

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

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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⁺ 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 10⁹ 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% CO₂ 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⁺/ 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 2×10^5 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% CO₂ 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 μ 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⁺ 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⁺ (TLR-4⁺) 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 ± 4 ; *M.*
6 *vaccae*: 41 ± 1 ; *M. obuense*: 55 ± 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- α 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 α 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- α , IFN- γ , 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 μ 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 μ 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 ± 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- α 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- α upon activation of monocytes (Damsgaard, et
25 al. 2009), the ability of *M. vaccae* to induce IL-10 and TNF- α production in cultures of isolated
26 monocytes (Baran, et al. 2004), and increased secretion of IL-10 and TNF- α 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 α . In contrast,
29 whole blood stimulation with HK mycobacteria did not induce the release of any of the signature
30 T cell cytokines, IFN- γ , 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- α , MCP-1, MIP-1 β (Silva, et al. 2013) and IFN- γ (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 β , 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- γ 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- γ 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- γ 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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4 **References**

- 5 Abbot, N.C., Beck, J.S., Feval, F., Weiss, F., Mobayen, M.H., Ghazi-Saidi, K., et al., 2002.
6 Immunotherapy with *Mycobacterium vaccae* and peripheral blood flow in long-treated leprosy
7 patients, a randomised, placebo-controlled trial. *Eur. J. Vasc. Endovasc. Surg.* 24 202-208.
- 8 Adams, V.C., Hunt, J.R., Martinelli, R., Palmer, R., Rook, G.A., Brunet, L.R., 2004.
9 *Mycobacterium vaccae* induces a population of pulmonary CD11c+ cells with regulatory
10 potential in allergic mice. *Eur. J. Immunol.* 34 631-638.
- 11 Antas, P.R., Cardoso, F.L., Oliveira, E.B., Gomes, P.K., Cunha, K.S., Sarno, E.N., et al., 2004.
12 Whole blood assay to access T cell-immune responses to *Mycobacterium tuberculosis* antigens
13 in healthy Brazilian individuals. *Mem. Inst. Oswaldo Cruz* 99 53-55.
- 14 Arkwright, P.D., David, T.J., 2001. Intradermal administration of a killed *Mycobacterium vaccae*
15 suspension (SRL 172) is associated with improvement in atopic dermatitis in children with
16 moderate-to-severe disease. *J. Allergy Clin. Immunol.* 107 531-534.
- 17 Asea, A., Kraeft, S.K., Kurt-Jones, E.A., Stevenson, M.A., Chen, L.B., Finberg, R.W., et al.,
18 2000. HSP70 stimulates cytokine production through a CD14-dependant pathway,
19 demonstrating its dual role as a chaperone and cytokine. *Nat. Med.* 6 435-442.
- 20 Bahr, G.M., De La Tribonniere, X., Darcissac, E., Ajana, F., Bocket, L., Sissoko, D., et al., 2003.
21 Clinical and immunological effects of a 6 week immunotherapy cycle with murabutide in HIV-1
22 patients with unsuccessful long-term antiretroviral treatment. *J. Antimicrob. Chemother.* 51
23 1377-1388.
- 24 Bahr, G.M., Stanford, J.L., Rook, G.A., Rees, R.J., Frayha, G.J., Abdelnoor, A.M., 1986. Skin
25 sensitization to mycobacteria amongst school children prior to a study of BCG vaccination in
26 North Lebanon. *Tubercle* 67 197-203.
- 27 Baran, J., Baj-Krzyworzeka, M., Weglarczyk, K., Ruggiero, I., Zembala, M., 2004. Modulation of
28 monocyte-tumour cell interactions by *Mycobacterium vaccae*. *Cancer Immunol. Immunother.* 53
29 1127-1134.
- 30 Barclay, A.N., Brown, M.H., Law, S.K.A., McKnight, A.I., Tomhnson, M.G., van der Merwe, P.,
31 1997. *The leucocyte antigen facts book*, 2nd ed. Academic Press, San Diego.
- 32 Behr, M.A., Divangahi, M., 2015. Freund's adjuvant, NOD2 and mycobacteria. *Curr. Opin.*
33 *Microbiol.* 23 126-132.

