Natural and Trained Innate Immunity against *Mycobacterium tuberculosis*

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Summary

Tuberculosis (TB), caused by *Mycobacterium tuberculosis* (*Mtb*) infection, remains a major global health emergency. It is estimated that one third of global population are affected, predominantly with latent granuloma form of the disease. *Mtb* co-evolved with humans, for its obligatory intra-macrophage phagosome habitat and slow replication, balanced against unique mycobacterial innate immunity, which appears to be highly complex. TB is transmitted via cough aerosol *Mtb* inhalation. Bovine TB attenuated Bacillus Calmette Guerin (BCG) live vaccine has been in practice for protection of young children from severe disseminated *Mtb* infection, but not sufficiently for their lungs, as obtained by trials in TB endemic community. To augment BCG vaccine-driven innate and adaptive immunity for neonates and better protection against adult pulmonary TB, a number of BCG pre-vaccination based, subset vaccine candidates have been tested via animal preclinical, followed by safe clinical trials. BCG also enhances innate macrophage trained immunity and memory, through primordial intracellular Toll-like receptors (TLRs) 7 and 9, which recognise distinct mycobacterial molecular pattern signature. This signature is transmitted by TLR signalling *via* nuclear factor-κB, for activating innate immune transcription and expression of gene profiling in a mycobacterial signature-specific manner. These are epigenetically imprinted in reprogramming of distinct chromatin areas for innate immune memory, to be recalled following lung reinfection. Unique TB innate immunity and its trained memory are considered independent from adaptive immune B and T cells. On the other hand, adaptive immunity is crucial in *Mtb* containment in granulomatous latency, supported by innate immune cell infiltration. In nearly 5-10 % of susceptible people, latent TB may be activated due to immune evasion by *Mtb* from intracellular phagosome within macrophage, perpetrating TB. However, BCG and new recombinant BCG vaccines have the capacity, as indicated in pre-and clinical trials, to overcome such *Mtb* evasion. Various strategies include pro-inflammatory-bactericidal type 1 polarisation (M1) phenotype of the infected macrophage, involving thrombospondin-TLR pathway. Saprophytic *M. smegmatis*-based recombinant vaccines are also promising candidates against TB. BCG vaccination of neonates/infants in TB endemic countries also reduced their pneumonia caused by various microbes independent of TB immunity. Here, we discuss host immune response against *Mtb*, its immune evasion strategies, and the important role innate immunity plays in the development of protection against TB.

Introduction

According to the World Health Organisation (WHO) Global Tuberculosis (TB) Report 2018/9, TB pandemic is larger than thought previously, owing to a better surveillance of the disease. In 2017, there were estimated 10.4 million new cases of TB worldwide; among men were 5.9 million, among women 3.5, and 1.0 million among children. People co-infected with HIV-1 and *Mtb* amounted to 1.2 million. Nonetheless, the infection incidence and deaths have been declining by 22%, as monitored between 2000 and 2015. This is in face of the first line multiple drug resistance (MDR), such as isoniazid and rifampicin, in certain areas. An accelerated incidence reduction rate of TB is called for by 2020, and further by 2030, for death reduction of 90%, and 80% for incidence reduction, as compared with 2015. Final goal is TB eradication.

A substantially lower TB infection rate in women seems to counterbalance a trade off in their higher (3 times over men) susceptibility to develop inflammatory autoimmune disease such as multiple sclerosis (MS), systemic lupus erythematosus (SLE), and rheumatoid arthritis (RA). The same immune cell mechanisms, including innate and adaptive immunity, may be engaged against TB. Much of this bias is biological in sex hormones. Oestrogen promotes production of pro-inflammatory cytokines such as IFN-γ, TNF-α and IL-12 by various immune cells, whereas testosterone induces IL-10, opposing IFN-γ secretion. This difference arises with puberty, confirming gender hormonal differences (Nhamoyebonde and Leslie, 2014).

TB is an ancient disease which co-evolved compatibly with humans, originating in Africa over millennia, traced back as early as 7,000 years. Together with demographic population migration all over the globe, *Mtb* co-diverged into distinct phylogenetic lineages. Such a view is based on the whole genome analysis of *Mtb* genotype variants, as compared with the founder pathogen complex (MTBC), which is still found in Africa (Russel, 2007; Comas et al. 2013). As a typical crowd disease, MTBC has adapted to Neolithic demographic transitions onto high density populations, associated more with virulent tuberculosis. However, in low density human settlements, *Mtb* can sustain a latent form of the disease for life. About 5-10% of infected people, *Mtb* can be reactivated and propagated to transmit TB. *Mtb* is a unique bacterial pathogen, and as an obligatory intracellular microbe, it has the ability to escape the host immune system (Comas et al., 2013; Gupta et al., 2012). Population density matters for the transmission of the disease, since Mtb released from pulmonary granuloma is transmitted via a close space contact, from a person suffering with active pulmonary TB, emitting *Mtb* in aerosol, which is inhaled by a susceptible host.

Mtb genotype polymorphisms have been found to be specific markers for diverging lineage strains. These include widespread Beijing/W strains characterised by large sequence polymorphisms-deletions (LSPs), which are associated with *Mtb* virulence (Tsolaki et al., 2004; 2005). In a separate study, Taiwan *Mtb* clinical isolates were genotyped for single nucleotide polymorphisms (SNPs), found also in Latin American, Mediterranean, ancient Beijing, modern Beijing, and Euro-Americans. Such data are important for the knowledge of *Mtb* transmission dynamics (Dou et al. 2017). For the same reason, people leaving in densely populated districts in Harris County, Texas that were exposed to active TB, their *Mtb* isolates were genotyped in clusters, as compared with that in low density regions with a low *Mtb* infection incidence (Agarwal et al., 2017). In low *Mtb*- demographic density countries, a large majority of infected people appear to be in a latent state for years. Such people, who are positive for tuberculin skin test (TST) and/or IFN-γ release test, have most likely sequestered *Mtb* infected macrophages, in forming immune granulomas-tubercles as a response, regulated mainly by

adaptive immune helper T (Th) cells (Figure 1). These dynamic structures comprise deposition of innate mononuclear immune cells, $CD4^+$ Th cells, epithelial, and foamy macrophages, enveloped in fibrous membranes. There is also recruitment of macrophages and polymorphs around the fibrous envelopment (Gupta et al. 2012; Russell, 2007). In approximately 10-20 % of infected people, the latent disease can be reactivated into full blown active disease, especially in immunocompromised people with a decline in $CD4^+$ Th cells due to HIV-1 infection/AIDS, malnutrition, physical stress, and/or by ageing. Primary *Mtb* infection proceeds mainly symptomless to be reactivated in some people later (Russell, 2007).

In contrast, up to 50% neonates/infants and adults, even though living in densely populated TB prevalent area, appear to be resistant to *Mtb* infection. Presumably, they may have immediate eradication of *Mtb* by innate phagocytes, epithelial cells, innate invariant T cells and natural killer (NK) cells, located in bronchial pulmonary airway mucosa and alveoli. They appear to have never been *Mtb*-infected. Accordingly, they are found to be negative for tuberculin skin test (TST) reaction, and there is an absence of granulomas. A total resistance of such individuals may suggest the power of innate cell immunity as a major natural effector against *Mtb* (Verrall et al., 2013).

BCG is so far the only approved vaccine, which is an attenuated *Mycobacterium bovis* strain. It has been protective in children from fatal *Mtb* dissemination, miliary TB, and partially from pulmonary TB. Most of the prophylactic clinical vaccine candidates undergoing trials require children to be pre-vaccinated with BCG (Triccas and Counoupas, 2016). This is followed by vaccination with its subset vaccines, targeting *Mtb* antigen-specific dominant virulence proteins, including early secreted antigenic target-6 (ESAT-6), as recombinant attenuated viral, or other pathogen vector (Kaufmann et al. 2017). Such BCG/subset vaccination combination is expected to augment innate as well adaptive immunity against TB. There are good results with respect to protective innate and adaptive immune response in children from these vaccines, which is vital for the safety of *Mtb* containment in their granuloma latency. Polyfunctional Th1 secretion of anti-bacterial cytokines is usually upregulated, including IFN-γ, TNF-α and IL-1β (McShane et al. 2004).

BCG vaccination has recently been demonstrated, in mice and humans, to augment anti-*Mtb* innate immune protection, through the primordial phenomenon, called 'innate trained immunity and memory'. It entails macrophage intracellular TLR-7 recognizing intracellular *Mtb* molecular signature, which is epigenetically imprinted on chromatin remodelling for its augmented memory, which can be recalled upon subsequent *Mtb* re-challenge. *Mtb* molecular pattern is also recognised by cytoplasmic nucleotide-binding oligomerization domain (NOD) like receptors, which can be augmented by BCG vaccination (Netea et al. 2016; Akira et al. 2006; Kleinnijenhuis et al. 2014).

Pathology and symptoms of active TB

TB symptoms, which are not specific, include haematuria, unexplained weight loss, consumption, fatigue, back pain, night sweats and breathing obstruction by a loss of gas exchange area. *Mtb* is a unique intracellular human pathogen residing in macrophage phagosome vesicle for its replication. *Mtb* gains entry in to macrophage via C-type lectin, CD91 and membrane TLR receptors, which recognise the mycobacterium molecular patterns, such as cell wall lipoglycans and ManLAM (Gupta et al., 2012). *Mtb* possesses the ESX secretion system required for its virulence. ESX-1 substrate, ESAT-6, induces phagosome-

disruption, and thus, giving *Mtb* the access into the cytosol exhibiting its pathological activity (Wong 2007; Houben et al. 2012; Pathak et al. 2007). ESAT-6 enters the endoplasmic reticulum (ER), where it interacts with β2 macroglobulin (a protein associated with MHC Class I molecule), inhibiting its association with MHC Class I molecule, possibly to delay or prevent the onset of anti-*Mtb* adaptive immune response (Sreejit et. al. 2014). As discussed above, the hallmarks of TB are tubercles-granulomas, primarily in the lungs, which may mature in their size and state, exhibiting central caseous necrosis accumulating necrotic cell, *Mtb* mycolic acid and other lipid casein-like debris, which still harbour viable pathogen. Necrosis arises from central hypoxic environment through decline in neovascularisation at this stage. Such granulomas may rupture, releasing free *Mtb* into airways for its aerosol transmission. TB granulomas tend to be located in apical lung regions. *Mtb* prefers oxygenated environments, which might explain its preference for ventilated apical lung region. Large granulomas, after discharging their content, appear as cavitation lesions. There is a variety of granuloma stages in a single person. *Mtb* cuticle and other constituents have immune cell inhibitory evasion, inflammatory and tissue destructing properties. In active TB, *Mtb* upregulates and activates macrophage matrix metalloprotease-1 (MMP-1), which destroys lung collagen architecture, because such collagen is a good substrate of MMP-1 (Russell 2007; Brace et al. 2017). Active disease develops in weeks or in longer periods due to *Mtb* slow replication within macrophages, as well as variation in strain virulence.

