The CD14 receptor does not mediate entry of \textit{Mycobacterium tuberculosis} into human mononuclear phagocytes

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Abstract

Prior reports have suggested that CD14 mediates uptake of \textit{Mycobacterium tuberculosis} into porcine alveolar macrophages and human fetal microglia, but the contribution of CD14 to cell entry in human macrophages has not been studied. To address this question, we used flow cytometry to quantify uptake by human monocytes and alveolar macrophages of \textit{M. tuberculosis} expressing green fluorescent protein. Neutralizing anti-CD14 antibodies did not affect bacillary uptake and the efficiency of bacillary entry was similar in THP-1 cells expressing low and high levels of CD14. However, most internalized bacteria were found in CD14+ but not in CD14− monocytes because \textit{M. tuberculosis} infection upregulated CD14 expression. We conclude that: (1) CD14 does not mediate cellular entry by \textit{M. tuberculosis}; (2) \textit{M. tuberculosis} infection upregulates CD14 expression on mononuclear phagocytes, and this may facilitate the pathogen’s capacity to modulate the immune response.

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1. Introduction

\textit{Mycobacterium tuberculosis} is a highly successful human pathogen, infecting one-third of the world’s population and causing 1.9 million deaths annually [1]. A distinctive characteristic of \textit{M. tuberculosis} is its capability to enter and replicate in macrophages. Eludicitation of the mechanism by which \textit{M. tuberculosis} enters macrophages will yield insight into a central feature of disease pathogenesis that may facilitate development of therapeautic interventions. Studies to date have revealed that \textit{M. tuberculosis} can enter human macrophages through complement receptors, the mannose receptor, scavenger receptors and the surfactant protein A receptor [2,3].

CD14 is a membrane-bound glycoprotein of mononuclear phagocytes that is the receptor for lipopolysaccharide of Gram-negative bacteria and for the mycobacterial homolog lipoarabinomannan [4–6]. Limited evidence suggests that CD14 mediates uptake of \textit{M. tuberculosis} into macrophages. Anti-CD14 antibodies inhibited phagocytosis of \textit{M. tuberculosis} by fetal microglia [7] and uptake of \textit{Mycobacterium bovis} by porcine alveolar macrophages [8]. To determine if CD14 is a receptor that facilitates entry of \textit{M. tuberculosis} into human mononuclear phagocytes, we studied entry of \textit{M. tuberculosis} expressing green fluorescent protein (GFP) into cells with variable degrees of CD14 expression.
2. Materials and methods

2.1. Isolation of mononuclear phagocytes

Blood was obtained from 10 healthy tuberculin-negative donors, peripheral blood mononuclear cells (PBMC) were centrifuged on Ficoll-Paque (Amersham Biosciences AB, Uppsala, Sweden) and CD14+ cells were isolated from the monocyte fraction with immunomagnetic beads conjugated to anti-CD14 (Miltenyi Biotech, Auburn, CA, USA).

Bronchoalveolar lavage was performed in three patients who had bronchoscopy for possible cancer. No patient had tuberculosis. Lavage was performed in the lung, where there was no bronchoscopic or radiographic evidence of cancer. Cell preparations that were >98% alveolar macrophages were obtained from bronchoalveolar lavage fluid, as described in [9].

2.2. Preparation of M. tuberculosis expressing GFP

*M. tuberculosis* H37Ra was transfected with a plasmid encoding the gene for GFP [10], with kanamycin as the selection marker. Transformants were selected on Middlebrook 7H10 medium with 0.5% glycerol and 50 µg ml⁻¹ of kanamycin. Single colonies of brightly fluorescent bacilli were picked and cultured in Middlebrook 7H9 broth with 0.2% glycerol and kanamycin for 5–10 days. Single-cell suspensions of *M. tuberculosis* were prepared as previously described [11] and were used to infect monocytes. The number of bacteria was estimated by counting in a Petroff–Hauser chamber. The inoculum was also plated on Middlebrook 7H10 medium, and mycobacterial viability was 65–70%.

2.3. Infection of mononuclear phagocytes with GFP-expressing M. tuberculosis

CD14+ monocytes were cultured in Teflon-coated PetriPERM plates (Heraeus Instruments, Hapkinton, MA, USA) at 10⁶ cells ml⁻¹ in RPMI-1640 (Gibco, Fredrick, MD, USA) with 10% heat-inactivated human serum. Under these conditions, cells remained non-adherent for 72–96 h, at which time CD14 was expressed on approximately 50% of the cells.

