-0885, SHDNR. 09/513), Department of Infectious Diseases, OUS, Ullevål. Written informed consent was obtained from all participants before inclusion.

RESULTS

Study Participants Characteristics

Thirty patients with TB disease (14 patients with EPTB and 16 patients with PTB) and 17 patients with LTBI, were included. Demographic and clinical variables of *Mtb* infected patients are listed in **Table 1**.

Levels of Plasma Eicosanoids During Different Stages of *Mtb* Infection

Plasma levels of PGE2, LXA4, and LTB4 were analyzed at diagnosis of TB disease (PTB and EPTB) and compared to LTBI. No significant differences in either PGE2 or LTB4 levels between the groups were observed (**Figure 2A**). In contrast, concentrations of the anti-inflammatory LXA4 were elevated in PTB compared to LTBI ($p < 0.01$). Concentrations of PTB and EPTB were comparable. Next, the effects of eicosanoids may depend on their relative contribution rather than absolute levels. We therefore investigated the following eicosanoid ratios: PGE2:LXA4, PGE2:LBT4 and LTB4:LXA4 (**Figure 2B**). However, there were no significant differences in either PGE2:LXA4, PGE2:LTB4 or LTB4/LXA4 ratios between the various groups at baseline.

During TB treatment, LTB4 levels decreased significantly in PTB patients ($p < 0.05$), whereas LXA4 and PGE2 levels did not change (**Figure 2C**). LTB4/LXA4 ratio was reduced during treatment ($p < 0.01$), while PGE2/LXA4 and PGE2/LTB4 ratio showed no difference (**Figure 2D**).

Eicosanoid balance may be affected by host genomic background and genetic polymorphism (28), we therefore investigated levels and ratios of eicosanoids by stratifying patients according to ethnicity (Caucasian, African, and Asian), but no differences were detected (**Supplementary Figure 2**).

Monocytes, Monocyte Subsets, and Expression of COX-2, EP2, and 5-LOX

We found increased frequencies of total monocytes in PTB at diagnoses compared to LTBI (11 vs 6.5%, $p < 0.05$) and with a significant reduction at end-of-treatment ($p < 0.05$) (**Figure 3A**). The distribution of monocyte subsets revealed higher frequencies of $CD14^{++}CD16^{-}$ classical monocytes (CM) in PTB at diagnosis compared to LTBI, with a significant reduction during treatment ($p < 0.05$, median 76% in PTB at time of diagnosis vs median 66% in PTB at end-of –treatment). Frequencies of both $CD14^{++}CD16^{+}$ intermediate monocytes (IM) and $CD14^{+}CD16^{++}$non-classical monocytes (NCM) were low in PTB at diagnosis, however with a significant increase in both IM and NCM at end-of-treatment ($p < 0.01$ and $p < 0.001$, respectively). LTBI had significantly higher frequencies of IM compared to PTB at diagnosis ($p < 0.01$) (**Figure 3A**).

We further analyzed the expression of COX-2, 5-LOX, and EP2 in total monocytes in different states of *Mtb* infection (**Figure 3B**). %COX-2 expression was low and comparable in PTB at diagnosis (5.5%) and LTBI (4.8%), and with no significant changes during TB treatment (PTB post: 2.5%). Levels of EP2 were higher (PTB Pre: 23.1%, PTB post: 32.9%, and LTBI 25.6%), while levels of 5-LOX were comparable to COX-2 (PTB Pre: 11.55%, PTB post: 11.2%, and LTBI 5.9%), with no significant differences between patient groups (**Figure 3B**). When comparing MFI levels, PTB at diagnosis and LTBI

TABLE 1 | Demographic and clinical variables in patients with Mtb infection and disease.

	EPTB n = 14	PTB N = 16	LTBI N = 17
Gender, male (n, %)	9 (64)	13 (81)	6 (35)
Age (median, range)	30 (18–40)	32 (18–64)	29 (20–54)
Ethnicity (n, %)			
Caucasian	3 (21)	6 (37)	0 (0)
African	8 (57)	3 (19)	5 (29)
Asian	2 (14)	6 (37)	9 (52)
Unknown	1 (7)	1 (6)	3 (18)
Previous TB treatment (n, %)			
Yes	1 (7)	2 (13)	0 (0)
Unknown	2 (14)	1 (6)	0 (0)
Confirmed *Mtb* complex in culture/PCR (%)			
Yes	8 (57)	13 (81)	0 (0)
Resistance (%)			
Monoresistant TB[1]	5 (36)	2 (13)	
No of TB localizations (%)			
1	12 (86)	13 (81)	
2	2 (14)	3 (19)	
Low: High symptom score[2]	7:7	5:11	16:1
QuantiFERON-TB Gold (positive: negative: no data)	12:0:2	9:3:4	17:0:0
ESR[3] at baseline (mm/hour, range)	32 (5–82)	35 (3–109)	21 (13–40)
CRP[3] at baseline (mg/L, range)	17 (1–89)	19 (0.6–97)	3 (0.7–10)

[1]*Monoresistance is defined as resistance to one first-line anti-TB drug only.*

[2]*High symptom score is defined as 2 or more of the following symptoms: Fever (>38.0°), weight loss, cough, lymphadenopathy, night sweat. Low symptom score is defined as one of the symptoms listed or asymptomatic.*

[3]*ESR, erythrocyte sedimentation rate; CRP, C- reactive protein.*

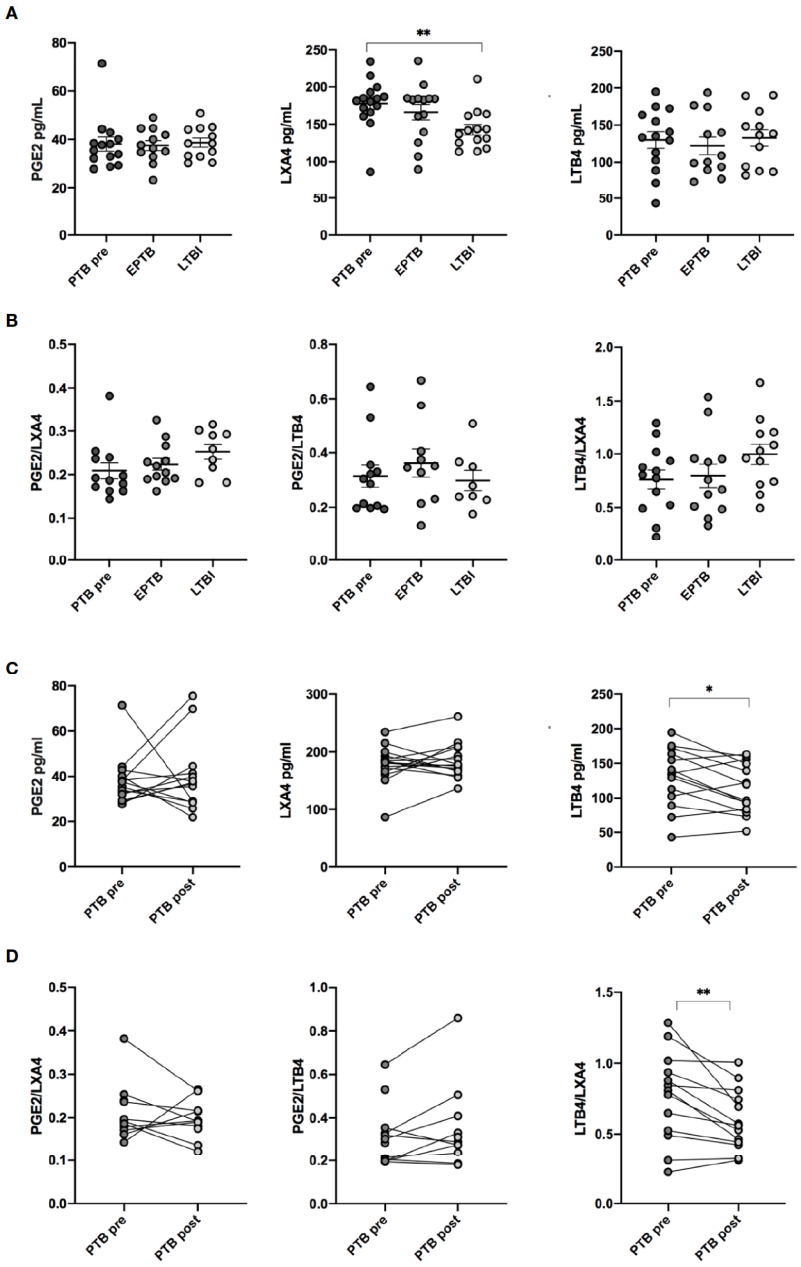

FIGURE 2 | Concentration and ratio of LXA4, PGE2 and LTB4 in plasma. **(A)** Concentrations of eicosanoid metabolites in pg/ml in plasma measured by ELISA at different stages of *Mtb* infection: EPTB (N = 14), PTB at time of diagnosis (PTB pre, N = 16), and LTBI (N = 17) **(B)** Ratio of eicosanoid metabolites in plasma in different stages of *Mtb* infection. **(C)** Levels of eicosanoid metabolites before (PTB pre, N = 16) and after treatment (PTB post, N = 16). **(D)** Ratios of eicosanoids before and after treatment. Data presented as median with interquartile ranges. P-values were calculated using the Kruskal-Wallis test with Dunn's post-hoc for multiple comparisons and Wilcoxon signed rank test was used for paired samples. Statistical significance represented by asterisk: ns, not significant; *$p < 0.05$; **$p < 0.01$.

showed a trend of increased levels of COX-2 compared to PTB at end-of treatment ($p = 0.09$ and $p = 0.08$, respectively), while there were no differences in levels of EP2 and 5-LOX (**Figure 3C**).