Extra-pulmonary TB

Extra-pulmonary TB (EPTB) can occur in any parts of the body, following initial spreading of *Mtb* to pulmonary draining lymphatic system. It is an important clinical issue (Gupta et al. 2012). Upon inhalation, *Mtb* may silently bypass lungs to be taken up by draining lymphatic vessel non-professional phagocytes such as endothelial cells and fibroblasts. From there, they may spread *via* lymph to other organ lymphatic systems (Lerner et al. 2016). *Mtb* DNA has been detected in pulmonary non-professional phagocytic cells in the absence of any histological lesions (Hernandez-Pando et al. 2000).

Lymphatic-endothelial vascular cells support *Mtb* growth, which is important for MHC class II cross-presentation of *Mtb*-specific antigen by follicular DCs to Th and B cells, although DCs themselves do not permit *Mtb* multiplication (Lerner et al. 2016). This pathway is important for pulmonary granuloma pathology and *Mtb* transmission. These events regulate, *via* Th1 cell produced IFN-γ, *Mtb* granuloma formation (Behr and Waters, 2014). Macrophage subsets, resistant to *Mtb* infection, have also been studied (Verralle 2013). Symptomatic EPTB occurs in nearly 10% of infected people. Overall, pulmonary TB prevails, since alveoli and airways are the main gateway of *Mtb* air-borne infection and its transmission (Gupta et al. 2012). EPTB also undergoes latency and active disease independent of pulmonary TB. Besides lymphatic system, TB lesions include peritoneum, kidneys, bone marrow, spleen, pleural, meningeal, peritoneal membranes, skin and genito-urinary tract, but not in microvascular endothelial cells of the brain that seem to reject Mtb and BCG proliferation (in mice) (Chen et al. 2015).

Comparative studies on disease risk for EPTB, as compared with pulmonary disease, indicated variations dependent on demographic population density, economic factors, gender and age. For example, in Nepal, which is a high burden TB country, EPTB is associated with lymphatic, peritoneal and intestinal TB, with almost equal incidence of pulmonary TB. People under 25, and female gender, were most susceptible to EPTB (Sreeramareddy et al. 2008). An American

demographic study also found that young women TB patients were at a higher risk to develop EPTB, but in priority to bone/joint TB, closely followed by cervical lymphatic and other organ disease, including disseminated miliary TB (Yang et al. 2004). Recently, in a Turkish region, a retrospective analysis of EPTB with young adults, revealed that women were most frequently affected with EPTB, while men suffered more often with pulmonary TB. It involved lymph nodes, followed by pleura, peritoneum and bone lesions (Sunnetcioglu et al. 2015).

Miliary TB, so called because the tubercles-granulomas resemble millet seeds, is a global organ-disseminated severe disease. In pre-antibiotic era, miliary TB affected mainly infants and children, but later on it appeared also in adults. Like other forms of TB, It is subject to innate and adaptive immune regulation. It may stem from pulmonary mature granuloma caseation necrosis, which after bursting, disseminates a large number of free *Mtb* via lymph and blood circulation, to organs preferably with fast blood flow such as lungs, spleen, kidneys, and liver (again not permissive in brain vascular endothelia proliferation) (Chen, 2015). Pulmonary and disseminated miliary TB may erupt owing to a local decline in Th cell regulation. A diagnostic sign is choroidal miliary tubercles (Sharmaat al. 2005). The co-infection of late HIV/AIDS (acquired immunodeficiency syndrome) with miliary TB showed a defective granuloma formation, and a substantial $CD4^+$ Th cell reduction, in accord with their role in TB latency (Sharma and Mohan, 2004).

TB and HIV/AIDS

Endemic TB demographic regions such as sub-Saharan countries, South Africa and Asia, people have been afflicted with another deadly epidemics, the AIDS, caused by human immunodeficiency virus-1 (HIV-1). As a new human disease, not having been co-evolved, AIDS seems to be in a total dis-equilibrium with humans. Since HIV-1 was first discovered in early 1980s, the HIV/AIDS disease has been on increase worldwide, together with TB co-infection (Sharma and Mohan, 2004). Persons with HIV-1 infection, especially when progressed to AIDS, are highly at risk to develop active TB primary infection, reinfection, or a latent granuloma reactivation. Such fulminant pathology, including necrosis and caseation, is due to Th1-produced IFN-γ deficiency, which normally regulates granuloma formation and its equilibrium. This HIV-1: *Mtb* combination is often associated with disseminated disease (Sharma and Mohan, 2004). In HIV-1 infection as well as and other forms of immunodeficiency, BCG is viewed as an opportunistic pathogen. For example, 1,950 male workers in South African gold mines, contracting HIV-1 infection, i.e. from their seroconversion to HIV, were cohort studied over several years for their increase in new active pulmonary TB. HIV-1 caused immunodeficiency increased with time, as did *Mtb* transmission load. Within 2 years of HIV-1 infection, the TB incidence rose to 1.4/100 person, and after 11years, nearly half of the men number Infected with HIV-1 has had active TB. As compared with HIV- negative men (5,702), active TB incidence was around 0.5-1.0/100 during this period (Glynn et al. 2008).

Non-tubercular mycobacteria (NTM), such as *M. avium* complex (MAC) and *M. kansasii*, cause opportunistic infections in HIV-1 co-infected patients who undergo a similar active pathogenicity course, and a high mortality rate, if not treated with anti-retroviral and anti-tubercular drugs (Procop, 2017). There is also a pathogenic synergy between TB and other diseases including diabetes mellitus type 2 epidemic in China (Cheng et al. 2017). In addition, there is an impact especially in children, co-infected with TB and malaria (*Plasmodium*

falciparum) predominantly in sub-Saharan regions. This situation requires an adjustment in drug interaction for the treatment of both diseases.

TB and Diabetes Mellitus

Diabetes Mellitus (DM) has been shown to have a strong link with TB (O'Garra et.al, 2013). As per International Diabetes Federation the number of people with diabetes is expected to rise to at least 592 million by 2035. Drug–drug interactions can lead to a reduction in the effectiveness of both TB and DM treatment, and potential worsening of drug side-effects (IDF, 2013). TB patients have higher blood sugar, a condition called impaired glucose tolerance, making them more prone to develop pre-diabetic condition (Restrepo & Schlesinger, 2013; Singh et al. 1984; Jawad et al. 1995). Rifampicin can increase the metabolism of most oral anti-diabetic drugs, thus worsening the glucose control in DM patients (Ruslami et al. 2010). Metformin, the glucose-lowering drug of choice in TB patients, has no meaningful interaction with rifampicin and may reduce TB mortality (Crevel et al., 2018). DM patients are three times more susceptible to develop TB. Monocytes derived from DM patients show reduced level of *Mtb* phagocytosis possibly due to alteration in complement C3 levels (Dooley & Chaisson, 2009). DM-TB conditions shows altered level of several cytokines such as IFN-γ, IL-12, IL-2, and TNF-α (Pal et. al. 2016), together with inhibition of *Mtb* antigen-specific cytokines (type 1 and type 17) (Kumari & Meena, 2014).

Both DM and TB patients have increased risk of cardiovascular complications such as myocardial infarction (Huaman et. al, 2017; Chung et. al. 2014) and stroke (Sheu et al., 2014), leading to higher rate of deaths in the first few months of anti-TB treatment (Faurholt-Jepsen et. al, 2013; Reed et al., 2013). Thus, it becomes very important to closely monitor anti-TB treatment in patients with combined DM and TB to avoid higher risk of drug toxicity, side effects and drug-drug interactions (Crevel et. al., 2018). A meta-analysis study considering information from 15 different countries revealed significant association of DM with MDR-TB (Tegegne et al., 2018). Moreover, DM is associated with an enhanced risk of multiple outcomes of not only treatment failure, but relapse and death during TB treatment (Pal et. al. 2016). As per WHO report there were an estimated 1.03 million people worldwide with HIV-associated TB, and this was associated with an estimated 370 000 HIV-related TB deaths in the year 2016 (WHO, 2017). Thus, possibly a growing number of people may be affected by DM, HIV and active TB at the same time, causing even a higher risk of mortality (Crevel et al. 2018).

Natural Innate Immunity and TB

Key innate barriers to Mtb infection

First physical and phagocyte-led defence is mounted by respiratory tract mucosa (nasal, bronchial, alveolar), which is composed of lamina propria, airway epithelial cells (AECs), and luminal airway surface liquid (ASL). Lamina propria harbours phagocytic cells such as macrophages and dendritic cells DCs), as well as the invariant T cells (Gold et al. 2010; Lerneret al. 2015). ASL contains innate anti-bactericidal peptides, including lysozyme, defensins, human cathelicidin (LL-37), human β-defensin-2, and hepcidin. Hepcidin expression is upregulated in human alveolar epithelial cells by *Mtb* cell wall components, mannose-capped lipoarabinomannan (ManLAM) and phosphatidyl-myo-inositol mannosides.

Live BCG had similar effects. Airway respiratory tract and lamina propria macrophages produce a range of pro-inflammatory cytokines and chemokines in response and defence against mycobacteria (Tsolaki, 2009; Li et al. 2012; Verrall et al., 2013; Lerner et al., 2015).

