The viable *Mycobacterium tuberculosis* H37Ra strain induces a stronger mouse macrophage response compared to the heat-inactivated H37Rv strain

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Abstract. Macrophages are the target cells for *Mycobacterium tuberculosis* (*M. tuberculosis*) as well as key effector cells for clearance of this pathogen. The aim of the present study was to measure and compare the responses of mouse peritoneal macrophages following exposure to the live *M. tuberculosis* H37Ra and heat-inactivated H37Rv strains. *In vitro* phagocytosis assays indicated that the macrophages had a higher capacity to engulf the live H37Ra strain compared to the inactivated H37Rv strain. Enzyme-linked immunosorbent assay (ELISA) demonstrated that H37Ra-stimulated macrophages produced significantly increased concentrations of interleukin-12p40 (IL-12p40), tumor necrosis factor-α (TNF-α) and interferon-γ (IFN-γ) compared to the untreated control cells. However, H37Rv exposure induced little to no increase in the levels of the cytokines examined. The results from ELISA were confirmed by reverse transcription-polymerase chain reaction (RT-PCR) at the mRNA level. There was a dose-dependent increase in nitric oxide (NO) and hydrogen peroxide (H_2O_2) production from the H37Ra-stimulated macrophages compared to the H37Rv-stimulated ones. Confocal microscopy and flow cytometric analysis indicated that the IFN-γ-stimulated macrophages from viable H37Ra-immunized mice had an enhanced surface expression of CD40 ligand (CD40L) compared to those from inactivated H37Rv-immunized mice. Our data collectively indicate that exposure to the viable H37Ra strain induces a stronger macrophage response compared to exposure to the heat-inactivated H37Rv strain, which may be associated with the increased surface expression of CD40L in activated macrophages.

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Introduction

Macrophages comprise an essential part of the innate immune system and provide a first line of defense against microbial infections (1). However, due to their highly phagocytic properties, macrophages are often targeted by pathogenic bacteria. They may even be exploited as host cells by some microorganisms such as *Mycobacterium tuberculosis* (*M. tuberculosis*) and *Rickettsia akari* (2,3). To avoid becoming a reservoir of infection, a number of defense mechanisms have evolved to either kill or prevent the growth of bacteria.

Upon stimulation from bacterial antigens, macrophages are activated and, in turn, release a wide range of cytokines, including inferferon-γ (IFN-γ), tumor necrosis factor-α (TNF-α), as well as interleukin (IL)-1, -6 and -12 (4). IFN-γ is a typical T helper 1 cytokine that is known as a key cytokine for protective immunity against *M. tuberculosis* (5). This cytokine stimulates nitric oxide (NO) production in macrophages leading to the clearance of invading pathogens. The impairment of IFN-γ synthesis by T cells has been found to enhance susceptibility to tuberculosis in complement C5-deficient mice (6). TNF-α also acts as a protective cytokine in the resistance to *M. tuberculosis* (7). It has been shown that IFN-γ, TNF-α and IL-18 cooperate to control the growth of *M. tuberculosis* in human macrophages (8).

The release of reactive nitrogen (RNI) and reactive oxygen intermediates (ROI) is an additional important mechanism for macrophages to combat invading pathogens. These mechanisms kill bacteria by damaging macromolecules such as bacterial DNA. Inducible NO synthase-deficient mice, which do not produce RNI, and phagocyte oxidase-deficient mice, which do not produce ROI, are more susceptible to *M. tuberculosis* infection compared to wild-type mice (9,10).

The attenuated *M. tuberculosis* H37Ra strain and its virulent counterpart, H37Rv, are derived from the parent strain H37, which was originally isolated from a 19-year-old male patient with chronic pulmonary tuberculosis by Edward R. Baldwin in 1905 (11). Over the past decades, studies on the virulence of *M. tuberculosis* have frequently involved comparisons of the H37Rv and H37Ra strains. Comparative genomic analysis has indicated genetic differences between the 2 strains, providing extensive insight into the basis of

Table I. Sequences of PCR primers used in this study.

