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IMMUNOPATHOGENESIS OF *MYCOBACTERIUM AVIUM* INFECTION

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1. ABSTRACT

One of the most obvious problems one perceives when working with *Mycobacterium avium* isolates is the vast array of phenotypes expressed with regard to colonial morphotype, serovar and particularly virulence. Thus whenever experimental data derived from different MAC isolates is compared the variety of this group of mycobacteria must always be considered.

Another issue of concern is the extrapolation of *in vitro* data to the *in vivo* disease. We have reported, in the past, that survival in murine macrophage culture does not always correlate with survival *in vivo* (23). It is plausible therefore, that the pathways outlined in section 5.2 and figure 3 play a crucial role in the initiation of the innate immune response in general and that there are components of this response which are not expressed by IFN-gamma activated macrophages but which are necessary for bacterial control.

In conclusion, we suggest that the initial control of MAC infection requires a healthy lung (or gut) architecture and that control by unactivated macrophages includes respiratory burst activity and also the sequestration of free iron away from the mycobacterial phagosome. Acquired immunity is important in controlling bacteria which have overcome the innate response and this control is mediated by cytokine activation of infected macrophages. Finally, we have described an animal model of infection in which uncontrolled bacterial growth occurs and in which lesions similar to those seen in AIDS patients develop.

2. INTRODUCTION

The recent increase in cases of mycobacterial disease caused by members of the *Mycobacterium avium*-complex (MAC) has lead to renewed interest in the nature of the interaction between these bacteria and the immune response of the host. While this interest has lead to increased knowledge of this interaction, the precise nature of the protective immune response to MAC remains enigmatic.

The HIV/AIDS epidemic has dramatically contributed to the increase in MAC disease in recent years; however, the number of non-AIDS related MAC infections has also been increasing, particularly in the older female population. In addition, while the prevalence of MAC in AIDS patients suggests that protection from this disease requires an antigen-specific T cell response, the nature of the immune defect in many of the non-AIDS patients remains unclear and may be related to altered lung architecture affecting innate responses. However, the predisposing condition(s) which allows this opportunistic infection to become established and cause disease have not been fully elucidated.

By comparing MAC disease to disease caused by the more virulent *Mycobacterium tuberculosis*, it would be reasonable to predict that MAC infection is controlled by antigen-specific T cell production of the macrophage activating cytokine interferon-gamma (IFN-gamma). While this is almost certainly true there are several key questions which remain unanswered in the delineation of the immunopathogenesis of MAC disease.

A primary question is what are the physical and innate responses which stop natural exposure progressing to colonization and infection? In addition, what are the crucial

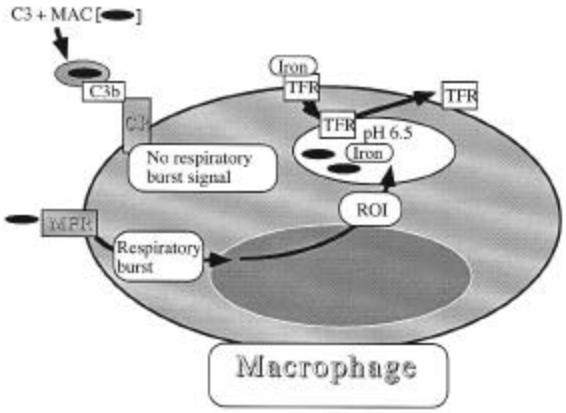

Figure 1. MAC activate complement and use the complement receptor for entry into the macrophage. The importance of complement activation (C3 to C3b) in determining survival within the macrophage has not been formally demonstrated. However, recent data suggest that the respiratory burst may play a role in controlling mycobacteria and it is known that the complement receptors (CR)(i.e. those receptors used by particles which have activated complement) do not stimulate a respiratory burst unlike other receptors (i.e. the mannose-fucose receptor MFR) which do. The phagosome which contains MAC within the non-activated macrophage is in contact with the transferrin receptor (TFR) and is at pH which allows dissociation of iron from the TFR.

anti-mycobacterial elements of the innate response which control initial infection? Finally, once infection has been established, what are the critical components of the acquired immune response which limit infection and dissemination?

This review will use current knowledge of the murine model of MAC infection to address these questions and in addition, comment on the importance of this knowledge in the study of the human disease.

