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The role of cytokines in the initiation, expansion, and control of cellular immunity to tuberculosis

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Summary

Tuberculosis (TB) results from an interaction between a potent immune response and a chronically persistent pathogen. The ability of *Mycobacterium tuberculosis* (Mtb) to induce a strong immune response while being able to resist the ability of the host to clear bacteria provides an excellent tool with which to investigate the role of specific cytokine pathways on the induction, expansion, and control of the effector T-cell response. In this review, the role of interleukin-12p40 (IL-12p40), IL-12p70, IL-23, and IL-27 in the immune response to Mtb are described. We show that IL-12(p40)₂ acts to mediate the activation of dendritic cells to become responsive to homeostatic chemokines. We also show that IL-12p70 is required for the optimal interferon- γ (IFN- γ) T-cell response, which is required for control of Mtb growth. IL-23 can induce IFN- γ responses in the lung if IL-12 is not present, but its major role is in supporting the IL-17 response within the lung. Neither IL-23 nor IL-17 is required for early control of Mtb in the lung. IL-23 and IL-17, however, can be instrumental in vaccine-induced protection. Finally, IL-27 limits protective immunity in the lung, but it is also required for long-term survival. These cytokines are therefore key players in the immune response to TB.

Keywords

cytokine; Th1/Th2/Th17; infectious disease; cell activation; lung; memory

Introduction

Tuberculosis (TB) is an important disease both in terms of its public health implications and because it provides a fascinating insight into the host's ability to respond to and control a chronic pathogen. TB is caused by a pathogen that is wide-spread throughout the human population, and yet it causes disease in only a small percentage (1, 2). The widespread nature of this disease is dependent upon its ability to be spread by aerosol transmission, which is facilitated by immune-dependent tissue-damaging inflammation. The disease is characterized by a substantial specific cellular immune response, which while it can stop the

growth of the pathogen is unable to completely clear infection (3). This ability of the pathogen to co-opt the cellular immune response to provide an aid to transmission while being able to persist provides a model with which to probe the mechanisms that mediate initiation, expansion, and control of the immune response during persistent antigen exposure and inflammation in general.

Many individuals that are exposed to the causative agent of TB, *Mycobacterium tuberculosis* (Mtb), generate and maintain a delayed-type hypersensitivity (DTH) response to purified protein antigens of the pathogen and yet fail to develop clinical disease (1). The basis for this discrepancy between infection and disease is a fascinating area for study but is as yet undefined; this is not, however, the focus of this review. Rather, here the cytokine pathways that are activated during the primary and secondary responses to a productive infection in the mouse model are discussed. Long-term cellular response to a non-productive infection in humans demonstrates that even a modest non-disease causing exposure can lead to the generation of a strong and lasting cellular reactivity. Indeed, the ability of Mtb to initiate strong cellular immune responses has been advantageous to the immunologist in the form of complete Freund's adjuvant; in this reagent, the immunostimulatory ingredient is inactivated Mtb. Long-lived cellular immunity is also generated by bacille Calmette–Guérin (BCG), the current vaccine against TB. While the immunity induced by BCG is successful in limiting disseminated disease such as tuberculous meningitis, it is not reproducibly protective against aerosol infection (4, 5). To improve upon current vaccination strategies, we need to dissect the mechanisms mediating protection and pathogenesis of this disease.

Understanding of the role of specific molecules in Mtb and TB has been enhanced by the use of gene-deficient mice in infectious disease models; this tool led to the discovery that interleukin-12 (IL-12) induction of interferon- γ (IFN- γ) is a key pathway in the control of TB (6-10). While our observations demonstrated the pathway in mice, other studies showed that gene deficiencies in this same pathway lead to enhanced susceptibility of humans to mycobacterial disease (11). It is the induction, expansion, and control of this axis of the cellular response as well as the associated immune pathways during TB that is the focus of this review.

Experimental model of TB

Most of the work discussed within this review involves the mouse model of TB. This highly tractable model allows specific questions of mechanism and causal interactions between events to be satisfactorily tested. Once these mechanisms have been identified, they then allow for contrasting and detailed analysis within non-human primates and humans.

In the low-dose aerosol mouse model, bacteria are delivered via an aerosol cloud of 3–5 μm particles to the lower airways of the lung (12); this approach is taken to be analogous to the natural mode of delivery. In our own work, bacteria are delivered while mice are loose in a chamber and the bacterial cloud surrounds them. This type of delivery has positive and negative aspects. The positive is that all mice are equally exposed, mice are not under stress, and up to 125 mice per chamber can be infected at the same time. This mode of delivery is highly reproducible once the bacterial suspension used to inoculate the mice has been

standardized (12). It has the disadvantage that the mice are covered in bacteria and may obtain a high oral dose as a result of grooming. In alternative aerosol models, a nose-only chamber is used, and this approach delivers suitably sized particles to the lower airway without depositing bacteria on the surface of the mouse. The disadvantages of this model are that the mice are restrained while receiving the bacteria and may be subject to a stress response and that only a limited number of mice can be challenged in one delivery. These caveats aside, it appears that chamber and nose-only delivery results in similar outcomes in regards to bacterial growth and induction of immunity.

Once bacteria are deposited in the lungs, they have a 3-day lag period when they do not grow; subsequently, they grow with an average of a 28 h doubling time and reach approximately 5 logs by 20 days (Fig. 1). The cessation of bacterial growth correlates with the arrival of IFN- γ -producing antigen-specific major histocompatibility complex (MHC) class II-restricted T cells, the expression of IFN- γ mRNA, and the upregulation of activation markers such as MHC class II on the CD11c⁺ population in the lung (13) (Fig. 1). Once bacterial growth has ceased, the immune response keeps bacteria in check for a prolonged period of time; bacterial re-growth can occur if inhibitors of immunity are delivered. These inhibitors include anti-CD4 T-cell antibody, anti-tumor necrosis factor (TNF) antibody, and inhibitors of nitric oxide synthase (14). Cessation of bacterial growth does not occur if the cytokines IFN- γ , IL-12p40, or TNF are absent (6, 8, 10, 15). Control does occur for a short period if IL-12p35 is absent but is incomplete (10). Although bacterial growth is slowed in $\alpha\beta$ T-cell-deficient mice and in class II MHC-deficient mice, this effect is not sustained, and mice eventually succumb to infection. Conversely, class I MHC-deficient mice are only modestly more susceptible to aerosol infection than wildtype mice (3). Similarly, mice lacking the mediators of anti-mycobacterial macrophage functions, such as expression of inducible nitric oxide synthase (16) or LRG-47 (17), are unable to control bacterial growth.

