



The Lack of Relationship between Serum Content of MBL, sCD14, Anti-PPD and Anti-Hsp65 IgG and Ingestion of *Mycobacterium bovis* BCG Bacilli by Phagocytes

BEATA PAZIAK-DOMAŃSKA¹, AGNIESZKA BONAR¹, MAGDALENA KOWALEWICZ-KULBAT¹, MAGDALENA KLINK², MICHAŁ KOWALSKI³, JARI KARHUKORPI⁴, RITTA KARTTUNEN⁵, MAGDALENA JURKIEWICZ¹, BARBARA RÓŻAŁSKA¹ and WIESŁAWA RUDNICKA^{1*}

¹ Department of Immunology and Biology of Infections, University of Łódź, Łódź, Poland, ² Microbiology and Virology Center, Polish Academy of Science, Łódź, Poland, ³ Health Centre for High Schools, Łódź, Poland, ⁴ Clinical Microbiology Laboratory, University Hospital of Oulu, Oulu, Finland, ⁵ Department of Medical Microbiology, University of Oulu, Oulu, Finland

Abstract. Prophylactic vaccination against tuberculosis (TB) with a live attenuated strain of *Mycobacterium bovis* bacille Calmette-Guérin (BCG) has been used worldwide. However, TB remains one of the most significant diseases of humans and animals. Better understanding of the mechanisms of human immunity to mycobacteria is essential for the development of new vaccines and the estimation of their efficacy. In this study we determined the levels of known humoral mediators of mycobacterial phagocytosis, i.e. mannose-binding lectin (MBL), soluble CD14 (sCD14), antibodies of the immunoglobulin G (IgG) class against mycobacterial purified protein derivative (PPD), and mycobacterial Hsp65 antigen, in the sera from healthy young volunteers vaccinated with BCG and presenting positive and negative Mantoux responses to PPD. Then we asked the question as to whether macrophages and polymorphonuclear leukocytes (PMNs) from the individuals with positive tuberculin test (TT(+)) and negative tuberculin test (TT(–)) differed in their ability to ingest mycobacteria. We also looked for a relationship between the intensity of mycobacterial ingestion by phagocytes in a medium of autologous sera containing different concentrations of MBL, sCD14 and anti-mycobacterial IgG. We found no significant differences between the investigated parameters for TT(+) and TT(–) volunteers. Our result suggest that the ability of macrophages and PMNs to ingest mycobacteria depends on an individual, intrinsic capacity of the phagocytes.

Key words: tuberculin test; macrophages; polymorphonuclear leukocytes.

Introduction

Since 1921 more than 1 billion children in more than 182 countries throughout the world have been vaccinated with *Mycobacterium bovis* bacille Calmette-Guérin

(BCG). Despite this, in the last 15 years an increase in the number of tuberculosis (TB) cases occurring in developed countries, an increase in the drug resistance of isolated mycobacteria, and outbreaks of multidrug-resistant TB, as well as an indication of TB, being an

Abbreviations used: TB – tuberculosis, BCG – bacille Calmette-Guérin, TT – tuberculin test, MBL – mannose-binding lectin, PPD – purified protein derivative, Hsp – heat shock protein, PMN – polymorphonuclear leukocyte.

*Correspondence to: Prof. Wiesława Rudnicka, Department of Immunology and Biology of Infections, University of Łódź, Banacha 12, 90-237 Łódź, Poland, fax: +48 42 678 49 32, e-mail: rudw@biol.uni.lodz.pl

infectious agent responsible for about 40% of the deaths related to HIV, have led to an unusual commitment of resources to TB research⁷. However, BCG vaccination is still recognized as the best tool in TB prophylaxis, decreasing the prevalence of this disease in children and young people¹² and providing a substantial protection against death⁵.

Mycobacteria are facultative intracellular pathogens able to successfully parasitize phagocytes. Both macrophages and neutrophils are capable of phagocytosis and microbial killing of mycobacteria and occur together in the early infiltrates of granulomas. The role of neutrophils in resistance to pathogenic mycobacteria was neglected because of their short life span. Recently, it has been suggested that they perform a scavenging function in mycobacteria granuloma and that their secretory products have the ability to enhance macrophage bactericidal activity¹⁰. When encountered with mycobacteria, neutrophils exhibit the typical early bactericidal response, including phagocytosis and generation of reactive oxygen intermediates¹⁶.

