

1           **Analysis of the immunomodulatory properties of two heat-killed mycobacterial**  
2           **preparations in a human whole blood model**

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4   Samer Bazzi <sup>a,b</sup>, Helmut Modjtahedi <sup>a</sup>, Satvinder Mudan <sup>c,d</sup>, Charles Akle <sup>e</sup>, Georges M. Bahr <sup>b,\*</sup>

5  
6   <sup>a</sup>School of Life Sciences, Faculty of Science, Engineering and Computing, Kingston University,  
7   Kingston upon Thames, Surrey *KT1 2EE*, United Kingdom.

8   <sup>b</sup>Faculty of Medicine and Medical Sciences, University of Balamand, 33 Amioun, Al Kurah,  
9   Lebanon.

10   <sup>c</sup>Division of Clinical Sciences, St George's, University of London, London *SW170RE*, United  
11   Kingdom.

12   <sup>d</sup>Department of Academic Surgery, Royal Marsden Hospital, London *SW3 6JJ*, United Kingdom.

13   <sup>e</sup>The London Clinic, London *W1G 6JA*, United Kingdom.

14  
15   **Email addresses**

16   Samer Bazzi: k1170034@kingston.ac.uk

17   Helmut Modjtahedi: H.Modjtahedi@kingston.ac.uk.com

18   Satvinder Mudan: mudans@aol.com

19   Charles Akle: cacle@me.com

20   Georges Bahr: georges.bahr@balamand.edu.lb

21  
22   **\*Corresponding author**

23   Georges M. Bahr, Faculty of Medicine and Medical Sciences, University of Balamand, PO BOX:  
24   33, Amioun, Al Kurah, Lebanon. Tel: 009613370780; Fax: 009616931956.

1 **Abstract**

2           The significant role played by mycobacteria in modulating immune responses through  
3 enhancing the crosstalk between innate and adaptive immunity has been highlighted in several  
4 studies. Owing to their unique antigenic profile, heat killed (HK) preparations of rapid-growing  
5 mycobacteria, currently undergoing clinical development, have been assessed as adjuvant  
6 therapy in various diseases. The purpose of this study is to investigate the regulation of  
7 leukocyte surface receptors, in whole blood from healthy donors, following *in vitro* stimulation  
8 with HK *Mycobacterium vaccae* (*M. vaccae*) or *M. obuense*. We have demonstrated the ability  
9 of both mycobacterial preparations to target monocytes and neutrophils and to regulate the  
10 surface expression of selected adhesion receptors, antigen-presenting and costimulatory  
11 receptors, pattern recognition receptors, complement and Fc receptors, as well as  
12 cytokine/chemokine receptors. Toll-like receptors (TLRs) 1 and 2 were also shown to be  
13 involved in mediating the *M. obuense*-induced upregulation of selected surface receptors on  
14 monocytes. Whole blood stimulation with *M. vaccae* or *M. obuense* resulted in a significant  
15 increase in the secretion of a specific set of cytokines and chemokines. Both mycobacterial  
16 preparations induced strong antigen-specific proliferative responses in peripheral blood  
17 mononuclear cells. Collectively, our data shows that *M. vaccae* and *M. obuense* have the  
18 potential to act as potent immunomodulators. Future research based on these findings may  
19 reveal novel immune pathways induced by these preparations with potential implication for their  
20 use in diverse immunotherapeutic approaches.

21 **Keywords:** Mycobacteria; immunomodulation; human leukocytes; CD antigens

22 **Abbreviations:** APC: antigen presenting cell, BCG: bacillus calmette-guerin, CCR: CC  
23 chemokine receptor, cpm: counts per minute, CR: complement receptor; CXCL: chemokine C-  
24 X-C motif ligand, CXCR: chemokine C-X-C motif receptor, DC: dendritic cell, ECMR:  
25 extracellular matrix receptor, FSC: forward scatter, GM-CSFR: granulocyte–macrophage  
26 colony-stimulating factor receptor, GP: glycoprotein, HK: heat killed, ICAM: intercellular  
27 adhesion molecule, LCA: leukocyte common antigen, LFA: lymphocyte function associated  
28 antigen, LPSR: lipopolysaccharide receptor, MFI: mean fluorescence intensity, MHC: major  
29 histocompatibility complex, MIP: macrophage inflammatory protein, MMR: macrophage  
30 mannose receptor, PBMCs: peripheral blood mononuclear cells, PECAM: platelet endothelial  
31 cell adhesion molecule, RANTES: regulated upon activation normal T cell expressed and  
32 secreted, SSC: side scatter, SI: stimulation index, TB: tuberculosis, TLR: toll-like receptor,  
33 TNFRSF: tumor necrosis factor receptor superfamily member.

## 1 Introduction

2 Mycobacteria, due to their diverse and complex cell wall structures, play a significant role in  
3 modulating immune responses (Rook, et al. 2004; Stanford, et al. 2009) For several years,  
4 Bacillus Calmette-Guerin (BCG), a live attenuated derivative of the slow-growing species  
5 *Mycobacterium bovis* (*M. bovis*), has been used as a prophylactic vaccine against tuberculosis  
6 (TB); though, with variable efficacy (Romano and Huygen 2012). Moreover, BCG has proven to  
7 be successful in the treatment of early non-invasive bladder cancer (Kawai, et al. 2013). The  
8 investigation of inactivated rapid-growing mycobacteria to be employed as immunomodulators  
9 has led to the selection of *M. vaccae* which was later evaluated as an immunomodulating  
10 therapeutic agent in various diseases including TB (Dlugovitzky, et al. 2006; Johnson, et al.  
11 2000; Yang, et al. 2011), leprosy (Abbot, et al. 2002; Truoc, et al. 2001), psoriasis (Lehrer, et  
12 al. 1998), dermatitis (Arkwright and David 2001), asthma (Camporota, et al. 2003) and a range  
13 of cancers (Cananzi, et al. 2013; Eaton, et al. 2002; O'Brien, et al. 2004; Patel, et al. 2008;  
14 Stanford, et al. 2008). *M. vaccae* was also found to be an effective vaccine that could confer a  
15 significant level of protection against TB among HIV-infected individuals who had received BCG  
16 vaccination during childhood (von Reyn, et al. 2010). In all these studies, *M. vaccae* was used in  
17 the form of a heat-killed (HK) preparation that retained its antigenic properties. In spite of the  
18 growing interest in the therapeutic implications of HK mycobacterial preparations, the nature of  
19 the host immune receptors regulated by these mycobacterial preparations has not been fully  
20 defined. Moreover, the exact mechanism by which HK mycobacterial preparations exert their  
21 immunomodulatory effects in humans is not fully understood. Nevertheless, in experimental  
22 models, immunization with HK *M. vaccae* was reported to generate CD8<sup>+</sup> T cells against *M.*  
23 *tuberculosis*-infected macrophages (Skinner, et al. 1997) and to downregulate T helper type 2  
24 (Th2) responses in murine models of allergic pulmonary inflammation via the induction of  
25 regulatory T cells (Zuany-Amorim, et al. 2002) and the priming of dendritic cells (DCs)  
26 consistent with a regulatory profile (Adams, et al. 2004). *In vitro* studies with human monocyte  
27 derived DCs have confirmed the ability of *M. vaccae* to dampen Th2 responses, via a  
28 mechanism dependent on DCs (Le Bert, et al. 2011). Furthermore, *M. vaccae* and *M. obuense*  
29 have been shown to promote the anti-tumor activity of human  $\gamma\delta$ T-cells via cytokine release  
30 from type-1 myeloid DCs (Fowler, et al. 2012). *M. obuense* has gained attention over the past  
31 few years as a potential immunotherapeutic agent against cancer and, in a phase 1 study in  
32 melanoma patients, it was shown to be safe and well tolerated (Stebbing, et al. 2012). More  
33 recently, in a phase 2 study, the use of *M. obuense* (NCTC13365) as adjunctive immunotherapy

1 for advanced pancreatic cancer resulted in clinically meaningful increases in overall survival as  
2 well as in progression-free survival of patients (Dalglish, A.G. and The IMAGE I Trial  
3 Investigators 2015). Currently, *M. obuense* (NCTC13365) is being investigated in a phase 2  
4 study involving patients with melanoma (NCT01559818). The interactions between different  
5 immune cells and between immune cells and soluble factors are mediated by a number of cell  
6 surface receptors involved in cellular adhesion, antigen presentation and co-stimulation,  
7 cytokine-mediated signaling, and cellular activation (Barclay, et al. 1997). Therefore, variations  
8 in the expression of these receptors would affect the immune cell function thus leading to the  
9 modulation of immune responses. Immune responses from healthy volunteers, assessed by the  
10 profile of released cytokines, have been extensively analyzed using short-term incubation of  
11 human whole blood with a large spectrum of microbial immunostimulants, immunomodulators,  
12 or specific antigens (Darcissac, et al. 1996a; Darcissac, et al. 1996b; Duffy, et al. 2014; Kassa,  
13 et al. 2012; Reichenbach, et al. 2006). The immunomodulatory properties of HK *M. vaccae* or  
14 *M. obuense* in the human whole blood model have not been previously evaluated. In this study,  
15 both HK mycobacterial preparations were assessed for their ability to modulate *in vitro* the  
16 expression of immunologically relevant leukocyte surface receptors and to alter cytokine and  
17 chemokine secretion in human whole blood. We have also addressed the role of toll-like  
18 receptors (TLRs) in mediating the immunomodulatory effects of *M. obuense*, and evaluated the  
19 ability of *M. vaccae* and *M. obuense* to induce lymphoproliferative responses in human  
20 peripheral blood mononuclear cells (PBMCs) from healthy volunteers.