3. EXPOSURE

The primary route of infection for MAC is as yet undetermined in human disease although the gut and the lung are the two primary candidates. The case for the gut as the portal of entry comes from the examination of AIDS patients who have heavy colonization of the lymphoid tissues and epithelial cells of this organ (1-3) . In addition, MAC in food particles can erode in gut epithelial cells (4) and the dissemination of MAC from a caecal injection to the spleen and liver suggests that colonization can be induced in the gut in the mouse model (5) . That MAC bacteria are present in the alimentary tract can be seen by the presence of MAC isolates in the stool of healthy individuals (6, 7)

The evidence for aerosol exposure leading to infection is less clear when AIDS is a confounding factor, (8) as few bacteria are seen in this organ in MAC/AIDS co-infected patients (1) . However, respiratory symptoms occur in a majority of AIDS patients progressing to disseminated MAC infection (8) . In addition, the majority of non-AIDS, MAC patients have pulmonary disease (9) suggesting that this organ is susceptible to MAC infection.

The absence of lung disease in AIDS patients may not preclude this organ as the portal of entry of MAC infection. It is quite possible that in the absence of an antigen-specific tissue response, dissemination is not restricted and poor recruitment of monocytes to the site may result in a lack of host cells thus resulting in a low level of infection in this organ.

The source of MAC aerosol infection is not thought to be from person to person as the number of caseated lung patients is low as a percentage of the infected population. It is more likely that aerosols of MAC are created in the environment by disturbance of water containing these bacteria. It has been suggested that the high incidence of non-AIDS MAC in the coastal regions results from the wave action of brakish waters resulting in the aerosolising of MAC (10) . In addition, the recent identification of identical MAC isolates from both patient samples and from the hot water system of the hospital suggest that the potable water was the source of the exposure (11) .

4. COLONIZATION

Once exposure has occurred the majority of the population does not succumb to infection and disease. This is clearly seen in the United States where skin test positivity ranges from 12% (12) to 80% (13) but the incidence of disease is as low as 1.3 cases per 100,000 (14) .

In the non-AIDS/MAC patient, there is generally a predisposing condition which leads to increased susceptibility, the commonest of which is the bronchiectasis caused by physical disruption of the elasticity of the lung tissue by bronchitis and emphysema (15). This loss of elasticity in the lungs may result in poor clearance mechanisms and the slow turnover of airway surface fluid (ASF). One of the important components of the ASF are defensins which are toxic to many bacteria including MAC (16). If the ASF has poor antimicrobial activity due to poor ASF turnover, then increased susceptibility to bacterial infections may be expected to occur. Indeed patients with cystic fibrosis, who have very poor anti-bacterial activity in their ASF, are becoming increasingly infected with MAC as other anti-bacterial treatments result in their improved long term survival (17) . It is not known at what point the MAC/AIDS patient becomes infected; it is possible however, that other concurrent infections predispose the AIDS patient to colonization by MAC.

Progression to a disease state after colonization is different in the AIDS versus non-AIDS patient. In the latter case the acquired immune response will recognize the pathogen and as in tuberculosis, the accumulation of monocytes and lymphocytes will result in a granuloma which will contain the infection within the lung. In the AIDS patient the loss of acquired immune responses will result in the rapid dissemination of the bacteria to other organs of the reticulo-endothelial system via the bloodstream. This bacteremia has been noted early in infection when it is sporadic but as disease

progresses the bacteremia becomes persistent (18) . At this stage the liver and spleen, which contain high numbers of the macrophage host cell, become heavily infected as does the alimentary tract (1, 2).

Although the consequences of infection are different in the immunocompetent and immunocompromised patient this does not mean that the initial colonization was different. The preponderance of pulmonary infection in the non-AIDS patient supports the contention that the lung is one of the main sites of initial infection and that it is the lack of acquired immunity which results in the disseminated disease of the MAC/AIDS patient.

5. BACTERIAL VIRULENCE

It is not just the susceptibility of the host but also the virulence of the bacteria which will contribute to the disease potential of the initial exposure. This is particularly true of MAC exposure as there are at least two species of mycobacterium represented in this grouping and within each species there are many strains, serotypes, morphotypes and sequevars.