In some experiments, CD14+ monocytes were adhered to tissue culture plates and incubated for 7 days to obtain monocyte-derived macrophages prior to infection. Alveolar macrophages were cultured in the plates for 72–96 h prior to infection.

In some aliquots of THP-1 cells (ATCC TIB-202), <10% of cells was CD14+, and in other aliquots, 30% was CD14+. The latter was further enriched for CD14 by positive selection with anti-CD14-conjugated magnetic beads. We previously obtained stable transfectants of THP-1 cells containing either empty vector (pRc/RSV) or RSV containing wild-type human full-length CD14, which were cultured using previously published methods [12].

Monocytes, monocyte-derived macrophages, alveolar macrophages, THP-1 cells and THP-1 transfectants were washed three times with serum-free medium, and then infected with GFP-expressing *M. tuberculosis* at a multiplicity of infection of 20:1. Infected and uninfected control cells were incubated in RPMI-1640 without serum for 4–24 h.

2.4. Effect of anti-CD14 antibodies

Three anti-CD14 antibodies were used: MY4 (Coulter, Hialeah, FL, USA), 63D3 and 60bca [13,14]. A polyclonal anti-CD14 neutralizing antibody (R&D Systems, Minneapolis, MN, USA) produced in sheep was also used. Previous studies have shown that 5–25 µg ml⁻¹ of anti-CD14 monoclonal antibodies effectively blocks CD14 [7,8]. Anti-CD14 antibodies were added in concentrations of 5–35 µg ml⁻¹ to monocytes 60 min prior to infection, and the percentages of infected monocytes were determined after 4 and 24 h, as outlined below.

2.5. Cytofluorometric and fluorescent microscopy analysis

Aliquots of mononuclear phagocytes were washed and stained with phycoerythrin (PE)-labeled anti-CD14 antibodies (Becton Dickinson Immunocytometry Systems, San Jose, CA, USA). For each sample, 5 × 10⁷ cells were incubated with PE-labeled anti-CD14 antibodies at a final concentration of 20 µg ml⁻¹ for 20 min on ice. FACS buffer (phosphate-buffered saline with 10 g l⁻¹ bovine serum albumin and 0.1 g l⁻¹ sodium azide) was used to wash the cells and to dilute the reagents. After incubation with PE-labeled anti-CD14 antibodies, cells were washed three times, and fixed in 1% paraformaldehyde. Samples were analyzed in an EPICS C flow cytometer (Coulter). The isotype control was PE-labeled rat IgG (BD Pharmingen, San Diego, CA, USA). To determine the percentage of infected cells, we gated on live cells and measured the percentage of GFP-expressing cells.

For fluorescent microscopy, infected and uninfected control cells were washed with FACS buffer and fixed in 1% paraformaldehyde. A fluorescent microscope (Olympus BX40, Olympus America, Lake Success, NY, USA) was used to visualize the internalized bacilli, which were counted in a minimum of 300 cells.

2.6. Statistical analysis

Results are shown as the mean ± standard error, and values were compared by the Student’s t-test or the Wilcoxon rank-sum test, as appropriate.
3. Results

3.1. Effect of neutralizing antibodies on cellular entry by M. tuberculosis

Monocytes are often isolated from PBMC by adherence. However, adherent cell preparations may be contaminated with other cells. To avoid this problem, highly purified monocytes were isolated from PBMC by positive selection with anti-CD14-conjugated magnetic beads. These cells were 96–100% CD14+, as assessed by flow cytometry.

After infection with GFP-expressing M. tuberculosis, fluorescent microscopy showed that >95% of the GFP-expressing M. tuberculosis was intracellular.

To determine if CD14 plays a role in mediating entry of M. tuberculosis into mononuclear phagocytes, we incubated monocytes with or without each of the monoclonal anti-CD14 antibodies, MY4, 60bca, and 63D3, or a polyclonal neutralizing anti-CD14 antibody for 60 min, prior to infection with GFP-expressing bacilli. Four and 24 h later, the percentage of infected (GFP+) monocytes was determined.

Fig. 1. Effect of anti-CD14 antibodies on entry of M. tuberculosis into human monocytes. CD14+ monocytes were incubated for 60 min with anti-CD14 monoclonal antibodies (MY4, 60bca and 63D3; panel A), a mixture of MY4, 60bca and 63D3 or polyclonal neutralizing anti-CD14 (panel B). Control cells were untreated. Monocytes were then infected with GFP-expressing M. tuberculosis, and flow cytometry was performed after 4 h and 24 h. The values shown are the means and standard errors for two to five independent experiments. C: A representative flow cytometry histogram of the monocytes 4 h (top panels) and 24 h (bottom panels) after infection with GFP-expressing M. tuberculosis. Untreated control cells (left panels) and MY4-treated cells (right panels) are shown.