- 1 Black, G.F., Dockrell, H.M., Crampin, A.C., Floyd, S., Weir, R.E., Bliss, L., et al., 2001. Patterns
2 and implications of naturally acquired immune responses to environmental and tuberculous
3 mycobacterial antigens in northern Malawi. *J. Infect. Dis.* 184 322-329.
- 4 Camporota, L., Corkhill, A., Long, H., Lordan, J., Stanciu, L., Tuckwell, N., et al., 2003. The
5 effects of *Mycobacterium vaccae* on allergen-induced airway responses in atopic asthma. *Eur.*
6 *Respir. J.* 21 287-293.
- 7 Cananzi, F.C., Mudan, S., Dunne, M., Belonwu, N., Dalgleish, A.G., 2013. Long-term survival
8 and outcome of patients originally given *Mycobacterium vaccae* for metastatic malignant
9 melanoma. *Hum. Vaccin Immunother.* 9 2427-2433.
- 10 Capece, D., Verzella, D., Fischietti, M., Zazzeroni, F., Alesse, E., 2012. Targeting costimulatory
11 molecules to improve antitumor immunity. *J. Biomed. Biotechnol.* 2012 926321.
- 12 Dalgleish, A.G. and The IMAGE I Trial Investigators, 2015. A multicenter randomized, open-
13 label, proof-of-concept, phase II trial comparing gemcitabine with and without IMM-101 in
14 advanced pancreatic cancer, Anonymous 2015, pp. 336.
- 15 Damsgaard, C.T., Lauritzen, L., Calder, P.C., Kjaer, T.M., Frokiaer, H., 2009. Whole-blood
16 culture is a valid low-cost method to measure monocytic cytokines - a comparison of cytokine
17 production in cultures of human whole-blood, mononuclear cells and monocytes. *J. Immunol.*
18 *Methods* 340 95-101.
- 19 Darcissac, E.C., Bahr, G.M., Parant, M.A., Chedid, L.A., Riveau, G.J., 1996a. Selective
20 induction of CD11a,b,c/CD18 and CD54 expression at the cell surface of human leukocytes by
21 muramyl peptides. *Cell. Immunol.* 169 294-301.
- 22 Darcissac, E.C., Bahr, G.M., Pouillart, P.R., Riveau, G.J., Parant, M.A., 1996b. Selective
23 potentiation of cytokine expression in human whole blood by murabutide, a muramyl dipeptide
24 analogue. *Cytokine* 8 658-666.
- 25 Deenadayalan, A., Maddineni, P., Raja, A., 2013. Comparison of whole blood and PBMC
26 assays for T-cell functional analysis. *BMC Res. Notes* 6 120-0500-6-120.
- 27 Dlugovitzky, D., Fiorenza, G., Farroni, M., Bogue, C., Stanford, C., Stanford, J., 2006.
28 Immunological consequences of three doses of heat-killed *Mycobacterium vaccae* in the
29 immunotherapy of tuberculosis. *Respir. Med.* 100 1079-1087.
- 30 Driessens, G., Kline, J., Gajewski, T.F., 2009. Costimulatory and coinhibitory receptors in anti-
31 tumor immunity. *Immunol. Rev.* 229 126-144.
- 32 Duffy, D., Rouilly, V., Libri, V., Hasan, M., Beitz, B., David, M., et al., 2014. Functional analysis
33 via standardized whole-blood stimulation systems defines the boundaries of a healthy immune
34 response to complex stimuli. *Immunity* 40 436-450.
- 35 Eaton, J.D., Perry, M.J., Nicholson, S., Guckian, M., Russell, N., Whelan, M., et al., 2002.
36 Allogeneic whole-cell vaccine: a phase I/II study in men with hormone-refractory prostate
37 cancer. *BJU Int.* 89 19-26.

- 1 Elia, A., Lincoln, L., Brunet, L., Hagemann, T., 2013. Treatment with IMM-101 induces
2 protective CD8+ T cell responses in clinically relevant models of pancreatic cancer. *Journal for*
3 *ImmunoTherapy of Cancer* 1 P215.
- 4 Flo, T.H., Halaas, O., Torp, S., Ryan, L., Lien, E., Dybdahl, B., et al., 2001. Differential
5 expression of Toll-like receptor 2 in human cells. *J. Leukoc. Biol.* 69 474-481.
- 6 Fowler, D.W., Copier, J., Wilson, N., Dalgleish, A.G., Bodman-Smith, M.D., 2012. Mycobacteria
7 activate gammadelta T-cell anti-tumour responses via cytokines from type 1 myeloid dendritic
8 cells: a mechanism of action for cancer immunotherapy. *Cancer Immunol. Immunother.* 61 535-
9 547.
- 10 Gaglia, J.L., Greenfield, E.A., Mattoo, A., Sharpe, A.H., Freeman, G.J., Kuchroo, V.K., 2000.
11 Intercellular adhesion molecule 1 is critical for activation of CD28-deficient T cells. *J. Immunol.*
12 165 6091-6098.
- 13 Gramaglia, I., Cooper, D., Miner, K.T., Kwon, B.S., Croft, M., 2000. Co-stimulation of antigen-
14 specific CD4 T cells by 4-1BB ligand. *Eur. J. Immunol.* 30 392-402.
- 15 Hermann, C., von Aulock, S., Graf, K., Hartung, T., 2003. A model of human whole blood
16 lymphokine release for in vitro and ex vivo use. *J. Immunol. Methods* 275 69-79.
- 17 Hogarth, P.M., Pietersz, G.A., 2012. Fc receptor-targeted therapies for the treatment of
18 inflammation, cancer and beyond. *Nat. Rev. Drug Discov.* 11 311-331.
- 19 Ivetic, A., 2013. Signals regulating L-selectin-dependent leucocyte adhesion and transmigration.
20 *Int. J. Biochem. Cell Biol.* 45 550-555.
- 21 Janke, M., Poth, J., Wimmenauer, V., Giese, T., Coch, C., Barchet, W., et al., 2009. Selective
22 and direct activation of human neutrophils but not eosinophils by Toll-like receptor 8. *J. Allergy*
23 *Clin. Immunol.* 123 1026-1033.
- 24 Johnson, J.L., Kanya, R.M., Okwera, A., Loughlin, A.M., Nyole, S., Hom, D.L., et al., 2000.
25 Randomized controlled trial of Mycobacterium vaccae immunotherapy in non-human
26 immunodeficiency virus-infected ugandan adults with newly diagnosed pulmonary tuberculosis.
27 The Uganda-Case Western Reserve University Research Collaboration. *J. Infect. Dis.* 181
28 1304-1312.
- 29 Kartalija, M., Ovrutsky, A.R., Bryan, C.L., Pott, G.B., Fantuzzi, G., Thomas, J., et al., 2013.
30 Patients with nontuberculous mycobacterial lung disease exhibit unique body and immune
31 phenotypes. *Am. J. Respir. Crit. Care Med.* 187 197-205.
- 32 Kassa, D., Ran, L., Geberemeskel, W., Tebeje, M., Alemu, A., Selase, A., et al., 2012. Analysis
33 of immune responses against a wide range of Mycobacterium tuberculosis antigens in patients
34 with active pulmonary tuberculosis. *Clin. Vaccine Immunol.* 19 1907-1915.
- 35 Kato, M., Neil, T.K., Fearnley, D.B., McLellan, A.D., Vuckovic, S., Hart, D.N., 2000. Expression
36 of multilectin receptors and comparative FITC-dextran uptake by human dendritic cells. *Int.*
37 *Immunol.* 12 1511-1519.