Human AECs, alveolar macrophage, polymorphs and monocyte-derived macrophages (MDM), upon *Mtb* infection, express cathelicidin LL-37 via TLR2, TLR4 and TLR9 activation at an early stage of infection, but not in *Mtb* granulomas, suggesting an important role of LL-37 in the innate immune responses in *Mtb* infection (Rivas-Santiago et al., 2008). However, *Mtb* may subvert cAMP-signalling on which cathelicidin expression depends, as evident from CRAMP lacking mice that exhibited an uncontrolled *Mtb* lung and spleen proliferation, CRAMP being a murine homologue CRAMP of human CAMP (Lim et al., 2016).

Alveoli possess epithelial type 1 cells required for air/blood capillary gas exchange. Type 2 epithelial cells act similar to the resident macrophages and DCs in response to pathogens, producing bactericidal peptides and cytokines. Type 2 epithelial cells also secrete hydrophobic surfactant proteins, relieving air/fluid tension, as well as hydrophilic surfactant proteins of collectin family, SP-A and SP-D, which can act on a range of lung pathogens including *Mtb* (Kishore et al. 1996; 2006; Ferguson et al. 1999; Le Vine and Whitsett 2001; Lerner et al. 2015).

Airway and alveolar epithelial cells, macrophages, lymphocytes, and DCs, may constitutively express innate immune receptors for pathogen molecular pattern recognition and effector function. These receptors include pro-inflammatory TLRs, C-type lectin receptors (CLRs), Dectin-1, NOD-2-like receptor, inflammasome-IL-1β activator, and DC-SIGN. Alveolar type-2 epithelial cell also secrete hydrolases which can modulate pulmonary interstitial lining, and can potentially alter Mycobacterial cell wall (Li Y et al. 2012; Lerner TR et al. 2015).

The C type lectin receptor, CLECSF8 (CLEC4D, MCL) is considered to be a vital non-redundant immune molecule against *Mtb*. CLECSF8 belongs to Dectin-2 cluster. It is constitutively expressed on myeloid cells such as macrophages, polymorphs and various DC subsets, when complexed with its cell signalling receptor FcRγ chain bearing ITIM motif. CLECSF8 recognises uniquely *Mtb*-molecular virulent carbohydrate pattern, the trehalose-6 dimycolate (TDM) (Graham et al. 2012; Miyake et al. 2013; Wilson et al, 2015). In a murine model, CLECSF8, through its receptor FcRγ chain intracellular signalling, downregulated an excessive recruitment by *Mtb*, of inflammatory neutrophils, to spare the host. CLECSF8 deficient mice, following aerosol *Mtb* infection, showed an enhanced pulmonary neutrophil infiltration, and production of pro-inflammatory cytokines, such as TNF-α, IFN-γ and G-CSF. In humans, a CLECSF8 polymorphism was associated with susceptibility to lung TB (Graham LM et al. 2012; Wilson GJ et al. 2015). CLECSF8 promoted innate part of granuloma formation through Mtb-TDM (Miyake Y et al. 2013).

Modulation of macrophage polarisation by Mtb

Following its macrophage internalisation in phagosome, *Mtb* pathogen has several complex mechanisms of evading its destruction, by inhibiting phagosome maturation *via* its early secretion antigenic target-6 (ESAT6). A key *Mtb* evasion event is its macrophage inflammatory type 1 (M1) polarisation switching into M2 anti-inflammatory phenotype (expressing IL-10 and TGF-β1) for its replicative residence. M2 phenotype is associated with TB, leprosy and other chronic inflammatory diseases such as asthma and parasites, but also with resolution of non-infectious inflammatory lesions. *Mtb* virulence factors such as ESAT-6 of ESX-1 system apparently drive M2 polarisation (Flynn and Chan, 2003; Russell et al. 2009, Gupta et al. 2012).

A critical macrophage polarisation factor is transcription factor Kruppel-like factor 4 (KLF4), which belongs to zinc-finger family proteins (Liao X et al. 2011) (Figure 1).

Recently, human and mice macrophage polarisation after *Mtb* infection has been studied via transcriptional, global gene expression and micro RNA-26 (miR-26a) analyses. Macrophage upregulated expression of miR-26a, inducing M1 polarisation, while suppressing KLF4 expression, and that of transcription factor CRAB induced C/EBPβ, favoured M2 polarisation. It is a balance which intracellular *Mtb* may exploit for its survival and replication, and to escape its early innate immune eradication (Sahu et al. 2017). In TB-susceptible children and adults, *Mtb*-infected M2 macrophages upregulate KLF4 and C/EBPβ, induce secretion of IL-10, CCL-24, arginase-1, while inhibiting anti-bacterial soluble factors (TNF-α, IL-12, IL-6 and NO). KLF4 signalling inhibit phagosome-lysosome fusion, their vacuole trafficking to lysosome, and prevent their autophagy, which may otherwise clear the *Mtb*. It has been suggested that miR-26a may possibly serve as a therapeutic drug in TB (Sahu et al. 2017). *Mtb* infection can suppress microRNA let-7, which is an inhibitor of A20, which in turn, inhibits NF-κB signalling (Kumar et al. 2015). M2 macrophages, expressing IL-10, IL-13 and arginase-1 induced by CREB-C/EBPβ signalling, can promote repair of injured muscle. Arginase-1 competes with iNOS for the common arginine substrate (Ruffell et al. 2009). Cyclic AMP (cAMP) signalling subversion by *Mtb*, *via* human cathelicidin gene CAMP or LL-37, also supports the pathogen's proliferation by inhibiting bactericidal cathelicidin production. Mice, deficient in their bone-marrow derived macrophages of homologue *Cramp* $^{-/-}$ mice, were tested for their pulmonary *Mtb* burden level and survival rate, after their *Mtb* infection. *Cramp*$^{-/-}$ mice showed a higher *Mtb* lung burden and died sooner than the wild-type mice (Gupta et al. 2017).

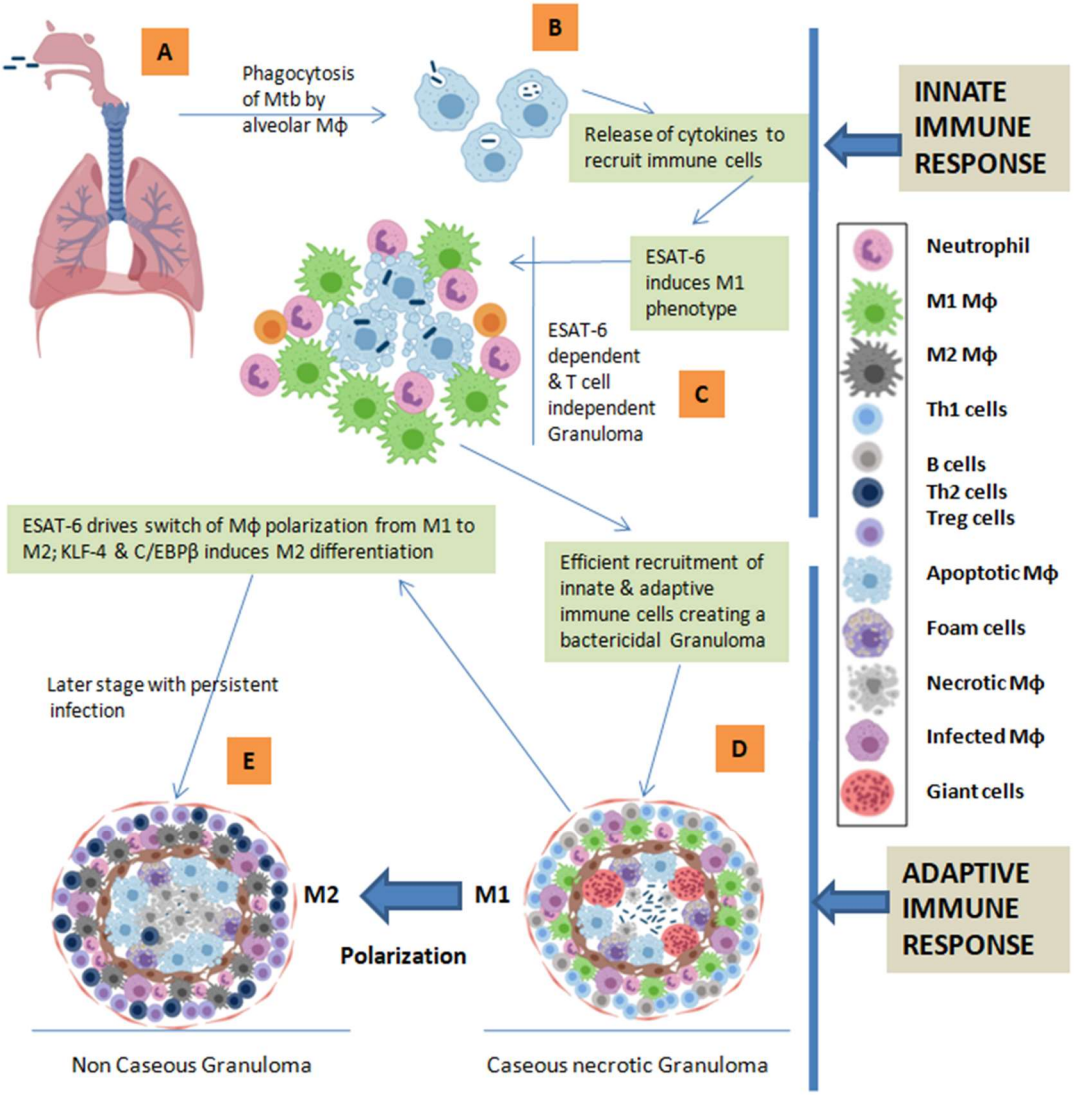

Figure 1: Mtb invasion and granuloma formation: A) Mtb entering through airway mucosa are phagocytosed by alveolar Mϕs; B) These alveolar Mϕs then release various cytokines recruiting NK cells, DCs, γδT cells, and innate invariant T cells (orange in colour), providing first line of defence against aerosol-based Mtb transmission; C) Upon internalization Mtb pathogen inhibits phagosome maturation, and induces M1 phenotypic differentiation via ESAT-6. This leads to the formation of primary innate granuloma, which is ESAT-6 dependent & T cell independent; D) Eventually a pro-inflammatory environment is generated enhancing efficient recruitment of adaptive immune-competent cells (Th1 & B cells) forming a bactericidal granuloma (caseous necrotic); E) With persistent infection for a longer period, ESAT-6 drives polarization of M1 towards M2 phenotype, inducing foamy cells differentiation (necrotic granuloma). Th2 and Treg cells surround the granuloma. Mtb infected M2 Mϕs upregulates KLF4 and C/EBPβ. KLF4 signalling inhibits phagosome/lysosome fusion preventing autophagy and thus helps in Mtb survival.

