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ORIGINAL RESEARCH

Mycobacterium marinum: a potential immunotherapy for *Mycobacterium tuberculosis* infection

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Purpose: The aim of the present study was to investigate the immune response induced by *Mycobacterium marinum* infection in vitro and the potential of *M. marinum* as an immunotherapy for *M. tuberculosis* infection.

Methods: The potential human immune response to certain bacillus infections was investigated in an immune cell–bacillus coculture system in vitro. As a potential novel immunotherapy, *M. marinum* was studied and compared with two other bacilli, *Bacillus Calmette-Guérin* (BCG) and live attenuated *M. tuberculosis*. We examined the changes in both the bacilli and immune cells, especially the time course of the viability of mycobacteria in the coculture system and host immune responses including multinuclear giant cell formation by Wright–Giemsa modified staining, macrophage polarization by cell surface antigen expression, and cytokines/chemokine production by both mRNA expression and protein secretion.

Results: The *M. marinum* stimulated coculture group showed more expression of CD209, CD68, CD80, and CD86 than the BCG and *M. tuberculosis* (an attenuated strain, H37Ra) groups, although the differences were not statistically significant. Moreover, the *M. marinum* group expressed more interleukin (IL)-1B and IL-12p40 on day 3 (IL-1B: $P = 0.003$ and 0.004, respectively; IL-12p40: $P = 0.001$ and 0.011, respectively), a higher level of CXCL10 on day 1 ($P = 0.006$ and 0.026, respectively), and higher levels of chemokine (C-X-C motif) ligand (CXCL) 8 and chemokine (C motif) ligand (XCL) 1 on day 3 (CXCL8: $P = 0.012$ and 0.014, respectively; XCL1: $P = 0.000$ and 0.000, respectively). The *M. marinum* stimulated coculture group also secreted more tumor necrosis factor (TNF)-α, IL-1β, and IL-10 on day 1 (TNF-α: $P = 0.000$ and 0.000, respectively; IL-1β: $P = 0.000$ and 0.000, respectively; IL-10: $P = 0.002$ and 0.019, respectively) and day 3 (TNF-α: $P = 0.000$ and 0.000, respectively; IL-1β: $P = 0.000$ and 0.001, respectively; IL-10: $P = 0.000$ and 0.000, respectively). In addition, the colony-forming units (an index of viability) of *M. marinum* in the *M. marinum* stimulated coculture group was significantly less than that of BCG and H37Ra in their corresponding bacillus stimulated groups ($P = 0.037$ and 0.013, respectively).

Conclusion: Our results indicated that *M. marinum* could be a potentially safe and effective immunotherapy.

Keywords: immunotherapy, *Mycobacterium marinum,* bacilli, immunity

Introduction

Immunotherapy, via modulation of the host immune response to infection, tumor, and autoantigen in autoimmune diseases, has been increasingly applied in treatment of many diseases, especially in combination with other therapeutic approaches. Tuberculosis (TB) is a global infectious disease caused by *Mycobacterium tuberculosis* (Mtb) infection and is usually treated with a combination of different

antibiotics for more than 6 months. However, the recent appearance of drug resistant Mtb strains has led to treatment failure and increased incidence of TB worldwide.[1] Nowadays, immunotherapy is the focus of research for new effective treatment of TB. Cytokines and immune cells have been explored as potential immunotherapies with limited or disappointing efficacy.[2] Meanwhile, studies have shown that the prophylactic and therapeutic vaccines, including heat-killed and live attenuated mycobacteria, and DNA/proteins of mycobacterial origin, appear to be more safe and effective.[2–4] These vaccines have been used both as adjuvants for immunization and immunotherapies for several diseases such as Mtb infection and bladder cancer;[5–9] however, all of these vaccines were known to control, but not to eradicate, Mtb infection.[10,11] Theoretically speaking, mycobacterial priming vaccines of an improved whole organism could potentially induce a more efficient immune response than other vaccines such as those from bacterial components.[12] We chose to focus our study on *M. marinum* (Mm) considering its reported safety that (1) most exposures in humans do not result in infectious disease and (2) the infected individual only shows several skin lesions at local sites without dispersal to the entire body or severe systemic responses.[13] Thus Mm is possibly a safe whole organism vaccine that can potentially be used as immunotherapy to treat TB infection.

Host immunity against mycobacterial infections involves complex interactions between the bacterium and various components of the host immune system including immune cells, cytokines, and chemokines. Macrophages are the most important immune cells against mycobacterial infections. Macrophages can phagocytose the bacilli at the local sites but are unable to eradicate them.[14,15] Studies have shown that macrophages can polarize into two subtypes (M1 and M2) in response to infections, autoimmune diseases, and tumors.[16,17] However, the function of its polarization in mycobacterial infection remains unclear. In addition to the immune cells, several cytokines such as interferon (IFN)-γ, tumor necrosis factor (TNF)-α, and interleukin (IL)-12 play critical roles in immune reactions against mycobacterial infections.[18] Moreover, chemokines such as chemokine (C–C motif) ligand (CCL) 3 and 5, chemokine (C-X-C motif) ligand (CXCL) 2, 9, and 10, and chemokine (C motif) ligand (XCL) 1 were reported to be involved in the type 1 response to mycobacterial infection and the recruitment of monocytes in local sites to constitute granuloma formation

during mycobacterial infection.[19] Although many studies used animal models to study mycobacterial infection, extrapolation of results obtained in animals (eg, mice) to humans was usually difficult because the immune response could be drastically different in various species. In vitro studies using human cells do not have this issue and are useful tools for studying human immunity.[20] Various human immune cells such as human monocytes, macrophages, and peripheral blood mononuclear cells (PBMCs) have been examined in the study of infections caused by various bacteria such as Mtb, *Bacillus Calmette-Guérin* (BCG), *M. avium*, and Mm.[20,21] In mycobacterial infection, local macrophages that engulf the bacilli are the most important cells in the innate immune response which induce the recruitment of monocytes/macrophages and lymphocytes to local sites to constitute granuloma formation and stimulate adaptive immunity.[14]

In the present study, we used a macrophage, PBMC, and mycobacterium coculture system as a tool to study the immune response to mycobacterial infections in vitro. We examined various components of the immune response including immune cells, cytokines, and chemokines stimulated by Mm (TMC 1218), BCG (TMC 1010), and Mtb (H37Ra). We found that Mm (TMC 1218) induced more significant Th1 and Th2 cell immunity with better safety compared with BCG and Mtb. Our results suggested that Mm had potential value as an immunotherapy for TB patients.