While we have clear understanding of what limits bacterial growth, we do not see elimination of Mtb in most vertebrate hosts that are experimentally exposed (18). Indeed in the mouse model, bacteria persist at a fairly constant level for months without outward ill effect, although the cellular inflammation within the lung progressively deteriorates over time (19). The ability of the bacteria to persist suggests that Mtb, although unable to grow in an immune environment, is able to survive within that same environment. The inability of the immune response to clear bacteria makes the kinetics of the cellular response a key element in determining the bacterial burden following aerosol infection. Specifically, as bacteria grow in the lung until cellular immunity is expressed, the earlier this expression occurs, the lower the bacterial burden will be. Unfortunately, the accumulation of antigen-specific cells in the lungs is slow, taking up to 20 days to reach an effective level (Fig. 1). Clearly, the most straightforward way of accelerating the immune response is to generate memory cells by vaccination. This approach can reduce the bacterial burden in vaccinated hosts, but it does not do so efficiently, as it accelerates the response by 5 days, leaving 15 days of bacterial growth to occur before cessation (20, 21). It is possible that it is the slowness of the cellular response to aerosol infection in BCG-vaccinated humans that is the cause of the poor protective nature of this vaccine. Determining how vaccine-induced

memory is expressed in the lung in response to aerosol challenge may therefore allow for more rational design of vaccines to combat this disease.

While the mouse model of Mtb infection does not result in the same type of immunopathologic consequences seen in humans and non-human primates, it does provide a tractable model for the investigation of the regulatory interactions of specific cytokines during immune responses. Indeed, comparative analysis of the role of cytokines such as IL-12p70, IL-23, IL-27, and IL-35 (Fig. 2) using infectious disease models such as Mtb, *Toxoplasma gondii*, *Leishmania* spp., and *Listeria monocytogenes* have highlighted the different roles these apparently similar cytokines play when confronted with unique challenges (22-24). It is the role of these cytokines that we discuss here.

Induction of cellular responses to Mtb

The cellular response to Mtb following a low-dose aerosol infection is relatively slow, when compared with responses to viral or fast growing bacterial infection. This slowness may reflect an immunomodulatory activity of the pathogen or simply be a result of the low dose and mode of challenge. When Mtb enters the lung in the 3–5 μ m droplets generated by a cough of a diseased individual, these droplets contain only a few Mtb that deposit in the alveolar space wherein the density of host cells is low. The bacterium is also a fairly slow grower taking approximately 28 h to double, even *in vivo* (25). One might imagine, therefore, that the chances of a bacterium encountering the conditions required to generate a host cellular response are low and are only increased as the number of bacteria increases or as the bacteria arrive at a lymph node. Indeed, several recent reports show conclusively that the very first elements of T-cell activation occur in the lymph nodes draining the lung and not within the lung itself. The first report in this regard used the development of effector function as an indicator of T-cell activation and by this method showed that functional cells occurred in the draining lymph node before any other organ and coincided with the arrival of bacteria in the node (26). More recently, the activation of naive T cells has been measured using T-cell receptor transgenic (TCRTg) T cells specific for an IAb-restricted epitope of the early secreted antigenic target 6 kDa protein (ESAT-6) or for an IAb-restricted epitope of the essential mycolyl transferase of Mtb, Ag85. Using these cells, which allow for the detection of the earliest activation events of naive T cells, it is clear that naive T cells upregulate CD69 (a sign of TCR ligation) (27) and begin to proliferate (28) in the draining nodes of the lung. Indeed, just as when effector function was used to detect early T-cell activation, the timing of this activation coincides with the arrival of bacteria in the lymph node (27, 28). The problem is, however, that the bacteria do not arrive in the lymph node until day 10 following low-dose aerosol infection (27), and thus, the bacterial burden in the lung is already established when T cells are first activated and gets even higher as the T cells take time to proliferate and become effector cells. The activated effectors traffic back to the infected lung tissue by day 15 and only stop bacterial growth by day 20 (27). In our own studies, we were able to detect an acceleration of bacterial arrival in the lymph node and T-cell activation if a higher aerosol dose was delivered; however, this acceleration was only by 2 days, despite a 16-fold increase in dose (27).

In studies using the Ag85-specific TCRTg T cells, an association between bacterial challenge dose and kinetics of the T-cell response was not readily apparent, and the authors hypothesized that the bacteria were infecting a cell that was either unable to migrate from the lung to the lymph node or was being inhibited (28) from doing so by the bacteria. As we were able to see some impact of bacterial dose upon kinetics, it seems likely that it is the initial location of the bacteria within a non-motile cell rather than an active inhibition of cell migration that slows the initiation of the T-cell response. Whatever the mechanism, it is clear that this delay in T-cell activation is a key element in the ability of Mtb to establish infection within the lung.

Migration of Mtb-stimulated dendritic cells from the lung to the lymph node

The route of infection and the low dose of natural challenge compromise our ability to detect bacteria directly following delivery to the lung. However, when fluorescent bacteria are delivered to the lung, these bacteria can be seen in dendritic cells (DCs) and cannot be detected in the lymph node if the chemokine pathways responsible for DC migration to the lymph node are absent (29). Delivery of Mtb-infected DCs to the lung also results in the DCs expressing CCR7 and migrating to the lymph node (30). While not yet proven, it is likely that DCs draining the lung are responsible for the migration of the bacteria to the lymph node, but it is also likely that these are not the first cells infected and that infection of these cells requires growth within the alveolar macrophages which are the most common cell in the alveolar space into which the bacteria are delivered. We have been investigating the role of IL-12 in the regulation of DC migration from the lung to the lymph node and the subsequent initiation of T-cell responses.

