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

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ABSTRACT

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

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

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1. Introduction

Mycobacteria, due to their diverse and complex cell wall structures, play a significant role in modulating immune responses (Rook et al., 2004; Stanford et al., 2009) For several years, Bacillus Calmette-Guerin (BCG), a live attenuated derivative of the slow-growing species *Mycobacterium bovis* (*M. bovis*), has been used as a prophylactic vaccine against tuberculosis (TB); though, with variable efficacy (Romano and Huygen 2012). Moreover, BCG has proven to be successful in the treatment of early non-invasive bladder cancer (Kawai et al., 2013). The investigation of inactivated rapid-growing mycobacteria to be employed as immunomodulators has led to the selection of *M. vaccae* which was later evaluated as an immunomodulating therapeutic agent in various diseases including TB (Dlugovitzky et al., 2006; Johnson et al., 2000; Yang et al., 2011), leprosy (Abbot et al., 2002; Truoc et al., 2001), psoriasis (Lehrer et al., 1998), dermatitis (Arkwright and David 2001), asthma (Camporota et al., 2003) and a range of cancers (Cananzi et al., 2013; Eaton et al., 2002; O'Brien et al., 2004; Patel et al., 2008; Stanford et al., 2008). *M. vaccae* was also found to be an effective vaccine that could confer a significant level of protection against TB among HIV-infected individuals who had received BCG vaccination during childhood (von Reyn et al., 2010). In all these studies, *M. vaccae* was used in the form of a heat-killed (HK) preparation that retained its antigenic properties. In spite of the growing interest in the therapeutic implications of HK mycobacterial preparations, the nature of the host immune receptors regulated by these mycobacterial preparations has not been fully defined. Moreover, the exact mechanism by which HK mycobacterial preparations exert their immunomodulatory effects in humans is not fully understood. Nevertheless, in experimental models, immunization with HK *M. vaccae* was reported to generate $CD8^+$ T cells against *M. tuberculosis*-infected macrophages (Skinner et al., 1997) and to downregulate T helper type 2 (Th2) responses in murine models of allergic pulmonary inflammation via the induction of regulatory T cells (Zuany-Amorim et al., 2002) and the priming of dendritic cells (DCs) consistent with a regulatory profile (Adams et al., 2004). *In vitro* studies with human monocyte derived DCs have confirmed the ability of *M. vaccae* to dampen Th2 responses, via a mechanism dependent on DCs (Le Bert et al., 2011). Furthermore, *M. vaccae* and *M. obuense* have been shown to promote the anti-tumor activity of human γδT-cells via cytokine release from type-1 myeloid DCs (Fowler et al., 2012). *M. obuense* has gained attention over the past few years as a potential immunotherapeutic agent against cancer and, in a phase 1 study in melanoma patients, it was shown to be safe and well tolerated (Stebbing et al., 2012). More recently, in a phase 2 study, the use of *M. obuense* (NCTC13365) as adjunctive immunotherapy for advanced pancreatic cancer resulted in clinically meaningful increases in overall survival as well as in progression-free survival of patients (Dalgleish, A.G. and The IMAGE I Trial Investigators 2015). Currently, *M. obuense* (NCTC13365) is being investigated in a phase 2 study involving patients with melanoma (NCT01559818). The interactions between different immune cells and between immune cells and soluble factors are mediated by a number of cell surface receptors involved in cellular adhesion, antigen presentation and co-stimulation, cytokine-mediated signaling, and cellular activation (Barclay et al., 1997). Therefore, variations in the expression of these receptors would affect the immune cell function thus leading to the modulation of immune responses. Immune responses from healthy volunteers, assessed by the profile of released cytokines, have been extensively analyzed using short-term incubation of human whole blood with a large spectrum of microbial immunostimulants, immunomodulators, or specific antigens (Darcissac et al., 1996a,b; Duffy et al., 2014; Kassa et al., 2012; Reichenbach et al., 2006). The immunomodulatory properties of HK *M. vaccae* or *M. obuense* in the human whole blood model have not been previously evaluated. In this study, both HK mycobacterial preparations were assessed for their ability to modulate *in vitro* the expression of immunologically relevant leukocyte surface receptors and to alter cytokine and chemokine secretion in human whole blood. We have also addressed the role of toll-like receptors (TLRs) in mediating the immunomodulatory effects of *M. obuense*, and evaluated the ability of *M. vaccae* and *M. obuense* to induce lymphoproliferative responses in human peripheral blood mononuclear cells (PBMCs) from healthy volunteers.

2. Materials and methods

2.1. Antibodies

Mouse monoclonal anti-human antibodies purchased from BD Biosciences (San Jose, CA, USA) included: FITC-conjugated CD2 (clone RPA-2.10), CD11a (clone HI111), CD35 (clone E11), CD40 (clone 5C3), CD44 (clone L178), CD45 (clone 2D1), CD50 (clone TU41), CD64 (clone 10.1), CD80 (clone L307.4), CD95 (clone DX2), CD195 (clone 2D7/CCR5), HLA-DP,DQ,DR (clone Tu39); PE-conjugated CD11b (clone ICRF44), (clone WM59), CD36 (clone CB38), CD58 (clone 1C3), CD89 (clone A59), CD102 (clone CBR-1C2/2), CD114 (clone LMM741), CD116 (clone hGMCSFR-M1), CD119 (clone GIR-208), CD122 (clone TU27), CD127 (clone HIL-7R-M21), CD132 (clone AG184), CD137L (clone C65-485), CD206 (clone 19.2), PerCP-conjugated CD14 (clone M5E2), HLA-DR (clone G46-6); PE-Cy7-conjugated CD16 (clone 3G8); APC-conjugated CD5 (clone UCHT2), CD11c (clone B-ly6), CD18 (clone 6.7), CD21 (clone B-ly4), CD25 (clone M-A251), CD32 (clone FLI8.26), CD54 (clone HA58), CD62L (clone DREG-56), CD86 (clone 2331), CD184 (clone 12G5), HLA-A,B,C (clone G46-2.6); Alexa Fluor 647-conjugated CD197 (clone 3D12) and isotype control antibodies: FITC-conjugated mouse IgG1 (clone MOPC-21), IgG2a (clone G155-178), IgG2b (clone MPC-11); PE-conjugated mouse IgG1 (clone MOPC-21), IgG2a (clone G155-178), IgM (clone G155-228); PerCP-conjugated mouse IgG2a (clone X39); PE-Cy7-conjugated mouse IgG1 (clone MOPC-21); APC-conjugated mouse IgG1 (clone MOPC-21), IgG2a (clone G155-178), IgG2b (clone 27-35); Alexa Fluor 647 rat IgG2a (clone R35-95). PE-conjugated mouse monoclonal antibodies against human CD282 (clone TL2.1) and CD284 (clone HTA125) and isotype control antibody PE-conjugated mouse IgG2a (clone eBM2a) were obtained from eBioscience (San Diego, CA, USA). 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 isotype control human IgA2 (clone T9C6) and mouse IgG1 (clone T8E5) were purchased from Invivogen (Toulouse, France).

2.2. HK mycobacterial preparations

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

2.3. Blood collection

Human peripheral blood was obtained by venipuncture from 36 Caucasian healthy volunteers and was collected into K2 ethylene diamine tetraacetic acid (K2-EDTA) vacutainer tubes (BD Biosciences). The study population had a mean age of 28 ± 8.5 years and included 14 females and 22 males. A written informed consent

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