Material and methods
Immune cells and bacteria
Peripheral blood was obtained from healthy Chinese donors with informed consent. In the People's Republic of China, BCG vaccination is compulsory for children (0–4 years). PBMCs were isolated using gradient centrifugation on Ficoll-Paque PREMIUM (GE Healthcare, Piscataway, NJ, USA), and monocytes were further enriched by the adherence method in AIM-V medium (Invitrogen, Grand Island, NY, USA). Macrophages were differentiated from monocytes using GM-CSF (Peprotech, Rocky Hill, NJ, USA) as previously described[22] and cultured for 5 days in RPMI 1640 medium and 20% fetal calf serum (HyClone, Waltham, MA, USA) at 37°C.

Mm (TMC 1218), BCG (TMC 1010), and Mtb (H37Ra) were cultured on modified were cultured on modifed Lowenstein-Jensen Medium Base. All bacteria were collected in Middlebrook 7H9 Broth (BD Difco, Sparks, MD, USA) and mixed with syringe needles. The bacteria were cultured with a serial dilution on modified Lowenstein-Jensen medium

and the viability was monitored by counting the colony-forming units (CFU) on the base.

Coculture of macrophages, PBMCs, and bacteria

Macrophages differentiated from monocytes were transferred into 24 well tissue culture plates (Corning, Tewksbury, MA, USA) (1×10^5 cells per well). Freshly prepared Mm, BCG, and H37Ra were subsequently added to each well with a multiplicity of infection (MOI) of 0.5 based on trial results with different MOIs. After 24 hours, autologous PBMCs were added into macrophages infected with bacteria (M+P+B) at a ratio of 5:1. Meanwhile, macrophages infected with different bacteria without PBMCs (M+B) and macrophages cocultured with PBMCs without bacteria (M+P) were included as controls. The cells were cultured for periods from 24 hours to 10 days at optimal growth temperatures of different bacilli (35°C for Mm and 37°C for the other two bacilli) with medium changed every other day.

Microscopy and cell examination

The cocultured cells were observed under an inverted microscope (Nikon, Chiyoda-ku, Tokyo, Japan). Photographs were taken with a Nikon capture system. Cells were stained with Wright–Giemsa (W–G) modified staining (Sigma-Aldrich, St Louis, MO, USA) according to the manufacturer's instructions after 1, 3, and 7 days cultivation, and multinuclear giant cells (MGCs) with different numbers of nuclei were observed and counted using a light microscope up to a fixed total number of cells.

Analysis of cell surface antigens by flow cytometry

Cells were collected after 1 and 7 days cultivation and the expression of cell surface antigens on macrophages, especially those associated with macrophage polarization, was analyzed using a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA, USA). Dead cells stained positive with 7-amino actinomycin D stain were excluded from the analysis. The monoclonal antibodies (mAbs) used in the antigen analysis included fluorescein isothiocyanate-conjugated mAb against CD68 (Y1/82A), phycoerythrin-conjugated mAb against CD163 (GHI/61) and CD80 (L307.4), and PerCP-CyTM 5.5-conjugated mAb against CD209 (DCN46), CD86 (FUN-1), and CD14 (M5E2) from BD Biosciences. The percentages in specific quadrants indicated the ratio of the cells stained positively with the variable antigens.

Real time polymerase chain reaction (RT-PCR) and ELISA assays

The expression of different cytokines and chemokines was detected by RT-PCR. RNAs were extracted using RNeasy Mini kit (QIAGEN, Düsseldorf, NW, Germany) and reverse transcribed by a reverse transcription system (Promega, Madison, WI, USA). RT-PCR was performed using SYBR Green PCR Master Mix (Promega) according to the manufacturer's instructions. The mRNA expression was determined on the ABI 7300 instrument with the ABI software (Applied Biosystems, Grand Island, NY, USA). Various cytokines in the culture supernatants including IFN-γ, TNF-α, IL1-β, IL-4, IL-10, and IL-12p70 were quantified using human double-antibody sandwich indirect ELISA (enzyme-linked immunosorbent assay) kits (NeoBioscience, Shenzhen, Guangdong, People's Republic of China). The quantities of cytokines were presented in terms of protein concentration (pg/mL).

Determination of bacterial viability

The three bacteria were recovered from the macrophages with 0.1 M sodium hydroxide and their viability was determined by culturing on Middlebrook 7H10 Agar (BD Bioscience). Mm was cultured at 32°C, and BCG and H37Ra at 37°C.

Statistical analysis

All experiments were carried out in triplicate. The relative mRNA expression of target genes was calculated with the $2^{-\Delta\Delta Ct}$ method. Data were analyzed using SPSS 13.0 (SPSS Inc, Chicago, IL, USA). The statistical significance of the data was analyzed using univariate ANOVA and the general linear model. Error bars represent the standard deviations of the triplicate values.

Results
Granuloma-like aggregate formation

When macrophages infected by Mm, BCG, or H37Ra were cocultured with autologous PBMCs (M+P+B-M, M+P+B-B, and M+P+B-H) in a 24 well tissue culture plate, some cells aggregated to form granuloma-like aggregates on day 7 as shown in Figure 1A. No aggregates were observed in the M+P and M+B control groups at this stage (Figure 1B and C). The cellular structure of the granuloma-like aggregates resembled that observed in histopathologic specimens of the tuberculoid leprosy lesion in which lymphocytes were on the outskirts surrounding the cellular aggregates (Figure 1D). The sizes of cellular aggregates in the three M+P+B groups showed no visible differences under the microscope.
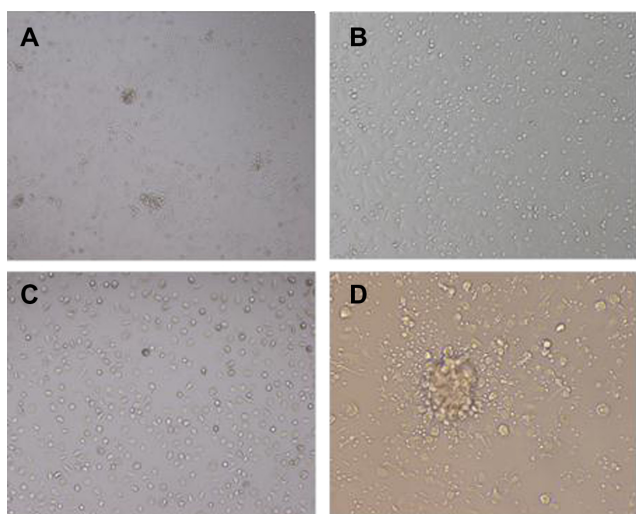
Figure 1 Granuloma-like aggregates formed by cocultivation of three different bacilli and two kinds of immune cells.
Notes: (**A**) Cells in M+P+B groups aggregated to form granuloma-like aggregates on day 7 (×100). (**B**) Cells in the M+P control group formed no aggregates at this stage (×100). (**C**) Cells in M+B control groups formed no aggregates at this stage (×100). (**D**) Granuloma formed in M+P+B experimental groups showing lymphocytes surrounding the unedged cell aggregates (×400). The group M+P, M+P+B, and M+B refer to the control group without bacilli, experimental groups, and control groups without peripheral blood mononuclear cells, respectively, while the bacilli B, M, and H refer to BCG, M. marinum, and H37Ra, respectively.