We were prompted in our investigation by the fact that while mice that lacked IL-12p70 or IL-23 could accumulate activated CD4⁺ T cells in the lung by day 21 of infection, mice that lacked IL-12p40 could not (31). IL-12p40 is thought primarily to be an antagonistic cytokine that inhibits the action of IL-12p70 and likely IL-23 by competitive binding to the common receptor molecule for these cytokines, IL-12R β 1 (Fig. 2). This concept was, however, always a little counter-intuitive, as IL-12p40 is often produced in excess of and before IL-12p70 when cells were stimulated with bacterial ligands, and this would appear counterproductive in terms of initiating a protective response. We therefore investigated the role of IL-12p40 in the initiation of the T-cell response in the lung.

In our first analysis, we found that when we delivered a bacterial stimulus such as Mtb or lipopolysaccharide (LPS) to the lung in the presence of a fluorescent dye, CD11c⁺ fluorescently labeled cells appeared in the lymph node at a higher frequency than when the fluorescent dye was delivered alone (31). This increased migration resulting from bacterial stimulation occurred in intact mice and in mice lacking either IL-12p35 or IL-23p19 but not in mice lacking IL-12p40, suggesting that this migration was related to the expression of IL-12p40 but not IL-12p70, IL-35, or IL-23 (31) (Fig. 2). To investigate this response *in vitro*, we cultured DCs from intact and gene-deficient mice, stimulated them with Mtb for 3 h, and analyzed the subsequent ability of these stimulated DCs to migrate toward the homeostatic chemokines associated with recruitment to the lymph node, CCL19 and CCL21. If the DC lacked IL-12p40, then migration in response to CCL19 and CCL21 remained at

the level of the non-activated DC, despite exposure to Mtb (31). In contrast, DCs lacking IL-12p35, IL-23p19, or both of these cytokine components became responsive to CCL19 and CCL21 when exposed to Mtb (31). These data suggested that bacterially induced responsiveness to homeostatic chemokines was dependent upon IL-12p40 and not IL-12p70, IL-35, or IL-23. To determine which cytokine was mediating this bacterially induced responsiveness, we delivered recombinant cytokine to the IL-12p40-deficient DCs during the period of Mtb exposure before assessment of chemokine responsiveness. Importantly, we found that we could restore the ability of IL-12p40 gene-deficient DCs to migrate toward CCL19 if we provided recombinant IL-12(p40)₂ to the DCs; this response was not restored if the homodimer was denatured or IL-12p70 was used (31). Owing to the rapid nature of the response, it is not clear how the IL-12(p40)₂ is acting. It is unlikely that any new proteins are being produced, but it is possible that receptor-mediated signaling may be being altered. We are currently investigating the cellular events that occur upon IL-12(p40)₂ exposure, and we have found that nuclear factor- κ B nuclear migration is rapid in response to this cytokine (unpublished data).

The IL-12(p40)₂-induced migratory response is likely regulated and limited to bacterial or other pathogen-related signals. If this were not the case, then DCs would deliver many inert particles to the lymph node and the likelihood of inappropriate stimulation of immunity would be increased. IL-10 is a likely candidate for this regulation, as in the absence of IL-12p40, IL-10 is readily expressed by bacterially stimulated DCs and the delivery of IL-12(p40)₂ reduces this IL-10 response (31). Further, in IL-10-deficient mice, the initiation of cellular response to aerosol BCG is more rapid than in intact mice, suggesting that IL-10 may be regulating this response (32). We hypothesize that the DCs within the lung naturally produce IL-10, which limits the responsiveness of DCs to migration, upon bacterial exposure the DCs generate excess IL-12(p40)₂, and this acts in an autocrine manner via IL-12R β 1 to allow the DCs to become responsive to chemokine and migrate to the lymph node. We are investigating this hypothesis. There is a growing data set supporting an agonistic role for IL-12(p40)₂ in initiation of cellular responses, and this cytokine should be considered as an active component of the response whenever it is detected (33).

Initiation of cellular responses by Mtb-stimulated DCs

While DC migration to the lymph node allows for the immune system to sample the environment, the most important activity of the bacterially stimulated DC is to drive the activation of naive T cells. To assess the role of IL-12p40 in the ability of bacterially stimulated DCs to initiate naive T-cell activation in the lymph node, we used Mtb-activated antigen-exposed *in vitro* cultured DCs to traffic antigen from the lung to the lymph node. We then monitored the ability of naive antigen-specific T cells to become activated, expand, and become effector cells in the lymph node. We found that while intact, IL-12p35-deficient and IL-23p19-deficient DCs were able to initiate an effector T-cell response to antigen delivered in this way, IL-12p40-deficient DCs were unable to do so (31). These data suggested that while IL-12p70, IL-35, and IL-23 were not required for this activity, IL-12p40 was. Delivery of IL-12(p40)₂ during the period of activation before delivery to the lung resulted in a restoration of the ability of the IL-12p40-deficient DCs to activate and expand a population of antigen-specific T cells in the lymph node (31). Importantly, we

assessed the ability of IL-12p40-deficient DCs to efficiently activate T cells without the need for migration and found that these cells were as good as intact DCs at initiating activation and expansion of naive T cells when used *in vitro* or when delivered systemically (31). These data suggest that failure of IL-12p40-deficient DCs to initiate T-cell activation following delivery to the lung was due to their failure to migrate rather than an innate inability of these DCs to activate T cells.