- 1 Kawai, K., Miyazaki, J., Joraku, A., Nishiyama, H., Akaza, H., 2013. Bacillus Calmette-Guerin
2 (BCG) immunotherapy for bladder cancer: current understanding and perspectives on
3 engineered BCG vaccine. *Cancer. Sci.* 104 22-27.
- 4 Killick, K.E., Ni Cheallaigh, C., O'Farrelly, C., Hokamp, K., MacHugh, D.E., Harris, J., 2013.
5 Receptor-mediated recognition of mycobacterial pathogens. *Cell. Microbiol.* 15 1484-1495.
- 6 Kleinnijenhuis, J., Oosting, M., Joosten, L.A., Netea, M.G., Van Crevel, R., 2011. Innate immune
7 recognition of *Mycobacterium tuberculosis*. *Clin. Dev. Immunol.* 2011 405310.
- 8 Lagrelius, M., Jones, P., Franck, K., Gaines, H., 2006. Cytokine detection by multiplex
9 technology useful for assessing antigen specific cytokine profiles and kinetics in whole blood
10 cultured up to seven days. *Cytokine* 33 156-165.
- 11 Le Bert, N., Chain, B.M., Rook, G., Noursadeghi, M., 2011. DC priming by *M. vaccae* inhibits
12 Th2 responses in contrast to specific TLR2 priming and is associated with selective activation of
13 the CREB pathway. *PLoS One* 6 e18346.
- 14 Lehrer, A., Bressanelli, A., Wachsmann, V., Bottasso, O., Bay, M.L., Singh, M., et al., 1998.
15 Immunotherapy with *Mycobacterium vaccae* in the treatment of psoriasis. *FEMS Immunol. Med.*
16 *Microbiol.* 21 71-77.
- 17 Lopez, R.D., Waller, E.K., Lu, P.H., Negrin, R.S., 2001. CD58/LFA-3 and IL-12 provided by
18 activated monocytes are critical in the in vitro expansion of CD56+ T cells. *Cancer Immunol.*
19 *Immunother.* 49 629-640.
- 20 Martinelli, R., Adams, V.C., Rook, G.A.W., Brunet, L.R., 2004. Effects of *Mycobacterium vaccae*
21 on toll-like receptor expression. *J. Allergy Clin. Immunol.* 113 S187.
- 22 Moreira, J., Aragao-Filho, W.C., Barillas, S.G., Barbosa, S.M., Pedroza, L.A., Condino-Neto, A.,
23 2012. Human leucocytes response to viable, extended freeze-drying or heat-killed
24 *Mycobacterium bovis* bacillus Calmette-Guerin. *Scand. J. Immunol.* 75 96-101.
- 25 Myers, N.D., Chantratita, N., Berrington, W.R., Chierakul, W., Limmathurotsakul, D.,
26 Wuthiekanun, V., et al., 2014. The role of NOD2 in murine and human melioidosis. *J. Immunol.*
27 192 300-307.
- 28 Nijhuis, M.M., Pasterkamp, G., Sluis, N.I., de Kleijn, D.P., Laman, J.D., Ulfman, L.H., 2007.
29 Peptidoglycan increases firm adhesion of monocytes under flow conditions and primes
30 monocyte chemotaxis. *J. Vasc. Res.* 44 214-222.
- 31 Nimmerjahn, F., Ravetch, J.V., 2008. Fcγ receptors as regulators of immune responses.
32 *Nat. Rev. Immunol.* 8 34-47.
- 33 Noorman, F., Braat, E.A., Barrett-Bergshoeff, M., Barbe, E., van Leeuwen, A., Lindeman, J., et
34 al., 1997. Monoclonal antibodies against the human mannose receptor as a specific marker in
35 flow cytometry and immunohistochemistry for macrophages. *J. Leukoc. Biol.* 61 63-72.

