TB does not respect artificial barriers, whether they are prisons, walls, or international frontiers

Navigator – Stephen Williams
Installation
Plastic containers, paper
(Variable dimensions)

'I don't want anything for me.
I only wish for the *possible impossible*:
A world without victims.'

The End of the Century **(Poem) – Jose Emilio Pacheco**

Stephen Williams

Stephen Williams has participated in solo and group exhibitions in Belgium, England, Japan, Poland, Spain, the United States, and Wales.

He has been curator and coordinator of numerous exhibitions. His works can be found in the Jac ft Del Blacker Collection (London); Zoe and Nabil Debs Collection (London); D K Edwards Collection (New York); J and M L Flavin (Cornwall); Penine Hart Collection (New York); The Miles Collection (Richmond); Museum of Modern Art (Lodz); Museum Narodowe (Wroclaw); Paul Smith Collection (London); Power Collection, Sydney Museum (Sydney); The Reston Collection (Cheshire); Sztuki Museum (Lublin); the Van den Boogaard Collection (Bordeaux); Toni and Michel Vert (Perpignan); Madeleine Williams (London), among others.

CHAPTER 5

T Cell Protective Immune Responses against Tuberculosis

Hazel M Dockrell

Introduction

T cell immunity to MTB is both straightforward and incredibly complex. This chapter will focus on results obtained from studies of human T cells, but draw on studies in murine models where appropriate. It will provide a brief overview of the main roles played by T cells in immunity to MTB, highlight some areas of recent research, discuss some of the ways in which T cell immune responses can be measured in vaccine trials, and identify areas where further research is needed.

T Cell Immunity to MTB

There is no question that T cells play a central and essential role in protection against progressive infection with MTB. On a simplistic level, activation of CD4+ T cells leads to secretion of cytokines such as IFN-γ and TNF-α that induce macrophage activation, with induction of bactericidal products such as nitric oxide and reactive oxygen intermediates (1, 2). The central role of CD4+ T cells in protection against MTB infection is also illustrated by the marked susceptibility to TB in individuals whose CD4+ T cell function and CD4+ T cell numbers are reduced due to HIV infection (3), and depletion of mycobacteria-specific CD4+ T cells is detectable shortly after HIV infection

(4). However, CD4+ T cells may also orchestrate responses within other T cell subsets, as discussed below.

Secretion of chemokines such as CCL2, CCL3, CCL5, and TNF-α by infected macrophages attracts T cells to the site of infection (5). Perturbations of the balance between cytokine and chemokine production may occur in different forms of TB (6). In murine models, both CD4+ and CD8+ T cells are present within the granulomas that result, together with the classic hallmark cells of such granulomas, macrophages, and giant cells (7, 8). New technologies allow the imaging of granulomas within both animal and human lungs and in zebrafish (9, 10), and this is an active area of research.

The Tuberculin Skin Test

The most widely used test of T cell immunity to MTB is the Tuberculin Skin Test (TST), which measures induration two to three days after intradermal injection of purified protein derivative (PPD). This response involves monocytes and previously primed memory T cells, which are attracted to the skin where they proliferate and secrete cytokines (11). The resulting induration provides a read-out of immunity that is, however, not MTB specific, as indurations of <10mm may be caused by prior BCG vaccination. Although large indurations of >15mm are associated with a greater risk of developing clinical TB, smaller indurations may be associated with protection from the disease (12)—one example of how T cell activation in the 'right' amounts can be protective but excessive responses may be associated with pathology. A lower cut-off to identify 'positive' responses, of ≥5mm rather than ≥10mm, is usually adopted in HIV+ subjects. Mantoux skin test indurations can be associated with T cell immunity measured in vitro, for example, using secretion of IFN-γ following stimulation of diluted whole blood cultures with PPD for six to seven days, but this association is not perfect, with some individuals who show production of IFN-γ yet no detectable skin test induration, or large skin test indurations without detectable IFN-γ secretion; the association between the two tests may be weakened rather than strengthened by BCG vaccination (13, 14) and is less strong in infants (15). Other types of skin test can be performed using PPD, including the multipuncture Heaf test. It is worth noting that skin testing may boost an individual's in vitro T cell response to mycobacterial antigens (16), and a blood draw for in vitro T cell functional assays should be performed at the time of administration of the skin test, rather than when it is read.