MGC formation

The cells were collected after 1, 3, and 7 days cocultivation, plated on glass slides, and stained with W–G modified staining for examination under the microscope. It was observed that several cells aggregated and fused in the M+P+B groups (Figure 2A). A significant difference was observed between the M+P+B-M and M+B-M groups ($P = 0.039$). While most MGCs formed in the three M+P+B groups had two nuclei, the MGCs in the M+P+B-M group on average had more nuclei than those in the M+B-M group ($P = 0.023$) (Figure 2C). On day 7, there were more MGCs observed in the M+P+B-M group than in the M+P+B-B and M+P+B-H groups, and the MGCs in the M+P+B-M group showed a higher number of nuclei as well, although the differences were not statistically significant ($P = 0.266$ and 0.334 for the number of MGCs, respectively; $P = 0.230$ and 0.444 for the number of nuclei per MGC, respectively) (Figure 2B and C).

Cell surface antigen expression

The expression of macrophage/monocyte cell surface antigens was investigated on days 1 and 7. Seven antigens were detected in the study, which were grouped into three types: monocyte (CD14), M1 type (CD68, 80, and 86), and M2 type (163, 206, and 209). On day 1, no significant differences were detected in the expression of most antigens between any two groups.

Cells in the M+P+B (-B, -M, and -H) groups seemed to express more CD86 on day 1 and less CD163 on day 7 than cells in the M+P group (CD86: $P = 0.041$, 0.029, and 0.041, respectively; CD163: $P = 0.003$, 0.000, and 0.001, respectively) (Table 1). However, on day 7, cells in the M+B (-B, -M, and -H) groups expressed less CD209 and CD80 than cells in both the M+P+B (-B, -M, and -H) groups (CD209: $P = 0.037$, 0.007 and 0.042, respectively; CD80: $P = 0.002$, 0.002 and 0.006, respectively) and M+P groups (CD209: $P = 0.001$, 0.000 and 0.000, respectively; CD80: $P = 0.000$, 0.000 and 0.000, respectively). On day 7, cells in the M+P+B-M group showed more CD206, CD209, CD68, CD80, and CD86 expression than cells in the M+P+B-B and M+P+B-H groups (Table 1). However, no significant differences in the antigens were observed between any two of the M+P+B groups.

mRNA expression of cytokines and chemokines

The cellular mRNA expression of the cytokines TNF-α, IFN-γ, IL-12p40, and IL-1B was detected by RT-PCR. The TNF-α expression in the M+P+B groups peaked on day 3 with a level higher in the M+P+B-M group than in the M+B-M group ($P = 0.017$). The IFN-γ expression in the M+P+B groups also peaked on day 3 with a much higher expression in the M+P+B-M group than in the M+P and M+B-M groups ($P = 0.000$ and 0.000, respectively). The M+P+B-H group also had a higher IFN-γ expression than the M+P and the M+B-H groups ($P = 0.000$ and 0.000, respectively). Among the three M+P+B groups, the M+P+B-M and the M+P+B-H groups exhibited higher IFN-γ expression than the M+P+B-B group ($P = 0.000$ and 0.000, respectively). On day 3 and 7, the M+P+B-M group expressed higher levels of IL-12p40 than the M+P+B-B and M+P+B-H groups (day 3: $P = 0.001$ and 0.011, respectively; day 7: $P = 0.001$ and 0.000, respectively). The IL-1B expression was highest on day 1 in the M+P+B groups, and higher IL-1B expression was observed in the M+P+B-M group when compared with the M+P, M+B-M, M+P+B-B, and M+P+B-H groups on day 3 ($P = 0.021$, 0.000, 0.003, and 0.004, respectively) (Table 2 and Figure 3A).

Expression of chemokines was investigated by detecting their mRNA levels on day 1 and 3. On day 1, cells in the M+P+B-M group showed higher levels of CCL3, CCL5, CXCL2, CXCL8, CXCL9, CXCL10, and XCL1 than cells in the M+B-M group ($P = 0.031$, 0.005, 0.000, 0.004, 0.002, 0.000, and 0.036, respectively). On day 3, cells in the M+P+B-M group expressed higher levels of CCL5, CXCL2, CXCL8, CXCL10, and XCL1 than cells in the M+B-M group ($P = 0.023$, 0.012, 0.001, 0.005, and 0.000, respectively).