Macrophages derived from C57BL/6 mice, when infected either with attenuated *Mtb*-H37Ra strain or with virulent *Mtb*-H37Rv strain, induce M1 or M2 polarisation, respectively (Figure 2). The virulent strain may induce M2 phenotype through ESAT-6, upregulating STAT3, STAT6, arginase-1 and KLF4. TLR2 and TLR4 signalling is essential to modulate macrophage polarisation. M1 phenotype is associated with smooth endoplasmic reticulum stress, i.e. in their misfolding caused by production of NO and oxygen reactive intermediates. They may induce apoptosis of infected macrophages through activation of caspase-3, Bax and cytochrome C, which is a way of clearing the infection. Apparently, *Mtb* interferes with macrophage polarization signalling in favour of its M2 intracellular habitat; however, it can be bypassed by other events such as by cytosol- reticulum stress and Mtb infected phagocyte apoptosis (Lim et al., 2016).

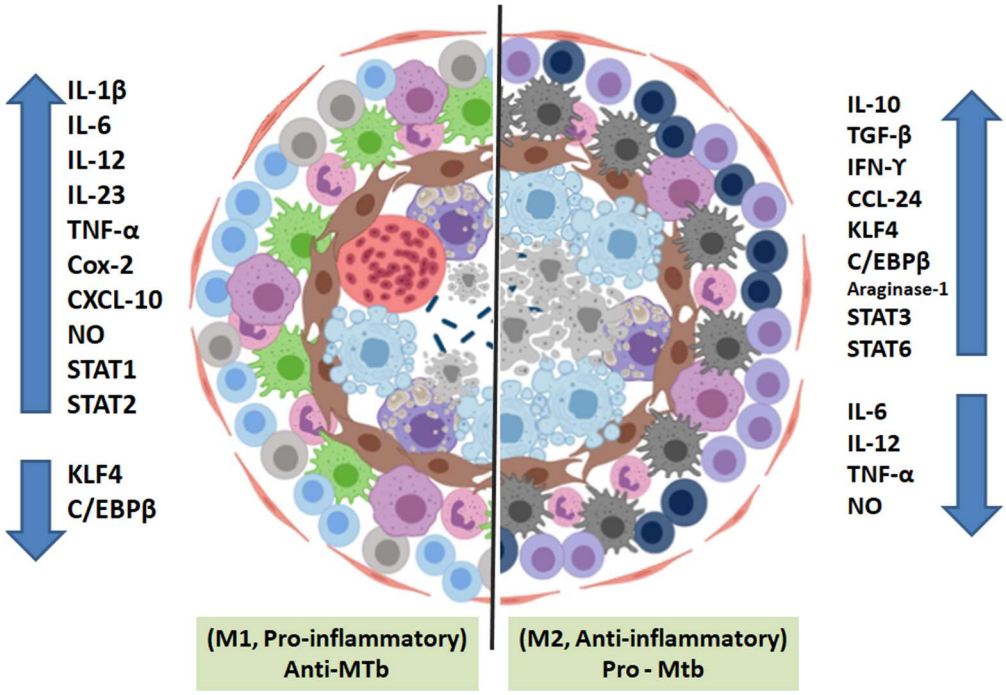

Figure 2: Cytokines and mediators involved in Anti-Mtb response & Pro-Mtb response. The upregulated cytokines and mediators are shown with upward arrows and the downregulated ones with downward arrows during M1 and M2 polarization.

Since granuloma formation is the most crucial part in the establishment of TB, the animal models have been attempted. Mouse strains commonly used for TB research are BALB/c mice or C57BL/6 mice, which fail to show lung cavitation, a key characteristics for disease transmission in human, and also to recapitulate the exact granuloma organisation, as observed in human pathological condition related to TB (Apt & Karamnik, 2009; Fortin et. 2007; Kaori et al, 2017; Singh & Gupta, 2018). The granuloma in mice model differs with human in being exclusively cellular, lacking hypoxia, show low bacterial count, and thus do not represent latent TB as the mice die with progressive infection (Barry et al, 2009). Necrotizing responses to *Mtb* occurs in other mouse strains (Kramnik et al, 2016), indicating the impact of genetic variability on the outcome of infection (Kaori et al, 2017). CBA/J, DBA/2 and C3H strains show lung lesions that are more similar to human TB infection (Apt &

Karamnik, 2009; Fortin et. 2007; Mitso et al, 2000; Chackerian et al, 2003). $NOS2^{-/-}$ mice, incapable of NO production, show serine protease activity contributing to the control of *Mtb* in hypoxic lung granulomas (Reece et al 2010; Gengenbacher et al. 2017).

Innate immune defence against Mtb and cytosol reticulum stress

Airway mucosa containing epithelial cells, macrophages, DCs, natural killer (NK), γδT, and innate invariant T cells, are the first line of defence against aerosol-borne *Mtb* transmission. Together with M1, over M2 polarisation regulated by TLRs, this is the deciding point, whether the microbe will be eradicated within macrophages, as may be the case in TB-resistant individuals, or it will spread and proliferate in lung mesenchymal cells, macrophages, draining mediastinal lymphatics, and other organs.

An attenuated *Mtb* strain has been shown to induce in mouse macrophages M1 polarisation, together with dysregulated cytosol reticulum (CR), resulting in stress and their apoptosis (Lim at al., 2016). Apoptosis of infected macrophages is a strong innate host defence tool against intracellular pathogens including *Mtb* (Behar et al., 2011). Such macrophage apoptosis may result from misfolded/unfolded protein defects, owing to CR impairment, called CR stress. CR stress can be triggered by *Mtb*-responding M1 macrophage via- iNOS-RNS, ROS, as well as by TNFα and IL-6, associated with TLR 2/4 pathways including activation of MAPK and Jun. Such CR stress in murine bone marrow-derived macrophages inducing apoptosis, has been demonstrated in response to Mtb 38 kDa antigen (Lim et al. 2016). A severe CR stress may activate macrophage apoptotic pathways, such as that of C/EBP homologous protein (CHOP), and growth arrest and DNA damage-inducible gene 153 (GADD153). Caspase-3- mediated pathway is modulated positively by pro-apoptotic BCL-2 family members, Bax and Bak. These proteins release mitochondrial cytochrome c, thus impairing macrophage energy-dependent functions, including cell membrane integrity (Lim et al. 2016; Wei et al. 2001; Oyadomari and Mori, 2004).

A possible modulatory role of calreticulin (CRT) involving *Mtb*-ER-stress in infected macrophages was investigated (Joet al, 2017). CRT is a calcium-binding chaperone protein, a subset of CR family. Upon *Mtb* H37Ra strain infection, a large part of CRT was translocated from endoplasmic reticulum to plasma membrane, which was induced by ROS (and reduced by ROS scavengers). This process included activation of CHOP, caspase-3 and of other CR stress markers such as GRP78 and p-eIF2a. Macrophage surface CRT interacted with CXCR1 and TNFR1, the complex that induces apoptosis in *Mtb*-infected macrophages in order to be recognised and cleared by uninfected phagocytes. The early phase apoptotic cell surface, CRT, is a major ligand of macrophage signalling scavenger receptor, CD91. It would appear that Mtb-induced ER stress response and apoptosis, as seen in animal models, may greatly diminish the infection, a notion explaining the fact that nearly half of the population in endemic TB areas can apparently withstand *Mtb* infection, by immediate killing and clearance by phagocytes (Morrison et al. 2008).

Invariant $CD8^+$ T cells

A further "innate" non-classical system is a T-cell subset, the lung and intestinal mucosa-associated invariant $CD8^+$ T cells (MAITs), which can rapidly and effectively respond to intracellular *Mtb*, well before the classical adaptive T cell response sets in (Huang et al. 2009; Gold et al. 2010). MAITs secrete IFN-γ, which can induce pro-inflammatory TNF-α, reactive

oxygen species (ROS) and N0 radicals. Human mucosa epithelial cell lining in lung upper airways contains an enriched population of the MAITs, which express the Vα7.2 T cell receptor (TCR). MAITs are restricted by MHC-related non-classical HLA-Ib molecule (MR1) (Gold et al. 2010; Harriff et al 2014). *Mtb*-infected lung epithelial cell line as well as its primary cell counterpart strongly activate MR1-restricted MAIT cells for their IFN-γ secretion (Gold et al. 2010). *Mtb*-specific antigens may also be expressed on surface of infected epithelial cells in complex with MR1, restricting their presentation to $CD8^+$ MAITs, similar to that by mucosal infected DCs. Presumably, by analogy with classical killer $CD8^+$ TCRs, MAITs receptor may only recognize specific *Mtb* antigen in complex with its restrictive MR1 on mucosa target epithelial cell surface to induce their activation, which may include their apoptosis.

MAITs are abundantly found in non-classically restricted $CD8^+$ T cell clones in the lungs of normal (*Mtb*- non-infected) persons; lower levels of MAITs are found in people suffering with active lung TB disease. In the middle percentages group were people with latent TB. *Mtb*-reactive MAIT cells are also found in people who may never had been infected with TB, suggesting an innate response by their MAIT (Gold et al. 2010). Genetic studies demonstrate that mucosal HLA-MR1-mediated antigen presentation to phagocytes is highly conserved in mammals. It evolved before the adaptive immunity. MR1 restricts antigens presentation to polyclonal MAIT cells which recognise various pathogen molecular motifs, rather than specific antigens, and are thus, less antigen discriminating than the classical TCR clones. MAITs are also less dependent on an antigen-specific clonal expansion. However, they have an advantage: they can act as memory cell phenotypes and be already activated for a robust immediate immune response. They are cross-reactive with various pathogens bearing similar conserved motifs such as between mycobacteria, or gut flora. Other than MR1 Ib molecular members have similar antigen restrictive T cell properties, such as CD1d restricted iNKT cells and H2-M3 to $CD8^+$ T cells (Huang et al., 2009). This was found to constantly augment MAIT cells. Lung mucosal epithelial cells, infected by *Mtb*, allow proliferation in late endosomal vacuoles, although less efficiently than DCs. Nonetheless, such *Mtb*-infected epithelial cells are very efficiently recognised by MAIT clones, inducing secretion of IFN-γ (Harriff et al. 2014).