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The percentages of infected monocytes in the absence or in the presence of anti-CD14 antibodies were similar (Fig. 1). Isotype control antibodies did not affect the percentage of infected monocytes (data not shown). Serum-free medium was used to infect the cells, so that complement receptors should not play a significant role in mediating intracellular entry of *M. tuberculosis* under these conditions.

Because previous studies using microglia and porcine alveolar macrophages suggested that CD14 mediated intracellular entry of mycobacteria [7,8], we considered the possibility that CD14 played a role in differentiated macrophages but not in monocytes. Therefore, monocyte-derived macrophages from three individuals were infected with GFP-expressing *M. tuberculosis*, in the presence or absence of the anti-CD14 antibody MY4, and the percentage of infected cells was measured by flow cytometry. Anti-CD14 did not affect entry of *M. tuberculosis* into the monocyte-derived macrophages (Fig. 2A). Alveolar macrophages were obtained from three persons, and the percentages of infected cells were counted, using a fluorescence microscope. Anti-CD14 antibodies did not affect the percentages of infected cells (Fig. 2B). The MY4 antibody was functional, as it effectively blocked TNF-α production by monocytes in response to lipopolysaccharide, a process that is CD14-dependent (5828 ± 64 pg ml⁻¹ versus 1865 ± 73 pg ml⁻¹ of TNF-α in the absence and presence of anti-CD14 antibody, respectively). These results are consistent with previously published results on the effects of MY4 on TNF-α production [15].

### 3.2. Entry of *M. tuberculosis* into mononuclear phagocytes expressing variable degrees of CD14

As an alternative means to evaluate the role of CD14 in the entry of *M. tuberculosis* into human monocyte-derived macrophages and alveolar macrophages, monocyte-derived macrophages (panel A) and alveolar macrophages (panel B) were incubated with anti-CD14 (MY4) for 60 min and control cells were untreated. Monocytes were then infected with GFP-expressing *M. tuberculosis*, and flow cytometry (panel A) and fluorescent microscopy (panel B) were performed after 4 and 24 h. The means and standard errors for three independent experiments are shown.

![Fig. 2. Effect of anti-CD14 antibodies on entry of *M. tuberculosis* into human monocyte-derived macrophages and alveolar macrophages. Monocyte-derived macrophages (panel A) and alveolar macrophages (panel B) were incubated with anti-CD14 (MY4) for 60 min and control cells were untreated. Monocytes were then infected with GFP-expressing *M. tuberculosis*, and flow cytometry (panel A) and fluorescent microscopy (panel B) were performed after 4 and 24 h. The means and standard errors for three independent experiments are shown.](image-url)

![Fig. 3. Effect of CD14 expression on entry of *M. tuberculosis* into THP-1 cells. THP-1 cells naturally expressing high (55%) and low (7%) levels of CD14 (panel A), and THP-1 cells transfected with CD14 or empty vector (panel B) were infected with GFP-expressing *M. tuberculosis*, and flow cytometry was performed after 4 and 24 h. The means and standard errors of three independent experiments are shown.](image-url)
entry of *M. tuberculosis* into mononuclear phagocytes, we isolated THP-1 cells expressing low (7%) or high (55%) levels of CD14. Four and 24 h after infection with *M. tuberculosis*, the percentages of infected cells were similar in THP-1 cells expressing low or high levels of CD14, suggesting that CD14 is not required for cellular entry of *M. tuberculosis* (Fig. 3A).

To more definitively demonstrate that CD14 is not required for entry of *M. tuberculosis* into mononuclear phagocytes, we studied stable transfectants of THP-1 cells containing either full-length CD14 or an empty vector (RSV) which were 96% and 4% CD14+, respectively. After 4 and 24 h, the percentages of infected cells in CD14-THP-1 transfectants and RSV-THP-1 transfectants were comparable (Fig. 3B).