One of the major methods for characterizing MAC strains is the serotyping based upon the recognition by antibody of a series of unique surface glycopeptidolipids (serovar specific glycopeptidolipid, ssGPL). Serovars 1, 4 and 8 have been associated with MAC disease in AIDS patients (19) and it is interesting that GPL's from one of these serovars is capable of immunomodulatory activity (20) . In a recent comprehensive study of 144 AIDS and 40 non-AIDS patient isolates, serovars 4 and 8 were predominant in both groups (J. B. Torrelles, personal communication) however, there was also a strong geographical effect such that patients within a small area all became infected with the same serovar. Thus, while the certain serovars appear to predominate in human infection, a direct link between the nature of GPL's expressed by those serovars and virulence is not yet clear.

The morphotype (i.e. colony morphology) of a particular isolate can vary depending on culture conditions; however, there is strong evidence that colonies, derived from the same isolate, which have a smooth transparent (SmT) appearance on agar are more virulent than those which are smooth and opaque (SmO) The chemical basis for this difference is not clear; however, there are differences in proportions of polar and apolar lipids (21) and some protein difference (22) . The role of this morphotypic differences is less clear in a study of 23 isolates which were tested for their virulence in mice (23). In that study, isolates from the environment, from animals, and from AIDS and non-AIDS patients were cultured *in vitro* and the SmT colonies used to infect mice. The animal isolates were uniformly virulent while the patient isolates were of varying virulence and the environmental isolates were avirulent. Thus, the presence of an SmT morphotype does not always predict virulence. From the same study it can be inferred that there is selection pressure for virulence factors consequent upon infection of a vertebrate but that these are less in an immunocompromised host.

6. INNATE CELLULAR RESPONSE

6.1. Phagocytosis and macrophage activation

The cellular immune response is generally considered to be the mediator of protection in mycobacterial disease. Indeed the majority of MAC bacteria reside in the macrophage populations of the host. In the primary interaction of the bacteria with a non-activated macrophage, receptor mediated phagocytosis occurs and results in an intracellular infection. The principal signal that the macrophage receives at this stage is that mediated by receptor ligation. Several lines of evidence point to a role for complement in this initial interaction. Macrophages are able to generate, locally, all the components of the alternative complement cascade (24) and MAC can activate C3 to C3b on its surface (25, 26) possibly via a GPL molecule (J. S. Schorey, personal communication). As a confirmation of the role of complement in this initial interaction, the role of the complement receptors (CR) in the phagocytosis of MAC has been demonstrated by the use of CR-specific monoclonal antibody inhibition of receptor mediated uptake (25) . The importance of particular receptor usage lies in the signals induced by each receptor; i.e. CR1 and CR3 fail to initiate a respiratory burst when they are ligated, unlike the IgG FcR and some sugar specific receptors (figure 1).

The role of the respiratory burst (RB) in the control of mycobacterial infections has been overlooked in recent years largely due to *in vitro* studies which demonstrated no role for this molecule in macrophage control of bacteria (27, 28) . However the recent reports of mycobacterial disease [both MAC and tuberculosis] in chronic granulomatous disease patients (29, 30) and the increased susceptibility of gp47-phox gene disrupted mice to both MAC and M. tuberculosis infection (M. Doherty, personal communication; A. Cooper, unpublished results) suggests that reactive oxygen intermediates play a role in early defense against mycobacterial disease. As mentioned above the macrophage complement receptors 1 and 3 fail to induce a RB and this taken together with the fact that the RB has anti-mycobacterial activity suggests that the ability to activate complement may be a virulence factor in MAC (figure 1).

Once within the macrophage MAC resides in a vacuole which is within the host cells recycling endosomal system. This MAC vacuole equilibrates at pH 6.3-6.5 (31) and has reduced hydrolytic activity despite the presence of proteins associated with the lysosomal compartments (31, 32). Of particular interest is the accessibility of this compartment to the iron transporting transferrin receptor (TFR) (33, 34). Within this compartment then is a moderate pH, a lack of degradative enzymes and a plentiful supply of iron from the TFR and a healthy, growing MAC population (figure 1).

