

Mycobacteria and allergies

Graham A.W. Rook^{a,*}, Eckard Hamelmann^b, L. Rosa Brunet^a

^a*Centre for Infectious Diseases and International Health, Windeyer Institute of Medical Sciences, Royal Free and University College Medical School, 46 Cleveland Street, London W1T 4JF, UK*

^b*Charité Universitätsmedizin Berlin, Department of Pediatric Pneumology and Allergology, Augustenburger Platz 1, 13353 Berlin, Germany*

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Abstract

Exposure to mycobacteria was inevitable throughout mammalian evolution. Most mycobacteria are saprophytic environmental organisms that are enormously abundant in soil and untreated water and evoke immune responses in the residents of developing countries. A few species are pathogens. For example *Mycobacterium tuberculosis*, the causative agent of tuberculosis (TB), infects approximately 1/3 of the world's population. Many individuals also receive vaccination with the Bacille Calmette Guérin (BCG), which is an attenuated form of the organism causing bovine TB. In order to understand the possible role that mycobacteria might have in the increases in allergic disorders over the last decades, it is necessary to dissect out these different mycobacterial influences. Above all it is essential, when analysing tuberculin test results, to distinguish between individuals who have latent TB and those who do not. Only then can probable effects of diverse types of exposure emerge. There is no doubt that in animal models mycobacteria can both prevent and treat allergic responses either by boosting Th1 or by driving allergen-specific regulatory T cells (RegT). Clinical trials in man remain inconclusive.

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Introduction: the evolution of the Hygiene Hypothesis

The Hygiene Hypothesis was first proposed in the late 1980s to explain the rise in allergic conditions (reviewed in [Rook et al., 2004](#); [Strachan, 1989](#)). The incidence of these disorders in the USA and in Europe increased from the late 19th Century, and appears to have doubled in some decades, particularly during the 1960s and 1970s. Epidemiological correlations with modern ways

of life prompted the suggestion that modern hygiene requirements were reducing contact with pathogens that prime Th1 immune responses. At that time, it was believed that lack of Th1 stimulation would result in the compensatory increase in the Th2 activity that characterizes allergic disorders.

This concept, requiring Th1-inducing infections to control Th2-mediated allergic conditions, ignored the simultaneous increase in several Th1-mediated disorders, such as Crohn's disease, Type-1 diabetes and multiple sclerosis. The incidences of allergic disorders (Th2) and of Type-1 diabetes (Th1), for example, correlate closely both within and outside Europe ([Stene and Nafstad, 2001](#)). The alternative hypothesis, that may explain the simultaneous

*Corresponding author. Tel.: +44 20 7679 9489;
fax: +44 20 7679 9434.

E-mail address: g.rook@ucl.ac.uk (G.A.W. Rook).

increases of autoimmunity and inflammatory bowel disease (IBD), which are Th1-mediated, and allergies, which are Th2-mediated, is that modern living conditions can lead to a defective maturation of regulatory T cells (RegT) and regulatory antigen-presenting cells (APCreg). So rather than Th1/Th2 balance, the crucial factor is likely to be T effector/RegT balance (Rook and Stanford, 1998; Wills-Karp et al., 2001). This version of the hypothesis states that in the absence of optimal levels of immunoregulation, the individual may develop a Th1- or a Th2-mediated inflammatory disorder, depending on his/her own particular Th1/Th2 bias, immunological history, and genetic and environmental background. The argument that a similar lack of RegT activity could underlie the increases in such diverse disorders as allergies, IBD and autoimmunity, is given added weight by the observation that mice or humans who have genetic defects of the transcription factor Foxp3, which is closely linked to some RegT functions, have a syndrome that includes components of all these disease types (Wildin et al., 2002). Evidence for underlying defects in RegT functions has also come from studies of allergy (Akdis et al., 2004), multiple sclerosis (Viglietta et al., 2004), autoimmune polyglandular syndromes (Kriegel et al., 2004), cow's milk intolerance (Karlsson et al., 2004) and IBD (Duchmann et al., 1995; Kraus et al., 2004; Powrie et al., 2003).

A further suggestion has been that the microorganisms relevant to the Hygiene Hypothesis might not only be those that cause overt infections, but also relatively harmless organisms that are part of our evolutionary history. There are several types of organism that have been so much a part of our environment that they, or their components, can be considered as part of our physiology. This is obvious where bowel flora or endotoxin (lipopolysaccharides, LPS) are concerned, but might also apply to organisms that are tolerated by the immune system, such as some helminths that have been co-existing with humans for millennia, or organisms such as the mycobacteria that were until recently ubiquitous in soil and water (Table 1). The Hygiene Hypothesis has therefore broadened to include not only the original concept of diminished exposure to infections, but also the alternative view that exposure to certain relatively harmless environmental organisms or components that the immune system has “learnt” to tolerate, might be necessary for priming the correct levels of RegT in the host. This variant of the Hygiene Hypothesis has been referred to elsewhere as the “Old Friends” hypothesis (Rook et al., 2004).

The mycobacteria are potentially relevant to all variants of the hypothesis because exposure to them is complex. Hence, we will consider not only *Mycobacterium tuberculosis* but also harmless environmental organisms that are now more or less depleted from our environment.

