Original Article

Immunomodulatory effects of heat killed *Mycobacterium obuense* on human blood dendritic cells

Samer Bazzi1,2 , Helmout Modjtahedi1 , Satvinder Mudan3 , Marcel Achkar4 , Charles Akle5 , Georges M. Bahr6

1School of Life Sciences, Faculty of Science, Engineering and Computing, Kingston University, Kingston upon Thames, United Kingdom.

2Biology Department, Faculty of Sciences, University of Balamand, Al Kurah, Lebanon.

3St George's University of London, Imperial College, London and The Royal Marsden Hospital, London, United Kingdom.

4Clinical Laboratory Department, Nini Hospital, Tripoli, Lebanon

5Immodulon Therapeutics Ltd., 6-9 The Square, Stockley Park, Uxbridge, United Kingdom

6Faculty of Medicine and Medical Sciences, University of Balamand, Al Kurah, Lebanon.

Corresponding author:

Georges M. Bahr (PhD, FRCPath), Faculty of Medicine and Medical Sciences,

University of Balamand, PO BOX: 33, Amioun, Al-Koura, Lebanon. Email:

georges.bahr@balamand.edu.lb;

Tel: +9613370780; Fax: +9616931956.

Abstract

Heat-killed (HK) *Mycobacterium obuense* is a novel immunomodulator, currently undergoing clinical evaluation as an immunotherapeutic agent in the treatment of cancer. Here, we examined the effect of *in vitro* exposure to HK *M. obuense* on the expression of different categories of surface receptors on human blood myeloid (m) and plasmacytoid (p) DCs. Moreover, we have characterized the cytokine and chemokine secretion patterns of purified total blood DCs stimulated with HK *M. obuense*. HK *M. obuense* significantly up-regulated the expression of CD11c, CD80, CD83, CD86, CD274 and MHC class II on whole blood mDCs and CD80, CD123 and MHC class II on whole blood pDCs. Down-regulation of CD195 expression on both DC subpopulations was also noted. Further analysis showed that HK *M. obuense* up-regulated the expression of CD80, CD83 and MHC class II on purified blood DC subpopulations. TLR 2 and 1 were also identified to be engaged in mediating the HK *M. obuense*-induced upregulation of surface receptor expression on whole blood mDCs. In addition, our data demonstrated that HK *M. obuense* augmented the secretion of CCL4, CCL5, CCL22, CXCL8, IL-6, IL-12p40 and TNF-α by purified total blood DCs. Taken together, our data suggest that HK *M. obuense* exerts potent differential immunomodulatory effects on human DC subpopulations.

Keywords

Dendritic cells, *Mycobacterium obuense*, immunomodulator, CD molecules, toll-like receptors, cytokines, chemokines

Introduction

Dendritic cells (DCs) are crucial modulators of the immune response whereby they act as a bridge between the innate and adaptive immune systems.[1] DCs are potent antigen presenting cells (APCs) that are in continuous surveillance for antigens while being in an immature state. Upon exposure to an antigen (self/foreign) or to a pro-inflammatory stimulus, DCs undergo a maturation process and migrate towards secondary lymphoid organs where they interact with T cells, consequently inducing an antigen-specific immune response or initiating immune tolerance.[2,3] The hallmarks of DC maturation include upregulation of co-stimulatory (CD80, CD86), antigen presentation (MHC class I and II) and maturation (CD83) receptors in addition to the release of pro-inflammatory cytokines (IL-6, IL-12).[4,5] To date, DCs are broadly categorized into two phenotypically and functionally distinct subpopulations defined as the conventional or myeloid (m) and plasmacytoid (p) DCs. Both subpopulations exist in peripheral blood and they comprise < 1% of peripheral blood mononuclear cells (PBMCs).[6,7]

The vital requirement for an effective immunotherapeutic approach for the treatment of various diseases has resulted in the development of novel strategies which exploit the biological properties of DCs.[8,9] DCs are equipped with a specialized set of receptors known as pattern recognition receptors (PRRs) which identify specific microbe or damage associated molecular patterns (MAMPs or DAMPs).[10] Therefore, this unique and pivotal characteristic of DCs makes them a potential target for bacteria-based immunomodulatory agents which, upon binding to PRRs on DCs, trigger a chain of events that leads to transcriptional activation, alterations in DC's cytokine/chemokine release, and a variation in surface receptor expression, thus affecting the interaction between DCs and other immune cells.[11,12] Among the bacteria-based immunomodulators, heat killed (HK) *Mycobacterium vaccae* (*M. vaccae*) preparation has demonstrated immunotherapeutic and vaccine potentials when evaluated in different disease settings.[13-17] In recent years, there has been a growing interest in assessing additional HK mycobacterial preparations, as potential immunotherapeutic agents, particularly in cancer. HK *M. obuense* (NCTC13365) has been shown to be safe and well tolerated among stage III/IV melanoma patients.[18] A recent randomized phase II study has demonstrated that the use of HK *M. obuense* as an adjunctive immunotherapeutic agent

for treating advanced pancreatic cancer was well tolerated and led to a significant extension in the overall and progression-free survival of patients with metastatic disease.[19] Research into the mode of action of HK *M. vaccae* and *M. obuense* has demonstrated their abilities to directly affect the innate arm of the immune system. In a study in allergic mice, *M. vaccae* was reported to promote the development of $CD11c^+$ antigen presenting cells (APCs) demonstrating a possible regulatory role.[20] Evidence from *in vitro* studies on human blood cells clearly indicated that both HK *M. vaccae* and *M. obuense* are able to modulate the expression of various cell surface receptors on monocytes and neutrophils[21] and to stimulate cytokine release from type-1 mDCs thus leading to the enhancement of the anti-tumor activity of γδ T cells.[22] Moreover, priming of monocyte-derived (Mo)-DCs with *M. vaccae* was reported to correlate with a significant attenuation of T helper type 2 (Th2) responses.[23]

Despite the emerging clinical interest in HK *M. obuense*, little is known about the outcome of its interaction with human innate immune cells, particularly primary blood DCs. The purpose of our study was to analyze the effect of HK *M. obuense* stimulation on the expression of different categories of surface receptors on whole blood and purified blood DCs and to evaluate whether toll-like receptors (TLRs) are involved in the *M. obuense*-induced upregulation of surface receptor expression on whole blood mDCs. In addition, we examined the secretion of cytokines and chemokines by purified total blood DCs stimulated with HK *M. obuense*. Our results showed that HK *M. obuense* differentially regulates the expression of surface receptors related to antigen presentation, co-stimulation and maturation on blood DCs. Furthermore, our data revealed that TLR-2 and TLR-1 contribute to the *M. obuense*-induced upregulation of CD11c, CD80 and MHC class II expression on whole blood mDCs. Finally, HK *M. obuense* demonstrated an ability to induce cytokine and chemokine production by purified total blood DCs.

Materials and methods

Blood Sampling

Peripheral whole blood samples were drawn, at the clinical laboratory department, Nini, hospital, Lebanon, from healthy donors and collected into K2 ethylene diamine tetraacetic acid (K2-EDTA) vacutainer tubes (BD Biosciences). Written informed

consents were obtained from all blood donors and the study procedure gained approval from the institutional review board at the University of Balamand and from the research ethics committee at the Faculty of Science, Engineering and Computing at Kingston University.

TLR blocking antibodies and reagents

Anti-human monoclonal blocking antibodies against TLR-1 (clone H2G2), TLR-2 (clone B4H2), TLR-4 (clone W7C11), TLR-5 (clone Q2G4), TLR-6 (clone C5C8) and matching isotype control mouse IgG1 (clone T8E5) and human IgA2 (clone T9C6) antibodies were purchased from Invivogen (Toulouse, France). Sterile vials of HK *M. obuense* (NCTC13365) suspended in borate-buffered saline (BBS; pH 8.0) at 50 mg/mL were manufactured by BioElpida (Lyon, France), whereby each 1 mg wet-weight of bacilli is roughly equivalent to 10^9 HK organisms.

Immunophenotyping of DCs

Whole blood cultures (total culture volume = 2 mL) were either left unstimulated or were stimulated with 30, 100 or 300 μg/ml of HK *M. obuense* for 3 and 6 h at 37 °C in a 5% CO2 humidified atmosphere. For purified total blood DCs, viable cells were resuspended at 10^5 cells/ml in complete RPMI 1640 medium (Lonza) supplemented with 10% heat inactivated human AB serum and were stimulated with *M. obuense* for 6 h, as mentioned above. Whole blood cultures or purified blood DCs incubated with equivalent amounts of BBS served as unstimulated control. Both mDC and pDC subpopulations were analyzed in whole blood and as purified cells for the expression of a group of cell surface receptors. Whole blood (100 μl) was incubated for 25 min at room temperature (RT) in the dark with optimized concentrations of antigen-specific or matching isotype control monoclonal antibodies (Supplementary Table S1) and red blood cells were then lysed by the addition of FACS lysing solution (BD Biosciences). After 15 min of incubation at RT, cells were pelleted and washed once with Cell Wash solution (BD Biosciences) and finally resuspended in Cell Fix solution (BD Biosciences). For the immunophenotyping of purified blood DCs, a similar procedure was adopted; however, the incubation and centrifugation steps were carried out at 4 °C and an Fc receptor

blocking step with 10% heat inactivated human AB serum (Lonza, Slough, UK) was introduced (15 min incubation period) prior to incubating cells with antibodies. Fixed cells were run on a FACSCalibur flow cytometer (BD Biosciences) and the generated data was acquired and analyzed using Cell Quest Pro software (BD Biosciences). A total of 2.5-3 x 10^5 leukocytes or 5 x 10^3 purified total blood DCs were acquired per sample. Single-color stained controls were used to set compensation settings. Total blood dendritic cells (DCs) were identified as Lineage$^-$/HLA-DR$^+$, while blood mDCs and pDCs were identified as Lineage$^-$/HLA-DR$^+$/CD11c$^+$/CD123$^-$ and Lin$^-$/HLA-DR$^+$/CD123$^+$/CD11c$^-$, respectively. The expression of each cell surface receptor was indicated as the percentage of cells positive or a particular surface receptor within a gated cell population or as the raw geometric mean fluorescence intensity (MFI) of the receptor-positive cell population.