As expression of eicosanoid enzymes may be unevenly distributed in different monocyte subsets, we further explored the eicosanoid expression between the CM, IM, NCM monocyte subsets in the different stages of *Mtb* infection. Overall, as shown

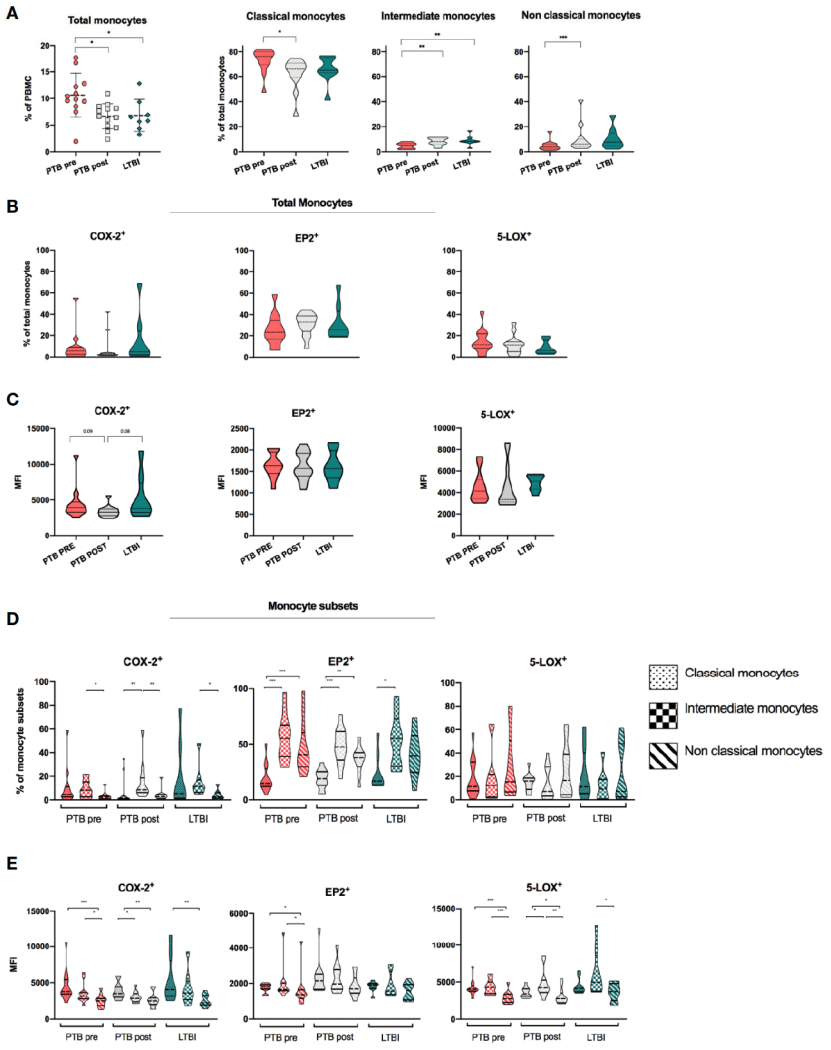

FIGURE 3 | Expression of monocytes, monocyte subsets and eicosanoid expression in stages of *Mtb* infection. **(A)** Distribution and frequencies of monocytes and monocyte subsets in different clinical stages of *Mtb* infection. PTB at time of diagnosis (PTB pre, pink, N = 13), PTB at end- of- treatment (PTB post, grey, N = 13), and LTBI (green, n = 8). Monocyte subsets were defined as classical monocytes ($CD14^{++}CD16^{-}$), Intermediate monocytes ($CD14^{++}CD16^{+}$) and non-classical monocytes ($CD14^{+}CD16^{++}$) (Gating strategy **Supplementary Figure 1A**) (29). **(B)** Frequencies of COX-2, EP2, and 5-LOX in unstimulated samples in total monocytes. **(C)** Mean fluorescence intensity (MFI) of COX-2, EP2, and 5-LOX in unstimulated samples in total monocytes. Frequencies **(D)** and MFI **(E)** of COX-2, EP2, and 5-LOX in unstimulated classical, intermediate, and non-classical monocyte subsets, presented by fill pattern, stratified by patient group (color). Mann-Whitney test was used for unpaired samples, Wilcoxon signed rank test used for paired samples. Statistical significance represented by asterisk: *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

for total monocytes, COX-2 expressing cells were low in all monocyte subsets, but significantly higher in IM subsets (8.3%) compared to NCM (2.3%) ($P < 0.05$) in PTB at diagnosis (**Figure 3D**). Expression of EP2 was modest in CM in all patient groups (PTB pre: 14.9, PTB post: 19.0, LTBI: 16.8). Interestingly, levels of EP2 were significantly elevated in both the IM and NCM subsets compared to CM in PTB at diagnosis and at end-of treatment ($p < 0.001$ and $p < 0.01$) (**Figure 3D**). There were no differences in frequencies of COX-2, EP2 and 5-LOX-expressing cells between patient groups (**Supplementary Figure 3**). When investigating MFI levels, COX-2 levels were higher in CM compared to NCM in all groups ($p < 0.001$, $p < 0.01$, and $p < 0.01$ for PTB pre, PTB post, and LTBI), while levels of 5-LOX were higher in CM and IM subsets compared to NCM in PTB at diagnosis and at end-of treatment ($p < 0.001$ and $p < 0.05$) (**Figure 3E**).

Dynamics of COX-2, EP2, and 5-LOX in Monocytes Upon *In Vitro* PPD Stimulation

We then investigated how PPD-stimulation affected the expression of COX-2, 5-LOX and EP2 in monocytes and

monocyte subsets. In total monocytes, COX-2-expression was significantly increased by PPD ($p < 0.01$), while EP2-expression was significantly downregulated for all groups ($p < 0.01$) except in PTB at end-of- treatment (**Figure 4A** and **Supplementary Figure 4**). 5-LOX mimicked COX-2 responses and was significantly up-regulated in PTB at diagnosis and at end- of-treatment ($p < 0.05$ and $p < 0.001$) (**Figure 4A** and **Supplementary Figure 4**). Next, we investigated co-expression of COX-2 and 5-LOX in unstimulated and stimulated monocytes. Frequency of double positive monocytes were low in all groups at baseline, but with significant upregulation by PPD in all groups ($p < 0.001$, $p < 0.001$, $p < 0.05$ for PTB pre, PTB post, and LTBI, respectively) (**Figure 4A**).

Within the various monocyte subsets, PPD stimulation induced a significant increase in COX-2 expression regardless of group ($p < 0.01$), however most pronounced for CM and IM monocytes ($p < 0.05$) (**Figure 4B** and **Supplementary Figure 4**). Downregulation of EP2 upon PPD stimulation was seen for all monocyte subsets in all patient groups, however most pronounced for the intermediate monocytes in PTB at diagnosis ($p < 0.001$) (**Figure 4B** and **Supplementary Figure 4**). Although 5-LOX was upregulated upon PPD stimulation in several of the monocyte subsets, there were no differences in induced 5-LOX expression between subsets in any of the clinical states of *Mtb* infection (**Figure 4B** and **Supplementary Figure 4**). Co-expression of COX-2/5-LOX was significantly upregulated in

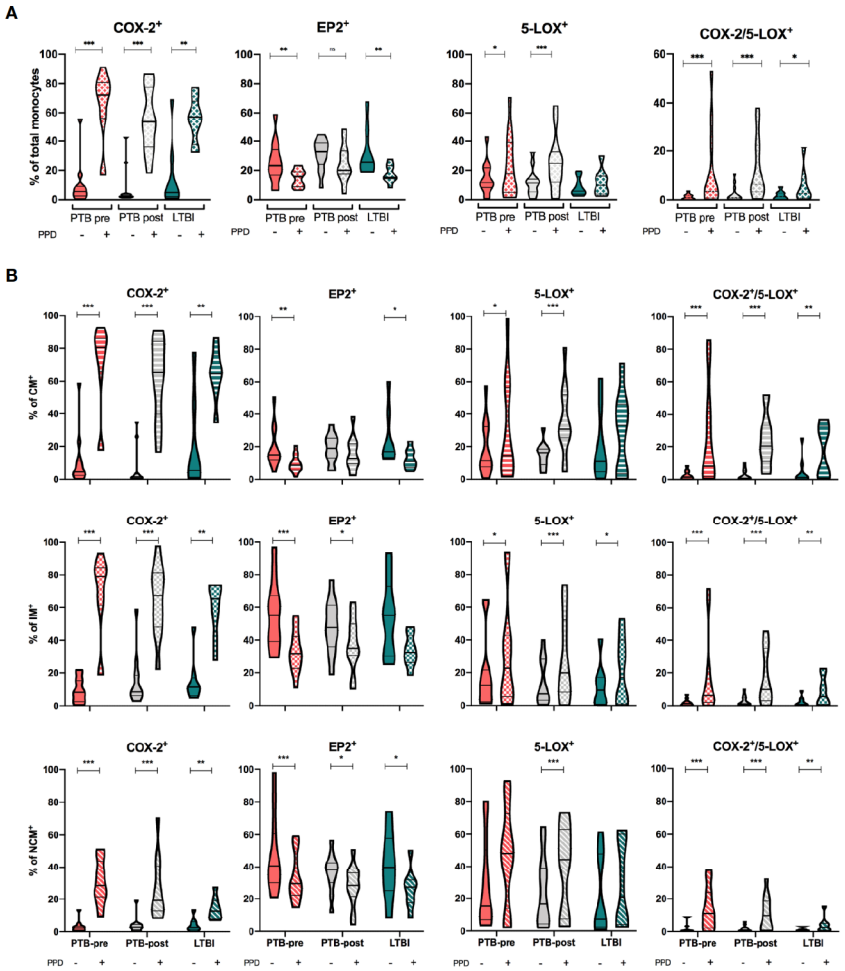

FIGURE 4 | Eicosanoid expression in unstimulated and PPD-stimulated monocytes. **(A)** Expression of COX-2, EP2, 5-LOX and double positive (COX-2/5-LOX) expression in HLA-DR$^+$ Total monocytes in unstimulated (filled bar) and stimulated (hatched bar) samples, stratified by patient group: PTB-pre (PTB at diagnosis, pink, n = 13), PTB post (PTB at end- of- treatment, grey, n = 13), and LTBI (latent TB, green, n = 8). **(B)** Expression of COX-2, EP2, 5-LOX, and double positive (COX-2/5-LOX) expression in unstimulated (filled bar) and stimulated (hatched bar) Classical (CD14^{++}CD16$^-$), Intermediate (CD14^{++}CD16$^+$) and non-classical monocytes (CD14$^+$CD16^{++}) stratified by patient group (color). Gating strategy is shown in **Supplementary Figure 1**. Mann-Whitney test was used for unpaired samples, Wilcoxon signed rank test used for paired samples. Statistical significance represented by asterisk: *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

all subsets by PPD (p = 0.001, p = 0.001, and p = 0.01 in PTB pre, PTB post, and LTBI respectively) (**Figure 4B** and **Supplementary Figure 4**).

Next, we evaluated the monocyte population in unstimulated and stimulated samples from PTB patients at diagnosis by the t-distributed stochatic neighbouring embedding algorithm (t-SNE) for unbiased visualization of COX-2, EP2, and 5-LOX-expressing subpopulations (**Figure 5**). This showed that EP2 is expressed by small clusters of cells in both unstimulated and stimulated regions, while 5-LOX was widely expressed throughout the monocyte populations. COX-2 is highly expressed in stimulated regions that also expressed high levels of CD14, and low levels of CD16. HLA-DR and CD14 were found in both stimulated and unstimulated regions of the monocyte population, while CD16 is mostly present in unstimulated samples (**Figure 5**).

Expression of Eicosanoid Enzymes in CD4 T Cells

Since effector CD4 T-cells are important in anti-*Mtb* host defense we explored the relevance of this cell subset in eicosanoid biosynthesis by analyzing their expression of COX-2, EP2, and 5-LOX. Basal levels of COX-2 and EP2 expression in CD4 T cells were generally low for both PTB at diagnosis and LTBI, with even lower frequencies in the PTB group at end-of-treatment ($p < 0.05$). In contrast, 5-LOX–expression was high in all clinical groups, especially in LTBI (**Figure 6A**). Basal co-expression of COX-2/5-LOX was low in all groups with no significant differences between patient groups (**Figure 6A**). Next, we investigated the capacity of T cells to induce eicosanoid enzymes upon PPD-stimulation (**Figure 6B** and **Supplementary Figure 5**). For all groups, COX-2 was modestly, but significantly induced ($p < 0.05$, $p < 0.01$ and $p < 0.05$ for PTB pre, PTB post and LTBI, respectively), while EP2 expression decreased in all groups ($p < 0.05$, $p < 0.001$, and $p < 0.05$). PPD-stimulation did not affect 5-LOX expression, but double positive COX-2/5-LOX T cells were significantly induced by stimulation ($p < 0.05$, $p < 0.01$, and $p < 0.05$ for PTB pre, PTB post, and LTBI, respectively) (**Figure 6B**).