## 21 **Materials and Methods**

### 22 **Antibodies**

23 Mouse monoclonal anti-human antibodies purchased from BD Biosciences (San Jose,  
24 CA, USA) included: FITC-conjugated CD2 (clone RPA-2.10), CD11a (clone HI111), CD35  
25 (clone E11), CD40 (clone 5C3), CD44 (clone L178), CD45 (clone 2D1), CD50 (clone TU41),  
26 CD64 (clone 10.1), CD80 (clone L307.4), CD95 (clone DX2), CD195 (clone 2D7/CCR5), HLA-  
27 DP,DQ,DR (clone Tu39); PEconjugated CD11b (clone ICRF44), (clone WM59), CD36 (clone  
28 CB38), CD58 (clone 1C3), CD89 (clone A59), CD102 (clone CBR- 1C2/2), CD114 (clone  
29 LMM741), CD116 (clone hGMCSFR-M1), CD119 (clone GIR-208), CD122 (clone TU27),  
30 CD127 (clone HIL- 7R-M21), CD132 (clone AG184), CD137L (clone C65-485), CD206 (clone  
31 19.2), PerCP-conjugated CD14 (clone M5E2), HLA-DR (clone G46-6); PE-Cy7-conjugated  
32 CD16 (clone 3G8); APC-conjugated CD5 (clone UCHT2), CD11c (clone B-ly6), CD18 (clone  
33 6.7), CD21 (clone B-ly4), CD25 (clone M-A251), CD32 (clone FLI8.26), CD54 (clone HA58),

1 CD62L (clone DREG-56), CD86 (clone 2331), CD184 (clone 12G5), HLA-A,B,C (clone G46-  
2 2.6); Alexa Fluor 647- conjugated CD197 (clone 3D12) and isotype control antibodies: FITC-  
3 conjugated mouse IgG1 (clone MOPC-21), IgG2a (clone G155- 178), IgG2b (clone MPC-11);  
4 PE-conjugated mouse IgG1 (clone MOPC-21), IgG2a (clone G155-178), IgM (clone G155-228);  
5 PerCPconjugated mouse IgG2a (clone X39); PE-Cy7-conjugated mouse IgG1 (clone MOPC-  
6 21); APC-conjugated mouse IgG1 (clone MOPC- 21), IgG2a (clone G155-178), IgG2b (clone  
7 27-35); Alexa Fluor 647 rat IgG2a (clone R35-95). PE-conjugated mouse monoclonal antibodies  
8 against human CD282 (clone TL2.1) and CD284 (clone HTA125) and isotype control antibody  
9 PE-conjugated mouse IgG2a (clone eBM2a) were obtained from eBioscience (San Diego, CA,  
10 USA). Anti-human monoclonal blocking antibodies against TLR-1 (clone H2G2), TLR-2 (clone  
11 B4H2), TLR-4 (clone W7C11), TLR-5 (clone Q2G4), TLR-6 (clone C5C8) and isotype control  
12 human IgA2 (clone T9C6) and mouse IgG1 (clone T8E5) were purchased from Invivogen  
13 (Toulouse, France).

#### 14 **HK mycobacterial preparations**

15 Sterile vials of HK *M. vaccae* (NCTC11659, rough strains) and *M. obuense*  
16 (NCTC13365, rough strains) preparations were manufactured, respectively, by Eden Biodesign  
17 (Liverpool, UK) and Bio Elpida (Lyon, France), whereby each 1 mg wet-weight of bacilli is  
18 equivalent to 10<sup>9</sup> HK organisms. Briefly, both preparations were suspended in borate-buffered  
19 saline (BBS; pH 8.0) at 50 mg/ml and autoclaved for 15 min at 121 °C.

#### 20 **Blood collection**

21 Human peripheral blood was obtained by venipuncture from 36 Caucasian healthy  
22 volunteers and was collected into K2 ethylene diamine tetraacetic acid (K2-EDTA) vacutainer  
23 tubes (BD Biosciences). The study population had a mean age of 28 ± 8.5 years and included  
24 14 females and 22 males. A written informed consent was obtained from each donor and the  
25 study protocol was approved by the Faculty Research Ethics Committees at Kingston University  
26 and University of Balamand.

#### 27 **Stimulation of whole blood cultures**

28 Preliminary experiments using 3 and 6 h time points to stimulate whole blood with  
29 different concentrations (30, 100 or 300 µg/ml) of HK *M. vaccae* or *M. obuense* preparations  
30 have revealed that the optimal regulation of surface receptors expression by either  
31 mycobacterial preparation was observable at 300 µg/ml (Supplementary Figure 1A) and after 3  
32 h of stimulation (Supplementary Figure 1B). Therefore, these optimized stimulation conditions

1 were employed in all investigations on the expression of surface receptors in which whole blood  
2 cultures (total culture volume = 3 ml) were either left unstimulated or were stimulated at 37 °C  
3 in a 5% CO<sub>2</sub> humidified atmosphere. A similar protocol was adopted for the analysis of  
4 cytokines and chemokines levels, except that the stimulation period was extended to 24 h.  
5 Whole blood incubated with equivalent amounts of BBS (vehicle) served as unstimulated  
6 control.

### 7 **Immunophenotyping of leukocytes**

8 After 3 h stimulation, blood leukocyte populations were immunophenotyped for a set of  
9 cell surface receptors. A volume of 100 µl of whole blood was incubated for 30 min at room  
10 temperature (RT) in the dark with optimized concentrations of the previously mentioned  
11 antibodies or isotype control antibodies. Erythrocytes were then lysed by incubation with FACS  
12 lysing solution (BD Biosciences) for 10-15 min at RT in the dark. Leukocytes were collected by  
13 centrifugation at 350 x g, for 5 min at RT and washed once with Cell Wash solution (BD  
14 Biosciences), then re-collected by centrifugation at 350 x g, for 5 min at RT, and finally  
15 resuspended in Cell Fix solution (BD Biosciences). Fixed samples were run on a FACSCalibur  
16 flow cytometer (BD Biosciences) equipped with an argon ion laser (488nm) and a red diode  
17 laser (635nm) and the obtained data was analyzed by means of CellQuestPro software (BD  
18 Biosciences). A minimum of 20,000 cells were acquired per sample. The signals were obtained  
19 in a linear mode for forward scatter (FSC) and side scatter (SSC) channels and in a logarithmic  
20 mode for fluorescence channels. The intensity and color compensation were set by using  
21 Calibrite beads (BD Biosciences) in combination with FACSComp software (BD Biosciences),  
22 and by running single-color stained blood cells. Lymphocytes and neutrophils were identified by  
23 their FSC and SSC properties. Monocytes were identified by their CD14<sup>+</sup>/ SSC profile, whereas  
24 in TLR blocking experiments, monocytes were gated according to their FSC and SSC profile.  
25 Bivariate dot plots were generated to determine the percentage of cells positive for the particular  
26 surface receptor within a gated cell population. Histogram plots were used to indicate the  
27 density of cell surface receptors which were expressed as geometric mean fluorescence  
28 intensity (MFI) on receptor-positive cell population.

### 29 **Lymphocyte proliferation assay**

30 Peripheral blood was diluted with an equal volume of RPMI 1640 medium (Lonza,  
31 Slough, UK) supplemented with standard concentrations of L-glutamine (Lonza), penicillin and  
32 streptomycin (Lonza). Blood was then layered over Ficoll-Paque plus (GE Healthcare, Little

1 Chalfont, UK) and centrifuged at 400 x *g* for 35 min at 19 °C. Peripheral blood mononuclear  
2 cells (PBMCs) were then collected from the interface and were washed twice. Finally, PBMCs  
3 were resuspended in RPMI medium supplemented with 10% heat-inactivated pooled human AB  
4 serum (Lonza). PBMCs were checked for viability (>95%), using the trypan blue exclusion  
5 method, were seeded, in triplicates, in 96-well flat-bottom plates (Corning, Tewksbury, USA) at  
6 a density of 2x10<sup>5</sup> cells/well, and were stimulated in the presence or absence of increasing  
7 concentrations of HK *M. vaccae* or *M. obuense* (1, 3, 10, 30, 100 and 300 µg/ml) or 10 µg/ml of  
8 *Candida albicans* (*C. albicans*) soluble antigen (Greer Laboratories, Lenoir, NC, USA). Cultures  
9 were maintained for a period of 7 days at 37 °C in a 5% CO<sub>2</sub> humidified incubator. On day 6,  
10 PBMC cultures were pulsed with 1µCi/well tritiated thymidine (Perkin Elmer, San Jose, CA,  
11 USA) for 16 h. Cells were then harvested onto glass fiber filter disks (Connectorate AG,  
12 Dietikon, Switzerland) using a cell harvester (Inotech Biotechnologies, Basel, Switzerland) and  
13 radioactivity was measured in a liquid scintillation counter (Perkin Elmer). Unstimulated cultures  
14 lacking the antigen and containing equivalent amounts of BBS (vehicle) served as negative  
15 controls. The radioactivity of incorporated thymidine was obtained as counts per minute (cpm).  
16 Results are presented as stimulation index (SI), which is defined as follows: mean cpm of  
17 antigen-stimulated cultures divided by the mean cpm of unstimulated cultures. A donor is  
18 considered as a responder to a given antigen if the SI is ≥ 3.

### 19 **Determination of cytokines, chemokines and sCD62L levels**

20 The release of soluble mediators, chemokine C-X-C motif ligand 8 (CXCL8)/ IL-8, IFN-α,  
21 IFN-γ, IL-2, IL-4, IL-5, IL-6, IL-10, IL-12 (p70), IL-13, IL-23 (p19/p40), macrophage inflammatory  
22 protein 1 (MIP1)-α, regulated on activation normal T cell expressed and secreted (RANTES),  
23 soluble (s) CD62L and TNF-α, was measured in supernatants from whole blood cultures using  
24 ELISA (R&D Systems, Abingdon, UK) in accordance with the manufacturer's instructions.  
25 Samples were tested in duplicates and the optical densities were read using Multiskan Ascent  
26 micro plate reader (Thermo, Waltham, MA, USA). In a restricted set of experiments, sCD62L  
27 levels were also assayed in supernatants of purified PBMCs that were cultured in RPMI medium  
28 supplemented with 10% heat-inactivated fetal bovine serum (FBS; Lonza) and were left either  
29 unstimulated or stimulated with 300 µg/ml of HK *M. vaccae* or *M. obuense* for 3 and 6 h.

### 30 **TLR blocking**

31 Whole blood was incubated with 10µg/ml of cell culture grade, azide-free blocking  
32 monoclonal antibodies against TLR1, TLR2, TLR4, TLR5, TLR6 or isotype control antibodies

1 (listed above) for 1 h prior to stimulating whole blood with HK *M. obuesne* for 3 h. As control  
2 experiments for testing the efficiency of TLR blocking, specific TLR ligands (Invivogen) were  
3 used. TLR agonists consisted of: Pam3CSk4 (300 ng/ml), HK *Listeria monocytogenes* ( $10^8$   
4 cells/ml), lipopolysaccharide (LPS; purified from *Escherichia coli* K12; 100 ng/ml), flagellin  
5 (purified from *Bacillus subtilis*; 10  $\mu$ g/ml) and FSL-1 (100 ng/ml) which signal through TLR2/1,  
6 TLR2, TLR4, TLR5 and TLR2/6 respectively.

## 7 **Statistical analysis**

8 All statistical analyses were conducted by means of GraphPad Prism software (version  
9 6; GraphPad Software, San Diego, CA, USA) by applying paired t-test, Wilcoxon signed rank  
10 test or Friedman test followed by Dunn's multiple comparison post hoc test. Data are expressed  
11 as mean values  $\pm$  standard error of the mean (SEM) and differences were considered to be  
12 statistically significant at  $p$  values  $< 0.05$ .

