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Complement factor H interferes with *Mycobacterium bovis* BCG entry into macrophages and modulates the pro-inflammatory cytokine response

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ABSTRACT

Mycobacterium tuberculosis is an accomplished intracellular pathogen, particularly within the macrophage and this is of the utmost importance in the host-pathogen stand-off observed in the granuloma during latent tuberculosis. Contact with innate immune molecules is one of the primary interactions that can occur with the pathogen *M. tuberculosis* once inhaled. Complement proteins may play a role in facilitating *M. tuberculosis* interactions with macrophages. Here, we demonstrate that factor H, a complement regulatory protein that down-regulates complement alternative pathway activation, binds directly to the model organism *M. bovis* BCG. Binding of factor H reaches saturation at 5–10 μg of factor H/ml, well below the plasma level. C4 binding protein (C4BP) competed with factor H for binding to mycobacteria. Factor H was also found to inhibit uptake of *M. bovis* BCG by THP-1 macrophage cells in a dose-dependent manner. Real-time qPCR analysis showed stark differential responses of pro- and anti-inflammatory cytokines during the early stages of phagocytosis, as evident from elevated levels of TNF-α, IL-1β and IL-6, and a concomitant decrease in IL-10, TGF-β and IL-12 levels, when THP-1:BCG interaction took place in the presence of factor H. Our results suggest that factor H can interfere with mycobacterial entry into macrophages and modulate inflammatory cytokine responses, particularly during the initial stages of infection, thus affecting the extracellular survival of the pathogen. Our results offer novel insights into complement activation-independent functions of factor H during the host-pathogen interaction in tuberculosis.

1. Introduction

Tuberculosis (TB) remains a major global health problem with the majority of cases of active TB disease and TB-related deaths occurring in developing countries (Zumla et al., 2013). Currently, there are 8.7 million new cases of active TB and 1.4 million deaths per year, with one in every three people infected worldwide with latent TB infection (Zumla et al., 2013). Once the causative agent *Mycobacterium tuberculosis* has infected the host through aerosol inhalation, it is able to evade and circumvent immune responses allowing it to establish a long-term latent infection through its ability to persist as an intracellular pathogen within phagocytes. *M. tuberculosis* prevents phagosomal maturation and modulates acidity within the phagolysosome, resulting in intracellular survival of the pathogen within phagocytes for an extended period of time, thus establishing a life-long infection (Gupta et al., 2012). During latency, *M. tuberculosis* resides within a granuloma, a dense collection of immune cells that is a key pathological feature of TB which not only limits the growth of the pathogen, but also provides a niche from which the pathogen can reactivate (Ehlers and Schaible, 2012).

Abbreviations: TB, tuberculosis; BCG, Bacillus Calmette-Guerin; C4BP, C4b-binding protein; CCP, complement control protein; CR, complement receptors; M^{++}, divalent metal ions; MAC, membrane attack complex.

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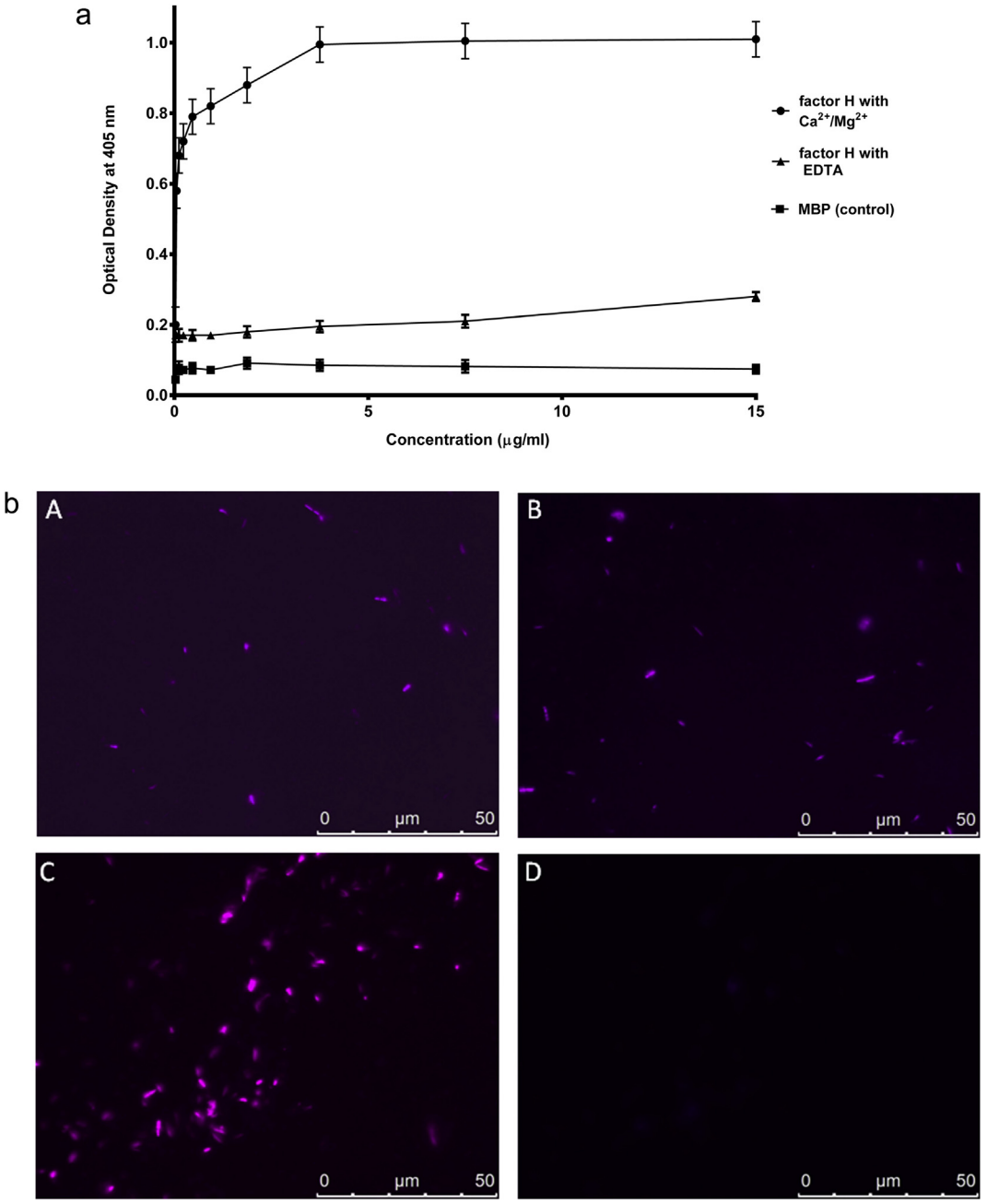