Antigen Recognition by Mycobacteria-Specific T Cells

The majority of studies investigating T cell immunity to mycobacteria have used PPD, produced from culture filtrates of MTB. PPD is used in the TST, in a preparation containing preservative, but preparations lacking preservative can be obtained for in vitro use. Equivalent PPDs can be produced from other mycobacterial species. As the constituent proteins in PPD are extensively degraded during its preparation, PPD does not require much antigen processing and activates predominantly CD4+ T cells.

Whole live mycobacteria can also be used to probe the T cell response. Live MTB will most closely mimic the situation occurring in vivo, and induces stronger activation of the CD8+, γδ+, and NKT subsets than soluble antigens (17). Live and killed mycobacteria may induce quantitatively and qualitatively different responses, although suspensions of live mycobacteria will also contain many dead bacteria. Live mycobacteria may also induce different immune responses in peripheral blood mononuclear cells and whole blood cultures, as in whole blood cultures many bacteria will be phagocytosed by neutrophils, which then leads to apoptosis and cross-priming via macrophages.

MTB has a genome of 4.4 Mb which contains over 4,000 genes (18), providing a wealth of potential antigenic targets to be recognized by T cells. An earlier focus on heat shock proteins has now shifted to interest in proteins expressed during latency, including those induced as part of the dosR regulon (19–21).

There is continued interest in proteins secreted by MTB. The proteins that are encoded within the RD1 region in the MTB genome have been used in many recent studies of T cell function, particularly as the loss of the RD1 region during the attenuation of *M. bovis* to form *M. bovis* BCG has resulted in ESAT-6 not being expressed by BCG (22), and thus T cell responses induced by BCG vaccination and MTB infection can be distinguished. ESAT-6 genes are found in *M. marinum*, *M. kansasii, M. flavescens*, and *M. sulzgai* in addition to MTB; *M. bovis* and *M. leprae* also contain an ESAT-6 homologue (23). Patients infected with *M. kansasii* or *M. marinum* can respond to ESAT-6 from MTB (24), but it is not known to what extent environmental exposure to these other mycobacterial species can interfere with the specificity of the two currently available commercial assays for TB, QuantiFERON-TB and T-SPOT-TB, which utilize peptides from ESAT-6 and CFP-10 (25). ESAT-6 can also have additional effects on T cell immunity, including inhibition of IFN-γ production by MTB-specific human T cells (26).

Activated CD8+ T cells recognizing shorter peptides of approximately 9 amino acids are also induced by infection with MTB (27–29). Debate continues about the mechanism through which mycobacterial antigens access the MHC

Class I pathway. This may be a result of the exocytosis of phagosomes (30), the uptake of apoptotic blebs (31, 32), or the recycling of major histocompatibility complex (MHC) molecules with peptide replacement. Whether MTB escapes into the cytoplasm is still controversial (33) but MTB does express a haemolysin (34) and other ESR-1 proteins such as ESAT-6 induce pore formation within the membrane of the phagocytic vacuole in *M. marinum* (35); thus antigens could also egress into the cytoplasm of the antigen presenting cell (APC) through pores in the phagosome membrane and in this way access the MHC Class I pathway.

It is not just protein antigens or their constituent peptides that are recognized by T cells; there has been increased interest in the recognition of lipid antigens by CD4+ T cells (36) and 'unconventional' CD1-restricted T cells (37). Mycobacterial lipopeptides (36) can also be recognized by antigen-specific CD4+ T cells, while the γδ T cell subset can recognize phosphoantigens, possibly presented by novel antigen-presenting molecules (38). The lack of classical MHC restriction in the recognition of these non-protein antigens may give them added value as vaccine candidates.

Cytokine Secretion by T Cells: How Good is IFN-γ as a Correlate of Protection?

In early studies of human T cell responses to mycobacteria, the lymphocyte transformation test was used, assessing the incorporation of tritiated thymidine into proliferating cells. However more recently, measurement of cytokines has become the norm, and among the many products made and secreted by T cells, IFN-γ has received the most attention.

There are good reasons why so many studies have measured the production of IFN-γ. It is the signature cytokine of the Th1 or Type 1 T cell, and plays a key role in macrophage activation. Mice, in which the gene for IFN-γ has been knocked out, become very susceptible to infection with MTB (39–41). IFN-γ can activate macrophages to become more bactericidal, producing greater quantities of ROI and RNI (2, 42, 43), although there have also been reports that IFN-γ-activated human macrophages do not become more mycobactericidal (44).