Another consequence of phagocytosis is the induction of the cytokine, tumor necrosis factor-alpha (TNF-alpha). This macrophage activating molecule can act in an autocrine manner, priming the macrophage for anti-bacterial activities. A close correlation between morphotype and virulence is demonstrated by the increased ability of SmO MAC to induce TNF-alpha in an *in vitro* macrophage population in comparison to the SmT morphotype (35, 36). The SmT bacteria limit TNF-alpha production by interfering

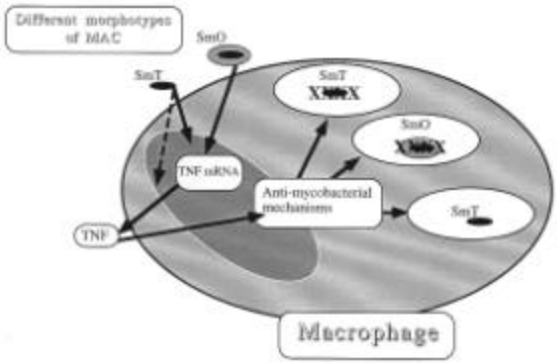

Figure 2. The expression of TNF by macrophages depends upon the morphotype and the isolate of MAC. The smooth transparent (SmT) and smooth opaque (SmO) morphotypes both induce mRNA for TNF however, the SmT morphotype reduces the stability of the TNF mRNA (dashed arrow) which results in reduced TNF expression. While some isolates (SmT and SmO crossed out) are sensitive to the anti-mycobacterial effects of TNF activated macrophages some are not (SmT not crossed out).

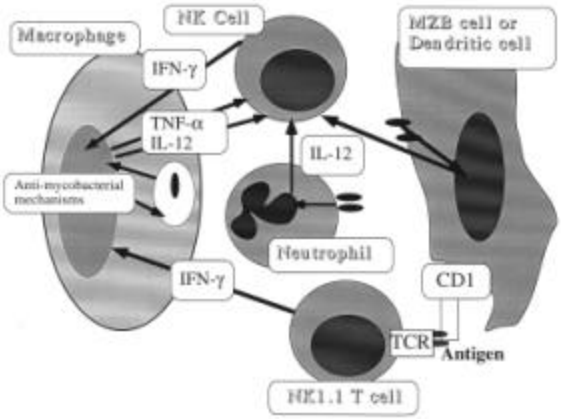

Figure 3. There are several ways in which cells can activate the innate response to produce macrophage activating cytokines. Infected macrophages (on the left) can express TNF and IL-12 which act upon natural killer (NK) cells to make IFN-gamma. Neutrophils (center), which are kept alive by IFN-gamma, can respond to pathogens by the production of IL-12 which could also act on NK cells. Dendritic cells (on the right) have recently been shown to react directly to foreign antigen by secreting IL-12. Both dendritic cells and marginal zone B (MZB) cells express CD1 which in conjunction with antigen (altered self or foreign) stimulates NK1.1 T cells (via their T cell receptor, TCR) to rapidly release cytokines such as IFN-gamma.

with the survival of TNF-alpha mRNA (37, 38). The question remains however, does the presence or absence of TNF-alpha correlate with the survival of the bacteria? If exogenous TNF-alpha is added to the SmT cultures many of them fail to grow (35) . In addition, the depletion of TNF-alpha *in vivo* results in increased bacterial growth in a TNF-alpha sensitive MAC isolate (39) (figure 2).

There is one gene which has been strongly associated with resistance to MAC infections in mice (40, 41). This is the natural resistance associated membrane protein-1 (NRAMP-1) which is expressed in tissue macrophages. The effect of this gene is much more strongly expressed in this mycobacterial infection than in any other however, the exact nature of the defect in the susceptible allele is unclear. The gene has pleiotropic effects including a role as an iron transporter. When an intravenous infection is given to a mouse the gene appears to act most strongly in the liver and spleen with the congenic resistant mouse being able to limit even the most virulent MAC infection easily (39) . Following an aerosol infection however the growth in the lungs of both the resistant and susceptible mice was similar (A.M. Cooper, unpublished results). In an attempt to determine if iron starvation played a role in the control of bacterial growth resistant mice were overloaded with iron and the bacterial counts in the liver and spleen following an intravenous infection became similar to those seen in the susceptible mice (S. Gomes and R. Appelberg, unpublished results). This change was not noted in the lung suggesting that the NRAMP-1 gene is not active in the lungs of mice or that iron levels in this tissue are high enough to compensate.

The observation of iron dependency of the NRAMP gene and the accessibility of the TFR to the MAC endosome in the non-activated macrophage (figure 1) together suggest that MAC survival is dependent to some extent on the presence of iron. This is supported by the observation that in the activated macrophage the MAC phagosome matures and becomes refractory to the transferrin receptor pathway and the MAC stops growing (42) .