Multiple host – pathogen interactions allow mycobacteria to enter phagocytes. The interactions between mycobacterial cell-wall components and receptor structures on the surfaces of macrophages may influence the intracellular fate of the pathogen and be critical in determining the outcome of the infection. Phagocytosis of mycobacteria is mediated by complement receptors (CRs), CR1, CR3 and CR4²¹. CR1 (CD35) recognizes C3b- and C4b-coated particles, whereas CR3 (CD11b/CD18) and CR4 (CD11c/CD18), which are members of the β_2 (leukocyte) integrin family, primarily recognize C3bi-coated particles and a variety of microbial ligands and other glycoproteins. The phagocytosis of mycobacteria also depends on the interaction between phagocyte mannose receptors and mannose-binding lectin (MBL) recognized as an opsonin and complement-activating molecule^{13, 24}. The binding of MBL to the surface of mycobacteria has been proved¹⁹. MBL and its associated serine protease can activate both the classical and the alternative complement pathways. Thus, MBL-mediated activation of complement in this fashion may also result in C3 opsonization of bacteria²¹.

M. tuberculosis is an intracellular pathogen that replicates within host macrophages, and host defenses are believed to be largely dependent on T lymphocytes, with antibodies being of only minor importance¹¹. However, it has recently been shown that the classical pathway plays a major role in complement activation induced by mycobacteria and that antimicrobial immunoglobulin G (IgG) on the bacilli can mediate this re-

sponse⁹. IgGs to various mycobacterial antigens, e.g. purified protein derivative (PPD), lipoarabinomannan (LAM), heat shock protein (Hsp) 65, Hsp71 and others, are produced in the response to *M. tuberculosis* infection¹⁴, BCG vaccination³ or BCG treatment in patients with superficial bladder cancer²⁸. The binding of the CD14 molecule, a pattern recognition receptor involved in the interaction with LAM of the mycobacterial wall, may also be used by mycobacteria to enter the intracellular compartment¹⁷. This glycosyl-phosphatidylinositol-linked glycoprotein is constitutively expressed on monocytes, macrophages and neutrophils and it is also present in serum in a soluble form (sCD14).

In this study, we asked whether the ingestion of mycobacteria by macrophages and polymorphonuclear leukocytes (PMN) could be related to a low or high concentration of MBL, sCD14 and anti-mycobacterial IgG in the culture media or rather the uptake of these bacilli is determined by an intrinsic capacity of the phagocytes. The intensity of ingestion of *M. bovis* BCG bacilli by blood-derived macrophages and PMNs was observed in a medium with 10% autologous serum containing various concentrations of MBL, sCD14, anti-PPD and anti-Hsp65 IgG. Additionally, the intensities of phagocytosis of mycobacteria by macrophages and PMNs from healthy volunteers with positive tuberculin test (TT(+)) and negative tuberculin test (TT(-)) were compared. All volunteers were BCG vaccinated in early childhood.

Materials and Methods

Mantoux responses to PPD. A total of 31 young (18–29 years old) healthy volunteers were under investigation. All of them were vaccinated with BCG in infancy and then in early childhood. At the time of this study, peripheral blood was collected and current Mantoux responses to 10 μ g PPD were estimated. The tuberculin tests were considered positive when skin induration (at 72 h after challenge) was ≥ 10 mm. In this, positive tuberculin tests were observed for 20 of the 31 volunteers (65%) and average skin induration was 14.5 ± 37 mm. Negative responses to PPD were recorded for 11 of the 31 volunteers (35%), despite inoculating them with 3–5 additional doses of BCG (at the age of 18, 22 and 27 years). All participants gave written informed consent for the studies, which were approved by the Local Ethics Committee.

Blood leukocytes. Peripheral blood was collected into heparin as an anticoagulant. The fractions of mono-

nuclear and polymorphonuclear leukocytes were separated by centrifugation on Gradisol G (AQUA-MEDICA) free from lipopolysaccharide (LPS) as estimated by E-TOXATE, PART1 Limulus Amebocyte Lysate (Sigma, St. Louis, USA). Neutrophils were washed and suspended in RPMI-1640 medium with 10% autologous plasma. To obtain monocytes, the mononuclear cell fractions were incubated for 90 min at 37°C, in RPMI-1640 medium with 10% heat-inactivated fetal calf serum (FCS). After washing, the adherent cells were rested for 18 h at 37°C, 5% CO₂, in RPMI-1640 medium with 10% autologous plasma before they were used for phagocytosis assay.