Fig. 1. Divalent metal ions increase the binding of human factor H to *M. bovis* BCG. (a) Factor H binding to mycobacteria in 10 mM HEPES, 60 mM NaCl, 100 μg/ml hen ovalbumin pH 7.5 containing either 0.5 mM EDTA or 0.5 mM $CaCl_2$/0.5 mM $MgCl_2$. Serial dilutions of factor H were added to the wells containing mycobacteria followed by mouse mAb anti-factor H, anti-mouse IgG conjugated with alkaline phosphatase. Maltose binding protein (MBP) was used as a negative control. Assay is conducted in quadruplicate. Error bars represent ± standard deviation. (b) To confirm binding results, fluorescence microscopy was performed to show binding of factor H at different concentrations to *M. bovis* BCG cells. Bacterial cells were incubated with either A) 2.5 μg/ml, B) 5 μg/ml, C) 10 μg/ml of factor H or D) 10 μg/ml of Maltose binding protein (MBP) (used as a negative control). Mouse anti-human factor H (MRCOX23) was used as the primary antibody and goat anti-mouse conjugated with AlexaFluor488 as the secondary antibody. Scale bar, 50 μm.

Although *M. tuberculosis* is known to interact with components of the innate immunity such as toll-like receptors, complement, surfactant proteins SP-A and SP-D (Tsolaki, 2009), the initial stages of TB pathogenesis remain poorly understood. The complement system is a key player in innate immunity, providing protection against pathogens without the need for previous exposure and/or immunization, in addition to potentiating adaptive immunity (Carroll and Sim, 2011). Complement is activated upon encounter with pathogens via one or more of three pathways: classical, lectin, and alternative. Activation of complement by any of the three pathways results in the formation of a C3 convertase and the formation of C3b, which opsonises pathogens and initiates a number of immune mechanisms, including the generation of the lytic membrane attack complex (MAC). The complement system is tightly regulated by several inhibitor proteins that are either surface-bound or present in the fluid-phase. A key regulator of complement activation is factor H, which regulates the alternative pathway (Sim et al., 1993) and the classical pathway (Kishore and Sim, 2012). Human factor H is a 155 kDa elongated glycoprotein and can bind simultaneously to C3b and suitable charge-cluster ligands on complement alternative pathway activator and non-activator surfaces (Kuhn and Zipfel, 1996).

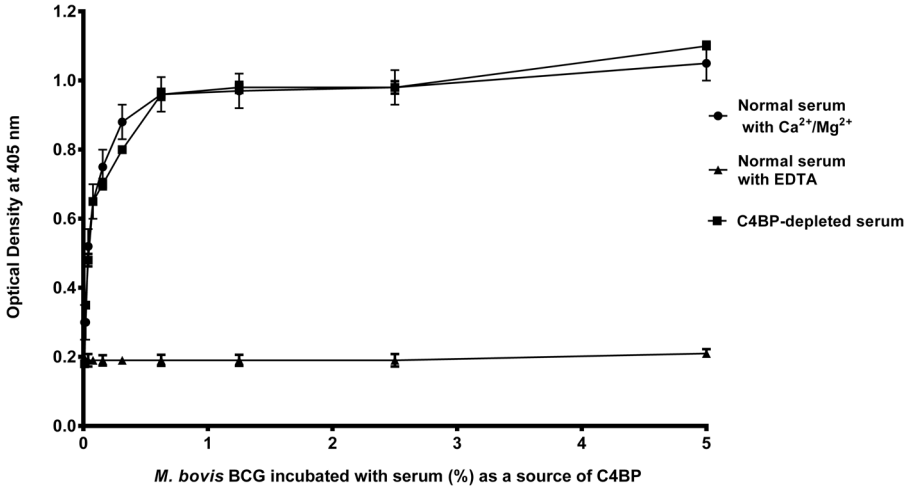

Fig. 2. Human C4BP from serum binds immobilised *M. bovis* BCG in EDTA and in $Ca^{2+} + Mg^{2+}$. Different concentrations of serum (as a source of C4BP) were added to mycobacteria. Binding of C4BP was determined using anti-C4BP polyclonal antibody. C4BP-depleted serum was used as negative control for binding specificity. Assays were conducted in quadruplicate. Error bars represent ± standard deviation.

Many bacteria have evolved mechanisms to evade complement, which include proteolytic cleavage of complement proteins, having their own complement inhibitors (Rooijakkers and van Strijp, 2007), or binding of host's complement regulatory proteins. Since factor H is a soluble down-regulator of the complement system, its sequestration on the bacterial cell surfaces may help pathogens resist complement attack. For instance, binding of factor H and/or C4 binding protein (C4BP), another complement regulator, was shown to decrease C3b and C4b deposition on the surface of Streptococci and reduce phagocytosis by macrophages (Thern et al., 1995). Factor H binds directly to many bacterial species e.g Yersinia, Hemophilus, Borrelia and Streptococcus (China et al., 1993; Hallstrom et al., 2008; Hellwage et al., 2001; Horstmann et al., 1988).

M. tuberculosis is highly specialised at residing within phagocytes and may exploit complement proteins to gain entry into macrophages. Although it has been shown that *M. tuberculosis* can activate both the classical and the alternative pathways (Ferguson et al., 2004), it is not clear how the pathogen uses complement proteins in TB pathogenesis. *M. tuberculosis* has been shown to bind to complement receptors CR1, CR3 and CR4 and gain entry into macrophages (Schlesinger et al., 1990). Enhanced phagocytosis of *M. tuberculosis* by human alveolar and monocyte-derived macrophages is due to opsonization by C3 (Hirsch et al., 1994). However, CR3 is not the dominant route for *M. tuberculosis* to gain access inside the phagocyte (Hu et al., 2000; Schlesinger et al., 1990). We have also previously shown multiple routes of complement activation by *Mycobacterium bovis* BCG which directly activates the classical, lectin and alternative pathways, resulting in direct binding of C3b onto the mycobacterial surface (Carroll et al., 2009). *M. bovis* BCG was also shown to bind factor H, suggesting a possible regulation of complement activation on the bacterial surface (Carroll et al., 2009).