Using the DC transfer model, it was shown that that Mtb-stimulated DCs delivered to the lung could effectively initiate T-helper 1 (Th1) cell development (30), and this study further supports the concept that Mtb is an efficient initiator of cellular immunity. It also provides an excellent tool to investigate the need for the expression of specific cytokines in the initiation of IFN- γ -producing T cells in a defined model *in vivo*. We, therefore, determined the ability of gene-deficient DCs to activate cells to an effector state and found that while the IL-12p35-deficient DCs delivered to the lung could activate and expand a T-cell population in the lymph node, they induced a low number of antigen-specific IFN- γ -producing cells (31). In contrast, intact DCs and DCs lacking only IL-23p19 were able to activate, expand, and generate an IFN- γ -producing effector cell population in the lymph node when activated by Mtb and delivered to the lung (31). The inability of IL-12p35-deficient DCs to drive IFN- γ production in the naive T cells despite the fact that the host animal was intact for IL-12p35 was illuminating, as it suggested that only the Mtb-activated DCs were involved in the response and that these DCs did not drive bystander cytokine release from other cells within the node. As the IL-12p40-deficient DCs were not able to expand or activate naive T cells when delivered by this route, we did not see any induction of IFN- γ -producing cells. Indeed when IL-12(p40)₂ was used to restore the ability of the DCs to stimulate naive T cells, it still failed to induce IFN- γ -producing cells, as these DCs were not able to produce IL-12p70 (31). These data further support the concept that the development of T-cell effector function in this model is directly dependent upon the nature of the DC delivered to the lung and could not be compensated for by the intact host cells within the node.

In an expansion of the IL-12p40 dependency of bacterially induced chemokine responsiveness in DCs, we have recently seen that *Yersinia pestis* induces IL-12(p40)₂ in DCs and that this cytokine is required for the induction of DC migration. In a notable difference from the Mtb model, this induction occurs only when the bacterium is grown at a temperature mimicking the invertebrate host and not when grown at vertebrate host temperature. At the low temperature, the lipid A molecule is different from that produced when grown at vertebrate temperature (34). Specifically, we found that when grown at low temperature, the bacteria stimulate DC responsiveness to chemokines via an IL-12p40 and TLR4-dependent mechanism, whereas when it is grown at the vertebrate temperature, it fails to initiate this response (35). It is tempting to think that *Y. pestis* finds it useful to induce DC migration when entering from the flea host and then turns off this activation as it reaches 37 °C within the lymph node of the host. The fact that both Mtb and *Y. pestis* are able to modulate the chemokine responsiveness of DCs in an IL-12p40-dependent mechanism speaks to a broad impact of this mechanism in the induction of cellular responses. It is also

important to remember that IL-12p40 can act as an antagonist and may modulate IL-12p70 and IL-23 activity later in infection as was seen recently in a *Leishmania major* model (36).

Expansion of effector T cells

Once T cells have been activated by Mtb, they progress to become effectors with apparently normal kinetics (27), and this observation is counter to the hypothesis that Mtb is a poor inducer of T cells. This normalcy of the response does not negate the possibility that infected cells at the site of infection may be less able to promote effector cell survival or function. Indeed, recent data suggest that the cells at the effector site are poor stimulators of antigen-specific T cells (29).

Once T cells are activated, they expand and are recruited to the infected lung where they are thought to mediate control of bacterial growth via IFN- γ -induced activation of infected phagocytes. This pathway of control is based on the data showing that the primary correlate of bacterial control is the expression of IFN- γ mRNA in the lung and that antigen-specific CD4⁺ T cells capable of making IFN- γ arrive at the same time as the cessation of bacterial growth in the lung (*Fig. 1*). The accumulation of IFN- γ -producing CD4⁺ T cells in the lung is dependent upon IL-12p70, and in its absence, the numbers of such cells are reduced (10). Despite this low number of cells, reduced growth of bacteria does occur in the absence of IL-12p70 (i.e. IL-12p35 gene-deficient mice), but this response cannot be maintained for a prolonged period of time. We have shown that in the absence of IL-12p35, the resulting antigen-specific IFN- γ -producing cells are dependent upon IL-23, as IL-12p35 IL-23p19 double gene-deficient mice are as unable as IL-12p40-deficient mice to generate IFN- γ -producing cells capable of limiting bacterial growth within the lung (37). The ability of IL-23 to mediate an IFN- γ response in the absence of IL-12p35 is intriguing, as the absence of IL-23 in the presence of IL-12p70 does not result in any compromise of the IFN- γ response, suggesting that this response is maximal in the presence of IL-12p70 and does not require IL-23 (37). It may also reflect an as yet unknown role for IL-35 [which utilizes IL-12p35 (24, 38, 39)] in regulating the IL-23-induced IFN- γ response, but this possibility has not been addressed in the Mtb model. We do know, however, that in the absence of IL-12p35, there is an enhanced antigen-specific IL-17-producing population, which may reflect a regulatory role for either IL-12p70 or IL-35 in this response (37) (*Fig. 3A*).

There are several models wherein a limited IFN- γ response is capable of stopping bacterial growth for some time but which subsequently fails to limit bacterial growth. In humans, the absence of efficient IL-12p40, IL-12R β 1, or IFN- γ -mediated mechanisms also leads to increased susceptibility to mycobacterial infection (40). In our own studies, we identified a model wherein an IFN- γ -producing T-cell response to a limited repertoire of antigens was expressed. In this model, while bacterial growth was initially controlled and macrophage activation was seen in the lung, the ability of the mice to contain the infection long term was compromised (41). Further, it appears that when IL-12p40-deficient mice are provided with IL-12p70, they are able to limit bacterial growth, but this response is lost if IL-12p70 is removed (42). IL-12p70 is also required for the ability of antigen-specific memory T cells to maintain their efficacy *in vivo* (42). These data demonstrate that IL-12p70 is required not only for the optimal initiation of the cellular response but also for its long-term expression.

Understanding how IL-12p70 acts to maintain IFN- γ -effector cell function will be highly informative with regard to our understanding of how effector T cells maintain their function in the face of chronic antigen exposure and inflammation. It is clear that long-term control of the infection in the lung requires broad antigen specificity and constant maintenance of the factors required in effector cell generation.

The ability of IL-23 to impact the generation of effector T cells during TB has been of interest due to the fact that IL-12p40 and IL-12R β 1-deficient humans are susceptible to *mycobacterial* disease and that their T cells are defective in their response to both IL-12p70 and IL-23. In mice, the absence of the p19 subunit of IL-23 does not result in substantial differences in early disease progression, with bacterial burden in the lung and dissemination to other organs being identical in the presence or absence of this subunit (37). This inability of IL-23 to impact bacterial burden was also seen for mice infected with BCG (43). There is a substantial reduction in the induction of IL-17 mRNA within the lung of infected IL-23p19-deficient mice compared with wildtype mice, and this is also reflected in the loss of the IAb-restricted IL-17-producing cellular response (37). Importantly, the consequences of this absence are minimal during the early primary response to Mtb infection, although there is a modest alteration in the nature of the inflammatory response as infection progresses (37). Others have delivered IL-23 to the lung early in infection and have seen improvement in terms of bacterial burden, suggesting that the IL-23 response is suboptimal (44). Importantly, IL-23 is able to drive IFN- γ -producing T cells (37), and thus, any protective effect may be through induction of IFN- γ rather than via induction of IL-17 responses.