In humans, there is evidence that individuals with mutations in their IFN-γR genes become susceptible to infections with mycobacteria (45–47). Mutations in other genes on the pathways that would lead to or enhance IFN-γ secretion such as IL-12Rβ1 or IL-12p40 (46), or in the signalling pathways downstream of IFN-γ also induce similar susceptibility (47).

IFN-γ can be measured easily in immunological assays, being secreted in large quantities by T cells. If peripheral blood mononuclear cells are stimulated

with mycobacterial antigens, IFN-γ is produced and accumulates in culture supernatants over the first seven days of culture, and its stability in culture supernatants has made it an easy cytokine to detect. IFN-γ released into culture supernatants can be measured by enzyme-linked immunosorbent assay (ELISA), or in bead array assays using flow cytometry; individual T cells secreting IFN-γ can be counted by enzyme-linked immunosorbent spot (ELISPOT) assay or by using intracellular flow cytometry (48).

In vitro, shorter incubations of cultured cells for 6–24 hours are increasingly used, on the basis that to detect T cell activation or cytokine secretion over such a short time period requires the presence of a previously activated effector T cell. Longer incubation periods may provide a more sensitive read-out of memory responses, including those induced by past latent infection (49, 50), as the longer period of antigenic stimulation allows the activation and expansion of resting central memory T cells into effector T cells (51). To detect IFN-γ production following a short period of antigenic stimulation requires a sensitive detection assay, such as ELISPOT or intracellular cytokine staining.

IFN-γ is currently the main read-out of immunogenicity in TB vaccine trials (52, 53); but although IFN-γ is a component of protective immunity, there is an increasing body of evidence that it does not provide a read-out of protective immunity. This evidence includes a number of animal studies in mice, guinea pigs, and cattle which show that although IFN-γ may be required for protective immunity, the quantities of IFN-γ produced do not parallel control of bacterial growth (54–57). Not only does more IFN-γ production not mean more immunity, in some animal models greater IFN-γ production is associated with more pathology (58). These observations, coupled with the availability of techniques that allow the simultaneous assessment of larger numbers of immune parameters, have led to intense interest in the measurement of immune signatures or profiles that might indicate protective immunity (59–61).

The use of multiparameter flow cytometry is one such approach. The production of a number of cytokines or chemokines within a single cell can be combined with the assessment of surface phenotypic markers (62, 63). Such an approach can confirm, for example, the relative contribution of CD4+ and CD8+ subsets to the production of key cytokines as well as the activation of other T cell subsets such as γδ, CD1 restricted, and NKT cells. The main cytokines usually assessed in addition to IFN-γ are TNF-α and IL-2. The current view is that cells capable of making IFN-γ, TNF-α, and IL-2 (termed multifunctional or polyfunctional) are a better indicator of protective immunity than the measurement of IFN-γ alone (64); some studies have shown that such polyfunctional T cells produce greater quantities of IFN-γ (as assessed by mean fluorescence intensity) than T cells making IFN-γ alone (65, 66). One theory of memory T cell maturation proposes that cells secreting IFN-γ alone may

represent terminally differentiated cells that have lost the ability to proliferate (64). Other studies have shown production of GM-CSF by memory T cells that also produce IFN-γ and TNF (67).

Multiparameter flow cytometry can be taken one stage further by using MHC-peptide tetramers or pentamers. This allows the frequency of T cells recognizing individual peptide epitopes to be assessed (68) and such staining can be combined with both surface phenotyping and intracellular cytokine staining. Proliferation of tetramer-positive T cells can also be assessed using incorporation of the dye CFSE (69).

These multiparameter flow cytometry techniques allow both the quantitation and phenotyping of antigen-specific T cells. However, they are likely to be exploratory research tools, or assays that could be performed on selected subjects for whom frozen peripheral blood mononuclear cells have been banked during a vaccine trial, for example, once these subjects have developed TB or have remained healthy (and thus are presumed to be protected). The cost and complexity of these assays make it unlikely that they could be performed on very large numbers of subjects during vaccine trials.