- 1 O'Brien, M.E., Anderson, H., Kaukel, E., O'Byrne, K., Pawlicki, M., Von Pawel, J., et al., 2004.
2 SRL172 (killed *Mycobacterium vaccae*) in addition to standard chemotherapy improves quality
3 of life without affecting survival, in patients with advanced non-small-cell lung cancer: phase III
4 results. *Ann. Oncol.* 15 906-914.
- 5 Oftung, F., Borka, E., Kvalheim, G., Mustafa, A.S., 1998. Mycobacterial crossreactivity of M.
6 tuberculosis reactive T cell clones from naturally converted PPD positive healthy subjects.
7 *FEMS Immunol. Med. Microbiol.* 20 231-238.
- 8 Patel, P.M., Sim, S., O'Donnell, D.O., Protheroe, A., Beirne, D., Stanley, A., et al., 2008. An
9 evaluation of a preparation of *Mycobacterium vaccae* (SRL172) as an immunotherapeutic agent
10 in renal cancer. *Eur. J. Cancer* 44 216-223.
- 11 Popa, C., Barrera, P., Joosten, L.A., van Riel, P.L., Kullberg, B.J., van der Meer, J.W., et al.,
12 2009. Cytokine production from stimulated whole blood cultures in rheumatoid arthritis patients
13 treated with various TNF blocking agents. *Eur. Cytokine Netw.* 20 88-93.
- 14 Reichenbach, J., Schubert, R., Feinberg, J., Beck, O., Rosewich, M., Rose, M.A., et al., 2006.
15 Impaired interferon-gamma production in response to live bacteria and Toll-like receptor
16 agonists in patients with ataxia telangiectasia. *Clin. Exp. Immunol.* 146 381-389.
- 17 Romano, M., Huygen, K., 2012. An update on vaccines for tuberculosis - there is more to it than
18 just waning of BCG efficacy with time. *Expert Opin. Biol. Ther.* 12 1601-1610.
- 19 Rook, G.A., Adams, V., Hunt, J., Palmer, R., Martinelli, R., Brunet, L.R., 2004. Mycobacteria
20 and other environmental organisms as immunomodulators for immunoregulatory disorders.
21 *Springer Semin. Immunopathol.* 25 237-255.
- 22 Santucci, M.B., Amicosante, M., Cicconi, R., Montesano, C., Casarini, M., Giosue, S., et al.,
23 2000. Mycobacterium tuberculosis-induced apoptosis in monocytes/macrophages: early
24 membrane modifications and intracellular mycobacterial viability. *J. Infect. Dis.* 181 1506-1509.
- 25 Schleiffenbaum, B., Spertini, O., Tedder, T.F., 1992. Soluble L-selectin is present in human
26 plasma at high levels and retains functional activity. *J. Cell Biol.* 119 229-238.
- 27 Sieling, P.A., Torrelles, J.B., Stenger, S., Chung, W., Burdick, A.E., Rea, T.H., et al., 2005. The
28 human CD1-restricted T cell repertoire is limited to cross-reactive antigens: implications for host
29 responses against immunologically related pathogens. *J. Immunol.* 174 2637-2644.
- 30 Silva, D., Ponte, C.G., Hacker, M.A., Antas, P.R., 2013. A whole blood assay as a simple, broad
31 assessment of cytokines and chemokines to evaluate human immune responses to
32 *Mycobacterium tuberculosis* antigens. *Acta Trop.* 127 75-81.
- 33 Skinner, M.A., Yuan, S., Prestidge, R., Chuk, D., Watson, J.D., Tan, P.L., 1997. Immunization
34 with heat-killed *Mycobacterium vaccae* stimulates CD8+ cytotoxic T cells specific for
35 macrophages infected with *Mycobacterium tuberculosis*. *Infect. Immun.* 65 4525-4530.
- 36 Smalley, D.M., Ley, K., 2005. L-selectin: mechanisms and physiological significance of
37 ectodomain cleavage. *J. Cell. Mol. Med.* 9 255-266.

- 1 Stanford, J., Stanford, C., Stansby, G., Bottasso, O., Bahr, G., Grange, J., 2009. The common
2 mycobacterial antigens and their importance in the treatment of disease. *Curr. Pharm. Des.* 15
3 1248-1260.
- 4 Stanford, J.L., Stanford, C.A., O'Brien, M.E., Grange, J.M., 2008. Successful immunotherapy
5 with *Mycobacterium vaccae* in the treatment of adenocarcinoma of the lung. *Eur. J. Cancer* 44
6 224-227.
- 7 Stebbing, J., Dalgleish, A., Gifford-Moore, A., Martin, A., Gleeson, C., Wilson, G., et al., 2012.
8 An intra-patient placebo-controlled phase I trial to evaluate the safety and tolerability of
9 intradermal IMM-101 in melanoma. *Ann. Oncol.* 23 1314-1319.
- 10 Thibodeau, J., Bourgeois-Daigneault, M.C., Lapointe, R., 2012. Targeting the MHC Class II
11 antigen presentation pathway in cancer immunotherapy. *Oncoimmunology* 1 908-916.
- 12 Truoc, L.V., Ly, H.M., Thuy, N.K., Trach, D.D., Stanford, C.A., Stanford, J.L., 2001. Vaccination
13 against leprosy at Ben San Leprosy Centre, Ho Chi Minh City, Vietnam. *Vaccine* 19 3451-3458.
- 14 Tsuyuguchi, I., Kawasumi, H., Takashima, T., Tsuyuguchi, T., Kishimoto, S., 1990.
15 *Mycobacterium avium*-*Mycobacterium* intracellular complex-induced suppression of T-cell
16 proliferation in vitro by regulation of monocyte accessory cell activity. *Infect. Immun.* 58 1369-
17 1378.
- 18 van Egmond, M., Bakema, J.E., 2013. Neutrophils as effector cells for antibody-based
19 immunotherapy of cancer. *Semin. Cancer Biol.* 23 190-199.
- 20 von Reyn, C.F., Mtei, L., Arbeit, R.D., Waddell, R., Cole, B., Mackenzie, T., et al., 2010.
21 Prevention of tuberculosis in Bacille Calmette-Guerin-primed, HIV-infected adults boosted with
22 an inactivated whole-cell mycobacterial vaccine. *AIDS* 24 675-685.
- 23 Wang, Y., Kelly, C.G., Singh, M., McGowan, E.G., Carrara, A.S., Bergmeier, L.A., et al., 2002.
24 Stimulation of Th1-polarizing cytokines, C-C chemokines, maturation of dendritic cells, and
25 adjuvant function by the peptide binding fragment of heat shock protein 70. *J. Immunol.* 169
26 2422-2429.
- 27 Weir, R.E., Fine, P.E., Nazareth, B., Floyd, S., Black, G.F., King, E., et al., 2003. Interferon-
28 gamma and skin test responses of schoolchildren in southeast England to purified protein
29 derivatives from *Mycobacterium tuberculosis* and other species of mycobacteria. *Clin. Exp.*
30 *Immunol.* 134 285-294.
- 31 Wingren, A.G., Parra, E., Varga, M., Kalland, T., Sjogren, H.O., Hedlund, G., et al., 1995. T cell
32 activation pathways: B7, LFA-3, and ICAM-1 shape unique T cell profiles. *Crit. Rev. Immunol.*
33 15 235-253.
- 34 Wollenberg, A., Mommaas, M., Oppel, T., Schottdorf, E.M., Gunther, S., Moderer, M., 2002.
35 Expression and function of the mannose receptor CD206 on epidermal dendritic cells in
36 inflammatory skin diseases. *J. Invest. Dermatol.* 118 327-334.

