MiniReview

Cytotoxic T cells and mycobacteria

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Abstract

How the immune system kills *Mycobacterium tuberculosis* is still a puzzle. The classical picture of killing due to phagocytosis by activated macrophages may be only partly correct. Based on recent evidence, we express here the view that cytotoxic T lymphocytes also make an important contribution and suggest that DNA vaccines might be a good way to enhance this. © 2001 Federation of European Microbiological Societies. Published by Elsevier Science B.V. All rights reserved.

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1. Introduction

Mycobacterium tuberculosis is one of the most successful bacterial parasites of humans, infecting over one-third of the population of the world. This remarkable success is because pathogenic mycobacteria can survive in the hostile habitat of a macrophage, even in the face of a specific T cell immune response. As a result, a small number of viable mycobacteria may persist at the site of infection. After years of dormancy, this organism may start to replicate, leading to the reactivation of infection and clinical disease. Despite many years of research, the effector mechanisms by which *M. tuberculosis* is killed, when the immune response mounts its most successful form of defence, remain contentious. Bacteriostasis is the most prominent feature of immunity, and is essential in the absence of effective bactericidal processes, but it probably also contributes to bacterial dormancy and persistence. Stronger bactericidal processes would be preferred in order to minimize the problems posed by reactivation of dormant infection many years later.

Adoptive transfer experiments have established beyond doubt that protection is cell-mediated and not antibody-mediated in tuberculosis. However, the evidence that protection requires activation of macrophages by antigen-specific T lymphocytes so that the macrophages acquire an ability to kill the mycobacteria remains less than compelling. New evidence suggests that cytotoxic T cells may also directly kill the bacteria, depending on the ability to deliver potent bactericidal proteins such as granulysin from their granules. Elucidation of the host–pathogen interactions involved during the disease process is critical to the development of new antimycobacterial treatment options. The development of new strategies to inhibit mycobacterial pathogenesis or augment the host response against mycobacteria is crucial in order to curtail this global health crisis. Here we discuss that a DNA vaccine used therapeutically provides a new means of exploring these processes showing its ability to induce the immune system to kill both actively multiplying and dormant bacteria.

2. Pathogenesis and the role of macrophages

M. tuberculosis infections are acquired through inhala-

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tion of infective bacilli. Bacteria are internalized by alveolar macrophages and set up infection foci in the lung tissue. These foci expand through bacterial growth and recruitment of macrophages and lymphocytes that build the granuloma that defines this infection. The granuloma seems to support limited bacterial growth and prevents metastasis of the infection. Nonetheless, the granuloma also protects the bacterium from the immune response and is probably responsible for the persistent or latent nature of the infection. Clinical disease develops when this immune-mediated constriction is abrogated through immune compromise. Even in individuals in whom infection is controlled at the granulomatous state or earlier, any later imbalance of the host's immune system may promote reactivation of the disease.

Macrophages play a multiplicity of roles in tuberculosis including antigen processing and presentation and effector cell functions. Amongst the best characterized antimicrobial effector functions of macrophages are lysosomal enzymes that are delivered to the phagosome during phagosome–lysosome fusion, the generation of reactive oxygen intermediates (ROIs) by the oxidative burst, the production of reactive nitrogen intermediates (RNIs) and apoptosis [1].

Recently, two transgenic murine models of chronic granulomatous disease have been developed. These transgenic mice are deficient in gp91phox or p47phox, phagosome oxidase components critical for the activity or assembly of the functional oxidase, respectively [2,3]. Cooper et al. [4] reported 10-fold higher bacterial numbers in the lungs of p47phox knockout mice, compared to wild-type controls, after aerosol challenge with *M. tuberculosis*. These findings provide evidence that ROI-mediated control is important early during infection. In particular, they are consistent with a role for ROIs in host defense against *M. tuberculosis* prior to the emergence of interferon-γ (IFN-γ)-mediated macrophage activation, nitric oxide (NO) production and phagosome acidification. However, *M. tuberculosis* has evolved strategies to avoid oxidative killing mechanisms. For example, CR1- or CR3-dependent uptake does not trigger the oxidative burst [5]. Mycobacteria also produce catalase and superoxide dismutase, two gene products capable of degrading reactive oxygen species [6], and deficiency in the *katG* gene encoding the mycobacterial catalase results in increased susceptibility to peroxidative killing [7].