DISCUSSION

Several studies have emphasized the role of eicosanoids and lipid mediators in TB pathogenesis (30–32). We hypothesized that the AA-derived metabolites PGE2, LXA4, and LTB4 reflects the ongoing inflammation and resolution processes orchestrated by eicosanoids in TB. We report absolute levels and ratios of eicosanoids, and the expression of key enzymes and proteins within the eicosanoid network in monocyte subsets and CD4 T cells, in different states of *Mtb* infection of interest to future HDT strategies.

We show that patients with TB disease have elevated plasma levels of the anti-inflammatory mediator LXA4 compared to LTBI. Levels of LTB4 decreased during treatment, whereas LXA4-levels remained unchanged and consequently the ratio of LTB4/LXA4 was lower after anti-TB therapy. Further, patients with TB disease had higher frequencies of predominately classical monocytes. The monocyte subsets in all patient groups expressed low basal levels of eicosanoid proteins, but while COX-2 and 5-LOX increased upon PPD stimulation, EP2 was reduced. Our data demonstrates dynamic changes and an imbalance in eicosanoids in TB that is not fully restored after treatment.

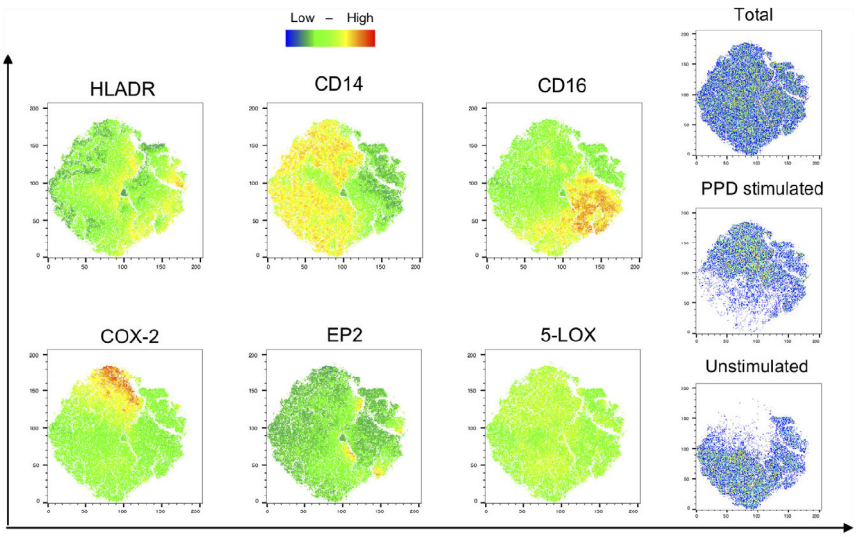

FIGURE 5 | Visualization of phenotypic changes with T-sne. Visualization of monocyte-related markers (HLA-DR, CD14, CD16) and the eicosanoid enzymes/proteins (COX-2, EP2, and 5-LOX) were analyzed with an unbiased approach using t-Distributed Stochastic Neighbor Embedding (tSNE) algorithm. Unstimulated and PPD-stimulated samples from 1 patient with PTB at diagnosis were concatenated and downsampled to $3*10^5$ cells/group. Color scale denotes low (blue) to high (red) density of the markers.

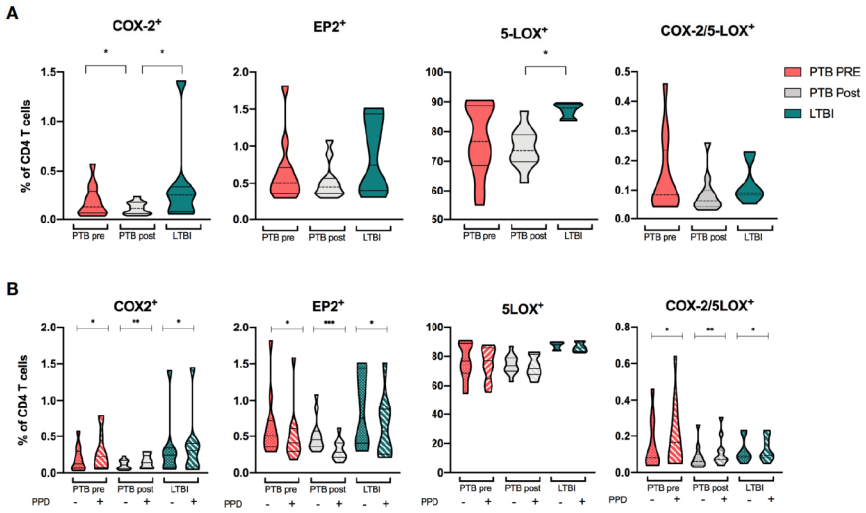

FIGURE 6 | Eicosanoid expression in CD4 T cells. **(A)** COX-2, EP2, 5-LOX, and double positive (COX-2/5-LOX) expression in unstimulated samples from patients with PTB at diagnosis (pink, n = 13), PTB at end-of-treatment (grey, n = 13), and LTBI (green, n = 8) in CD4 T cells. **(B)** Unstimulated (filled bar) and PPD-induced expression (hatched bar) of COX-2, EP2, 5-LOX, and COX-2/5-LOX in CD4 T cells. Wilcoxon matched-pairs signed rank test was used for paired data, Mann-Whitney for unpaired group comparisons. Statistical significance represented by asterisk: ns, not significant; *p < 0.05; **p < 0.01; ***p < 0.001.

Pulmonary TB Patients Exhibit a Relative Increase in Anti-Inflammatory Eicosanoids

We report that PTB patients exhibit a relative increase in the anti-inflammatory eicosanoid LXA4 compared to LTBI at diagnosis, which extends beyond treatment. This finding has also been reported in previous studies and may propose a role for LXA4 as a potential biomarker for TB disease (21, 22). Moreover, LXA4 has been linked to increased bacterial survival due to abrogated host immunity caused by promoted necrosis of infected macrophages and suppressed production of TNF-α (14, 18), suggesting a role for LXA4 in TB progression. By contrast, the pro-inflammatory mediator PGE2 has been suggested to confer protective responses during TB disease, by acting in a cross-regulatory network with IL-1 and promoting apoptosis of infected macrophages (20). Strict classification of PGE2 with regard to anti- or pro-inflammatory capacity is however less evident as PGE2 has been shown to harbor both pro- and anti-inflammatory effects depending on concentration and timing at local sites of infection (33–35). In contrast to other reports showing increased levels of PGE2 in TB disease (20–22), we observed no difference in PGE2-levels or PGE2:LXA4 ratio between patient groups. However, we observed reduced ratios of LTB4:LXA4 upon treatment, likely reflecting the reduced levels of LTB4 while LXA4 remained unchanged. In TB, an optimal balance of LTB4 is considered preferable, as high levels of this mediator tips the balance towards excess production of TNF-α, macrophage necrosis and enhanced growth of *Mtb* (18). Our data with no significant differences in levels of LXA4 and PGE2 during treatment of PTB, may be linked to treatment induced inflammatory imprinting of eicosanoid profiles, as shown in other studies (36, 37): Even though the inciting stimulus is cleared, the production of anti-inflammatory mediators may be extended and occur months after initial exposure (33, 37). Hence, future studies on dynamics of eicosanoids in *Mtb* infection should preferably secure samples 6–12 months after finalized TB treatment.

Monocyte Heterogeneity and Eicosanoid Signaling in *Mtb* Infection

We report increased frequencies of total monocytes in PTB at diagnosis compared to end-of-treatment and LTBI. Classical monocytes constituted the major subset regardless of clinical state, while frequencies of intermediate monocytes and non-classical monocytes were low but increase moderately during treatment. Monocytes are reported to expand during TB progression (38), and our data suggests that this expansion is mostly due to increased frequency of classical monocytes. However data are conflicting, and some report an association between $CD16^+$ expansion (i.e. intermediate and non-classical monocytes) and disease severity (25, 38–40), whilst others have shown higher levels of $CD16^+$ monocytes in LTBI patients (41). Notably, our study did not assess severity within PTB patients.

Although functions of monocyte subsets in *Mtb* infection are reported by others (42), evidence is scarce concerning their contribution in eicosanoid regulation. We report low basal expression of COX-2 in all monocyte subsets, but significantly higher expression in intermediate monocytes, possibly indicating higher production of PGE2 in this subset. Other studies have shown that intermediate monocytes produce high levels of TNF-α, IL-6 and IL-1, but reduced levels of IL-10 (42–44). Thus, the

finding that intermediate monocytes express high frequencies of COX-2, as well as elevated expression of EP2 compared to classical subsets, may support the notion that these cells are important producers of inflammatory mediators in general. Further, high levels of EP2 in intermediate and non-classical monocytes suggests that these cells are more susceptible to PGE2 signaling, compared to classical monocytes, indicating a pivotal role for intermediate and non-classical monocytes in eicosanoid signaling.

COX-2 and 5-LOX in total monocytes and monocyte subsets are clearly increased upon mycobacterial stimulation, while expression of EP2 is reduced. As classical monocytes and intermediate monocytes are considered superior phagocytes and important for secreting pro-inflammatory cytokines, it is not surprising that these subsets show elevated levels of COX-2 upon stimulation (24, 45). However, the finding that 5-LOX mimics the upregulated response of COX-2, indicates that mycobacterial antigens promote parallel induction of both enzymes also reflected in the increased levels of the anti-inflammatory LXA4 in PTB patients, as shown in our study. Our results therefore support suggestions of mycobacterial antigens manipulating the host response toward further bacterial spread through eicosanoid imbalance resulting from 5-LOX-induction and generation of LXA4 (14). Further, we found that basal levels of monocytes co-expressing COX-2 and 5-LOX were low, albeit with a significant upregulation by PPD. This strengthens our findings that monocyte subsets differ in their contribution to eicosanoid production. Reduced EP2 expression upon PPD stimulation may indicate a negative feedback loop in *Mtb* infection resulting in reduced PGE2-effects, as suggested by others describing a strong regulatory connection between COX-2 and EP2 in PGE2 synthesis (46). EP2 is reported to be distinctly upregulated upon LPS stimulation (47), and is normally not internalized or desensitized upon stimulation (48, 49). Thus, our findings of a marked reduction in EP2 receptor upon PPD stimulation raise questions regarding the potential for COX inhibitors as HDT in TB as the natural increase in EP2 expression observed in other bacterial infections seems to be abrogated by *Mtb*.

Eicosanoid Expression in Cells of the Adaptive Immune Response

In accordance with others (50, 51), we demonstrate that eicosanoid enzymes are also expressed by CD4 T cells. The COX-2-gene is transcriptionally upregulated in human T cells during T cell receptor signaling *in vitro* (48). Although CD4 T cells express low basal COX-2 activity, expression is modestly induced upon stimulation with PPD, indicating a functional role in response to *Mtb* infection with CD4 T cells capable of PGE2 production. Surprisingly, CD4 T cells show very high levels of basal 5-LOX activity, which is seemingly unaffected by PPD stimulation. Although it has been shown that 5-LOX is expressed by T cells (51), to our knowledge, this has not previously been shown in the context of TB. 5-LOX expression in T cells may possibly suggest a capacity for CD4 T cells to produce the anti-inflammatory mediator LXA4 or the pro-inflammatory LTB4. The unexpectedly high levels of 5-LOX in CD4 T cells should be explored in future studies.