1 Yang, X.Y., Chen, Q.F., Li, Y.P., Wu, S.M., 2011. Mycobacterium vaccae as adjuvant therapy to
 2 anti-tuberculosis chemotherapy in never-treated tuberculosis patients: a meta-analysis. PLoS
 3 One 6 e23826.

4 Zipfel, P.F., Skerka, C., 2009. Complement regulators and inhibitory proteins. Nat. Rev.
 5 Immunol. 9 729-740.

6 Zuany-Amorim, C., Sawicka, E., Manlius, C., Le Moine, A., Brunet, L.R., Kemeny, D.M., et al.,
 7 2002. Suppression of airway eosinophilia by killed Mycobacterium vaccae-induced allergen-
 8 specific regulatory T-cells. Nat. Med. 8 625-629.

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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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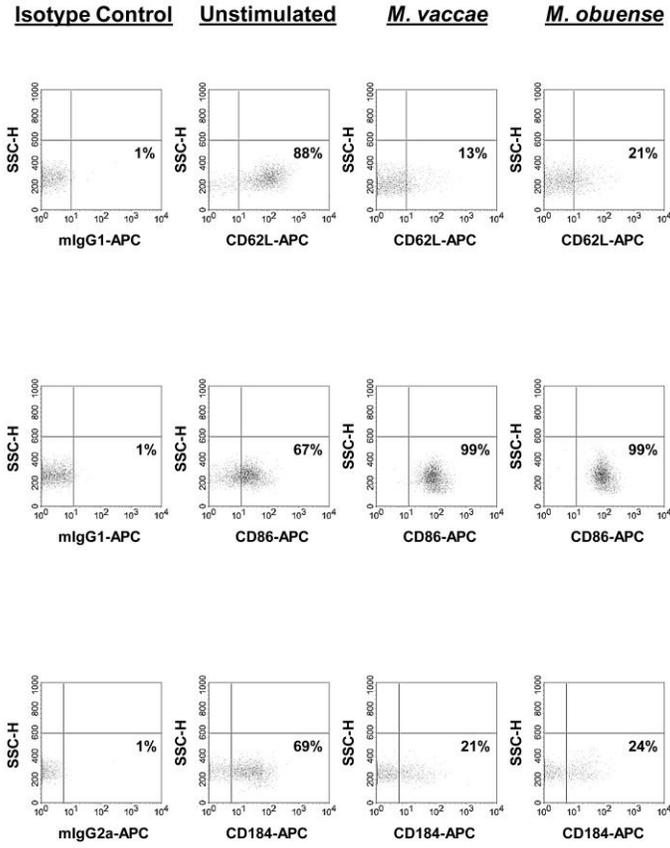
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Fig. 3. Cytokine and chemokine levels in supernatants of whole blood cultures following stimulation with *M. vaccae* or *M. obuense*. Scatter plots showing IL-6, IL-10, TNF- α , CXCL8/IL-8, MIP-1 α , and RANTES levels in supernatants of whole blood cultures that were left unstimulated or stimulated with 300 μ 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).