Mycobacteria

The mycobacteria are a genus of more than 80 species, within a complex of related and poorly studied organisms, the *Rhodococcus–Nocardia–Mycobacterium–Gordona–Tsukamurella–Dietzia* cluster (Rainey et al., 1995). Almost all of these are harmless saprophytes, particularly common in soil and water. The majority have not been formally classified and the taxonomy remains incomplete, partly because most cannot be cultured in the laboratory. Large numbers of mycobacteria (or of other organisms from the cluster with similar staining properties) can be observed when applying the Ziehl–Neelsen stain to soil samples, or to filtrates of untreated water, or to scum from *Sphagnum* bogs. It is not unusual for untreated water supplies in developing countries to contain 10^9 or more mycobacteria per litre. Therefore it is inevitable that residents of developing countries are sensitized to a multitude of environmental mycobacterial species (Black et al., 2001a; Floyd et al., 2002; Weir et al., 2003), which can be detected by specific IFN- γ responses in vitro (Black et al., 2001b), or by skin-testing (Stanford et al., 1988). When the same methods are applied to samples from people living in the UK, much lower sensitization rates are found (Dockrell, H.M., Black, G.F., Weir, R.E., personal communication). This is because mycobacteria are relatively depleted from concrete environments and from clean chlorinated water. Therefore in the discussion that follows, we consider separately the possible relevance to the increasing prevalence of allergic disorders of changing patterns of exposure to *M. tuberculosis*, Bacille Calmette Guérin (BCG) vaccination and environmental saprophytes. We also distinguish between mechanisms involving Th1/Th2 balance and those attributable to reduced induction of immunoregulatory pathways.

Tuberculosis and the Hygiene Hypothesis

The prevalence of allergic disorders is inversely related to the incidence of tuberculosis (TB) in the same country (von Mutius et al., 2000). Following exposure to *M. tuberculosis*, a small percentage (~5%) of those infected goes on to develop progressive TB during the subsequent 3–4 years. However, in the majority, the infection persists as latent tuberculosis (LTBI) with no clinical manifestations. The relevance to allergic disorders of these two outcomes needs to be considered separately.

Does active tuberculosis protect from allergies?

On theoretical grounds, it is unlikely that active clinical TB can protect from allergic disorders, for

Table 1. A possible scheme of some of the major changes in microbial exposure, emphasizing those likely to affect the prevalence of allergic disorders

Distant past	Hunter–gatherer	Farming/livestock (~10,000 BC)	The environment in developed countries Comment
Lactobacilli			Reduced ^a
Vegetation, early types of fermentation and food storage			
Saprophytic mycobacteria			Reduced ^a
Enormously abundant in untreated water and mud			
Tuberculosis			Reduced ^a
Mild forms (Canetti strains) co- evolved with man in the “horn” of Africa. At some point, more virulent modern TB strains were imported back into Africa (Gutierrez et al., 2005). Currently 1/3 of the world’s population is latently infected			
Endotoxin			Still present in gut ? ^a Less inhaled LPS
	Anthrax		(Sporadic, fatal)
	Rabies		(Sporadic, fatal)
	Typhus		(Sporadic, fatal)
		Influenza	Not protective
		Rhinovirus	Not protective
		Smallpox	Not protective
		Measles	Not protective
		Hepatitis A	Reduced ^a
		Salmonella	?
		Ascaris	Absent ^a
		Microfilariae	Absent ^a
		Enterobius	Reduced ^a
		Schistosomiasis	Absent ^a

? indicates that the role or change in exposure are uncertain.

^aOrganisms that are depleted from the environment in developed countries, and that are thought to modulate Th1/Th2 balance (tuberculosis, HepA; Umetsu et al., 2005), or more frequently, the balance between effector and regulatory T cells.

reasons reviewed elsewhere (Rook et al., 2005a). Briefly, although the dominant response to *M. tuberculosis* is Th1, there is also a prominent Th2 component, particularly in patients in developing countries close to the equator (Rook et al., 2005b). It is not unusual for such patients to have high circulating IL-4 levels, which are easily assayed by ELISA in serum (Rook et al., 2005b). In developed countries, such high IL-4 levels are very unusual, even in Th2-mediated disorders such as asthma. Similarly, some TB patients have raised levels of IgE (Adams et al., 1999; Ellertsen et al., 2005; Suzuki et al., 2001), which decline following successful treatment (Adams et al., 1999), (Fig. 1). It is therefore not surprising that a significantly higher level of allergen-specific IgE (detected by the Phadiatop kit from Pharmacia) was also found in TB patients compared with controls (Ellertsen et al., 2005). There is strong evidence to suggest that although *M. tuberculosis* is a relatively Th1-biased stimulus, the IL-4 response to the

organisms increases susceptibility to disease (Rook et al., 2005a). Therefore the 5–10% of infected individuals who develop progressive TB might be genetically predisposed to make inappropriate Th2 responses, whether to *M. tuberculosis* or to allergens.