Autophagy and apoptosis as innate immune anti-*Mtb* strategies

Human innate immunity, including autophagy, is an immediate defence mechanism against *Mtb* in their clearance (Lerner and Borel, 2015; Lam et al. 2017). IFN-γ is a key molecule in controlling intracellular *Mtb* growth and their killing. IFN-γ is produced by lung mucosal MAIT $CD8^+$ cell subset, NK cells, NKT cells and $CD3^+$ T cells. Secreted IFN-γ activates its effectors for inducing macrophage autophagy pathways, i.e. a process of the fusion of autosome vacuole with *Mtb*-infected phagosomes into double membrane auto-phagosomes, which merge with lysosomes. Such autophagy pathway can override the blockade by *Mtb* for phagosome's direct fusion with lysosomes by inhibiting phagosome maturation (Gutierrez et al. 2004; Deretic and Levine, 2009).

Autophagy is a primordial process conserved in eukaryotic cells and metazoans, involved in its canonical intracellular anti-microbial immunity (Deretic and Levine, 2009; Mizushima et al., 2008). Lysosome fusion with autophagosome on its part, provides acidic environment for hydrolases including cathepsin D, oxidases, iNO-synthase, oxygen radicals, and anti-bacterial peptides for *Mtb* killing and its degradation. However, lysosome hydrolases and acidification depend on phosphatidylinositol-3 kinase (PI3K), which *Mtb* can inhibit by stimulating lipids

and phosphatidylinositol substrates, thus blocking PI3K ligation. The main markers of autophagy are ATG genes such as ATG5, and the microtubule-associated protein-1 (LC3) (Gutierrez et al. 2004). A further study on autophagy showed that *Mtb*-containing phagosomes are targets of bacterial ESX-1 secretion system, mediating phagosome permeability. This event exposes on the phagosome extracellular bacterial DNA which is membrane bound, and thus sensed by cytosolic STING-pathway engaged in tagging *Mtb* with ubiquitin. Ubiquitin is a general marker and effector for autophagy in delivering various damaged cell constituents to lysosome for their degradation and energy metabolism. In this process, the adaptor protein p62, ligating ubiquitin as well as the autophagosome marker LD3, are required. *Mtb* ESX-1 is a virulence factor, which BCG lacks (Abdallah et al, 2007; Watson et al, 2012; Behar and Baehrrecke, 2015).

A recent study found unexpectedly that autophagy protects the *Mtb*-infected host from an excessive polymorphonuclear cell (PMN)-elicited tissue damage. Mice defective in ATG5 factor succumb to TB within a month with a severe inflammatory damage mediated by activated PMNs. Such an ATG5-mediated cell protection is unique, since a lack of other factors produced a modest inflammatory PMN phenotype and a rise in pathogen replication. Thus, ATG5 protein substantially regulates TB pathology and bacillus replication (Kimmey et al, 2015).

The role of autophagy in the regulation of *Mtb*-induced DC maturation and MHC class II-restricted specific antigen presentation has been investigated. A virulent *Mtb* strain marked with ESAT-6-ESX-1 type VII secretion system was able to inhibit phagolysosome fusion. This process contrasted with an *Mtb* strain lacking this virulent system. Such DC impairment could be surmounted by the pro-inflammatory rapamycin-activating mTOR signalling system, inducing IL-12 and Th1 cytokines (Romagnoli et al, 2012).

A collaboration between autophagy and IFN-γ to target Mtb

Activated vitamin D3 (1, 25-D3) induces autophagy in human monocytes and macrophages, as well as direct *Mtb* killing via cathelicidin activation. Cathelicidin recruits autophagy-related Beclin-1 and ATG5. Cathelicidin co-localises during autophagosome formation and fusion with lysosome. Human cationic anti-microbial protein-18 (hCAP-18), and its C-terminal cleavage part, LL-37, mediates these activities. Vitamin D3 and LL-37 are considered to be essential defence mechanism against *Mtb* (Yuk et al. 2009). The role of cytosol-nucleotide-binding oligomerization domain 2 (NOD2) like receptor in human alveolar macrophages in response to *Mtb* muramyl-dipeptide (MDP) antigen was analysed. NOD2 can sense MDP, and their ligation upregulated in these cells expression of LL37, which in turn, induced pro-inflammatory cytokine expression such as IL-1β, IL-6 and TNF-α. In addition, LL37 also recruited autophagy regulatory proteins *ATGM,* LC3 and ATG16L1 in the *Mtb*-infected autophagosome for their lysosome fusion and destruction. NOD2 appears to be efficient in warding off a primary *Mtb* lung infection (Juarez et al., 2012). IFN-γ can enhance autophagy also in patients against active TB antigens. A positive correlation between IFN-γ-producing Th1 cells and with *Mtb*-infected monocyte autophagy in high responder patients has been demonstrated. Exogenous addition of IFN-γ to low responder monocytes augmented their autophagy, suggesting a collaboration between autophagy and IFN-γ (Rovetta et al., 2014).

Innate immunity-related GTPase sand TB susceptibility through autophagy

Additional IFN-γ-inducible autophagy players are immunity-related GTPases (IRG), known previously as 47-GTPases, and the 65 kDa guanylate binding proteins (Lerner et al. 2015). These proteins, acting independently from IFN-γ induced iNO-synthase, are vitally involved in phagosome maturation and trafficking. IRG-47 gene-deficient mice could not control *Mtb* replication, neither did IFN-γ-deficient mice (MacMicking et al., 2003; Taylor et al., 2004; Feng et al., 2004). However, in contrast with mice genome containing 23 IRGs, human *IRG* genes encode for only three IRG proteins, i.e. IRGC, IRGQ and IRGM, of which IRGM is functional. IRGM appears to be not induced by IFN-γ (Che et al., 2010; Lerner et al., 2015).

In human TB, the IRGM gene allele polymorphism (10065172C/T) has been identified as a potential disease susceptibility link. A Chinese population study found a single nucleotide polymorphism (SNP) (1208A/G) within the 1.7 kb IRGM promoter region to be associated with the risk for TB. Such finding suggested a protective role played by an autophagy regulator, IRGM, against TB (Che et al. 2010). In African-American patients with mainly pulmonary TB, three common polymorphisms found (in nearly 100% linkage disequilibrium) are rs10065172 C/T,rs13361189 T/C and a 20-kb deletion upstream of the gene coding region. The TB risk allele, rs10065172, frequency in African-American patients, as compared with non-infected cases, were significant- 43.8% over the 33.6% controls. In Caucasian TB patients and controls, the values were 10.7%/8.7%, which were not significant. Immune responses to TB may influence the IRGM1 polymorphism bias. Genetic factors and digestive tract cell environment (including gut microbiome) may also be involved (King CY et al. 2011). On the other hand, among Ghanaian population, the European/American clade of *Mtb* became rooted, on which the autophagy gene variant IRGM-261T exhibits protective effects from the disease. However, against African TB clades, the IRGM-261T allele was ineffective, nor where other clades including Beijing strain. Haplotypes of IRGM-261T variants were also analysed for significance with controls (Intemann et al., 2009).

Apoptosis of *Mtb*-infected phagocytes, to be taken up by bystander macrophage through efferocytosis for autophagy, is an efficient means of killing of the intracellular bacilli and disposing off debris (Srinivasan et al., 2014; Lam et al., 2017). Apoptosis of *Mtb*-containing macrophages may result from ER stress due to the accumulation of misfolded bacillus antigens. ER stress may release ER-Ca^{2+}reserves, and raise the cell redox state. ER stress also induces apoptosis via macrophage signalling factors such as inositol-requiring-1α, (IRE-1α), double-stranded RNA-dependent protein kinase (PKR)-like ER kinase (PERK), and activating transcription factor-6 (ATF-6). Further host ER stress regulators include AMP-activated protein kinase (AMPK), the ubiquitin-like microtubule-associated protein-1 light chain 3 (LC3) and autophagy protein 5 (ATG-5)-ATG12, which contribute to the double membrane autophagosome structure (Lamet al. 2017). Secreted IFN-γ has a similar role in autophagosome maturation and has anti-microbial property. However, the virulent *Mtb* strain H37Rv can selectively inhibit the autophagosome maturation of human macrophage cell line THP-1, by producing its own IL-6. IL-6 negatively regulates p38 and JNK pathways engaged in autophagy process (Dutta et al., 2012). On the other hand, *Mtb*-secreted virulence factor ESAT-6 inhibited the production of IFN-γ by human T cells in response to *Mtb* (Wang et al., 2009).

In a recent clinical trial study with 185 TB patients, it was revealed that their clinical Mtb isolates/strains were able to induce autophagosome formation in macrophages, but to a variable degree. Autophagy was estimated by macrophage expression of the autophagy-associated LC3II marker protein. Severity of the active disease was inversely correlated with LC3II production, i.e. in TB patients with severe radiographic monitored pulmonary disease, and with poor treatment outcome. Some virulent *Mtb* strains were more likely to produce only a little of LC3II-*Mtb* autophagy in macrophage response, *and vice versa*. The results show a significant protection-mitigation in humans from TB disease, owing to macrophage autophagy response (Li et al. 2016).

Ubiquitination and autophagy

Ubiquitination of *Mtb* proteins, following their processing to antigen level in macrophage autophagy vacuoles, has a prominent role in innate host protection. Recently, the ubiquitin ligase Smurf1 (SMAD-specific E3 ubiquitin-ligase protein 1) has been shown to selectively control through autophagy, *Mtb* replication in human macrophages and in mice. In *Mtb*-infected macrophages, Smurf1 recruited K48 ubiquitin, the autophagosomal membrane associated-protein LC3, and the lysosomal membrane protein, LAMP-1, to be finally degraded. Smurf1 chains also ligated several cytoplasmic proteins for their degradation in proteasomes, such as Smad 1/5, RhoA, transforming growth factor β (TGF-β) receptor, transcription factors MEKK2, JunB, STAT1 and TLRs MyD88 (Cao and Zhang, 2013; Franko et al. 2017). Smarf1 ligation and disposal of STAT1 protein negatively regulated IFN-γ receptor signalling pathways in infection, and potentially in autophagy. Conversely, IFN-γ can upregulate low levels of Smurf1 expression (Yuan et al. 2012). Smarf1 appears to be an important leading protein of innate immune system in early protection of the host from *Mtb* infection (Franko et al. 2017).