Isolation of CD4$^+$ T cells and blood DCs

PBMCs were isolated from peripheral blood using the standard Ficoll-Paque gradient method, as previously described.[21] CD4$^+$ T cells and total blood DCs were purified from PBMCs by negative selection using the Dynabeads untouched human CD4 T cells and human DC enrichment kits, respectively, and following the manufacturer's instructions (Thermo Fisher Scientific, Waltham, MA, USA). For the depletion of platelets, biotin-conjugated mouse anti-human CD41 antibody (clone M148; Leinco Technologies, St. Louis, MO, USA) was added to the antibody cocktail present in the DC enrichment kit. Isolated cells were checked for purity by flow cytometry and were consistently found to be > 95% and ~ 80% for CD4$^+$ T cells and DCs, respectively. Cells were routinely analyzed for viability with trypan blue exclusion method and showed > 90% viability.

Antigen presentation assay

Freshly purified total blood DCs were seeded in a 96-well flat-bottom plate at a density of 1 x 10^4 viable cells/well in complete RPMI medium supplemented with 10% heat inactivated human AB serum and were either left unstimulated or were stimulated with 300 μg/ml of HK *M. obuense* in the presence or absence of 10 μg/ml *Candida*

albicans (*C. albicans*) soluble antigen (Greer Laboratories, Lenoir, NC USA), for 7 days at 37 °C in a 5% CO2 humidified atmosphere. After 1 day in culture, DCs were co-cultured with 1 x 10^5 purified autologous $CD4^+$ T cells to obtain a T-cell/DC ratio of 10:1. Co-cultures were maintained for a period of 6 days at 37 °C in a 5% CO2 humidified atmosphere. Cells were pulsed with 1 μCi/well tritiated thymidine (Perkin Elmer, San Jose, CA, USA) during the last 16 h of co-culture period. Cells were then harvested onto glass fiber filter disks (Connectorate AG, Dietikon, Switzerland) using a cell harvester (Inotech Biotechnologies, Basel, Switzerland). Proliferation of $CD4^+$ T cells was assessed by measuring the radioactivity of incorporated methyl-tritiated-thymidine in a liquid scintillation counter (PerkinElmer) and which was obtained as counts per minute (cpm). Unstimulated (containing equivalent amounts of BBS as vehicle) DCs co-cultured with $CD4^+$ T cells as well as $CD4^+$ T cells, without DCs, stimulated with HK *M. obuense* plus *C. albicans* served as negative controls. Stimulation index (SI) was calculated by dividing the mean cpm value of DC-$CD4^+$ T cell co-cultures (triplicate) stimulated with HK *M. obuense* and/or *C. albicans* over the mean cpm value of unstimulated DC-$CD4^+$ T cell co-cultures (triplicate).

TLR blocking

Whole blood was incubated with 10 μg/mL of cell culture grade, azide-free blocking monoclonal antibodies against TLR1, TLR2, TLR4, TLR5, TLR6 or isotype control antibodies (mentioned above) for 1 h prior to stimulating whole blood with 300 μg/ml of HK *M. obuense* for 3 h. The efficiency of TLR blocking antibodies has been previously validated.[21]

Detection of chemokine and cytokine production

Purified total blood DCs (5 x 10^4 cells/ml) were either left unstimulated or were stimulated with 300 μg/ml of HK *M. obuense* for 24 h at 37 °C in a 5% CO2 humidified atmosphere. DC culture supernatants were collected after 24 h and were stored at −20°C until further analysis. Levels of CCL4, CCL5, CCL22, CXCL8, IFN-α, IL-6, IL-10, IL-12p40 and TNF-α were determined in DC culture supernatants using commercially

available sandwich enzyme-linked immunosorbent assay (ELISA) kits (R&D Systems, Abingdon, UK) and performed according to the manufacturer's protocol.

Statistical analysis

Statistical analysis of the data was carried out using GraphPad Prism software (version 6; GraphPad Software, San Diego, CA, USA). Data are presented as mean values ± standard error of the mean (SEM). Statistical differences were determined by paired t-test, Wilcoxon matched-pairs signed rank test or one-way ANOVA test followed by the Dunn's post-hoc test and were considered to be statistically significant at *p*-values < 0.05.

Results

HK *M. obuense* modulates the expression of different surface receptors on whole blood mDCs and pDCs

Flow cytometric analysis of DC subpopulations in whole blood was done initially by gating viable leukocytes and excluding debris and dead cells on the basis of their FSC/SSC characteristics at 3-, 6- and 24-h time points. Within this gate, total DCs were recognized as Lineage$^-$/HLA-DR$^+$ and the mDC and pDC subpopulations were further defined in the gated DC population as Lineage$^-$/HLA-DR$^+$/CD11c$^+$/CD123$^-$ and Lineage$^-$/HLA-DR$^+$/CD123$^+$/CD11c$^-$, respectively (Figure 1a). Preliminary experiments over a 3-, 6- and 24-h time course indicated that blood DCs tend to lose their phenotype as Lineage$^-$/HLA-DR$^+$ cells in 24-h unstimulated and *M. obuense*-stimulated whole blood cultures (Figure 1b). We performed a dose-response analysis to assess the effect of whole blood stimulation with HK *M. obuense* for 3 and 6 h on the surface expression of MHC class II on blood DCs. HK *M. obuense* upregulated the surface expression of MHC class II on blood DCs in a dose-dependent manner and this increase was apparently more prominent in mDCs than pDCs (Figure 1c). Based on these results, 300 μg/ml of HK *M. obuense* was selected as an optimal concentration for regulating surface receptor expression in blood DCs and was employed in subsequent experiments.

In order to determine whether *in vitro* whole blood stimulation with HK *M. obuense* would influence the phenotype of whole blood mDC and pDC subpopulations,

we measured the expression level of different categories of surface receptors on both DC subpopulations following a 3- and/or 6-h stimulation period. Representative flow cytometry figures of the percentage or MFI of receptor-positive mDCs are shown in Supplementary Figure S1. Among the antigen presentation receptors, only the MFI of MHC class II^+ cells was significantly elevated ($p < 0.05$) on mDCs with a 1.75- and ~2.5-fold change at 3 and 6 h after stimulation with HK *M.obuense*, respectively (Figure 2). mDCs responded to stimulation with HK *M. obuense* by significantly upregulating ($p < 0.05$) the expression of the B7 family receptors where the percentages of $CD80^+$ and $CD86^+$ mDCs were significantly increased 7- and 2-fold, respectively, after 3 h of stimulation as well as 38- and 2.5-fold, respectively, after 6 h of stimulation. However, a significant increase in the MFIs of $CD80^+$ and $CD86^+$ mDCs was noted only after 6 h of mycobacterial stimulation (Figure 2). The percentage and MFI of $CD274^+$ mDCs were significantly induced ($p < 0.05$) after 3 h of stimulation and these were even more evident at the 6-h time point (Figure 2). The expression of complement receptors was significantly enhanced on mDCs following stimulation with HK *M. obuense*. A significant upregulation ($p < 0.05$) in the percentage of $CD11b^+$ mDCs and in the MFI of $CD11c^+$ mDCs was observed at 3 h and at both incubation time points after *M. obuense* stimulation, respectively (Figure 2). Analysis of the expression of cytokine and chemokine receptors revealed that in response to HK *M. obuense* stimulation, the MFI of $CD116^+$ mDCs and the percentage of $CD253^+$ mDCs were significantly upregulated ($p < 0.05$) at 3 and 6 h, respectively (Figure 2). On the other hand, the expression of CD195 was significantly ($p < 0.05$) downregulated on mDCs at 3 and 6 h. Members of the PRRs demonstrated an enhanced expression on mDCs after stimulation with HK *M. obuense*. The percentages of $CD282^+$ and $CD284^+$ mDCs were significantly enhanced at both stimulation time points, whereas only the percentage of $CD206^+$ mDCs and the MFI of $CD282^+$ mDCs were significantly enhanced at the 3-h time point (Figure 2). Whole blood stimulation with HK *M. obuense* for 3 and 6 h also resulted in a marked increase ($p < 0.05$) in the expression of the DC maturation marker, CD83, on mDCs (Figure 2).