The immunology of latent tuberculosis infection (LTBI) compared to progressive TB

Compared to TB patients, individuals who control the infection and develop LTBI are immunologically very different. Rather than increased levels of IL-4, these individuals tend to express increased levels of an antagonist of IL-4, the splice variant IL-4δ2 (Fig. 1) (Demissie et al., 2004; Fletcher et al., 2004). In order to identify individuals who have LTBI, tests have been developed that rely on the ability of partially activated effector T cells that have recently encountered antigen

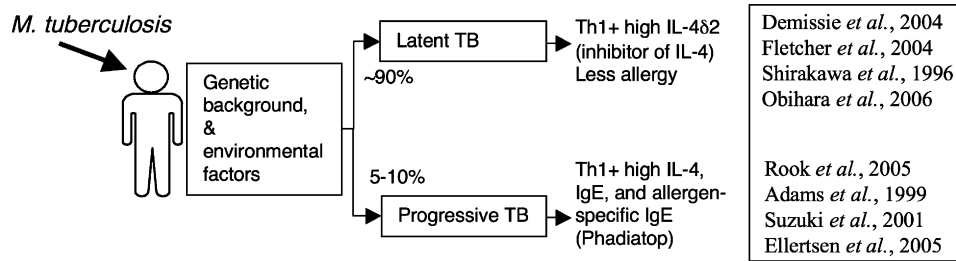


Fig. 1. Tuberculosis and allergic responses. Following exposure to *M. tuberculosis* a few individuals develop progressive disease, but in most the organisms persist in a latent form without causing disease. This outcome is partly determined by the extent to which the patient makes a Th2 response to *M. tuberculosis* in addition to the dominant Th1 response. Patients have increased IL-4, IgE, IgG4, and also increased responses to allergens. Individuals who control the infection so that it stays latent sometimes have raised expression of mRNA encoding IL-4δ2 (an inhibitor of IL-4) and two studies suggest that they might have less susceptibility to allergic disorders. This might mean that latent TB protects from allergic disorders, but more probably, the outcome of infection with *M. tuberculosis* is a “test” that defines individuals with more or less tendency to make inappropriate Th2 responses.

in vivo to respond to specific antigen in vitro by releasing IFN- γ within 24 h. This is detected by ELISA of the supernatant or by ELISPOT in overnight assays (Richeldi et al., 2004). Non-activated memory T cells will release IL-2 or start to proliferate, but will not release IFN- γ in 24 h assays. Unfortunately 24 h ELISPOTs, or other equivalent assays, have never, to our knowledge, been used to distinguish individuals with LTBI from uninfected individuals in any studies of allergic disorder or of atopic sensitization in relation to tuberculin positivity. There are several studies using the tuberculin skin test (TST) to assess mycobacterial exposure, and 12 of these were recently reviewed (Matricardi and Yazdanbakhsh, 2003); interpretation of the data needs to be reconsidered in the light of the different immunological correlates of TST positivity due to different causes (Fig. 1). The TST can be positive because of exposure to *M. tuberculosis* that has been cleared, or because of LTBI, or because of BCG vaccination, or because of exposure to other cross-reactive mycobacteria in the environment. It is therefore not surprising that studies of the relationship between TST responses and atopy or allergy have provided conflicting results. This is discussed in the next section.

Tuberculin skin test (TST) responses due to latent tuberculosis (LTBI) and the Hygiene Hypothesis

There is some reason to suppose that LTBI does correlate with resistance to atopy, though this cannot be stated with certainty until 24 h ELISPOT studies are used to confirm LTBI. In a large study of Japanese schoolchildren, there was an inverse association between TST responses and atopic disorder (Shirakawa et al., 1996). The TST responses were so large in this study that they were probably due to LTBI, even though this was not confirmed by any of the methods described

above. Another study was performed in an area of South Africa where there is a very high incidence of TB, and the median TST response was 18 mm, which strongly suggests that most subjects tested had LTBI. In this community, TST-positive children were significantly less likely to have recent atopic rhinitis than were TST-negative children (Obihara et al., 2005). There was also a significant inverse relationship between the size of the TST response and the frequency of allergic disease symptoms, in particular allergic rhinitis ($P < 0.001$) (Obihara et al., 2006). There is no way of determining at present whether this association is because LTBI protects from allergic disorders, or because people who encounter *M. tuberculosis* and control it without developing progressive TB have an inherently lower tendency to develop inappropriate Th2 responses (Fig. 1). However the latter interpretation would make sense, and is the reverse of the argument we applied above to people who develop progressive TB.

TST responses not due to LTBI, and allergic disorders

In countries where the presence of a positive TST usually does not denote LTBI, the inverse relationship between TST positivity and atopy is not seen. For instance, in The Gambia, about 60% of TST-positive individuals are negative by ELISPOT (Philip Hill, personal communication), and so probably do not have LTBI. In this environment, the TST positivity is not inversely related to atopy or allergic disorders (Ota et al., 2003). Several other studies have been performed in areas where the positive TST is likely to have been due to BCG vaccination or to exposure to environmental mycobacteria rather than to LTBI. Inverse correlations, when present, tend to be weak and require confusing subgroup analyses (Annus et al., 2004;

Matricardi and Yazdanbakhsh, 2003). One study found a significant relationship between a negative TST and the risk of having an allergic history at the age of 2 years in Turkey and Thailand but not in Argentina (Townley et al., 2004). This negative, or inconsistent association between TST positivity and atopy or allergic disorders was also noted when skin-test responsiveness to four other species of mycobacteria (*M. avium*, *M. gordonae*, *M. chelonae* and *M. fortuitum*) was tested (Bibakis et al., 2005).