## 13 **Results**

### 14 ***M. vaccae* and *M. obuense* modulate, *in vitro*, the expression of surface receptors on** 15 **monocytes and neutrophils in whole blood**

16 The expression levels of a panel of surface receptors on monocytes (Fig. 1, Table 1,  
17 Supplementary Table 1), neutrophils (Table 2, Supplementary Table 2) and lymphocytes in  
18 whole blood obtained from 15 donors and stimulated *in vitro* with HK *M. vaccae* or *M. obuense*  
19 were evaluated. Monocytes and neutrophils exhibited substantial variations in the expression  
20 level of different categories of cell surface receptors following whole blood stimulation with *M.*  
21 *vaccae* or *M. obuense*. Among the adhesion receptors analyzed, a significant 6.5- and 5-fold  
22 remarkable reduction ( $p < 0.05$ ) in the percentage of monocytes expressing CD62L was  
23 detected following stimulation with *M. vaccae* and *M. obuense*, respectively. This reduction in  
24 CD62L expression was also accompanied by a significant 4-fold decrease ( $p < 0.05$ ) in the MFI  
25 of CD62L (Fig. 1A, Table 1). Conversely, a 2.75-fold elevation ( $p < 0.05$ ) in the percentage of  
26 monocytes expressing CD2 was observed following mycobacterial stimulation. Moreover, the  
27 intensity of receptor expression, measured as MFI, of CD18 (Fig. 1B), CD11a, CD44, CD54 and  
28 CD58 was significantly upregulated ( $p < 0.05$ ) on monocytes post stimulation with either  
29 mycobacterial preparation (Table 1). On the other hand, both mycobacteria affected the  
30 expression of a restricted number of adhesion receptors on neutrophils. While the expression of  
31 CD62L was significantly reduced, a significant increase ( $p < 0.05$ ) in the expression of only  
32 CD18 and CD54 could be detected (Table 2).



1 Significant variations in the expression of antigen presentation and co-stimulatory  
2 receptors on monocytes and neutrophils subsequent to stimulation with mycobacterial  
3 preparations were also detected. In unstimulated blood cultures, monocytes lacked the  
4 expression of CD80, but stimulation with either mycobacterial preparation induced monocytes to  
5 express CD80 (Table 1). A significant upregulation ( $p < 0.05$ ) in the expression of CD86  
6 (Fig.1A), HLA-DPDQDR (Fig. 1B) and CD45 was observed on monocytes (Table 1). We have  
7 also noted, using a smaller sample size ( $n = 9$ ), a significant increase ( $p < 0.05$ ) in the  
8 percentage of CD137L<sup>+</sup> monocytes following stimulation with *M. vaccae* or *M. obuense* from  
9 10±1.5 % to 38±5 % and 51±4.1 %, respectively. Stimulation with either mycobacterial  
10 preparation caused an increase in the MFI of CD45 on neutrophils; however, this increase was  
11 only statistically significant ( $p < 0.05$ ) with *M. vaccae*. On the contrary, stimulation with *M.*  
12 *vaccae*, but not with *M. obuense*, significantly decreased ( $p < 0.05$ ) the MFI of HLA-ABC on  
13 neutrophils (Table 2). Members of the pattern recognition receptors (PRRs), CD14, CD36, and  
14 CD206, displayed an upregulated expression ( $p < 0.05$ ) on monocytes after stimulation with  
15 either mycobacterial preparation (Table 1). In a set of preliminary experiments conducted on 5  
16 donors, both *M. vaccae* and *M. obuense* were able to highly up-regulate TLR expression on  
17 monocytes with approximately a 2-fold increase in the MFI of CD282 (TLR-2) (unstimulated:  
18 77±8.4; *M. vaccae*: 155±9.0; *M. obuense*: 173±10;  $p < 0.05$  vs. unstimulated) and approximately  
19 a 7-fold increase in the percentage of CD284<sup>+</sup> (TLR-4<sup>+</sup>) monocytes (unstimulated: 8±1 %; *M.*  
20 *vaccae*: 53±6 %; *M. obuense*: 59±6 % ;  $p < 0.05$  vs. unstimulated). The expression of  
21 complement and Fc receptors was significantly modified on monocytes and neutrophils in  
22 response to stimulation with either mycobacterial preparation. A substantial increase ( $p < 0.05$ )  
23 in the expression of CD11c (Fig. 1B), CD11b and CD35 on monocytes was shown after  
24 stimulation with *M. vaccae* or *M. obuense* (Table 1). On the other hand, both mycobacterial  
25 preparations induced a modest but significant enhancement ( $p < 0.05$ ) in the expression of  
26 CD64 and CD89 on monocytes (Table 1). On neutrophils, a significant elevation ( $p < 0.05$ ) in  
27 the expression of CD11c, CD16, CD35 and CD64 was observed following stimulation with either  
28 mycobacterial preparation, whereas a significant elevation ( $p < 0.05$ ) in the expression of  
29 CD11b and CD89 was only noted following stimulation with *M. vaccae* (Table 2).

30 Significant variations in the expression of cytokine and chemokine receptors, on  
31 monocytes and neutrophils, were detected following stimulation with either of the HK  
32 mycobacteria. Both preparations were equipotent at significantly upregulating ( $p < 0.05$ ), on  
33 monocytes, the expression of CD116, CD127 and CD132, as well as at significantly

1 downregulating ( $p < 0.05$ ) the expression of CD184 (Fig. 1A) and CD119 (Table 1) In  
2 neutrophils, stimulation with either *M. vaccae* or *M. obuense* significantly reduced ( $p < 0.05$ ) the  
3 percentage of cells expressing CD184 and significantly downregulated ( $p < 0.05$ ) the MFI of  
4 CD119 (Table 2). In addition, when tested on a smaller sample of 9 donors, the MFI of CD114  
5 was found to be downregulated following mycobacterial stimulation (unstimulated:  $97 \pm 4$ ; *M.*  
6 *vaccae*:  $41 \pm 1$ ; *M. obuense*:  $55 \pm 4$ ). However, this effect only attained statistical significance ( $p <$   
7  $0.05$ ) with *M. vaccae*. In this short-term *in vitro* assay, neither *M. vaccae* nor *M. obuense*  
8 induced statistically significant variations in the expression level of any of the screened surface  
9 receptors on lymphocytes (data not shown).

#### 10 **TLR-1 and TLR-2 contribute to the *M. obuense*-induced upregulation of CD11c and HLA-** 11 **DR expression on monocytes**

12 TLR-2 has been previously shown to play a role in mediating the main transcriptional  
13 responses occurring after DC stimulation with HK *M. vaccae* (Le Bert, et al. 2011). In order to  
14 examine whether TLR-2 and other TLRs (TLR-1, 4, 5 and 6) are involved in mediating the *M.*  
15 *obuense*-induced upregulation of surface receptors on monocytes in whole blood, TLR blocking  
16 experiments ( $n = 6$ ) with specific antibodies against TLR-1, TLR-2, TLR-4, TLR-5 and TLR-6  
17 were performed on the expression of one adhesion receptor (CD11c) and another receptor  
18 involved in antigen presentation (HLA-DR), both of which displayed significant variations on  
19 monocytes upon mycobacterial stimulation. The blocking capacity of these antibodies was  
20 initially confirmed in control experiments using stimulation with specific TLR ligands, as  
21 described in materials and methods (Fig. 2A).

22 Blocking either TLR-1 or TLR-2 significantly abrogated ( $p < 0.05$ ) the *M. obuense*-induced  
23 upregulation of the two tested receptors (Fig. 2B). Analysis of combined blocking with anti-TLR1  
24 and anti-TLR2, versus the blocking induced by either antibody separately, resulted in an  
25 improved blocking effect which reached statistical significance ( $p < 0.05$ ) versus either antibody  
26 in the case of CD11c, but only against anti-TLR2 in the case of HLA-DR. However,  
27 simultaneous blocking of TLR-2 and TLR-6 was comparable to the effect observed with blocking  
28 TLR-2 alone in reversing the *M. obuense*-induced expression of CD11c and HLA-DR (Fig. 2B).  
29 Blocking of TLR-4, 5 and 6 did not cause any significant reduction in the *M. obuense*-induced  
30 upregulation of CD11c and HLA-DR (Fig. 2B). Taken together, these results indicated a major  
31 contribution for both TLR-1 and TLR-2 in mediating the *M. obuense*-induced regulation of  
32 CD11c and HLA-DR expression on monocytes.

## 1 **Whole blood stimulation with *M. vaccae* or *M. obuense* induces the secretion of a** 2 **specific set of cytokines and chemokines**

3 A panel of 12 cytokines and 3 chemokines was analyzed in supernatants of whole blood  
4 cultures from 15 different donors that were stimulated with *M. vaccae* or *M. obuense* for a period  
5 of 24 h. Among the 12 screened cytokines, IL-6 and TNF- $\alpha$  levels were markedly increased ( $p <$   
6 0.05) in response to stimulation with *M. vaccae* or *M. obuense*, whereas IL-10 levels were  
7 significantly elevated ( $p < 0.05$ ) only after stimulation with *M. vaccae* (Fig. 3). The secreted  
8 levels of the 3 studied chemokines also showed a significant increase following mycobacterial  
9 stimulation. A 45- and 42-fold increase ( $p < 0.05$ ) in CXCL8/IL-8 levels was detected after  
10 stimulation with *M. vaccae* and *M. obuense*, respectively (Fig. 3). Moreover, blood stimulation  
11 with *M. vaccae* or *M. obuense* significantly induced ( $p < 0.05$ ) MIP-1 $\alpha$  production (Fig. 3).  
12 Likewise, RANTES levels were increased in response to stimulation with *M. vaccae* or *M.*  
13 *obuense*; however, this increase attained statistical significance ( $p < 0.05$ ) only for the latter  
14 (Fig. 3). No significant difference in IL-18 levels was observed between unstimulated (612 $\pm$ 71  
15 pg/ml) and *M. vaccae*- (638 $\pm$ 65 pg/ml) or *M. obuense*- (641 $\pm$ 70 pg/ml) stimulated blood cultures.  
16 Moreover, IFN- $\alpha$ , IFN- $\gamma$ , IL-2, IL-4, IL-5, IL-12 (p70), IL-13 and IL-23 (p19/p40) levels in  
17 unstimulated and mycobacteria-stimulated blood cultures were below the detection limit of the  
18 employed assays.

19 In order to determine whether the observed *M. vaccae*- and *M. obuense*-induced decrease in  
20 CD62L surface expression was associated with an increase in the release of sCD62L,  
21 supernatants from unstimulated and mycobacteria-stimulated whole blood cultures were  
22 collected. There was no significant difference ( $p > 0.05$ ) in sCD62L levels in culture  
23 supernatants after 3, 6 or 24 h of mycobacterial stimulation when compared with their respective  
24 unstimulated controls (Supplementary Figure 2). Moreover, using purified PBMC cultures from 6  
25 different donors, we could not detect sCD62L levels in the supernatants of unstimulated or  
26 mycobacteria-stimulated PBMCs tested after 3 and 6 h culture periods.

