Role of complement activation and antibody in the interaction between Mycobacterium tuberculosis and human macrophages

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Mycobacterium tuberculosis-specific antibodies possess immunomodulatory effects during tuberculosis infection. Prior sensitization to environmental mycobacteria is known to suppress immune responses against BCG and M. tuberculosis. Mycobacteria-induced antibodies can influence events such as complement activation and phagocytosis during infectious process. In the present study role of anti-M. tuberculosis IgG (anti-M. tb IgG) antibody during interaction between M. tuberculosis and human macrophages mediated through complement has been examined in vitro. Anti-M. tb IgG antibody significantly enhanced complement activation by M. tuberculosis. Phagocytosis of M. tuberculosis by macrophages increased significantly in the presence of complement and/or antibody. Moreover, antibody enhanced phagocytosis in the presence of complement. Addition of antibody alone or in combination with complement also augmented intracellular viability of bacilli within macrophages. Results of this study showed that anti-mycobacterial antibody enhances complement activation and anti-M. tb IgG antibody probably modulates effects of complement during early stages of tuberculosis infection.

Keywords: Complement, Immunoglobulin, M. tuberculosis, Phagocytosis

Mycobacterium tuberculosis remains one of the most common causes of infectious disease morbidity worldwide. It is estimated that 9.2 million new tuberculosis (TB) cases and approximately 2 million tuberculosis-related deaths are caused by this pathogen. One-third of the world’s population is infected with M. tuberculosis. M. tuberculosis, the causative agent of tuberculosis, is a facultative intracellular pathogen, which uses macrophages as its primary host cell and survives and replicates inside these cells. Therefore, invasion of macrophages by tubercle bacillus is a vital aspect in the establishment of tuberculosis infection; consequently, M. tuberculosis has evolved several strategies to parasitise its host cell. Multiple distinct macrophage receptors have the potential to recognise and bind M. tuberculosis; these include complement receptors (CRs) 1, 3 and 4; mannose receptor; CD14; surfactant protein A receptors and scavenger receptors.

Complement system comprises a network of more than 30 proteins, belonging to both innate and adaptive arms of the immune system. Complement receptor 1 (CR1) (CD35) is a single-chain glycoprotein that binds complement fragments C3b and C4b. CR3 (CD11b/CD18) and CR4 (CD11c/CD18) are heterodimers belonging to the leukocyte β2-integrin family. These two receptors bind complement fragment C3bi and also contain a polysaccharide-binding site. M. tuberculosis can bind to complement receptors via both complement-dependent and -independent pathways and is subsequently phagocytosed by the phagocytic cell. Presence of human serum containing active complement components was found to enhance binding of M. tuberculosis to CR1, CR3 and CR4 on the surface of human monocytes and monocyte-derived macrophages (MDMs). Complement component C3 identified as the major component in human serum is involved in enhancing the adherence and uptake of M. tuberculosis by mononuclear phagocytes.

It is well documented that mycobacteria can activate the complement system. Antibodies are known to enhance complement activation. It has been demonstrated that intra-dermal BCG vaccination induces antibodies of immunoglobulin G1 (IgG1), IgG2 and IgG3 isotypes. An important target of antibody responses induced by intra-dermal BCG
vaccination was found to be lipoarabinomannan (LAM), a major component of the mycobacterial cell wall. Several studies suggest that anti-LAM antibodies may have an important protective role. Passively administered monoclonal anti-arabinomannan antibody increased survival of mice after challenge with *M. tuberculosis*. Antibodies induced by vaccination with arabinomannan-protein conjugates were partially protective in experimentally infected animals. BCG could induce secretory mycobacterium-specific antibodies. In the context of infections with other microorganisms it has been shown that antibodies could enhance immunity through many mechanisms, including neutralisation of toxins, opsonisation, activation of complement, promotion of cytokine release, enhanced antibody-dependent cellular cytotoxicity and enhanced antigen presentation. Anti-mycobacterial antibodies were found to have enhancing effect on complement component C3 binding to the mycobacteria. The homologous antibodies should have more enhancing effect in comparison to heterologous antibodies.

Therefore, the aim of the present investigation was to study *in vitro* the role of anti-*M. tb* IgG antibody in interaction of *M. tuberculosis* with human macrophages in the context of complement system.