The aim of the present study was to investigate further the role of factor H in TB pathogenesis, by using the model organism *M. bovis* BCG. We show that factor H may have functions which are distinct from its role as a regulator of complement activation. Purified human factor H binds to the surface of mycobacterial cells, and inhibits uptake of *M. bovis* BCG by macrophages in a dose dependent manner. Factor H: mycobacteria interaction also alters the pro- and anti-inflammatory cytokine responses of macrophages, indicating that factor H may play a role in shaping the initial immune response against mycobacteria. This study highlights novel non-complement-related roles of factor H in the initial stages of TB infection. It is likely that factor H has an important role to play in influencing cellular immunity during latent TB infection.

2. Materials and methods

2.1. Proteins, serum and antibodies

Human factor H was purified from plasma as described previously (Sim et al., 1993), using a monoclonal anti-factor H column. C4BP-depleted human serum was prepared as described by Sim et al. (Sim et al., 1981). Rabbit anti-human factor H and anti-human C4BP polyclonal antibodies were generated within the MRC Immunochemistry Unit, Oxford.

2.2. Mycobacterial cell culture

M. bovis BCG (Pasteur strain) was grown in liquid culture using Middlebrook 7H9 media (Sigma-Aldrich), supplemented with 0.2% (v/v) glycerol, 0.05% (v/v) Tween-80, and 10% (v/v) albumin-dextrose-catalase (ADC) (BD BBL, Becton Dickinson). Cultures were incubated at 37°C with agitation (~120 rpm) for 7–10 days until the bacteria had reached the exponential growth phase (OD_{600nm} = 0.80–1.00), which is equivalent to $0.8–1 \times 10^9$ bacteria/ml.

2.3. THP-1 cell culture

THP-1 macrophage cells derived from a human acute monocyte leukemia cell line (ATCC TIB-202) were cultured in complete RPMI-1640 (Gibco) (cRPMI) consisting of 10% (v/v) fetal bovine serum (FBS) (Sigma-Aldrich), 2 mM L-glutamine (Sigma-Aldrich), 100 U/ml penicillin (Sigma-Aldrich), 100 μg/ml streptomycin (Sigma-Aldrich) and 1 mM sodium pyruvate (Sigma-Aldrich) and left to grow in 5% CO_2 incubator at 37°C for approximately 3 days before passaging. To count cells and assess cell death, equal volumes of cell suspension and Trypan Blue (0.4% w/v solution) were mixed and cells were counted using a haemocytometer with Neubauer rulings. Cells were then re-suspended in cRPMI.

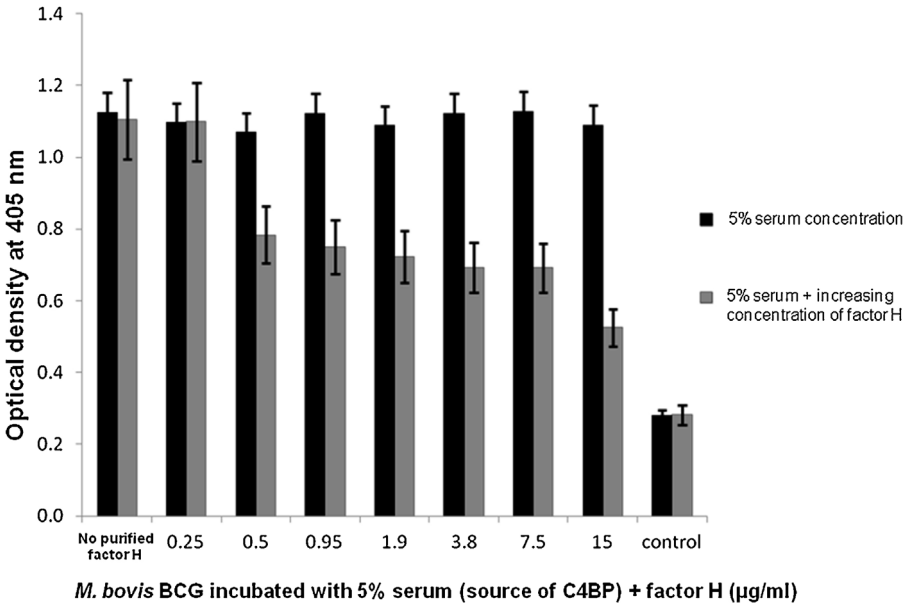

Fig. 3. Binding of C4BP to the surface of *M. bovis* BCG in the presence of different concentrations of factor H. *M. bovis* BCG-coated wells were incubated with 5% human serum as a source of C4BP and with human serum to which increasing quantities of factor H were added. Binding of C4BP was determined using anti-C4BP polyclonal antibody. Controls are incubations of serum without mycobacteria, and serum with or without 15 μg/ml of added factor H. Assay was conducted in quadruplicate. Error bars represent ± standard deviation.

2.4. Assay for measuring purified human factor H binding to mycobacteria

Mycobacterial cells were harvested and washed in PBS and the concentration was adjusted to 1.25×10^9 cells/ml in PBS by measuring the OD_{600nm} (using the approximation that an $OD_{600nm} = 1$ is equivalent to 1×10^9 cell/ml). Then, 200 μl of *M. bovis* BCG bacterial suspension was dispensed into each well of a 96-well ELISA plate (Maxisorb™, Nunc). Plates were incubated at 37°C for 2 h, or 4°C overnight and washed with buffer I containing 10 mM HEPES, 60 mM NaCl, 50 μg/ml hen ovalbumin (Sigma), pH 7.5. Wells were blocked for 2 h at 37°C with buffer I containing 10% w/v milk powder (Marvel Dried Milk)

Serial two-fold dilutions of purified factor H were added, (maximum concentration 15 μg/ml, 100 μl/well, in 10 mM HEPES, 60 mM NaCl, 0.5 mM EDTA, 100 μg/ml hen ovalbumin pH 7.5) and incubated for 2 h at room temperature. Wells were washed 3 times with the same buffer. Then, MRCOX23 mouse anti-human factor H antibody (Sim et al., 1983) in the same buffer (2 μg in 100 μl) was added to the wells and incubated for 1 h h at room temperature. Plates were washed an additional 3 times and incubated with sheep anti-mouse IgG-alkaline phosphatase conjugate (Sigma-Aldrich, #A3562), diluted 1/5000 in the same buffer. The substrate p-nitrophenol phosphate (buffered tablets, Sigma-Aldrich, #N7660) was then added to each well and the colour was read at 405 nm. Additional assays were also performed, using the 10 mM HEPES, 60 mM NaCl buffer but with 0.5 mM $CaCl_2$, 0.5 mM $MgCl_2$, instead of EDTA. All assays were performed in quadruplicate.