## 27 ***M. vaccae* and *M. obuense* induce proliferative responses of PBMCs**

28 In order to determine the optimal concentrations of HK *M. vaccae* and *M. obuense* that  
29 are capable of inducing strong proliferative responses in PBMCs from healthy donors, different  
30 concentrations (1, 3, 10, 30, 100 and 300  $\mu$ g/ml) of either mycobacterial preparation were tested  
31 on 4 separate donors (Fig. 4A). At the lowest and highest concentrations, only weak or no  
32 responses could be detected to either preparation (Fig. 4A). To further verify our data, PBMCs  
33 from 8 additional donors were tested at concentrations ranging from 3-100  $\mu$ g/ml. Results of

1 proliferative responses from the 12 different donors tested revealed that the highest proliferative  
2 responses to *M. obuense* were induced at 3 µg/ml (Fig. 4B), which attained a statistically  
3 significant difference ( $p < 0.05$ ) versus 30 or 100 µg/ml. In contrast, maximal responses to *M.*  
4 *vaccae*, noted at 10 µg/ml, were nearly equivalent to those induced by 30 µg/ml and were  
5 significantly higher ( $p < 0.05$ ) than those induced by 100 µg/ml. At the 3 µg/ml concentration, the  
6 *M. obuense*-induced proliferative responses were significantly higher ( $p < 0.05$ ) than the  
7 responses induced by *M. vaccae*, whereas the reverse was true at the 30 µg/ml concentration  
8 (Fig. 4B). Soluble recall antigen of *C. albicans* (10 µg/ml) was included in the proliferation  
9 assays as a positive control on account of its known potency to induce strong PBMC  
10 proliferative responses. PBMCs from all tested donors showed high proliferative responses to *C.*  
11 *albicans* (Mean SI =  $134 \pm 39$ ) and these responses were significantly higher ( $p < 0.05$ ) than  
12 those induced by *M. vaccae* or *M. obuense*.

## 13 Discussion

14 Identifying the outcome of interaction of HK mycobacteria with immune cells is a crucial  
15 step in the process of assessing their immunomodulatory properties for potential  
16 immunotherapeutic applications. Our study is the first to utilize the established human whole  
17 blood model (Darcissac, et al. 1996a) to investigate the capacity of HK *M. vaccae* and *M.*  
18 *obuense* preparations to modulate the expression of an array of different categories of cell  
19 surface receptors expressed on human monocytes, neutrophils and lymphocytes. The reason  
20 behind employing the whole blood model in our study is that it closely resembles the status of  
21 circulating cells *in vivo* and it involves physiological concentrations of factors that affect  
22 leukocytes function. Our immunophenotyping results demonstrated that under the experimental  
23 conditions used, the target peripheral blood immune cells for HK *M. vaccae* and *M. obuense*  
24 appear to be the phagocytic cells; namely, neutrophils and monocytes. Whole blood stimulation  
25 with either mycobacterial preparation upregulated the expression of the adhesion receptors,  
26 CD2, CD11a, CD44 and CD58 on monocytes and of CD18 and CD54 on monocytes and  
27 neutrophils. These findings are similar to those previously reported following whole blood  
28 stimulation with the mycobacterial cell wall component muramyl dipeptide (MDP) or with  
29 endotoxin (Darcissac, et al. 1996a). The increased expression of CD2, and more importantly of  
30 its ligand CD58 on monocytes, may indicate an increased ability of monocyte-macrophage cells  
31 to adhere to T lymphocytes which constitutively express the CD2 receptor (Lopez, et al. 2001).  
32 Accordingly, the immunomodulating activity of mycobacterial preparations may depend on their  
33 ability to regulate CD58 expression on antigen presenting cells (APCs), thereby enhancing

1 antigen presentation and induction of adaptive immunity (Wingren, et al. 1995). Similarly,  
2 increased expression of CD54 on APCs may contribute to antigen-specific T cell activation by  
3 enhancing the adhesion of T cells to APCs (Gaglia, et al. 2000). The most intriguing observation  
4 was the dramatic reduction in the expression of L-selectin (CD62L) on monocytes, and to a  
5 lesser extent on neutrophils, following mycobacterial stimulation. This observation is in line with  
6 previous reports which demonstrated that in response to activation signals, L-selectin is shed  
7 from the surface of neutrophils and monocytes within minutes (Janke, et al. 2009; Nijhuis, et al.  
8 2007). Upon the ectodomain shedding of CD62L, its soluble form, sCD62L, is released into the  
9 extracellular environment (Ivetic 2013; Smalley and Ley 2005). Following whole blood  
10 stimulation with either mycobacterial preparation, we could not detect any significant increase in  
11 sCD62L levels. Nevertheless, the constitutive levels of sCD62L detected in all supernatants  
12 were quite high and exceeded 1000 ng/ml. Thus it is quite likely that such high constitutive  
13 levels of sCD62L could mask the detection of low levels of sCD62L shedding induced by  
14 mycobacterial stimulation (Schleiffenbaum, et al. 1992). In addition, our inability to detect  
15 sCD62L in unstimulated and mycobacteria-stimulated PBMC culture supernatants, despite the  
16 significant downregulation of CD62L expression on monocytes, might be attributed to low  
17 amounts of shed CD62L which are below the detection limit of our assay (0.3 ng/mL).

18 On the other hand, whole blood stimulation with *M. vaccae* or *M. obuense* has resulted  
19 in increased monocyte expression of key receptors involved in antigen presentation and  
20 costimulation including MHC-class II, CD80, CD86 and CD137L receptors. The observed  
21 upregulated expression of antigen presentation and costimulatory receptors strongly suggests a  
22 better antigen presentation function of mycobacteria-stimulated APC leading to improved T cell  
23 activation and survival (Gramaglia, et al. 2000). In fact, one of the mechanisms by which tumor  
24 cells escape immune detection is by down-regulating antigen presentation and costimulatory  
25 receptors which would generate anergic T cells incapable of initiating an effective antitumor  
26 immune response (Driessens, et al. 2009; Thibodeau, et al. 2012). Consequently, augmenting  
27 the expression of costimulatory receptors might contribute toward enhanced antitumor immunity  
28 (Capece, et al. 2012).

29 We have also shown that mycobacterial stimulation was associated with a significant  
30 upregulation of different PRRs, including TLR-2, TLR-4, CD14, CD36 and CD206, on  
31 monocytes. This is the first description of an induced expression of CD206 on monocytes in a  
32 whole blood model and after a short period of mycobacterial stimulation. Previous studies have  
33 demonstrated extremely low or no CD206 expression on resting monocytes, with the CD206

1 expression being restricted to immature DCs and macrophages (Kato, et al. 2000; Noorman, et  
2 al. 1997; Wollenberg, et al. 2002). Therefore, it is likely that mycobacterial stimulation could  
3 initiate the expression of markers on monocytes associated with a subsequent potential  
4 differentiation of these cells into macrophages. The elevated CD14 and CD282 (TLR-2) gene  
5 expression in mycobacteria-stimulated monocytes has been previously reported in *M. vaccae*-  
6 stimulated THP-1 human monocytic cell line (Martinelli, et al. 2004). Such an effect could well  
7 contribute towards boosting the recognition of pathogen-associated molecular patterns. On the  
8 contrary, some studies have reported a significant downregulation of CD14 expression on  
9 purified monocytes that were stimulated with live *M. tuberculosis* or HK *M. avium-M.*  
10 *intracellulare* complex for 16 or 48 h, respectively (Santucci, et al. 2000; Tsuyuguchi, et al.  
11 1990). The apparent contradiction in CD14 expression between our study and other studies  
12 previously reported might be attributed to differences in the culture systems, in the nature of  
13 stimulus, and/or in the stimulation periods utilized.

14 Moreover, both mycobacterial preparations demonstrated an evident potential to  
15 upregulate the expression of complement and Fc-receptors on monocytes and neutrophils, an  
16 effect that is tightly linked to improved phagocytosis and immune complex clearance  
17 (Nimmerjahn and Ravetch 2008; Zipfel and Skerka 2009). For instance, in monoclonal antibody-  
18 mediated therapy targeting tumor-associated antigens, upregulating the expression of  
19 complement and Fc receptors might enhance the process of tumor cell killing through antibody-  
20 dependent cell-mediated cytotoxicity or complement-dependent cytotoxicity, respectively  
21 (Hogarth and Pietersz 2012; van Egmond and Bakema 2013).

22 Although both mycobacterial preparations altered the expression of various surface  
23 receptors on neutrophils and monocytes, no significant effect could be observed on  
24 lymphocytes. The lack of a modulatory effect by both mycobacterial preparations on  
25 lymphocytes might be attributed to the short 3 h stimulation period adopted in our whole blood  
26 culture system. Using purified PBMCs and longer stimulation periods of 24 and 48 h, a previous  
27 study has shown that, *in vitro*, both HK *M. vaccae* and *M. obuense* upregulated the surface  
28 expression of various activation receptors on the  $\gamma\delta$  T lymphocyte subpopulation (Fowler, et al.  
29 2012). Moreover, *in vivo* treatment with these HK mycobacterial preparations has been reported  
30 to induce, few weeks later, a measurable activation of cytotoxic T lymphocytes (Elia, et al.  
31 2013). Another potential explanation is that blood lymphocytes fail to recognize both  
32 mycobacterial preparations due to the absence, or barely detectable surface expression of TLR-  
33 2 which is a crucial receptor for the recognition of mycobacteria (Flo, et al. 2001). This

1 explanation is further substantiated by the inability to detect the surface expression of TLR-2 on  
2 lymphocytes in our system (data not shown).

3 Mycobacteria are known to trigger host immune responses following initial interaction  
4 with innate immune cells through selected PRRs (Killick, et al. 2013; Kleinnijenhuis, et al. 2011).  
5 A HK preparation of *M. vaccae* has been previously reported to induce *in vitro* DC activation via  
6 TLR2 (Le Bert, et al. 2011). To determine whether the interaction between HK *M. obuense* and  
7 TLRs was responsible for the increased surface expression of receptors on monocytes, blocking  
8 experiments with antibodies against TLR-1, 2, 4, 5 and 6 were carried out. These experiments  
9 clearly indicated that in human whole blood, blocking of TLR-1 and/or TLR-2 significantly  
10 inhibited the *M. obuense*-induced upregulation of CD11c and HLA-DR surface expression on  
11 monocytes. Despite the blocking of TLR-1 and/or TLR-2, the expression levels of CD11c and  
12 HLA-DR remained higher than those of unstimulated monocytes ( $p < 0.05$ ) and therefore a  
13 possible participation of other receptors, such as nucleotide oligomerisation domain 2 (Nod-2)  
14 receptor, in mediating the biological effects of *M. obuense* cannot be excluded (Behr and  
15 Divangahi 2015). Differences in the average MFI values of MHC class II between TLR-blocking  
16 experiments (Fig. 2B) and immunophenotyping experiments (Table 1) can be explained by the  
17 fact that in TLR-blocking experiments, we used an antibody specific for only HLA-DR whereas in  
18 the immunophenotyping experiments an antibody recognizing the three MHC class II antigens,  
19 namely HLA-DPDQDR, was employed.