Materials and Methods

**Study subjects**—Thirty-five normal healthy laboratory volunteers aged 21 to 50 years (23 males and 12 females), having no clinical history of TB and other respiratory disorders, and 20 untreated pulmonary TB (PTB) patients (13 males and 7 females) aged 21 to 50 years were included in the study. All the PTB patients were naive for anti-tuberculosis treatment and were positive for sputum smears and culture. Examination of smear and culture was done according to the methods already established.

All the subjects recruited into this study were negative for HIV infection as ascertained by Tridot assay (J Mitra & Co., New Delhi, India) and Retroquic (Qualprodiagnostics, Goa, India). Informed consent was obtained from the subjects before drawing blood. This study was approved by the Institutional Ethics Committee.

**Growth and preparation of mycobacteria—**

*M. tuberculosis* H37Rv bacilli were cultured in Middle brook 7H9 broth medium enriched with albumin, glucose and catalase ADC (Difco Laboratories, Detroit, Mich., USA) at 37°C for 3–4 weeks and harvested by centrifugation. The bacilli were washed and aliquoted in vials containing phosphate-buffered saline (PBS; pH 7.4). These aliquots were frozen in PBS containing 10% glycerol. During the experiments, vials were thawed and enumerated for viable colony forming units (CFU) on Middle brook 7H11 agar plates. Before inoculation onto the agar plates, bacterial aliquots were thawed at 37°C and diluted in PBS to the desired concentration.

**Preparation of human sera for assaying complement activation**—Normal human serum (NHS) was prepared using 10 ml blood individually drawn from 15 normal subjects and 20 PTB patients. The obtained serum samples were stored for further use. During further experiments, demonstration of classical pathway (CP) and alternative pathway (AP) using sera samples was performed as described previously. Briefly, sera samples were diluted in phosphate buffers, namely, PBS-Tween 20 (0.05%; pH 7.4) (PBST) containing Ca"+"Mg"++" [supplemented as CaCl₂ (0.3 mM) and MgCl₂ (2 mM)] or PBST-MgEGTA to demonstrate, respectively, CP and AP. Addition of PBST-EDTA blocks complement activation and this was used as control.

*M. tuberculosis* sonicate antigen preparation—

*M. tuberculosis* H37Rv bacilli (100 ml packed volume) harvested from the broth cultures were subjected to lysozyme treatment (1 mg/5 ml of cell suspension) for 15 min at 37°C with gentle stirring. The bacilli were then suspended in twice the volume of phosphate buffer (20 mM Tris with 8.5% NaCl of pH 7.4 supplemented with 1 mM PMSF, 10 mM EDTA). The bacterial cells in the breaking buffer were disrupted using Fisher Sonic Dismembrator (Model 300) (Artek systems corporation, Port Washington NY, USA). The sonication was carried out for 30 cycles; each cycle lasting 1 min, with an interval of 1 min. The process of sonication was carried out under ice-cold conditions, since the procedure results in the release of heat that would otherwise denature the proteins.

**Measurement and evaluation of purity of anti-*M. tb* IgG antibody**—Antibody titration against *M. tuberculosis* sonicate antigen was done using ELISA technique described elsewhere. After titration, isolation of anti-*M. tb* IgG antibody from patient sera was done as described by Cox et al. using ammonium sulphate precipitation and affinity chromatography methods. Then, agarose column purification method was used to purify IgG.
antibodies. In addition, purity of anti-\textit{M. tb} IgG antibody was evaluated by electrophoresis and immunoblot analysis\textsuperscript{22}. Finally, the obtained anti-\textit{M. tb} antibody fractions were aliquoted and stored at −20 °C.

**Opsonisation of \textit{M. tuberculosis} with anti-\textit{M. tb} IgG antibody**—For complement binding and infection experiments, \textit{M. tuberculosis} bacilli were opsonised with anti-\textit{M. tb} IgG antibody. Briefly, 2×10\textsuperscript{7} \textit{M. tuberculosis} bacilli were incubated with 20 µg/mL anti-\textit{M. tb} IgG in RPMI-1640 for 1 h at 37°C. After incubation, the opsonised bacilli were washed with RPMI-1640 by centrifugation at 4000 g for 10 min. After washing, the bacterial pellet containing pre-opsonised bacilli was resuspended in 1ml of RPMI-1640 for use in complement activation and macrophage infection experiments.