Phagocytosis assay. *M. bovis* BCG bacilli were grown for 14 days in Middlebrook 7 H9 broth (Difco) + 0.05 % Tween 80. The bacilli were heat killed (80°C for 1 h) and labeled with fluorescein isothiocyanate (FITC; Sigma St. Louis, USA)⁴. Briefly, the bacilli were suspended in phosphate-buffered saline (PBS) containing FITC (100 µg/ml), vortexed for 30 s, sonicated for 15 s, and kept in darkness for 18 h at room temperature. After washing with PBS + 4% bovine serum albumin (BSA) the bacilli were suspended in RPMI-1640 medium with 20% FCS. The labeled bacilli could be stored at 4°C for 3 weeks without loss of fluorescence.

The 100 µl aliquots of the FITC-labeled bacterial suspensions (1×10^8 cells/ml) were mixed on microplate with 100 µl aliquots of neutrophil or macrophage suspensions (1×10^6 cells/ml), always in triplicate, and incubated for 2 h at 37°C in 5% CO₂⁴. The cells were incubated for 4 h at 37°C, 5% CO₂. The fluorescence of attached but not ingested bacilli was quenched with 100 µl/well 0.1% trypan blue in PBS. The fluorescence of intracellular bacilli was measured in a Wallac 1420 counter VICTOR² (Oy, Turku, Finland) at 485 nm excitation and 530 nm emission wavelengths and expressed as relative fluorescence units (RFU) per 10⁵ phagocytes.

Serum levels of MBL, sCD14 and anti-mycobacterial antibodies. Serum levels of MBL, anti-Hsp65 IgG and anti-PPD IgG were determined by ELISA on microtiter plates (Maxisorp; Nunc, Roskilde, Denmark) coated with 37 µg/well mannan (Sigma, St. Louis, USA), Hsp65 (0.1 µg/well) from *M. bovis* BCG (kindly supplied by Dr. K. Huygen, Pasteur Institut, Brussels, Belgium) or 5 µg/ml PPD (Statens Serum Institute, Copenhagen, Denmark), respectively. For the determination of MBL levels, rabbit polyclonal anti-MBL antibodies (kindly supplied by A. Ezekowitz, Harvard Medical School, Boston, USA) were used as capture antibodies and swine anti-rabbit IgG peroxidase con-

jugated antibodies (Dako) as detecting ones. Recombinant MBL (30–400 ng/ml) was used as a standard (supplied by A. Ezekowitz). Anti-Hsp65 and anti-PPD IgG were detected with rabbit anti-human IgG peroxidase conjugated antibodies (Dako). Peroxidase substrate, O-phenyldiamine dihydrochloride (OPD; Sigma, St. Louis, USA), was used and absorbance was measured at 490 nm in a Wallac 1420 multilabel counter VICTOR² (Oy, Turku, Finland). Soluble CD14 was measured with a Human sCD14 EIA kit (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions.

Statistical analysis. Calculations were performed using Statistica 5.0 (Statsoft). Student's *t*-test was applied to reveal significant differences in the values for *M. bovis* BCG ingestion and the levels of MBL, sCD14, and anti-mycobacterial IgG. A value of $p < 0.05$ was accepted as the level of significance. Correlations between investigated immunological parameters (*in vitro* and *in vivo*) were analyzed with Pearson's correlation *r*-test.

Results

The study was conducted on 31 healthy young (18–29 years old) individuals who were BCG vaccinated in early childhood. Eleven of them (over 35%) had never developed delayed hypersensitivity to PPD despite 3–5 BCG inoculations. The data in Fig. 1 demonstrate a great variation in the capacity of human macrophages and PMNs to ingest mycobacteria in the medium with autologous serum. There was no difference in the intensity of mycobacterial phagocytosis between granulocytes and macrophages nor between phagocytes from the volunteers with positive and negative skin reactivity to tuberculin. Also, a great variation was observed as regards the serum levels of MBL, sCD14, anti-Hsp65 and anti-PPD IgG for the volunteers under study (Fig. 2). However, there was no difference in the average content of MBL, sCD14, anti-PPD and anti-mycobacterial Hsp65 IgG in the sera from the volunteers with positive and negative delayed-type hypersensitivity (DTH) to tuberculin.