20 In the current work, we have measured a battery of cytokines and chemokines in  
21 supernatants of whole-blood cultures following 24 h of whole blood stimulation with *M. vaccae*  
22 or *M. obuense*. Among the cytokines tested, IL-6, IL-10, and TNF- $\alpha$  were significantly elevated  
23 following mycobacterial stimulation. This is in line with previous reports indicating the release of  
24 considerable amounts of IL-6, IL-10, and TNF- $\alpha$  upon activation of monocytes (Damsgaard, et  
25 al. 2009), the ability of *M. vaccae* to induce IL-10 and TNF- $\alpha$  production in cultures of isolated  
26 monocytes (Baran, et al. 2004), and increased secretion of IL-10 and TNF- $\alpha$  in PBMC cultures  
27 stimulated with HK *M. bovis* (Moreira, et al. 2012). Moreover, mycobacterial stimulation  
28 enhanced the production of the three tested chemokines: CXCL8/IL-8 and MIP-1 $\alpha$ . In contrast,  
29 whole blood stimulation with HK mycobacteria did not induce the release of any of the signature  
30 T cell cytokines, IFN- $\gamma$ , IL-2 or IL-4. Thus, the profile of HK mycobacteria-induced cytokines and  
31 chemokines in the *in vitro* system used is most likely monocyte-derived and triggered by  
32 structures common to the two organisms. Among such structures are cell wall components  
33 containing muramyl peptides as well as heat shock proteins (HSPs) which have been reported

1 to induce a range of proinflammatory cytokines and chemokines either *in vitro* or *in vivo* (Asea,  
2 et al. 2000; Bahr, et al. 2003; Darcissac, et al. 1996b; Stanford, et al. 2009; Wang, et al. 2002).  
3 This profile of cytokine release, dominated by IL-10 and proinflammatory cytokines, was also  
4 reported following whole blood stimulation with HK *Lactobacillus rhamnosus*, *Helicobacter*  
5 *pylori*, *Burkholderia pseudomallei*, MDP, and LPS (Darcissac, et al. 1996b; Duffy, et al. 2014;  
6 Myers, et al. 2014). On the other hand, the release of cytokines that play a direct role in the  
7 adaptive immune system has been reported to occur in whole blood cultures from healthy  
8 donors after stimulation with certain gram-negative and gram-positive HK bacteria, with fungi,  
9 with live BCG or with live *M. intracellulare*, but not with HK *M. tuberculosis* (Duffy, et al. 2014;  
10 Kartalija, et al. 2013; Popa, et al. 2009; Reichenbach, et al. 2006). It is noteworthy to mention  
11 that the cytokine responses to mycobacterial stimulation observed in the whole blood assay  
12 might differ from those detected in other culture systems. While the levels of some cytokines  
13 such as IL-2, IL-5, IL-6, IL-7, IL-13, TNF- $\alpha$ , MCP-1, MIP-1 $\beta$  (Silva, et al. 2013) and IFN- $\gamma$  (Antas,  
14 et al. 2004); Deenadayalan, et al. 2013; Silva, et al. 2013) were comparable between whole  
15 blood and PBMC cultures stimulated with specific *M. tuberculosis* antigens, levels of other  
16 cytokines like IL-1 $\beta$ , IL-4, IL-10, IL-12 (p70), IL-17, G-CSF and GM-CSF were significantly  
17 different between the two culture models (Silva, et al. 2013).

18 We have also examined the lymphoproliferative responses to HK *M. vaccae* and *M.*  
19 *obuense* through stimulation of PBMCs from healthy individuals. Distinct from cell proliferation  
20 occurring in cancers and other pathological processes, this *in vitro* assay is routinely used to  
21 assess the presence of mycobacteria-sensitized lymphocytes in peripheral blood and their  
22 ability to proliferate in response to mycobacterial antigens. Taking into consideration that *M.*  
23 *vaccae* and *M. obuense* are phylogenetically related, lack group iii antigens, possess their own  
24 species-specific group iv antigens, and are rich in group i common mycobacterial antigens  
25 (Stanford, et al. 2009), it can be assumed that the observed *M. vaccae* and *M. obuense* induced  
26 PBMC proliferative responses might be directed, mainly, against the common mycobacterial  
27 antigens. Previous studies, conducted to analyze the sensitization to environmental  
28 mycobacteria in north Lebanon, southeast England, and northern Malawi by skin testing and/or  
29 whole blood IFN- $\gamma$  release assay, revealed a very low level of exposure to some environmental  
30 mycobacteria including *M. vaccae* (Bahr, et al. 1986; Black, et al. 2001; Weir, et al. 2003).

31 Moreover, the healthy donors tested were not previously vaccinated with BCG in line  
32 with the policy adopted by the local health authorities. However, several studies have indicated  
33 that different mycobacterial species possess cross-reacting T-cell epitopes which could induce



1 the proliferation of sensitized T-cell clones (Oftung, et al. 1998; Sieling, et al. 2005). Therefore,  
2 *M. vaccae*- and *M. obuense*- specific T cells are likely to exhibit a broad spectrum of cross-  
3 reactivity with an as yet to be identified environmental mycobacterial species that could be  
4 abundant in the Lebanese environment and could elicit the observed lymphoproliferative  
5 responses.

6 As a limitation of the current study, our preliminary kinetics experiments were based on  
7 determining the optimal regulation of two surface receptors, and only at two time points, in  
8 response to mycobacterial stimulation. However, other receptors analyzed in this study might  
9 display optimal regulation at different stimulation periods. An additional limitation of our study  
10 was the analysis of cytokine levels at a single time point following mycobacterial stimulation of  
11 whole blood. Previous studies have emphasized the importance of identifying optimal  
12 stimulation time points correlating with peak levels of cytokines since these time points are  
13 cytokine- and stimulus-dependent (Hermann, et al. 2003; Lagrelius, et al. 2006). For instance,  
14 we could not detect IFN- $\gamma$  levels in 24 h mycobacteria-stimulated whole blood cultures.  
15 However, other studies employing longer stimulation periods of 5 and 6 days were capable of  
16 detecting IFN- $\gamma$  in whole blood cultures stimulated with specific *M. tuberculosis* antigens (Antas,  
17 et al. 2004; Deenadayalan, et al. 2013; Silva, et al. 2013). Thus, future kinetic studies employing  
18 additional and extended time points might be useful in addressing the effect of mycobacterial  
19 stimulation on the expression of other surface receptors and on the release of cytokines.

20 In conclusion, our study revealed a potent ability of HK cell preparations of *M. vaccae*  
21 and *M. obuense* to regulate the expression of several receptors on phagocytic cells, and to  
22 induce the release of a specific set of cytokines and chemokines in this whole blood *in vitro*  
23 system. The data provided could serve as a baseline to explain the outcome of interaction  
24 between HK mycobacterial preparations and immune cells which in additional future studies  
25 might be correlated with the previously known *in vivo* activities of HK mycobacterial preparations  
26 such as the adjuvant, the anti-infectious, and the anti-tumor effects

### 27 **Authors' contribution**

28 SB and GMB conceived and designed the experiments. SB performed the experiments. SB,  
29 HM, SM, CA and GMB analyzed the data. HM, SM and CA contributed reagents, materials, and  
30 analysis tools. SB and GMB wrote the paper.

### 31 **Conflict of interest**

32 The authors declare they have no conflicts of interest.

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## 10 **Figures Captions**

11 **Fig. 1.** Whole blood stimulation with *M. vaccae* or *M. obuense* modulates surface expression of  
12 various receptors on monocytes. (A) Representative flow cytometry dot plots demonstrating the  
13 expression of CD62L, CD86 and CD184 on gated monocytes in whole blood that was left  
14 unstimulated or stimulated with 300 µg/ml of *M. vaccae* or *M. obuense* for 3 h. Number within  
15 lower right quadrant indicates the percentage of receptor-positive monocytes out of the total  
16 monocyte population. (B) Representative flow cytometry histogram overlay plots showing the  
17 expression of CD11c, CD18 and HLA-DP,DQ,DR on gated monocytes in whole blood that was  
18 either left unstimulated or stimulated with 300 µg/ml of *M. vaccae* or *M. obuense* for 3 h.  
19 Numbers in the plots correspond to the geometric mean fluorescence intensity (MFI) of  
20 receptor-positive monocytes in each condition (filled gray histogram: isotype control; black line  
21 histogram: unstimulated; red line histogram: *M. vaccae*; green line histogram: *M. obuense*).

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23 **Fig. 2.** Toll-like receptor (TLR)-1 and/or TLR-2 blocking inhibit *M. obuense*-induced upregulation  
24 of CD11c and HLA-DR expression on monocytes in whole blood. Whole blood was  
25 preincubated with anti (α)-TLR-1, α-TLR-2, α-TLR-1/2, α-TLR6, α-TLR-2/6, α-TLR-4, α-TLR-5,  
26 isotype-matched control antibody IgA (for α-TLR-2 and 5) or IgG (for α-TLR-1, 4 and 6) for 1 h  
27 and then stimulated with (A) specific TLR2/1, TLR2, TLR4, TLR5, TLR2/6 ligands or (B) 300  
28 µg/ml of *M. obuense* (Mo) for 3 h. The surface expression of CD11c and HLA-DR was analyzed  
29 on monocytes. Columns indicate the geometric mean fluorescence intensity (MFI) of CD11c or  
30 HLA-DR on monocytes. Data obtained are (A) representative of 1 out of 2 independent donors  
31 and (B) mean ± SEM from 6 donors. Statistically significant differences were determined by  
32 paired t-test. (\**p* < 0.05 versus *M. obuense*).

