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Tuberculosis vaccination needs to avoid 'decoy' immune reactions. Running Title: Decoy reactions in tuberculosis Review

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List of Abbreviations:

Tuberculosis (TB); Bacillus Calmette-Guerin (BCG); dendritic cells (DC); mycobacterial glycolipoprotein (LprG); mycobacterial lipoarabinomannan (LAM); major histocompatibility complex (MHC);

Abstract

Current search for a new effective vaccine against tuberculosis involves selected antigens, vectors and adjuvants. These are being evaluated usually by their booster inoculation following priming with Bacillus Calmette-Guerin. The purpose of this article is to point out, that despite being attenuated of virulence, priming with BCG may still involve immune mechanisms, which are not favourable for protection against active disease. It is postulated, that the responsible 'decoy' constituents selected during the evolution of pathogenic tubercle bacilli may be involved in the evasion from bactericidal host resistance and stimulate immune responses of a cytokine phenotype, which lead to the transition from latent closed granulomas to reactivation with infectious lung cavities. The decoy mechanisms appear as favourable for most infected subjects but leading in a minority of cases to pathology which can effectively transmit the infection. It is proposed that construction and development of new vaccine candidates could benefit from avoiding decoy-type immune mechanisms.

1.0 Introduction

Prophylactic vaccination had eradicated smallpox and is eminently effective for a number of previously devastating viral infections (e.g. polio, measles or rubella) which get transmitted soon after infection. Protection by these vaccines as well as following natural infection are antibody mediated. In contrast, the host response to intracellular pathogens, including Mycobacterium tuberculosis (Mtb) has been attributed to Darwinian selection of 'decoy' constituents with low immunogenicity and lack of protective antibodies ¹. Unlike the viral transmission by most infected subjects prior to their immune response. Mtb is transmitted following a long asymptomatic latent phase by only about a 5 % fraction of subjects who reactivate into lung cavitary disease with efficient expectoration of bacilli. The duplicity of the immune responses is reflected by inducing initially beneficial latency in the majority of infected subjects, while also conditioning the host to reactivation toward pulmonary pathology, leading to transmission of infection. The latter aim, being a mandatory requirement for pathogenicity would have led to the evolution of the required Mtb constituents. Unlike the virulence factors of other microbial pathogens being often toxins for certain host cells, it has been proposed, that Mtb evolved its antigenic and immunomodulatory constituents as 'decoys' or 'secret trumps' to mislead the infected host by high-jacking its immune responses in favour of its persistence and transmission ^{2,3} which fits within the more widely formulated 'damage-response framework' of microbial pathogenesis.4

Prophylactic vaccination against TB with Bacillus Calmette-Guerin (BCG) is known to be protective only against TB meningitis and disseminated TB in children under 5 years of age⁵, but not against adult TB and it failed also in reducing the transmission of tuberculous infection. Protection by BCG vaccination has long been classified as T cell mediated, but none of the cytokine bioassays correlate reliably with protection^{6,7} and early protection in mice was attributed to T cell independent recruitment of CD11b+F4/80+ monocytes into the lungs. ⁷ The geographic variations in BCG protection have been associated with the gut microbiome, since its protection can be reduced by either non-tuberculous mycobacteria (e.g. *M*. avium)⁸, or by antibiotic induced gut dysbiosis⁹. Moreover, some degree of protection against other infections was attributed to non-specific 'trained' memory of cells of the innate immune system ^{10,11}. BCG vaccination is widely used in several countries, although it was never accepted in the USA, and has recently been labelled as 'antiquated and inadequate'¹². Despite such reservations, priming by BCG carries the advantage, that it abrogates the adjuvant dependency for the booster subunit antigen inoculation ¹³ and has still been retained within the prime-boost paradigm for the development of some of the multistage antigen construct based new vaccines.¹⁴ The following paragraphs discuss the decoy elements of host responses to tuberculous infection, particularly with a potential of being involved following BCG-prime/boost vaccination and proposing that their avoidance would be desirable for continuing research efforts toward an effective prophylactic vaccine against TB.