Data in Fig. 3 and 4 demonstrate the lack of a relationship between the intensity of uptake of mycobacteria by macrophages (Fig. 3) and PMNs (Fig. 4) and the content of MBL, sCD14, anti-PPD or anti-Hsp65 IgG in the sera added to the culture. This was observed for the volunteers with positive and negative skin reactivity to tuberculin.

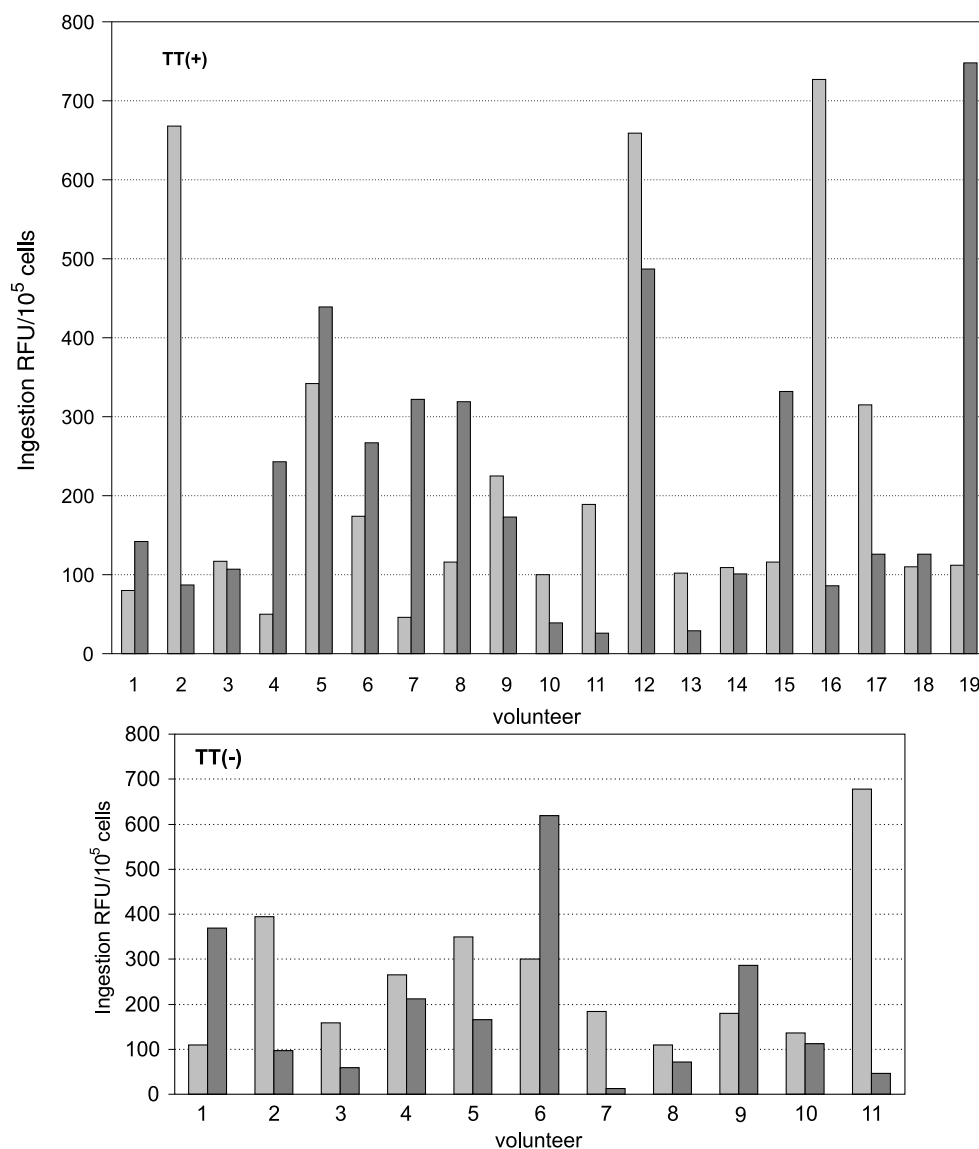


Fig. 1. Ingestion of *M. bovis* BCG bacilli by macrophages and PMNs from volunteers with positive (TT(+)) and negative (TT(-)) skin reactivity to tuberculin; □ macrophages, ■ PMN

