Early Clearance of *Mycobacterium tuberculosis*: a new frontier in prevention

Short title: Early Clearance of *Mycobacterium tuberculosis*

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Abbreviations used in this manuscript:

BCG Bacille Calmette-Guerin

DC-SIGN Dendritic cell specific intracellular adhesion molecule-3-grabbing non-integrin

EC Early Clearance

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Abstract

Early clearance (EC) is the successful eradication of inhaled *Mycobacterium tuberculosis* before an adaptive immune response develops. Evidence for early clearance comes from case contact studies that consistently show a proportion of heavily exposed individuals do not develop *M. tuberculosis* infection. Further support for the existence of this phenotype comes from genetic loci associated with tuberculin reactivity. In this review we discuss aspects of the innate response that may underpin EC and hypotheses that can be tested through field laboratory link studies in *M. tuberculosis* case contacts. Specifically, we consider mechanisms whereby alveolar macrophages recognize and kill intracellular *M. tuberculosis*, and how other cell types, such as neutrophils, NK T cells, MAIT and γδ T cells may assist. How EC maybe impaired by HIV infection or vitamin D deficiency is also explored. As EC is a form of protective immunity, further study may
advance the development of vaccines and immunotherapies to prevent *M. tuberculosis* infection.

**Introduction**

*Mycobacterium tuberculosis* is the second most common infectious killer worldwide: in 2013, 9 million people will have been diagnosed with tuberculosis (TB) and 1.2 – 1.4 million will die from the disease.(1) A lack of feasible preventive strategies means the public health response to *M. tuberculosis* focuses on case finding and management and has a modest impact on transmission. Better preventive strategies are desperately needed, yet efforts to develop a vaccine against infectious pulmonary TB have not been successful.(2)

Early Clearance (EC) of *M. tuberculosis* infection can be defined as the eradication of infecting *M. tuberculosis* before an adaptive response develops. This phenotype has long been speculated to exist, however its immunological correlates are yet to be described.(3) Identification of the immunological mechanisms behind EC could present new opportunities to prevent *M. tuberculosis* infection. Firstly EC may be influenced by modifiable host factors that could be therapeutic targets. Secondly EC is a model of protective immunity to *M. tuberculosis* and its study may yield sought after correlates of protection for vaccine development. (4) For these reasons EC should be prioritized as a focus of *M. tuberculosis* research. In this review, we discuss putative immunological mechanisms of EC that can serve as hypotheses to be tested in field-laboratory link studies.

**Evidence for early clearance**

Presently, both epidemiological and genetic data support the existence of EC of *M. tuberculosis*. Case contact studies repeatedly find exposure to *M. tuberculosis* does not always lead to a positive Interferon Gamma Release Assay (IGRA) or Tuberculin Skin Test (TST). In 1941 Israel remarked on heavily exposed nursing students: "the persistence of a negative reaction in these students, after the majority of other students had long been infected, is a striking phenomenon." (5) TB case contact studies show that approximately half of exposed persons are TST negative, even in high endemicity settings.(6) *M. tuberculosis* exposure is a function of duration, proximity and grade of sputum positivity, so EC can be best appreciated when these dimensions of exposure are maximal (Table 1).(7) Outbreaks in closed environments, such as a US naval ship where 66 sailors shared a cabin with seven others with pulmonary TB, and thirteen remained TST negative after six months, illustrate this best.(8) In such settings it is highly likely *M. tuberculosis* was inhaled, contained and cleared before an adaptive response developed.

Lurie demonstrated hereditable resistance to *M. bovis* infection in rabbits in the 1940s.(9) Today, genetic loci associated with TST reactivity have been identified in two populations, suggesting that host factors play an important role in failure to establish an infection after exposure. Recently, Cobat and others used a genome-wide linkage study
to show TST status is highly hereditable: a single locus accounted for 65% of TST variability in a Columbian population (10), and two loci determined TST status in South Africa (11). The first (TST1) is associated with a lack of TST responsiveness and the second (TST2) is associated with the degree of response. Additionally, two candidate gene studies compared TST positive to negative subjects and found associations with cytokine genes (12,13). The best explanation for these observations is EC. Alternative explanations can only be discounted by studies that address exposure level as a confounder and reduce the likelihood that a negative TST reflects anergy through post exposure follow up.