2.5. Fluorescence microscopy

M. bovis BCG bacteria (approximately 10^6 cells) were spotted on poly-L-lysine coated microscope slides and incubated at 37°C for cells to adhere. After washing twice with PBS, bacterial cells were then fixed with paraformaldehyde for 10 min. Slides were washed five times with PBS and then incubated at 37°C for 2 h with 2.5, 5 or 10 μg/ml of factor H, or 10 μg/ml of maltose binding protein (negative control) in 10 mM HEPES, 60 mM NaCl, 0.5 mM $CaCl_2$, 0.5 mM $MgCl_2$, 100 μg/ml hen ovalbumin, pH 7.5. Slides were washed five times with PBS, and then the primary antibody (MRCOX23 mouse anti-human factor H) added at 1/500 dilution and incubated for 30 min at room temperature. After washing five times with PBS, goat anti-mouse conjugated with AlexaFluor488 was added as the secondary antibody and incubated for 30 mins at room temperature. Slides were then washed five times with PBS and mounted with antifade (Citifluor AF3) PBS solution and viewed using a Leica DM4000 Fluorescence microscope. Images were processed using Image J (http:\\imagej.nih.gov/ij).

2.6. C4BP binding assay

To examine binding of C4BP to mycobacteria, microtitre wells were coated with *M. bovis* BCG, washed and blocked as described above. Two-fold serial dilutions of normal human serum or C4BP-depleted human serum were added into the wells in the HEPES buffer containing EDTA or Ca^{2+}/Mg^{2+} as described earlier and incubated for 2 h at room temperature prior to washing and addition of 1/1000 dilution of rabbit anti-C4BP polyclonal antiserum, diluted in the appropriate buffer. Next, goat anti-(rabbit IgG)-alkaline phosphatase conjugate (1/5000 dilution; Sigma Aldrich, # A3687) and substrate p-nitrophenol phosphates were added and the plate was read at 405 nm. Assays were performed in duplicate.

To test whether C4BP competes with factor H for its binding sites on the mycobacterial surface, we tested the binding of C4BP (from serum) to the surface of *M. bovis* BCG in the presence and absence of increasing concentrations of factor H in buffer with Ca^{2+}/Mg^{2+}. An ELISA plate was coated with *M. bovis* BCG and incubated (2 h, room temperature) with 5% human serum (as a source of C4BP), or 5% human serum containing added purified factor H (0.25–15 μg/ml). Bound C4BP was then detected using anti-C4BP polyclonal antibody. Assays were performed in quadruplicate.

2.7. Phagocytosis assay

50 ml culture of *M. bovis* BCG were harvested at mid exponential phase ($OD_{600nm} = 1$) and were centrifuged at 1000*g* for 10 min at 4°C. The cell pellet was re-suspended in 50 ml of 7H9 growth medium (with 10% ADC, 0.05% v/v Tween-80 and 0.2% v/v glycerol).

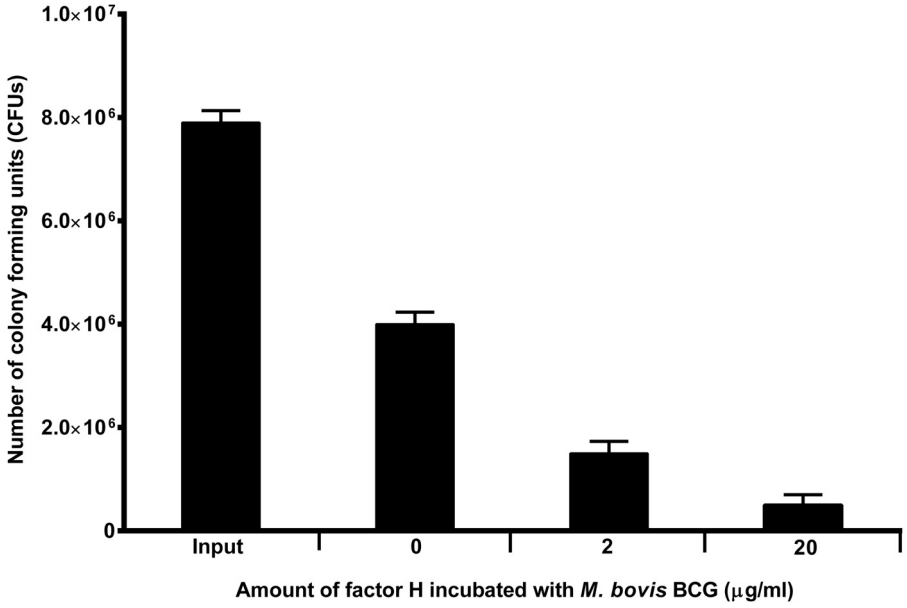

Fig. 4. Effect of factor H on the phagocytosis of *M. bovis* BCG by THP-1 cells. *M. bovis* BCG were pre-incubated with factor H at concentrations of 0, 2 and 20 μg/ml, and then incubated with THP-1 cells for 2 h. After THP-1 cell lysis, surviving *M. bovis* BCG were measured by plating lysate on 7H10 media in order to obtain colony forming units (CFUs). The input value was the starting number of untreated *M. bovis* BCG added to the THP-1 cells, pre-phagocytosis.