2.0 Early post-infection events

2.1. Evasion from host resistance

Tubercle bacilli escape destruction by the infected host by abrogating the initial bactericidal action of macrophages and then by subverting the antigen presenting function of dendritic cells by separate distinct mechanisms.^{15,16} The survival of Mtb in infected macrophages is facilitated through repression of their apoptosis ^{17,18}. However the complex metabolic reprogramming of phagocytic cells¹⁹, involving the triggering of different 'pattern recognition receptor' (PRR) mediated pathways by mycobacterial 'pathogen associated molecular patterns (PAMPs) ^{20,21} is beneficial not only to the host, but also to the pathogen.²²

Thus, potentially the protective Th1 immune response is delayed by the mycobacterial glycolipoprotein (LprG) induced TLR-2 mediated inhibition of MHC-II antigen processing by dendritic cells (DC),²³ and by lipoarabinomannan (LAM) mediated blocking of the expression of the DC-SIGN receptor and induction of immunosuppressive IL-10 and CCL18 production ²⁴.

Although BCG has reduced expression of virulence factors and activities, such as the Rv3097c-encoded lipase ²⁵ and dephosphorylation of host proteins ²⁶, it still produces both LAM and LprG, which inhibit the immunostimulatory CD-1 and IL-12 production and induces the immunosuppressive IL-10 even in the absence of DC-SIGN ²⁷. Hence, BCG retains despite its attenuated virulence, demonstrable activity *in vitro*, which is pertinent to immune evasion mechanisms and subversion of its vaccination potential. ²⁸ Delayed migration of antigen-bearing DCs from Mtb infected lungs of infected mice to draining lymph nodes results in the failure of Th1 cell activation and their recruitment to the lungs.²⁹ Furthermore, an infected phagosome coat protein TACO, preventing lysosomal transfer and degradation of mycobacteria allows their survival. ³⁰ Notably, both Mtb and BCG survive *in vivo* within phagosomes by initially resisting delivery to lysosomes and despite their subsequent lysosomal transfer ³¹.

2.2 Conditioning for reactivation

Experimental models of reactivation of Mtb infection in mice ('Cornell' models) have a number of variants, involving either spontaneous relapse following non-sterilizing chemotherapy with isoniazid and pyrazinamide or a rapid increase of bacterial counts from low level persistent infection, induced by immunosuppressive agents, such as hydrocortisone or anti-CD4 T cell antibodies. ³² The duration of the spontaneous relapse is much shorter than the recrudescence from chronic infection, but neither model reflects fully the unknown circumstances, which lead to the development of clinically active TB in the small minority of infected humans.

Both Cornell models of reactivation were shown to occur not only following pathogenic Mtb, but also following intravenous BCG Pasteur strain infection.^{33,34}, which could be explained by the presence of a number of virulence-related constituents (discussed in the preceding paragraph. ²⁵⁻²⁸ Using the post-chemotherapy spontaneous relapse model, the essential role of an early host response conditioning for the infection relapse has been suggested by the finding, that reactivation occurred only when the chemotherapy was started not earlier than three weeks post-infection. ³³ Since the innate immune mechanisms which condition the host toward reactivation remain to be unidentified, further research, could be targeted on: a) the granulocyte macrophage colony stimulating factor (GM-CSF) and DC-SIGN receptor mediated function of DCs²⁷; b) the influence of mycobacterial DosR(Rv3133c) genes which had been associated with extracellular Mtb persistence³⁵ and c) regulation of mRNA expression in monocytes, by the Toll-interacting ubiquitin binding protein (TOLLIP), which has been associated with BCG adjuvanticity and susceptibility to tuberculosis.³⁶ Though the relapse was demonstrated following intravenous inoculation of BCG, a lower risk from subcutaneous vaccination may depend from the vaccine dose³⁷ and could apply also to subsequent pathogenic Mtb infection. However, the early conditioning for reactivation is clearly different from the later mechanisms which can be alleviated by immunotherapy³⁸⁻⁴⁰ and different from the TNF α mediated exacerbation of lung granulomas without reducing the bacillary load ('Koch phenomenon'), caused by the administration of mycobacterial antigens following Mtb infection.⁴¹

3.0 Adaptive immune responses.

3.1.MHC-permissive epitopes.

