Review

Mycobacteria and innate cells: critical encounter for immunogenicity

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Protective immunity against mycobacterial infections such as *Mycobacterium tuberculosis* is mediated by interactions between specific T cells and activated macrophages. To date, many aspects of mycobacterial immunity have shown that innate cells are the key elements that substantially influence the subsequent adaptive host response. During the early phases of infection, phagocytic cells and innate lymphocyte subsets play a pivotal role. Here we summarize the findings of recent investigations on macrophages, dendritic cells and $\gamma\delta$ T lymphocytes in the response to mycobacteria.

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1. Introduction

Effective adaptive immune responses to pathogenic and commensal microorganisms require that T lymphocytes be endowed with effector properties appropriate to each challenge. In this context, CD4 T cells differentiate in the peripheral tissues to adopt a variety of fates: the T-helper (Th)1 cells, which produce interferon (IFN)-γ; and Th2 cells, which produce interleukin (IL)-4. Th1 cells control intracellular pathogens, including viruses and bacteria, and are involved in autoimmune diseases (Gately *et al* 1998). Specific cell-mediated immunity is critical in the host defence against mycobacteria, but many aspects of mycobacterial immunity involve other levels of responses (Houben *et al* 2006). In this context, innate cells play a pivotal role, particularly during the early phases of infection, and substantially influence adaptive immunity.

2. Monocyte/macrophages and the balance between apoptosis and reaction

Phagocytic cells play a key role in the initiation and direction of the adaptive immune response against mycobacteria through antigen presentation, co-stimulatory activity, cytokine and chemokine production (Mueller and Pieters 2006). In addition, their innate responses are important in the early containment of infection. Alveolar resident macrophages are the primary cell types involved in the initial uptake of mycobacteria as *Mycobacterium tuberculosis* (MTB). During the early stages of infection, the control of intracellular bacterial survival and proliferation depends on both the innate resistance of macrophages and cytokine-induced activation (Hunter and Reiner 2000). Mycobacteria may persist in inactivated macrophages and replicate inside the cells in part by modulating the phagosomal compartment, preventing the incorporation of the vesicular proton ATP-ase and subsequent acidification, and in part by moving into the cytoplasm or into non-fused vesicles (Kusner 2005).

Endocytosis of MTB involves different receptors on the phagocytic cells, which either bind to non-opsonized or opsonized mycobacteria (Hirsch *et al* 1994). Besides phagocytosis, recognition of mycobacteria or related products is a crucial step in the host reaction. Immune recognition of the major cell wall component, lipoarabinomannan (LAM), appears to resemble that of Gram-negative bacterial lipopolysaccharide (LPS) (Zhang

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Abbreviations used: APC, antigen-presenting cell; ATP, adenosine triphosphate; BCG, bacille Calmette-Guérin; DC, dendritic cell; DOXP, 1-deoxy-d-xylulose-5phosphate; GM-CSF, granulocyte–macrophage colony-stimulating factor; IFN, interferon; IL, interleukin; imDC, immature dendritic cell; LAM, lipoarabinomannan; LPS, lipopolysaccharide; MDM, monocyte-derived macrophage; MHC, major histocompatibility complex; MTB, *Mycobacterium tuberculosis*; MVA, mevalonate; Th, T-helper; TLR, toll-like receptors; TNF, tumour necrosis factor

et al 1993). In this context, toll-like receptors (TLR) are essential for microbial recognition of macrophages. Through TLRs, mycobacterial lysates or soluble mycobacterial cell wall-associated lipoproteins induce production of IL-12, a strong inflammatory cytokine (Jo *et al* 2007). Indeed, TLR-2, and not TLR-4, is necessary for the signalling of LAM. Among the lipoproteins, the 19 kDa lipoprotein acquired importance in the study of virulence of mycobacteria for its dual capacity to activate macrophages and induce their apoptosis.