In response to whole blood stimulation with HK *M. obuense*, pDCs exhibited a modest but statistically significant regulation in the expression of a restricted number of receptors belonging to the antigen presentation, B7 family and cytokine and chemokine

families of receptors, while no significant regulation in the expression of the studied complement receptor and PRR families was detected (Figure 3). A mild but significant ($p < 0.05$) increase in the MFI of MHC class II^+ pDCs was observed after stimulation with HK *M. obuense* for 3 and 6 h (Figure 3). The percentage of $CD80^+$ pDCs was slightly but significantly ($p < 0.05$) upregulated at 6 h after stimulation with HK *M. obuense* (Figure 3). Moreover, 3- and 6-h *M. obuense*-stimulated pDCs demonstrated a slight but significant increase in the MFI of $CD123^+$ cells and a significant decrease in the percentage and MFI of $CD195^+$ cells as compared to unstimulated pDCs (Figure 3). We could not detect any spontaneous or HK *M. obuense*-induced expression of CD1d, CD83 CD91, CD282, CD284, and CD274 on pDCs (Figure 3).

Additional receptors (CD40, CD197, CD209, CD273, CD275, and CD276) were also studied on both DC subpopulations and under similar conditions; however, their expression was neither constitutively expressed nor induced following mycobacterial stimulation. Taken together, our data reveal that whole blood stimulation with HK *M. obuense* resulted in significant variations in the expression of receptors on mDC and pDC subpopulations; however, these variations were more prominent on mDCs. Based on the significant upregulated expression of CD80, CD83, CD86 and MHC class II and downregulated expression of CD195 on *M. obuense*-stimulated mDCs, it can be inferred that HK *M. obuense* might drive mDCs towards acquiring a mature DC phenotype.

TLR-2 and TLR-1 blocking reduces the HK *M. obuense*-induced upregulation of CD11c, CD80 and MHC class II expression on whole blood mDCs

We have previously reported that TLR-2 and TLR-1 contribute to the *M. obuense*-induced upregulation of surface receptor expression of CD11c and MHC class II on monocytes in whole blood.[21] We therefore attempted to determine whether TLR-2 and TLR-1 in addition to other TLRs (TLR-4, 5 and 6) are also involved in mediating the *M. obuense*-induced expression of surface receptors on whole blood mDCs. TLR blocking experiments were carried out using specific blocking antibodies against TLR-1, 2, 4, 5 and 6 whereby the effect of TLR blocking on the *M. obuense*-induced expression of selected receptors was studied. The selected receptors included: CD11c, CD80 and MHC

class II which represented three categories of surface receptors that demonstrated significant variations in expression (% or MFI) on mDCs following whole blood stimulation with HK *M. obuense* (Figure 2). Both single and simultaneous blocking of TLR-2 and TLR-1 led to a significant reduction ($p < 0.05$) in the *M. obuense*-induced expression of CD80 (% of $CD80^+$ cells) and MHC class II (MFI of MHC class II^+ cells) on mDCs, whereas blocking of TLR-2 and TLR-2/1 resulted in a significant reduction ($p < 0.05$) in the *M. obuense*-induced expression of CD11c (MFI of $CD11c^+$ cells) on mDCs (Figure 4). We did observe a trend of an enhanced blocking effect with combined TLR-2/1 blocking versus separate TLR blocking; however, this effect achieved statistical significance ($p < 0.05$) only against single TLR-2 blocking in the case of MHC class II. Although there was a trend towards a decrease in the *M. obuense*-induced expression of CD11c and MHC class II in response to TLR-1 and TLR-2/6 blocking, respectively, this decrease did not attain statistical significance (Figure 4). Simultaneous blocking of TLR-2 and TLR-6 significantly reversed the *M. obuense*-induced expression of CD11c and CD80, an effect comparable to that observed with blocking TLR-2 alone (Figure 4). Blocking of TLR-4, 5 and 6 resulted in no significant reduction in the *M. obuense*-induced upregulation of the three surface receptors (Figure 4).

HK *M. obuense* modulates surface expression of different receptors on purified blood DCs

Our results have shown that whole blood stimulation with HK *M. obuense* induced significant variations in the expression of different surface receptors on mDCs and pDCs in whole blood. This finding has prompted us to investigate whether the detected variations in receptor expression were merely related to a direct stimulatory effect induced by HK *M. obuense* on DCs or due to an indirect stimulatory effect induced by the whole blood milieu (other blood cells, pro-inflammatory cytokines, etc.). To answer this question, we examined the effect of stimulating purified blood DCs with HK *M. obuense* on the expression of different receptors that were selected to represent those which showed significant variations on whole blood DCs upon stimulation with HK *M. obuense*. Following 6 h stimulation with HK *M. obuense*, purified blood mDC and pDC subpopulations significantly upregulated ($p < 0.05$) the MFI of MHC class II^+ cells

(Figure 5). A significant ($p < 0.05$) ~8.5- and 16.5-fold increase in the percentage of $CD80^+$ cells was observed on *M. obuense*-stimulated purified blood mDCs and pDCs, respectively; however, only *M. obuense*-stimulated purified blood pDCs showed a significant 2.8-fold increase in the MFI of $CD80^+$ cells (Figure 5). Unlike pDCs, *M. obuense*-stimulated purified blood mDCs revealed a significant elevation in the expression of $CD274^+$ cells. *M. obuense*-stimulated purified blood mDCs and pDCs demonstrated, respectively, a significant upregulation ($p < 0.05$) in the percentage of $CD253^+$ cells and in the MFI of $CD123^+$ cells (Figure 5). Both mDCs and pDCs enhanced the percentage of $CD83^+$ cells in response to stimulation with HK *M. obuense*.

HK *M. obuense* induces cytokine and chemokine secretion by purified total blood DCs

We next examined the cytokine and chemokine secretion patterns of purified total blood DCs stimulated with HK *M. obuense*. Stimulation of DCs with HK *M. obuense* for 24 h was shown to substantially induce the release of CCL22 (1248 ± 324 pg/ml) and CXCL8 (2703 ± 443 pg/ml) (Figure 6). Moreover, *M. obuense*-stimulated DCs exhibited a significant ($p < 0.05$) ~18- and ~2-fold increase in the production of CCL4 and CCL5, respectively, as compared to unstimulated DCs (Figure 6). Stimulation with HK *M. obuense* also resulted in a weak, but significant ($p < 0.05$) secretion of IL-6, IL-12p40 and TNF-α by DCs (Figure 6). On the other hand, levels of IFN-α and IL-10 were not detected in supernatants of either unstimulated or *M. obuense*-stimulated DC cultures.

Effect of DC stimulation with HK *M. obuense* and/or *C. albicans* on $CD4^+$ T-cell antigen-specific responses

We investigated the ability of purified total blood DCs to present HK *M. obuense* antigens and/or *C. albicans* soluble antigen to autologous purified $CD4^+$ T cells. DCs stimulated with HK *M. obuense* or *C. albicans* induced comparable autologous $CD4^+$ T-cell proliferation with SIs of 24 ± 14 and 25 ± 14, respectively. However, concomitant stimulation of DCs with HK *M. obuense* and *C. albicans* antigens elicited significantly ($p < 0.05$) higher $CD4^+$ T-cell proliferative responses (SI = 51± 26) than DCs stimulated separately with HK *M. obuense* or with *C. albicans* alone (Supplementary Figure S2).

Further analysis of our $CD4^+$ T-cell proliferation data clearly demonstrated that the autologous $CD4^+$ T-cell proliferative responses induced by DCs stimulated with HK *M. obuense* and *C. albicans* were not statistically different ($p > 0.05$) from the additive proliferative responses to each antigen separately. As expected, low $CD4^+$ T cell proliferation was noted when these lymphocytes were stimulated with both antigens, but without the presence of DCs (280 ± 66 cpm), or when co-cultured with DCs, but without the presence of antigens (504 ± 107 cpm).

Discussion

Shedding light on the outcome of interaction of an immunomodulator with immune cells is a vital step towards understanding the immunomodulator's mode of action. Over the past few years, HK *M. obuense*, a novel systemic immunomodulator, has attracted the attention of cancer researchers;[18,19] however, little is known about the consequences of its interaction with human innate immune cells. In this study we investigated the effect of HK *M. obuense* on the phenotype and cytokine/chemokine secretion profile of human blood DCs, which are limited in number,[24] and represent a challenge in the study of human DC biology. To the best of our knowledge, this is the first study to describe the effect of HK *M. obuense* on human whole blood and purified primary blood DCs.

Our first approach was aimed at examining the effect of *in vitro* exposure to HK *M. obuense* on the expression of a group of receptors belonging to six different categories on mDC and pDC subpopulations in a human whole blood model which represents a physiologically relevant system that has been used extensively to assess the effect of several immunomodulators on the phenotype, function and responses of blood DCs.[25-27] During initial time-course studies, we could not identify DCs either in unstimulated or in *M. obuense*-stimulated 24 h whole blood cultures due to the loss of their unique flow cytometric profile as $Lineage^-/HLA\text{-}DR^+$ cells. A previous study has also failed to measure intracellular cytokine production by human DC subpopulations in 24 h-whole blood cultures due to an inability to reliably detect different DC subpopulations.[28] On the contrary, other studies have reported the detection of human DC subpopulations after 20-

24 h culture period of whole blood and were able to assess DC's phenotype and function.[27,29] The reasons behind this discrepancy with our data are not clear.