IFN-γ is now known to be essential for protection [8] and is produced by CD4$^+$ T cells, CD8$^+$ T cells, NK cells and the *M. tuberculosis*-infected macrophage itself [9]. The importance of IFN-γ is demonstrated by the high susceptibility to mycobacterial infections of patients with defective IFN-γ receptors [10]. A major factor of IFN-γ and other Th1 cytokines in the antituberculous host response is their macrophage activating and recruiting properties. This could be largely due to its role in activating macro-

phages for mycobacteriostasis [11,12], rather than for killing, but it also impacts on many other aspects of immunity besides macrophage activation [13]. Similarly, there is compelling evidence for the importance of macrophage NO in protection [14,15], but this might be more due to a regulatory function of this key intracellular signaling agent [16,17], upstream of the actual lethal events, rather than due to direct toxicity of NO for the bacteria. However, there is evidence that RNIs do play an important role in controlling *M. tuberculosis* infection in the murine model [18]. At an in vivo level iNOS gene knockout mice, that are deficient in mounting a RNI response, exhibit chronic disease when infected with *M. tuberculosis*, in comparison to control mice [19]. Moreover, treatment of *M. tuberculosis*-infected mice with the iNOS inhibitor (L-NMMA) has also been shown to increase bacterial burden, tissue damage and mortality [18]. Recently, however, conflicting reports suggesting that RNIs are not beneficial in the murine response against mycobacteria have emerged [20] showing that the iNOS gene knockout mice has increased levels of IFN-γ in serum, increased granuloma formation and increased survival of CD4$^+$ T cells. Also, the actual production of NO by human macrophages remains a controversial issue. Previously it was thought that human macrophages did not produce NO and that this accounted for their general inability to control infection with virulent mycobacteria in contrast to mice that are innately resistant. However, with the onset of new and improved molecular technology, many reports have emerged discounting this theory [21]. Notwithstanding the controversy regarding the role of NO in mice or human host defence against tuberculosis, it seems highly likely that other macrophage antimycobacterial effector mechanisms are involved, since many reports show reduced viability of *M. tuberculosis* within human macrophages that are not affected by NO inhibitors [22]. Thus, we can question, in fact, whether active killing by macrophages has a major role in either the initial arrest of bacterial population growth or in the subsequent slow decline in population.