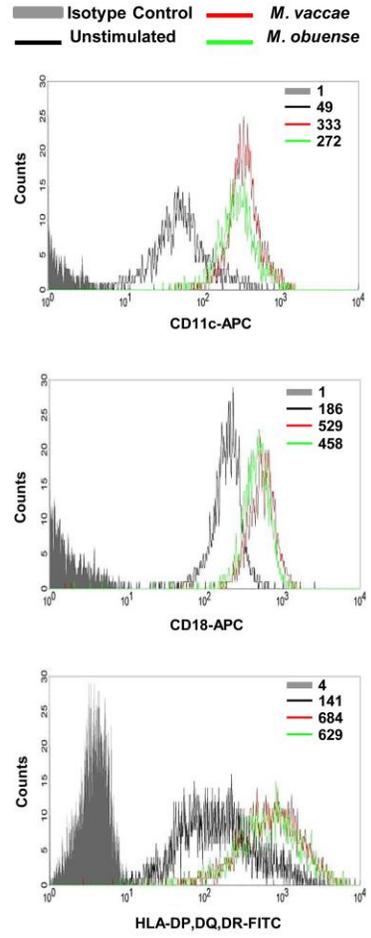
Fig. 4. Induction of peripheral blood mononuclear cells (PBMCs) proliferative responses to *M. vaccae* and *M. obuense*. PBMCs were stimulated with different concentrations (1, 3, 10, 30, 100 and 300 μ g/ml) of *M. vaccae* or *M. obuense* for 7 days. Data are reported as (A) symbols with error bars showings 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 μ g/ml of *M. vaccae*; $^{\circ}p < 0.05$ versus 3 μ 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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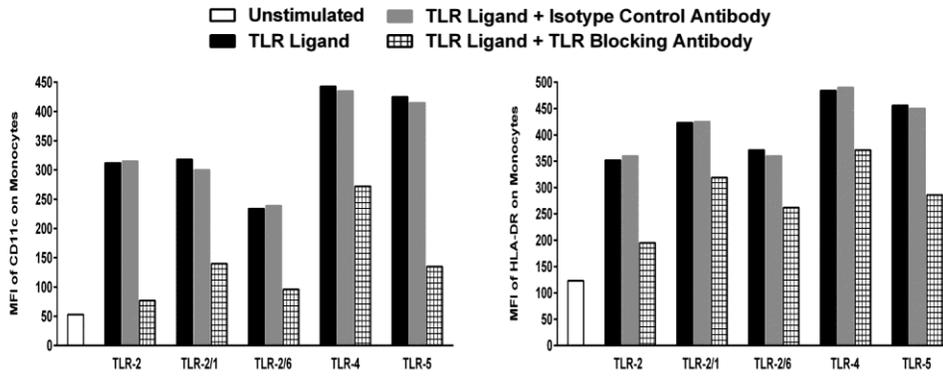
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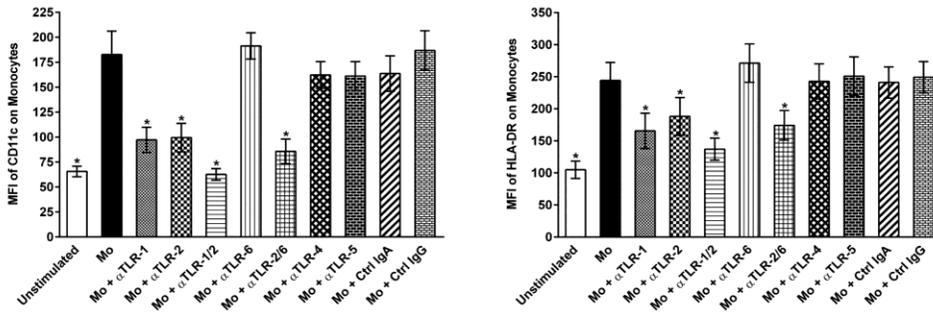
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1 **Fig. 2**
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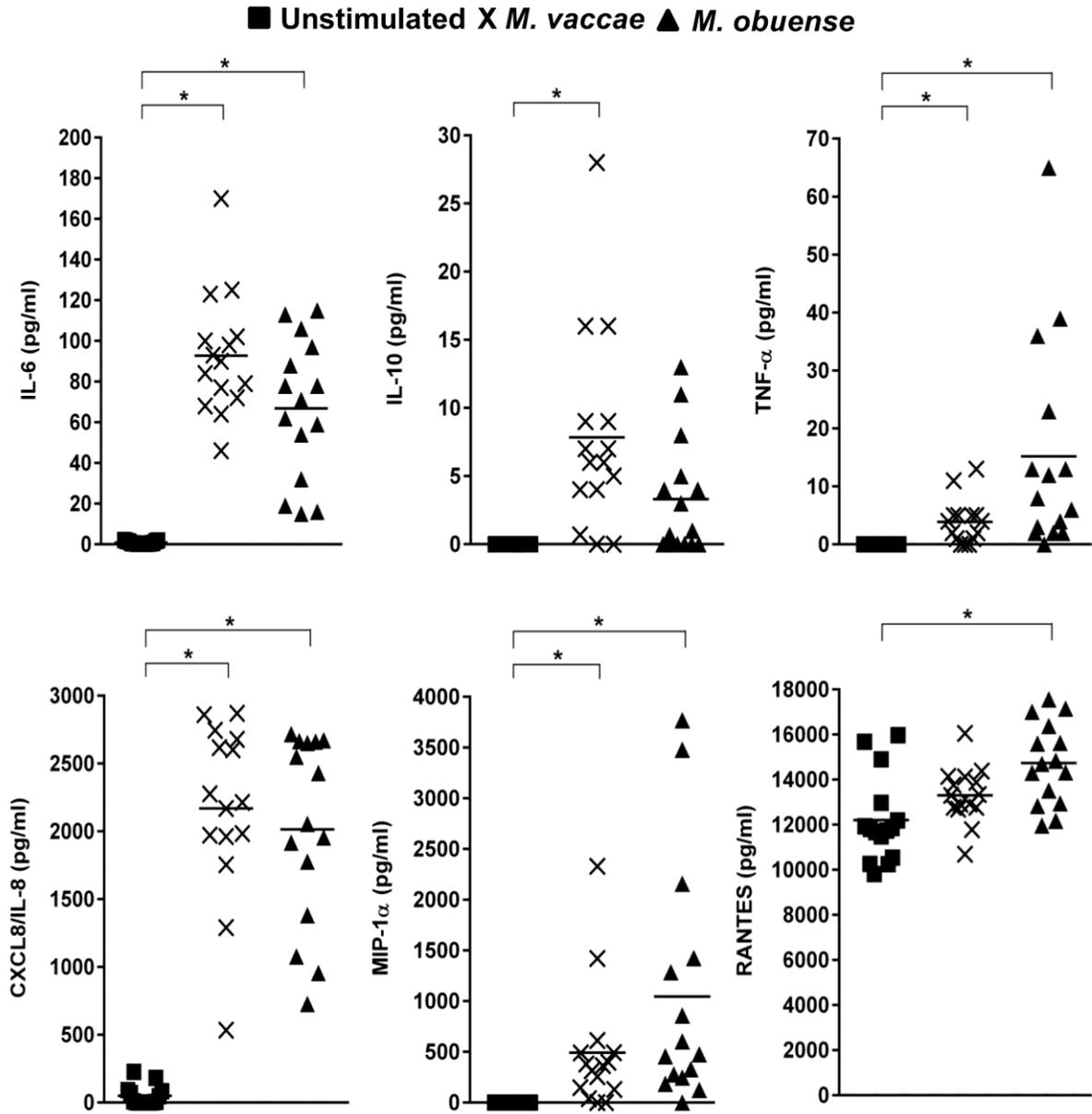