A key aspect of how any pathogen drives the effector T-cell population is the type of cytokines released by the antigen-presenting DCs during activation; this cytokine pattern is defined to a large degree by the ligation of various pattern recognition receptors (PRRs) by the pathogen. It is becoming increasingly clear that this cytokine response is regulated as a result of the sequence and type of PRR that are ligated by the pathogen (45). In recent studies using human DCs, the induction of IL-12 and IL-23 was measured in response to Mtb and various Toll-like receptor (TLR) ligands. It was shown that Mtb induced IL-23 in preference to IL-12 but that the presence of IFN- γ reversed this to an IL-12-dominant response. TLR2 ligation was found to promote IL-23 in this model (46). These data suggest that Mtb is a potent inducer of IL-23 in human cells and provides further support for investigation of this cytokine and its function in TB.

The ability of the mycobacteria-infected DCs to promote specific effector functions in T cells during infection has been investigated. Indeed, the comparison between *Mycobacterium bovis* BCG and Mtb may be informative, as the antigen-specific IL-17 response is downregulated by IFN- γ during BCG infection (47), whereas this regulation is not as strong during Mtb infection when both an IFN and an IL-17 antigen-specific response can be detected throughout infection (37). It is possible that BCG and Mtb activate DCs to slightly different degrees and that this is reflected in the relative ability of each bacterium to drive specific effector cells. In this regard, we have seen induction of IL-12p40, IL-12p35, and IL-23 mRNA in DCs exposed to Mtb (37), but when we measured cytokine production by DCs infected with *M. bovis* BCG, we found that while IL-23 was induced by DCs,

IL-12p70 was produced only when IFN- γ was present (47). Of course the level of other cytokines present during activation of naive T cells is instrumental in the differentiation process, specifically transforming growth factor- β (TGF- β) or IL-6, as these cytokines are crucial to the development of IL-17-producing T cells, and it is their relative levels which differentiate between regulatory and Th17 cells (48, 49). While we have not studied the role of TGF β or IL-6 in Th17 cell generation in TB, we have analyzed the IFN- γ response in the absence of IL-6 and have found this to be reduced (50). These data suggest that the interaction between IL-6, IL-17, and IFN- γ is not as straightforward as the data from other models might suggest. We are currently investigating the relative roles of these cytokines in the induction of Th1, Th17, and regulatory T cells in TB.

IL-27 was first considered to be an important component in the initiation of Th1 cells; however, it soon became clear that this cytokine is pleiotropic in function and acts to control cellular immunity to systemic pathogens such as *T. gondii* (23). With regard to Mtb infection in the absence of IL-27R activity, two separate reports have shown that in mice lacking IL-27R activity, the bacterial burden in the lung and other organs following aerosol infection with Mtb is significantly reduced (51, 52). In one study, this increased control of bacterial burden was correlated with increased inflammatory cytokine responses in the lung, increased macrophage function, and increased numbers of IFN- γ -producing cells (52). In our own study, we saw increased cellular accumulation within the lung lesions, while the IFN- γ and inflammatory cytokine response was not increased (51). In both studies, the inflammatory response was impacted, and when survival of infected mice was investigated, it was shown that mice lacking IL-27R activity became moribund and exhibited an accelerated development of the end-stage pathology associated with Mtb infection of the mouse (51, 52). Thus, although the mice had lower bacterial burdens, they were more susceptible to the pathological events that lead to death in this model. These data highlight the fact that the inability of the host to eliminate Mtb may be a survival advantage, as the immunologic response required to reduce bacteria may be more damaging than the actual infection.

The mechanisms mediating the altered response of IL-27R-deficient mice to Mtb are still under investigation. The pleiotropic nature of the molecule makes dissection of its function on T cells versus other cells essential for a clear understanding of its ability to impact T-cell activation. We have demonstrated recently that the absence of this receptor on CD8⁺ T cells severely compromises the ability of antigen-specific T cells to produce IFN- γ during both influenza and Toxoplasma infection. Importantly, this deficiency was seen when all other cells, apart from the CD8⁺ T cells, were sufficient for the receptor (53). We have not yet utilized this type of analysis in the Mtb infection, but in our studies of ex vivo antigen-induced IFN- γ production, we did determine that mice lacking IL-27R activity had IAb-restricted cells in the lung that produced significantly less IFN- γ on a per cell basis than did the same cells from wildtype mice (51). The dominant view of IL-27 activity on T-cell function is that it regulates Th1, Th2, and Th17 cells during infection and autoimmune inflammation (54). IL-27 has also been shown to be a potent inducer of IL-10 (55), and it is tempting to speculate that it is the absence of IL-27R-induced IL-10 in the Mtb model that allows the IL-27R-deficient mice to better control bacterial burden in the lung and also that

the absence of IL-10 leads to the premature immunopathologic morbidity of these mice. Alternatively, the ability of the IL-27 to regulate a potentially damaging IL-17 response may also be the cause of the accelerated morbidity. Unfortunately, clear data supporting either of these models has not so far been forthcoming, and the mechanisms mediating the observed Mtb phenotype in the IL-27R-deficient mice are as yet unclear.

Expression of memory response to Mtb infection in the lung

In contrast to the primary response, IL-23 and IL-17 appear to play an important role in the expression of vaccine-induced protection against pulmonary challenge with Mtb. To investigate CD4⁺ T-cell memory in the aerosol challenge model of TB, we have used a defined subunit vaccine wherein the only antigen is a peptide containing an IAb-restricted epitope. This tool allows us to assess the kinetics, nature, and efficacy of a strictly IAb-restricted protective memory T-cell response. Performing an analysis of the protective cellular response is much more difficult if one tries to examine memory in previously infected drug-cured mice or if a live attenuated vaccine such as BCG is used due to the variety of memory T cells induced as well the difficulty in accounting for the effects of previous infection on the host. We have used a subunit vaccine model in gene-deficient mice to investigate the role of the IL-12p40-dependent cytokines in the induction and expression of the protective CD4⁺ T-cell memory response to aerosol challenge with virulent Mtb.