**Early versus delayed clearance**

EC is one of several phenotypes in the progression to TB disease after exposure, (Figure 1). By definition, EC occurs before the development of a positive IGRA or TST, that is before the development of an adaptive immune response. Clearance could also occur later in the course of infection. However at present clearance in a person with a positive IGRA or TST could only be distinguished from LTBI by necropsy, thus it is difficult to study delayed clearance. If EC fails, either primary progressive TB or LTBI develops. These phenotypes are influenced by both innate and adaptive immune responses and are beyond the scope of this review.

**Early clearance of *M. tuberculosis* is most likely to be mediated by an effective innate immune response**

If individuals exposed to *M. tuberculosis* clear the infection, what could be the possible mechanism? The most plausible hypothesis is that EC is an innate immune response that occurs before adaptive immunity can develop. Inter-individual variability in quantitative assessment of innate immune responses, such as mycobacterial whole blood stimulation assays (14) and inhibition of mycobacterial growth in vitro, support the notion of variability in ability to clear *M. tuberculosis* between individuals. The Lubeck disaster in which 252 newborns were accidentally inoculated with virulent mycobacteria illustrates this variability most dramatically: approximately one third died, but another third remained well and the remainder were only slightly ill (15).

Others have argued waning *M. tuberculosis* specific T-cell responses following point source exposure suggest a role for T-cells in clearance of an acute infection (16). Similar claims have been made about IGRA "reversions" in serially tested health care workers (17). Both observations could result from bias, due to random variation in IGRA result (18). IGRA reversion was no more likely in the those treated for LTBI than controls in a clinical trial, so cannot be assumed to reflect EC (19).
How does early clearance occur?

The alveolar macrophage is the primary target cell of early *M. tuberculosis* infection. Recognition by alveolar macrophages, and possibly epithelial cells, leads to pro-inflammatory cytokine production, chemokine secretion and antimicrobial peptides.(20) This may influence the activation state of other target cells. Lurie's seminal rabbit studies identified infection of alveolar macrophages as the first stage of pulmonary TB, and point at which pathological findings in rabbits bred to resist infection diverged from susceptible rabbits.(21) However, if *M. tuberculosis* escapes the alveolar macrophage, a period of logarithmic growth (second stage) ensues with infection of newly recruited monocytes. More recently a zebrafish *M. marinum* model has indicated that granuloma formation occurs at this stage and facilitates further infection of new macrophages.(22) The arrival of the CD4+ T cells secreting IFN-γ to activate recruited macrophages marks the third stage and the start of an adaptive response. EC is more likely when intracellular mycobacterial killing in alveolar macrophages is successful in the first stage, and least likely once mycobacterial outgrowth and infection of recruited macrophages favour pathogen survival.

The role of alveolar macrophages in early clearance

As the dominant phagocyte in the healthy lung, alveolar macrophages probably play a key role in EC, by phagocytosis and immune recognition of *M. tuberculosis*, and the subsequent inflammatory response and *M. tuberculosis* killing. Mathematical models differ in their estimates of EC, due to differing assumptions about the killing capacity of alveolar macrophages.(23,24) Some view alveolar macrophages as alternatively activated or promoting anergy.(24,25) However Flynn and others argue that they may not fit either category, particularly in early stages of infection, prior to cytokine activation.(26) Notwithstanding these controversies, a model of *M. tuberculosis* infection of alveolar macrophages can be advanced drawing on murine studies and ex vivo human studies. Figure 2 describes how the outcome of infection can be influenced by different events including pathogen recognition, cytokine production, intracellular killing, and the mode of macrophage death.

Phagocytosis, pattern recognition of *M. tuberculosis* and induction of protective cytokines

Uptake of *M. tuberculosis* by phagocytes occurs via specific receptors that can influence subsequent events within the cell. The Mannose Receptor (MR) is heavily expressed by alveolar macrophages and the primary avenue for uptake of non-opsonised *M. tuberculosis* in the alvolar space where complement is rare. Ligation of the MR by mannose-capped lipoarabinomannan (Man-LAM) induces phagocytosis.