A polypeptide component of the 19 kDa lipoprotein has been shown to be responsible for apoptosis in macrophages (Lopez *et al* 2003). In addition, the active N-terminal peptide of the *M. leprae* 19 kDa lipoprotein can trigger apoptosis in Schwann cells via TLR-2, contributing to nerve damage in leprosy (Oliveira *et al* 2003). The 19 kDa lipoprotein is expressed by MTB and some other slow-growing mycobacteria, such as *M. bovis* bacille Calmette-Guérin (BCG) and *M. avium*-intracellulare, but not fast-growing mycobacteria such as *M. smegmatis* and *M. vaccae.* The function of the 19 kDa lipoprotein is still unclear, but its antigenic properties in both humans and mice (such as the production of specific antibodies or induction of T cell responses) have been described (Vordermeier *et al* 1997). Our group has intensively exploited the relevance of this lipoprotein in the past years. We have reported an experimental model resembling the macrophage response that occurs in the presence of high bacterial load, such as that observed during the course of HIV infection. In fact, the number of bacilli increases in the lungs of HIV-infected patients with tuberculosis with an increase in apoptosis of the alveolar macrophages (Placido *et al* 1997). Mycobacterial 19 kDa lipoprotein induces apoptosis in human monocyte-derived macrophages (MDMs) in a dose-dependent manner (Ciaramella *et al* 2000), extending previous results showing that the 19 kDa polypeptide, which lacks an acylation signal, is sufficient to induce cell death in macrophages and the THP-1 (human promonocytic) cell line (Lopez *et al* 2003). Indeed, after infection with MTB and BCG strains lacking the 19 kDa lipoprotein, MDM cell death was reduced (by nearly 70%), compared with apoptosis induced by wild-type strains (Ciaramella *et al* 2004). Moreover, when the 19 kDa lipoprotein was expressed in *M. smegmatis*, a mycobacterial strain unable to induce apoptosis, cell death took place in the infected macrophages. These results showed that the 19 kDa lipoprotein represents the main pro-apoptotic signal during the early phases of a high multiplicity of infection. Importantly, the rapid death signal induced by the 19 kDa lipoprotein does not require other apoptotic signals such as the tumour necrosis factor (TNF)-α system (Ciaramella *et al* 2002). Thus, apoptosis of macrophages may contribute to the anergic status observed in the course of high bacterial load and disseminated mycobacterial infection.

It is well established that mycobacteria display a wide spectrum of survival strategies to take advantage of internalization mechanisms and escape the host immune response. Studies concerning the effect of target cell apoptosis on the viability of infecting bacilli have provided only contrasting results. It has been shown that the addition of adenosine triphosphate (ATP) to MTB-infected macrophages culminates with cell suicide and elimination of bacilli (Lammas *et al* 1997). However, there is evidence that ATP-mediated activation of purinergic receptors promotes phagosome–lysosome fusion and induces significant changes in the intraphagosome pH, which are unsuitable for the growth of mycobacteria (Di Virgilio 1995). While H_2O_2-induced apoptosis of *M. avium*-intracellulare infected cells leads to the elimination of mycobacteria (Laochumroonvorapong *et a*l 1996), Fas-mediated T cell killing of MTB-infected macrophages is not always coupled with mycobacterial eradication (Thoma-Uszynski *et al* 2000). In contrast, the observation that the control of mycobacterial growth may depend on target cell apoptosis comes from Frattazzi *et al* (1997), who have shown that apoptosis, but not necrosis, of *M. avium*-infected cells prevents mycobacteria from spreading and induces their growth inhibition by uninfected bystander macrophages (Frattazzi *et al* 1997).

In vivo, apoptosis occurs in epithelioid granuloma, but few data are currently available. Keane *et al* (1997) found that, in the caseous areas of lung sections from clinical cases of tuberculosis, more than 50% of the cells were apoptotic. The presence of apoptotic cells in productive granulomas suggests that apoptosis is an active process able to modulate the local cell turnover, thereby limiting tissue damage and spread of infection. However, further characterization of apoptotic cells was not performed. Thus, infection-induced target cell apoptosis may be a strategy to eliminate pathogens and ensure host survival (Bocchino *et al* 2005). In addition, several studies considered infection-induced cell death to be a crucial step in cross-priming of CD8 T cells during tuberculosis (Winau *et al* 2006). The cross-priming would be beneficial to the host because it results in specific CD8 T cell activation when the pathogen inhibits the classical antigen presentation. Thus, the response of macrophages, resulting in a balance between active innate response and apoptosis, should be considered in the new vaccination strategies against tuberculosis (figure 1A).