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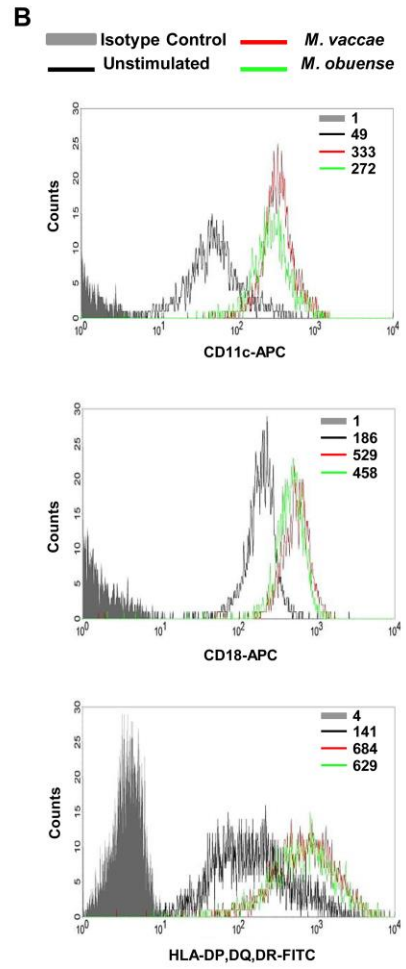
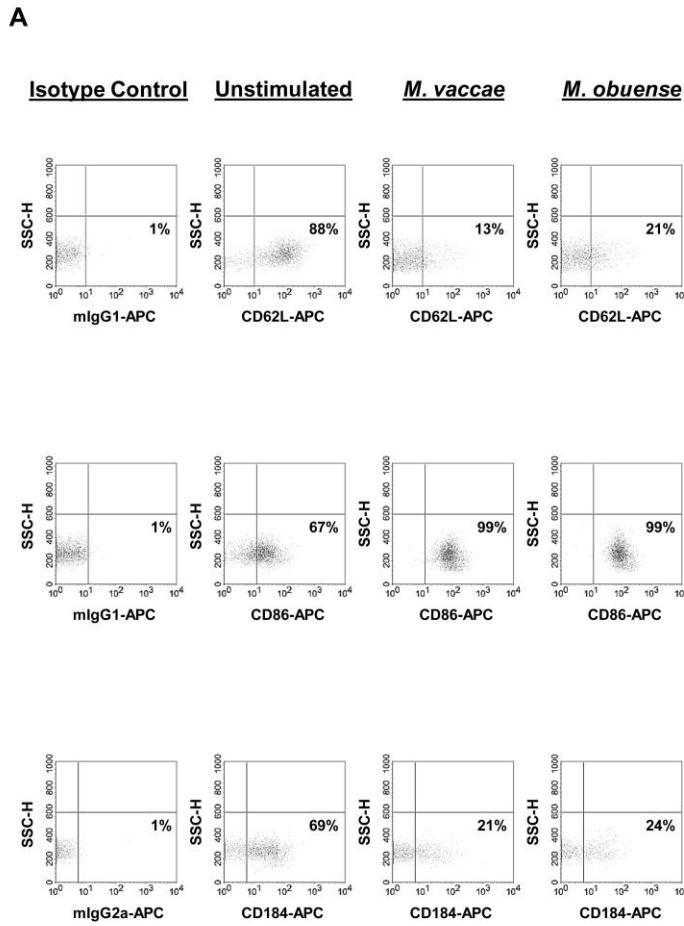


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**Fig. 3.** Cytokine and chemokine levels in supernatants of whole blood cultures following stimulation with *M. vaccae* or *M. obuense*. Scatter plots showing IL-6, IL-10, TNF- $\alpha$ , CXCL8/IL-8, MIP-1 $\alpha$ , and RANTES levels in supernatants of whole blood cultures that were left unstimulated or stimulated with 300  $\mu$ g/ml of *M. vaccae* or *M. obuense* for 24 h. Horizontal bars indicate group mean values of cytokine or chemokine concentration of 15 donors. Statistically significant differences were determined by Friedman test followed by Dunn's multiple comparison post hoc test ( $*p < 0.05$  versus unstimulated).

**Fig. 4.** Induction of peripheral blood mononuclear cells (PBMCs) proliferative responses to *M. vaccae* and *M. obuense*. PBMCs were stimulated with different concentrations (1, 3, 10, 30, 100 and 300  $\mu$ g/ml) of *M. vaccae* or *M. obuense* for 7 days. Data are reported as (A) symbols with error bars showings mean of stimulation index  $\pm$  SEM from 4 donors or as (B) columns with error bars indicating mean of stimulation index  $\pm$  SEM from 12 donors. Statistically significant differences were determined either by Friedman test followed by Dunn's multiple comparison post hoc test ( $*p < 0.05$  versus 10 or 30  $\mu$ g/ml of *M. vaccae*;  $^{\circ}p < 0.05$  versus 3  $\mu$ g/ml of *M. obuense*) or by Wilcoxon signed rank test ( $*p < 0.05$  versus *M. vaccae* at the same concentration).

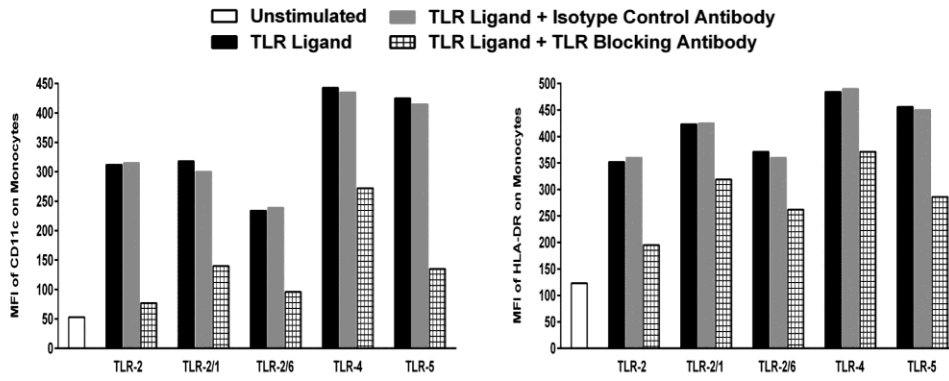
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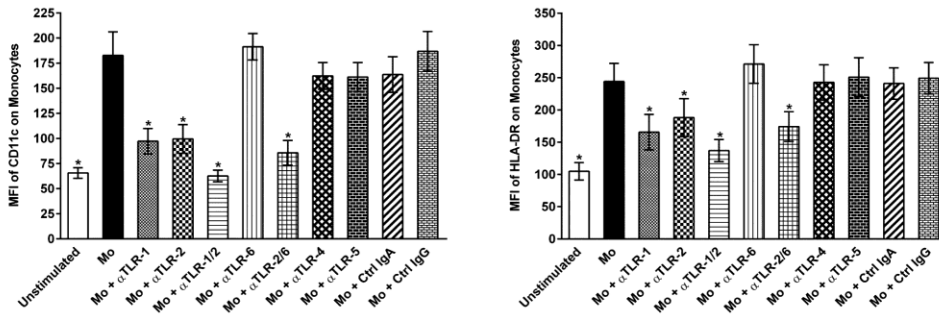
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1 **Fig. 2**  
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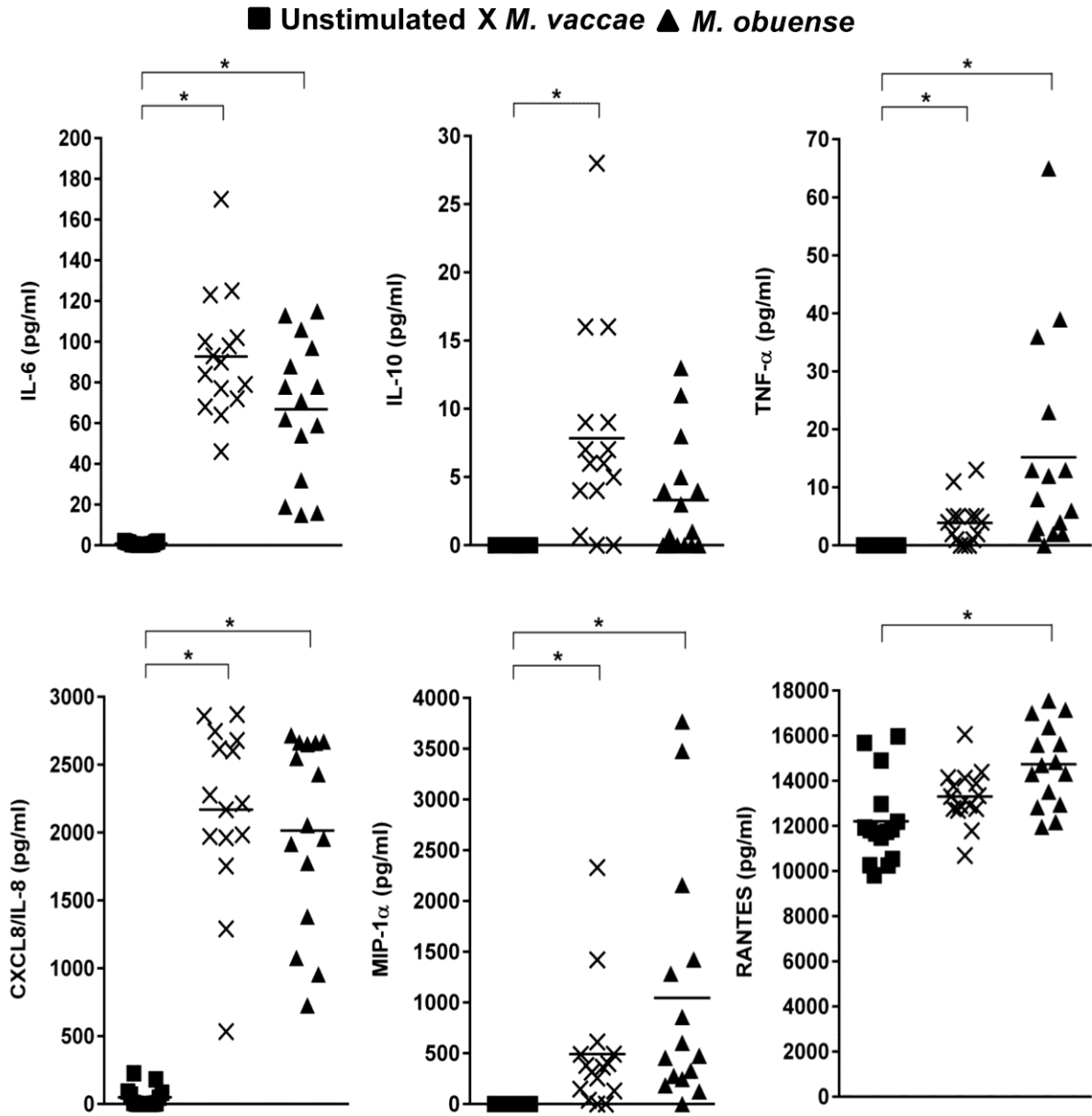