When certain *M. tuberculosis* cell wall components bind to Pattern Recognition Receptors (PRRs) on the surface or in the cytosol of host innate cells, this shapes the subsequent immune response. Toll like receptors (TLRs), NOD like receptors (NLRs)
and C-type Lectins recognise *M. tuberculosis* and drive cytokine signals that influence mycobacterial clearance. TLR 2 binds lipoarabinomannan, and as a dimer with TLR6 binds a 19-kDa *M. tuberculosis* lipoprotein. These signals are transduced through MyD88 and transcription of Nuclear Transcription Factor kappa β (NF-κβ) and thus promote inflammatory cytokine secretion, particularly Tumor Necrosis Factor alpha (TNF-α). TLR4 recognises heat-labile cell-associated factor and also activates MyD88 and NF-κβ, yet induces Interleukin-B1 production. TLR8 and 9 are endosomally located and thus able to detect the nucleic acids of intraphagosomal *M. tuberculosis*. Stimulation results in the expression of NF-κB dependent cytokines as well as type 1 interferons. TLR8 expression is increased in patients with active TB and in ex vivo cell stimulation experiments and genetic variants have been associated with susceptibility to active TB in two populations.

Similarly, ligation of nucleotide-binding oligomerisation domain 2 (NOD2), another PRR expressed by human alveolar macrophages, limits intracellular *M. tuberculosis* growth through up-regulation of autophagy and cathiliciden production. NOD2 polymorphisms have been associated with TB in some populations, albeit not in others. NLR or Interferon-inducible protein AIM2 (AIM2) receptor ligation also activates the inflammasome; the multiprotein complex containing caspase-1 that activates Interleukin-1β (IL-1β). Polymorphisms in the inflammasome pathway, specifically Caspase recruitment domain-containing protein 8, are associated with risk of TB in HIV infected individuals.

Besides MR, two other C type lectins may recognise *M. tuberculosis*: Dendritic cell specific intracellular adhesion molecule-3-grabbing non-integrin (DC-SIGN) and Macrophage C type Lectin (MCL). *M. tuberculosis* induces expression of DC-SIGN in alveolar macrophages, and binding of DC-SIGN to *M. tuberculosis* Man-LAM leads to an anti-inflammatory signal. MCL (also called Clec4d) has recently been identified as a functional receptor for *M. tuberculosis* in experimental models, but genetic association studies in humans are needed.

**Mycobacterial killing**

Macrophages have a number of mechanisms to kill intracellular mycobacteria. After phagocytosis the *M. tuberculosis* containing phagosome matures through interactions with endocytic pathway. This includes acidification of the phagosome through expression of H+ ATPase as well as the hydrolytic enzyme cathepsin D. Mycobacteria are subjected to further oxidative stress through up-regulation of Nicotinamide adenine dinucleotide phosphate oxidase and Nitric Oxide Synthetase to produce reactive nitrogen intermediates (RNI) and possibly reactive oxygen intermediates (ROI). In mice this process is up-regulated by IFN-γ acting in synergy with TNF-α whereas in humans 1,25-(OH)2 Vitamin D3 is also required. The importance of this defence for killing of intraphagosomal *M. tuberculosis* has driven both pathogen evasion strategies and host counter strategies to influence phagolysosomal fusion. Man-LAM, through interaction with the MR, inhibits phagolysosomal fusion and,
via peroxisome proliferator-activated receptor, down regulates transcription of NF-κB, AP-1, and Signal Transducer and Activator of Transcription (STAT), favouring an anti-inflammatory cytokine profile and blunted oxidative response. (45,46) Cell wall constituents from more virulent mycobacterial strains achieve greater inhibition of phagolysosomal fusion. (47) Macrophages may counter this inhibition though deploying autophagosomes that engulf phagosomes and force lysosomal fusion. (48,49) Autophagy is promoted by IFN-γ, vitamin D and TLR4 via Toll-interleukin-1 receptor domain-containing adaptor-inducing interferon-b (TRIF) signaling. (48,50). Finally, M. tuberculosis infected macrophages produce hepcidin and catheliciden, which have direct activity against M. tuberculosis. (51,52)