3. Dendritic cells as an immunity or escape target for mycobacteria

Dendritic cells (DCs) are a system of cells specialized for the presentation of antigen to T cells. They are the most potent antigen-presenting cells (APCs) and central to the

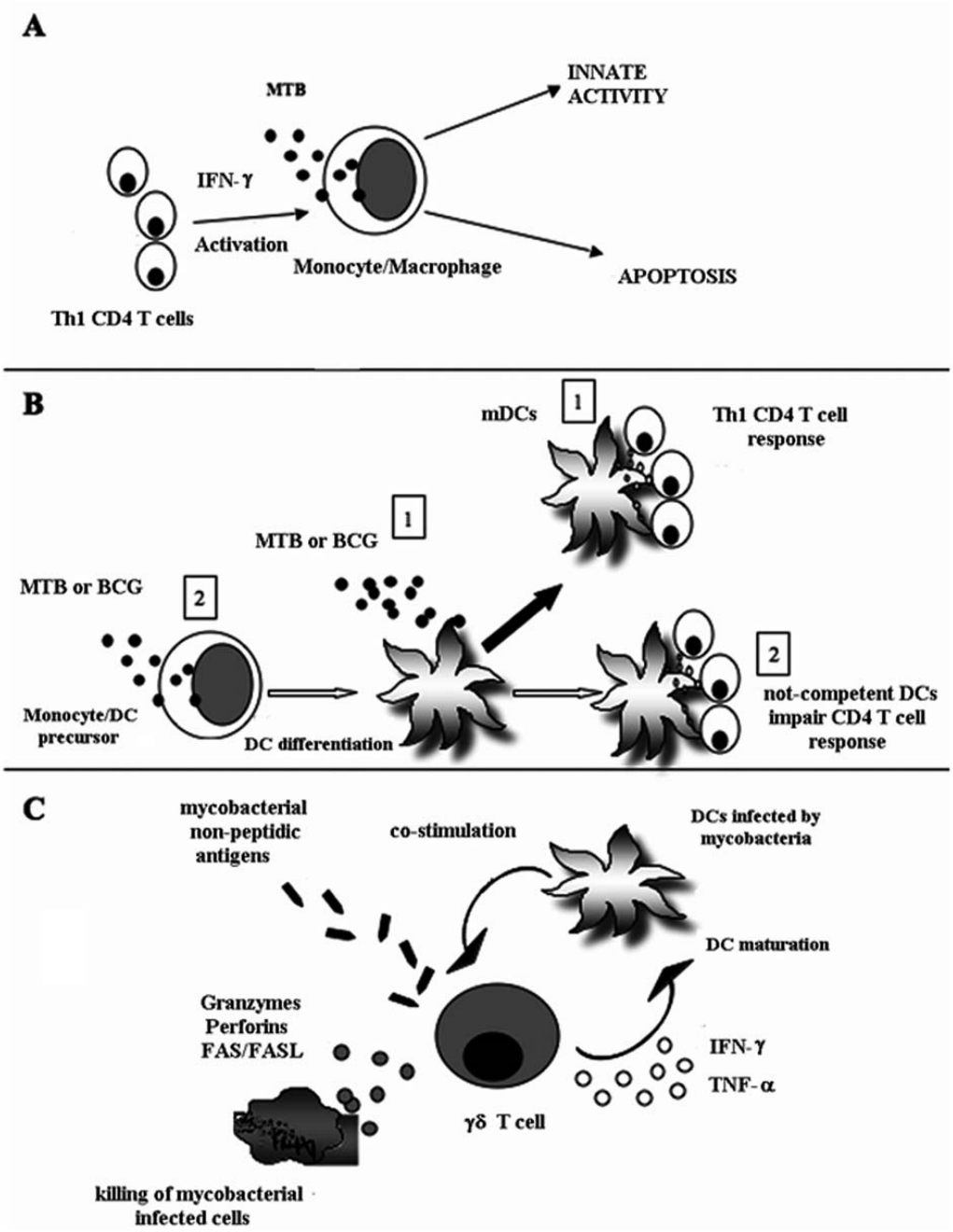

Figure 1. **(A)** Macrophage response to MTB. **(B)** Model of DC infection by mycobacteria: (1) Mycobacteria infect immature dendritic cells (imDCs) inducing their maturation programme (solid arrow) and expansion of T-helper (Th)1 cells: (2) Freshly recruited monocytes (DC precursors infected by mycobacteria differentiate into not fully competent DCs unable to expand Th1 cells (hollow arrow). **(C)** Role of human *γδ*T cells in the mycobacterial infection.