**C3, C4 and factor B binding assay for complement activation**—Solid phase ELISA was performed to assess the pattern of complement activation by \textit{M. tuberculosis} at levels of C3, C4 and factor B as described earlier\textsuperscript{9} and also to determine the effect of anti-\textit{M. tb} IgG antibody on complement activation. Briefly, heat-killed anti-\textit{M. tb} IgG-opsonised or-unopsonised mycobacteria (at 10\textsuperscript{8}/mL, 10\textsuperscript{7}/mL and 10\textsuperscript{6}/mL numbers) were coated onto microtiter plates and incubated overnight at 4 °C, followed by blocking the wells with 1% BSA in PBST. NHS diluted in various buffers (pH 7.4) viz., PBST-Ca\textsuperscript{++}Mg\textsuperscript{++} or PBST-MgEGTA or PBST-EDTA to demonstrate, respectively, CP, AP and as control was added to the wells and incubated for 1 h at 37 °C. C3 activation was measured through both CP and AP, with serum dilutions 1/50, 1/100 and 1/250 (for CP) and 1/10, 1/20 and 1/50 (for AP). C4 activation was measured through CP using 1/50, 1/100 and 1/250 serum dilutions. For factor B (through AP), serum dilutions used were undiluted sera, 1/2 and 1/10. Anti-human complement antibodies raised in rabbit (anti-C3c and anti-C4c; DAKO, Glostrup, Denmark) or goat (anti-factor B; R&D Systems, Minneapolis, MN, USA) conjugated with HRP were added and incubated at 37 °C for 1 h. After washing the plate, the substrate tetra methyl benzidine (TMB) was added and incubated for 15 min at room temperature in dark. Finally, the reaction was stopped using 0.5M H\textsubscript{2}SO\textsubscript{4} and the plate was read for OD values at 450 nm in an ELISA Reader (Spectramax 250).

**Macrophage culture and their infection with \textit{M. tuberculosis} and evaluation of phagocytosis**—Blood samples (40 mL) individually drawn from the remaining 20 normal healthy subjects were used for cell culture and infection experiments. Peripheral blood mononuclear cells (PBMCs) were isolated from each blood sample as previously described\textsuperscript{22}. For infection experiments, 2×10\textsuperscript{5} MDMs/coverslip were incubated in a microtiter plate with RPMI-1640 containing CP components (NHS 1/50 dilution in PBST-Ca\textsuperscript{++}Mg\textsuperscript{++}) or AP components (NHS 1/10 dilution in PBST-MgEGTA) or components to block complement activation (NHS 1/50 dilution in PBST-EDTA). The macrophages were infected with anti-\textit{M. tb} IgG-opsonised mycobacteria or -unopsonised mycobacteria at a multiplicity of infection (MOI) of 1:10 (macrophage:mycobacteria) and incubated for 2 h at 37°C, after which the macrophage monolayer was washed using warm RPMI-1640 to remove non-adherent bacilli. Plain MDMs (i.e., without treatment with complement or anti-\textit{M. tb} IgG) were used as control. Finally, after formalin fixation of the macrophages, phagocytosed bacteria were quantitated by light microscopy in triplicates for each experimental condition (~300 MDMs/cover slips).

Phagocytic index was calculated using the formula:

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\text{Phagocytic index} = \frac{\text{No. of macrophages containing intracellular mycobacteria} \times 100}{\text{Total no. of counted macrophages}}
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**Measurement of intracellular mycobacterial growth**—Intracellular growth of \textit{M. tuberculosis} in macrophages, in the presence or absence of complement and/or anti-\textit{M. tb} IgG antibody, was assessed as described elsewhere\textsuperscript{23}. MDMs at a concentration of 1×10\textsuperscript{5}/well were used for infecting \textit{M. tuberculosis} at an MOI of 1:10 under the same conditions as done for phagocytosis assay.

**Statistical analysis**—Statistical analysis of the data was carried out with the SPSS software program (SPSS 13, Chicago, IL, USA). The statistical significance of antibody titers and complement binding was determined by unpaired \textit{t} test. One-way ANOVA with Tukey’s test was used to compare the phagocytic ability of macrophages and intracellular viability of \textit{M. tuberculosis}. Results are expressed as mean ± standard errors. \textit{P} values less than 0.05 were considered as statistically significant.