**Macrophage death and survival of M. tuberculosis**

Macrophages may die through apoptosis or necrosis, and experiments in macrophage cell cultures show that apoptosis inhibits M. tuberculosis replication. The apoptotic macrophage expresses ATP and phosphatidyl serine to promote its efferocytosis by other phagocytes that kill the bacterium. (53) Virulent strains of M. tuberculosis may evade apoptosis and induce necrosis through production of lipoxin A4, which blocks prostaglandin E2 synthesis and prevents repair of the plasma membrane. (54) This necrotic death favours membrane disruption and mycobacterial outgrowth. A zebrafish model in which the balance of pro and antiinflammatory eicosanoids lipoxins was associated with macrophage necrosis and M. tuberculosis outgrowth. (55)

**Neutrophils**

To date neutrophil responses are the only aspect of EC studied in a prospective cohort of case contacts. Martineau et al found absolute neutrophil count associated with a negative IGRA response and greater killing capacity in a ex vivo Bacille Calmette-Guerin (BCG) lux whole blood stimulation assay. Killing was also associated with serum levels of the neutrophil antimicrobial peptides, cathelicidin, human neutrophil peptides 1 and 3 and Lipocalin 2. (56) Lowe and colleagues argue for an influential role of neutrophils in EC, (57) noting that early depletion or recruitment of neutrophils impacts the outcome of M. tuberculosis infection in animal models. (58-60) Kisich observed that neutrophils from some donors killed mycobacteria spontaneously whereas others required TNF-α activation; variability that may reflect varying capacities for EC. (61) Neutrophils phagocytose M. tuberculosis and are in turn phagocytosed by monocyte/macrophages recruited in the second stage of infection. At this point the neutrophil can influence whether the macrophage undergoes apoptotic or necrotic cell death, thereby influencing the likelihood of clearance. (57)

**NK and T cell subsets**

Even less is known about the role of other immune cells in EC. Natural Killer (NK), Mucosa Associated Invariant T (MAIT) cells and γδ T cells express germ-line encoded
PRRs that give them innate type functions. The early appearance of NK cells in *M. tuberculosis* suggests a possible role in EC. The germ line encoded receptors NKp46 on NK cells recognises infected monocytes. (62) NK cells produce IFN-γ and IL-22 that promote *M. tuberculosis* killing and macrophage apoptosis. (63,64) NK cells in experimentally infected animals have a variable impact on the course of infection. (65,66)

MAIT cells are a recently described T cell population found in mucosal tissue including the lung. As they do not depend on clonal expansion, MAIT cells respond rapidly to danger signals and provide an early innate source of IFN-γ to drive macrophage activation. MAIT cell deficient mice challenged with aerosolised *M. bovis* have a significantly higher burden of infection at day 10. (67) MAIT cell populations expanded early in infection in lungs and were required for timely recruitment of CD4 and CD8 cells in a *Francisella tularencis* vaccine – mouse model of acute infection. (68).

γδ T cells may also play a role in the early response to *M. tuberculosis* as they are present in alveoli, and recognize mycobacterial phosphoantigens expressed on the surface of infected macrophage. (69) Their activation results in killing of infected macrophages through cytotoxic granules, IFN-γ and TNF-α signalling, and cell contact dependent help. As with other T cell sub-sets, the role of γδ T cell in the response to early *M. tuberculosis* infection needs to be defined.

**Acquired defects of Early Clearance**

A variety of clinical conditions could impair clearance including diabetes mellitus, smoking, end stage renal disease, corticosteroid use and anti-TNF-α agents among others. Conditions where laboratory and clinical observations support impaired clearance are HIV infection and Vitamin D deficiency.