In response to whole blood stimulation with HK *M. obuense*, mDCs demonstrated a considerable increase in the expression of CD11c, CD80, CD83, CD86, CD274, CD282 (TLR-2), CD284 (TLR-4) and MHC class II with a concomitant decrease in the expression of CD195 (CCR5). On the other hand, pDCs showed a slight but significant increase in the expression of CD80 and MHC class II and a decrease in the expression of CD195. Based on the screened battery of surface receptors on mDCs, it was evident that whole blood stimulation with HK *M. obuense* favored the upregulation of a specific set of receptors (CD80, CD83, CD86, MHC class II) which are signature receptors correlating with the maturation status of DCs.[30] Similar induction of phenotypic maturation receptors has been reported on human Mo-DCs, phenotypically identical to mDCs,[31] by some mycobacterial species such as live or HK *M. bovis*, HK *M. vaccae*, and live, HK or γ-irradiated *M. tuberculosis*,[23,32-36] but not by HK *M. leprae*.[34] Moreover, our data showed a significant downregulation of CD195 (CCR5) expression on *M. obuense*-stimulated whole blood mDCs and pDCs. A similar rapid downregulation of CCR5 surface expression has been previously noted on Mo-DCs that are undergoing maturation.[37] Furthermore, HK *M. obuense* showed an ability to upregulate the expression of CD274 (PDL-1) on whole blood mDCs, a phenomenon which has been observed following stimulation of Mo-DCs with whole cell lysates of *M. tuberculosis*.[38]

The critical role of TLRs in mediating the recognition and response to mycobacterial antigens in innate immune cells has been emphasized in several studies. TLR-2 (as a heterodimer with either TLR-1 or TLR-6), TLR-4 and TLR-9 have been demonstrated to be engaged in sensing various mycobacterial cell wall components such as glyoclipids, glycoproteins, diacylated and triacylated lipoproteins, heat-shock proteins as well as unmethylated CpG motifs in mycobacterial DNA.[39,40] An earlier study from our group has suggested a role for TLR-2 and TLR-1 in mediating HK *M. obuense*-induced upregulation of CD11c and HLA-DR expression on monocytes in whole blood culture system.[21] The data presented here indicated that the *M. obuense*-induced upregulation of CD11c, CD80 and MHC class II expression on whole blood mDCs was mediated, at least in part, through the interaction of HK *M. obuense* with both TLR-2 and

TLR-1. Using a panel of HEK293 cells expressing different human TLRs, previous studies have shown that cellular activation of HEK293 cells by HK *M. vaccae* or HK *M. indicus pranii* was mediated through interaction with TLR-2 alone, with TLR-2/TLR-6 heterodimer and more potently with TLR-2/TLR-1 heterodimer.[23,41] Our data also point to a putative interaction between HK *M. obuense* and surface TLR-2/TLR-1 and which could be mediated by interaction with triacylated lipoproteins, known to constitute part of the mycobacterial cell wall components.[42-44] Moreover, mycobacterial triacylated lipoproteins have been reported to activate and to promote the maturation of human Mo-DCs.[45,46]

In this study, we also examined the effect of stimulating purified blood DCs with HK *M. obuense* for 6 h on the expression of surface receptors on purified mDC and pDC subpopulations. Similar to the observed effects on whole blood DCs, *M. obuense* stimulation of purified blood DCs induced a significant upregulation in the expression of CD80 and MHC class II on both subpopulations of DCs and of CD83 and CD253 only on mDCs. Unlike whole blood pDCs, purified blood pDCs expressed low but detectable levels of CD83 and CD274 and upregulated CD83 expression upon stimulation with HK *M. obuense*. Furthermore, in contrast to whole blood mDCs, purified blood mDCs displayed no significant change in the expression of CD11c upon stimulation with HK *M. obuense*. These observed variations in *M. obuense*-induced surface receptor expression between whole blood and purified blood DCs might be a consequence of the DC enrichment process which eliminated other immune cells with potential contribution to the immunomodulatory effects of HK *M. obuense* on DCs. The differential regulation in expression of surface receptors between the two major DC subpopulations might be attributed to variations in the expression pattern of TLRs thus leading to differential recognition of HK *M. obuense* by mDCs and pDCs. Findings from this study as well as from previous studies have clearly indicated that human blood mDCs express TLR-2 while blood pDCs lack TLR-2 expression.[47,48] Therefore, the absence of surface TLR-2 on pDCs might explain the differential regulation in surface receptors between mDCs and pDCs in response to whole blood stimulation with HK *M. obuense*. Despite the lack of TLR-2 surface expression on blood pDCs, the involvement of other receptor(s) such as Dectin-2,[49] and nucleotide-binding oligomerization domain-containing protein 2

(NOD2)[50,51] in mediating the above mentioned effects of HK *M. obuense* on blood pDCs should not be neglected. A putative role for human blood mDC-pDC cross-talk in mediating the observed pDCs responses to HK *M. obuense*, despite the absence of TLR-2 expression, should be also taken into consideration. This mDC-pDC cross-talk has been reported to reverse the absence of responsiveness of purified human blood pDCs to recombinant bacillus Calmette-Guerin (rBCG) infection when cultured alone, but to allow the upregulation of MHC class I, II, CD86 and CD123 expression when co-cultured with rBCG-infected mDCs.[52-54]

Cytokines and chemokines produced by DCs are critically essential in developing subsequent immune responses.[55,56] In this study, we found that purified total blood DCs stimulated with HK *M. obuense* mostly exhibited increased levels of secretion of several proinflammatory cytokines and chemokines including IL-6, IL-12p40, TNF-α, CCL4, CCL5 and CXCL8. Our observation is in accordance with a previous report whereby treating Mo-DCs with HK BCG or HK *M. bovis* for 24 h stimulated their production of CCL5 and TNF-α.[57] Given our data showing that TLR-1 and TLR-2 are involved in mediating DC responses to HK *M. obuense*, a previous study has also demonstrated the ability of the specific TLR1/2 ligand, Pam3CSK4, to enhance the production of CXCL8, IL-6 and TNF-α by purified total blood DCs.[58] IFN-α, a cytokine reported to be mainly released in high amounts by pDCs upon activation of their TLRs,[59] was not detectable in supernatants from both unstimulated and *M. obuense*-stimulated purified total blood DC cultures. This observation may suggest a lack of pDC's cytokine response to stimulation with HK *M. obuense*.

Our finding that HK *M. obuense* induces DC phenotypic changes consistent with a mature-like phenotype suggests that priming purified blood DCs with HK *M. obuense* might also result in an enhanced ability of DCs to present antigens and to activate T-cells. Mo-DCs primed with HK *M. vaccae* have been shown to enhance autologous $CD4^+$ T-cell responses to the recall antigen tetanus toxoid.[23] To address this hypothesis, we attempted to study the effect of priming purified blood DCs with HK *M. obuense* on the autologous $CD4^+$ T cell proliferative responses to the soluble recall antigen, *C. albicans*; however, our attempt was unsuccessful due to the following technical difficulties. First, the number of purified blood DCs from 50 ml of peripheral blood was low and not

achieving > 5 x 10^5 cells. This prohibited the ability of performing adequate bulk DC priming and comparing with unprimed DCs due to cell loss upon centrifugation and washing. Second, the continued presence of HK *M. obuense* organisms with DCs even after bulk stimulation, centrifugation and washing. Third, the continued presence of HK *M. obuense* organisms in wells of cultured DCs (1 x 10^4 cells/well) due to an inability to carry out vigorous washing dictated by the non-adherent nature of the purified blood DCs. Despite the above-mentioned limitations, we assessed the ability of purified blood DCs to present mycobacterial antigens and/or *C. albicans* antigen to autologous $CD4^+$ T cells. We noted significant proliferation of the $CD4^+$ T cells that were induced by *M. obuense*-stimulated DCs. This is in line with our previous report where we observed strong PBMC proliferative responses against HK *M. obuense* in healthy non-BCG-vaccinated individuals in Lebanon.[21] Our results also suggest no interference between the mycobacterial antigens and *C. albicans* antigen presentation mechanisms as evidenced by the additive $CD4^+$ T cell responses induced by purified blood DCs simultaneously stimulated with HK *M. obuense* and *C. albicans*.

Collectively our data provide a new insight into the outcome of interaction between HK *M. obuense* and human DCs and uncover the immunomodulatory effects of this mycobacterial preparation on DCs. Our findings might have significant implications in the design of immunotherapy protocols that could exploit the immunomodulatory properties of HK *M. obuense* to augment DC-mediated immune responses.