BCG upregulated innate trained immune cell memory

BCG vaccine is able to enhance macrophage functions, by inducing so called “innate trained immunity” and memory, increasing upon host reinfection, which is independent of adaptive immunity. This immunity is non-specific to some pathogen, including *Mtb*, as observed in BCG-vaccinated neonates, protected from severe TB, and independently, from various pneumonia, living in TB endemic region. Such protective innate memory is primordially conserved, across plants and mammals, relying basically on Toll, or Toll-like receptors, together with IL-1β receptor, for pathogen recognition and defence function. In humans, this system has been only recently described (Netea et al. 2011; 2016; Kleinnijenhuis et al 2012; 2014).

Trained innate immune cell memory is stored/imprinted in nucleus epigenetically, by reprogramming histone-chromatin for corresponding immune gene expression, or repression-silencing, through their demethylation and methylation, respectively, at the key lysine residue. These dual effects are regulated by methyl transferases. Gene acetylation function is regulated in a similar dual opposing way. PAMPs are recognised by macrophage PRRs, including signalling protein, such as TLR2 or TLR4 for extracellular, and cytoplasmic TLR7 for intracellular bacterial binding, such as *Mtb*. A further cytoplasmic PRR is NOD2 receptor, encompassing caspase-recruiting domain (CARD) and six leucine rich repeats (LRRs), which enhances innate trained immunity. This process engages chromatin gene-specific nucleosome

core histone 3 lysine 4 tri-methylation (H3K4m3), promoting via methyl transferase, TNF-α, IL-6 and TLR-4 gene expression, associated with macrophage M1 phenotype (Kleinnijenhuis et al. 2012; van der Heijden et al. 2017). Methylation-demethylation of DNA nucleotide 5-methylcytosine may be recruited in chromatin remodelling, towards the regulation of various cell processes (van der Heijden CD et al. 2017).

Similarly, TLR4 differentially regulates gene-specific macrophage M1 and M2 polarisation, in methylation-demethylation of gene chromatin regions, which encode for transcription factors for opposing immune effector proteins (Foster et al. 2007). Both chromatin modifications are reversible by demethylase and by deacetylase, respectively (van der Heijden et al, 2017). This bi-directional epigenetic mechanism also controls inflammation for host protection as a physiological homeostatic surveillance (Foster et al. 2007). Given a vast range of conserved pathogen PAMPs, recognised by PRRs, their influence on physiological function, and diversity of epigenetic chromatin modification, the trained innate immunity and memory appears as a dynamic adaptive system in host resistance to intracellular pathogens. On the other hand, trained macrophage immunity is also associated with chronic disease such as atherosclerosis and diabetes (van der Heijden et al. 2017). On the whole, trained monocytes/macrophage and DCs act against intracellular bacteria, whereas NK cells, also possessing a trained phenotype, are host-protective against viral infection (Netea et al. 2011; Shann 2013).

Activation of innate immune system and enhanced responsiveness to subsequent triggers is referred to as trained immunity, where various epigenetic and metabolic reprogramming activities take place (Koeken et al, 2019; Netea et al. 2020). Trained immunity has existed in plants, as systemic acquired resistance (SAR), and in invertebrates, well before the evolution of adaptive immunity (Netea et al. 2011). In mice, such antibody-independent, acquired cellular resistance against intracellular pathogens including *Listeria* and *Mycobacterium*, has been described (Mackaness 1964; 1969). Such resistance faints upon primary mycobacterial elimination, together with cross-protection of other pathogens such as fungi and infant respiratory pathogens (Mackaness, 1969). Recently, human monocyte-trained immune memory to BCG has been demonstrated as a heightened innate cell anti-bacterial cytokine response to a secondary infection (Kleinmijjenjuis et al. 2012). A recent study suggested that the control of *Mtb* growth strongly correlated in presence of $CD14^{dim}$ monocyte population and T cells; it also suggested involvement of CXCR3, CXCL9, CXCL10 and CXCL11 ligands in trained innate immunity (Joosten et al., 2018). An important association of glycolysis and glutamine metabolism with BCG-induced trained immunity in monocytes has been suggested. Histone markers, such as H3K4me3 and HK3K9me3, were affected while inhibition of trained immunity due to modulation of rate-limiting glycolysis enzymes, suggesting that cellular metabolism contributes to the long-term epigenetic reprogramming of trained monocytes (Netea et al., 2016).

Human BCG enhanced phagocyte trained immunity-memory

A study involving healthy volunteers vaccinated with BCG showed that their circulating monocytes became hyper-responsive to *Mtb* and to unrelated pathogens as tested on 2^{nd} and 3^{rd} week. This immune potentiation was monitored by secretion of their pro-inflammatory cytokines, upregulation of PRRs and distinct myeloid markers such as CD14, CD11b and TLR4. Their PBMCs were stimulated *ex vivo* with sonicated *Mtb* lysate (and heat-killed *S. aureus* and *Candida albicans*), which increased IFN-γ secretion proximally seven-fold, as compared with

basal level of donors before their BCG vaccination. Monocyte secretion of TNF-α and IL-1β was augmented 2-fold. Such trained immune effects associated with histone epigenetic reprogramming depended on the activation of NOD2 receptor, increasing methylation of histone 2 at lysine 4 (H3K4m3) through methyl-transferase. H3K4m3 was found to be upregulated *via* TNF-α, IL-6 and TLR-4 for their transcription. These events are blocked by methyl transferase inhibitors, thus confirming trained immune memory imprinting in histone-chromatin. T and B cell deficient mice (SCID), vaccinated with BCG, were protected through trained immunity against lethal doses of *C. albicans* infection. They all survived, as compared by 30% in controls. These results indicated BCG inducing in mice an all-innate non-specific trained immune protection via NOD2-epigenetic histone modifications, in immune resistance to these pathogens (Kleinnijenhuis J et al. 2012). BCG-enhanced innate trained immunity is mirrored by synthetic adjuvant muramyl dipeptide and by mycobacterial analogues against *Klebsiella pneumoniae* infection of mice in their protection (Chedid L et al. 1977). BCG-trained immunity can be long-lived and effective over one year in healthy volunteers post-vaccination. It can also recruit heterologous (non-specific) Th1 cells for IFN-γ, and that of Th-17 for IL-17, IL-22 responses to non-related pathogens, in humans. Their monocytes, stimulated with sonicated *Mtb*, increased production of IFN-γ as previously found after 2 weeks and 3 months, and further 10-fold after 1 year. There was a steady increase of IFN-γ by heat killed *S. aureus*, but a substantial decrease with *C. albicans*. There was an increase of IL-17 and IL-22 induced by these pathogens in Th cells after one year. A trained immune trait which strongly persisted was TLR4 receptor stimulation by bacterial LPS, which is associated with epigenetic modification by distinct monocyte phenotype, induced by such pathogens. Such trained monocytes upregulated their PRRs and other innate activation markers, such as myeloid $CD14^+$ Th cells, complement receptor 3 integrin- $CD11b^+$, TLR4, C-type -1 lectin mannose receptor (MR), whereas TLR2 and Dectin-1 markers remained steady over the year. TNF-α and IL-1β increased over the year only when stimulated by *E. coli* LPS, i.e., through TLR4 signalling pathways. It has been suggested that non-specific Th cells as well as trained monocytes may collaborate in host protection against *Mtb*, which may be relevant in vaccine design (Kleinnijenhous et al. 2014).

In a recent study with BCG-vaccinated infants, their whole blood leukocytes were analysed on a wide panel for secreted activation markers induced by non-specific stimuli, including *Mtb* lysate, *C. albicans*, *S. aureus*, and Pam3Cys, a ligand of TLR2 on NK cells. Infant marker profile differed from that of adult trained immunity, recognised by unspecific cross-protection of unrelated pathogens. There was no increase of TNF-α and IL-1β production over the non-vaccinated infants, although *Mtb* did enhance TNF-α secretion. INF-γ and IL-6 production was similar to the control, but secretion of regulatory epidermal growth factor (EGF), platelets derived growth factor (PDGF), and certain chemokines was significantly elevated. There was an enhanced NK cell activation (overexpressing CD69 and TLR2), mediating IL-10 and IL-12p40 secretion, monocyte markers being CD14 and CR3 receptor integrin-subunit CD11b. Infants were vaccinated with BCG intradermal injection at the age of cca 6 weeks, and their heparinised venous blood was analysed at 4 month post-vaccination. Mtb lysate-mediated innate cell stimulation appears to be predominant in cytokine production and NK cell activation, apart from Pam3Cys ligand of TLR2. Potentially, it may via TLR4 signalling induce epigenetic reprogramming of NK cells as well as monocytes in host protection from TB. On the whole, infants showed a new cytokine/chemokine signature for trained immunity to BCG. Newborns and infants rely on innate immunity which is well regulated, but is less

inflammatory to spare themselves (Smith et al. 2017). However, there is seemingly an overlap with earlier studies on an effective immediate innate immune cell response by phagocytes against microbes, which is conserved in plant and animal kingdom (Janeway and Medzhitov, 2002). Mycobacteria-induced innate trained immunity and memory may be seen as an extended arm of a basal innate conserved phagocyte system in an immediate primary infection response against pathogens, including *Mtb*. This response apparently does not require an immune memory in primary infection, as probably is the case with new-borns (Akira et al. 2006). At the same time, success and failure of *Mtb* in establishing an active long-term infection depends upon how innate lymphoid cells interact with the microbiota and the mucosal epithelium inducing tolerance (Gupta et al. 2018).