6.2. Other cells of the innate response

The vertebrate host must make a response to this invasion and the strength of this response depends upon its ability to recognize the invader as foreign. Where once this recognition was thought to be the responsibility of the acquired immune response it has recently become apparent that there are numerous pathogen recognition receptors which alert the innate immune response to “danger” (43) ; the types of molecules recognized include, but are not limited to, altered self, unusual bacterial carbohydrates, and bacterial DNA. These innate responses to "danger" separate the scavenging activities of the macrophage system from its defensive activities. Cells which are involved in this response network include dendritic cells (44) , natural killer [NK] cells (45) , NK-T cells (46) , neutrophils (47, 48) , marginal zone [MZ] B cells (49) and macrophages.

MAC infected macrophages can make TNF-alpha and IL-12, which when acting in concert can drive an IFN-gamma response from NK cells (45) . This innate IFN-gamma serves to prime the macrophage to produce more IL-12 and thereby potentiate the T cell response. The ability of NK-T cells (which can produce IFN-gamma upon stimulation by CD1 and antigen (46)) to respond to mycobacterial products in the context of the non-polymorphic MHC class I like molecule has yet to demonstated in the mouse however, CD1 specific cells in human *in vitro* models do respond to CD1 and do produce IFN-gamma (50) . Thus, these are two potential mechanisms whereby the innate immune response can

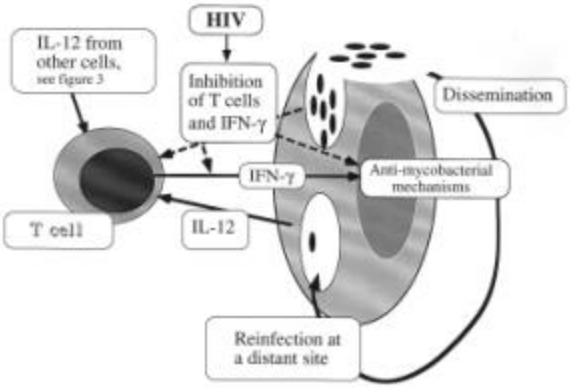

Figure 4. Antigen specific T-cell activation of infected macrophages controls bacterial replication in MAC infection. The IL-12 released by the cells as described in figure 3, acts upon antigen-specific T cells to drive them to an IFN-gamma producing phenotype. IL-12 can also come from infected macrophages (on the right). If T cell control falters, due to the inhibitory activity of HIV, then macrophages will become super-infected and burst resulting in dissemination. It is also possible that high numbers of MAC have a deleterious effect on T cell activation.

stimulate the protective IFN-gamma response (figure 3). That these mechanisms may play a role in MAC infection is demonstrated by a moderate increase in bacterial load when NK positive cells are depleted *in vivo* (51).

7. ACQUIRED IMMUNITY TO MAC

While the innate response can generate some of the IFN-gamma /IL-12/TNF-alpha required to control infection it cannot maintain this production in the absence of an acquired immune response. While both IFN-gamma and TNF-alpha are important in the early response, only continuous depletion of IFN-gamma results in a prolonged increase in susceptibility (52) . Indeed, in a recent study of several gene knock-out animals it was shown that only the IFN-gamma KO animal was significantly susceptible to increased bacterial growth (53) . Thus, the crucial component of the antigen-specific immune response would appear to be IFN-gamma.

The accepted progression to an antigen-specific T cell response involves the action of IL-12. That this cytokine is induced in MAC infections which come under T cell control has been shown by several investigators (53-55) . It is, of course, crucial to the production of the antigen-specific CD4 T cells IFN-gamma producing T cells which activate the infected macrophages which then control mycobacterial growth (54, 55) . The depletion of these CD4 T cells throughout infection results in the increase in bacterial growth when in the control animals the infection is becoming chronic (52, 56, 57) . Thus, the classical axis of antigen-specific, IFN-gamma producing CD4 T cells controlling infection by activating the infected macrophage holds true for both systemic and aerogenic infection models in the mouse (figure 4). Interestingly, recent data concerning unusual disseminated MAC infections in non-AIDS patients supports a role for this classical axis in human disease as these patients have defects in the IL-12 or IFN-gamma pathway (58) .