It seems likely that appropriately activated macrophages can indeed sometimes kill virulent *M. tuberculosis* [23,24] but that this is not usually sufficient for sterilizing immunity. Effects seen in vitro are generally modest and the target is clearly a difficult one for macrophages to kill. Extensive efforts have produced only sporadic claims to demonstrate substantial killing and independent confirmatory evidence is sparse, particularly for human cells [25]. Possibly the right combination and sequence of differentiation and activation signals and a high rate of replacement of expended macrophages occur in vivo, particularly in the high turnover granulomas characterizing the earlier stages of the immune response [1]. This might give major and sustained bactericidal action but has not yet been reproduced in vitro.
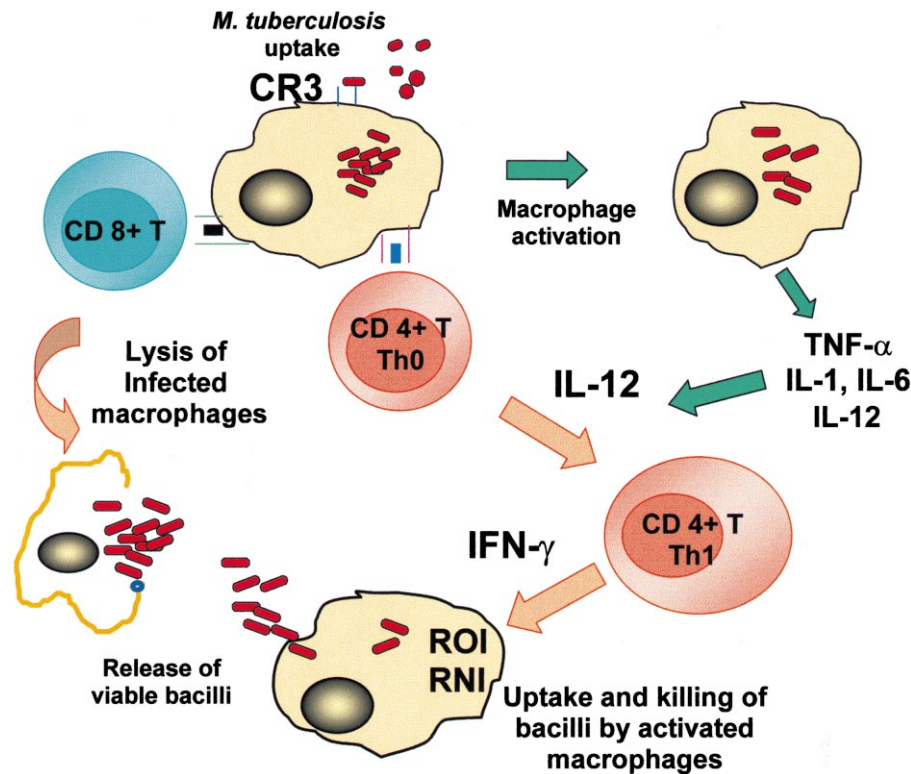
Fig. 1. The basic traditional view of the expression of acquired protective immunity against tuberculosis. The bacteria that are engulfed by monocytes and tissue macrophages prior to the onset of the specific immune response are able to multiply within those cells. When specifically sensitized T cells arrive, they release large amounts of IFN-γ on contact with antigen that is appropriately presented on cell surfaces. IFN-γ cannot activate the infected cell for killing, only for bacteriostasis, but it is able, probably in conjunction with other factors, to activate freshly arriving monocytes sufficiently to kill *M. tuberculosis* during phagocytosis. Killing may involve discharge of toxic macrophage products such as superoxide, NO and granule (lysosome) contents into the phagocytic vesicle. The bacteria are made available for phagocytic killing through lysis of the infected macrophages by antigen-specific cytotoxic T cells. Abbreviations: IFN-γ, interferon-γ; CD4+, T cell differentiated to CD4+ and release of a type 1 cytokine profile (Th1) on recognition of antigen; CD8+, T cell differentiated to CD8+ and expression of cytotoxicity (Tc1) on recognition of antigen.

3. Which T cells confer protection?

Although adoptive transfer of protection with T lymphocytes from infected or immunized rats into naive animals established over 20 years ago that acquired immunity against tuberculosis is cell-mediated [26], attempts to define the phenotype and function of the protective T cells have given conflicting results. CD4+, CD8+, CD1-restricted and γδ-TCR T cells have all been implicated, as have IFN-γ and cytotoxicity, without establishing how they actually contribute to protection [27]. The importance of the T cell response was also illustrated by Doherty and Sher [28], who reported that athymic nude mice, which have no mature T cells due to defective formation of the thymic stroma, show a similar pattern of increased susceptibility to mycobacterial infection to that of SCID (severe combined immunodeficiency) mice, in which neither antibody nor T cell responses are made. The importance of the CD4+ subset of T cells in antituberculous immunity is seen in HIV patients, in which depleted CD4+ T cell numbers correlate with extreme susceptibility to tuberculosis. The protective CD4+ T cell response in active tuberculosis in humans is of the Th1 type and demonstrates the characteristic Th1 cytokine profile (secretion of IFN-γ, IL-12, IL-2 and TNF-α) [29]. Thus, the accepted paradigm has been that protection is mainly due to antigen-specific CD4+ Th1 cells that produce IFN-γ to activate macrophages that then kill the mycobacteria during phagocytosis. Bacteria that are not killed by this process have their multiplication inhibited inside IFN-γ-activated macrophages. A subsidiary function is then served by cytotoxic CD8+ T cells that release intracellular bacteria from infected cells so that they can be killed during phagocytosis by activated macrophages [11] (Fig. 1). Even allowing for some crossover between the activating and cytotoxic functions of CD4+ and CD8+ T cells, it is now clear that this is not the whole story. How do these T cells really function in protection? First, there is general agreement that activation of antimicrobial activities in macrophages by T cell cytokines is involved. Accordingly, IFN-γ, which is a major macrophage activating cytokine, and other Th1 cytokines are critical as stated above. Second, direct killing of mycobacteria by T cells has been demonstrated. Third, mycobacteria-reactive T cells lyse infected macrophages. Macrophage lysis appears to be a prerequisite for killing by T cells of microbes residing inside macrophages. More-

over, lysis of infected macrophages could promote release of mycobacteria from incapacited macrophages to more proficient monocytes.