MHC-permissive T cell recognition of mycobacterial immunodominant epitopes in the context of both class II ⁴²⁻⁴⁴ and class I alleles⁴⁵ has been attributed <u>to</u> evolutionary selection on the grounds of their higher frequency in MHC- heterozygous than homozygous T cell hybridomas, ⁴² hence considered favourable for the pathogen's transmission. ⁴⁶ This interpretation has been confirmed by the highly conserved sequence of immunodominant epitopes between diverse Mtb strains.^{47,48} Recognition of lower peptide concentrations by the higher affinity of T cell receptors for these epitopes is conducive for host survival during the latent phase of infection. However, the decoy character of these hyperconserved epitopes and their evolutionary advantage to the pathogen has been proposed to be associated with mechanisms which lead to the subsequent reactivation in the minority of infected subjects, hence leading to efficient transmission of the infection in the population. ⁴⁹

3.2. Ambiguity of the T cell functions

Despite the broadly accepted protective role of T cells the corresponding effector functions have not been clarified and a reliable protection bioassay, based on Th1 cellular or cytokine markers, has not been found.⁵⁰ Moreover, subunit vaccination can elevate Th1 cytokine levels, but without protection ⁵¹, while mucosal vaccination can protect, without elevating Th1 cytokine levels.⁵² Some of this ambiguity may be due to IL-6 mediated blocking of bactericidal action of Th1 cytokines ⁵³. Though IFN γ can activate macrophages to become bactericidal and limits neutrophilic pulmonary inflammation,⁵⁴ it's levels failed to associate reliably with protection and adding IFN γ to tissue culture media was reported even to enhance Mtb growth in human macrophages ⁵⁵. Although protection in humans has been associated with the recognition of more antigen epitopes ^{56,57} and the production of several cytokines ⁶³.

Protection following BCG vaccination is considered compromised by inducing T cells mostly of the terminally exhausted 'effector' phenotype, instead of the better protective 'central' memory phenotype ⁶⁴ and by the initial localization of T cells to the un-infected areas of the lungs, leading to delayed granuloma formation and latent infection with a potential for reactivation. ⁶⁵ Hence, the immunity imparted by BCG, despite its attenuation, may still carry the decoy advantages for the transmission of infection. However, following peptide based vaccination, CD4+T cells also failed to target the Mtb infected cells, despite reaching the lung parenchyma ⁶⁶. Notably, in a murine model of diabetes, BCG or recombinant BCG vaccination was shown to be protective against reactivation of latent lymphatic TB infection by CD4 and CD8 T cell independent mechanisms.⁶⁷ Moreover, dysregulated CD4 T cell responses may have deleterious role, reflected by their amplification of infection and lung pathology in PD-1 receptor knockout mice ⁶⁸, by enhanced T cell responses in mitochondrial cytophilin D (CypD) deficient mice ⁶⁹ and by their influx to sites of lung pathology, mediated by the chemotactic CXCL10 chemokine. ⁷⁰

A protective role of CD8 T cells has been demonstrated in CD4- T cell depleted mice. ⁷¹ However, their limited role for protection was argued on the grounds that CD8 T cell lines binding the TB10.44-11 immunodominant epitope failed to recognise and to inhibit the growth of Mtb-infected macrophages, although they recognised the same epitope, when macrophages were pulsed with irradiated Mtb.⁷² The authors attributed the blocking of the CD8 response to TB10.44 by the live infection to the subversive decoy function of this major

epitope. The limited protective role of CD8 T cells, despite their robust response following infection is supported also by the Th2 cytokine profile human HLA-E-restricted CD8 cells ⁷³ and by their terminally-differentiated phenotype.⁷⁴.