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Declaration of Conflicting Interests

SM and CA are unsalaried directors and shareholders of Immodulon Therapeutics, Ltd, UK. GMB is a member of the Scientific Advisory Board for Immodulon Therapeutics, Ltd, UK. The remaining authors declare no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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Authors' contribution

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

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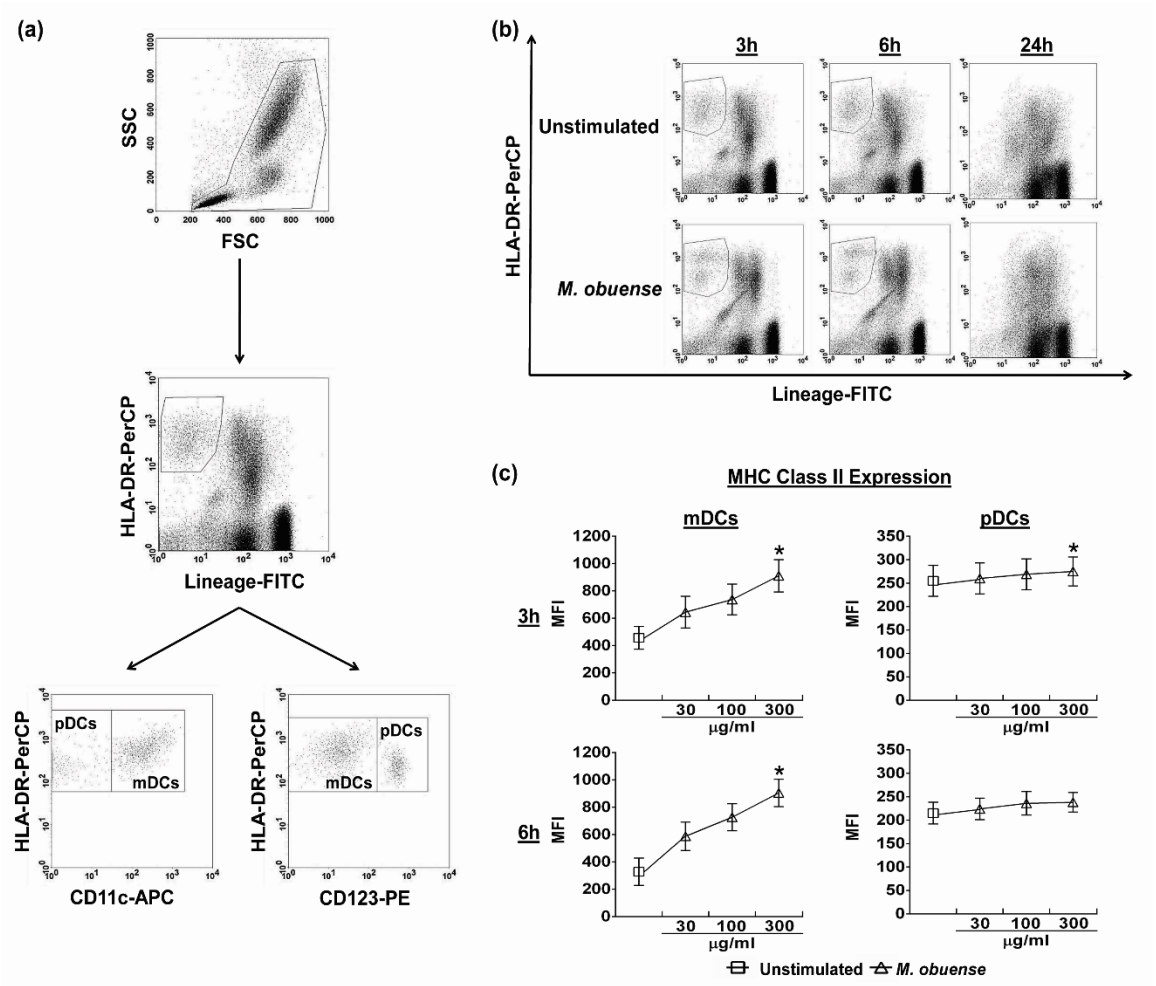

(a)
SSC
FSC
HLA-DR-PerCP
Lineage-FITC
pDCs
mDCs
CD11c-APC
CD123-PE
(b)
3h
6h
24h
Unstimulated
M. obuense
HLA-DR-PerCP
Lineage-FITC
(c)
MHC Class II Expression
mDCs
pDCs
3h
6h
MFI
μg/ml
Unstimulated
M. obuense

Figure 1. (a) Gating scheme for identification of myeloid (m) and plasmacytoid (p) dendritic cells (DCs) in whole blood. Viable leukocytes were gated based on forward and side scatter (FSC and SSC) properties. Among the gated leukocyte population, DCs were identified as Lineage$^-$/HLA-DR$^+$ and within this DC population, mDC and pDC subpopulations were identified as Lineage$^-$/HLA-DR$^+$/CD11c$^+$/CD123$^-$ and Lineage$^-$/HLA-DR$^+$/CD123$^+$/CD11c$^-$, respectively. (b) Loss of flow cytometric profile of total DCs (Lineage$^-$/HLA-DR$^+$) in 24 h whole blood cultures. Total DCs were identified in whole blood cultures that were either left unstimulated or were stimulated with 300 μg/ml of heat killed (HK) *M. obuense* for 3, 6 and 24 h. Flow cytometry graphs are representative of three independent experiments. (c) Dose-response assessment for the effect of whole blood stimulation with HK *M. obuense* for 3 and 6 h on the expression level of MHC class II on mDCs and pDCs. Whole blood was stimulated with various concentrations of HK *M. obuense* (30, 100 and 300 μg/ml) for 3 and 6 h and the expression level of MHC class II, presented as geometric mean fluorescence intensity (MFI) of MHC class II$^+$ DCs, was measured on mDC and pDC subpopulations. Symbols and error bars indicate mean values and SEM, respectively (n=4). Statistically significant differences were determined by one-way ANOVA test followed by the Dunn's post-hoc test (*$p < 0.05$ *versus* unstimulated).

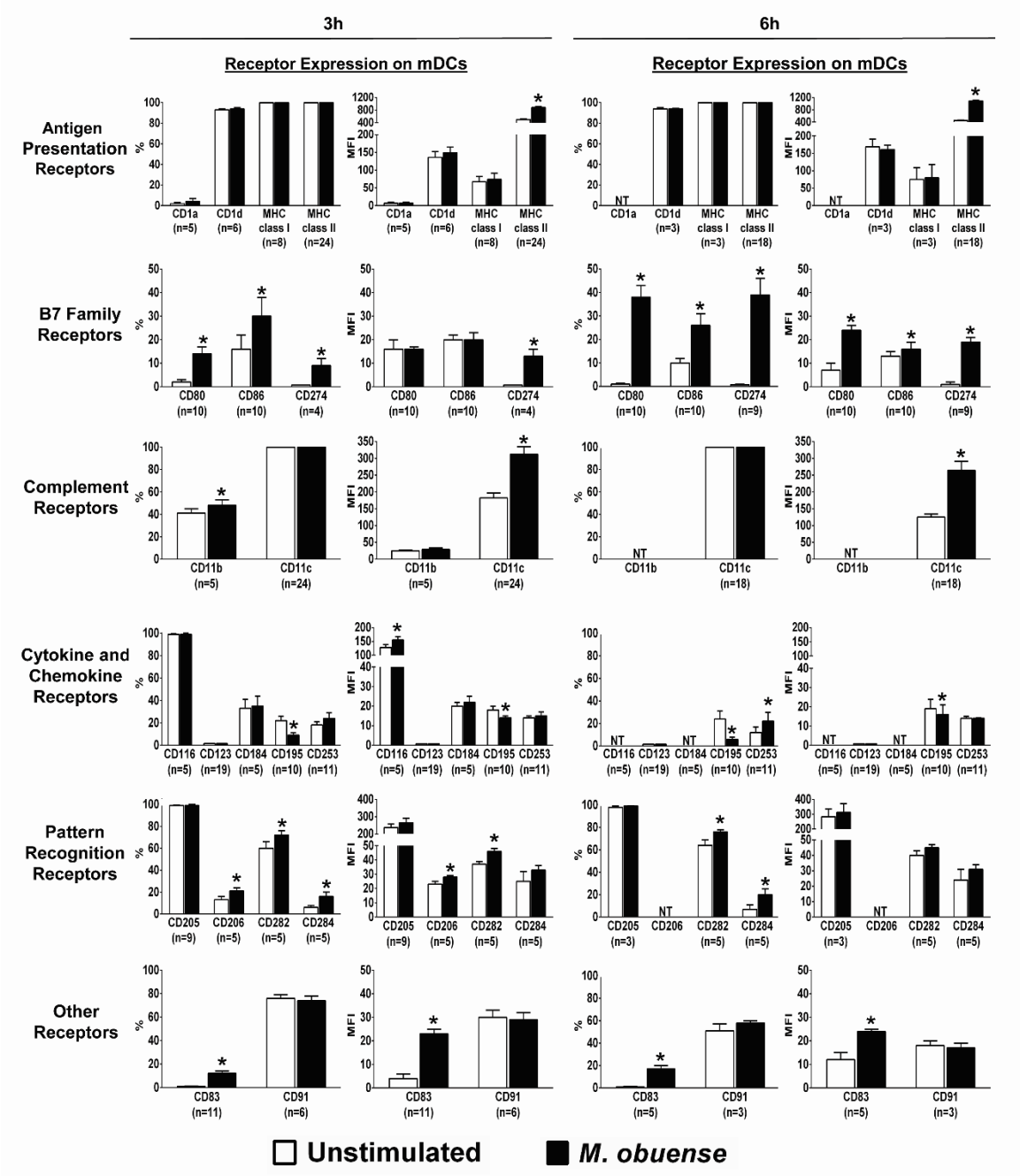


Figure 2. Whole blood stimulation with heat-killed (HK) *M. obuense* modulates surface expression of different receptors on myeloid (m) dendritic cells (DCs). Whole blood cultures were either left unstimulated or were stimulated with 300 μg/ml of HK *M. obuense* for 3 and 6 h and the expression level of different categories of cell surface receptors was measured on mDCs. Column bars represent mean values of the percentage (%) and geometric mean fluorescence intensity (MFI) of receptor-positive mDCs of at least 3 donors. Error bars represent SEM. Statistically significant differences were determined by paired t-test (*$p < 0.05$ *versus* unstimulated). NT: not tested.