A further complication that makes it difficult to interpret studies using TST reactivity is the accelerated waning of TST responses after BCG vaccination in tropical areas (Fig. 2). In a large study performed in Malawi, the diameter of TST responses peaked 2–3 months after BCG vaccination, yielding reactions that were similar to those observed after BCG vaccination in areas such as the UK or Denmark (Floyd et al., 2002). However, in Malawians, the sizes of the TST responses waned rapidly and were close to pre-vaccination levels after 2.5 years, whereas most individuals in temperate regions stay TST positive for several years (Black et al.,

2001a; Fine et al., 1994; Floyd et al., 2002). This accelerated waning was also observed in a very large study in South India (Baily, 1980) and is thought to be due to the induction of RegT as a result of the large oral intake of environmental saprophytes (S. Burl, R. Brookes, P. Hill, The VACSIS Study Group, unpublished observation). We will return to RegT in detail later. At this stage, it is sufficient to note that the immunological meaning of TST reactivity, even in individuals not infected with *M. tuberculosis*, is not the same in all individuals or in all countries. The complexities involved are illustrated in Fig. 2.

BCG vaccination and the Hygiene Hypothesis

If the TST response is uninterpretable in the context of atopy and allergic disorders, can we instead seek to determine the effects of BCG vaccination? Here again the obstacles to meaningful epidemiology based on BCG scars or BCG administration records are probably insurmountable, and the complexities shown in Fig. 2

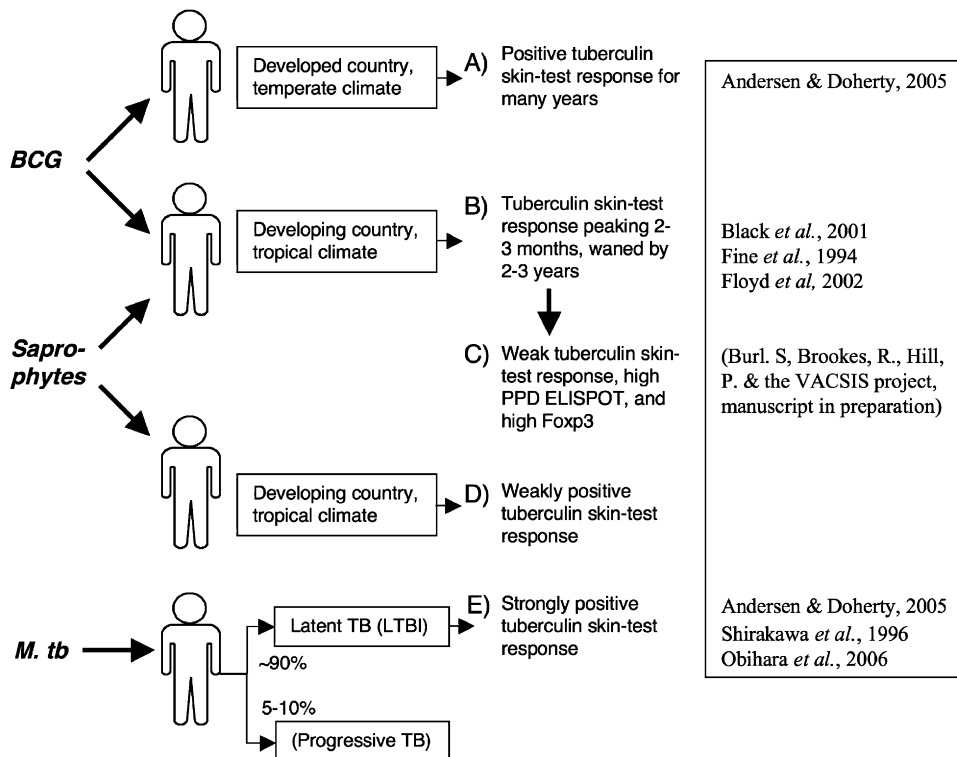


Fig. 2. Tuberculin test responses and the multiple types of exposure to mycobacteria. *M. tuberculosis*, BCG or environmental saprophytes can all induce positive TST. There is also evidence that high exposure to environmental saprophytes can turn off TST reactivity primed by BCG or Mtb, and the TST wanes rapidly in tropical countries. Surveys based on BCG scars do not distinguish between (A), (B) and (C). Surveys based on tuberculin test responses might be meaningful in a temperate climate, but in a tropical context, skin-test size calculations will tend to compare (C) and (D) with individuals harbouring latent TB (E). (Additional scenarios can result if *M. tuberculosis* is superimposed on scenarios (A), (B), (C) or (D). These have been omitted in the interests of clarity.) But as explained in the main text, several of these groups potentially have some mycobacterium-related protection from allergy, but for different reasons that will correlate neither with BCG scars nor with TST size. This might be manifested in the low levels of allergy seen in tropical and TB-endemic countries (von Mutius et al., 2000).

need to be taken into consideration. The immunological effects of BCG vaccination are very different in different populations (Andersen and Doherty, 2005). In some it protects against TB, and in others it does not. More specifically, as pointed out in the previous paragraph, BCG vaccination evokes TST responses that tend to persist in temperate climates and tend to wane in tropical ones. Moreover, it is thought that in tropical countries the response induced by previous contact with cross-reactive environmental mycobacteria can be sufficient to provide “immunity” to the BCG so that the organism is rapidly eliminated before it has replicated or exerted its immunological effects (Andersen and Doherty, 2005).