A
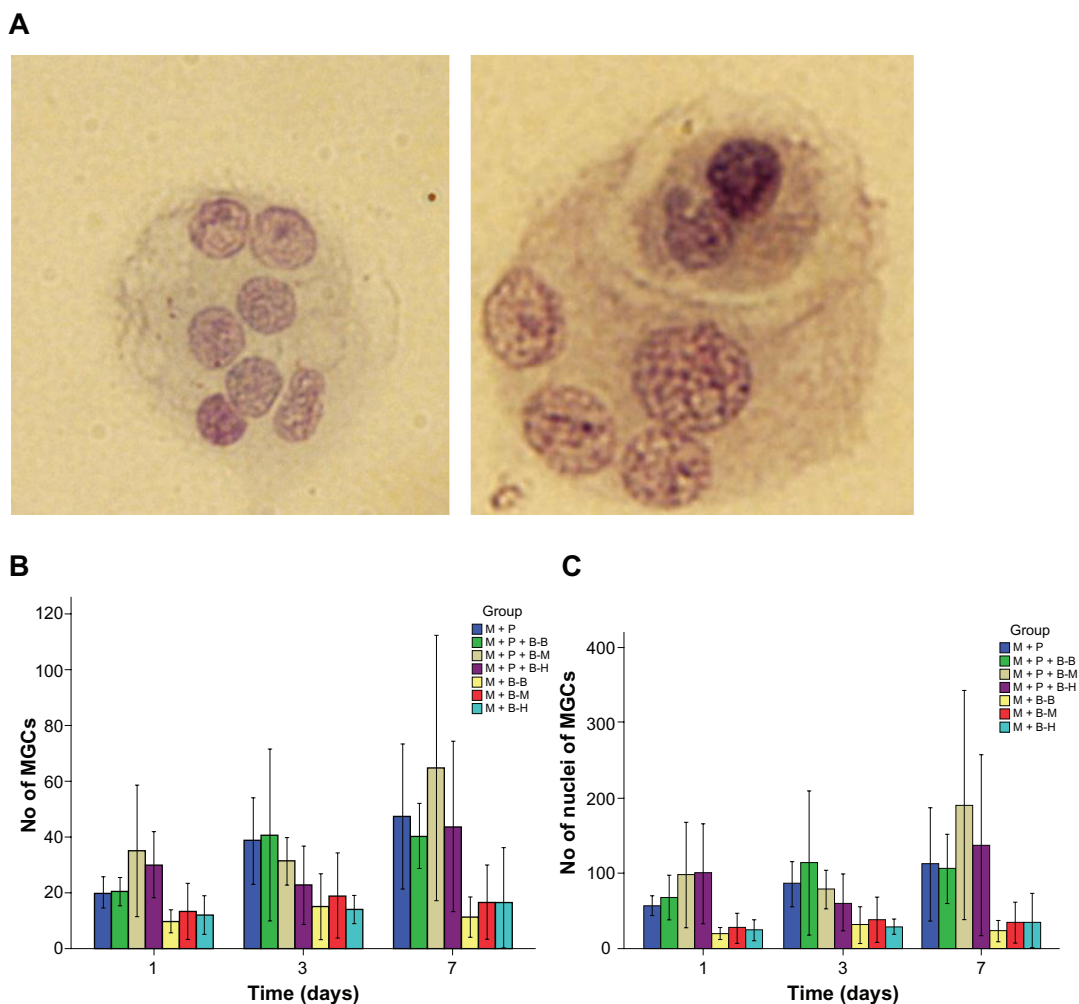

B

C

Figure 2 Characterization of multinuclear giant cells (MGCs) formed after stimulation with different bacilli.
Notes: (**A**) Images of MGCs formed in the study (×400). (**B**) Error bars showing the number of MGCs formed in various groups. (**C**) Error bars showing the total number of nuclei in the MGCs formed in various groups. The group M+P, M+P+B, and M+B refer to the control group without bacilli, experimental groups, and control groups without peripheral blood mononuclear cells, respectively, while the bacilli B, M, and H refer to *Bacillus Calmette-Guérin*, *M. marinum*, and H37Ra, respectively.

Table 1 The percentage of antigen (CD163, CD206, CD209, CD68, CD80, and CD86) positive cells in various groups on day 1 and day 7 (%)

Antigens	Days	Group						
		M+P	M+P+B-B	M+P+B-M	M+P+B-H	M+B-B	M+B-M	M+B-H
CD163	1D	4.480	5.460	4.353	4.700	4.378	3.425	5.062
	7D[f]	48.470	16.763[a,b]	9.260[a]	10.255[a]	5.680	6.408	8.160
CD206	1D	42.452	43.187	42.137	42.650	42.295	42.325	42.962
	7D[f]	77.792	45.632[a]	52.782	44.525[a]	41.415	37.238	45.038
CD209	1D	20.737	24.518	24.365	24.003	21.655	21.185	22.205
	7D	42.240	30.848[b]	37.250[b]	34.510[b]	12.215	12.053	16.362
CD68	1D	5.540	7.347	5.917	4.340	8.217	5.397	4.977
	7D[f]	7.807	11.413	19.520	14.490	9.740	12.087	10.897
CD80	1D	52.410	63.570	67.058	59.825	56.780	62.385	57.610
	7D	79.032	71.230[b]	80.830[b]	75.805[b]	42.968	48.605	45.210
CD86	1D	79.273	89.103[a]	89.927[a]	89.133[a]	87.493	88.917	88.757
	7D[f]	75.683	81.953	89.243	87.697	81.453	80.363	80.327

Notes: Superscript represents significant statistical difference between certain groups with *P* value below 0.05. [a]M+P+B compared with M+P; [b]M+P+B compared with M+B; [c]M+P+B-M or M+P+B-H compared to M+P+B-B; [d]M+P+B-B or M+P+B-H compared to M+P+B-M; [e]M+P+B-B or M+P+B-M compared to M+P+B-H; [f]the values on various days compared with day 1.
Abbreviations: B, *Bacillus Calmette-Guérin*; D, days; H, H37Ra; M+P, the control group without bacilli; M+P+B, experimental groups; M+B, control groups without peripheral blood mononuclear cells; M, *M. marinum*.

Table 2 The relative mRNA expression kinetics of different cytokines on day 1, 3, and 7

Cytokines	Days	Group						
		M+P	M+P+B-B	M+P+B-M	M+P+B-H	M+B-B	M+B-M	M+B-H
TNF-α	1D	1.000	2.387[a,b,d,e]	1.446[c]	1.345[c]	0.696	1.686	0.6
	3D[f]	3.082	5.660	6.563[b]	6.327[b]	1.76	0.867	1.446
	7D[f]	0.73	3.473[b]	3.889[a,b]	5.844[a,b]	0.35	0.539	1.051
IFN-γ	1D	1.000	17.201[a,b]	14.052[a]	14.358[a,b]	0.701	9.046	1.739
	3D[f]	2.671	32.758[d,e]	123.056[a,b,c]	145.237[a,b,c]	25.724	2.398	1.867
	7D[f]	6.154	23.857[b,e]	11.228[e]	69.651[a,b,c,d]	2.596	7.731	19.702
IL-1β	1D	1.000	1.643[b]	1.626[b]	0.983[b]	0.008	0.033	0.009
	3D[f]	0.556	0.354[d]	1.044[a,b,c,e]	0.394[d]	0.032	0.014	0.004
	7D[f]	1.273	0.516[a]	0.419[a]	0.596[a,b]	0.01	0.111	0.007
IL-10	1D	1.000	1.821[b]	1.450[b]	1.009	0.143	0.189	0.179
	3D	3.631	0.930[a]	1.736	1.194[a]	1.022	0.376	0.731
	7D	1.364	0.912	1.278	1.443	1.028	0.592	0.951
IL-12p40	1D	1.000	2.288[a,b]	1.636	1.590[b]	0.442	1.547	0.505
	3D	4.822	0.352[a,d]	4.790[b,c,e]	1.650[a,d]	0.635	0.550	0.417
	7D[f]	2.393	5.553[d]	22.237[a,b,c,e]	2.527[d]	0.211	10.289	1.346

Notes: Superscript represents significant statistical difference between certain groups with *P* value below 0.05. [a]M+P+B compared with M+P; [b]M+P+B compared with M+B; [c]M+P+B-M or M+P+B-H compared to M+P+B-B; [d]M+P+B-B or M+P+B-H compared to M+P+B-M; [e]M+P+B-B or M+B-M compared to M+P+B-H; [f]the values on various days compared with day 1.
Abbreviations: B, *Bacillus Calmette-Guérin*; D, days; H, H37Ra; IFN, interferon; IL, interleukin; M+P, the control group without bacilli; M+P+B, experimental groups; M+B, control groups without peripheral blood mononuclear cells; M, *M. marinum*; TNF, tumor necrosis factor.