**HIV**

HIV is an established risk factor for developing active TB, and probably also a risk for *M. tuberculosis* infection. HIV impairs cell-mediated immunity through depletion of CD4 T-cells. (70) Additionally, HIV impairs innate immunity in a manner that may impair EC: macrophage turnover is increased in HIV infection, and apoptosis is impaired (71,72); dendritic cell numbers are diminished and their autophagic and antigen presenting functions are suppressed (73,74); NK cells have low levels of glutathione and permit higher rates of mycobacterial growth in co-cultured monocytes. (75) Molecular epidemiological studies of *M. tuberculosis* suggest susceptibility to infection is a greater contributor to risk of TB in HIV positive persons than accelerated disease progression. HIV was strongly associated with reinfection in a study of previously treated South African miners. (76) Additionally, a metaanalysis of molecular epidemiological studies shows high rates of clustering of *M. tuberculosis* strains, supporting susceptibility to infection rather than reactivation of previously circulating strains. (77) A low baseline
A monocyte to lymphocyte ratio was associated with a higher risk of subsequent TB in HIV positive persons, and may reflect impaired clearance.(78)

**Vitamin D**

Vitamin D deficiency may be a modifiable risk factor for *M. tuberculosis* infection. Vitamin D is a potent modulator of innate immunity and was a treatment for TB in the pre-antibiotic era. Vitamin D promotes mycobacterial killing through modulation of the innate immune response including the production of antimicrobial peptides(52,79) and up-regulation of autophagy in macrophages.(79,80) The effect occurs at physiologic concentrations: a single oral dose of vitamin D increased the killing of BCG observed in whole blood assays performed on TB case contacts.(81) Vitamin D receptor polymorphisms and Vitamin D deficiency (VDD) interact to cause susceptibility to TB infection among Guajarati Indians living in the UK.(82)

Whereas clinical studies of vitamin D supplementation in active TB have been disappointing,(83) very few studies have considered whether VDD influences development of *M. tuberculosis* infection. In a small Spanish study following TST negative TB case contacts, all 12 who underwent TST conversion had VDD compared to 57 of 82 (69.5%) non-converters.(84) In a double blind placebo-controlled RCT among 120 Mongolian child case contacts, vitamin D supplementation seemed to protect against TST-conversion (RR 0.41; 95% CI 0.16 to 1.09; P=0.06).(85) Therefore, there is some evidence that Vitamin D influences EC, and more field studies in TB case contacts are needed.

**Early clearance and vaccine development**

EC is a genuine example of protective immunity to *M. tuberculosis* and could offer new avenues for vaccine development. Although IFN-γ and *M. tuberculosis* specific CD4+ T cells are clearly involved in the host response to *M. tuberculosis*, there is increasing awareness that these factors are not particularly robust correlates of protection.(86) Furthermore, the possibility of concurrent infection with two *M. tuberculosis* strains, and exogenous reinfection following *M. tuberculosis* treatment suggest the adaptive response to *M. tuberculosis* is not protective.(87,88) TB incidence in previously treated South Africans was estimated to be four times higher than the general population, implicating a deficit in innate immunity rather than a protective acquired response.(89) EC provides a model of protective natural immunity to *M. tuberculosis* that is likely to represent a more fruitful avenue for vaccine development than adaptive responses.

**Further research and conclusions**

EC is a priority for *M. tuberculosis* research as such studies have potential to discover new immunotherapies and vaccines to prevent infection. Case contact studies allow EC to be observed in humans, through the integration of immunological phenotyping with a
well established field study design. This approach is analogous to the study of highly exposed persistently negative sex workers to identify broadly neutralising antibodies to HIV, now recognised as a new avenue for HIV vaccine development. Case contact studies allow investigators to quantify and adjust for environmental and pathogen factors, so host factors can be studied without confounding. Specifically, duration of index case infectivity, proximity and frequency of contact, sputum bacillary burden and strain type. Long term follow up of putative ECs, potentially over decades, can strengthen confidence that persistent TST negativity is in fact clearance, not anergy.

Identifying and validating biomarkers is a necessary first step for EC research. This will provide supporting evidence for EC as a distinct phenotype, assist in easier identification of ECs in future studies, and suggest possible mechanisms of EC. Biomarkers may also enable study of EC in HIV, which is presently limited by the poor sensitivity of TST and IGRA. Other M. tuberculosis phenotypes have been studied in this way, for example, the cytokine and cell populations that distinguish LTBI progressors from non progressors. Additionally, the effect of ex vivo M. tuberculosis stimulation on cytokine production or gene transcription can be studied for its correlation with phenotype. A challenge in EC research will be to assess the events that occur in the lungs of healthy case contacts. Some biomarkers may be detectable in blood, whereas others might only be found on bronchoalveolar lavage. The latter has been proposed though its invasiveness and complexity limits its use in large studies.