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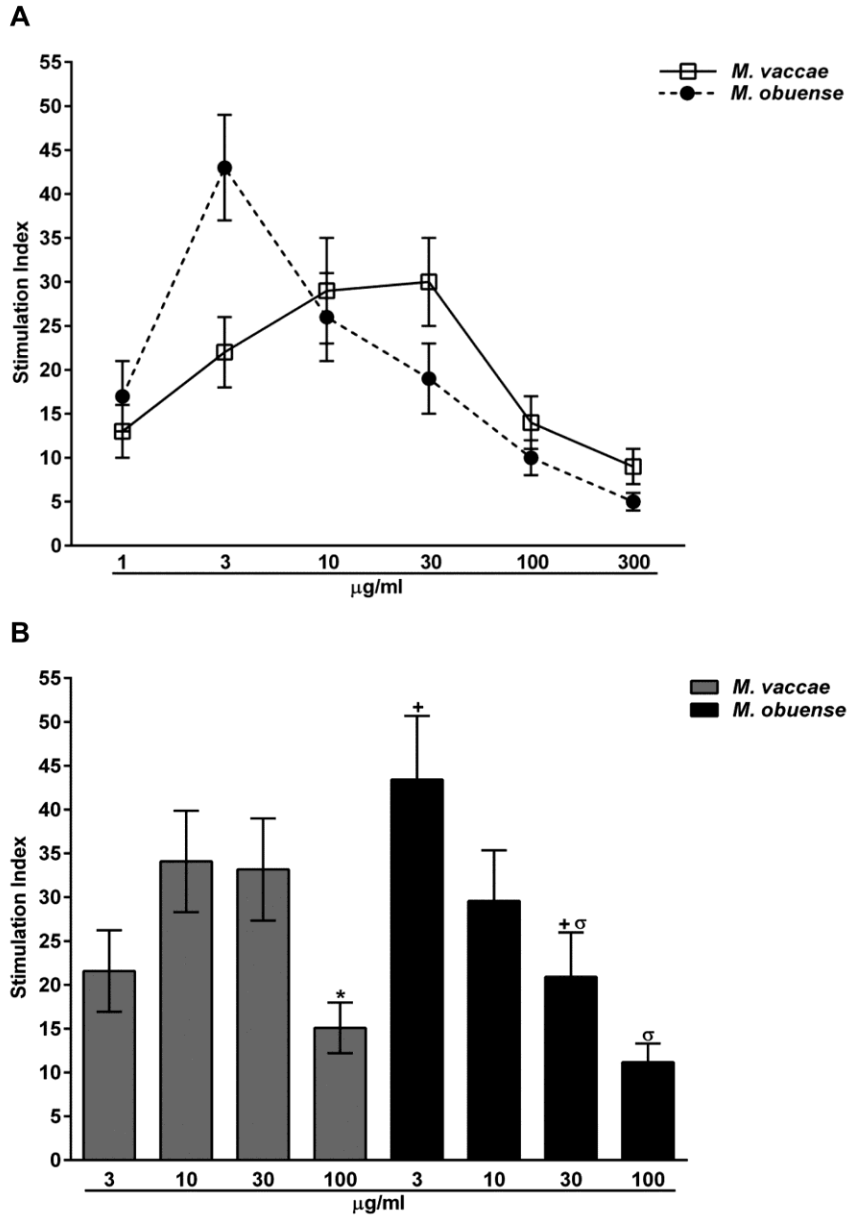
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1 **Fig. 3**  
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1 **Fig. 4**  
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**Table 1.**  
Expression levels of surface receptors on monocytes in whole blood.

Receptor Category	Surface Receptor Expression on Monocytes					
	% of Receptor-Positive Cells			MFI of Receptor-Positive Cells		
	Uns	<i>Mv</i>	<i>Mob</i>	Uns	<i>Mv</i>	<i>Mob</i>
<b>Adhesion</b>						
CD2 (LFA-2)	4±1	11±2*	11±1*	28±3	19±2	19±1
CD11a (LFA-1)	100±0	100±0	100±0	124±6	197±8*	193±8*
CD18 (β2 integrin)	100±0	100±0	100±0	207±15	602±44*	514±44*
CD44 (ECMRIII)	100±0	100±0	100±0	62±4	88±8*	97±10*
CD54 (ICAM-1)	99±0	100±0	100±0	93±6	328±23*	272±24*
CD58 (LFA-3)	99±0	100±0	100±0	99±7	138±9*	145±12*
CD62L (L-selectin)	86±2	13±5*	17±5*	64±8	16±3*	17±3*
<b>Antigen Presentation/ Costimulation/Signaling</b>						
CD45 (LCA)	100±0	100±0	100±0	291±11	647±26*	601±28*
CD80 (B7-1)	0±0	4±1*	5±1*	0±0	12±2*	13±2*
CD86 (B7-2)	61±6	97±1*	97±2*	19±2	67±9*	66±9*
HLA-DPDQDR (MHC II)	99±1	100±0	100±0	147±14	558±47*	524±49*
<b>Pattern Recognition</b>						
CD14 (LPSR)	100±0	100±0	100±0	295±9	570±14*	541±16*
CD36 (GPIIb)	64±5	85±3*	86±3*	74±17	98±17*	113±22*
CD206 (MMR)	0±0	4±1*	5±1*	0±0	37±2*	33±1*
<b>Complement/FcR</b>						
CD11b (CR3)	100±0	100±0	100±0	345±28	1212±65*	1033±66*
CD11c (CR4)	93±3	100±0*	99±1*	52±6	253±33*	200±30*
CD35 (CR1)	97±1	99±0	99±0	46±3	129±11*	114±9**
CD64 (FcγRI)	100±0	100±0	100±0	86±3	97±4*	96±4*
CD89 (FcaR)	98±0	99±0	99±0	205±12	330±21*	300±20*
<b>Cytokine/Chemokine</b>						
CD116 (GM-CSFRα)	100±0	100±0	100±0	186±5	291±9*	284±9*
CD119 (IFN-γRα)	100±0	100±0	100±0	219±5	150±5*	161±6*
CD127 (IL-7Rα)	65±6	86±3*	90±2*	25±1	36±2*	38±2*
CD132 (IL-2Rγ)	63±3	94±1*	93±1*	34±1	71±3*	64±4*
CD184 (CXCR4)	62±5	21±5*	23±3*	26±4	18±2	21±2

The table comprises all the CD antigens that showed statistically significant variations of expression on monocytes in whole blood that was left unstimulated (Uns) or stimulated with 300 µg/ml of *M. vaccae* (*Mv*) or *M. obuense* (*Mob*) for 3 h. Data represent mean ± SEM of the percentage (%) and geometric mean fluorescence intensity (MFI) of receptor-positive monocytes of 15 donors. Statistically significant differences were determined by Friedman test followed by Dunn's multiple comparison post hoc test (\**p* < 0.05 versus Uns; \*\**p* < 0.05 versus *Mv*).

1 **Table 2.**

2 Expression levels of surface receptors on neutrophils in whole blood.

Receptor Category	Surface Receptor Expression on Neutrophils					
	% of Receptor-Positive Cells			MFI of Receptor-Positive Cells		
	Uns	<i>Mv</i>	<i>Mob</i>	Uns	<i>Mv</i>	<i>Mob</i>
<b>Adhesion</b>						
CD18 (β2 integrin)	100±0	100±0	100±0	144±7	383±25*	251±16**
CD54 (ICAM-1)	81±3	98±1*	92±3*	27±3	46±3	36±2
CD62L (L-selectin)	100±0	80±3*	94±1*	141±13	53±5*	105±9**
<b>Antigen Presentation/ Costimulation/Signaling</b>						
CD45 (LCA)	100±0	100±0	100±0	87±4	246±14*	144±8*
HLA-A,B,C (MHC I)	100±0	99±0	100±0	104±14	78±11*	100±14*
<b>Complement/Fc</b>						
CD11b (CR3)	100±0	100±0	100±0	237±15	1074±76*	563±46*
CD11c (CR4)	89±3	99±1*	95±3*	30±2	78±7*	51±5**
CD16 (FCγRIII)	100±0	99±0	99±0	1731±96	2368±137*	2008±148**
CD35 (CR1)	95±1	100±0*	99±1	27±2	114±9*	54±5**
CD64 (FcγRI)	16±4	24±5*	21±5*	17±2	17±2	18±2
CD89 (FcαR)	100±0	100±0	100±0	182±16	332±30*	215±17*
<b>Cytokine/Chemokine</b>						
CD119 (IFN-γRα)	100±0	100±0	100±0	80±3	61±2*	69±3**
CD184 (CXCR4)	82±2	57±3*	71±3**	30±2	27±2	29±1

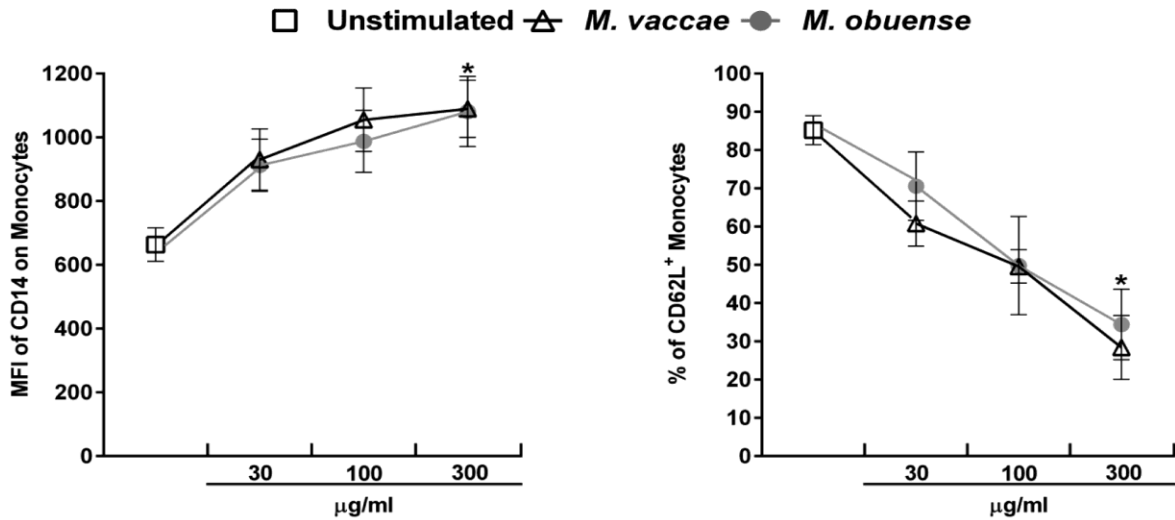
3 The table comprises all the CD antigens that showed statistically significant variations of  
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 6 percentage (%) and geometric mean fluorescence intensity (MFI) of receptor-positive  
 7 neutrophils of 15 donors. Statistically significant differences were determined by Friedman test  
 8 followed by Dunn's multiple comparison post hoc test (\**p* < 0.05 versus Uns; \**p* < 0.05 versus  
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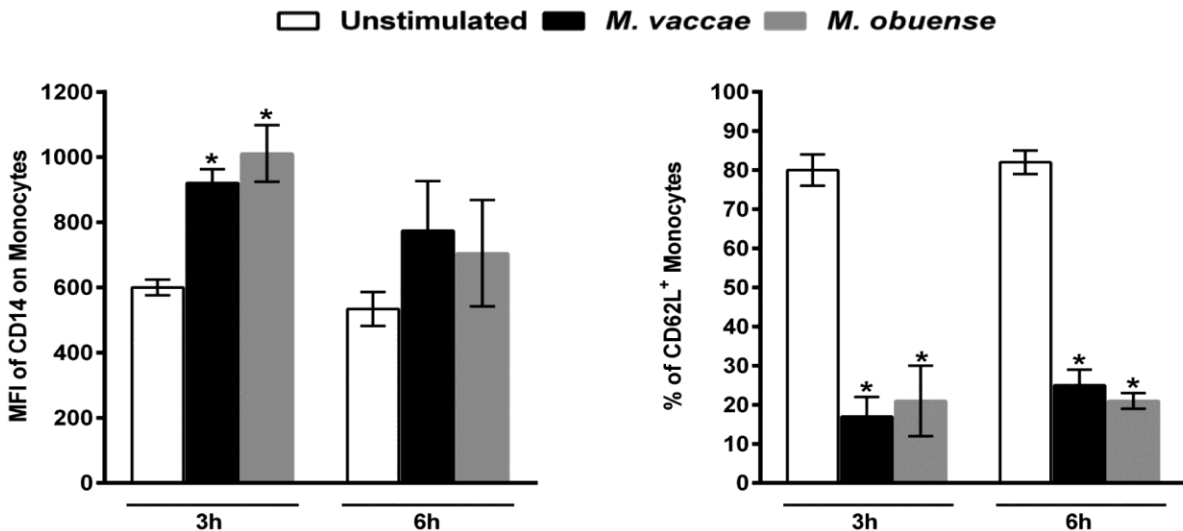
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### Supplementary data