Additional Cytokines of Interest

Although IFN-γ, TNF-α, and IL-2 have been the cytokines most studied to date, other cytokines and T cell subsets have generated recent interest. These include IL-17, produced by the Th-17 T cell subset (70). In one human study, the mycobacteria-specific T cells making IL-17 also made IFN-γ and had the phenotype of Th1 central memory or effector memory T cells (71), whereas in another study IL-17 producing T cells were distinct from Th1 T cells (72). In MTB-infected mice, it is γδ+ T cells rather than CD4+ T cells that are the major IL-17 producers (73).

It may also be useful to assess the balance between pro-inflammatory and anti-inflammatory cytokines. IL-4 production is induced during clinical TB (74, 75) and may also indicate developing infection (76). Reductions in IL-4 production can be detected shortly after the initiation of TB treatment (77, 78). Reduced IFN-γ production is also associated with the increased production of IL-10 which may be monocyte-derived (79). TGF-β production by regulatory T cells is also detected in TB infection (80, 81). Thus, the ratios between IFN-γ and other anti-inflammatory cytokines may be more informative than the measurement of a single cytokine in isolation.

Another way in which immune profiles can be assessed is using multiplex bead arrays. In these techniques a small volume of supernatant from antigen-stimulated cultures is assayed for a number of cytokines and chemokines

simultaneously. Kits allowing the measurement of up to 42 analytes are currently available. Although the cellular origin of the analytes cannot be identified (and such commercial kits are expensive which would again restrict their use to selected subjects within a vaccine trial) the ability to measure Th1 and Th2 associated cytokines, pro-inflammatory and anti-inflammatory cytokines, chemokines, and other products may allow the components of a protective biosignature to be identified. These assays are also very sensitive, allowing detection of cytokines such as IL-2 or IL-4 whose detection by ELISA may be problematic due to their rapid binding to their receptors and thus removal from cell cultures. A recent study from The Gambia using a 7-plex bead array showed that PPD-stimulated cultures from TB cases contained less IL-10, IL-13, and IL-17 than TB contacts (82) while a study from South Africa using a 29-plex kit showed marked changes in cytokine and chemokine profiles in TB patients with treatment (78); results from further studies using such bead arrays can be expected.

Cytotoxic Effector Function

Although CD4+ T cells usually play a central role in immunity to intracellular pathogens, CD8+ T cells are also important in immunity to MTB (27, 29, 83, 84). Murine studies have suggested that CD8+ T cells may be more important at later latent rather than early acute stages of MTB infection (85) but a recent study in non-human primates has demonstrated that removal of CD8+ T cells reduced BCG-induced immunity to MTB 42 days post-infection (86). MTB- specific CD8+ T cells can secrete cytokines such as IFN-γ and TNF-α, similar to CD4+ T cells (75). They can also act as cytotoxic effector cells, secreting cytolytic granules into the contact zone between the CD8+ T cell and the infected macrophage, thus causing cell death. The action of CD8+ cytotoxic T cells on infected macrophages can lead to the killing of intracellular bacteria and a reduction in CFU (87). The mediators produced by such CD8+ T cells include perforin, granzymes, hydrolases, granulysin, and extracellular ATP. Granulysin has a direct cytotoxic effect on mycobacteria as well as on other bacteria, parasites, and fungi (88), and is also expressed by human NKT cells that show antimycobacterial activity (89). The effects of granulysin cannot be studied in mice as they do not express this gene. Perforin does not act directly on the mycobacteria, but the pores it induces are required for mycobacterial killing by both CD8+ and γδ+ T cells (90, 91). Another effect of cytotoxic T cells on infected macrophages may be to release the viable intracellular mycobacteria (92); this may lead to their uptake by more effective microbicidal macrophages capable of killing the mycobacteria using nitric oxide and other mediators.

To measure the change in CFU following the incubation of infected monocytes/macrophages with appropriately activated T cells may therefore provide a highly relevant read-out of protective immunity. A number of assays have been described, in which stimulated T cells are added to mycobacteria-infected monocyte-derived macrophages, and the CFU measured (93, 94). These assays would, however, require simplification for use on a large scale. As with some of the other techniques discussed in this chapter, one way forward is to freeze peripheral blood mononuclear cells from all vaccinees within a trial and then test cells from selected subjects who subsequently do or do not develop disease. A simpler assay uses BCG or MTB expressing the *lux* gene, which is added to diluted whole blood cultures, and bacterial growth inhibition tested using a luminometer, as live and metabolizing BCG-*lux* bacteria will produce ATP enabling light to be detected (95). Further work is however needed to assess which of these methods is most useful for the measurement of antimycobacterial immunity induced by vaccination.