Although CD4$^+$ T cells are considered as the major source of IFN-γ, other T cell populations have also been shown to produce this and other Th1 cytokines. Similarly, CD8$^+$ T cells are mainly responsible for the killing activities although additional T cell sets, in particular CD1-restricted T cells, can perform these functions [30,31]. This redundancy should not be misinterpreted as meaning that a single T cell population would suffice for protection. These T cell populations differ in other capacities, including antigen specificity, genetic restriction and activation requirements. Hence, it appears unlikely that any one T cell population could fully compensate for another.

Involvement of CD8$^+$ T cells in protective immunity against tuberculosis has been recognized for some time [32–34] although the antigen specificity and function of these cells were not clear. More recently, gene-targeted mice with disrupted expression of key immunologic functions have provided new insights into the role of CD8$^+$ T cells in host defence. When β_2-m and TAP1 knockout mice, which cannot generate CD8$^+$ T cells, were infected with *M. tuberculosis*, this resulted in an exacerbated course of infection [35,36]. Moreover CD8$^+$ T cells from lungs of infected mice produced IFN-γ in response to recognition of antigen presented by *M. tuberculosis*-infected dendritic cells or macrophages [37]. Short-term culture of the lung T cells from infected mice with infected dendritic cells resulted in CD8$^+$ T cells capable of MHC class I-restricted specific lysis of macrophages infected with live, virulent *M. tuberculosis* [38]. CD8$^+$ T cells from PPD-reactive human subjects could also be expanded in vitro by macrophages infected with *M. tuberculosis* or BCG [39]. These CD8$^+$ T cells proliferated in response to live bacteria or mycobacterial antigens and produced IFN-γ. These data supported the hypothesis that MHC class I-restricted CD8$^+$ T cells are required for control of tuberculosis.

Recent studies also support the idea that stimulation of the CD8$^+$ T cell population must be considered in vaccine design against tuberculosis [40,41]. The discovery that immunization of mice with a single mycobacterial antigen (65-kDa heat-shock protein; hsp65) could give substantial protection against tuberculosis challenge [42,43] led to analyses in which the diversity of antigens recognized by T cells responding to *M. tuberculosis* was no longer a variable. The key to eliciting protection was in the use of immunization procedures favoring presentation as an endogenous antigen. Thus, in vivo expression from retroviral vector-transfected bone marrow cells or from a transfected macrophage-like cell line, or DNA vaccination of muscle or skin, or intravenous cationic liposome delivery of the protein were all effective [44]. Endogenous antigen favored responses from MHC class I-restricted CD8$^+$ T cells. Adoptive transfer of protection with hsp65-specific T cell lines or clones raised from such immunized animals

showed that, individually, the most protective were indeed CD8$^+$ cells, although CD4$^+$ and γ/δ T cells also protected and marked synergy occurred with all three types transferred together [45,46]. Protection partly reflected the ability of the cells to produce IFN-γ; IL-4 producing cells were not protective and protection with IFN-γ producing cells was decreased by administering antibody against IFN-γ. However, the most protective CD4$^+$ and CD8$^+$ T cell clones also displayed antigen-specific cytotoxicity in vitro and selectively lysed macrophages that were infected with *M. tuberculosis* [46,47]. This is consistent with the view that cytotoxicity also has a positive role in protection [11].

4. The microbicidal proteins of cytotoxic lymphocytes

Since killing by macrophages seems to be inefficient we should take note of the increasing evidence that some T lymphocytes can directly kill the bacteria. Macrophages may not even be the main source of bactericidal products in protection against tuberculosis after all. Perhaps the first pointer in this direction came from evidence that intracellular BCG could be killed when human monocytes containing the bacteria were lysed by specific lymphocytes that induced apoptosis by releasing ATP [48,49]. Killing of virulent *M. tuberculosis* has not been reported by this mechanism and other inducers of apoptosis or necrosis were ineffective. It has not been established what the toxic factor is which is generated under these conditions. Similarly, Oddo and associates [50] have reported that cytotoxic CD4$^+$ T lymphocytes from man killed virulent *M. tuberculosis* when they lysed infected macrophages by the Fas–FasL pathway. The bacteria were also killed when apoptosis was induced with TNF-α, suggesting that the lethal product came from the macrophage rather than the lymphocyte.