Clearly therefore studies investigating the effect of BCG vaccination on the development of allergic disorders are most likely to be meaningful in countries where the incidence of TB is very low, and exposure to environmental mycobacteria is limited. Under these conditions, weak protective effects have been recorded. A very large study of 38,808 children in Germany suggested that BCG vaccination afforded weak protection against the development of asthma (Gruber et al., 2002). By contrast, two smaller Scandinavian studies have failed to confirm these findings (Alm et al., 1997; Strannegard et al., 1998).

Interestingly, there are two studies that investigated the range of allergic symptoms in inherently allergic individuals and concluded that asthma was a less common manifestation if they had received BCG vaccination. In a study conducted in an area with a very low incidence of TB, BCG vaccination appeared to have no influence on allergic sensitization but among the subgroup of subjects with a family history of rhinitis or eczema, vaccination was associated with a lower prevalence of current asthma (Marks et al., 2003). Similarly, in a study in Brazil, neonatal BCG vaccination was not associated with reduction in the overall prevalence of reported wheezing or asthma. However, in the subgroup reporting current allergy and sneezing, neonatal BCG was associated with a 37% reduction in the lifetime prevalence of asthma (da Cunha et al., 2004).

In conclusion, it is conceivable that a small reduction in asthma is associated with BCG vaccination, particularly in allergic individuals. This might be revealed by very large studies (Gruber et al., 2002) or by analysis of the symptoms in the allergic subgroup (da Cunha et al., 2004; Marks et al., 2003). It is however apparent from Fig. 2 that analysis of TST responses in relation to allergic symptoms or to atopic sensitization is unlikely to be fruitful for two reasons. First, the skin-test response cannot distinguish between immunologically distinct subgroups such as LTBI, BCG vaccination or exposure to environmental mycobacteria. Secondly, individuals with the strongest TST (LTBI, discussed earlier) or the weakest TST (dominant RegT responses,

discussed later) are both potentially protected, though for different reasons.

Animal models: mycobacteria and regulation of allergic responses

As early as 1983 Ishizaka and colleagues were aware of the ability of mycobacterium-primed T cells to suppress IgE levels (Suemura et al., 1983). This early observation was probably due to IFN- γ production, and to the mutual antagonism between Th1 and Th2 responses that is seen in in vitro systems.

BCG in mouse models

In mouse models, a number of workers have shown that BCG infection prior to allergen sensitization (Herz et al., 1998), but also in many cases, given to previously sensitized animals, can reduce the allergic response to a subsequent allergen challenge (Choi et al., 2005; Erb et al., 1998; Hopfenspirger and Agrawal, 2002). Several authors reported a switch towards a Th1 pattern of response (Choi et al., 2005; Herz et al., 1998; Hopfenspirger and Agrawal, 2002), with little evidence of increased regulatory cell activity, since production of the regulatory cytokine IL-10 as well as production of IL-4 was suppressed (Choi et al., 2005; Herz et al., 1998).

It is possible that this is indeed how BCG infection works in mouse models: the induction of a strong Th1 response might inhibit the allergen-specific Th2 response. However, the Th1 response cannot merely be superimposed upon the existing Th2 response because polarized Th1 effector cells do not switch off the function of Th2 effector cells in lungs when they are transferred into allergic recipients but rather synergize with Th2 cells to produce severe immunopathology (Hansen et al., 1999).

M. vaccae, saprophytic environmental mycobacteria and the “Old friends” hypothesis

An alternative mode of action to explain a protective effect of treatment with mycobacteria in animal models of allergy is the induction of RegT, rather than of Th1 cells. This appears to be how heat-killed *Mycobacterium vaccae* works in these models. *M. vaccae* is an environmental saprophyte that originally came to the notice of a worker in the field of mycobacterial taxonomy because it was remarkably difficult to induce a good polyclonal antibody to it for use in immunodiffusion studies (John L. Stanford, personal communication). This might have been an early indication of its immunoregulatory properties. Therapeutic effects of autoclaved *M. vaccae* in a mouse model of allergy were first reported in 1998 by

Wang and colleagues (Wang and Rook, 1998). This was confirmed in other laboratories where it was shown to be active, whether given at birth (Ozdemir et al., 2003), or to adult animals before or after sensitization with ovalbumin (Hopfenspirger and Agrawal, 2002; Smit et al., 2003; Zuany-Amorim et al., 2002a,b). From a clinical perspective, it is interesting to note that it could also be given after ovalbumin-sensitized mice had received an ovalbumin challenge into the airways. In this model, which may mimic an already established asthmatic phenotype, treatment with *M. vaccae* still diminished the inflammatory and airway responses to a subsequent challenge (Hopfenspirger and Agrawal, 2002). Similarly in a guinea pig model of asthma, pre-treatment with *M. vaccae* was found to improve lung function, inhibit airway inflammation and airway hyper-responsiveness. Interestingly, both early and late asthma reactions were affected (Zhao et al., 2003).