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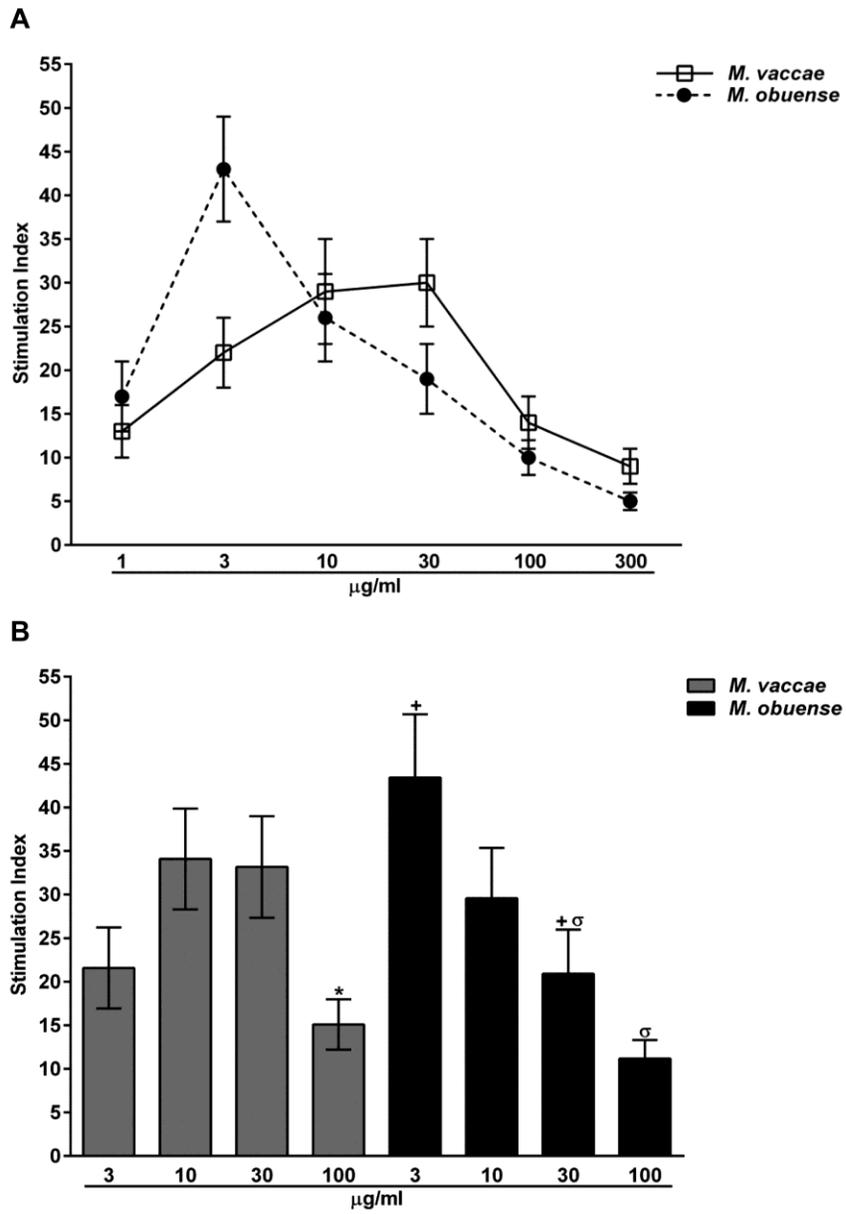
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1 **Fig. 3**
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1 **Fig. 4**
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Table 1.
Expression levels of surface receptors on monocytes in whole blood.

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>
Adhesion						
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*
Antigen Presentation/ Costimulation/Signaling						
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*
Pattern Recognition						
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*
Complement/FcR						
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*
Cytokine/Chemokine						
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>
Adhesion						
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**
Antigen Presentation/ Costimulation/Signaling						
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*
Complement/Fc						
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*
Cytokine/Chemokine						
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

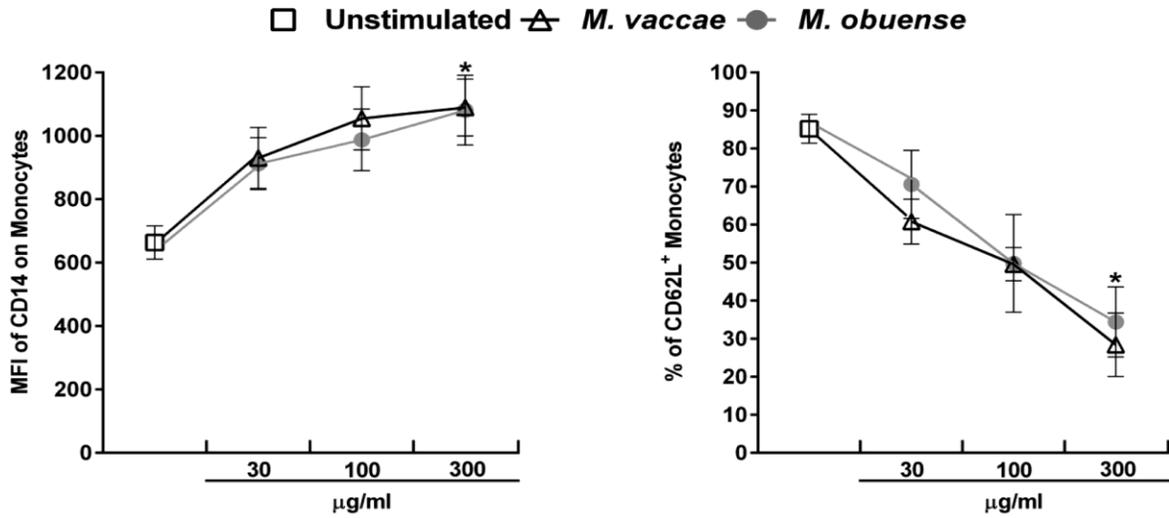
3 The table comprises all the CD antigens that showed statistically significant variations of
 4 expression on neutrophils in whole blood that was left unstimulated (Uns) or stimulated with
 5 300 µg/ml of *M. vaccae* (*Mv*) or *M. obuense* (*Mob*) for 3 h. Data represent mean ± SEM of the
 6 percentage (%) 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
 9 *Mv*).