An approach from a completely different direction by Robert Modlin and co-workers [31] has recently revealed the existence, in man, of categories of cytotoxic CD8$^+$ T lymphocytes that deliver highly microbicidal proteins into infected macrophages. In so doing, they kill virulent *M. tuberculosis*. These lethal cells can either be conventionally MHC class I-restricted or recognize lipophilic antigens (a prominent feature of mycobacteria) presented on CD1 structures. Their essential feature is that they lyse the target macrophages by the granule-mediated perforin mechanism of apoptosis and co-deliver bactericidal proteins. In contrast to the findings of the Meylan group [50], T cells that lysed targets by the Fas–FasL pathway to apoptosis were not bactericidal. A key mycobactericidal protein delivered by the T cells has been identified as granulysin, present in the same granules with perforin [51]. This protein is also found in NK cell granules and has known potent lethal action against a range of microorganisms and tumor cells.
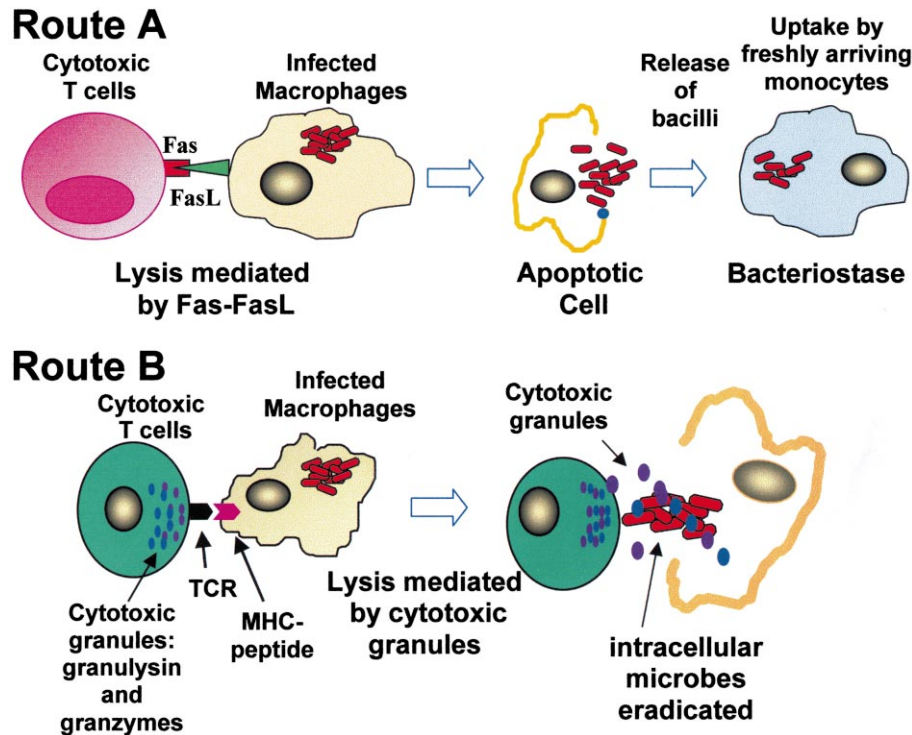
Fig. 2. Two potential routes to concomitant killing of intracellular *M. tuberculosis* during the death of infected macrophages. Route A: lysis mediated by most CD4$^+$ cytotoxic T lymphocytes (CTL). The crosslinking of Fas on the CTL and FasL on the target cell induces lysis of the infected cell but does not affect significantly the viability of the intracellular bacteria, characterizing as a bacteriostase. Route B: lysis mediated by most CD8$^+$ CTL. *M. tuberculosis*-reactive CTLs recognize bacterial antigen on the surface of an infected cell. Granular contents gain access to the intracellular compartment via the polyperforin pore. Effector molecules such as granulysin and granzymes can now exert their antimycobacterial activity and contribute to the eradication of intracellular microbes.