There are some limitations to our study. First, a small number of patients give reduced power in statistical calculations and small differences may not have been detected. Further, we have not analyzed genes important in eicosanoid biosynthesis, e.g *lta4h* locus polymorphism as shown in previous reports (18). Future studies on eicosanoid enzyme and receptor polymorphism, as well as expression of other key markers in eicosanoid biosynthesis involved in TB pathogenesis (e.g. LTA4H, EP1-3, 15-LOX, and PTGES), are warranted. Changes and dynamics in peripheral blood may not reflect immune responses at local sites of *Mtb* infection. Thus, our study design limits our possibility to conclude on causative factors for TB progression, but still highlights important aspects of eicosanoid biosynthesis in *Mtb* infection.

Concluding Remarks

In this study, we show that levels of eicosanoids in plasma vary between clinical states of *Mtb* infection with an increased anti-inflammatory profile in active TB disease. Imbalance of eicosanoids extends beyond treatment. Monocytes and CD4 T cells express mediators involved in eicosanoid signaling, but monocyte subgroups differ with regard to their responsiveness and contribution to eicosanoid mediators. PPD-stimulation induced changes in eicosanoid mediators that could represent a *Mtb* strategy to divert host immune responses, but this need further investigation. Causative or not, monocyte heterogeneity is likely to impact on TB pathogenesis by influencing the balance of mediators of the eicosanoid pathway, but more mechanistic in-depth studies are warranted to understand the full network interaction. Therefore, our data support future studies exploring the role of eicosanoid mediators in immunopathogenesis in *Mtb* infection and as potential targets for HDT strategies in TB.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Regional Committees for Ethics in Medical Research (REK-Sør-Øst 2016/2123). Biobank samples were collected and stored in the "Research Biobank Infectious Diseases" ("Forskningsbiobank Infeksjonssykdommer" (REK 1.2006.181-S-0885, SHDNR. 09/513), Department of Infectious Diseases, OUS, Ullevål. The ethics committee waived the requirement of written informed consent for participation.

AUTHOR CONTRIBUTIONS

Conceived and designed the experiments: KN, MJ, KT, SJ, and AD-R. Recruited patients and collected clinical data: SJ, AD-R, and KT. Data acquisition: KN and MJ. Analyzed the data: KN, MJ, SJ, AD-R, and KT. Drafted and reviewed the manuscript: KN, MJ, SJ, AD-R, and KT. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2020.579849/full#supplementary-material

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II

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Plasma LOX-Products and Monocyte Signaling Is Reduced by Adjunctive Cyclooxygenase-2 Inhibitor in a Phase I Clinical Trial of Tuberculosis Patients

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Introduction: Eicosanoids and intracellular signaling pathways are potential targets for host-directed therapy (HDT) in tuberculosis (TB). We have explored the effect of cyclooxygenase 2 inhibitor (COX-2i) treatment on eicosanoid levels and signaling pathways in monocytes.

Methods: Peripheral blood mononuclear cells isolated from TB patients included in a randomized phase I clinical trial of standard TB treatment with (n=21) or without (n=18) adjunctive COX-2i (etoricoxib) were analyzed at baseline, day 14 and day 56. Plasma eicosanoids were analyzed by ELISA and liquid chromatography-mass spectrometry (LC-MS), plasma cytokines by multiplex, and monocyte signaling by phospho-flow with a defined set of phospho-specific antibodies.

Results: Lipoxygenase (LOX)-derived products (LXA4 and 12-HETE) and pro-inflammatory cytokines were associated with TB disease severity and were reduced during TB therapy, possibly accelerated by adjunctive COX-2i. Phosphorylation of p38 MAPK, NFkB, Erk1/2, and Akt in monocytes as well as plasma levels of MIG/CXCL9 and procalcitonin were reduced in the COX-2i group compared to controls.

Conclusion: COX-2i may reduce excess inflammation in TB *via* the LOX-pathway in addition to modulation of phosphorylation patterns in monocytes. Immunomodulatory effects of adjunctive COX-2i in TB should be further investigated before recommended for use as a HDT strategy.

Keywords: host-directed therapy (HDT), eicosanoids, cyclooxygenase-2 inhibitor, tuberculosis, monocytes, cytokines, innate immunity, lipooxygenase

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INTRODUCTION

Tuberculosis (TB), caused by *Mycobacterium tuberculosis* (*Mtb*) is responsible for an estimated 1.5 million deaths annually (WHO, 2020). Although a curable disease, effective TB treatment is challenged by increasing incidence of multi-drug resistant TB (MDR-TB). Host-directed therapy (HDT) has emerged as an alternative treatment strategy, aiming to increase treatment efficacy and shorten treatment duration by modulation of host immunity (Kolloli and Subbian, 2017).

The eicosanoid system, encompassing several biologically active lipid mediators, have been proposed to play an important role in the pathophysiology of *Mtb* infection (Peres et al., 2007; Dennis and Norris, 2015; Sorgi et al., 2020). Their synthesis is predominantly regulated by two families of intracellular enzymes, Cyclooxygenase (COX) and Lipoxygenase (LOX), of which there are several different subclasses. COX-2 is upregulated by inflammation and generates prostanoids including prostaglandin E2 (PGE2) while 5-LOX, 12-LOX, 15-LOX and 8-LOX produce lipoxins, leukotrienes and intermediate metabolites such as hydroxyeicosatetraenoic acids (HETEs) (**Figure 1**) (Dennis and Norris, 2015). Recent reports highlight a dysregulation of the eicosanoid network, with a skewed balance toward LOX products, promoting tissue damage and mycobacterial survival (Chen et al., 2008; O'connor et al., 2016; Pedruzzi et al., 2016). Lipoxin A4 (LXA4) seems to induce macrophage death while 12/15-HETE drive neutrophilic inflammation with subsequent tissue damage (Chen et al., 2008; Mishra et al., 2017), but effects of other products of the LOX pathway in TB pathogenesis are unclear.

Approved drugs that augment PGE2 levels have been suggested as a possible HDT-strategy in TB as PGE2 has been reported to limit detrimental type I interferon (IFN)-production in *Mtb* infected mice (Mayer-Barber et al., 2014) and induce macrophage apoptosis rather than necrosis (Divangahi et al., 2013). However, excess levels of PGE2 may also contribute to disease progression by inhibiting cell-mediated immunity (Rangel Moreno et al., 2002). COX-2 inhibitors (COX-2i) that inhibit PGE2 production reduce bacterial burden and increase survival in some animal models (Vilaplana et al., 2013; Sorgi et al., 2020), although the effect of COX-2i seems to be dependent on route of infection (Mortensen et al., 2019). Thus, COX-2i is of interest as potential HDT.

Monocytes and macrophages are key cellular players in TB pathogenesis and major producers of inflammatory mediators and eicosanoids (Rangel Moreno et al., 2002; Chen et al., 2008; Dennis and Norris, 2015). Toll like receptors (TLRs) and other bacterial pattern recognition receptors on the cell surface recognize foreign pathogens and initiate downstream signaling, resulting in the initiation of the early immune responses. Upon binding to the receptor, protein phosphorylation induces a set of transcription factors leading to production of pro-inflammatory cytokines such as PGE2, TNF- α, IL-12, IL-1 and IL-6 (Guha and Mackman, 2001; Basu et al., 2012).

The signaling pathways of p38 mitogen-activating protein kinase (MAPK), inhibitory κB kinase (IkK) and nuclear factor-κB (NFκB), interferon regulatory factors (IRF) and extracellular signal-regulated kinase (ERK) seem to be involved in monocyte/macrophage-derived cytokine signaling in response to mycobacterial antigens (Barnes and Karin, 1997;

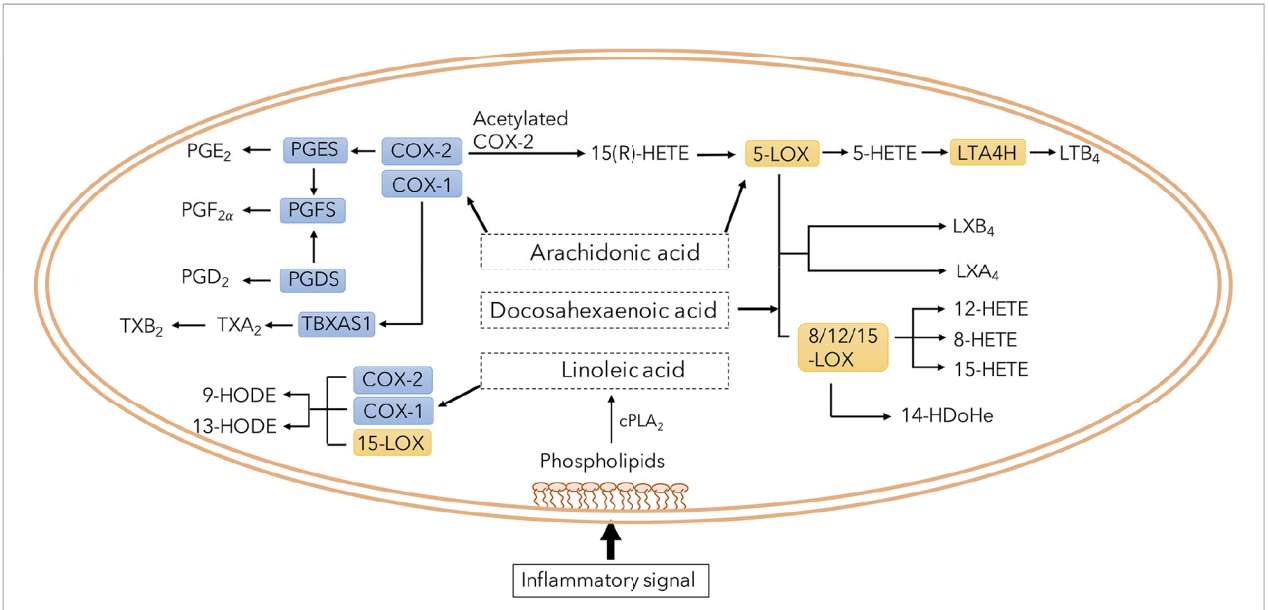


FIGURE 1 | Overview of eicosanoid pathway and its metabolites. Phospholipids in the plasma membrane is converted to several specific fatty acids. Among them, arachidonic acid is a common substrate for both COX and LOX enzymes generating immune modulatory prostaglandins, lipoxins and leukotrienes.

Zingarelli et al., 2003; Jo et al., 2007). *Mtb* infected macrophages produce high levels of PGE_2, partly mediated through TLR2/p38 MAPK signaling, thereby inducing apoptosis of *Mtb* infected macrophages (Nishimura et al., 2013). Translocation of NFκB to the nucleus and transcription of pro-inflammatory genes plays a key role in TB control, and NFκB has been suggested as a possible therapeutic target (Zingarelli et al., 2003; Tay et al., 2010; Fallahi-Sichani et al., 2012; Bai et al., 2013). During TB infection, eicosanoids seem to exert immunomodulatory functions by affecting the production of cytokines such as IL-1, IFN-γ and TNF-α (Tobin et al., 2010; Braverman et al., 2016; Cadena et al., 2016) through altered intracellular signaling, hereby representing a potential HDT target (Almeida et al., 2014). However, the effects of COX-2i treatment on eicosanoid production and monocyte signaling during TB disease remain unknown.