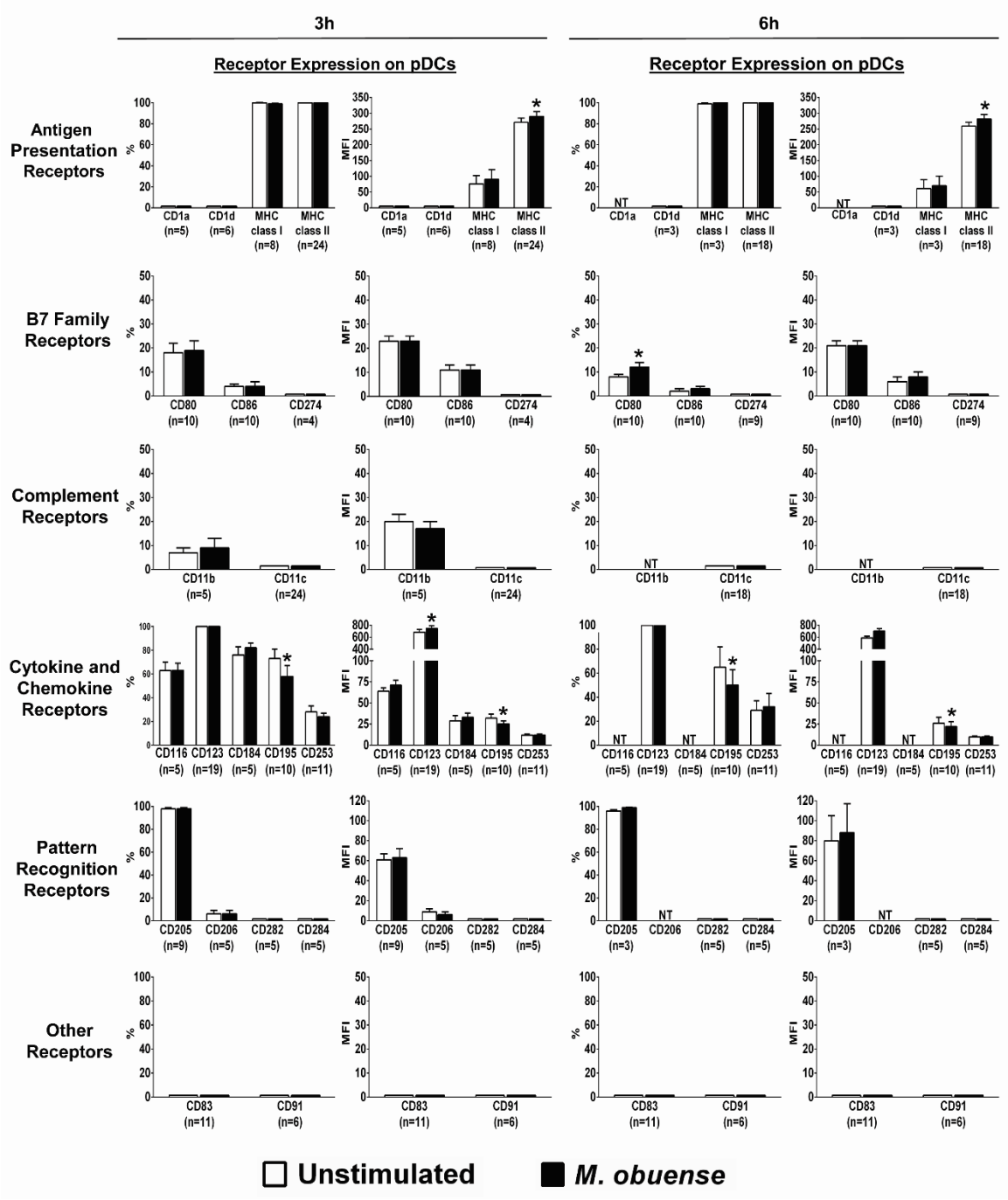


Figure 3. Whole blood stimulation with heat-killed (HK) *M. obuense* modulates surface expression of different receptors on plasmacytoid (p) dendritic cells (DCs). Whole blood cultures were either left unstimulated or were stimulated with 300 μg/ml of HK *M. obuense* for 3 and 6 h and the expression level of different categories of cell surface receptors was measured on pDCs. Column bars represent mean values of the percentage (%) and geometric mean fluorescence intensity (MFI) of receptor-positive pDCs of at least 3 donors. Error bars represent SEM. Statistically significant differences were determined by paired t-test (*$p < 0.05$ *versus* unstimulated). NT: not tested.

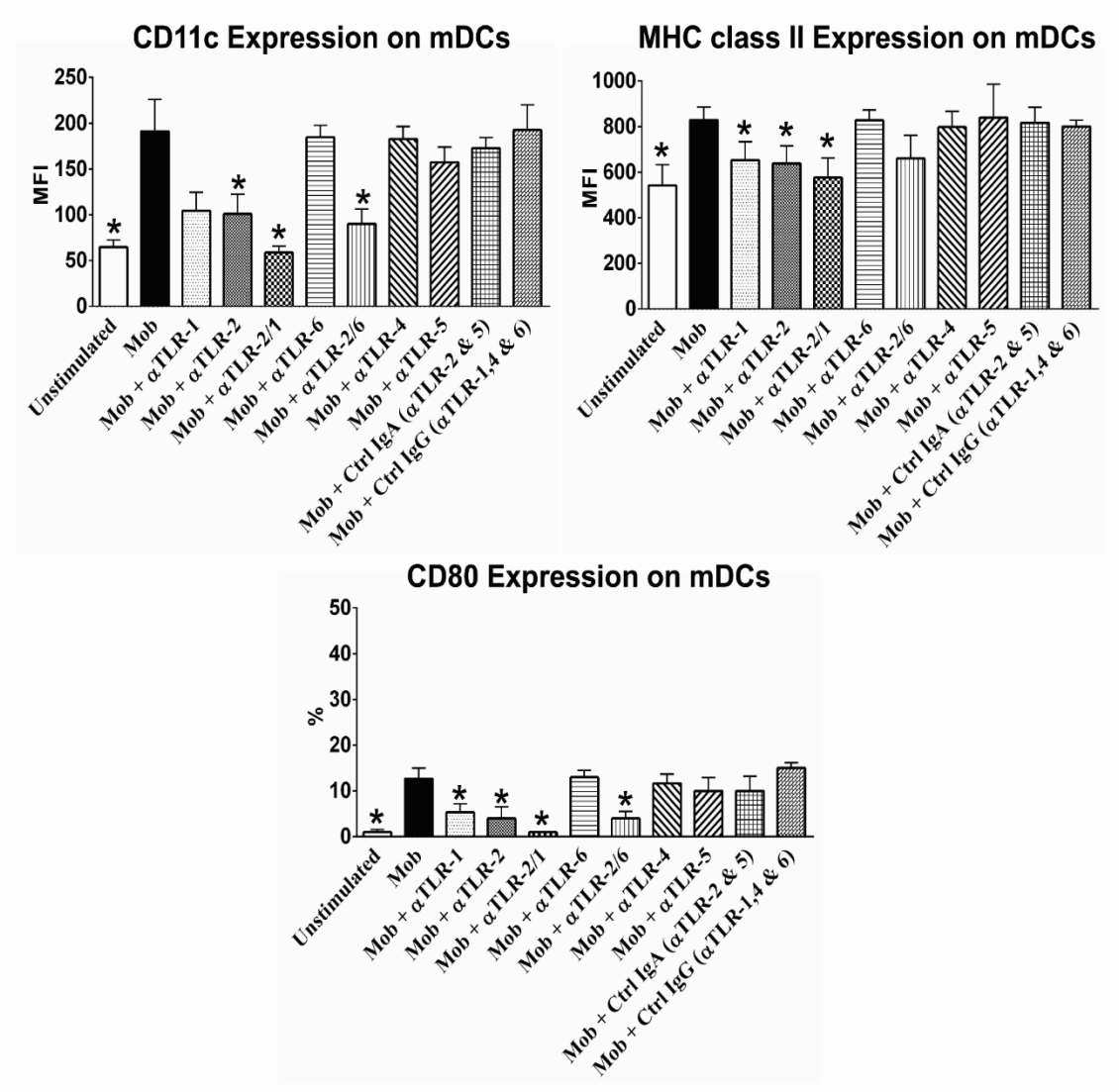


Figure 4. Blocking of toll-like receptor (TLR)-2 and/or TLR-1 reduces the *M. obuense*-induced upregulation of CD11c, CD80 and MHC class II expression on myeloid (m) dendritic cells (DCs) in whole blood. Whole blood cultures were pre-incubated with anti (α)-TLR-1, α-TLR-2, α-TLR-1/2, α-TLR6, α-TLR-2/6, α-TLR-4, α-TLR-5 or isotype-matched control antibodies: IgA (for α-TLR-2 and 5) or IgG (for α-TLR-1, 4 and 6) for 1 h and then stimulated with 300 μg/ml of heat-killed (HK) *M. obuense* (Mob) for 3 h followed by the analysis of the expression level of CD11c, CD80 and MHC class II on mDCs. Column bars represent mean values of the percentage (%) of $CD80^+$ mDCs or the geometric mean fluorescence intensity (MFI) of $CD11c^+$ and MHC class II^+ mDCs of 3 donors. Error bars represent SEM. Statistically significant differences were determined by Wilcoxon matched-pairs signed rank test (*$p < 0.05$ *versus* Mob).