The differences in the findings between these various studies may be due to differences in the way that macrophages were prepared from peripheral blood monocytes. These cells can proceed from being undifferentiated immature monocytes through into widely diverse forms, including dendritic cells [52], in vivo and in vitro depending on the environmental stimuli encountered. Cell adherence to different surfaces and exposure to different cytokines prior to apoptosis doubtless resulted in biochemically and functionally different cells in these studies. Hence we hypothesize that in some differentiated states the cells have a mycobactericidal potential that is mobilized during apoptosis, perhaps through autolytic generation of toxic products (Fig. 2A). In other states they do not and killing during apoptosis then depends on delivery of the toxic agent by the lymphocyte (Fig. 2B).

Despite the in vitro evidence from human cells, published studies with genetically modified mice have failed to support a role for cytotoxicity in protection against tuberculosis. Immunity during the first few weeks of infection was unimpaired in mice in which the genes for either perforin or granzyme B (a cytotoxic granule protease that contributes to apoptosis) had been knocked out [53]. Protection against BCG also appeared normal in Fas-defective mice [54]. However, since there are multiple pathways of cytotoxicity and multiple lytic and cytotoxic proteins in cytotoxic granules, some redundancy might be

expected. Furthermore, three research groups are reputed to have evidence that, at later stages of infection, immunity is impaired in perforin knockout mice [51]. Thus, on balance, it seems likely that similar systems contribute to protection in man and mouse but that the relative importance of each may differ at different stages of infection.

5. Implications for vaccines and immunotherapy

Although CD4$^+$ T cells play a major role in the build-up of an optimal protective immune response against tuberculosis, CD8$^+$ T cells are needed as well. Thus, if cytotoxicity can make a contribution to protection, tuberculosis vaccines should be designed to promote this aspect of cellular immunity, not just Th1 responses and macrophage activation. In particular, antigen-specific delivery of cytotoxic mycobactericidal proteins may be highly desirable. Unfortunately, little is currently known of the mechanisms of regulation of the development of cytotoxicity [55], still less of the differential development of cells expressing the Fas–FasL versus the perforin pathways. However, DNA vaccination seems pre-disposed to generate responses with a strong bias towards cytotoxicity [56], including the phenotype most protective against tuberculosis. DNA vaccines actually generate antigens that are presented via endogenous and exogenous pathways and also,

the induced cross-priming events where exogenous antigen released by myocytes is taken up by antigen presenting cells is an important mechanism for either stimulating CD4$^+$ and CD8$^+$ T lymphocytes and B cells to produce antibodies. Huygen et al. [41] have recently shown that a highly active tuberculosis DNA vaccine protects CD4 knockout mice but is not protective in CD8 knockout mice. This result suggests that DNA vaccine induced protective CD8$^+$ T cells. We have also found precursors of the protective hsp65-specific CD8$^+$/IFN-γ producing/cytotoxic/CD44^{hi} phenotype to persist at elevated frequency in spleens in parallel with persistence of protection for at least 8 months after DNA vaccination [44]. This also occurs after BCG vaccination, or during *M. tuberculosis* infection, but then the persistent response is dominated by T cells with the non-protective Th2 phenotype (IL-4 producing, CD44^{lo}, non-cytotoxic) [47]. To better understand the role of T cells in protection against tuberculosis we further characterized 28 CD4$^+$ and 28 CD8$^+$ hsp65-specific T cell clones in vitro and in vivo and test whether lysis of *M. tuberculosis*-infected target macrophages by these clones can cause death of the bacteria by either the perforin- or Fas–FasL-dependent pathway [57].