To obtain more insight into eicosanoid biology in TB we first studied the association between different eicosanoid mediators and severity of TB disease. To further evaluate the potential of COX-2i as HDT we investigated the effects of COX-2i on eicosanoid and cytokine levels in plasma from TB patient recruited into a phase I/II clinical trial assessing the safety and immunogenicity of adjunctive COX-2i in TB disease (TBCOX2 study). Finally, to explore novel HDT targets related to innate immunity we analyzed various signaling pathways in monocytes and the *in vitro* and *ex vivo* effects of COX-2i on signal transduction.

MATERIALS AND METHODS

Study Participants

Samples were collected from a total of 39 patients with culture confirmed drug sensitive TB recruited from a phase I/II/clinical trial at Oslo University Hospital, Norway in the period 2015-2019 (TBCOX2, *NCT02503839*). 18 patients received adjunctive COX-2i treatment (etoricoxib) for 140 days in addition to standard TB treatment and 21 patients received only standard TB treatment (**Table 1**). All participants experienced clinical improvement and culture conversion after 2 months of treatment. In addition, five patients (age 18-70) with pulmonary TB were included in a pilot study with blood sampling before TB treatment initiation. All participants were HIV negative. Clinical examination, symptoms, analyses of erythrocyte sedimentation rates (ESR), monocytes and lymphocytes (ML) ratio in peripheral blood, and chest X-ray performed at baseline were recorded. For an overview of the patients included in the different assays see **Supplementary Figure S1**.

Sample Collection and Preparation

Peripheral blood was drawn at baseline, day 14 and day 56. Blood samples were collected in CPT™ Cell Preparation tube (BD Biosciences), using Sodium-Heparin as anti-coagulant, and immediately centrifuged 15 minutes at 1700 g. Plasma was snap-frozen and stored at -80°C until analysis. Peripheral

TABLE 1 | Patient characteristics.

	Total (n = 39)	COX-2i (n = 18)	Control (n = 21)
Age (median)	27 (18-52)	29 (19-49)	26 (18-52)
Male (%)	21 (54)	9 (50)	12 (57)
Origin			
Black	20 (51)	8 (44)	12 (57)
Asian	11 (28)	5 (28)	6 (29)
Caucasian	6 (15)	3 (17)	3 (14)
Other	2 (5)	2 (11)	0
Clinical presentation			
Pulmonary	28[a]	14[b]	16[c]
Cavity	9	5	4
Extrapulmonary	7	4	3
Symptoms			
Cough (%)	20 (51)	10 (56)	10 (48)
Night-Sweat (%)	18 (46)	7 (39)	11 (52)
Weight loss (%)	15 (39)	9 (50)	6 (29)
Fever (%)	9 (23)	5 (28)	4 (19)
Chest pain (%)	11 (28)	4 (22)	7 (33)
Low:high symptom score[d]	17:22	8:10	9:12
Findings			
BMI[e] (min-max)	21 (16-30)	21 (16-30)	21 (17-27)
ML ratio[f] (min-max)	0.33 (0.13-1.36)	0.36 (0.13-1.36)	0.33 (0.17-1.14)
ESR[g] (mm/hour, min-max)	20 (1-116)	26 (2-105)	20 (1-116)
TTP[h] (min-max)	12.2 (2.7-42.1)	12.8 (2.71-24.9)	12.2 (4.7-42.1)
Ct values[i] (min-max)	36 (31-46)	34 (31-46)	41 (31-46)

[a]4/28, [b]2/14 and [c]2/16 with both PTB and EPTB.
[d]High =≥2 of the following symptoms: Cough, night-sweat, weightloss and fever (>38°C). Low = 1 symptom or asymptomatic/detected by screening.
[e]Body mass index (n= 34).
[f]Myeloid:lymphocyte ratio (n = 34).
[g]Erythrocyte Sedimentation Rate (n=34).
[h]Time to Mtb positive culture, days (n=30).
[i]Cycle threshold values.

blood mononuclear cells (PBMC) were isolated and frozen containing freezing media with 10% DMSO. Sputum or relevant tissue specimens were incubated at 37°C for minimum 42 days in Mycobacteria Growth Indicator Tube (MGIT, BD biosciences, New Jersey, USA) and the number of days to detection of bacteria can be measured as time to positive sample (TTP). Cycle threshold (Ct) values were obtained from analysis with quantitative PCR assay (Xpert MTB/RIF) for rapid detection of *Mtb*-specific nucleic acids.

Chemicals and Reagents

Commercially available EIA kits were used to measure PGE2 (cat.no. 514010, Cayman chemical, Ann Harbour, Michigan, USA) and LXA4 (cat.no. EA45 Oxford Biomedical Research, Oxford, Michigan, USA). Samples underwent extraction protocols using C18-SPE Cartridges (Cat.no WAT023501, Waters Inc, Massachusetts, USA) prior to EIA analysis. Cytokines were analyzed using Magnetic Luminex assay (cat.no. LXSAHM-24, RnD systems, Minneapolis, Canada and SAA Human ProCartaPlex™ Simplex Kit (cat.no. EPX01A-12136-901, Thermo Fisher Scientific, Massachusetts, USA)

Directly conjugated monoclonal antibodies for staining monocyte surface markers were directed to HLA-DR FITC (cat.no. 307604, Biolegend, San Diego, USA) and anti-CD14 PE antibodies (cat.no. 345785, BD Bioscience), antibodies for intracellular phosphoflow staining were anti – p38 mitogen activated protein kinase (MAPK) (pS180/S182) (cat.no. 612595), extracellular signal-regulated kinase (ERK) 1/2 (pT202/pY204) (cat.no. 6125939), Protein kinase B (Akt) (pS473) (cat.no. 560343), Nuclear factor κB (NFκB) p65 (pS529) (cat.no. 5584229, interferon regulatory factor (IRF)-7 (pS477) (cat.no. 558630), Cyclic AMP- response element binding protein (CREB) (pS133) ATF-1 (pS63) (cat.no. 558434), protein kinase A (PKA) RIIb (pS114) (cat.no. 560205) all from BD, Biosciences, San Jose, CA, USA. Fluorescent cell barcoding reagents were Pacific Blue Succinimidyl Ester (cat.no. P10163, Thermo Fisher Scientific, Massachusetts, USA) and Pacific Orange Succinimidyl Ester (cat.no. P30253, Thermo Fisher Scientific). Cells were fixed and permeabilized using BD Phosphoflow™ Fix Buffer I (BD Bioscience, cat.no. 557870) and BD Perm/Wash (BD Bioscience, cat.no. 554723).

COX-1/2 inhibitor used in the *in vitro* signaling assay were Indomethacin (20uM, cat.no. I7378-100G, Sigma Aldrich, Saint Louis, Missouri, USA). Cells were counted using Trypan Blue Solution 0.4% (cat.no. 15250061, Gibco™, Thermo Fisher Scientific) and stimulated with either 10ug/mL PPD (SSI, Denmark), 10ng/mL lipopolysaccharide (LPS) or 10mM Prostaglandin E2 (cat.no. HY101952, MedChemExpress, New Jersey, USA).

Enzyme-Linked Immunosorbent Assay

Using a competitive parameter immunoassay, human plasma concentrations of PGE2 and LXA4 from TB patients treated with or without adjunctive COX-2i, at diagnosis and day 14 was quantified using commercial EIA. All assays were performed according to manufacturer's instructions. Briefly, samples underwent extraction protocols using C18-SPE Cartridges. Samples were run in duplicates and optical density was determined at 450 nm or 650 nm using a Spectramax Abs plus microplate reader (Molecular devices Corporation).

Liquid Chromatography – Mass Spectrometry

Eicosanoid concentrations in plasma from patients treated with or without adjunctive COX-2i were analyzed at baseline, day 14 and day 56. Quantification of 5 – hydroxyeicosatetraenoic acid (HETE), 8-, 12 and 15 – HETE, 9-hydroxyoctadecadienoic acid (HODE), 13 – HODE, 14 Hydroxydocosahexaenoic acid (HDoHe) using liquid chromatography – mass spectrometry (LC-MS) was performed as previously described (Le Faouder et al., 2013) at the MetaToul Lipidomic Core Facility (I2MC, Inserm 1048, Toulouse, France, MetaboHUBANR-11-INSB-0010). In the panel, 19 metabolites were not detectable in plasma [resolvin (RV) E1, D1, D2, D3, D5, thromboxane B2, 11B-prostaglandin (PG) F2a, PGE3, PGF2a, PGE2, PGD2, PGA1, 8-iso-PGA2, 6-keto PGF1a, 15-deoxy-delta PGJ2, LXB4, LXA4, LTB5, 7-Maresin 1, 18-hyrdoxyeicosapentanoic acid (HEPE), 5,6 DiHETE, 17-HDoHe, 14,15-epoxy eicosatrienoic acid (EET), 5-oxo-EET, 11,12-EET, 8,9-EET, 5,6-EET]. Briefly, methanol and internal standard (Deuterium labeled compounds) was added before centrifugation (2000 g for 15 min at 4°C). Supernatants were transferred into 96-well deep plates and diluted in H2O. Samples were then submitted to solid phase extraction (SPE) using OASIS HLB 96-well plate (30 mg/well, Waters) and reconstituted in MeOH. Lipid mediators were separated on a ZorBAX SB-C18 column (Agilent Technologies) using Agilent 1290 Infinity HPLC system (Technologies) coupled to an ESI-triple quadruple G6460 mass spectrometer (Agilent Technologies). Data were acquired in Multiple Reaction Monitoring (MRM) mode with optimized conditions (ion optics and collision energy). Peak detection, integration and quantitative analysis were done using Mass Hunter Quantitative analysis software (Agilent Technologies) based on calibration lines built with commercially available eicosanoids standards (Cayman Chemicals). Metabolites that were not detectable in more than 30% of the samples were excluded for further analysis.

Cytokine Analysis

Measurements of cytokines in plasma collected from the COX-2i and control groups at baseline, day 14 and day 56 were performed using a Magnetic Luminex assay with a Luminex IS200 instrument (Bio-Rad). Measurements of chemokine (C-C motif) ligand 1 (CCL1), macrophage inflammatory protein-1α (MIP-1α/CCL3), MIP-1β/CCL4, interferon (IFN) IFN-α, IFN-β, IFN-γ, macrophage-derived chemokine (MDC/CCL22), Monokine induced by gamma (MIG/CXCL9), granulocyte colony stimulating factor (G-CSF), monocyte chemoattractant protein-1 (MCP-1/CCL2), interleukin (IL)-1β /IL-1F2, IL-2, IL-12p70, IL-1rα, IL-4rα, IL-8/CXCL8, IL-18/IL-1F4, CD25/IL-2rα, pentraxin 3, S100 calcium-binding protein A9 (S100A9), IFN-γ inducible protein (IP-10/CXCL10), matrix metalloproteinase-1 (MMP-1), procalcitonin and tumor necrosis factor (TNF)-α were analyzed using 24-plex kit while serum amyloid A(SAA) were analyzed using ProCartaPlex.