In wildtype mice that have been vaccinated once subcutaneously with the defined subunit vaccine or mice that have been BCG vaccinated or which have experienced drug-cured previous infection, there is an accelerated IFN- γ response in the lung (13). This accelerated response means that bacteria are stopped from growing 5 days sooner than in a naive animal, and an approximately 10-fold reduction in bacterial burden occurs. Despite this earlier response, the bacterial burden is not reduced past this 10-fold level, and thus, the presence of memory T-cell responses to Mtb does not result in a very effective level of protection in the lung. One hypothesis for this apparent inefficient response is that the memory induced by these various mechanisms (live attenuated vaccine, previous drug-cured infection, or subunit vaccine) is innately compromised. However, when these same mice are challenged via the systemic route, bacterial growth is stopped almost immediately, suggesting that if memory is activated and recruited efficiently, then it can stop bacterial growth effectively (20). We hypothesize that the inefficient expression of memory in vaccinated mice is a result of the route and dose of infection and that memory responses are not inhibited by the bacteria within the lung but that they are simply not stimulated by the infection. We also hypothesize that this is why BCG vaccination is efficient at controlling disseminated disease in humans but why it is only variably effective against pulmonary disease (4, 5). To begin to test these hypotheses, we have used the subunit vaccine to monitor the antigen-specific CD4⁺ memory T-cell response and found that antigen-specific IFN- γ T cells do not accumulate in the lung until day 15 of infection and that this correlates exactly with increased class II MHC expression on CD11c⁺ cells and cessation of bacterial growth in the lung (13). These data lead us to postulate that if we could determine how the memory IFN- γ -producing CD4⁺ T cells arrive in the lung, we would be better placed to induce them to accumulate more rapidly. Knowing that IL-12 and IL-23 were involved in the induction of specific effector

cell types that responded to Mtb infection, we therefore determined the role of these cytokines in the induction and expression of CD4⁺ T-cell memory.

Vaccinated IL-12p35-deficient mice were able to express the accelerated antigen-specific CD4⁺ T-cell response, activation of CD11c⁺ cells, and cessation of bacterial growth seen in the vaccinated wildtype mice. However, due to the absence of IL-12p70, the number of antigen-specific IFN- γ -producing cells both in the primary and secondary response was reduced, and therefore although bacterial burden was reduced by vaccination, it remained higher both in the naive and vaccinated IL-12p35 mice than in the vaccinated wildtype mice (13). These data suggest that either the absence of IL-35 or the presence of compensatory IL-23 resulted in an IFN- γ memory response but that IL-12p70 was required for an optimum response. In contrast, when IL-12p40 was absent, no sign of antigen-specific IFN- γ -producing responses were seen in either primary or recall responses (13). This finding is not surprising, as neither IL-12 nor IL-23 were present, and we have no evidence that the activated CD4⁺ T cells induced in the presence of IL-12p40 are able to mediate any protective function. Based on the data from the primary response to Mtb in IL-23p19-deficient mice, we did not expect to see a phenotype in this mouse in the subunit vaccine model; however, we persevered with the experiment. We were surprised to see that while expression of IFN- γ -producing cells, CD11c⁺ activation, and cessation of bacterial growth occurred as expected during the primary response, there was no vaccine-induced acceleration of this response in the absence of IL-23p19. To investigate the reason for this observation, we examined the cellular response to vaccination and found that in the absence of IL-23p19, vaccinated mice generate an IFN- γ -producing IAb-restricted memory population, whereas they fail to generate an equivalent IL-17-producing response (13). In contrast, vaccinated wildtype and IL-12p35-deficient mice generated an IL-17-producing memory population, not only in the spleen and lymph nodes but also in the non-inflamed lung (13). When wildtype mice are vaccinated and the antigen-specific cells expanded *in vitro* with IL-23 and then transferred into wildtype or IL-23p19-deficient mice, the transferred cells can respond to infection regardless of the presence of IL-23 (13). These data suggest that IL-23 is required during priming and expansion of the protective memory response rather than during the effector stage. In another surprising observation, we found that the vaccine-induced IL-17-producing cells transferred into either wildtype or IL-23p19-deficient hosts populate the lung much more efficiently than do the IFN- γ -producing memory cells. We think that this result suggests that these two cell populations have different abilities to populate normal peripheral tissues. In this regard, we have demonstrated that the cells populating the lung express the CCR4 chemokine receptor that has been associated with migration of cells into tissue (13) (*Fig. 3B*).

Upon challenge, we knew that the IFN- γ -producing cells expanded to effective levels by day 15; however, we were surprised to see even more dramatic acceleration of the antigen-specific IAb-restricted IL-17 response in the wildtype vaccinated mice. This response reached detectable levels by day 12 postinfection (13). Importantly, it is this early IL-17 response that is lost in the vaccinated IL-23p19-deficient mice, and along with the loss of this response, there is no early granuloma formation or accumulation of activated CD4⁺ T cells (13). To determine what aspect of the response was lost in the absence of IL-23p19, we

performed a focused comparative array analysis of genes expressed at day 12 in the lungs of challenged vaccinated wildtype and IL-23p19-deficient mice. Along with the significant expression of IL-17 (but not IFN- γ), we found that CXCL9, CXCL10, and CXCL11 were induced in the vaccinated wildtype but not in the vaccinated IL-23p19 gene-deficient lungs (13). Indeed, if we blocked IL-17 in the vaccinated wildtype mice, we were able to significantly reduce the induction of chemokine mRNA. Delivery of IL-17 to the lungs of the vaccinated IL-23p19-deficient mice restored the early accumulation of IFN- γ -producing memory cells (13), supporting the hypothesis that early expression of IL-17 acted as a mediator of cellular recruitment. This is not an unusual function for IL-17, but it is more often associated with granulocytes and induction of chemokines associated with their recruitment. Although we did not demonstrate direct induction of CXCL, CXCL10, and CXCL11 by IL-17, all three chemokines have IL-17 responsive elements in their promoter region.