**Results**

**Determination of antibody titer against \textit{M. tuberculosis} sonicate**—Antibody titration against \textit{M. tuberculosis} sonicate was done using ELISA. As a preliminary step, the titers of patient serum antibodies against \textit{M. tuberculosis} sonicate were estimated and
these were compared with those from the NHS samples. Both IgG and IgM antibody titers against *M. tuberculosis* sonicate were higher in PTB patients compared to NHS (Fig. 1A and B). The antibody titers were significantly higher in PTB patients than normal healthy controls (*P* < 0.001). It was also found that IgG antibody titers were higher than IgM titers in serum samples from both patients and controls (*P*<0.05).

**Isolation of anti-*M. tb* IgG antibody**—Sera from 20 active PTB patients were used to prepare anti-*M. tb* IgG antibody. A total of 4 mg anti-*M. tb* antibody was obtained and this was checked for purity on SDS-PAGE. Heavy and light chains characteristic of IgG antibody in PTB patient sera are shown in Fig. 2A. On Western blot analysis of this preparation, it was found that the antibodies from the patient sera were predominantly against 24, 30, 45, 65, 71, 82 and 96 kDa antigens (Fig. 2B).

Fig. 2—(A)—Affinity purification of anti-*M. tb* IgG antibody. IgG antibodies against *M. tuberculosis* in pulmonary tuberculosis patients' serum were purified by affinity purification. The eluted IgG was analysed by Coomassie brilliant blue R-250 staining after SDS-PAGE; (B)—Western blot analysis of *M. tuberculosis*-specific IgG antibody. Bands from low molecular weight (24kDa) to the highest (96kDa) were observed for *M. tb* sonicate proteins probed with purified IgG. These were compared with protein profiles of *M. tb* sonicate and MW markers (in KDa), indicated on the left. [MW: Molecular weight marker; *M. tb*: *M. tuberculosis* sonicate protein; IgM: IgM antibody; IgG: Purified IgG].

Fig. 1—Levels of antibody responses to *M. tuberculosis* sonicate: (A)—IgG; (B)—IgM. Sera were collected from untreated pulmonary tuberculosis patients (n=20) and normal healthy volunteers (n=15). Dotted line: 3 SD ± mean, *P*<0.05.
Complement activation pattern by *M. tuberculosis*—Results of ELISA experiments to assess complement activation are shown in Fig. 3. Both C3 binding (through both CP and AP) and C4 binding (through CP) to *M. tuberculosis* are dependent on serum concentration as well as on the number of bacilli (Fig. 3A–C). Results of factor B binding to *M. tuberculosis* showed that factor B binding was highest only at $10^8$/ml numbers of bacilli with all 3 serum dilutions as shown in Fig. 3D. There was negligible or almost no factor B binding at $10^7$/ml and $10^6$/ml bacilli numbers for all the 3 serum dilutions.

Effect of anti-*M. tb* IgG antibody on activation of complement by *M. tuberculosis*—To study the effect of anti-*M. tb* IgG antibody on complement binding, $10^8$/ml of heat-killed bacilli (opsonised or unopsonised) were used to activate C3, C4 and factor B using NHS 1/50 dilution for CP, 1/10 for AP and undiluted sera for factor B binding using ELISA (Fig. 4). Anti-*M. tb* IgG antibody significantly enhanced the binding of C3 through both CP ($P<0.01$) and AP ($P<0.05$) and C4 ($P<0.05$) compared to that observed with unopsonised bacilli. However, the level of binding of factor B was not enhanced by prior

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**Fig. 3**—Solid-phase ELISA was performed to determine patterns of complement binding to *M. tuberculosis* assessed by C3 binding through (A)-classical and (B)-alternative pathways, (C)-C4 binding through CP and (D)-factor B binding. [Shaded bar: Limits of complement binding measured using NHS diluted in PBST-EDTA. Complement binding was directly proportional to serum concentration and numbers of bacilli].

**Fig. 4**—Effect of anti-*M. tb* IgG antibody on complement binding of *M. tuberculosis* at levels of C3, C4 and factor B. [Shaded bar: Limits of complement binding measured using NHS diluted in PBST-EDTA. Anti-*M. tb* IgG-opsonised *M. tuberculosis* showed a significant increase of complement binding compared to unopsonised bacilli. *$P<0.05$. CP: Classical pathway; AP: Alternative pathway].
opsonisation and showed similar results to that obtained without *M. tuberculosis* opsonisation.