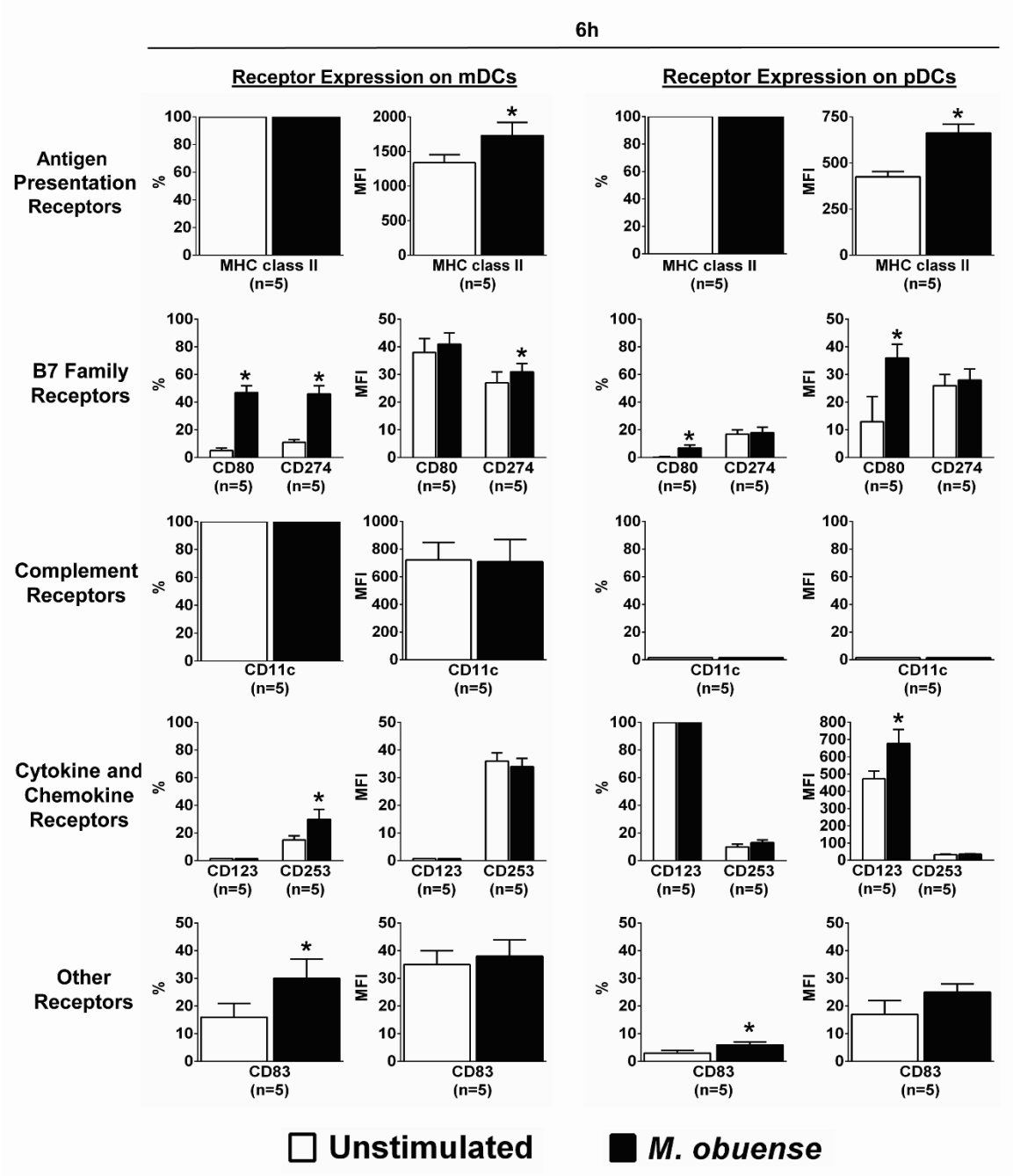


Figure 5. Heat-killed (HK) *M. obuense* modulates surface expression of different receptors on purified dendritic cells (DCs). Purified total blood DCs were either left unstimulated or were stimulated with 300 μg/ml of HK *M. obuense* for 6 h and the expression level of different categories of cell surface receptors was measured on the mDC and pDC subpopulations. Column bars represent mean values of the percentage (%) and geometric mean fluorescence intensity (MFI) of receptor-positive mDCs and pDCs of 5 donors. Error bars represent SEM. Statistically significant differences were determined by paired t-test (*$p < 0.05$ *versus* unstimulated).