Mode of action of *M. vaccae*

In the BALB/c mouse model of pulmonary allergic inflammation, it emerged that *M. vaccae* does not cause a switch to a Th1-dominated response (Adams et al., 2004; Zuany-Amorim et al., 2002a,b). Rather, it acts by inducing a population of allergen-specific RegT (Zuany-Amorim et al., 2002a,b). The regulatory activity was found within the CD4+CD45RB^{Lo} population. These cells, when isolated from the spleens of *M. vaccae*-treated allergic animals, and transferred into further allergic recipients prior to allergen airway challenge, blocked the response of the recipients to allergen challenge, which could be prevented by co-administration of neutralizing antibodies to IL-10 and TGF- β (Zuany-Amorim et al., 2002a). In contrast, antibody to IFN- γ had no effect. Importantly, these RegT were allergen specific (Zuany-Amorim et al., 2002b) since spleen cells from ovalbumin-immunized, *M. vaccae*-treated donors failed to have a protective effect in Cockroach Extract Antigen (CEA)-allergic recipients. This proved that the RegT have specificity, since identical cell suspensions were active in ovalbumin-allergic recipients. Finally, the experiment was repeated, but this time the CEA-allergic recipients were challenged with a mixture of ovalbumin and CEA. Under these conditions, ovalbumin-specific RegT were active in the CEA-sensitized recipients. This shows that if the allergen for which RegT are specific is present, their regulatory properties are triggered, and they will exert bystander inhibition on responses to other allergens.

The precise type of RegT induced by *M. vaccae* is not yet clear, but *M. tuberculosis* can evoke several types of RegT, and this might provide some clues. T cells from some patients with weak Th1 responses and severe progressive TB secrete IL-10 (Boussiotis et al., 2000;

Delgado et al., 2002; Guerkov et al., 2003). These IL-10-secreting cells could be TR1 regulatory cells. TB is also accompanied by increased expression of Foxp3, characteristic of the “constitutive” CD25+ Foxp3+T_{reg} (Guyot-Revol et al., 2006) (note that it has become usual to use the term T_{reg} for this type of RegT, but we use “RegT” to refer collectively to all types of regulatory T cell). Moreover levels of TGF- β are raised in serum of TB patients and TGF- β is released by patients’ cells in vitro suggesting the possibility that there might also be Th3 RegT (Fiorenza et al., 2005; Toossi and Ellner, 1998). A study using heat-killed *Listeria monocytogenes* as an immunomodulator in a mouse allergy model identified Th1-like RegT expressing TBet (Stock et al., 2004). These Th1/T_{reg} cells suppress Th2 responses, suggesting that they might be of particular interest in the context of allergic diseases (Stock et al., 2004). It is not yet known whether mycobacteria can also induce this type of RegT.

How does *M. vaccae* induce RegT? Further studies revealed the presence in the lungs of *M. vaccae*-treated allergic mice of a CD11c+ cell population that expressed high levels of IFN- α , IL-10 and TGF- β (Adams et al., 2004). These cells are likely to be plasmacytoid DC (pDC), a cell type shown recently to be crucially involved in driving regulatory responses in mouse lungs (De Heer et al., 2004). Thus it may be that *M. vaccae* activates pDC, but this needs to be confirmed and further work to address this issue is in progress.

M. vaccae by the oral route

In developing countries, exposure to high levels of these harmless saprophytes occurs mostly via the oral route. Hence, the effects of oral administration of heat-killed *M. vaccae* were investigated in BALB/c mice that were pre-immunized twice with allergen before receiving a single dose of *M. vaccae* by gavage. Following two further immunizations with allergen, mice were challenged by the intratracheal route 40 days after the intragastric dose of *M. vaccae*. Interestingly, *M. vaccae* was as effective by this route as it was by the subcutaneous route, and at precisely the same doses (Hunt et al., 2005). Since much of an oral dose must be lost, this implies that the oral route is particularly efficient, in agreement with the observations that mycobacteria are easily picked up by M cells on Peyer’s patches (Fujimura, 1986) and that the gut may be a major site of induction of RegT (Powrie et al., 2003).

Mycobacterial components, innate immunity and induction of RegT

A number of mycobacterial components have been shown to modulate immune responses, and some have

been tested in models of allergy. First, like all bacteria, the mycobacteria contain ligands for many Toll-like receptors (TLR). However when whole live organisms are added to TLR-expressing cells, the dominant signals from mycobacteria trigger via TLR2 (Heldwein and Fenton, 2002). Mycobacterial components that do this include the cell wall skeleton of BCG (Seya et al., 2002), lipoarabinomannan (LAM; from those species, mostly harmless saprophytes, that have LAM capped with arabinose rather than mannose), phosphatidyl inositol mannosides (PIM) and the triacylated mycobacterial 19 kDa lipoprotein (Heldwein and Fenton, 2002). Interestingly, the triacylated mycobacterial lipopeptide is recognized by a dimer of TLR2 and TLR1, whereas similar diacylated compounds are recognized by dimers of TLR2 and TLR6 (Morr et al., 2002; Takeuchi et al., 2002).