### 3.3. Effect of *M. tuberculosis* infection on CD14 expression by monocytes

The findings above indicate that CD14 does not enhance entry of *M. tuberculosis* into mononuclear phagocytes. Nevertheless, others have suggested that *M. bovis*-infected alveolar macrophages express higher levels of CD14 than uninfected macrophages [8]. In addition, 4 h after infection of monocytes from seven donors with GFP-expressing *M. tuberculosis*, 34±9% of the CD14+ cells was infected with *M. tuberculosis*, compared to 5.5±2.7% of CD14- cells (*P* = 0.01, data not shown). To determine if *M. tuberculosis* infection enhances expression of CD14, we isolated CD14+ monocytes from PBMC of three donors. These cells were 99% CD14+, but after incubation for 3-4 days in Teflon-coated plates, CD14 expression was reduced to 50±7%. These monocytes were then exposed to GFP-expressing *M. tuberculosis*, which infected a subpopulation of the monocytes. After 4 h, cells were stained with PE-labeled anti-CD14 prior to infection and 4 h after infection. The percentages of CD14+ cells prior to infection are shown on the left hand side of the figure. Four hours after infection, we gated on infected GFP+ cells and uninfected GFP- cells, and the percentages of infected or uninfected cells that expressed CD14 were calculated. Each symbol (circle, square, triangle) shows a result for one person. The closed symbols and solid lines represent values for infected monocytes, the open circles and dashed lines represent values for uninfected monocytes.

### 4. Discussion

The data in this report suggest that: (1) the CD14 receptor does not play an essential role for entry of *M. tuberculosis* to human mononuclear phagocytes; (2) *M. tuberculosis* infection upregulates CD14 expression on mononuclear phagocytes, and this may represent an important mechanism by which the pathogen modulates the immune response.

The question of whether mycobacteria utilize the CD14 receptor to enter mononuclear phagocytes is controversial. Anti-CD14 antibodies inhibit uptake of *M. tuberculosis* by human fetal microglia, which are derived from monocyte precursors and are phagocytic [7]. In addition, *M. bovis* preferentially infects porcine alveolar macrophages that express high levels of CD14, and anti-CD14 antibodies inhibit bacillary entry [8]. In contrast, Reiling and colleagues reported that macrophages from CD14+/+ and CD14−/− mice are equally susceptible to entry by *Mycobacterium avium* [16]. We found that the efficiency of bacillary entry was similar in THP-1 cells expressing low and high levels of CD14, and three different neutralizing monoclonal antibodies to CD14 and a neutralizing polyclonal anti-CD14 antibody did not reduce the capacity of *M. tuberculosis* to infect monocytes, monocyte-derived macrophages or alveolar macrophages. We believe that the most likely explanation for these results is that CD14 is not an essential receptor for entry of *M. tuberculosis* into human mononuclear phagocytes. However, we cannot formally exclude the possibility that all the mononuclear phagocytes that we studied expressed very low levels of CD14, and that these low levels were sufficient to mediate mycobacterial entry.

Several factors may explain why our results differ from those of other studies evaluating members of the *M. tuberculosis* complex. First, the rate of entry of *M. tuberculosis*
into microglia is much slower than that into monocytes or alveolar macrophages [7], suggesting that the receptors used for bacillary entry differ in these cell populations. Second, Khanna and colleagues studied entry of *M. bovis* into porcine cells, whereas we evaluated entry of *M. tuberculosis* into human cells [8]. These species differences may explain differences in receptors used by mycobacteria to enter phagocytic cells. For example, closely related *Chlamydia* species show differential usage of mannose receptors to enter macrophages [17]. We demonstrated that the avirulent H37Ra strain of *M. tuberculosis* did not utilize CD14 to enter human mononuclear phagocytes. However, our findings may also apply to virulent strains, as both CD14-deficient and wild-type mice have comparable levels of infection in the lungs after aerosol infection with H37Rv [18].

Although our results did not suggest that CD14 was necessary for *M. tuberculosis* to enter human monocytes, 4 h after infection the majority of infected cells were CD14+. This may imply that *M. tuberculosis* infection upregulated surface CD14 expression in infected monocytes. CD14 binds to the mannosenriched polysaccharide lipoarabinomannan containing highly branched arabinofuranosyl side chains, which is a major component of the mycobacterial cell envelope of *Mycobacterium smegmatis* [6]. Lipoarabinomannan in *M. tuberculosis* strains is a homologous structure that is capped with terminal mannose residues [19]. Mannosylated lipoarabinomannan stimulates significant production of the immunosuppressive cytokine transforming growth factor (TGF)-β, but not of the proinflammatory cytokines such as TNF-α and IL-6 [20]. In patients with tuberculosis, enhanced TGF-β production markedly inhibits T-cell responses to *M. tuberculosis* [21], and TGF-β enhances mycobacterial growth in monocytes [22]. It is intriguing to speculate that upregulation of CD14 expression by *M. tuberculosis* may contribute to the immunopathogenesis of tuberculosis by facilitating interactions with mannosylated lipoarabinomannan, leading to increased TGF-β production and suppression of the immune response.

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**References**