3.3 Antibody markers

The association of prominently elevated antibody levels to the PstS1 glycoprotein⁷⁵ with the HLA-DR2 haplotype ^{76,77} and with the multibacillary, infectious type of active TB qualifies the PstS1 antigen as a decoy constituent .⁷⁸ The pathogenic mechanisms may involve epitope presentation by B cells instead of dendritic cells, thus diverting T cell maturation from a protective Th1 to a pathogenic (as well as B cell helper) Th2 phenotype.⁷⁹ Thus, despite the MHC-permissive strong immunogenicity of PstS1, its tendency toward inducing a Th2 response seems discouraging for further vaccine development, without removing its Th2-stimulatory epitope moieties. The protective potential of antibodies against the α -crystallin (Acr) antigen was suggested following passive vaccination with the IgA (TBA61) monoclonal antibody combined with recombinant IFN γ and anti-IL-4 treatment.⁸⁰⁻⁸² and by *in vitro* killing of intracellular Mtb by human antibodies with a distinct glycosylation profile.⁸³ However, a possible inference toward active vaccination would need the removal from the antigens their potentially pathogenic IL-4 producing Th2 stimulatory moiety.

4.0 Mediators and mechanisms of pathology

The promotion of the development of destructive granulomatous lung lesions by T cell responses has been noted previously ⁸⁴. Since the initial finding of elevated IL-4 production in active TB ⁸⁵, the association of Th2 cytokines with pathogenic mechanisms has been suggested by: (1) Interference with the bactericidal action of Th1 cell cytokines ^{86,87}, involving elevated intracellular (SOCS and IRAK-M) and extracellular (IL-10 and TGF_βRII, IL-1Rn, and IDO) mediators.⁸⁸; (2) Alternative activation of macrophages, which supports the persistence of Mtb ⁸⁹; (3) TNF α mediated change from apoptosis to necrosis of infected cells ⁹⁰ and cAMP initiated granuloma formation ⁹¹; (4) Inhibition of TB-resistance by intestinal microbiota induced IL-10⁹² and by (5) regulatory T cell mediated suppression of protective CD4 T cells ⁸⁷.

Neutrophils recruited to the infected lungs can counteract the cathelicidin and lipocalin mediated mycobactericidal action of Th1 cells ⁹³. Low-density neutrophils in TB lesions have deficient phagocytosis and oxidative burst 94 , while necrotic neutrophils reduce TNF α and increase IL-10 production ⁹⁵. Infected macrophages upregulate the MCL1 gene expression, which interferes with Mtb killing ⁹⁶, while increased Eis gene expression in Mtb-containing autophagosomes enhances Mtb survival by attenuating TLR mediated autophagy, modulating the cell death and suppressing the host innate immune defenses.⁹⁷ Subsequent translocation of Mtb-infected alveolar macrophages from airways to the lung interstitium involves the Mtb ESX-1 secretion system and MyD88/IL-1 receptor inflammasome signalling ⁹⁸. The quoted mechanisms in neutrophils and macrophages support the maturation of lung granulomas to cavitation with liquefaction, resulting in efficient transmission of the infection to susceptible hosts. The IL-1 cytokine can initially be protective against Mtb infection, but it's persistent production aggravates disease by contributing to neutrophil accumulation, which can be alleviated by co-administration of the IL-1 receptor antagonist protein with the linezolid antibiotic ⁹⁹. This example shows, that identifying decoy mechanisms can be supportive for the development of 'host directed therapies' ¹⁰⁰

5.0 Predominance of priming over response to challenge

The key importance of the sequence of antigen exposure and the potential for a deleterious impact was demonstrated by the finding that immunization with various mycobacterial antigens can aggravate lung pathology in Mtb pre-infected mice. ⁴¹ The 'original antigenic sin' (OAS) phenomenon, reflecting the predominance of immunity to the first priming antigen following booster immunization, has been described following infection and vaccination with influenza virus and several other infections,¹⁰¹ thus overriding the antigenic specificity of the booster antigen.^{102,103} The subversive role of OAS as a decoy mechanism was proposed for the generation of non-protective antibodies against HIV-1 and Ebola virus infections.¹ The OAS could also explain, why BCG vaccination showed preferential stimulation to *M. avium* antigens in children who were pre-exposed to environmental mycobacteria,¹⁰⁴ the failure of boosting protection by antigen challenge of BCG primed macaques,¹⁰⁵ and the blocking of BCG protection by prior exposure to environmental species of mycobacteria in mice,¹⁰⁶ and in human populations.¹⁰⁷ The mandatory role of the sequence of antigens for heterologous prime/boost vaccination has been documented also by the finding, that priming with the Ag85A-coding DNA yielded protection in mice when administered before, but not when given after BCG.¹⁰⁸