In addition, more work is needed to validate the two published studies examining genetic risk factors for M. tuberculosis infection. In this respect, household contact studies have particular advantages. Poor selection of cases and controls may bias many studies of M. tuberculosis susceptibility. Comparison of TB cases to poorly defined controls naturally yields a variety of different associations that could reflect susceptibility to infection or disease progression. A narrower definition of phenotype, that focuses just on the shorter chain of events between exposure and infection (or clearance) is more likely to yield loci associated with EC.

This review has suggested a mechanism for EC based on animal and ex-vivo human experiments, as well as genetic studies. Whether these pathways are in fact associated with EC must be tested in human studies. Understanding the mechanism of EC could enable its manipulation through drugs or vaccines, and open a new field in TB prevention.

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Table 1. Selected highly exposed populations and proportion TST negative

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<tr>
<th>Highly exposed population</th>
<th>Duration of exposure</th>
<th>Proportion TST negative</th>
<th>References</th>
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<tbody>
<tr>
<td>American sailors shared cabin with seven pulmonary TB cases.</td>
<td>6 months</td>
<td>19.7%</td>
<td>(8)</td>
</tr>
<tr>
<td>Nurses, in 1930s New York hospital</td>
<td>3 years</td>
<td>7%*</td>
<td>(96)</td>
</tr>
<tr>
<td>Household contacts in Jinan, China with average exposure 9 hours/day and proximity 30-40cm</td>
<td>Not stated</td>
<td>31%</td>
<td>(97)</td>
</tr>
<tr>
<td>Household case contacts in The Gambia sharing same bed</td>
<td></td>
<td>43%</td>
<td>(98)</td>
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<table>
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<th>Gambian adults sharing room with case</th>
<th>35%</th>
<th>(99)</th>
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<tbody>
<tr>
<td>Contact at least alternate days with a case of laryngeal TB</td>
<td>6 months</td>
<td>31%</td>
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* Boosting may occur in health care workers undergoing repeated TSTs.

Figure 1. Phenotypes in the progression to TB disease after exposure.

*M. tuberculosis* exposure leads to infection. EC occurs before the development of an adaptive immune response and is likely to be due to innate factors, during Stage I or II. If the pathogen evades EC mechanisms, an adaptive response develops (Stage III), measurable through a positive TST or IGRA. Early evasion of both innate and adaptive responses results in primary progressive TB. However in the majority of infected individuals *M. tuberculosis* is contained as LTBI with only 5% later reactivating disease. Others speculate clearance could also occur at the time of or after TST/IGRA conversion, we term this delayed clearance.

Figure 2. Hypothesized mechanisms of clearance versus infection during first and second stage of *M. tuberculosis* infection

In stage one infection is primarily of alveolar macrophages. Activation of macrophages may be important for EC, through recognition of *M. tuberculosis* via surface, cytosolic or phagosomal PRRs and/or TNF-α and IFN-γ secreted by other cells. This promotes killing of intracellular *M. tuberculosis* by phagosomal acidification, hydrolytic enzymes, and generation of RNI. Also, antimicrobial peptides and autophagy promote mycobacterial killing and are both induced by vitamin D. Alternatively, infection is favoured if the macrophage’s initial interaction with *M. tuberculosis* is via ligation of the MR. This promotes uptake without recognition and inhibition of phagolysosomal fusion. In stage two, infected macrophages undergo apoptosis and express adenosine triphosphate and phosphotidyl serine. This attracts monocytes and neutrophils that engulf the infected cell and deploy oxidative killing mechanisms to achieve clearance. Neutrophils activated in this fashion secrete antimicrobial peptides cathelicidin, human neutrophil peptides and Lipocalin 2 to kill infected monocytes. Sustained infection is most likely when infected macrophages undergo necrotic cell death. The disruption of the macrophage membrane facilitates mycobacterial outgrowth so newly of recruited monocytes are infected and logarithmic growth ensues.