This mycobacterial culture was then placed into three microfuge tubes and treated with either 2 or 20 μg/ml of purified factor H, or left untreated and incubated for 2 h at room temperature. The cell suspension was washed once in growth medium before re-suspending in RPMI-1640 containing 0.05% Tween-80 but without FBS, penicillin and streptomycin. 200 μl of the mycobacterial suspension was added to each well of THP-1 cells, as described below. Mycobacterial concentration was adjusted to give approximate bacteria: macrophage ratio of 10:1. To obtain an estimated input number of mycobacteria, 4 serial 1/10 dilutions were made and 10 μl of the concentrated mycobacterial suspension and diluted suspensions were spotted on to 7H10 media (Sigma-Aldrich) agar wells in 24-well plates. The plates were secured with parafilm tape and wrapped in aluminum foil to prevent dehydration, inverted and incubated at 37°C for up to 14 days before counting the colony forming units (CFUs).

THP-1 cells were counted and adjusted to 1×10^6 cells/well (in 1.8 ml) in wells of a 24-well plate. In order to induce adherence onto the wells, THP-1 cells in RPMI-1640 without FBS, penicillin or streptomycin were differentiated into macrophages by adding 50 ng/ml of phorbol 12-myristate 13-acetate (PMA) (Sigma-Aldrich #P1585) and left to settle for at least 30 min before adding 200 μl volume of bacterial culture (to give 1×10^7 bacteria/ml). Plates were gently swirled, secured with micropore tape and incubated in 5% CO_2 at 37°C for up to 6 h in order to allow mycobacterial uptake by the macrophages. THP-1 cells were collected at 15 min, 30 min, 45 min, 1 h, 2 h and 6 h. Plates were swirled gently to re-suspend mycobacteria from the bottom of the well and the medium was then removed to discard non-ingested bacteria and the THP-1 cells were washed once with FBS/antibiotic-free RPMI. THP-1 cells were lifted after adding 1 ml of 0.25% trypsin to the wells and incubated for 10 min at 37°C using 1.5 ml microfuge tubes and centrifuged at 1000*g* for 10 min at 4°C. In order to release the ingested mycobacteria, THP-1 cells were lysed by re-suspending the cell pellets in 1 ml of sterile water followed by a series of vortex-mixing for 10 min at room temperature. 24-well plates containing 2 ml solid 7H10 agar per well were prepared in advance. Four serial 1/10 dilutions of the cell lysate were made and 10 μl of the concentrated mycobacterial suspension and diluted suspensions from each time-point were spotted onto the 7H10 agar wells in 24-well plates. The 24-well plates were secured with parafilm and wrapped in aluminum foil, inverted and incubated at 37°C for 14 days as described above. Wells were photographed after approximately 2 weeks of incubation as described (Theus et al., 2004), and the CFU counted.

2.8. Total RNA preparation and cDNA synthesis

THP-1 cell pellets were collected and lysed as described above. RNA extraction was performed on the pellets using the GenElute Mammalian Total RNA Purification Kit (Sigma-Aldrich) according to the manufacturer's protocol. Samples were then treated with DNase (Sigma-Aldrich) to remove any contaminating DNA according to the manufacturer's protocol. Samples were heated at 70°C for 10 min to inactivate both the DNase I and the RNase, and subsequently chilled on ice. The amount of RNA was measured using a NanoDrop 2000/2000c spectrophotometer (Thermo Fisher Scientific) at 260 nm and the ratio of absorbance at 260 nm and 280 nm was used to assess the purity of the RNA. cDNA was synthesized using High Capacity RNA to cDNA Kit (Applied Biosystems) following the manufacturer's protocol.

2.9. Primers for cytokines

Primer sequences were designed and analyzed for specificity using the nucleotide Basic Local Alignment Search Tool and Primer-BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The following primers were used: *18S* forward (5′-ATGGCCGTTCTTAGTTGGTG-3′) and *18S* reverse (5′-CGCTGAGCCAGTCAGTGTAG-3′); *IL-1β* forward (5′-GGACAAGCTGAGGAAGATGC-3′) and *IL-1β* reverse (5′-TCGTTATCCCATGTGTCGAA-3′); *IL-6* forward (5′-GAAAGCAGCAAAGAGGCACT-3′) and *IL-6* reverse (5′-TTTCACCAGGCAAGTCTCCT-3′); *IL-10* forward (5′-TTACCTGGAGGAGGTGATGC-3′) and *IL-10* reverse (5′-GGCCTTGCTCTTGTTTTCAC-3′); *IL-12* forward (5′-AACTTGCAGCTGAAGCCATT-3′) and *IL-12* reverse (5′-GACCTGAACGCAGAATGTCA-3′); *TGF-β* for-