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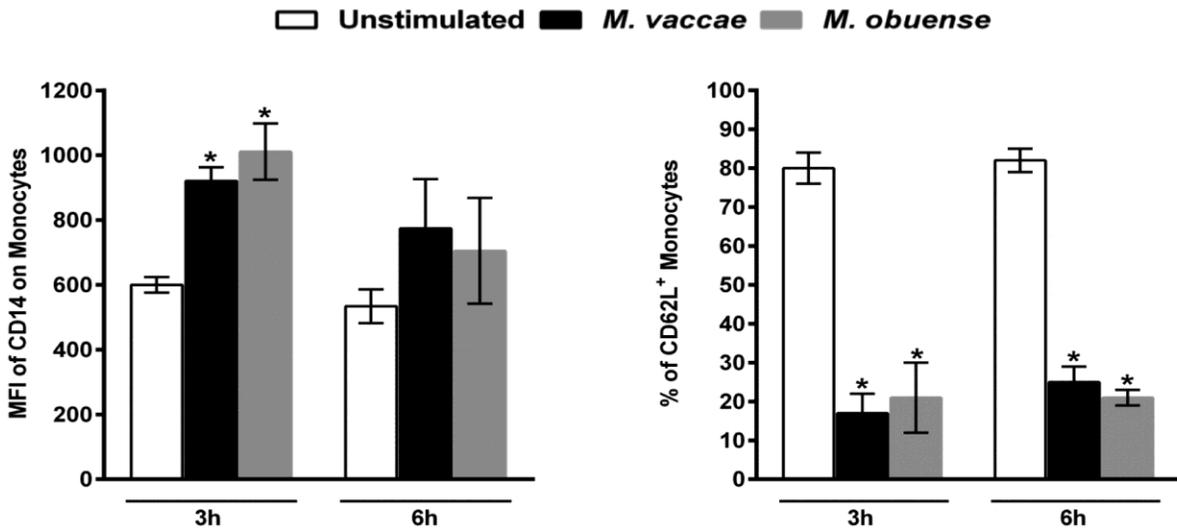
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Supplementary data

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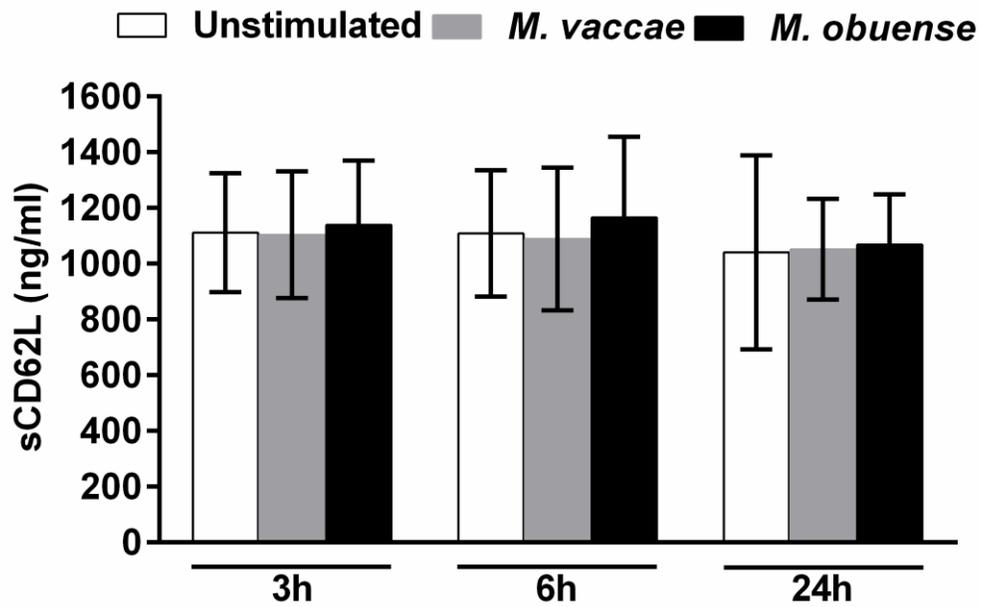


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Supplementary Figure 1. Optimization of whole blood stimulation conditions with *M. vaccae* or *M. obuense*. (A) Dose-response evaluation for the effect of whole blood stimulation with different concentrations of *M. vaccae* or *M. obuense* (30, 100 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>
Adhesion/Activation						
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
Antigen Presentation/ Costimulation/Signaling						
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
Complement/FcR						
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
Cytokine/Chemokine						
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
^a 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 ^aExperiments 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>
Adhesion/Activation						
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
Antigen Presentation/ Costimulation/Signaling						
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
^a 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
Pattern Recognition						
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
Complement/FcR						
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
Cytokine/Chemokine						
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.

^aExperiments conducted on 9 donors.