Cells in the M+P+B-M group demonstrated higher levels of CCL5, CXCL2, and CXCL9 on day 1 (*P* = 0.035, 0.002, and 0.000, respectively), and CXCL10 and XCL1 on day 1 and 3 than cells in the M+P group (day 1: *P* = 0.000 and 0.007, respectively; day 3: *P* = 0.014 and 0.000, respectively). On day 1, cells in the M+P+B-M and M+P+B-B groups expressed higher CXCL2 levels than cells in the M+P+B-H group (*P* = 0.002 and 0.001, respectively). When compared with cells in the M+P+B-B and M+P+B-H groups, cells in the M+P+B-M group expressed a higher level of CXCL10 on day 1 and higher levels of CXCL8 and XCL1 but a lower level of CXCL9 on day 3 (CXCL10: *P* = 0.006 and 0.026, respectively; CXCL8: *P* = 0.012 and 0.014, respectively; XCL1: *P* = 0.000 and 0.000, respectively; CXCL9: *P* = 0.005 and 0.028, respectively) (Table 3 and Figure 3B).

Cytokines in the culture supernatants

Cytokines were also detected by ELISA in the supernatants of various cell cultures on days 1, 3, and 7. In the M+P+B groups, TNF-α and IL-1β secretion peaked on day 1, IFN-γ was higher on days 3 and 7, whereas IL-10 showed higher levels on day 1 and 3 than on day 7. The culture supernatants from the M+P+B-M group contained higher levels of TNF-α, IL-1β, and IL-10 than those from the M+P, M+B-M, M+P+B-B, and M+P+B-H groups on days 1 and 3 (day 1, TNF-α: *P* = 0.000, 0.001, 0.000, and 0.000, respectively; IL-1β: *P* = 0.000, 0.000, 0.000, and 0.000, respectively; IL-10: *P* = 0.001, 0.001, 0.002, and 0.019, respectively; day 3, TNF-α: *P* = 0.000, 0.001, 0.000, and 0.000, respectively; IL-1β: *P* = 0.000, 0.000, 0.000, and 0.001, respectively; IL-10: *P* = 0.000, 0.000, 0.000, and 0.000, respectively). On day 1, a higher level of IFN-γ was observed in the culture supernatant from the M+P+B-M group than those from the M+P and M+B-M groups (*P* = 0.012 and 0.039, respectively). No significant differences in IFN-γ secretion were found between the M+P+B-M and the M+P+B-H group on day 1 and 3. However, higher IFN-γ secretion was observed in the M+P+B-H group when compared with the M+P+B-B group (*P* = 0.048 and 0.012, respectively) (Table 4 and Figure 4). The amount of IL-12/23 was surprisingly low in the supernatants of all groups (IL-12 < 1.5 pg/mL, IL-23 < 4 pg/mL); nonetheless, IL-12/23 levels were slightly higher in the culture supernatant from the M+P+B-M group (data not shown). The IL-4 levels in the culture supernatants were below the detection level of ELISA (data not shown).

Viability of bacilli in granuloma-like aggregates

We determined the viability of Mm, BCG, and H37Ra released from phagocytes on days 1, 3, and 7 by counting the CFUs on the 7H10 medium. The number of CFUs of Mm in the M+P+B-M group was less than that of BCG in the M+P+B-B group and H37Ra in the M+P+B-H group (*P* = 0.037 and 0.013, respectively). The viability of Mm from the M+P+B-M group dropped more significantly when compared with that of BCG and H37Ra from the other two groups. Surprisingly, the
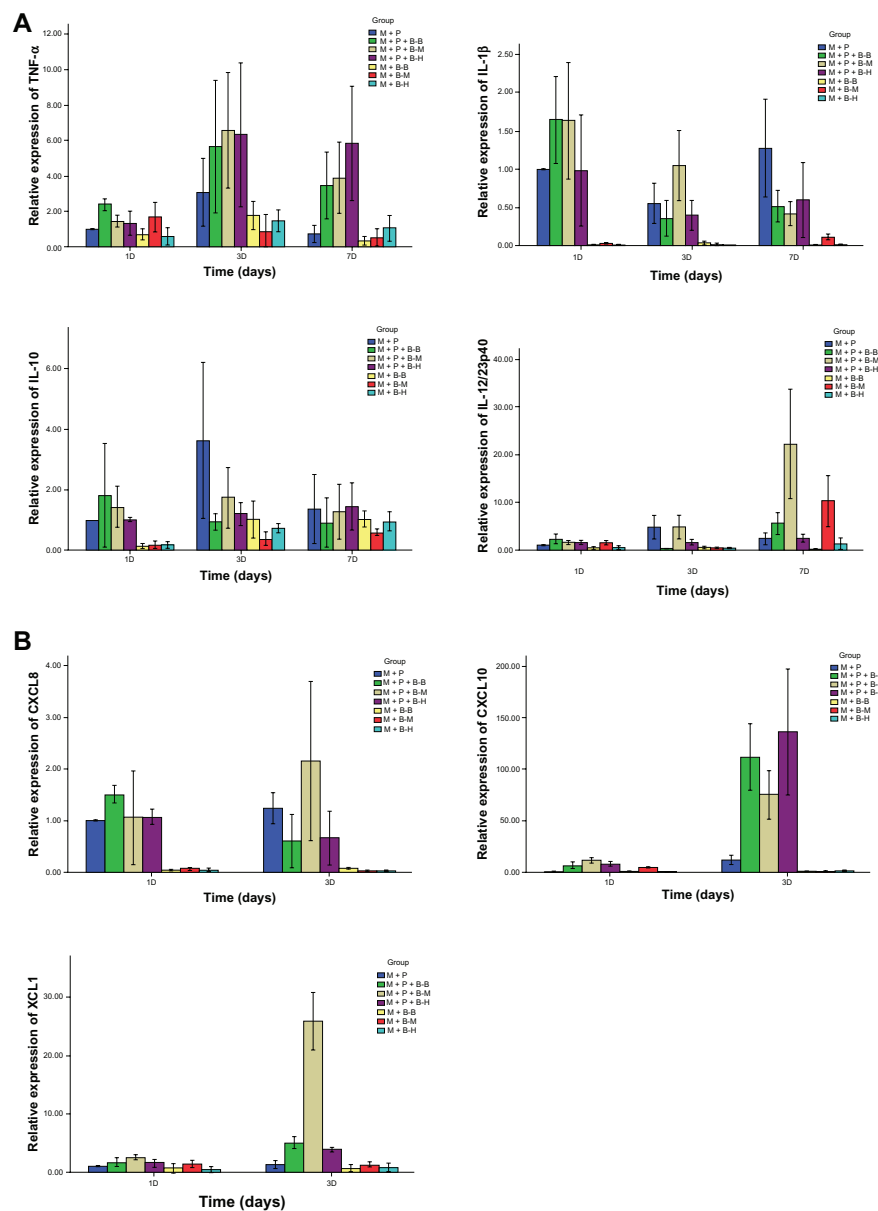
Figure 3 The mRNA expression kinetics of cytokines and chemokines was detected by RT-PCR.
Notes: (**A**) The mRNA expression of different cytokines (tumor necrosis factor-α, interleukin (IL)-1β, IL-10, and IL-12p40) was detected on day 1, 3, and 7. (**B**) The mRNA expression of different chemokines (chemokine (C-X-C motif) ligand [CXCL] 8, CXCL10, and chemokine (C motif) ligand 1) was detected on day 1 and 3. The group M+P, M+P+B, and M+B refer to the control group without bacilli, experimental groups, and control groups without peripheral blood mononuclear cells, respectively, while the bacilli B, M, and H refer to *Bacillus Calmette-Guérin*, *M. marinum*, and H37Ra, respectively.
Abbreviations: CXCL, chemokine (C-X-C motif) ligand; IL, interleukin; TNF, tumor necrosis factor; XCL, chemokine (C motif) ligand; RT-PCR, real time polymerase chain reaction.