Analyses were performed in duplicates and analyzed with the Bio-Plex manager Software version 6.2 (build 175). Out of range values (OOR)> were set to the highest measurable concentration and OOR< were set to zero. Values that were out of the standard range but stipulated from the standard curve were included. Levels of IFN-α, IFN-β, IFN-γ, MIP-1β/CCL4, IL-12p70 were not detectable in more than 30% of the samples and were therefore excluded for further analysis

Cell Culture

Cryopreserved PBMCs were thawed and rested for 1h in a 37°C w/5% CO_2 incubator. Thawed cells were manually counted by microscope, and viability was measured by using Trypan Blue Solution. All samples included had a viability above 80% and the majority of the samples had a viability above 90%. Next, cells were subjected to various stimulation conditions ranging from 0 to 60 min. In a pilot study, PBMCs from confirmed pulmonary TB patients were collected before initiation of TB treatment. Cells were either unstimulated or stimulated with 10ng/mL LPS, 10ug/mL PPD or PPD in combination with 20uM Indomethacin and immediately fixed at 0 min, 10, 30 and 60 min after stimulation. Indomethacin was added to cells 30 min prior to PPD stimulation. PBMCs from the TBCOX2 clinical trial (COX-2i group, n= 8, controls, n=6) were subjected to the same procedures as the pilot study and stimulated with either 10ng/mL LPS, 10ug/mL PPD or 10uM PGE2 for the same period of time, but with no addition of Indomethacin to cultures.

Flow Cytometry

Phosphoflow were performed as previously described (Hermansen et al., 2018; Skanland, 2018). Briefly, prior to permeabilization, the different stimuli conditions were barcoded with different combinations of Pacific Blue and Pacific Orange in room temperature for 20 min. After barcoding, cells were washed with PBS containing 2%FBS, pooled, permeabilized and stained with anti-HLA-DR FITC and anti-CD14 PE antibodies (BD Bioscience) and 6 different phospho – specific antibodies namely anti – p38 mitogen activated protein kinase (MAPK) (pS180/S182), extracellular signal-regulated kinase (ERK) 1/2 (pT202/pY204), Protein kinase B (Akt) (pS473), Nuclear factor κB (NFκB) p65 (pS529), interferon regulatory factor (IRF)-7 (pS477), Cyclic AMP-response element binding protein (CREB) (pS133) ATF-1 (pS63) and in samples from TB patients treated with or without adjunctive COX-2i: Protein kinase A (PKA) RIIb (pS114) was included. **Supplementary Table S1** display antibodies included in the experiments. After 30 min incubation, cells were subjected to flow cytometry analysis with BD FACS Canto II. Monocytes were defined as HLA-DR$^+$ and CD14$^+$, the gating strategy can be found in **Supplementary Figure S2**. Cell acquisition (<300,000 events) was performed on a FACS Canto II (BD Biosciences). Instrument calibration was performed according to manufacturer's instructions and compensation settings adjusted using antibody-capture beads (CompBeads, BD Biosciences).

Statistical Analysis

For plasma analytes, all data are expressed as median and interquartile range (IQR). Non-parametrical statistical methods were applied, Mann-Whitney U test was used for unpaired data and Wilcoxon for matched pairs, Spearman for correlation analysis. Due to the exploratory nature of the data, it was not corrected for multiple comparisons, but cation was taken when interpreting the results. For flow cytometry analysis, the pooled stimulated samples could be deconvoluted with the different barcoding signatures and analyzed individually. Samples were analyzed using Cytobank (https://cellmass.cytobank.org) and Graphpad Prism (LCC, San Diego, US) and phosphorylation intensities are displayed as arcsinh ratio of medians. Mann U Whitney test was used to compare two unrelated groups. Multiple comparison with Holm Sidak's correction was used to compare phosphorylation time courses between the control and COX-2i group.

RESULTS

Lipoxygenase (LOX)-Derived Metabolites Are Elevated in Cavitary TB Disease

To investigate the role of eicosanoid metabolites in TB pathology, we stratified our cohort based on clinical criteria for disease severity at diagnosis (**Table 1**). In our cohort of 39 TB patients, 23% (n=9) displayed pulmonary TB with cavitary disease and 18% (n=7) were defined as extrapulmonary TB. A total of 22 patients had a high symptom score while 17 patients had low symptom score. Clinical parameters such as erythrocyte sedimentation rate (ESR), monocyte-lymphocyte (ML) ratio, time-to-positive *Mtb* culture (TTP) and cycle threshold (Ct) values were comparable in both groups at diagnosis.

While there was no difference in the levels of PGE2 between the clinical groups at baseline, mediators of the LOX pathway were elevated (LXA4, p=0.006 and 12-HETE, p=0.042) in cavitary disease compared to non-cavitary disease (**Figure 2**). No difference in eicosanoid concentrations were found when patients were stratified by symptom score (**Supplementary Figure S3**). PGE2 levels did not correlate with any laboratory markers of disease severity (ESR, ML ratio, TTP and Ct values), whereas LXA4 (r = -0.413, p=0.052) and 12-HETE (r = -0.522, p = 0.018) correlated inversely with time to positive *Mtb* culture (TTP) (**Figure 2C**).

Lipoxygenase (LOX)-Derived Products Decline With Adjunctive COX-2i Treatment

To explore possible effects of adjunctive COX-2i treatment we analyzed eicosanoid metabolites in plasma after 14 days of treatment when etoricoxib was expected to reach a steady state. Etoricoxib concentrations were detectable in plasma in all patients (data not shown). We observed no significant decline in PGE2 levels (COX-2 derived) in the COX-2i-group nor in controls (**Figure 3A**). By contrast, LXA4 levels (5-LOX-derived) declined significantly (p = 0.024) in the COX-2i-group but not in controls. Although the median PGE2/LXA4 ratio was higher in the COX-2i group at baseline, no significant changes were observed for any of the groups after 14 days of treatment (**Figure 3A**).

We then analyzed longitudinal effects of adjunctive COX-2i on eicosanoid plasma profiles after 14 and 56 days in more detail

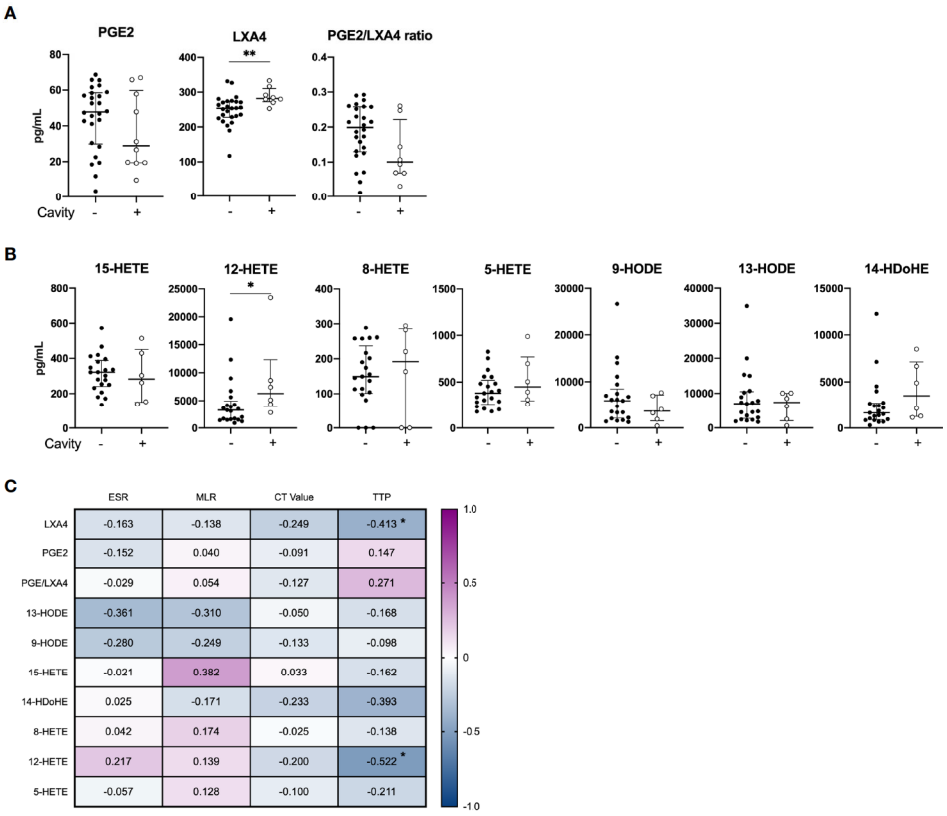


FIGURE 2 | Baseline plasma eicosanoid profiles in cavitary vs. non-cavitary TB disease and correlations to clinical markers. Plasma levels of **(A)** PGE2, LXA4 and PGE2/LXA4 ratio displaying cavitary (n = 8) and non-cavitary disease (n = 26) in TB patients at diagnosis measured by ELISA (included 16 patients in the control group and 18 patients in the COX-2i group) and **(B)** Eicosanoid metabolites comparing cavitary (n=6) and non-cavitary disease (n = 21) in TB patients at diagnosis measured by LC-MS (included 10 patients in control group and 18 patients in COX-2i group). One baseline sample was excluded due to limited plasma. **(C)** Eicosanoid correlations to clinical parameters erythrocyte sedimentation rate (ESR), monocyte lymphocyte (ML) ratio, Cycle threshold (CT) values and time to positive *Mtb* culture (TTP) collected at diagnosis. Significance calculated with Mann Whitney T test, *p < 0.05, **p < 0.01, Lines indicate median with interquartile range (IQR). Rho calculated with spearman correlation.

by LC-MS (**Figure 3B**). From diagnosis up to day 56, LOX metabolites such as 15-HETE (day 14, p = 0.034, day 56, p = 0.048), 8-HETE (day 14, p = 0.045), 12-HETE (day 56, p = 0.054) and 14-HDoHE (day 56, p = 0.01) were significantly reduced in the COX-2i-group. In controls, the only metabolite that was decreased at day 56 was 14-HDoHE (day 56, p = 0.01). Still, there were no significant differences at day 14 nor 56 when comparing the levels of metabolites between the COX-2i-group and controls at the respective time points (**Supplementary Figure S4**).

The Adjunctive Effects of COX-2i on Plasma Cytokines During TB Treatment

A broad specter of plasma cytokines was screened using a 24-plex kit and a single-plex Luminex Kit. The pro-inflammatory cytokines CCL1, Pentraxin3, CD25/IL-2ra, IP-10, S100A9 and MMP-1 correlated with markers of disease severity (**Supplementary Figure S5**). As COX-2i has anti-inflammatory properties (Kroesen et al., 2017) we investigated if COX-2i treatment influenced on plasma cytokines levels. In general, the inflammatory mediators declined during TB therapy in both the COX-2i group and in controls. Still, CXCL9/MIG and procalcitonin levels were significantly reduced after 56 days only in the COX-2i-group (**Figure 4A**). In contrast, CCL22/MDC, S100A9, IL-4Ra, CD25/IL-2ra, MMP-1, IP-10 and SAA were significantly reduced while CCL2/MCP-1 increased in both groups after 56 days.