An immediate innate type 1 phagocyte response in clearing TB infection

Transition from a primary Mtb infection, prompting an innate cell immediate defence, into delayed adaptive phagocyte responses, including innate cell memory, and the adaptive lymphocyte antigen specific memory, might represent certain timing events through a basic innate mechanism, regulated e.g., by TLRs. Various TLRs recognition and effects, exhibit a specificity to various PAMP (Kawai and Akira et al. 2010; vande Heijden et al. 2017). *Mtb* surface lipoprotein (*Mtb* LP), inducing through TLR2 ligation type 1 phagocyte phenotype, together with NOD-like receptor family, may contribute to such requirements (Brightbill et al. 1999; Aderem and Ulevitch, 2000).

Mtb-LP can induce in mice and human monocytes a rapid and strong production of anti-mycobacterial IL-12, mediated through TLRs (Brightbill et al. 1999). *Mtb*-LP (p19 kD), purified from the whole *Mtb* lysate subcellular fraction, was shown to trigger initiation of an early anti-microbial type 1 response, as tested in human and in murine monocyte/macrophage cell lines. *Mtb*-LP stimulated cell release of IL-12 promoter p40 protein in a TLR2-dependent manner. In addition, *Mtb*-LP induced transcription of inducible nitric oxide synthase (iNOS). IL-12/receptor is a key type 1 innate immune cell regulator. The L-arginine-iNOS dependent reactive NO intermediates (RNI) including NO_2 and HNO_2 appeared to be the only effective agents in killing intracellular *Mtb*, in accord with mice model lacking iNOS (Chan et al. 1992). IFN-γ augmented iNOS expression (Brightbill et al. 1999). Stimulation of IL-12 p40 promoter activity by *Mtb*-LP was, to a great extent, equal to that induced by bacterial LPS, suggesting an identical TLR signalling pathway via NF-κB transcription of immune cell type 1 protein coding gene profiling. However, *Mtb*- LP, engaging TLR2, appear to be the dominant macrophage response over those elicited by LPS-TLR4 pathway. A dominant negative deletion mutant of TLR2 inhibited both *Mtb*-LP and LPS induced IL-12p40 promoter activity. *Mtb*-LP-mediated innate cell stimulation depended on the LP-NH_2 terminal lipo amino acid, N-acyl-S-diacylglyceryl-cystein domain (Aliprantis et al. 1999). Thus, *Mtb*-LP have the capacity to induce in innate phagocytes an immediate mycobactericidal nitric oxide mediated response through TLR2 type signalling to eradicate the mycobacterium. These acute effects seem to be separate from the *Mtb*-LP monocyte/DC induction of IL-12-dependent adaptive immunity. Here, by stimulating Th1 cell propagation and IFN-γ production, IL-12 is regarded as a biological adjuvant. The soluble *Mtb* cell-wall protein fraction (SCWPs) also strongly stimulated monocytic IL-12 p40 secretion, which is associated with *Mtb* adjuvant property in vaccines (Brightbill et al. 1999, Kleinnijenhuiset al. 2014).

Mtb-LP as well as BCG can be viewed as collateral effectors to adaptive T cell type 1 response, induced by TB vaccines. Perhaps, this pathway may be interpreted in the randomized trial of BCG vaccination of low-birth-weight neonates/infants, suggesting survival benefits from TB infection (Kleinnijenhuis et al. 2012; Aaby et al. 2011). Such innate protection may be corroborated later by adaptive immunity, which is still developing after birth (Levy 2007; Jaganath and Mupere 2012). Besides, *Mtb*-LP induction of apoptosis of *Mtb*-infected macrophages (Aliprantis et al. 1999), IFN-γ was able to override phagosome-lysosome blockade to induce in *Mtb*-infected macrophages an early iNOS2 expression, which in turn, induced apoptosis of infected cells (Herpst et al. 2011).

In *Mtb*-infected mice in conjunction with IFN-γ, their *Mtb* lung burden was much reduced, suggesting Mtb elimination (Brightbill et al. 1999; Aliprantes et al. 1999). IFN-γ produced by T or NK cells, also induced in macrophages guanosine triphosphatases (LRG-47), controlling phagosome maturation and vesicular trafficking of Mtb and other microbes, for their disposal (MacMicking et al. 2003).

In mycobacteria, there are several virulence genes inhibiting phagocyte apoptosis. Recombinant BCG vaccines are largely devoid of such genes, including urease- *ureC* and *nuoG*, which are able to substantially reduce such anti-apoptosis blockade (Velmurugan K et al. 2007; Hinchey et al. 2007). Another *Mtb* virulence gene is *secA2* coding for bacillus superoxide dismutase. The secA2 deletion mutant strongly induced in mice infected macrophage apoptosis, and priming of $CD8^+$ T-cells (Hinchey et al. 2007). Macrophage Mtb apoptosis relied on multiplicity of infection (MOI) number. 2-4 bacillus per cell induces classical intrinsic apoptosis in infected macrophages. On the contrary, a delayed macrophage infection with 20/cell MOI, over a short time induced apoptosis, may switch to infected macrophage necrosis, independently from caspases pathway, dissipating TB infection, such as in casein-vacuolar granulomas in acute lung TB (Lee et al. 2006).

Macrophage co-expression of IL-12 and IL-18 for intracellular *Mtb* killing: host lung collateral damage and immune tolerance

A recent study on human monocyte cell line THP-1 and peripheral blood monocytes (PBMC)-derived macrophages (hMDMs) demonstrated that for their direct killing of intracellular mycobacteria such as BCG, *M. smegmatis*, and virulent H37Rv Mtb strain, a co-expression of IL-12 together withIL-18, was required (Yang et al. 2018). Human alveolar macrophages, infected with *Mtb*, produce an autogenous IFN-γ, together with TNF-α, which was augmented by IL-12. Such IFN-γ had autocrine and paracrine regulatory roles (Fenton et al. 1997). Human NK cell expression of IFN-γ was dependent on macrophage-derived IL-12 and IL-18 stimulation. For example, IFN-γ production by NK cells was highly augmented by macrophage IL-12 + IL-18 (Fegniger et al. 1999). Murine and hMDM IL-12 and IL-18, in response to mycobacterial infection, induced in such cells pro-inflammatory p38 MAPK and STAT4 signalling. These pathways were preferred for this purpose, rather than contribution of NF-κB transcription regulator of immune cytokine genes, which was negligible (Schindler et al. 2001; Yang et al. 2018). However, hMDM source of IFN-γ did not induce in infected macrophages caspase-mediated apoptosis in these microenvironment. Similar results were obtained with human lung epithelial cells. A synergy of IL-12 with IL-18 has been shown in Th1 differentiation mediated *via* DCs (Yang et al. 2018). Such inhibition of *Mtb* replication within human macrophages appears to suggest an immediate eradication of the mycobacterium in TB resistant infants in TB endemic population. Innate invariant T- cells can provide sufficient

pleiotropic IFN-γ production for killing intracellular *Mtb*. The bacillus-activated macrophages engage cytoplasmic TLR7 and ubiquitin system for their proteasome degradation beyond mycobacterial antigenic epitope capability. This process may underlie TB resistance of such individuals, in which apparently TB infection never occurred. Hence, they do not react to PPD skin test. A failure of such innate immune rapid mechanism may result in TB lung containment, thus crossing this border into adaptive immunity containment. There seems to be no direct evidence that boosting of pro-inflammatory Th type 1 immunity via TB vaccine, eradicates the disease, though its can ameliorate the disease, jointly in bacillus in granuloma containment. It can also, in some people, even aggravate lung TB *via* granuloma cavitation (Lerner et al. 2015; Kowalewicz-Kulbat and Locht, 2017; Divangahi and Behr, 2018).

To spare the host organs including lungs from collateral damage caused by pathogen as well as host pro-inflammatory response, immune tolerance has been suggested as a mechanism to reduce host susceptibility to TB. These include regulation of microbe burden, which is a defence strategy against *Mtb* in containment. Such pathogen tolerance can be sensed ahead from infection through olfactory and taste innervations (Medzhitov et al. 2012). In TB, containment granulomas of Th1 phenotype may be subdued by T-regulatory (Treg) cells, inducing IL-10 and TGF-β cytokines, which in turn, are regulated by mitochondrial protein cyclophilin D. TST reaction in people with latent TB, did not differ from that of a minority of people, 5-10 %, who progressed to acute TB, suggesting an unlikely role of adaptive immunity in eradication of TB disease. Instead, it affords a silent secure latent infection in granulomas, which is vulnerable in TB susceptible people, together with innate trained immunity (Netea et al. 2006; Kowalewicz-Kulbat and Locht 2017; Meunier et al. 2017; Divangahi and Behr 2018).

Innate immunity and BCG vaccine

Phagocyte type 1 induction by BCG

Bovine BCG vaccine differs from *Mtb* virulent strains largely owing to its gene deletion of RD1 (region of difference 1). RD1 region encodes several bacterial virulence genes including ESX-1 secretory system, ESAT-6 and CSP-10, which are associated with tissue lysis and destruction of pulmonary syncytial cells (Hsu et al. 2003; Tang et al. 2016). Instead, BCG has conserved innate immune PAMP, to be recognized by macrophage PRRs, which regulate expression via epigenetic reprograming of chromatin genes, and are associated with phagocyte's anti-bacterial type 1 inflammatory phenotype. Conserved BCG PAMP configuration has apparently not been diminished. Such ΔRD1mutants were able to protect mice from *Mtb* aerosol challenge, similarly to that by BCG (Hsu et al. 2003). As such, BCG vaccination exerts a prominent influence on innate cell anti-microbial immunity, through induction of type 1 phenotype in monocytes-macrophages, DCs and NK-cells in response to *Mtb*-LP and lipoglycans. Lipomannan (LM) is pro-inflammatory, opposing type 2 response induced by ManLAM for microbe intracellular survival. LM and LP may prevail in this balance, overcoming ESAP-6/ESX-1 blockade (Akira et al. 2006; Quesniaoux et al. 2004). An early study in West Africa with high child mortality found that BCG vaccination significantly reduced their mortality ratios, as assessed by the presence of their cutaneous scar and tuberculin delayed hypersensitivity reaction. This feature was compared with BCG scar-negative children in which the mortality ratio remained high. BCG has been suggested to be protective against other infections, independent of its effects on TB, and of other children vaccination, including

diphtheria-tetanus-pertussis (DTP) (Garly et al. 2003). Later reports from West Africa, where BCG vaccination against TB of infants is obligatory, also cited BCG reducing non-specifically and significantly their lower lung respiratory disease, caused by various bacteria, viral DNA-RNA, and sepsis-linked mortality (Shann 2013). Similarly, In Spain's Basque Country, where BCG vaccination at birth against TB is still a routine, the vaccine reduced infant hospitalization due to respiratory disease and sepsis, with a total prevention fraction (PF) of 41.4 %, as compared with the disease incidence in all of Spain (Castro et al. 2015). BCG and its subset vaccines, only partially protected neonates from bacterial pulmonary infection. It has been suggested that a live BCG-based vaccine may be designated for an upgraded protection of new-borns and adults from TB, and from unrelated respiratory disease (Tameris et al. 2013). However, BCG subset TB vaccine, MVA85A, strongly boosted poly-functional Th1 response, in producing IFN-γ, TNF-α, IL-2, and IL-17 cytokines, which are invaluable for mycobacteria containment in TB latency in infancy as well as adulthood (McShane et al. 2004). These results were obtained in large randomised trials in neonates/infants living in South Africa.