Discussion

The BCG vaccine has been available for over 70 years. However, tuberculosis remains one of the most significant diseases of humans and animals⁶. The efficacy of BCG vaccination has been differently estimated in various trials, from 77% protection in a Medical Research Council trial in the United Kingdom⁸, to zero protection in the largest clinical trial in India²³. This lack of protection has resulted in increased efforts to develop a new generation of TB vaccines^{5, 12}. However, the BCG vaccine remains the best and the only one available for the prevention of TB. One would think that before a new TB vaccine is elaborated it is worthwhile to explain why some BCG-vaccinated individuals are not protected from TB. The present study

showed that about 35% of BCG-vaccinated healthy young people remain tuberculin negative despite receiving 3–5 BCG inoculations. It is known that the mechanisms responsible for both DTH to tuberculin and increased resistance to a subsequent reinfection with mycobacteria rely on the activation of phagocytic cells. However, this is more easily demonstrated in animal models²⁰.

A first critical stage of the interaction of mycobacteria with macrophages and PMNs comprises the adhesion of bacilli to the phagocyte membrane. This process includes direct and indirect binding of various bacterial ligands to host receptors and it is mediated by many components of normal sera. Of these components, the most important is MBL, which binds to the phagocyte mannose receptor, driving a lectin phagocytosis^{13, 24}.