initiation of immune responses. DCs are a trace population in most tissues but notably form networks underlying major body surfaces such as the skin, trachea and intestine, where their functions are the uptake and presentation of processed antigens in lymphoid areas (Cella *et al* 1997). High levels of expression of major histocompatibility complex (MHC) class II and co-stimulatory molecules including CD80, CD86 and CD40 have been considered to contribute to the efficiency of DCs as APCs. In the periphery, DCs serve as "sentinels" monitoring the exposure of body surfaces

to antigens. In these sites, DCs are considered immature cells (imDCs) expressing low levels of MHC class II and co-stimulatory molecules, but with a high capacity to phagocytose invading microorganisms (Reis e Sousa 2004). These imDCs are stimulated to migrate away from the body surface following tissue injury, inflammation or infection (Dudziak *et al* 2007). The migration from the periphery via the afferent lymphatic vessels to the draining lymph nodes is associated with functional and phenotypic maturation. In the lymph nodes, DCs acquire the maturity to effectively stimulate naïve T lymphocytes and are considered mature cells (mDCs). This appears to be related to the upregulated expression of MHC II, CD80, CD86, CD40 and other surface molecules involved in the interaction with, and stimulation of, T lymphocytes (Dudziak *et al* 2007).

Considerable *in vitro* evidence exists that mycobacteria may modulate the functions and phenotype of DCs after their uptake. DCs lining the trachea may be the first cells to encounter mycobacteria and are therefore likely to be responsible for the ensuing immune response. Interaction of human DCs with MTB or BCG results in cell maturation and activation that is characterized by changes in the cell surface phenotype and stimulation of T cells (Hickman *et al* 2002).

The altered cytokine profile that is observed following mycobacterial infection is also of importance for the interaction with T cells and modulation of immune responses by DCs. Infection of DCs with either MTB or BCG is associated with increased expression of IL-12, TNF-α, IL-1 and IL-6 (Hickman *et al* 2002), which are considered important agents in the establishment of the protective antimycobacterial immune response. As noted above, IL-12 can potentiate IFN-γ and TNF-α secretion by the T cells and these in turn may serve to enhance the antimicrobial activity of macrophages to destroy invading bacilli. In addition to the production of pro-inflammatory cytokines, mycobacterial infection of DCs is also associated with the secretion of IL-10, which may inhibit the cellular response to mycobacteria through the downregulation of IL-12 secretion (Hickman *et al* 2002; Nigou *et al* 2001; Giacobini *et al* 2001). DCs are reported to provide an environment where mycobacteria can survive and replicate, albeit to a low extent (Bodnar *et al* 2001). It appears that the turnover of mycobacteria within DCs is not enough to kill the host cell and the outcome of slow replication is reflected by the constant availability of antigens for presentation to the T cells, which will therefore potentiate the immune response. In contrast to macrophages, DCs are able to control the replication of mycobacteria upon activation with IFN-γ but unable to eradicate them. Instead, mycobacteria appear to reside in vacuoles separated from the normal recycling pathway (Bodnar *et al* 2001). DCs may therefore be a reservoir for mycobacteria *in vivo*, particularly within the lymph nodes to which they migrate following the initial response to infection. The implication of these observations is that the uptake of bacteria by different APCs would influence the outcome of infection. Although survival and/or replication of mycobacteria within DCs is likely to induce T cell activation, the uptake by DCs could result in the transport of bacteria to the lymph nodes, leading to their persistence.