Although the readout of <u>the OAS</u> phenomenon has previously involved mostly antibody responses, the recognition mechanism has been attributed to T helper cell mediated cross-reactivity between taxonomically related antigens¹⁰⁹. Moreover, its impact could be even wider, due to non-reciprocal (immunodominant or cryptic) cross-recognition ('mimicry') of epitopes between taxonomically unrelated mycobacterial proteins.¹¹⁰ Such relationships could contribute to the predominance of the primary antigenic exposure in the BCG-prime/heterologous boost vaccine regimen, thus sustaining the specificity and phenotype of T cell response, initiated by the primary BCG, rather than of the boosting antigen subunit. Priming to several of the BCG antigens may include also the undesirable decoy antigens which evolved for initiating a host response for the ultimate advantage of the Mtb pathogen.

6.0 New vaccine research 6.1 Subunit constructs

Current research involves 'multistage' fusion of proteins, containing both replicating and latent stage antigens ^{111,112}, adjuvants, recombinant formulations and genetically modified BCG or attenuated Mtb ¹¹³⁻¹¹⁵, with emphasis on *in vivo* and latency expressed (IVE), DosR regulon and Rpf stage specific antigens, expressed on infected macrophages. In particular, the Rv2034 protein, strongly immunogenic in TB patients was found to be protective in HLA-DR3 transgenic mice by inducing peptide 31-50 specific IFN γ and TNF α producing CD4 T-cells as well as antibodies.¹¹⁶ Significance of a cluster of 17 IVE antigens has been argued on the grounds of inducing TNF α secreting T cells in TB patients.¹¹⁷ In contrast, another choice of protective antigen subunits considered their downregulated expression during infection,¹¹⁸ and other functions for recombinant expression in BCG, vaccinia virus and adenovirus vectors.¹¹⁹⁻¹²¹ Subdominant Mtb peptides of ESAT-6, though poorly recognized following Mtb infection were found to protect better than epitopes which are immunodominant during natural infection ¹²². On the other hand, it has been proposed to avoid antigens which are well expressed at early stage of infection, which may lead toward an exhaustion and dysfunction of T cell responses^{118,123}.

Several vaccine candidates reached various stages of evaluation in clinical trials. Significant protection without BCG priming was reported three years after intramuscular vaccination with two doses of the M72/AS01_E the recombinant fusion construct of the Mtb32A and

Mtb39A antigens¹²⁴. The case for avoiding BCG comes also from the finding, that the mycobacterial wall lipase LipY (Rv3097c) has been protective in mice when given as a purified protein in adjuvant, while its recombinant overexpression in BCG abrogated protection against Mtb challenge, probably by shifting the T cell response from Th1 to Th2 phenotype.²⁵ These considerations go along the suggestion that learning from past 'failed' trials and diversity of research need to 'embrace risk in persuit of vaccine development'.¹¹³

6.2 Route of vaccination

Mucosal vaccination by respiratory route delivery attracts interest with the aim of fast localizing the protective T cells to the lungs, ready for countering inhaled Mtb infection without dependence on the recruitment of systemically primed T cells, ^{125,126} which may also lack CCR5 and CXCR3 chemokine receptors needed for migration to the lung.¹²⁷ Oral inoculation of BCG in the original MRC Trial was abandoned due to cervical lymphadenopathy. Though intranasal BCG caused lung pathology in mice ¹²⁸, profoundly better protection by pulmonary mucosal than intradermal delivery in rhesus macaques was attributed to polyfunctional TH17 cells, interleukin-10 and immunoglobulin A ¹²⁹. Other proposed mechanisms involved the role of innate immunity in facilitating the homing of DCs and T cells^{130,131} and the recruitment and retention of protective CD8 T cells in the lungs ¹³². Interest in mucosal delivery is supported in a study of needle-free vaccination of mice and guinea pigs, combining oral prime with intranasal booster ⁵², the use of nanoparticles ¹³³or the use of tetramerized streptavidin core fused with biotinylated anti-DC antibodies ¹³⁴. Notably, protection without BCG priming, imparted by intranasal delivery of recombinant soluble DnaK (hsp70) antigen was attributed to lung resident IL-17 producing T cells, acting even after depleting circulating CD4 T cells ¹³⁵.