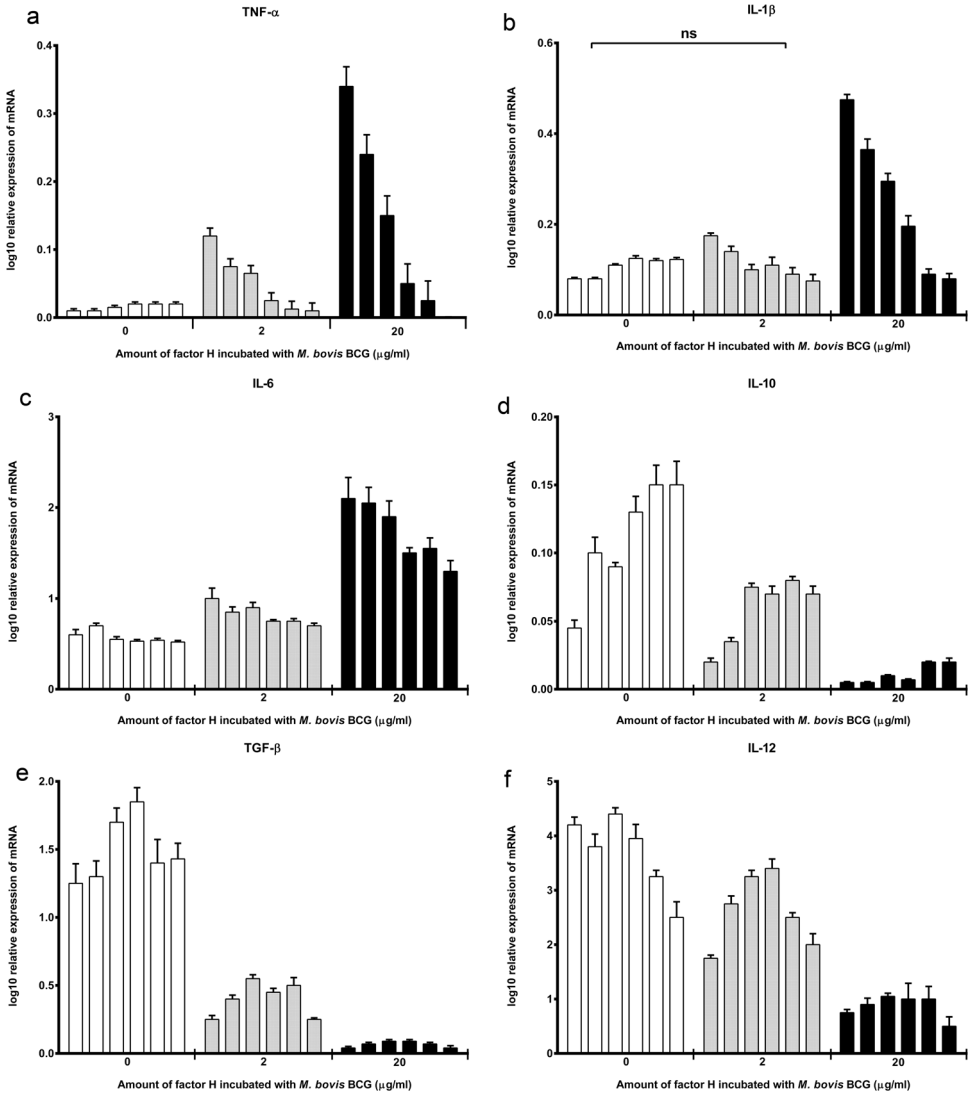

Fig. 5. Temporal expression of cytokines by THP-1 cells incubated with *M. bovis* BCG and different concentrations of factor H. (a) TNF-α; (b) IL-1β; (c) IL-6; (d) IL-10; (e) TGF-β and (f) IL-12. qPCR analysis of the expression of cytokines at the following time-points: 15, 30, 45, 60, 120 and 360 mins (not shown along the x-axis) was carried out. The calibrator sample used was THP-1 cells without *M. bovis* BCG and 0 μg/ml of factor H. The data was normalized to 18 S rRNA gene expression which was used as an endogenous control. Assays were conducted in triplicate. The RQ value was calculated using the formula: $RQ = 2^{-\Delta\Delta Ct}$. Error bars represent ± standard error of the mean. A 2-way ANOVA was performed on the data to compare differences in expression for each cytokine (e.g. between zero and 2 μg/ml of factor H and zero and 20 μg/ml of factor H). All comparisons are statistically significant ($p \leq 0.01$), except where shown (ns: not significant).

ward (5′-GTACCTGAACCCGTGTTGCT-3′) and *TGF-β* reverse (5′-GTATCGCCAGGAATTGTTGC-3′); *TNF-α* forward (5′-AGCCCATGTTGTAGCAAACC-3′) and *TNF-a* reverse (5′-TGAGGTACAGGCCCTCTGAT-3′).

2.10. qPCR assay for cytokine expression and data analysis

PCR was performed on all cDNA samples to assess the quality of the cDNA. The qPCR assay was performed for the expression of pro- and anti-inflammatory cytokines. The qPCR reaction consisted of 5 μl Power SYBR Green MasterMix (Applied Biosystems), 75 nM of forward and reverse primer and 500 ng template cDNA in a 10 μl final reaction volume. PCR was performed in a 7900HT Fast Real-Time PCR System (Applied Biosystems). The initial steps were 2 min incubation at 50°C followed by 10 min incubation at 95°C, the template was then amplified for 40 cycles under these conditions: 15 s incubation at 95°C and 1 min incubation at 60°C. Samples were normalized using the expression of human 18 S rRNA. Data was analyzed using the RQ Manager Version 1.2.1 (Applied Biosystems). Ct (cycle threshold) values for each cytokine target gene were calculated and the relative expression of each cytokine target gene was calculated using the Relative Quantification (RQ) value, using the formula: $RQ = 2^{-\Delta\Delta Ct}$ for each cytokine target gene, and comparing relative expression with that of the 18 S rRNA constitutive gene product. Assays were conducted twice in triplicate.

2.11. Statistical analysis

Statistical analysis was performed using GraphPad Prism version 6.0 (GraphPad Software). A 2-way ANOVA test was used to compare the means of the cytokine targets between zero and 2 μg/ml of factor H and zero and 20 μg/ml of factor H for any significant differences in gene expression over the given time-points. P values were computed, and graphs were compiled and analyzed.

3. Results

3.1. Human factor H binds to mycobacteria

In our previous study, we showed that *M. bovis* BCG was able to bind factor H (Carroll et al., 2009). Initial characterization of this binding was performed in the presence of EDTA, as nearly all previously observed factor H binding is not divalent metal ion (M^{++}) dependent. Further investigations showed that factor H binding to *M. bovis* BCG and was higher in the presence of Ca^{2+}/Mg^{2+} than in the presence of EDTA. A concentration of 5–10 μg/ml of factor H was sufficient to reach saturation of binding at the given number of bacteria, which was well below the *in vivo* plasma level of 128–654 μg/ml (Ansari et al., 2013) (Fig. 1(a). A 3- to 4-fold greater binding of factor H was observed in the presence of Ca^{2+}/Mg^{2+} than in EDTA. This may suggest at least 2 different types of binding site for factor H, namely M^{++}-dependent and M^{++}-independent. Fluorescence microscopy shows binding of factor H to *M. bovis* BCG cells; increasing signal intensity can be noticed with increasing concentrations of factor H (Fig. 1b).

3.2. Competition assay showed C4BP binding to similar ligands with factor H on mycobacterial surface

C4BP was observed to bind to *M. bovis* BCG and the degree of binding was very similar in the presence of EDTA and of Ca^{2+}/Mg^{2+} (Fig. 2). No binding of C4BP was observed when C4BP depleted serum was used as a control for assay specificity. A competitive assay confirmed that C4BP competed with factor H for binding on the mycobacterial cell surface (Fig. 3). The binding of C4BP to mycobacteria was also observed to be dose-dependently inhibited by factor H, suggesting that both complement proteins bound to the same or overlapping ligands on mycobacteria.