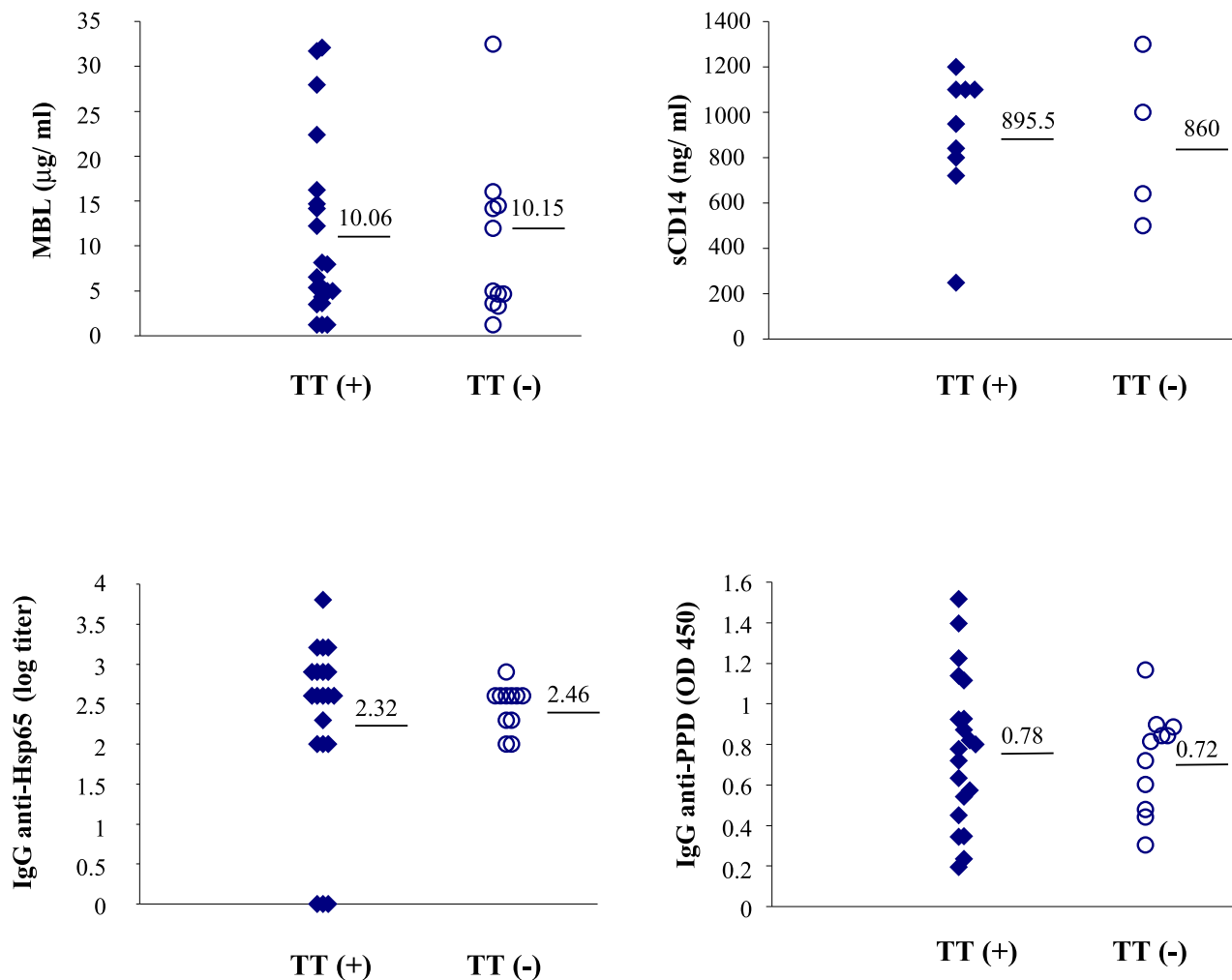


Fig. 2. The levels of MBL, sCD14, IgG anti-mycobacterial Hsp65 and IgG anti-PPD in sera from volunteers with positive (TT(+)) and negative (TT(-)) skin reactivity to tuberculin

Moreover, MBL as a complement activator facilitates opsonin phagocytosis with an involvement of phagocyte β_2 integrin receptors²¹. A critical role in the adhesion of mycobacteria to the phagocyte membrane should be attributed to CD14 molecule, which binds LAM, a surface virulence factor of the mycobacterial wall¹⁷. An involvement of the anti-mycobacterial IgGs should not be excluded, because they may also mediate opsonin phagocytosis via phagocyte Fc receptors⁹.

In this study, we showed that sera from healthy young people differ in MBL, sCD14, anti-mycobacterial Hsp65 and anti-PPD IgG content. However, we could see no differences in the serum concentrations of these humoral factors between volunteers with positive and negative Mantoux responses to PPD. We asked whether a low or a high concentration of these adhesion mediators in the sera may result in a less or more intensive ingestion of mycobacteria by autologous macrophages and PMNs. The presented results for as many as 31 individuals dem-

onstrated no relationship between the uptake of mycobacteria by macrophages or PMNs and the levels of MBL, sCD14 and anti-mycobacterial IgG in the sera. This suggests a simultaneous interaction between various mycobacterial ligands with appropriate phagocyte receptors. We observed a similar phenomena in the study of the interaction of *Helicobacter pylori* bacteria with phagocytes⁴. On the other hand, this suggests that the intensity of ingestion of mycobacteria by macrophages and PMNs depends on an individual intrinsic capacity of the phagocytes. As is known, a *Nramp1* (natural resistance-associated macrophage protein 1) gene has been postulated in mice as regulating the anti-mycobacterial capacity of macrophages^{15, 25} as well as their ability to present mycobacterial antigens to T lymphocytes²⁷. A polymorphism of the human homologue of the murine *Nramp1* gene was suggested to be associated with susceptibility to tuberculosis², although this suggestion was not confirmed in studies by other authors^{1, 22}.