Identification of Novel Genes: Analysis of Gene Expression in T Cells

A number of studies have used, or are currently using, gene expression as a way of assessing genes whose expression is induced by stimulation with MTB, during MTB infection, or by vaccination (96–98). Gene arrays can be used to identify novel genes, signalling pathways and metabolic processes that are induced by vaccination or during infection, using transcriptomics and new bioinformatics tools. As for the bead array approach, if blood or antigen-stimulated cells are used for RNA extraction, the cells expressing the differentially expressed genes cannot be identified without further work, unless selected T cell populations are used for the transcriptomics (96). In addition, these analyses are better performed on blood samples that do not have marked increases or decreases of cell numbers or proportions, such as may occur in clinical disease when deconfounding may be required (99).

Expression of a more limited set of genes can be evaluated by multiplex ligation-dependent probe amplification (MLPA) assays in which expression of up to about 60 genes can be quantitated simultaneously (100). As with gene array techniques, principal component analysis may be required to identify the most important components that can distinguish changes in vaccinees induced by vaccination, or gene activation patterns that can distinguish protected from non-protected individuals. It will also be important to confirm that any changes in gene expression are associated with changes in protein production.

From Small-Scale Studies to Field Trials

Once the components of a protective biosignature have been identified in small-scale in-depth studies, it will be necessary to modify the detection systems to allow their evaluation on a much larger scale in field trials, in settings where laboratory facilities may be limited. For such purposes, assays that utilize whole blood rather than separated peripheral blood mononuclear cells may be preferable. Cytokine secretion and quantitation using ELISA or multiplex bead array assays can be performed on undiluted whole blood samples stimulated overnight, or diluted whole blood assays stimulated for longer periods of up to six to seven days to allow the activation and expansion of resting memory T cells. A diluted whole blood assay has been used to show the changes in IFN-γ production following BCG vaccination using small blood samples from UK and Malawian infants (101) and from adolescents and young adults in the same settings (14). Flow cytometry and gene expression profiles can also be generated from whole blood samples.

One test parameter that should be monitored carefully in any such field studies is the time taken to get blood samples into culture. A number of recent studies have shown that the number of cytokine secreting cells detected in ELISPOT assays, the number of cells with intracellular cytokine production, and the gene expression profiles within whole blood or peripheral blood mononuclear cells are profoundly affected by the time taken to get blood into culture (102–104) (Smith, Dockrell—unpublished results). Thus, to ensure interpretable results with any of these techniques, blood needs to be drawn into tubes containing heparin and stimuli and kept at 37 °C (102) or to be placed directly into PAXgene™ tubes or another suitable RNA stabilization reagent. If this is not possible, the blood should be separated if required and cells stimulated within the minimum time period that can be realistically achieved, and details recorded of the time blood was taken and the time it was put into culture. For future deconfounding it may also be useful to have full blood counts performed.

The Future: Towards Biosignatures

It is clear that measurement of one component of immunity will not be sufficient to identify protective immune responses, even if there is good evidence that this component is part of a protective immune response (59–61, 105). The additional components that are required may be identified by a range of newer techniques including bead array assays or gene expression techniques. Results from studies evaluating changes in immune profiles with TB treatment may also

inform the choice of components (61, 77, 78, 106). Comparisons of immunity in vaccinees pre- and post-vaccination can identify changes in immune status induced by vaccination, and the immunogenicity of both the existing BCG vaccine and new candidate TB vaccines can be compared in this way. However, identification of a biosignature of protective immunity will be more complex, requiring large trials in which blood samples are drawn following vaccination, but prior to the development of disease, as in the large infant BCG studies ongoing in South Africa (107). Once the key components of immunity have been clarified, simpler assays to evaluate these genes or their products could be developed, enabling them to be assessed in a larger number of subjects within vaccine trials. In the meantime, these new approaches will contribute to a better understanding of the features of a protective cellular immune response that enable effective control of mycobacterial growth.

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