Next, we investigated the association between eicosanoid and cytokine levels in plasma (**Figure 4B**). Interestingly, CXCL9/MIG showed a diverse relationship with products of the two eicosanoid pathways, with a weak positive correlation with PGE2 (r = 0.373, p = 0.050) and a moderate negative correlation with LXA4 (r = -0.497, p = 0.007), 13-HODE (r = -0.417, p = 0.031), 14-HDoHE (r = -0.630 p = 0.0001), 12-HETE (r = -0.552, p = 0.003) and 5-HETE (r = -0.372, p = 0.056). Further, LXA4 correlated positively with Pentraxin 3 (r = 0.369, p = 0.053) and IL-18 (r = 0.0389, p = 0.040). The LOX-products 15-HETE,

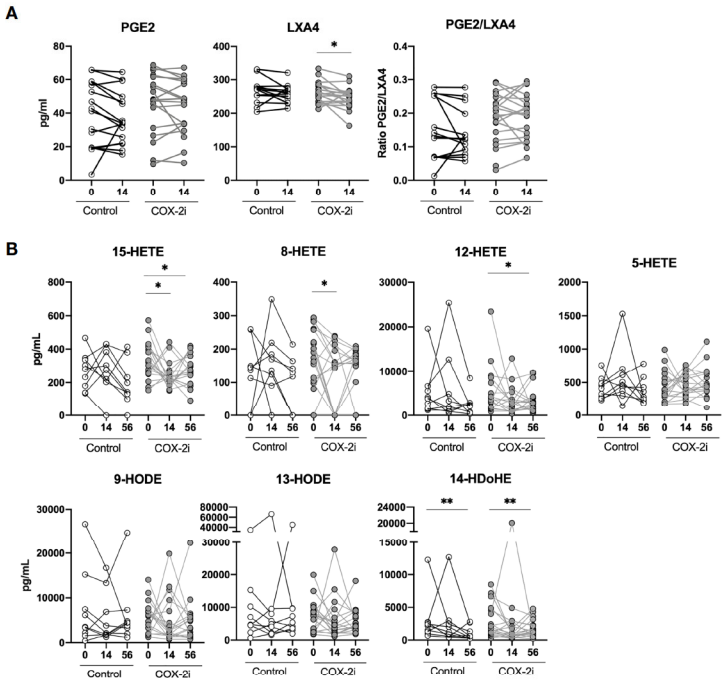


FIGURE 3 | Plasma eicosanoids levels during standard TB therapy alone and with adjunctive COX-2i. **(A)** Plasma levels of PGE2, LXA4 and PGE2/LXA4 ratio measured by ELISA comparing 14 days of treatment without (n = 16) and with (n = 18) COX-2i therapy. **(B)** eicosanoid metabolites measured by LC-MS at diagnosis, 14 and 56 days after treatment without (open circles, n = 10) and with (grey circles, n = 18) COX-2i. Significance calculated with Wilcoxon test comparing baseline and day 14 and baseline and day 56. *p < 0.05, **p < 0.01. Lines indicate median with interquartile range (IQR).

12-HETE and 5-HETE all showed positive correlations with CCL1, Pentraxin3, CD25/IL-2ra and IP-10, respectively.

Signaling Pathways in Peripheral Monocytes Induced by Lipopolysaccharide and Mycobacterial Antigens

Lipopolysaccharide and mycobacterial antigens bind TLRs in monocytes and induce signaling cascades with immune-modulatory effects. Thus, to further explore targets for HDT in a pilot study, we analyzed by phospho-flow cytometry the phosphorylation patterns in peripheral blood monocytes from another prospective cohort of TB patients before start of TB therapy (**Supplementary Figure S6**). We detected several phospho-epitopes following *in vitro* stimulation with endotoxin lipopolysaccharide (LPS) and mycobacterial antigens (purified protein derivative, PPD), but no effects on phosphorylation was observed by adding COX-2i to the cell cultures (**Supplementary Figure S6**). We then investigated the same phospho-epitopes during stimulation with either PGE2, LPS or PPD in samples collected from the TBCOX2 study after 14 days of standard TB treatment (**Figure 5**). *In vitro* stimulation by LPS induced phosphorylation of p38 MAPK (pS180/S182), Erk1/2 (pT202/Y204), Akt (pS473) with significantly higher intensities compared to PPD and PGE2 stimulation. In contrast, PGE2 induced higher intensity of PKA RIIb (pS114) phosphorylation than LPS and PPD as expected (**Figure 5**), indicating that LPS and PPD induce similar signaling cascades while PGE2 induce distinct pathways involving PKA. A schematic overview of the signaling pathways and the potential targets is illustrated in **Supplementary Figure S7**.

Adjunctive COX-2i Influences Phosphorylation in Peripheral Monocytes

The phosphorylation signaling pathways are upstream of the transcription of pro-inflammatory cytokines. Therefore, we investigated if adjunctive COX-2i for 14 days influenced monocyte signaling and responsiveness to *Mtb* antigens in TB patients from the TBCOX2 trial. The phosphorylation kinetics of p38 MAPK (pS180/S182), NFκB (pS529), Erk1/2 (pT202/Y204), Akt (pS473), CREB (pS133)/ATF-1 (pS63) and IRF-7 (pS477/479) showed a similar pattern in these patients as that seen in the pilot study (**Supplementary Figure S6**). Overall, we observed lower levels of phosphorylation in the COX-2i group compared to controls, especially pronounced for LPS induced phosphorylation of p38 MAPK (pS180/S182) ($p<0.001$), NFκB (pS529) ($p<0.01$), Erk1/2 (pT202/Y204) ($p<0.05$) and Akt (pS473) ($p<0.05$) (**Figure 6A**). The intensity of PPD-induced phosphorylation of p38 MAPK (**Figures 6A, B**) was significantly lower in the COX-2i group compared to controls. Interestingly, the phospho-sites that were induced by PGE2 (IRF-7 (pS477/479), CREB (pS133)/ATF-1 (pS63) and PKA RIIb (pS114) displayed more pronounced

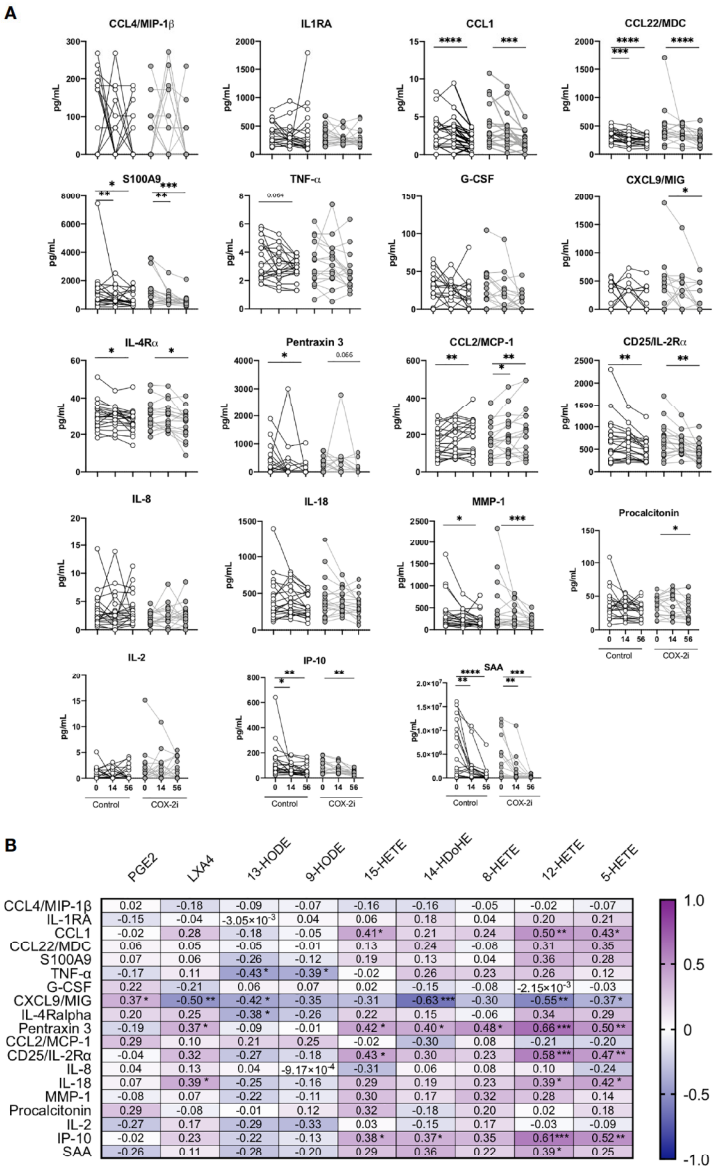


FIGURE 4 | Plasma cytokine levels during standard TB therapy alone and with adjunctive COX-2i. **(A)** Levels of cytokines in plasma in TB patients without (n = 21) and with (n = 18) COX-2i therapy. Significance calculated with Wilcoxon test comparing baseline and day 14 and baseline and day 56. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001, Lines indicate median with interquartile range (IQR). **(B)** Relationship between eicosanoids and cytokines levels in plasma from TB patients at diagnosis. Correlations are displayed using the Rho-value calculated with spearman correlation.

phosphorylation in the COX-2i-group compared to controls, although differences were not significant.

DISCUSSION

Targeted immunomodulating therapy may improve TB treatment strategies. COX-2i could possibly reduce excess inflammation and tissue damage in chronic stages of TB infection with clinical benefits for patients. Still, for some patients this might come at a cost of reduced bacterial clearance due to reduced pro-inflammatory responses (Kroesen et al., 2017). In the context of a phase I/II clinical trial assessing the safety of COX-2i given adjunctive to standard TB treatment we observed that the LOX-derived products LXA4 and 12-HETE were associated with disease severity at diagnosis. Several

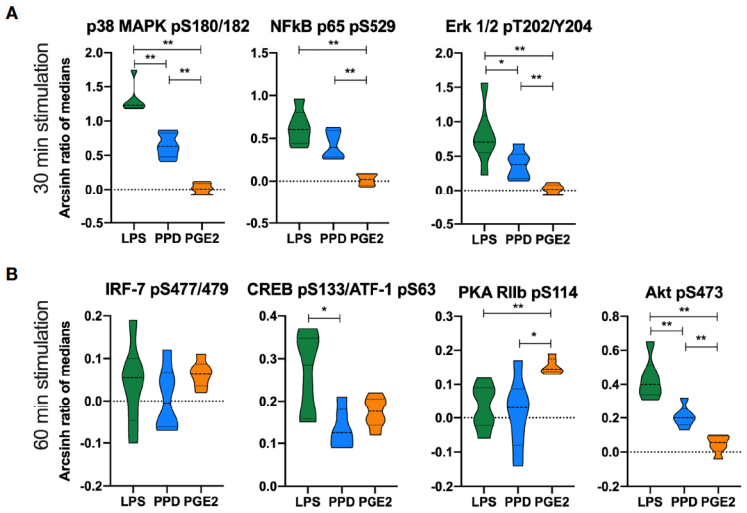
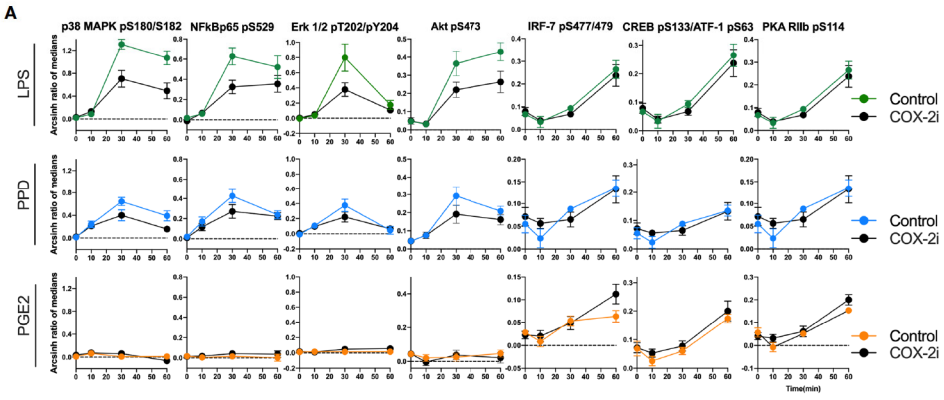


FIGURE 5 | Differential phosphorylation responses in monocytes to *in vitro* stimulation with LPS, PPD and PGE2. Phosphorylation intensities measured as arcsinh ratio of medians from patients receiving standard TB treatment for 14 days (n=6) after stimulation with LPS (green), PPD (blue) and PGE2 (orange) for **(A)** 30 min and **(B)** 60 min. Significance calculated with Mann Whitney test *p < 0.05, **p < 0.01. Violin plot displaying line at median and quartiles.