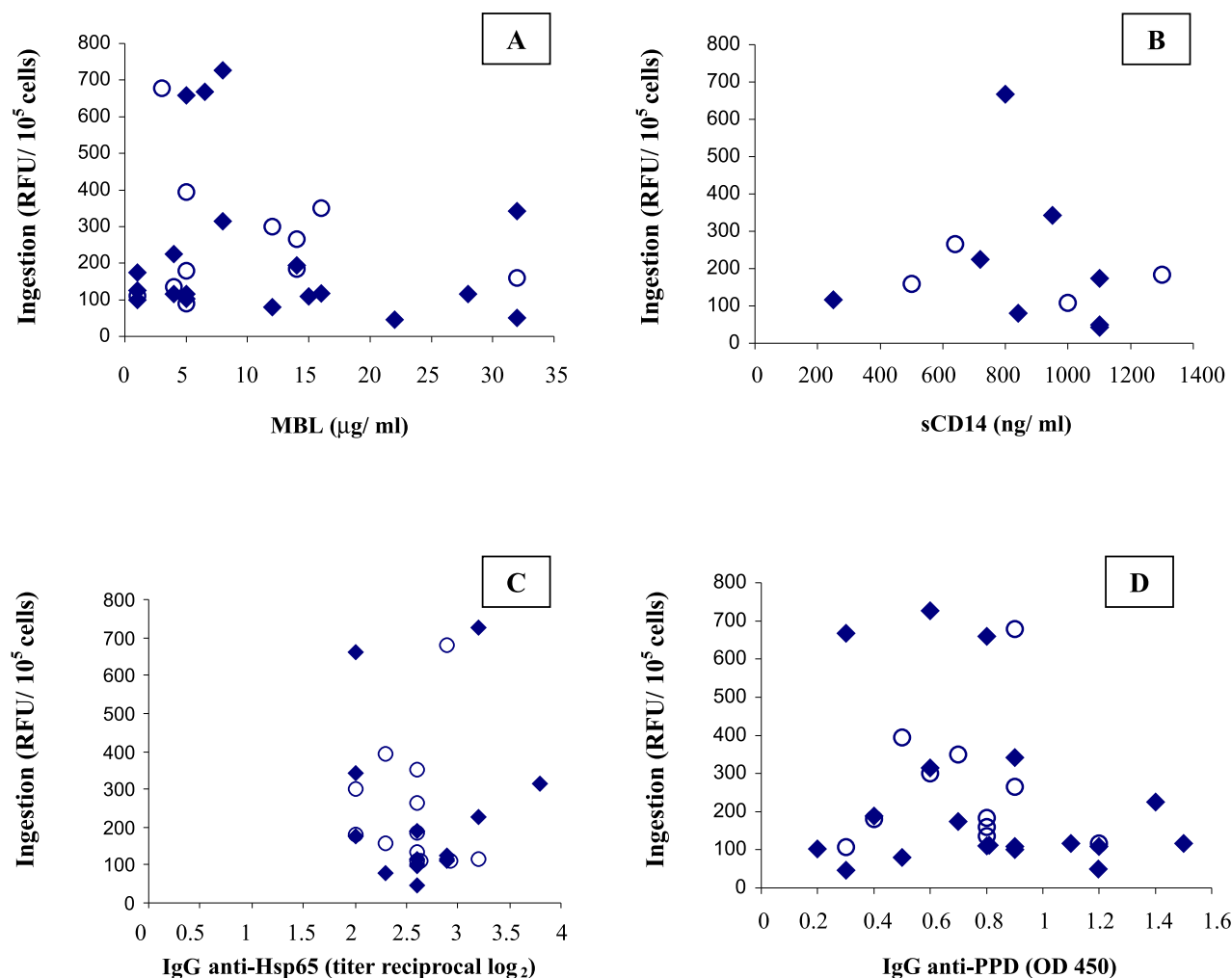


Fig. 3. The lack of relationship between the intensity of uptake of *M. bovis* BCG by macrophages and serum levels of MBL (A), sCD14 (B), anti-mycobacterial Hsp65 IgG (C) and anti-PPD IgG (D) in volunteers with positive (black diamonds) and negative (white circles) skin reactivity to tuberculin

In the present study, the suggestion of the role of an individual, intrinsic capacity of phagocytes in the ingestion of mycobacteria by macrophages and PMNs is confirmed by the lack of any significant differences in the uptake of *M. bovis* BCG bacilli by phagocytes from individuals with positive and negative skin reactivity to tuberculin. It should be stressed that adhesion followed by ingestion of mycobacteria by macrophages and PMNs determines the further stages of phagocyte activation, resulting in the killing of bacilli and the release of various cytokines regulating immune response to mycobacteria and their products. So it is worth mentioning that we could see no difference in the ingestion of mycobacteria by mononuclear macrophages recognized as the main consumer of mycobacteria in infected hosts and by the rather neglected PMNs. Both macrophages²⁶ and PMNs^{10, 18}, when activated, produce

a large number of cytokines: TNF- α , IL-1 β , IL-6, IL-8, IL-10, IL-12, and GM-CSF. These cytokines are known for their inflammatory and immunoregulatory functions. A collaboration between macrophages and PMNs may be critical for the expression of the immune response to mycobacterial products. It is possible that the anti-mycobacterial capacity of macrophages and PMNs depends rather on the responses of these cells to cytokines than on the concentration of humoral mediators of phagocytosis in the sera. A better understanding of the mechanisms of human immunity to mycobacteria is essential for the development of new vaccines and an estimation of their efficacy.