Strikingly, only the T cell clones using the granule-dependent pathway showed clear evidence of killing intracellular *M. tuberculosis* when they lysed infected macrophages [57]. Those clones using the Fas–FasL pathway had small effects, equivalent to bacteriostasis. These might be partly attributable to discharge of the bacteria into the less favorable growth environment provided by the tissue culture medium and partly to activation of non-lysed macrophages by IFN-γ. The close correlation between the degree of killing and the granule content of the clone, and the selective inhibition of killing by prior degranulation, are consistent with killing by the granule contents [57]. The mechanism is likely to be similar to that which was recently revealed in studies of human cells [51]. Thus the granule enzyme perforin may lyse macrophage membranes to allow access of potent microbicidal granule enzymes such as granulysin to the target mycobacteria. Attempts to identify the mycobactericidal agent in the mouse cells are underway. The correlation between the ability of the cytotoxic clones to protect against challenge with *M. tuberculosis* in vivo and the clone's granule content is a further indication that this mycobacterial killing mechanism has a role in protective immunity. It may account for the major component of the protective effect of these clones that was resistant to neutralization in vivo by injection of antibody against IFN-γ [46]. Although others found that knockout mice that do not express the perforin gene did not have decreased resistance to the early stages of tuberculosis [53,54], this defence mechanism may be more important later in infection. Our high-dose intravenous challenge model probably resembles late-stage rather than early-stage tuberculosis.

It was also striking that most of the cells lysing targets using the cytotoxic granule pathway were CD8$^+$ cells, whereas most of cells that lysed targets using the Fas–FasL pathway were CD4$^+$ cells. This suggests that cytotoxicity may generally serve different purposes in tuberculosis depending on whether it is triggered by endogenous or exogenous antigen. Thus the cytotoxic CD8$^+$ T cells may have a predominantly antimicrobial function against this intracellular pathogen, whereas the cytotoxic CD4$^+$ T cells may have a predominantly immunomodulatory role in removing the cells that sustain immune 'help' by presenting antigen on MHC class II [31]. However, this division of labor is not absolute, since a minority of cytotoxic CD4$^+$ clones used the granule pathway and a minority of the CD8$^+$ clones used the Fas–FasL pathway. Furthermore, at least in studies of human T cells, lysis of infected target macrophages by non-granule-dependent pathways may sometimes have direct antimycobacterial effects [48–50] and non-MHC-restricted cells may contribute significantly to granule-dependent killing [51]. These diverse and somewhat contradictory findings may reflect the plasticity of the target cells as stated above. Hence we hypothesize that in some differentiated states the cells have a mycobactericidal potential that is mobilized during apoptosis, perhaps through autolytic generation of toxic products. In other states they do not and bacterial killing during cytolysis then depends on delivery of the toxic agent by the lymphocyte.

6. Concluding remarks

In summary, several distinct bactericidal mechanisms may operate in cellular immunity against tuberculosis. Different mechanisms may feature at different stages of infection, to kill both actively multiplying and dormant bacteria, and cumulatively decrease the duration of persistence of the infection. It is likely that both cytokines, released in response to specific antigens, and direct cell:cell interactions modulate differentiation of these mechanisms. DNA vaccines used either preventive or therapeutically provide a new means of exploring these processes and may lead to practical vaccines or immunotherapy that bring bactericidal rather than bacteriostatic mechanisms to the fore. The clinical impact of such developments could be substantial. A recent publication of our group reported successful therapy of tuberculosis in mice by treatment with a DNA construct encoding hsp65 antigen [40]. For tuberculosis, these issues deserve some reconsideration, one-third of the world population is already infected with *M. tuberculosis*, and thus living with a time bomb, so there is a sufficiently large group to be considered as targets for a post-infection immunotherapy. Moreover, with the increasing risk of multi-drug-resistant tuberculosis and co-infection with HIV and tuberculosis, therapeutic vaccines may warrant specific consideration.

It can also be argued that microbial stress proteins do

not make good candidates as vaccines because of the implication of the homologous mammalian proteins in autoimmune diseases [58]. However, there are possible alternatives to leaving these major antigens out of vaccines: the epitopes involved might be readily engineered out of the DNA, or the formulation and delivery might be selected to direct the response away from a harmful type and towards a beneficial one. Indeed, T cells recognizing mycobacterial HSPs can either protect against or potentiate autoimmune disease, depending on their phenotype [59]. It was recently demonstrated that DNA vaccination with mycobacterial hsp65 protected Lewis rats against induction of adjuvant arthritis [60]. This may be the best approach in the long run because, to the extent that an acquired immune response depends on recognition of 'altered self' rather than 'foreign', essentially all antigens may have the potential for autoimmune involvement.

Acknowledgements

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