viability of BCG and H37Ra gradually increased with cocultivation time following an almost identical ascending trend. No significant difference in the viability of Mm was observed between the M+P+B-M and M+B-M groups (Figure 5).

Discussion

Mm is the most common nontuberculous mycobacterium, causing skin infection with no vector in humans. Most Mm exposure in humans does not cause infectious diseases.

Despite causing various lesions on the skin of extremities, Mm infections rarely induce a severe systemic response. No fatal cases have thus far been reported.[13] These observations indicate that Mm is able to induce a sufficient host immune response while being relatively safe in humans, endowing it potential value as an agent for immunotherapy. All current immunotherapies for tuberculosis, while being able to reduce the bacterial load, fail to eradicate the bacteria. New and more effective therapies are needed for better therapeutic outcome.

Table 3 The relative mRNA expression kinetics of different chemokines on day 1 and day 3

CKs	Days	Group						
		M+P	M+P+B-B	M+P+B-M	M+P+B-H	M+B-B	M+B-M	M+B-H
CCL5	1D	1.000	1.529[b]	1.477[b]	0.887[b]	0.334	0.476	0.220
	3D	0.559	1.123	1.935[a,b]	1.281	0.250	0.433	0.296
CXCL2	1D	1.000	2.364[a,b,e]	2.445[a,b,e]	0.920[b,c,d]	0.068	0.091	0.061
	3D[f]	1.162	0.657	1.204[b]	0.436	0.074	0.059	0.095
CXCL8	1D	1.000	1.511[b]	1.060[b]	1.076[b]	0.041	0.068	0.048
	3D	1.241	0.611[d]	2.162[b,c,e]	0.659[d]	0.082	0.022	0.023
CXCL9	1D	1.000	10.633[a]	18.635[a,b]	17.518[a,b]	8.354	4.183	2.732
	3D	13.753	128.434[a,b,d]	50.261[c,e]	107.808[a,b,d]	17.261	1.976	3.422
CXCL10	1D	1.000	7.262[a,b,d]	11.985[a,b,c,e]	8.385[a,b,d]	0.642	5.322	0.486
	3D	12.137	111.953[a,b]	75.709[a,b,e]	136.675[a,b,d]	0.799	0.988	1.792
XCL1	1D	1.000	1.639	2.528[a,b]	1.528[b]	0.612	1.411	0.360
	3D	1.177	4.925[a,b,d]	25.888[a,b,c,e]	3.879[d]	0.561	1.220	0.750

Notes: Superscript represents significant statistical difference between certain groups with P value below 0.05. [a]M+P+B compared with M+P; [b]M+P+B compared with M+B; [c]M+P+B-M or M+P+B-H compared to M+P+B-B; [d]M+P+B-B or M+P+B-H compared to M+P+B-M; [e]M+P+B-B or M+P+B-M compared to M+P+B-H; [f]the values on various days compared with day 1.
Abbreviations: B, *Bacillus Calmette-Guérin*; CCL5, chemokine (C-C motif) ligand; CXCL, chemokine (C-X-C motif) ligand; CK, chemokines; D, days; H, H37Ra; M+P, the control group without bacilli; M+P+B, experimental groups; M+B, control groups without peripheral blood mononuclear cells; M, *M. marinum*; XCL, chemokine (C motif) ligand.

Research on Mm and other potential vaccines may lead to the discovery of more effective immunotherapies for better disease control.

In the present study, we used an in vitro system of macrophage, PBMC, and mycobacterium cocultivation as a tool to study the immune response to infections caused by Mm in comparison with BCG and H37Ra. The safety of the bacilli was also tested in these in vitro systems by measuring bacterial viability during cocultivation. We found that Mm was the safest among the three bacilli, exhibiting the lowest viability after cocultivation with human immune cells. The viability of bacilli in the host in the presence of host immune cells determines the severity of infection. Culturing results in our study demonstrated that Mm from the Mm-stimulated M+P+B-M

and M+B-M groups exhibited much lower CFUs than BCG and H37Ra from their corresponding bacillus-stimulated groups. These differences were statistically significant on day 1, 3, and 7. The CFUs of Mm declined throughout the cocultivation period and approached zero on day 7, whereas those of the other two bacilli gradually increased. Our results indicated that Mm infection could be well controlled in our in vitro model while BCG and H37Ra could not.

Mm induced macrophage polarization to both M1 and M2 phenotypes. Monocytes/macrophages can be polarized into two subsets, the classical (M1) and the alternative (M2) one, in response to infections, solid tumors, and autoantigens in autoimmune diseases.[23] Previous studies have indicated that M1 exhibits CD80 and CD86 on the

Table 4 The protein level of tumor necrosis factor-α, interferon-γ, interleukin (IL)-1β, and IL-10 on day 1, 3, and 7 (pg/mL)

Cytokines	Days	Group						
		M+P	M+P+B-B	M+P+B-M	M+P+B-H	M+B-B	M+B-M	M+B-H
TNF-α	1D	55.576	110.640[d]	520.694[a,b,c,e]	161.803[d]	42.997	247.482	47.260
	3D[f]	38.609	83.103[d]	425.465[a,b,c,e]	129.440[d]	27.393	194.385	38.570
	7D[f]	26.235	226.587[a,b]	299.847[a,b,e]	160.386[b,d]	24.905	114.936	20.020
IFN-γ	1D	0.722	13.830[e]	27.773[ab]	34.219[a,b,c]	6.877	6.301	5.233
	3D[f]	6.206	282.822[e]	442.367[a]	839.651[a,b,c]	3.574	27.018	3.348
	7D[f]	4.778	581.242	397.056[e]	1184.000[a,b,d]	6.083	9.248	0.569
IL-1β	1D	100.429	269.302[d]	1162.000[a,b,c,e]	426.270[a,b,d]	0.450	4.889	1.021
	3D[f]	59.247	188.445[d]	842.345[a,b,c,e]	333.163[a,b,d]	0.272	6.350	0
	7D[f]	74.175	213.014[d]	743.801[a,b,c,e]	291.789[d]	2.661	6.631	0.495
IL-10	1D	1.442	4.262[d]	19.761[a,b,c,e]	8.866[d]	0.684	1.840	0.327
	3D	1.105	5.673[b,d]	17.280[a,b,c,e]	6.463[a,b,d]	0.518	0.937	0.682
	7D[f]	2.768	3.157[b]	2.982[b]	2.886[b]	0.931	0.831	0.407