Extensive affinity chromatography experiments using immobilised factor H and *M. bovis* BCG lysates (in Triton X100) failed to detect any protein/glycoprotein ligand(s) for factor H (not shown) by SDS-PAGE analysis. This suggests that the mycobacterial ligands for factor H may not be proteinaceous. An alternative explanation could be that the mycobacterial ligand(s) is not solubilized by Triton X100.

3.3. Factor H inhibits uptake of M. bovis BCG by THP-1 macrophages

Following phagocytosis assay using PMA-differentiated THP-1 cells, CFU counts of *M. bovis* BCG showed that factor H dose-dependently inhibited mycobacterial uptake by THP-1 cells (Fig. 4). The input number of mycobacteria (unchallenged with THP-1) was about 78×10^6 CFU/ml. From this number, 40×10^6 CFU/ml (about 50%) of mycobacteria were phagocytosed from the control well (untreated with factor H). The uptake by macrophage was reduced by ~60% (15×10^6 CFU/ml) when mycobacteria were incubated with a low dose of purified factor H (2 μg/ml). The uptake was reduced by ~80% (6×10^6 CFU/ml) when mycobacteria were incubated with a higher dose of purified factor H (20 μg/ml). Thus, factor H, once bound to *M. bovis* BCG, can interfere with phagocytosis, highlighting a non-complement related role for factor H in host-pathogen interaction.

3.4. Factor H modulates cytokine responses during phagocytosis of M. bovis BCG by THP-1 macrophages

Our quantitative (qPCR) data revealed that there was a broad up-regulation by factor H of pro-inflammatory cytokines TNF-α, IL-1β, and IL-6 (Fig. 5a, b and c), particularly at the initial stage of macrophage uptake (15 min) which decreased gradually towards the later stages of phagocytosis. It is well known that macrophage activation by TNF-α is particularly critical for killing of intracellular mycobacteria and is a key mediator in granuloma formation. In contrast, there was an extensive down-regulation of anti-inflammatory IL-10 and TGF-β (Fig. 5d and e). The cytokine IL-12 also appeared to be down-regulated (Fig. 5f), suggesting a likely suppression of Th1 activated $CD4^+$ T-cells, resulting in reduced levels of IFN-γ production, a key cytokine important in activating macrophages to kill intracellular mycobacteria and the recruitment of immune cells during granuloma formation. Factor H, therefore has a marked influence on the cytokines produced by macrophages, which may play an important role in shaping the adaptive immune response during *M. tuberculosis* infection.

4. Discussion

We have previously shown that human factor H can bind to *M. bovis* BCG and that the bacterium can trigger antibody-independent complement activation by all three pathways (Carroll et al., 2009). However, very little is known about the role of individual complement proteins in tuberculosis infection, particularly complement control proteins such as factor H. In the present study, our goal was to further characterise the interactions of factor H with mycobacteria, with a view to examining its possible role in the pathogenesis of tuberculosis. Factor H has been shown to bind to a number of microorganisms with probable roles in pathogenesis. This has been particularly highlighted for *N. meningitidis* via structural characterisation of the pathogen ligand and its binding site on factor H (Schneider et al., 2009). The central premise in most studies is that factor H is utilised by pathogens to evade complement attack, since factor H is a downregulator of complement. However in the present study, we present data suggesting that factor H may have other non-complement related functions in host-pathogen interaction too, particularly in modulating and shaping the localisation of the bacteria in tuberculosis infection.

We observe that human factor H binds to *M. bovis* BCG (Fig. 1a and b). Binding is dose dependent; saturation of binding is observed at 5–10 μg/ml of factor H, which is far below the plasma level of 128–654 μg/ml (Ansari et al., 2013). Binding is greater in the presence of divalent metal ions (Ca^{2+} and Mg^{2+}) than in the presence of EDTA. This has not been previously observed in the binding of factor H to microorganisms and in most other ligands, such binding is generally divalent metal ion-independent. C1q is known to compete with factor H for binding to a range of ligands (Kishore and Sim, 2012), whilst β2-glycoprotein 1, a structural homologue of factor H, behaves similarly to factor H in binding anionic phospholipids (Kertesz et al., 1995). Separate binding tests with C1q and β2-glycoprotein 1 show that both bind to *M. bovis* BCG (Carroll et al., 2009), but neither competes with factor H for binding to the bacterium. However, C4BP was also shown to bind to the mycobacteria (Fig. 3), and factor H competed significantly with C4BP binding (Fig. 4), suggesting that both complement proteins may bind to the same or overlapping mycobacterial ligands. C4BP is also a close structural and functional homologue of factor H and is also known to bind to several bacteria (Blom et al., 1999; Pietikainen et al., 2010).

The recruitment of factor H by mycobacteria may be particularly critical in the initial stages of tuberculosis infection, when after inhalation, the first host cell *M. tuberculosis* encounters is the alveolar macrophage. In the present study, we show that coating of *M. bovis* BCG with factor H significantly inhibits uptake of the bacterium by THP-1 macrophages (Fig. 4). In vivo, alveolar macrophages phagocytose mycobacteria, but ultimately fail to destroy them, and in the process produce important chemoattractants that recuit inflammatory cells such as neutrophils, γδ-T