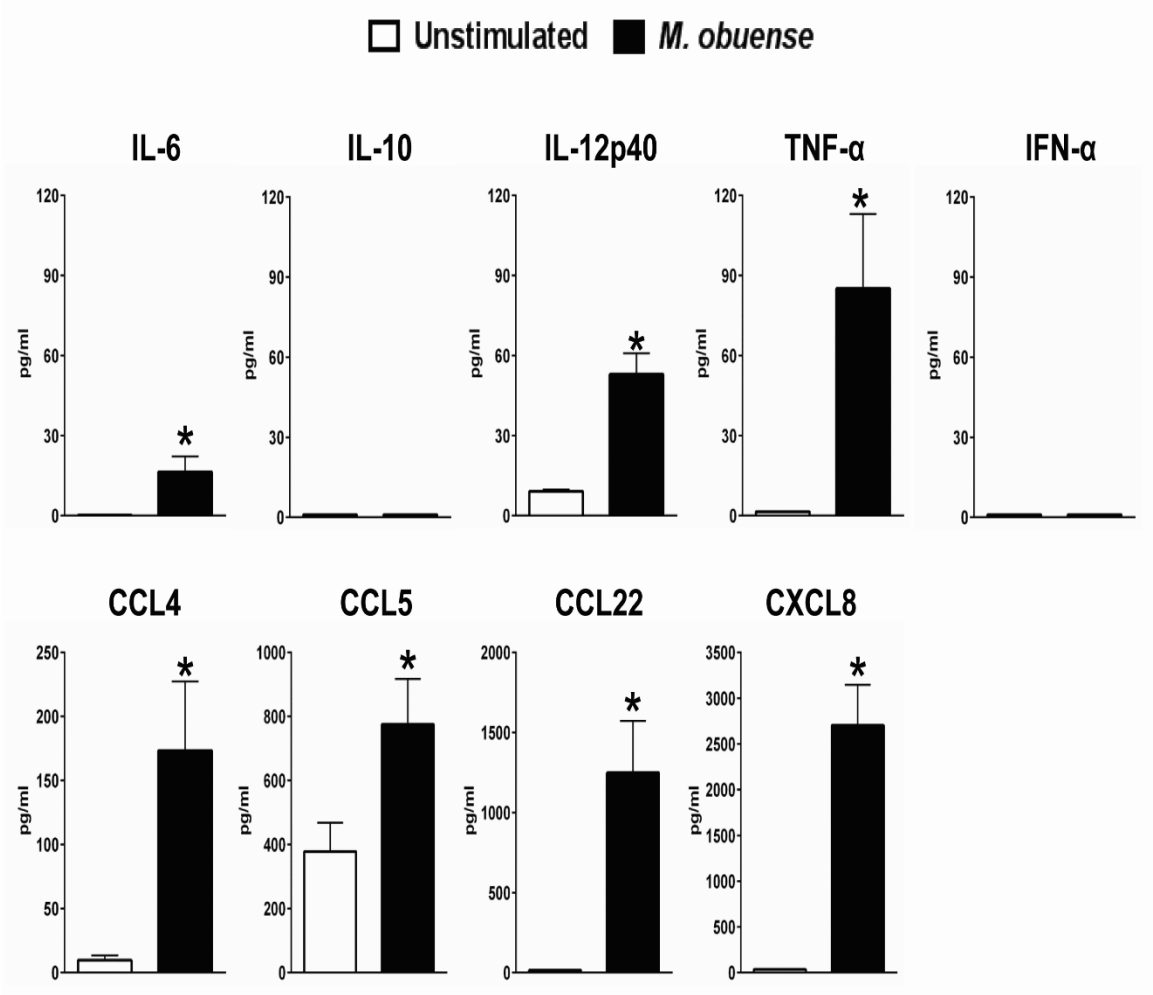


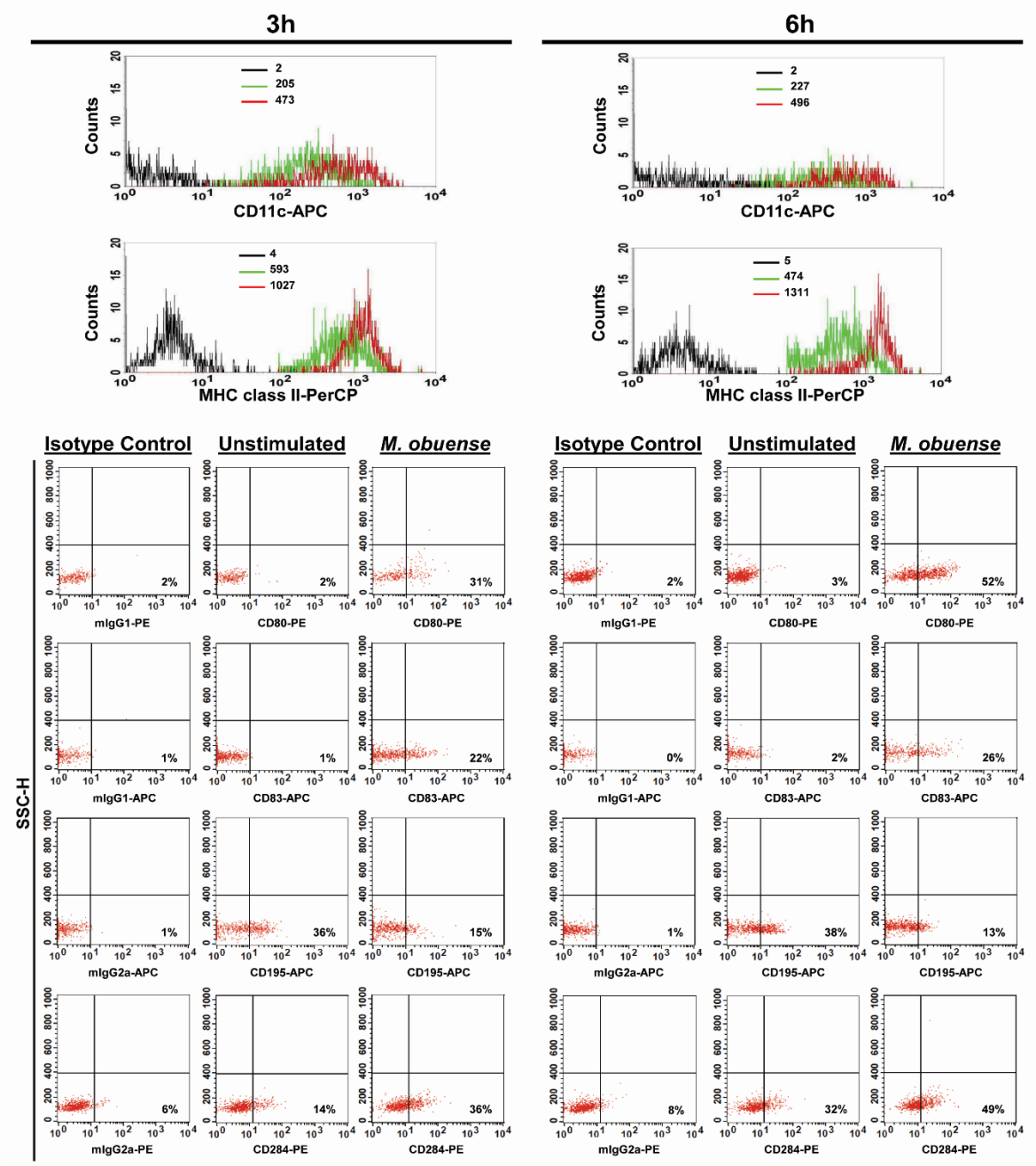
Figure 6. Heat-killed (HK) *M. obuense* induces the secretion of cytokines and chemokines by purified dendritic cells (DCs). Purified total blood DCs were either left unstimulated or were stimulated with 300 μg/ml of HK *M. obuense* for 24 h. Levels of CCL4, CCL5, CCL22, CXCL8, IFN-α, IL-6, IL-10, IL-12p40 and TNF-α were measured in DC culture supernatants. Column bars represent mean values of cytokine or chemokine concentration in DC culture supernatants of 7 donors. Error bars represent SEM. Statistically significant differences were determined by paired t-test (*$p < 0.05$ *versus* unstimulated).

Supplementary Table S1. List of antigen-specific and isotype control antibodies used for immunophenotyping of DCs

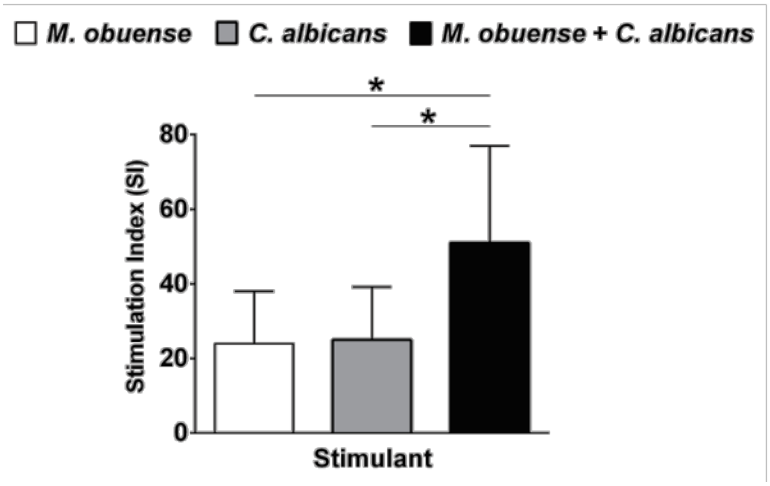
Antigen-Specific Antibody	**Name**	**Clone**	**Conjugate**	**Manufacturer**
CD1a	T6/Leu-6	HI149	APC	BD Biosciences
CD1d	R3G1	CD1d42	PE	
CD4	T-cell surface glycoprotein	RPA-T4	PE	
CD11b	CR3	ICRF44	PE	
CD11c	CR4	B-ly6	APC	
CD32	FcγRII	FLI8.26	APC	
CD40	TNFRSF5	5C3	APC	
CD80	B7-1	L307.4	PE	
CD83	HB15	HB15e	APC	
CD86	B7-2	2331	APC	
CD91	HSPR	A2MR-α2	PE	
CD116	GM-CSFRα	hGMCSFR-M1	PE	
CD123	IL23R	9F5	PE	
CD184	CXCR4	12G5	APC	
CD195	CCR5	2D7/CCR5	APC	
CD197	CCR7	3D12	Alexa Fluor-647	
CD205	DEC-205	MG38	PE	
CD206	MMR	19.2	PE	
CD209	DC-SIGN	DCN46	APC	
CD253	TRAIL	RIK-2	PE	

CD273	B7-DC/PD-L2	MIH18	APC	
CD274	B7H1/PD-L1	MIH1	PE	
CD275	B7H2	2D3/B7-H2	PE	
CD276	B7-H3	185504	APC	R & D Systems
CD282	TLR-2	TL2.1	PE	eBioscience
CD284	TLR-4	HTA125	PE	
HLA-ABC	MHC I	G46-2.6	PE	BD Biosciences
Lin 1 antibody cocktail: CD3; CD14; CD16; CD19; CD20; CD56	Lineage 1	SK7; MφP9; 3G8; SJ25C1; L27; NCAM16.2	FITC	
HLA-DR	MHC II	L243	PerCP	
Isotype Control Antibody	**Name**	**Clone**	**Conjugate**	**Manufacturer**
Mouse IgG1	*NA*	MOPC-21	PE	BD Biosciences
Mouse IgG1	*NA*	MOPC-21	APC	
Mouse IgG2a	*NA*	X39	PerCP	
Mouse IgG2a	*NA*	G155-178	APC	
Mouse IgG2b	*NA*	clone 27-35	PE	
Mouse IgG2b	*NA*	27-35	APC	
Rat IgG2a	*NA*	R35-95	Alexa Fluor-647	
Mouse IgG1	*NA*	11711	APC	R & D Systems
Mouse IgG2a	*NA*	eBM2a	PE	eBioscience

NA: not applicable



Supplementary Figure S1. Whole blood stimulation with heat killed (HK) *M. obuense* modulates surface expression of various receptors on myeloid (m) dendritic cells (DCs). Representative flow cytometry graphs demonstrating the expression of CD11c, MHC class II (presented as histogram plots), CD80, CD83, CD195 and CD284 (presented as dot plots) on whole blood mDCs that were left unstimulated or stimulated with 300 μg/ml of HK *M. obuense* for 3 and 6 h. Numbers in histogram plots correspond to the geometric mean fluorescence intensity (MFI) of receptor-positive mDCs in each condition (black line histogram: isotype control; green line histogram: unstimulated; red line histogram: *M. obuense*). Numbers within lower right quadrant of dot plots indicate the percentage of receptor-positive mDCs out of the total mDC population. SSC-H: side scatter height.



Supplementary Figure S2. Effect of stimulating DCs with heat-killed (HK) *M. obuense* and/or *C. albicans* on $CD4^+$ T cell antigen- specific responses. Purified total blood dendritic cells (DCs) were either left unstimulated or were stimulated with 300 μg/ml of HK *M. obuense* and/or 10 μg/ml of the soluble recall antigen *C. albicans* for 7 days. After 1 day in culture, DCs were co-cultured with autologous $CD4^+$ T cells at a T cell/DC ratio of 10:1 for 6 days. Tritiated-thymidine incorporation, expressed as counts per minute (cpm), was measured after 6 days of co-culture. Unstimulated DCs co-cultured with $CD4^+$ T cells as well as $CD4^+$ T cells, without DCs, stimulated with HK *M. obuense* and *C. albicans* served as negative controls. Stimulation index (SI) was calculated by dividing the mean cpm value of DC-$CD4^+$ T cell co-cultures stimulated with HK *M. obuense* and/or C. albicans over the mean cpm value of unstimulated DC-$CD4^+$ T cell co-cultures. Column bars represent mean SI of 6 donors. Error bars represent SEM. Statistically significant differences were determined by Wilcoxon matched-pairs signed rank test (*$p < 0.05$).