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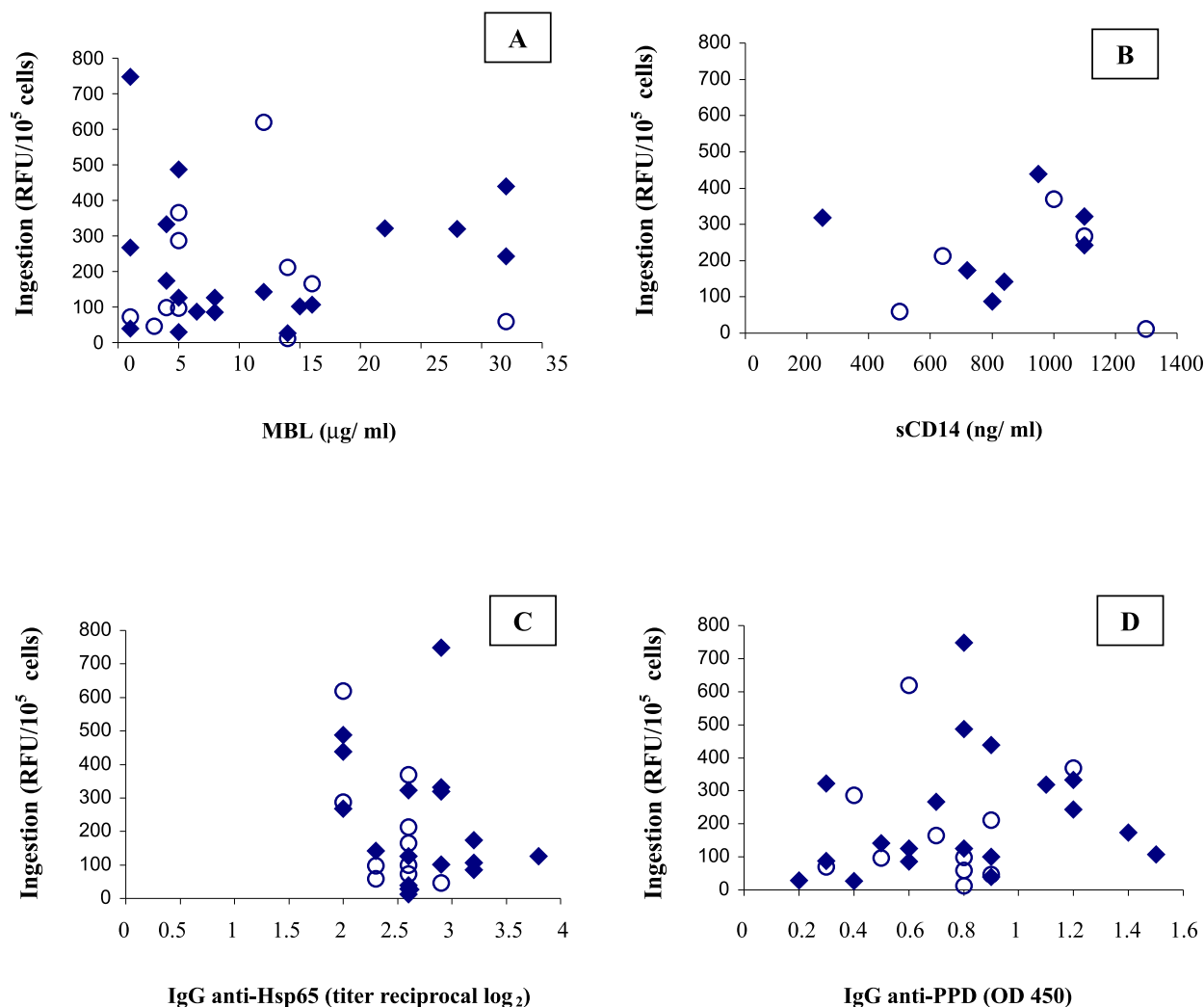


Fig. 4. The lack of relationship between the intensity of uptake of *M. bovis* BCG by PMNs and serum levels of MBL (A), sCD14 (B), anti-mycobacterial Hsp65 IgG (C) and anti-PPD IgG (D) in volunteers with positive (black diamonds) and negative (white circles) skin reactivity to tuberculin

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