B

Phosphosite	LPS	PPD	PGE2
p38 MAPK pS180/182	***	**	ns
NFkB p65 pS529	**	ns	ns
Erk 1/2 pT202/204	*	ns	ns
Akt pS473	*	ns	ns
IRF-7 pS477/479	ns	ns	ns
CREB pS133/ATF-1 pS63	ns	ns	ns
PKA RIIb pS114	ns	ns	ns

FIGURE 6 | Distinct signal intensities in TB patients receiving COX-2i as adjunctive treatment. **(A)** Phosphorylation intensity induced by LPS (green), PPD (blue) and PGE2 (orange) in comparing COX-2i group (n = 8, black circles) and controls (n = 6, colored circles) after 0, 10, 30 and 60 min of stimulation. Phosphorylation intensity is measured as archsin ratio of medians. **(B)** Table of statistically significant differences between phosphorylation of the various sites in the control and COX-2i group after LPS, PPD and PGE2 stimulation. Significance calculated with multiple comparison with Holm Sidak's correction, asterix indicate significance p-value (*p < 0.05, **p < 0.01, ***p < 0.001, ns: not significant). Error bars indicate Mean±SEM.

eicosanoid metabolites were significantly reduced after 56 days of standard TB treatment, some already after 14 days, with a possible accelerated effect of COX-2i. Independent of COX-2i, pro-inflammatory plasma cytokines were reduced during the first two months of standard TB treatment, many already after 14 days while experiencing clinical improvement. Of interest, CXCL9/MIG and procalcitonin were significantly reduced only in the COX-2i-group indicating a possible adjunctive effect of COX-2i. In our *in vitro* monocyte signaling assay, LPS and mycobacterial antigens induced phosphorylation of the same phospho-epitopes. However, our findings suggest that IRF-7 is not activated by mycobacterial antigens, but rather by eicosanoids such as PGE2. We show novel data that TB patients treated with adjunctive COX-2i displayed an overall lowered signaling potential by LPS and PPD induced phosphorylation compared to controls suggesting reduced transcription of inflammatory cytokines in monocytes.

Clinical markers of TB disease severity and bacterial burden such as cavitary disease and number of days to *Mtb* positive culture (TTP) were both associated with levels of the LOX-derived metabolites LXA4, 12-HETE and 8-HETE in plasma. A detrimental role of 5/12-LOX derived metabolites in TB have been suggested due to LXA4-mediated necrosis of macrophages (Chen et al., 2008; Behar et al., 2010). Further, 12/15-LOX-derived products found in cavitary lesions may facilitate mycobacterial spread by driving neutrophilic inflammation, granuloma disintegration and tissue damage contributing to bacterial dissemination (Chen et al., 2008; Divangahi et al., 2010; Divangahi et al., 2013; Lau et al., 2015; Mishra et al., 2017). Our data are in accordance with a previous study reporting no association between PGE2 and TB disease severity, but rather an increase of LXA4, 15-epi LXA4 and LTB4 in cavitary TB disease correlating to bacterial burden (Pavan Kumar et al., 2019).

We further evaluated the effect of adjunctive COX-2i in TB patients by measuring plasma eicosanoids levels. We show that selective COX-2 inhibition influences the LOX arm of the eicosanoid system as several LOX-metabolites (LXA4, 15-HETE, 12-HETE, 8-HETE and 14-HDoHE) were reduced in the COX-2i-group, but not in controls. Our findings suggest possible beneficial effects of reduced levels of unfavorable LOX-metabolites, although it has previous been shown that COX-2 inhibition may also increase the activity of LOX-enzymes and its products (Dennis and Norris, 2015). Surprisingly, we observed no effect of COX-2i on PGE2 levels in plasma. This might be due to a suboptimal inhibitor dose or that 14 days are too early to detect possible effects. Further, we observed no association with disease severity, indicating a limited role of PGE2 than initially hypothesized in this stage of TB disease (Rangel Moreno et al., 2002). Therefore, selectively targeting the LOX-products by LOX inhibitors such as Zileuton or MK886 might be a better approach to avoid inhibiting potentially beneficial effects of the COX-2/PGE2 axis (Kaul et al., 2012; Sorgi et al., 2020).

A balanced and timely coordinated cytokine response is paramount in host immune defenses. Elucidating the systemic inflammatory milieu could expose novel HDT targets (Cicchese et al., 2018) as well as biomarkers for disease severity and treatment efficacy (Walzl et al., 2014; Kumar et al., 2019). We and others have previously reported that CCL1 and IP-10 could serve such a purpose (Tonby et al., 2015; Wergeland et al., 2015; Xiong et al., 2016; Kumar et al., 2019). Intriguingly, the cytokines pentraxin 3, IL-18, CD25 (IL-2R) and IP-10 associated with TB disease severity, were positively correlated with LOX-derived metabolites. The levels of several of these pro-inflammatory cytokines were reduced after 56 days of TB treatment, independent on COX-2i intervention, indicating that standard TB treatment is the main contributor to reduced bacterial load and inflammation. Still, there was reduced CXCL9/MIG and procalcitonin after 56 days of COX-2i treatment, not found in controls. The LOX-pathway produces both pro- and anti-inflammatory mediators (Dennis and Norris, 2015) often induced simultaneously. COX-2 induction by NFκB leads to conversion of 15-HETE and induction of 5-LOX, ultimately promoting lipoxin production. Thus, prolonged and excess inflammation facilitate *Mtb* survival and result in increased TB pathology (Stek et al., 2018; Vinhaes et al., 2019).

We further explored the relationship between monocytes and COX-2i by investigating monocytes signaling induced by the endotoxin LPS, that engages TLR4, and by mycobacterial antigens (PPD) that engage both TLR4 and TLR2 (Jo et al., 2007). Several of the investigated proteins, such as p38 MAPK (Balboa et al., 2013), NFκB (Bai et al., 2013), and Akt (Singh and Subbian, 2018) have been suggested as therapeutic targets in TB as they confer regulatory roles of infection and inflammation (Blumenthal et al., 2002; Yadav et al., 2004; Basu et al., 2012). TNFα-induced NFκB phosphorylation is crucial in conferring mycobacterial control and granuloma formation (Fallahi-Sichani et al., 2012). We observed increased NFκB p65 (pS529) phosphorylation after 30 min stimulation with LPS and PPD. PGE2 stimulation was also investigated to study potential indirect effects of COX-2i, such as altered PGE2 responsiveness. PGE2 induced phosphorylation of PKA RIIb (pS114), IRF-7 (pS477/479) and CREB (pS133)/ATF-1 (pS63) but not NFκB p65 (pS529), p38 MAPK (pS180/182) and Erk1/2 (pT202/Y204), indicating that PGE2 induces distinct pathways compared to LPS and/or PPD stimulation. Bound to transmembrane EP receptors, PGE2 induce accumulation of cAMP and thus activation of the PKA signaling pathway (Diaz-Munoz et al., 2012) while IRF-7 has a multifaceted role in *Mtb* infection as it can either promote or impair pathogen control (Manca et al., 2005; Mayer-Barber et al., 2011). Our findings suggest that PGE2 rather than LPS and PPD activate IRF-7 and PKA.

To the best of our knowledge, we present for the first time novel data on the effects of COX-2i on phosphorylation patterns in peripheral blood monocytes from TB patients harvested 14 days following initiation of adjunctive COX-2i. In line with already known anti-inflammatory properties of COX-2i (Williams et al., 1999), we observed reduced LPS-induced phosphorylation of p38 MAPK (pS180/182), NFκB p65 (pS529), Erk1/2 (pT202/Y204) and Akt (pS473) in the COX-2i-group possibly indicating reduced responsiveness of monocytes in patients treated with COX-2i. As several of these signaling pathways regulate transcription of pro-inflammatory cytokines (Jo et al., 2007), adjunctive COX-2i

potentially reduces pro-inflammatory responses in monocytes. However, whether this reduction is beneficial or detrimental for the patients with chronic TB must be further explored. A trend of higher PGE2-induced phosphorylation was observed in the COX-2i-group compared to controls, indicating COX-2i-driven susceptibly for PGE2 in monocytes. A possible explanation is a rescue mechanism to maintain PGE2 effects in the cells possibly by upregulation of EP receptors on the cell surface (Nishimura et al., 2013). This could also explain why we observed no effect of adjunctive COX-2i on plasma PGE2 levels. The mechanism could be upregulation of EP receptors with a simultaneous lowered ability to phosphorylate components of LPS and/or PPD induced pathways.

The major limitation of our study is the small sample size due to the phase I clinical trial design. Thus, our study is exploratory and the results hypothesis generating concerning possible effects of COX-2i on the eicosanoid pathways and monocytes in TB. Also, different tissue compartments must be studied to increase the understanding of eicosanoid metabolites and cellular interplay in TB pathogenesis. Future investigations on the effects of LOX-inhibitors on cell signaling and eicosanoid pathways are needed, to illuminate their potential role as HDT-targets. In addition, the potential efficacy of both COX-2 and LOX inhibitors as adjunctive HDT in TB should be investigated in larger patient cohorts with various clinical presentations where modest differences in cell behavior can be detected.

In conclusion, we show that LOX-derived products are associated with disease severity in untreated TB, while PGE2 seem to play a less important role during the first 14 days of TB treatment. While COX-2i primarily targets the prostaglandin pathways we observed an early reduction in potentially harmful effects of LOX-derived products. COX-2i seemed to reduce pro-inflammatory responses reflected in reduced phosphorylation potential and signal transduction in monocytes. These data provide knowledge on the possible benefits and disadvantages of using adjunctive COX-2i as an HDT strategy in TB disease.

DATA AVAILABILITY STATEMENT

The datasets presented in this article are not readily available because of the privacy of the research participants included in the study. Requests to access the datasets should be directed to Professor AM-DR, email: a.m.d.riise@medisin.uio.no.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by The Regional Committees for Medical and Health Research Ethics (REK SØ 2015/692, EudraCT nr: 2014-004986-26). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

Study concept and design, AM-DR, KTa, and MJ. Funding, AM-DR. Recruitment of participants, KTo, SJ, and AM-DR. Laboratory analyses and acquisition of data, multiplex (HCDA), flow cytometry (MJ and KN), LC-MS (EL and JN). Statistical analyses MJ and KN. Interpretation of data, MJ, KN, KTo, SJ, RM, KTa, DK, and AM-DR. Drafting of the manuscript, MJ and KN. Critical revision of the manuscript and intellectual content: KTo, SJ, AM-DR, RM, KTa, DK, HA, EL, and JN. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcimb.2021.669623/full#supplementary-material

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III

The Cyclooxygenase 2 inhibitor etoricoxib as adjunctive therapy in Tuberculosis impairs macrophage control of mycobacterial growth

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