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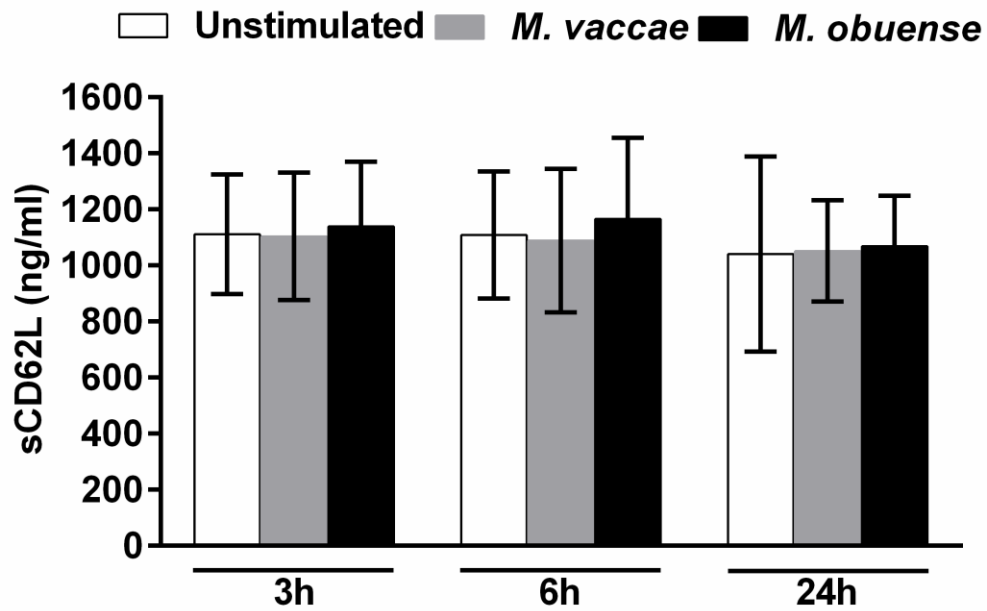
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**Supplementary Figure 1.** Optimization of whole blood stimulation conditions with *M. vaccae* or *M. obuense*. (A) Dose-response evaluation for the effect of whole blood stimulation with different concentrations of *M. vaccae* or *M. obuense* (30, 100 and 300 µg/ml) for 3 h on the expression levels of CD14 and CD62L presented as geometric mean fluorescence intensity (MFI) and percentage of positive monocytes, respectively. Symbols and error bars indicate mean values and SEM (n=5). (B) Time-response evaluation for the effect of whole blood stimulation with 300 µg/ml of *M. vaccae* or *M. obuense* for 3 or 6 h on the expression levels of CD14 and CD62L presented as geometric MFI and percentage of positive monocytes, respectively. Columns and error bars indicate mean values and SEM (n=3). \* $p < 0.05$  versus unstimulated.





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2 **Supplementary Figure 2.** Soluble (s) CD62L levels in supernatants of whole blood cultures.  
3 sCD62L levels in supernatants of whole blood cultures that were left unstimulated or stimulated  
4 with 300 µg/ml of *M. vaccae* or *M. obuense* for 3, 6, or 24 h. Columns indicate the mean  
5 sCD62L levels of 4 donors. Error bars represent SEM.  
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1 **Supplementary Table 1.**

2 Surface receptors with unchanged expression levels on monocytes in whole blood.

Receptor Category	Surface Receptor Expression on Monocytes					
	% of Receptor-Positive Cells			MFI of Receptor-Positive Cells		
	Uns	<i>Mv</i>	<i>Mob</i>	Uns	<i>Mv</i>	<i>Mob</i>
<b>Adhesion/Activation</b>						
CD31 (PECAM-1)	100±0	100±0	100±0	895±10	972±10	1041±40
CD50 (ICAM-3)	100±0	100±0	100±0	245±10	250±10	244±10
CD102 (ICAM-2)	99±0	99±0	99±0	144±5	148±6	157±8
<b>Antigen Presentation/ Costimulation/Signaling</b>						
CD5 (T1/Leu-1)	0±0	0±0	0±0	0±0	0±0	0±0
HLA-ABC (MHC I)	100±0	100±0	100±0	655±101	606±113	691±118
<b>Complement/FcR</b>						
CD21 (CR2)	0±0	0±0	0±0	0±0	0±0	0±0
CD32 (FcγRII)	82±3	79±5	76±5	24±3	27±5	24±4
<b>Cytokine/Chemokine</b>						
CD25 (IL-2Rα)	0±0	0±0	0±0	0±0	0±0	0±0
CD40 (TNFRS5)	11±4	10±3	15±4	18±2	21±2	20±2
CD95 (TNFRSF6)	97±1	98±1	98±1	21±1	23±1	23±1
<sup>a</sup> CD114 (G-CSFR)	100±0	100±0	100±0	62±3	57±4	63±4
CD122 (IL-2Rβ)	0±0	0±0	0±0	0±0	0±0	0±0
CD195 (CCR5)	17±1	20±2	23±2	21±1	20±1	21±1
CD197 (CCR7)	0±0	0±0	0±0	0±0	0±0	0±0

3 The table comprises all the receptors which did not show any significant variation of expression  
 4 on monocytes in whole blood that was left unstimulated (Uns) or stimulated with 300 µg/ml of *M.*  
 5 *vaccae* (*Mv*) or *M. obuense* (*Mob*) for 3 h. Data represent mean ± SEM of the percentage (%)  
 6 and geometric mean fluorescence intensity (MFI) of receptor-positive monocytes of 15 donors.  
 7 <sup>a</sup>Experiments conducted on 9 donors.

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**Supplementary Table 2.**

Surface receptors with unchanged expression levels on neutrophils in whole blood.

Receptor Category	Surface Receptor Expression on Neutrophils					
	% of Receptor-Positive Cells			MFI of Receptor-Positive Cells		
	Uns	<i>Mv</i>	<i>Mob</i>	Uns	<i>Mv</i>	<i>Mob</i>
<b>Adhesion/Activation</b>						
CD2 (LFA-2)	0±0	0±0	0±0	0±0	0±0	0±0
CD11a (LFA-1)	100±0	100±0	100±0	38±2	43±2	44±2
CD31 (PECAM-1)	100±0	100±0	100±0	384±26	401±21	423±21
CD44 (ECMRIII)	64±6	62±6	66±6	16±1	16±1	17±2
CD50 (ICAM-3)	100±0	100±0	100±0	331±11	245±11	306±11
CD58 (LFA-3)	100±0	100±0	100±0	72±4	72±4	76±7
CD102 (ICAM-2)	0±0	0±0	0±0	0±0	0±0	0±0
<b>Antigen Presentation/ Costimulation/Signaling</b>						
CD5 (T1/Leu-1)	0±0	0±0	0±0	0±0	0±0	0±0
CD80 (B7-1)	0±0	0±0	0±0	0±0	0±0	0±0
CD86 (B7-2)	0±0	0±0	0±0	0±0	0±0	0±0
<sup>a</sup> CD137L (4-1BBL)	0±0	0±0	0±0	0±0	0±0	0±0
HLA-DPDR (MHC II)	0±0	0±0	0±0	0±0	0±0	0±0
<b>Pattern Recognition</b>						
CD36 (GPIIb)	0±0	0±0	0±0	0±0	0±0	0±0
CD206 (MMR)	0±0	0±0	0±0	0±0	0±0	0±0
<b>Complement/FcR</b>						
CD21 (CR2)	0±0	0±0	0±0	0±0	0±0	0±0
CD32 (FcγRII)	100±0	100±0	100±0	50±3	46±4	52±4
<b>Cytokine/Chemokine</b>						
CD25 (IL-2Rα)	0±0	0±0	0±0	0±0	0±0	0±0
CD40 (TNFRS5)	0±0	0±0	0±0	0±0	0±0	0±0
CD95 (TNFRSF6)	95±2	94±2	95±1	20±1	19±1	20±1
CD116 (GM-CSFRα)	98±1	92±2	97±1	32±1	34±1	36±1
CD122 (IL-2Rβ)	0±0	0±0	0±0	0±0	0±0	0±0
CD127 (IL-7Rα)	0±0	0±0	0±0	0±0	0±0	0±0
CD132 (IL-2Rγ)	58±4	51±5	60±4	18±1	20±1	21±1
CD195 (CCR5)	0±0	0±0	0±0	0±0	0±0	0±0
CD197 (CCR7)	0±0	0±0	0±0	0±0	0±0	0±0

The table comprises all the receptors which did not show any significant variation of expression on neutrophils in whole blood that was left unstimulated (Uns) or stimulated with 300 µg/ml of *M. vaccae* (*Mv*) or *M. obuense* (*Mob*) for 3 h. Data represent mean ± SEM of the percentage (%) and geometric mean fluorescence intensity (MFI) of receptor-positive neutrophils of 15 donors.

<sup>a</sup>Experiments conducted on 9 donors.