In terms of immunity, it may be advantageous to have an extended range of cells that are targets for infection, each with differing functions which should allow more effective clearance of the invading pathogens. Another facet of DC immunity has been described recently. It is well established that DCs represent a dynamic system of migrating cells that change during the course of the infection. At the sites of inflammation, cytokines and chemokines promote the activation of resident DCs and the recruitment of DC precursors, which may permeate the peripheral tissues including the skin, where they receive stimuli from local infection or inflammation. Peripheral blood monocytes differentiate into DCs or macrophages depending on the environmental factors encountered in the peripheral tissues. Upon contact with granulocyte–macrophage colony-stimulating factor (GM-CSF) and IL-4, cytokines produced by tissue mast cells, CD14+ monocytes differentiate into competent imDCs (Zhou and Tedder 1996; Chapuis *et al* 1997). Different signals of infection, inflammatory cytokines such as TNF-α or phagocytosis have been demonstrated to interfere with DC differentiation (Lyakh *et al* 2000). Pathogens or their components can interact with imDCs or their precursors, influencing DC generation. MTB has been reported to be able to subvert the differentiation of infected monocytes into DCs, suggesting an escape mechanism, which contributes to mycobacterial intracellular persistence (Mariotti *et al* 2002). In fact, MTB-infected peripheral monocytes, in the presence of GM-CSF and IL-4, differentiate into a population of DCs lacking the CD1 molecules on their surface and are able to produce IL-10 but not IL-12. This cytokine profile leads to the polarization of naïve CD4 T cells towards differentiated T cells unable to produce IFN-γ or IL-4 (Mariotti *et al* 2002). This implies that during the infection, DC precursors recruited from peripheral blood to the site of infection differentiate into not fully competent DCs, contributing to the persistence of MTB in the host. In addition, by infecting human monocytes with BCG, we have shown that BCG infection leads to a DC population very close to that generated by MTB, which produces IL-10 and is not able to support the polarization towards IFN-γ producing cells (Martino *et al* 2004, 2006).

The differentiation of DCs from monocytes has been extensively studied in recent years; these suggest a target of escape for different pathogens and tumours. In fact, cytokines, tumour supernatants, gangliosides and, in particular, microorganisms interfere in the early steps of DC differentiation from monocyte precursors, generating

semiprofessional APCs characterized by a less stimulatory mode (Chomorat *et al* 2000; Delneste *et al* 2003). Since this mechanism is common to both MTB and BCG, we asked whether the infection of monocytes with the environmental non-pathogenic *Mycobacterium smegmatis* had the same effects on DC differentiation and polarization of T cells. Monocytes infected with *M. smegmatis* differentiated into DCs showing an early mature phenotype not completely comparable to that induced by MTB or BCG (Martino *et al* 2005). DCs derived from *M. smegmatis*-infected monocytes, upon LPS stimulation, produced high levels of IL-10 and retained the capacity to produce IL-12, which plays a major role in triggering the Th1 immune response that correlates with the protection against mycobacterial infection. Consequently, the co-culture of DCs, derived from *M. smegmatis*-infected monocytes and naïve CD4 T cells leads to the expansion of mixed Th1 and Th2 cells (Martino *et al* 2005). These results suggest a possible correlation between mycobacterial virulence and their capacity to subvert DC differentiation. Differentially, this could be explained by the fact that during persistent infection as that caused by MTB or BCG, the immune system could acquire the capacity to downmodulate several immune functions leading to a balance between bacterial containment and host damage (figure 1B).

4. $\gamma\delta$ T cells as special guests in the antimycobacterial response

$\gamma\delta$ T cells participate in the early immune response against MTB infection. They are rapidly recruited to the lungs of mice infected with BCG, produce IFN-γ and are cytotoxic against an array of infected cells.

Human Vγ9Vδ2 T cells are the main blood/lymphoid organ $\gamma\delta$ T cell subpopulation and typically recognize phosphomonoester molecules synthesized in the mevalonate (MVA) and 1-deoxy-d-xylulose-5phosphate (DOXP) metabolic pathways. These low molecular-weight, phosphate-containing, non-processed antigens are called "phosphoantigens" and were first described in mycobacteria. Recently, they have been shown to be produced by different Gram-positive and Gram-negative bacteria (as well as by eukaryote parasites or those derived from abnormal metabolic routes of eukaryotic cells) (Tanaka *et al* 1994). These unique features characterize Vγ9Vδ2 T cells as a "special" peripheral lymphoid subset with a "sentinel" function showing broad antitumour and antimicrobial reactivity *in vitro* and *in vivo* (Girardi 2006). While pyrophosphomonoester antigens may directly activate Vγ9Vδ2 T cells through T cell receptor (TCR) engagement, other classes of stimulatory molecules such as the bisphosphonates and alkylamines may activate Vγ9Vδ2 T cells indirectly through the blocking of the MVA pathway in eukaryotic cells and leading to phosphantigen accumulation (Gober *et al* 2003).