cells, monocyte-derived macrophages and NK cells that encourages inflammation and tissue remodelling (Feng et al., 2006). During interaction and phagocytosis of factor H-treated *M. bovis* BCG by THP-1 cells, there was a striking difference in the levels of pro- and anti-inflammatory cytokines produced by the THP-1 cells (Fig. 5). TNF-α expression was heightened significantly in the presence of factor H, particularly in the early stages of phagocytosis. This enhanced pro-inflammatory response was also mirrored in elevated levels of IL-1β and IL-6 cytokines. IL-1β is a potent cytokine required for host resistance to tuberculosis (Mayer-Barber et al., 2010), whilst IL-6 is required for an optimal T cell response against *M. tuberculosis* infection (Leal et al., 1999). In contrast, there was a downregulation of IL-12 by factor H, which *in vivo* may downregulate the Th1 response and the production of IFN-γ reducing inflammation. The cytokines IL-10 and TGF-β were also observed to be downregulated significantly in the presence of factor H, suggesting the suppression of the anti-inflammatory cytokine response. Taken together, these data may mirror the early inflammatory processes in vivo, which results in granuloma formation, a lesion of inflammatory mononuclear cell infiltrate that cordons off *M. tuberculosis* infection and limits its growth, but also provides a niche for the pathogen to persist as a latent infection. TNF-α is a key mediator in granuloma development; both TNF-α and IFN-γ accelerate the recuitment of cells in the granuloma (Smith et al., 1997). Factor H may, therefore, play a role in promoting the pro-inflammatory cytokine responses that results in granuloma formation and its maintenance. It is known that in order for a protective homeostatic granuloma to be maintained, a balance of inflammation is required, primarily the Th1/Th2 balance (Ehlers and Schaible, 2012). This is driven by the balance in IFN-γ/TNF-α versus IL-4/IL-10/TGF-β within the granuloma. It is possible, from the data in the present study, that factor H can play a role in perpetuating this balance. Within the granuloma, *M. tuberculosis* is sequestered intracellularly and undergoes metabolic adaption limiting its growth. It is unknown if any complement proteins reside within the granuloma, but it is likely that significant quantities of factor H are present. Factor H expression is also enhanced in monocytes by IFN-γ (Schwaeble et al., 1987). Therefore factor H, if resident within the granuloma, may be a key signalling molecule that plays a role in preventing Th1/Th2 imbalance, which ultimately results in granuloma necrosis, *M. tuberculosis* recrudescence and growth, which is precipitated by $CD4^+$ T cell activation (Dannenberg, 1991). It may therefore not be surprising that innate immune molecules would support granuloma/latency, targetting macrophages instead of allowing T cell involvement. Since macrophages can locally produce complement proteins such as C1q, they may be the primary source for factor H in the granuloma.

The suppression of IL-10 and TGF-β by factor H may promote mycobacterial clearance by the host during the early stages of *M. tuberculosis* infection, as discussed below. Upon phagocytosis of *M. tuberculosis*, alveolar macrophages produce IL-10 (Shaw et al., 2000), which *M. tuberculosis* may exploit to circumvent anti-microbial responses. IL-10 has been shown to block phagosome maturation, which facilitates *M. tuberculosis* survival (O'Leary et al., 2011). Furthermore, IL-10 produced during phagocytosis may also block antigen presentation via downregulation of the major histocompatibility complex. IL-10 has also been implicated in down-regulating dentritic cell activation (Demangel et al., 2002), which leads to a weakened Th1 response with increased mycobacterial growth (Tian et al., 2005). Clinically, IL-10 and TGF-β have also been shown to be elevated in the lungs of active tuberculosis patients, indicating a limited immune response to *M. tuberculosis* and a role in pathogenesis and disease progression (Gong et al., 1996). Thus, factor H-influenced phagocytosis of mycobacteria may act to diminish these evasion mechanisms, enhancing clearance in the early stages of infection and facilitating a protective response against mycobacterial infection.

The supression of IL-12 by factor H may be a direct result of the reduction in phagocytosis of *M. bovis* BCG and poor antigen presentation, which would lead to a downregulation in Th1 response and the mobilization of $CD4^+$ T cells, producing IFN-γ. This would be particularly crucial in contributing to the Th1/Th2 balance within the protective granuloma, where studies have shown that T-cell activation can kick start granuloma necrosis (Ehlers et al., 2001).

The mycobacterial ligand for factor H is unknown, but affinity chromatography suggested that it may not be a protein or glycoprotein. Given the substantial and complex lipid composition of the mycobacterial cell wall, the ligand may be lipid in nature and may be comparable to known interactions of factor H with lipid A and anionic phospholipids (Kishore and Sim, 2012).

The quantity of factor H in the lungs of healthy individuals is unknown. It is likely to be low, but would probably increase upon inflammation, as observed in lung cancer cells (Ajona et al., 2004) and would probably be produced locally by phagocytes such as the alveolar macrophage or alveolar epithelium (Ajona et al., 2007). It would be also be interesting to determine the concentation of factor H in immunocompromised (e.g. HIV-infected) individuals as well as those with chronic obstructive pulmonary disease (COPD) or underlying lung disease e.g. asthma. The local levels of factor H may be a key element in influencing opportunistic infections. Similarly, it is also worth considering the role of factor H in extrapulmonary TB, since extracellular *M. tuberculosis* in the blood would come into contact with copious amounts of factor H, potentially facililating the dissemination of TB infection to other organs.

Intriguingly, factor H has been identified previously as having a possible role in granuloma-like pathology. During *Echinococcus granulosus* infection, the parasite forms a hydatid cyst and is present in mammals and humans in a condition called hydatid disease, that is analoguous to a latent infection, surviving in the host for decades. It has been found that the cyst wall binds substantial amounts of factor H, effectively surrounding the organism with a high concentration of factor H (Diaz et al., 1997). During this chronic infection, a granuloma-like layer forms around the cyst wall, containing multinucleated giant cells and macrophages which take on osteoclast-like properties (Diaz et al., 2011, 2000); factor H mediates this host-pathogen interaction.

In conclusion, the data in this study suggest that factor H is involved in modulating the host-pathogen interaction in the early stages of tuberculosis infection. The consequences are that factor H may have a dual role: firstly, the enhancement of clearing of mycobacterial infection, by downplaying pathogen immune evasion strategies via upregulating the cellular immune response against infection; and secondly by promoting the formation and maintenance of the protective granuloma. This study provides insights into previously unknown involvement of complement regulatory protein factor H in shaping the cellular immune response against mycobacteria. Further studies are required to fully characterise the nature and extent of involvement of factor H in tuberculosis pathogenesis, particularly in the early stages of granuloma formation and the maintainance of immune balance. Should a significant role be confirmed for factor H in this intricate host-pathogen stand-off, then this will provide a new rationale for the design of therapies against this most complex of diseases. Clearly, this study needs further investigation using pathogenic *M. tuberculosis.*

Conflict of interest

None.

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