Notes: Superscript represents significant statistical difference between certain groups with *P* value below 0.05. [a]M+P+B compared with M+P; [b]M+P+B compared with M+B; [c]compared with M+P+B-B; [d]compared with M+P+B-M; [e]compared with M+P+B-H; [f]the values on various days compared with day 1.
Abbreviations: B, *Bacillus Calmette-Guérin*; D, days; H, H37Ra; IL, interleukin; M+P, the control group without bacilli; M+P+B, experimental groups; M+B, control groups without peripheral blood mononuclear cells; M, *M. marinum*; TNF, tumor necrosis factor.
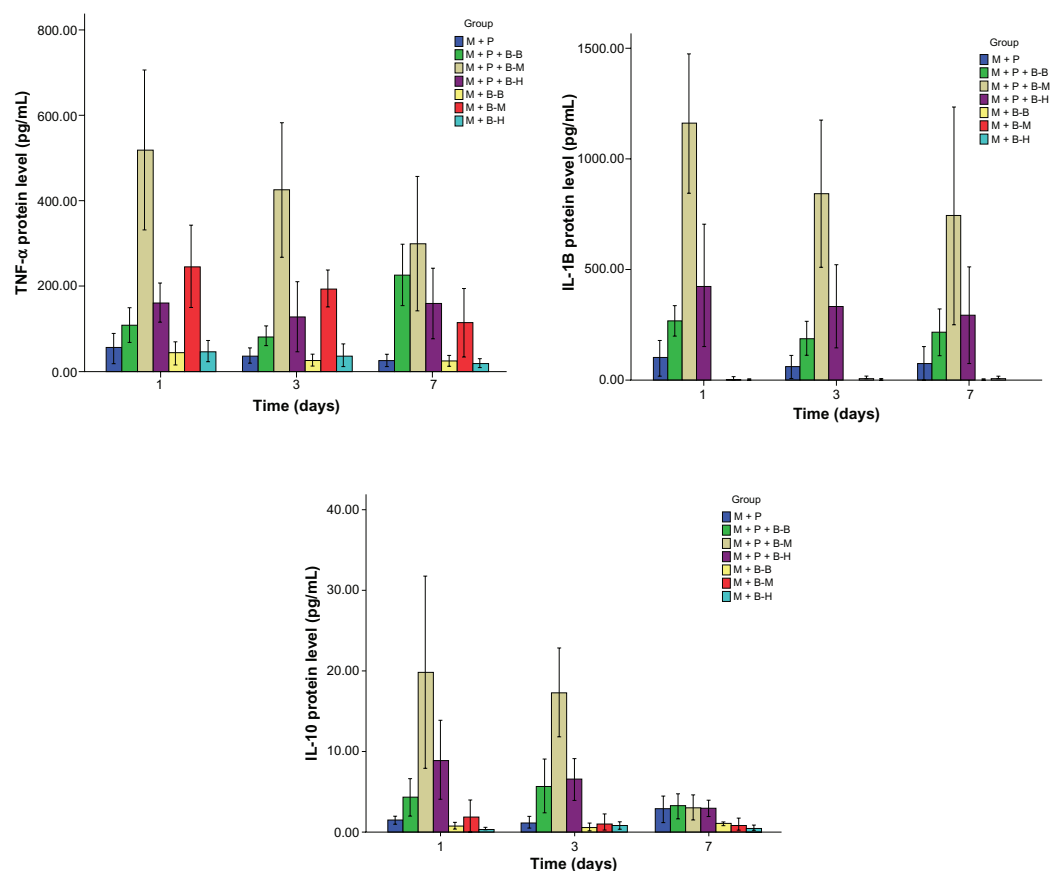
Figure 4 The levels of cytokines secreted in the culture supernatant were detected by ELISA on day 1, 3, and 7.
Notes: The group M+P, M+P+B, and M+B refer to the control group without bacilli, experimental groups, and control groups without peripheral blood mononuclear cells, respectively, while the bacilli B, M, and H refer to *Bacillus Calmette-Guérin*, *M. marinum*, and H37Ra, respectively.
Abbreviations: IL, interleukin; TNF, tumor necrosis factor; ELISA, enzyme-linked immunosorbent assay.

cell surface, whereas M2 expresses CD163, CD206, and CD209 which are diverse among various subtypes of the M2 phenotype.[24] However, unique or restrictive markers for identification of different subsets of macrophages have not been fully determined. In the present study, we investigated the polarization of macrophages by measuring several CD molecules on the cell surface. Previous studies validated the function of the M1 subtype against various infections,[14,16,23,25] which remained unconfirmed against Mtb infection. In our study, the M1 and M2 macrophages seemed to coexist with a fine balance in the bacillus-stimulated and control groups in the early phase of infection. As the infection progressed, the expression of M2 type antigens increased at a slower rate while the M1 type increased at a faster rate in the M+P+B groups when compared with the M+P group, suggesting that mycobacterial infection gradually stimulated the M1 and inhibited the M2 polarization of macrophages. Notably, Mm induced higher expression of CD80, CD86, CD206, and CD209 than the other two bacilli, suggesting that Mm promoted both M1 and M2 programming more effectively.

Since M1 and M2 macrophage polarization has been found to be analogous to Th1 and Th2 division of CD4+ T cells in a previous study,[17] our findings suggested that Mm could potentially be more effective in inducing both Th1- and Th2-type immune reactions than the other two bacilli. The lack of statistical difference might be attributed to the limited samples in the study.