Although the loss of T cell immunity in AIDS patients is an obvious reason for their increased susceptibility to MAC it is curious that the number of T cells required to control tuberculosis is much greater than that required to control MAC infection. This raises the question of whether an innate immune defect, present only in late stage AIDS patient, is required for MAC colonization to become established. An alternative hypothesis would be that MAC bacteria are simply less virulent and require a less strong antigen-specific response to be kept under control. It is our opinion that MAC infection occurs earlier in AIDS than the occurrence of the bacteremia and that the infection progresses slowly in a largely undetectable manner. The increased incidence of MAC specific IgG and IgA in AIDS patients who progressed to MAC infection supports this hypothesis (59) . As mentioned above, bacteremia is not a constant in the early stages of MAC disease, it appears to fluctuate for some time before becoming constant. This fluctuation along with the serology data suggest that the AIDS patient and the MAC are in a running battle the success of which [for the patient] depends upon a good CD4 T cell response which of course eventually wanes due to HIV activity.

In this regard, recent work in our laboratory suggests that not only the virus is reducing the T cell response but that MAC infection itself may result in the loss of the antigen-specific CD4 responses. In the mouse model increasing numbers of bacteria fail to result in increasing numbers of antigen-specific T cells, and in fact these cells are gradually lost in infections using certain MAC strains (A.M. Cooper, unpublished observations). This loss of reactivity could be antigen-specific or a result of general immunosuppression (53) . This model of T cell specific immunosuppression by MAC in the mouse may explain the fluctuating bacteremia seen in the patient. Bacteremia may result from local high numbers of bacteria in an infected tissue causing immunosuppression, allowing the dissemination of bacteria to distant sites where new stimulation of the CD4 T cells could occur which would then control the dissemination. This cycle would continue until there were too few naive CD4 T cells left to be stimulated [i.e. late stage AIDS] and MAC bacteremia and tissue infection would be fully established (figure 4).

8. THE PATHOGENIC NATURE OF THE IMMUNE RESPONSE TO MAC

While a strong protective immune response to mycobacterial infection is something to be heartily wished for, too much of a good thing can cause trouble. This is particularly true in *M. tuberculosis* infections when in a mouse a bacterial load of 10^{7-8} in the lung is sufficient to kill the mouse not from bacterial numbers alone but from the inflammatory response which occludes much of the functional lung tissue (60) . In MAC infections however, bacterial loads of upto 10^{9-10} can be tolerated as less inflammation appears to be induced by this pathogen (61) . The inflammation induced in response to MAC is dependent to some extent on the TNF-alpha/nitric oxide axis as the mice which lack the TNF-Receptor (TNFR) and the gene for the inducible nitric oxide synthase (iNOS) have reduced spleen sizes (53). As

mentioned above the ability of different MAC isolates to induce TNF-alpha is very variable (35) ; in addition, the sensitivity of different isolates to TNF-alpha is also variable (39) . It is plausible to suggest therefore that the level of inflammation induced by individual isolates will vary. In the absence of TNF-alpha there will be reduced expression of the chemokines responsible for much of the cellular influx and subsequent inflammation in mycobacterial disease (60) . Interestingly, the bacterial growth in the mice lacking the TNFR and iNOS genes is not increased, suggesting that for MAC (as in tuberculosis) the protective and granulomatous responses are dissociated (60) .

One strain of MAC (ATCC 724) does not enter the chronic stage of infection in mice but continues growing until lung burdens of 10^{10} bacteria per organ are reached (61) . There appears to be little protective immune response and minimal inflammatory response until quite late in infection. Recent work using this strain in an aerosol model has demonstrated a role for gamma-delta T cells in the eventual inflammation seen in this model. In that study, gamma-delta gene disrupted mice had a delayed inflammatory response, compared to control mice, which limited the tissue damage. Whether the gamma-delta T cells respond to mycobacterial antigens (62) or the altered self resulting from the high bacterial burden (63) is not clear, however they appear to be necessary for the mononuclear influx in MAC infections just as they are in tuberculosis (64). In AIDS/MAC patients the type of lesion which develops is similar to that seen in the ATCC 724 infection i.e. a high bacterial burden accompanied by extensive tissue necrosis and fibrosis (65, 66) suggesting that in the absence of a protective T cell response the accumulation of mononuclear cells contributes to the pathogenic development of these lesions.

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