Signalling via TLR2 is relevant to pathogenesis. A mutation in TLR2 is associated with susceptibility to the lepromatous form of leprosy caused by *M. leprae*, and results in a failure of signalling for IL-12 production (Kang et al., 2002), and activation of NF-kappaB (Bochud et al., 2003). In a mouse model of TB, absence of a functional TLR2 gene had no effect following infection with 100 *M. tuberculosis*, but high-dose aerosol challenge with 2000 organisms revealed that TLR2-, but not TLR4-defective mice are more susceptible than control mice (Abel et al., 2002; Reiling et al., 2002). There is also some evidence that killing of *M. tuberculosis* can be triggered via TLR2 (Stenger and Modlin, 2002).

These TLR2-mediated functions are also relevant to the immunomodulatory effects of mycobacteria. TLR2 is expressed on constitutive CD25^{high} T_{reg} (Caramalho et al., 2003), and Suttmüller et al. have revealed that TLR2 agonists from the pathogen, together with APC and antigens, drive expansion of TLR2⁺ T_{reg}, while simultaneously preventing their function so that they do not inhibit the effector mechanisms that are required to combat the infection. When the pathogens have been cleared so no more TLR2 agonist is released, the blocking stimulus is lifted and the expanded population of T_{reg} terminates the inflammatory response and limits tissue damage (Suttmüller et al., 2006). The mycobacterial cell wall lipoglycans, LAM and PIM, are TLR2 agonists that have been identified as components of mycobacteria capable of suppressing airway disease in the mouse model. The mechanism involved production of IL-10 rather than a switch to Th1 (Sayers et al., 2004). Indeed there is evidence that these compounds tend to suppress Th1, because their interactions with TLR2 and DC-SIGN lead to reduced release of IL-12 from APC, with increased release of IL-10 (reviewed in Briken et al., 2000).

Bacterial chaperonins (Cpn) and heat shock proteins have often been found to have immunoregulatory

properties. Two bacterial Cpn (Cpn60.1 and Cpn10) have been shown to suppress eosinophil recruitment and bronchial hyper-responsiveness in a murine model of allergic inflammation, when administered before sensitization (Riffo-Vasquez et al., 2004).

A single treatment with a CpG-containing immunostimulatory DNA sequence given before allergen challenge can inhibit Th2-mediated airway responses in animal models of allergic asthma (Hessel et al., 2005). It is probable that the DNA of mycobacteria is released and contains appropriate CpG sequences, but we are not aware that this point has been investigated.

Finally, a lipid fraction was isolated from *M. vaccae* that was as effective as the whole organism using the BALB/c model of allergic pulmonary inflammation. This fraction contained an unusual fatty acid with the double bond in position C10-11. A single dose of 2–5 µg of a synthetic version of this compound, given as a triacylglycerol to mice presensitized with ovalbumin, is able to inhibit the allergic response to an intratracheal challenge given 40 days later (L. Rosa Brunet, R. Martinelli, J. Hunt, G. Rook, manuscript in preparation). Spleen cells from treated animals, but not from untreated allergic controls, release IL-10 when cultured with allergen in vitro, suggesting the hypothesis that the mode of action is the generation of allergen-specific RegT, as is the case for the organism in which the compound was first observed (Zuany-Amorim et al., 2002b). This material is currently under investigation as a potential anti-allergic agent.

Clinical trials

BCG

There has been a study of repeated intradermal injections of heat-inactivated BCG in adults suffering from asthma. TST-negative adults with stable moderately severe asthma who were skin prick test positive to house dust mite were randomized to receive one injection of heat-inactivated BCG every week for 4 weeks. Markers of asthma severity, reliever drug use and blood eosinophil and IgE levels were monitored for 3 months. The killed BCG had no effect and recruitment was halted early due to excessive local reactions to BCG administration (Shirtcliffe et al., 2004).

An interesting possibility is the use of living BCG as an adjuvant for sublingual immunotherapy (SLIT). BCG was given intradermally to some patients before they underwent a course of SLIT. The PBMC from the patients who received both SLIT and BCG released more IL-12 in response to house dust mite allergen in vitro, but the patients did not show any improvement in clinical outcome compared to that seen in patients receiving SLIT alone (Arikan et al., 2004). To our

knowledge, sublingual co-administration of mycobacteria mixed with desensitizing allergen has not yet been tested but may theoretically have improved immunomodulatory effects compared to the two-compartment strategy.

A double-blind study of the effects of a single large intradermal dose (58.2×10^7 CFU) of BCG on the first day of a 12-week treatment period revealed significantly increased pulmonary function tests at early time points. Asthma symptom scores were not significantly changed, but the weekly medication usage was significantly decreased, and there was evidence for a suppressed IL-4 response (Choi and Koh, 2002). To investigate whether revaccination would further improve lung function, BCG vaccine was given again 1 year later. Current lung function tended to improve in the group receiving a second dose of BCG (Choi and Koh, 2003).