Mm also induced higher production of TNF-α, IL1β, IL-10, and higher IL-12p40 expression than the other two bacilli in the M+P+B groups. TNF-α was found to be associated with host resistance to various species of mycobacteria such as Mtb, *M. bovis*, and Mm, and TNF-α deficiency caused defects in phagocyte activation, chemokine expression, and influx of various cells into the granuloma.[17,21] TNF-α was also found to be associated with long-term maintenance of granuloma and control of bacterial growth,[26,27] and has been used as a marker of immune response against mycobacterial infections.[28] Among the three M+P+B groups, the M+P+B-M group showed highest TNF-α secretion throughout the cocultivation experiment. In addition, the M+P+B-M
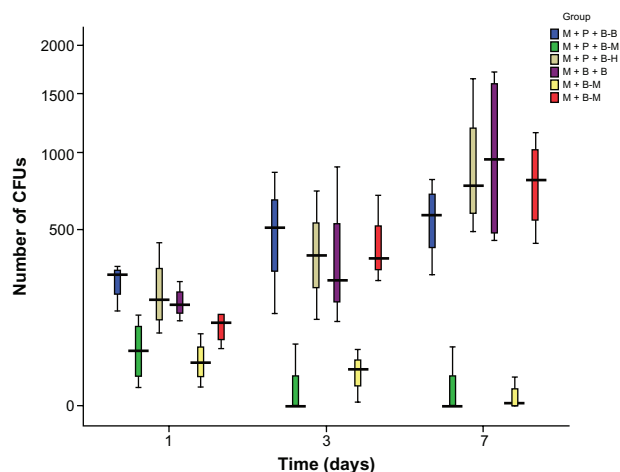
Figure 5 The viability of bacilli in various groups on day 1, 3, and 7. The number of colony-forming units on the agar was counted.
Notes: The group M+P, M+P+B, and M+B refer to the control group without bacilli, experimental groups, and control groups without peripheral blood mononuclear cells, respectively, while the bacilli B, M, and H refer to *Bacillus Calmette-Guérin*, *M. marinum*, and H37Ra, respectively.
Abbreviation: CFU, colony-forming unit.

group also had the highest TNF-α mRNA expression on day 3 among the three M+P+B groups. The observation that the M+P+B-M group, which had the highest mRNA expression and protein secretion of TNF-α, showed the lowest bacillus viability suggested that TNF-α played a significant role in controlling bacillus infection in our in vitro model.

The IL-1 family of cytokines, especially IL-1β, is also required for host resistance against mycobacterial infection. IL-1B deficient mice demonstrated acute susceptibility to Mtb.[29] In the present study, the M+P+B-M group also showed higher IL-1B mRNA expression and protein secretion than the M+P+B-B and M+P+B-H groups. Similar results were observed in levels of IL-12, another pro-inflammatory and Th1-type cytokine induced by mycobacterial stimulation. IL-12p40 is rapidly induced after Mtb, or its components are ligated with Toll-like receptor TLR2 and TLR9 in vitro.[18,30] In this study, the IL-12p40 mRNA expression was higher in the M+P+B-M group on days 3 and 7 than in the other two M+P+B groups (*P* < 0.05). However, the level of secreted IL-12p70 was surprisingly low in the supernatants. The IL12p40 expression was higher when compared with the low secretion level of IL-12p70. Our results confirmed the bioactivity of IL-12p40 rather than IL-12p70, and previous studies indicated both the existence of free IL-12p40 subunit and formation of IL-12p40 homodimers (known as IL-12p80) in mycobacterial infection,[31,32] which in this study needed further investigation.

The three aforementioned cytokines (TNF-α, IL-1β, and IL-12) are known to be associated with the Th1 immune response against various mycobacteria. Our cytokine expression results indicated that Mm induced a stronger Th1 immune response than BCG and H37Ra in our in vitro model. Mm unexpectedly induced increased production of IL-10. IL-10 is a typical anti-inflammatory and Th2 cytokine that modulates the immune response to mycobacteria. Elevated IL-10 was observed in TB patients and and TB-infected mice.[33,34] IL-10 was also reported to reduce the expression of both TNF-α and IL-12/23 p40.[33,34] In the present study, although the mRNA expression of IL-10 was lower in the M+P+B groups than in the M+P group on day 3, IL-10 secretion appeared to be easily detectable on day 1 and 3 in both the M+P+B and M+P groups, and cells in the M+P+B groups secreted more IL-10 than cells in the M+P group at the early stage of infection. Among the three M+P+B groups, cells in the M+P+B-M group secreted much more IL-10 than cells in the M+P+B-B and M+P+B-H groups. Taken together, our results have shown that mycobacterial infection induced stronger Th1 and Th2 type immune responses at the early stage of infection, highlighting the importance of balance between pro- and anti-inflammatory responses. Other studies have also stressed the fine balance between TNF-α and IL-10 (the TNF-α/IL-10 ratio) in determining the protective effect against TB.[18] The balance between TNF-α and IL-10 was maintained in the M+P+B-M group with higher levels of both cytokines induced by Mm.

Mm also caused higher expression of chemokines including CXCL8, CXCL10, and XCL1. Chemokines present at appropriate levels may prevent cells from migrating out of the granuloma, and hence, contribute to the maintenance of granuloma.[35] They have been demonstrated to be associated with mycobacterial infections. CXCL8 can induce neutrophil chemotaxis in TB patients, and exogenous CXCL8 reduced Mtb survival, while its inhibition caused Mtb proliferation.[36] In leprosy patients, mycobacterium-induced CXCL8 was 2 to 3 fold lower than in TB patients.[37] Increased chemokine levels of CCL3, 4, 5, and 12 and CXCL9 and 10 were also found in Mtb infected mice.[35] XCL1 was reported to be involved in chronic infection of Mtb and suppressed the induction of IFN-γ,[38] which might partly explain the lack of high levels of IFN-γ in the M+P+B-M group. Chiu et al proposed the hypothesis that expression patterns of chemokines were associated with upstream cytokine signaling.[39] They found that transcripts of CXCL10 and XCL1 were largely enhanced by Th1-related cytokines, which is consistent with our hypothesis that Mm might induce a more significant Th1 response.

Conclusion

In the present study, Mm was found to induce higher production of important cytokines (TNF-α, IL1β, and IL-10) and chemokines (CXCL8, CXCL10, and XCL1), suggesting that Mm may significantly induce both Th1 and Th2 cellular immunity. In contrast to BCG and H37Ra, Mm completely lost its viability in the cocultivation system with the immune cells, demonstrating the effective and superior immune response induced by Mm. Our findings also suggested that human exposure to Mm would be more tolerable than exposure to the same amount of BCG and H37Ra, implying that Mm could be superior to BCG and H37Ra with regard to the immunotherapy associated safety profile.[11,12] The fact that Mm infections rarely lead to severe systemic involvement indicates that the immune system of a healthy human is able to provide sufficient defense against this specific pathogen. The safety of Mm can be attributed to its unique characteristics such as the proteins of the proline–glutamic acid (PE) and the proline–proline–glutamic acid (PPE) families mentioned in the previous studies.[40] However, further studies on the pathogenesis of Mm needs to be conducted to verify and extend our findings. The possible roles that various components of the bacillus play in inducing an immune response are not clear and require investigation. Our future research is to focus on the study of pathogenesis of Mm.

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Disclosure

The authors report no conflict of interest in this work.

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