**Effect of anti-** *M. tb** IgG on phagocytosis of *M. tuberculosis*—Effect of anti-*M. tb* IgG antibody on the phagocytosis of *M. tuberculosis* by human MDMs under various conditions is shown in Fig. 5. Phagocytosis of unopsonized bacilli was >45%, while addition of CP components significantly increased the percentage phagocytosis to >70% (*P*<0.05) and with AP components it significantly increased to >60% (*P*<0.05), compared to that of the unopsonised bacilli. Addition of *anti-M. tb* IgG antibody alone also significantly increased the phagocytic ability of macrophages to about 60% (*P*<0.05%). Further, addition of anti-*M. tb* IgG antibody along with CP and AP components significantly increased phagocytosis by >80% and >70%, respectively compared to unopsonised bacilli. These results indicate that complement and antibody independently act as good augmenters of phagocytosis of *M. tuberculosis* and when in combination, the two opsonins probably have a synergistic effect.

**Intracellular growth of *M. tuberculosis* in macrophages**—Intracellular growth of *M. tuberculosis* in MDMs was assessed by counting the CFU on the 7H11 agar plates on which the lysed macrophages containing the bacilli were inoculated and grown. At 1 h post-infection, it was found that there were significantly more viable *M. tuberculosis* bacilli (*P*<0.05) in the macrophages treated with *M. tb* + CP, *M. tb* + IgG and/or CP and AP compared to the macrophages treated with *M. tuberculosis* alone (Fig. 6A). At 24 h post-infection, significantly higher number of viable bacilli were found inside the macrophages (*P*<0.05) at all conditions, compared to the macrophages treated with *M. tuberculosis* alone (Fig. 6B). Moreover, addition of anti-*M. tb* IgG in the presence of complement (CP and AP) significantly increased the intracellular growth of *M. tuberculosis* (Fig. 6C).

*Fig. 6—Effect of anti-*M. tb* IgG antibody on intracellular viability of *M. tuberculosis* measured at (A)-1 h, (B)-24 h and (C)-48 h post-infection of normal human macrophages under various conditions. A significant increase in the viability of bacilli was observed in the macrophages infected with *M. tuberculosis* and treated with complement and/or anti-*M. tb* IgG compared to those infected with *M. tuberculosis* alone (*P*<0.05). *P*<0.05 implies statistically significant augmenting effect of anti-*M. tb* IgG on increase in the viability mediated through complement. Viability was also significantly increased upon addition of both antibody and complement, compared to complement alone (*P*<0.05).*


Discussion

One of the reasons proposed for the failure of BCG vaccination to protect against post primary forms of TB is that a prior sensitisation with non-tuberculous environmental mycobacteria, which downregulates subsequent response to both BCG and M. tuberculosis14,24,25.

The presence of moderate amounts of anti-mycobacterial antibodies in BCG unvaccinated and vaccinated individuals in endemic normal subjects is well documented26,27. Although a possible therapeutic role for immune sera in the control of tuberculosis has been conferred28, little attention has been paid to a possible role for antibody in protection from M. tuberculosis infection. Mycobacteria coated with specific antibodies were more effectively processed and presented by dendritic cells for stimulation of CD4+ and CD8+ T-cell responses16. Mice treated with mAb IgG3 specific for arabinomannan and then challenged with M. tuberculosis localised the pathogen within the granuloma centers, suggesting that the mAb conferred protection by enhancing a cellular immune response14. Joller et al.17 also reported the protective effects of antibodies mediated through Fc receptors using Legionella pneumophila and Mycobacterium bovis BCG. These findings indicate that some mAbs to M. tuberculosis are able to modify the course of experimental TB infection. Few studies have also documented the effect of antibody on complement activation by mycobacteria29,30.