Responses of human Vγ9Vδ2 T cells to MTB were described as early as 1989 (Janis *et al* 1989). Later, a range of studies described a marked expansion of this subset in the blood of tuberculosis patients and also in those with other mycobacterial infections such as leprosy or BCG vaccination (Boom *et al* 1999; Lee *et al* 2004). The response to this variety of mycobacterial agents is the direct result of the recognition of shared non-peptide compounds. In humans, phosphoantigen-specific T cells express TCR Vγ9Vδ2-encoded receptors with, on average, 1–3% of circulating T lymphocytes in adults, representing the highest frequency of MTB-specific T lymphocytes in the blood. In the presence of mycobacterial phosphoantigens and IL-2, these cells proliferate, express chemokine receptors, secrete IFN-γ as well as other Th1 cytokines and kill MTB-infected cells (Hayday 2000). Interestingly, despite the absence of defined microbial antigens recognized by murine $\gamma\delta$ TCRs, mouse $\gamma\delta$ T cells can expand during the early phases of infection with mycobacteria and other pathogens. The expansion usually peaks a week after infection and lasts for a short time (Girardi 2006).

Adaptive immune responses of Vγ9Vδ2 T cells during mycobacterial infections have also been demonstrated in macaque animal models. Primary BCG infection induces major clonal expansion of phosphoantigen-specific Vγ9Vδ2 T cells. Surprisingly, these antigen-specific $\gamma\delta$ T cells can have immune memory and mount rapid, prolonged and high-magnitude recall expansion after BCG or MTB reinfection (Shen *et al* 2002). The immune memory responses of antigen-specific Vγ9Vδ2 T cells have also been seen in human studies (Hoft *et al* 1998).

A new way to induce adaptive responses by $\gamma\delta$ T cells has been shown by several groups. Indeed, a strict relationship exists between human $\gamma\delta$ T cells and DCs (Martino *et al* 2005; Martino and Poccia 2005). The co-culture of BCG-infected DCs with human phosphoantigen-activated $\gamma\delta$ T cells increases their status of maturation and cytokine production as TNF-α, showing a stronger capacity to elicit a local inflammatory immune response (Martino *et al* 2007). Furthermore, we observed that activated $\gamma\delta$ T cells induce a significant amount of IL-15 in BCG-infected DCs. This last represents a cytokine that has important functions in lymphocyte differentiation, homeostasis and expansion of CD8 memory T cells. On the other hand, a new result has been found by our investigations to analyse the effect of DCs infected by BCG on Vγ9Vδ2 T cell properties. Although the role of DCs in the differentiation of Vγ9Vδ2 T cells following infection is still unclear, we demonstrated that BCG infection of human monocyte-derived DCs leads to a rapid and strong activation of co-cultured Vγ9Vδ2 T cells without further stimulation, expanding a functional and competent cytotoxic subset, even if phenotypically immature. Indeed, they are central memory cells but they

can be distinguished in two subsets on the basis of perforin expression: perforin ^{low} or perforin $^{high+}$ Vγ9Vδ2 T cells. A strong cytotoxic activity is associated with this phenotype, which is able to kill freshly added mycobacterial-infected monocytes. Interestingly, they preserve the viability of co-cultured BCG-infected DCs, contributing to the phenomenon called "DC editing" described in other cellular models (Martino *et al* 2007). Although human and murine $\gamma\delta$ T cells have been shown to target mycobacteria-infected macrophages, it is clear that their role is not limited to the cytotoxic function. Recently, it has been shown that $\gamma\delta$ T cells provide an essential early burst of IFN-γ that conditions DCs for an efficient priming of CD8 T cells and for the full development of a protective response (Caccamo *et al* 2006). These findings not only shed light on the role of $\gamma\delta$ T cells and DCs at the interplay between early innate and late adaptive immune response but may also help in the design of novel approaches to the development of an efficient mucosal tuberculosis vaccine via manipulation of $\gamma\delta$ T cells. (figure 1C).

5. Conclusions

The interplay between mycobacteria and the immune system determines the outcome of the infection. Both innate and adaptive immune responses are involved, with different but cooperating mechanisms. The current concept of a strict relationship between innate and adaptive immune responses changes our point of view about infections. Here we have summarized some examples of the complex innate response to mycobacteria and how these elements may interfere with the adaptive immunity. It is expected that greater understanding of the role of innate response in mycobacterial infections will help to design such adjunctive treatment to limit their spread.

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