There is however a trade-off for such BCG-mediated protection, the clinical reactogenicity specific to mycobacteria in infants and adolescents in the form of innate skin blister, and ulceration scar response upon intradermal vaccination (Hoft et al.1999). Such BCG inoculum is captured by DCs migrating to local lymphatic system for their processing and T cell priming. Skin lesions attract inflammatory granulocytes, monocytes, and $CD3^+$ T cells, leading to lymphocyte proliferation and IFN-γ secretion. BCG-DNA appeared in blood specimens 3 days after vaccination. The bacillus was present in ulcer drainage for 2 months thereafter, potentially posing a danger of lymph node draining and spreading, especially in immune compromised persons, such as those suffering with AIDS.

Previously, BCG reactogenicity has been suggested as an important part of any clinical vaccines to prevent TB, and a search for vaccine collaterals *Mtb* immunity has been recommended (Hoft et al. 1999; Minassian et al. 2012; Bhatt et al. 2015). However, BCG-reactogenicity (delayed hypersensitivity) is now regarded not to be correlated with BCG-mediated immune protection against *Mtb* infection (Kowalewicz-Kulbat and Locht, 2017). Another BCG property is its ability to ameliorate autoimmune diseases such as type 1 diabetes and multiple sclerosis. Recently, a new approach to boost BCG-induced trained innate immunity for a strong protection against TB has been tried using mice. This involved BCG targeting the bone marrow-derived haematopoietic stem cells (HSCs), rather than progeny monocytes/macrophages. BCG/HSC chromatin epigenetic imprint was found to be more durable and efficient, compared to monocytes (Kaufmann et al. 2018).

BCG-mediated bone marrow hematopoietic stem cell 'education'

A shortcomings of BCG prophylactic vaccination against TB is its insufficient protection of infant lungs, soon fading towards adulthood. This is thought to be due to their numerical decline, and that of innate cell trained immunity (Minassian et al. 2012; Netea et al. 2011; Akira et al. 2006). Monocytes/macrophages have relatively short life-span for epigenetically storing BCG molecular pattern imprints. However, when assessed in mice bone-marrow *via* intravenous vaccination, BCG trained innate immunity phenomenon could be much upgraded, through "educating" bone marrow HSCs, which are self-renewing (Kaufmann et al. 2018). These cellular events are largely regulated by TLR2 and TLR4 adaptor, Myd88 and cofactors, which are already expressed on HSCs and their early progenitor lineage c-Kit^+ Sca-

1^+ (LKS^+) cell population. Upon recognition and ligation of their PAMPs such as BCG, shared by *Mtb* and other mycobacteria, TLRs activate HSC/LKS^+ cell cycle entry, and their proliferative expansion. In this way, TLR's flexible signalling network could epigenetically reprogram BCG chromatin for their unique profile imprint, which was shown to be durable. The BCG-HSC imprint could be passed on, through generating multipotent progenitors (MPP), their differentiated myeloid progenitors, and finally into monocytes-macrophage lineage, which are effectors of trained innate immunity. Myeloid lineage bias *versus* lymphocyte adaptive immune lineages, and its expansion, bypassing growth and differentiation factors, is apparently dictated by TLR/BCG primordial conserved recognition. It is capable of inducing an immediate response for host first line protection (Nagai et al. 2006; King and Goodell, 2011; Kaufman et al. 2018). The innate cell myeloid MPPs have been studied on monocyte-macrophage transcriptome level, which revealed differentially expressed transcriptome as compared with BM HSCs and other lineages. It revealed a vast participating number of genes, such as 4774 versus HSC- 3914, and 2511 shared, while downregulated lymphoid cell markers such as Rag2, Rag3, and Pax5. It unveiled a vast network connection such as with DNA and RNA processes in metabolism, cell cycle and responsiveness to IFN-β and IFN-γ. IFN-γ, induced by BCG in bone marrow environment, is required by HSC and progenitors for their activation, proliferation and myeloid bias. BCG, thus, induces the unique epigenetic and transcription signature-based memory in macrophages in a rapid response to *Mtb* re-challenge.

In BCG-intravenously vaccinated mice (of wild type C57BL/6), the BCG/HSC trained phagocyte immunity very strongly enhanced protection against their pulmonary *Mtb* infection by killing the mycobacterium, as detected by CFUs decline. This immune protection in mice was mediated by circulatory BCG-trained monocytes (Kaufmann et al. 2018). As extrapolated from BCG-induced protection against *Mtb* as well as unrelated pulmonary pathogens, one might expect, that BCG-HSC educated trained immunity would augment such non-specific infant protection from TB. In non-human primates, it was augmenting adaptive immunity against *Mtb* (Sharpe et al. 2016). The number in bone marrow-educated HSC was directly correlated with expansion of HSC-LKS^+ population. HSCs were found to be resistant to BCG infection, and that of several intracellular pathogens.

Previous studies have demonstrated that besides TLRs, complement activation due to infection is able to mobilize bone marrow HSCs for their expansion and egress into peripheral blood circulation. They may be retained in local tissue infection or injury, to give rise to monocytes, macrophages, granulocytes, and DCs for defence and repair. Such innate effectors may also balance cell homeostatic conditions, including turn-over of HSC-progenies (Ratajczak et al. 2010; Lee et al. 2010). Pleiotropic IFN-γ has the capacity to activate HSCs and progeny such as common myeloid progenitors (CMP) in a murine model of chronic infection with *Mycobacterium avium* (Baldridge et al. 2010).

Trained immunity and memory phenomenon is an adaptive feature of innate immunity which is evolutionarily conserved (Kleinnijenhuiset al. 2012); its epigenetic reprogramming of chromatin imprint profiles are pathogen unique such as in mycobacteria (Kaufmann et al. 2018). There seems an adaptable plasticity of innate phagocyte receptor systems, their signalling variant combination, engaged in a complex interaction as a network that recognises specific bacterial molecular pattern targets. Their macrophage receptor pathogen specific signalling may be epigenetically imprinted in chromatin modification as innate trained immune memory. On recall by *Mtb* reinfection, they may transmit intensively augmented

bactericidal response *via* effector signalling of macrophage receptors (Kishore, 2018; Kaufmann et al., 2018). On these grounds perhaps, innate cell immunity may be viewed as pathogen-pattern specific and adaptive, immune gene transcription variation, when compared with adaptive immune somatic variation and pathogen antigen specific clonal expansion. Its effectors, including bactericidal cytokines and their cell type polarisation, are shared with cellular adaptive immunity.

BCG and recombinant BCG-induced neonate immunity

Neonates depend mainly on innate immunity for their survival (Levy, 2007). Their BCG vaccination may serve as partial TB protection, but also as a general non-specific enhancer of innate immune immediate responses in their protection against various pathogens, especially causing lower lung pneumonia (Kowalewicz-Kulbat and Locht, 2017). Perhaps, recombinant BCG (rBCG) vaccines may also further augment paternal BCG vaccine for infant protection against various microbes as well as from TB. rBCG may, in infants and adults, also upgrade *Mtb* innate trained immunity and memory (Kleinnijenhuis et al. 2012; Kaufmann et al. 2017a). However, such imprints may be sensitive to monocyte energy metabolism (Arts et al. 2016). The ability of BCG vaccine to polarize *Mtb*-residing macrophages into M1 phenotype may be enhanced, facilitated by complement components such as thrombospondin-containing properdin interaction with BCG involving TLR signalling (Al-Mozainiet al.2018). BCG vaccine efficacy is apparently not needed in TB resistant individuals. Children, living in TB endemic environment, may have developed naturally an immune resistance to TB and to other bacteria upon their constant *Mtb* cough aerosol challenge. BCG may mask this effective innate immunity, which apparently has a conserved memory via TLR and IL-1β receptor (Whitham et al. 1994; Kowalewicz-Kulbat and Locht, 2017).

Concluding remarks

TB continued to be a major epidemic disease worldwide, but its conquest is on the horizon. It has emerged that innate phagocyte-mediated host protective immunity against intra-macrophage-dwelling mycobacteria, including *Mycobacterium tuberculosis* (*Mtb*), is unique. *Mtb's* distinct molecular pattern/signature is recognised by macrophage's flexible and adaptive TLRs in conjunction with IL-1βR, including cytosolic TLR7, TLR9, NOD-like receptors and AIM2 inflammasome receptors. They transmit signalling through the transcription factor NF-κB, for *Mtb* signature to be epigenetically imprinted in chromatin. They may be augmented by innate trained immunity and memory. A deciding battle ground appears to be apoptosis of *Mtb*-infected macrophages to be removed by bystander non-infected macrophages for their autophagy, to be degraded in proteasomes together with cellular debris pyrocytosis. These processes are opposed by phagosome-residing *Mtb*, in blocking these events. Next generation of prophylactic TB vaccines, based on recombinant attenuated BCG, have been constructed to overcome the *Mtb* blockade. Thus, the likelihood of developing strong lung protective vaccines against TB for children and adults has sharply increased, owing to a better understanding of the unique innate immunity of phagocytic cells against intracellular mycobacteria, both of primordial origins. New generation of recombinant-BCG vaccines possess a potent bactericidal capacity for protection of people living in TB endemic areas, as indicated in vaccine pre-clinical and clinical trials.

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