Owing to the current interest in determining how IL-17-producing T cells are induced, we also examined the role of specific adjuvant components in the induction of the IFN- γ and IL-17-producing memory cells. The small cationic liposome dimethyl dioctadecylammonium bromide (DDA), used as an antigen depot in the adjuvant formulation, was able to induce IL-17 and IFN- γ -producing cells *in vitro* and indeed was the only component that induced TGF γ mRNA in DCs (13). The other components of the adjuvant, monophosphoryl lipid A and trehalose dimycolate, were effective inducers of IL-12p35, IL-23p19, and IL-12p40, as well as IL-6 and IL-1, and the combination of these components with DDA resulted in the most potent induction of memory cells *in vivo* (13). We are currently investigating the most potent inducer of IL-17-producing cells capable of populating the non-inflamed lung; this study will be informative not only for the vaccine field but also for those studying autoimmune disease.

We also examined the cellular response within the lymph node following vaccination and found that IL-23R mRNA was expressed earlier than was IL-12R β 2 mRNA on the CD4⁺ T cells in the lymph node and that IL-23p19 mRNA was expressed in the IL-12p40-expressing CD11c⁺ cells in the node (13). Interestingly, the expression of IL-17 mRNA by CD4⁺ T cells preceded the mRNA for IFN- γ , but this message was not dependent upon the presence of IL-23 in the host. These data show that while IL-23 and the ability of CD4⁺ T cells to respond to IL-23 is present early during activation of the cellular response to vaccination, IL-23 is not directly responsible for the induction of the CD4⁺ T-cell IL-17 response. When proliferation of naive T cells in response to vaccination is examined within the presence or absence of IL-23, however, the absence of IL-23 reduces the proliferation of the naive T cells (13), suggesting that IL-23 is a growth factor for the newly responding cells. The data demonstrating a reduced population of antigen-specific IL-17 producing cells in the IL-23p19-deficient hosts suggest that while IL-23 is not required for initiation of this T-cell population following subunit vaccination, this population does require IL-23 to become established.

Our working model of the role of IL-23 and IL-17 in vaccine-induced protection is that during subunit vaccination, ligation of certain PRRs induces cytokines in DCs that promote differentiation of different subsets of memory T cells. One subset is the expected IFN- γ -

producing memory cell that is dependent upon IL-12p70 for optimal induction, and this subset populates the spleen and lymph nodes and is recruited to inflamed tissue to mediate macrophage activation and thereby cessation of mycobacterial growth. In contrast, a second subset makes IL-17 upon restimulation and is induced by adjuvant-activated DCs, likely via the expression of IL-6 and TGF β . This population of cells is able to populate the spleen, lymph node, and normal lung interstitium and requires IL-23 to become an established long-lived population. We propose that these IL-17-producing memory cells then act as a surveillance population, and when Mtb enter the lung, the memory cells can respond rapidly by dividing and producing IL-17. This IL-17 then induces CXCL chemokines capable of recruiting CXCR3 expressing IFN- γ -producing memory cells, which then activate infected phagocytes and mediate cessation of bacterial growth (Fig. 3B). We are testing this working model.

Control of chronic inflammation

Despite the ability of vaccination to mediate a modest level of protection, bacterial persistence is a key aspect of Mtb infection in the presence or absence of vaccination. This persistence is a product of the inability of the vertebrate immune system to dramatically reduce the viability of Mtb once the infection is established, and therefore, clearance of the infection is rarely achieved. The balance between host protection and pathogen-induced immunopathologic consequences is therefore one that must be maintained for a prolonged period. Importantly, this balance is also maintained within the lung, which is an organ that must resist inflammatory responses to function effectively. In addition to this balance, transmission of Mtb relies on the development of a caseating lesion within the lung and delivery of bacteria into the sputum for aerosolization when the patient coughs. These factors make for a complex and dynamic interaction between host immune response and the pathogen. The role of cytokines in mediating the balance and limiting tissue damage is not yet fully defined; in particular, it is difficult to isolate an effect of a cytokine on bacterial growth versus a direct effect on the immune response.

In the absence of IFN- γ , there is uncontrolled growth of Mtb in the lung, and death is rapid (6). Despite the absence of this cytokine, however, activated CD4⁺ T cells can be detected within the lung, and associated with this exuberant T-cell response is a rapid and extensive accumulation of granulocytes at the lesional sites (9) with most of these cells being eosinophils (6, 9). While in this situation it is difficult to distinguish the impact of rampant bacterial growth from the regulation of immunity, other less virulent mycobacterial infections are more informative. In the absence of IFN- γ , *M. leprae* infections of the murine footpad result in a much increased level of inflammation, while there is little effect on the bacterial growth (56). Further, in a murine model utilizing *M. avium*, there is little impact on bacterial growth but a substantial change in the nature of the inflammatory response (57). Together these data suggest that IFN- γ promotes a mononuclear response to mycobacterial infection but that it also acts to regulate the T-cell response and thereby limit immunopathologic consequences (Fig. 3C).

While not strictly cytokines, the enzymes required for generation of nitrogen and oxygen radicals, inducible nitric oxide synthase (iNOS) and p47phox, are also instrumental in the

control of immunopathology. Mice lacking these enzymes and infected with either Mtb or *M. avium* exhibit increased pathologic responses and an increase in neutrophil recruitment and airway damage (57). One other regulatory pathway implicated in limiting control of Mtb is that mediated by the lipoxins, whose absence leads to increased anti-Mtb activity both during primary and secondary responses (58). Whether this regulatory pathway is important in survival of chronic infection has not yet been reported.

As discussed above, mice lacking IL-27R are able to control bacterial growth better than wildtype mice; however, these mice die more rapidly than did the wildtype mice (52). These data suggest that the host accommodates a certain level of bacterial burden as a means to maintain lung function and limit inflammation. In this regard, recent data have shown that regulatory T cells accumulate within the lungs of Mtb-infected mice. Using an elegant depletion model, it was shown that these regulatory T cells were serving to limit the ability of the host to control bacterial growth (59). Whether this reduced regulatory activity altered the long-term survival of the mice or indeed the immunopathologic consequences has not yet been fully addressed.