The inconsistent effects observed with BCG administration suggest that the factors that affect the ability of BCG to protect from TB (protection varies from 80% to 0%) might also affect its relevance to allergic responses.

***M. vaccae* and asthma**

Several small clinical studies suggested a beneficial effect of treatment with *M. vaccae* in allergic diseases (Arkwright and David, 2001; Camporota et al., 2003; Hopkin, 2001). These observations motivated a multi-centre phase II, randomized, placebo-controlled study of the safety and efficacy of heat-killed *M. vaccae* in the treatment of adults with moderate persistent asthma. In all, 178 patients were recruited, albeit with milder symptoms than planned. Treatment with heat-killed *M. vaccae* failed to significantly improve mean weekly asthma symptoms as recorded in the patients' diary cards (primary efficacy analysis), though there was a trend towards improvement. However, further statistical analysis revealed a significant effect of intradermal heat-killed *M. vaccae*. When the data were adjusted for variations between treatment groups at baseline (a correction that had inadvertently been omitted from the statistical analysis plan), patients receiving two high doses of heat-killed *M. vaccae* showed a significant reduction in asthma symptoms score ($P = 0.0436$). In addition, compared to placebo, heat-killed *M. vaccae* significantly decreased asthma exacerbations ($P = 0.0251$) (S.R. Pharma; unpublished data from a phase II study).

As discussed earlier, there is evidence that the active components of mycobacteria are lipids and glycolipids. A study using a preparation of *M. vaccae* from which lipids had been removed showed no effect of treatment. Thirty-seven adults with stable moderately severe asthma who were skin prick test positive to house

dust mite were randomized to receive two doses 2 weeks apart of delipidated acid-treated *M. vaccae* (first dose 0.4 mg and second dose 0.8 mg) or phosphate-buffered saline, given as drops intranasally. There were no statistically significant differences between the treatment group and placebo for any of the outcome variables (Shirtcliffe et al., 2003).

Intradermal *M. vaccae* and eczema

A pilot study of intradermal administration of *M. vaccae* in atopic dermatitis gave encouraging results (Arkwright and David, 2001) and led to a study of its effect on cytokine responses in these children. A 5–10-fold increase in Th1-type activity was found 1 month after *M. vaccae* administration ($P < 0.05$), but it had returned to baseline by 3 months. The results did not support the hypothesis that *M. vaccae* reduces Th2-type or increases TGF- β activity of PBMC isolated from children with atopic dermatitis (Hadley et al., 2005).

The pilot studies in moderate/severe atopic dermatitis (Arkwright and David, 2001, 2003) also motivated a phase II study in 166 children. The primary endpoint was an improvement in the six-area six-sign atopic dermatitis (SASSAD) scores 12 weeks after the intradermal injection. All patients, regardless of treatment, showed more than 30% improvement in these scores, and against this background no effects of *M. vaccae* could be detected (Berth-Jones et al., in preparation).

In conclusion, the therapeutic value of intradermal administration of heat-killed *M. vaccae* remains unproven, but appears more promising in asthma than in eczema. Future studies will concentrate on the oral route, which is more acceptable to patients, allows multiple doses, and is effective in animal models. The oral route also makes more sense from an evolutionary perspective, because the oral route was the major route of exposure throughout mammalian evolution. Further, no clinical data are available for treatment of high-risk children early in life with *M. vaccae* in order to prevent the development of allergic sensitization and/or disease prior to first manifestation.

Conclusions

The mycobacteria, together with the helminths, remain strong candidates as mediators of the Hygiene Hypothesis. However, definitive proof is still lacking for several reasons. First, simultaneous changes in the load of helminths in rich societies make it difficult to isolate effects attributable to mycobacteria. Secondly, the multiple types of mycobacterial exposure (TB leading to latent infection, BCG vaccination and contact with a variety of environmental saprophytes, mostly via the

oral route) are difficult to distinguish by routine methods such as TST positivity, and are likely to have different effects on the regulation of the immune response (Figs. 1 and 2).

The outcome of exposure to *M. tuberculosis* (progression versus latency) might act as an indicator of individuals with a greater or lesser tendency to make inappropriate Th2 responses. Thus TB patients have increased correlates of allergy (Adams et al., 1999; Ellertsen et al., 2005; Suzuki et al., 2001), but in individuals with non-progressing latent TB (LTBI) the reverse might be true. In populations where most TST positivity is due to LTBI, there is an inverse correlation between allergic manifestations and TST reactions (Obihara et al., 2005, 2006; Shirakawa et al., 1996).

The effects of BCG vaccination are contradictory, at least partly because the immunological consequences of BCG vaccination differ markedly in different environments (Andersen and Doherty, 2005). The data are mostly uninterpretable, but a very large retrospective study did indicate a weak beneficial effect in Europe, where interference by other mycobacteria might be minimal (Gruber et al., 2002). More studies are in progress.

Environmental saprophytes such as *M. vaccae* might help to prime regulatory responses. This is clearly the case in the mouse model (Zuany-Amorim et al., 2002a), though clinical trials have so far been inconsistent. More studies are needed, preferably using the oral route, since this was the mode of exposure throughout human evolutionary history, and has proved particularly effective in the mouse model (Hunt et al., 2005).

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