6.3 Testing for protection against reactivation

Preclinical testing of novel TB vaccines in animal models is done routinely in reference to protection against primary challenge, thus ignoring that the predominant disease in adult humans is the post-primary, reactivated form of TB. However, significant contributions have been made by testing of different recombinant vaccines on the reactivation of latent TB infection in murine models which mimic the susceptibility of HIV-infected humans by anti-CD4 antibody treatment ⁶⁷ or by diet-induced type-2 diabetes.¹³⁶ The substantial difference between the primary and secondary disease has recently been pointed out, while emphasizing the need to test novel vaccines in available animal models of human post-primary TB ¹³⁷. This concept is to be supported, since it would focus research on such host responses which can prevent reactivation, unlike natural infection which leads merely to latency, but with a potential for recrudescence in the minority of subjects.

There is a dilemma of choosing suitable stimuli for reactivating a persisting latent infection in animal models. Though HIV or immunosuppressive therapy are well known to reactivate human TB, the vast majority of adults develop pulmonary disease without any known immune compromise, but associated with a broad range of causes, such as poverty, malnutrition, endocrine and life-habit related factors. Decoy immune mechanisms have been indicated by the association of reactivation of chronic infection in mice with overexpressed IL-10 production ¹³⁸ and by the finding of higher cortisone-induced reactivation in Bcg-resistant than Bcg-susceptible mice ³⁴. SIV-coinfection induced reactivation of lung TB in rhesus macaques showed elevated proinflammatory cytokines and chemokines, thus associating increased T cell activity with a decoy, rather than protective function ¹³⁹.

The role of neuro-endocrine regulation by the hypothalamic-pituitary-adrenal axis was demonstrated by triggering the reactivation of TB infection by physical exertion of mice.¹⁴⁰ The increase in cortisol/DHEA in association with the Th1 to Th2 shift in pulmonary TB ¹⁴¹ also indicated the role of dysregulation of the hypothalamic-pituitary-adrenal axis.¹⁴², while an even wider signature distinguishing between latent and active TB was identified by immunometabolomic and RNA sequence analysis ¹⁴³. Future preclinical research is desirable to select from this broad list of available approaches, which biomarkers would be most suitable for testing of vaccine candidates for protection against the reactivation form of active TB in humans.

7.0 Ethical, safety and funding issues

Protection against disseminated TB in children and also cross-protection against other microbial pathogens ¹⁴⁴ gives ethical justification for continued neonatal BCG vaccination, established in several countries despite a reported risk of aggravated reactions in HIV co-infection ¹⁴⁵. However, research toward novel vaccine constructs, aiming to reduce TB transmission should reconsider if priming by BCG is suitable, in view of its potential decoy capacities, as discussed in this article. This aspect seems pertinent also to the new genetically attenuated strains of Mtb and recombinant BCG constructs, despite their rigorous conventional testing for attenuation ¹⁴⁶. The proposed testing of candidate vaccines for protection against TB reactivation would extend the housing of experimental animals in containment facilities, thus substantially increasing the cost of pre-clinical research.

8.0. Conclusions

The decoy reactions to tuberculous infection are represented by initial conditioning of innate immunity followed by subsequent Th2 cell cytokine responses to HLA-permissive epitopes. These host reactions are in favour of the pathogen, since following a latent infection, they lead to reactivation in a minority of subjects to a type of pathology which effectively transmits the infection. Therefore, pre-clinical testing would benefit from extended evaluation for protection against recrudescence from dormancy. The predominant influence of priming (e.g. BCG) carrying a potential risk for conditioning for reactivation, deserves a consideration for optimising future strategies for new subunit vaccines.

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AUTHOR CONTRIBUTIONS STATEMENT

Being the single author, I confirm writing this Review Article.