One of the strongest inhibitors of the IL-12/IFN- γ pathway is IL-10; however, the role of this cytokine in TB is somewhat disputed. In humans, an increased ability of macrophages to produce IL-10 when stimulated with TLR ligands such as LPS is associated with an increased tendency to develop primary progressive TB (60). This observation suggests that the level of IL-10 expressed by the innate response to Mtb may either limit the ability of the host to initiate an acquired response or that the IL-10 could inhibit the expression of the acquired response. In support of the first potential defect, the absence of IL-10 results in an autocrine-induced acceleration of DC migration from the lung to the draining lymph node upon mycobacterial infection (32). In support of the second potential defect, we have found that in Mtb-infected CBA mice, macrophages in the lung express high levels of IL-10, and this expression correlates with the inability of this strain of mouse to control bacteria as efficiently as B6 mice (61). B6 mice do not make a strong IL-10 response to Mtb, and in the absence of this cytokine (i.e. B6.il10^{-/-} mice), there is little impact on disease development (62). In contrast, reduction of IL-10 activity in CBA mice does allow the mice to reduce bacterial burden during the chronic phase of disease (63). We hypothesized that we could make B6 mice more susceptible to Mtb infection if we made them over-express IL-10. We made use of an IL-10 transgenic strain, wherein IL-10 was expressed under the IL-2 promoter. In this model, we saw that the over-expression of IL-10 resulted in a reduced ability of mice to control the bacteria compared with wildtype B6 mice (61). These data suggest that while IL-10 is not an automatic player in the immune response to Mtb, it can be, and when it is, it is detrimental.

The regulation of the cellular response during chronic Mtb infection is very difficult to dissect, and as can be seen from the discussion above and *Fig. 3C*, there are several potentially active components that may be regulating immunity in the lung. One important aspect of the inflammatory response to Mtb that has received some attention, however, is the adverse response of infected mice to re-exposure to mycobacterial antigens either in the form of DNA vaccination or as a result therapeutic delivery of BCG (64, 65). In these studies, the delivery of antigen or live BCG resulted in no changes in bacterial burden in the

lung but increased inflammatory involvement in the lung tissue and an increased pyogranulomaotus response (64, 65). The fact that IL-17-producing cells are induced by Mtb infection (13, 37, 66) and the fact that IL-17 is associated with neutrophil recruitment (67) support the hypothesis that the pyogranulomatous cellular response following repeated antigen exposure may be associated with the IL-23/IL-17 cytokine axis. Thus while IL-23 and IL-17 are not important players during the primary response, they may play a role when antigen challenge is increased or repeatedly delivered. One aspect of the pathologic response that occurs in the repeatedly challenged mice is that it resembles the late stage pathologic response in end-stage disease in the mice (64, 65); we are therefore also investigating the role of IL-17 and IL-23 in the later stages of disease progression (*Fig. 3C*).

Conclusion

The mechanisms mediating development of specific subsets of T cells can be carefully delineated using *in vitro* studies. It is clear, however, that the complexity and counterbalancing nature of these subsets can only be studied by comparing and contrasting the behavior of these cells as they respond to pathogens. Indeed, it is also clear that the activity of any one cytokine may be protective or detrimental depending upon when it is acting (*Fig. 3*). Further, it is essential to note that the response of one subset of cells to one pathogen does not define the factors required for the induction, expansion, and effector function of that same subset of cells responding to a different pathogen. Indeed, as immunologists, we choose to subset cells for our own convenience, but at every turn, the immune response to pathogens *in vivo* is able to surprise us.

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Fig. 1. Low-dose aerosol infection with Mtb results in growth of bacteria and slow induction of immunity in the lung

(A) Bacterial colonization of the lung occurs upon infection with approximately 75 bacterial colony-forming units within aerosol particles of 3–5 μm diameter. After 3 days, bacteria begin to grow logarithmically until day 20, whereupon their growth is slowed. (B) Ex vivo stimulation of cells with peptide containing an IAb-restricted epitope demonstrates that the accumulation of IFN- γ -producing cells within the lung is slow but correlates with cessation of bacterial growth. (C) The cessation of bacterial growth and arrival of 10^4 IFN- γ -producing T cells also correlates in time with the upregulation of class II MHC on CD11c⁺ cells within the lung.

Fig. 2. IL-12-related cytokines and their receptors

Dimeric cytokines utilizing the IL-12p40, IL-12p35, IL-23p19, EB13, and p28 subunits share subunits and receptor components. All subunits are induced during Mtb infection in mice, and most play a role in the initiation, expansion, and regulation of the cellular response to Mtb.

Fig. 3. Cytokines play an intrinsic role in the cellular response to Mtb infection in the lung

(A) Migration of Mtb-infected DCs can be influenced by IL-12p40 and the development of effector subpopulations of antigen-specific cells depends upon IL-12p70, IL-23, and IL-27. The Th17 population is regulated by IL-12p35 possibly as a result of IL-35. Effector T-cell populations are required for the generation of the mononuclear granuloma and the control of bacterial growth. (B) Following subunit vaccination, a population of IL-17-producing IL-23-dependent antigen-specific memory cells can populate the lung. These cells respond to infection and accumulate in the lung. A circulating population of IFN- γ -producing IL-12p70-requiring antigen-specific memory cells sees antigen (likely in the lymph node), expands, and is recruited to the lung as a result of a chemokine response that is dependent upon the IL-17-producing memory population. The accelerated accumulation of cells in the lung results in earlier cessation of bacterial growth and a reduced bacterial burden. (C) Failure of the immune response to eliminate bacteria results in chronic infection and stimulation of antigen-specific immune responses. Repeated antigen exposure results in detrimental changes, specifically increase in size of the lesion and an increase in granulocyte

accumulation; this effect may be the result of an altered balance in the subsets of effector T cells. The maintenance of the mononuclear granuloma is dependent upon a variety of factors, the loss of anyone of which can result in development of damaging inflammation and resurgence of bacterial growth.