In the present study, effect of both opsonins, anti-M. tb IgG antibody and complement, in the early interaction of M. tuberculosis with human macrophages was investigated. It was observed that pre-opsonisation of M. tuberculosis with anti-M. tb IgG resulted in enhanced activation of C3 (through both pathways) and C4 (through CP), but not factor B. This indicates that binding of both C3 and C4 is augmented by antibodies without influencing factor B. Initial contact between intracellular microorganisms and phagocytes can be mediated by opsonic30 and non-opsonic interactions31. The former is mediated by either Igs or C3b/C4b complement fractions that interact with Fc receptors and CRs, respectively. These processes bring about phagocytosis of the pathogen by the professional phagocytes. It was observed in the present study that opsonisation with complement and/or antibody increases the phagocytosis of M. tuberculosis by macrophages. Addition of anti-M. tb antibody alone had a significantly enhancing effect on phagocytosis of mycobacteria by macrophages. This effect was further significantly enhanced in the presence of complement components (especially CP components). Similar observations were reported by Hostetter et al.32, who showed that phagocytic index of macrophages was maximum in immunised and naive sera compared to that of heat-inactivated immune/naïve sera.

The increased phagocytosis may be due to predominant CR-mediated uptake or alternatively the interaction between Fc and CRs as earlier suggested33. In addition, there was an increased binding of C3 and C4 by the anti-M. tb IgG-opsonised bacilli, which might account for the increased uptake of bacilli through phagocytosis by the macrophages. Use of multiple receptor types for entry of M. tuberculosis into macrophages has been described previously and it is hypothesised that in vivo this may be the most relevant mechanism of uptake. In the presence of complement, a subset of CD4+ T cells carrying CR1 and CR2 were infected with HIV at a higher efficiency in vitro34. Also monocyte and macrophage-derived cell lines, which are CR3 positive, become more permissive for HIV infection in the presence of complement35.

The role of CRs on monocytes in the course of tuberculous infection has been a subject of much controversy. Schlesinger et al. reported that in the presence of antibodies against CR1 and CR3, a significant reduction in the adherence of M. tuberculosis and M. leprae to human MDMs was observed3. However, Hu et al.36 observed that CR3-deficient mice did not alter the course of tuberculous infection.

Assessment of intracellular viability of M. tuberculosis in the present investigation showed that both complement and antibody had an enhancing effect on the viability of bacilli. However, there was no significant change in the number of CFU at 48 h post-infection when the macrophages were treated with the

\(^{8}P<0.05\), Fig. 6B). At 48 h post-infection, a robust increase in the number of viable bacilli was observed at all conditions, except in the macrophages treated with CP components alone (Fig. 6C). Addition of antibody significantly increased the viability of bacilli alone \(^{8}P<0.05\) and also in combination with complement (CP components) \(^{8}P<0.05\); Fig. 6C). Thus, it was interesting to note in this experiment that intracellular growth of M. tuberculosis did not increase in the presence of complement alone after 48 h of infection, compared to that observed at 24 h post-infection.
bacilli and CP components alone, compared to that observed at 24 h post-infection. This is an interesting finding, which indicates a probable protective role of complement-mediated phagocytosis. Nevertheless, antibody alone or in combination with complement (both CP and AP components) significantly increased the growth of bacilli in the macrophages (Fig. 4). However, it was also earlier reported that entry of *M. tuberculosis* into macrophages through CRs promotes poor phagosome–lysosome fusion. The study has few drawbacks. In the present study, the complement binding was not quantitated, which could have helped to assess the actual levels of complement uptake by the bacilli. In addition, apoptosis and necrosis were not assessed, which could have thrown more light on the macrophage–mycobacteria interaction and intracellular viability aspects.

The observations of this study might corroborate the hypothesis that prior exposure to environmental mycobacteria is one of the reasons for the failure of BCG vaccination in adult forms of TB. The present findings indicate that *M. tuberculosis*-specific antibody, despite enhancing complement activation, might suppress the complement-mediated immune response against tuberculosis, which was evidenced by the increased viability of bacilli in the macrophages. Furthermore, increased phagocytosis mediated through complement and/or antibody might account for the immunopathological response, which could imply that exposure to environmental mycobacteria can induce immunosuppression. Further studies are needed to delineate the exact role of mycobacteria-specific antibody and to decipher the molecular mechanisms involved in the complement and/or Fc receptor–mediated routes of entry of the bacilli into the macrophages.

Therefore, the present study indicates that the role of humoral and innate immunity, at the level of complement system, during the early stages of tuberculosis infection is an essential component to be considered in the development of novel drug therapies against TB and thus devise methods by which the antibody-induced immunosuppression can be obviated.

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**References**