Gene	Sequence	Product size (bp)
IL-12p40	F: 5'-TGCTGGTGTCTCCACTCATG-3' R: 5'-CCAAGGCACAGGGTCATCATC-3'	302
TNF-α	F: 5'-CTGAGACAGAGCCTGCCTTA-3' R: 5'-GTCTGAGAGCCGAAGACTGA-3'	449
IFN-γ	F: 5'-AGGCCATCAGCAACAACATAAGTG-3' R: 5'-GACAGCTTTGTGCTGGATCTGTG-3'	140
GAPDH	F: 5'-AGGGCCGGTGCTGAGTATGTC-3' R: 5'-TGCCTGCTTCACCACCTTCT-3'	530

the attenuation of virulence in H37Ra (12). However, there is limited knowledge available as regards their ability to initiate the macrophage activation program. Thus, the aim of the present study was to measure and compare the responses of mouse peritoneal macrophages following exposure to the live H37Ra or heat-inactivated H37Rv strains. Since CD40 signaling has been well established to participate in macrophage activation (13,14), we investigated whether exposure to the H37Ra and H37Rv strains affects the surface expression of CD40 ligand (CD40L) in activated macrophages.

Materials and methods

Animals, bacterial strains and cell culture. Specific-pathogen-free, 6 to 8-week-old BALB/c mice (weighing 18±2 g) were purchased from the Laboratory Animal Center, Chongqing Medical University (Chongqing, China) and housed in a pathogen-free environment. All the experiments involving animals were approved by the Institutional Animal Care and Use Committee of Chongqing Medical University.

The *M. tuberculosis* H37Ra strain (ATCC 25177) was obtained from the Department of Immunology (Chongqing Medical University), and the H37Rv strain (ATCC 27294) was obtained from the Central Laboratory of Genetic Diagnosis of Tuberculosis of Chongqing (Chongqing, China). Bacterial cultures were grown in Middlebrook 7H9 medium (BioMerieux, La Balme-les-Grottes, France) at 37˚C for 2-3 weeks. The mid-log phase cultures were pelleted, resuspended in sterile saline containing 0.05% Tween-80, and titered. Heat-killed H37Rv was prepared by heating the bacteria at 80˚C for 5 min. Suspensions of bacteria were then supplemented with 5% glycerol and stored at -80˚C until use.

Macrophage isolation and infection. Peritoneal macrophages were isolated from 10 normal BALB/c mice as previously described (15). Briefly, isolated peritoneal cells were pelleted, resuspended in RPMI-1640 medium (HyClone, Logan, UT, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Gibco, Carlsbad, CA, USA), and plated onto 24-well plates. After a 4-h culture at 37˚C, the non-adherent cells were removed and adherent cells (macrophage-rich population) were maintained in fresh culture medium. For phagocytosis assay, macrophages were seeded onto 24-well plates at a density of $5x10^5$ cells/well. The cells were infected with a multiplicity of infection of 10:1 (10 bacteria to 1 cell) for 12 h. The ingested bacteria were detected using acid-fast staining. The percentage of phagocytosis and the phagocytic index were determined by counting 400 macrophages/glass slide under a light microscope. The percentage of phagocytosis was defined as the percentage of macrophages containing ≥1 ingested bacterium. The phagocytic index was the mean number of bacteria ingested/macrophage. For secretion assay, macrophages seeded at a density of $5x10^5$ cells/well were added with 5-65 CFU/ml bacteria and incubated for 24 h. The culture supernatants were collected and assessed for the production of cytokines, NO and hydrogen peroxide (H_2O_2).

Enzyme-linked immunosorbent assay (ELISA). The levels of IFN-γ, TNF-α and IL-12p40 in the culture supernatants were measured using ELISA kits (eBioscience, San Diego, CA, USA) according to the manufacturer's instructions.

Measurement of NO and H_2O_2. NO levels were determined with the Griess method using a commercially available kit (Jiancheng Bioengineering Institute, Nanjing, China) according to the manufacturer's instructions. The concentrations of H_2O_2 were measured using standard biochemical methods with a commercially available kit (Jiancheng Bioengineering Institute).

Immunization. BALB/c mice were intraperitoneally injected with an equal volume of physical saline (used as the control; n=10), H37Ra (n=10) or H37Rv suspension (n=10). Thirty days following immunization, the animals were sacrificed and peritoneal macrophages were collected as described above. Isolated macrophages were subjected to gene expression analysis by reverse transcription-polymerase chain reaction (RT-PCR), flow cytometry and confocal microscopy.

RNA isolation and RT-PCR. Total RNA was isolated from the macrophages using a total RNA extraction kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Reverse transcription was performed with the First-Strand cDNA Synthesis kit (Takara, Dalian, China). PCR amplification was conducted under the following conditions: initial denaturation at 94˚C for 5 min, followed by 28 cycles of denaturation at 94˚C for 30 sec, annealing at 55-62˚C for 30-45 sec, and elongation at 72˚C for 1 min. The PCR primers used in this study are listed in Table I. PCR products were sepa-

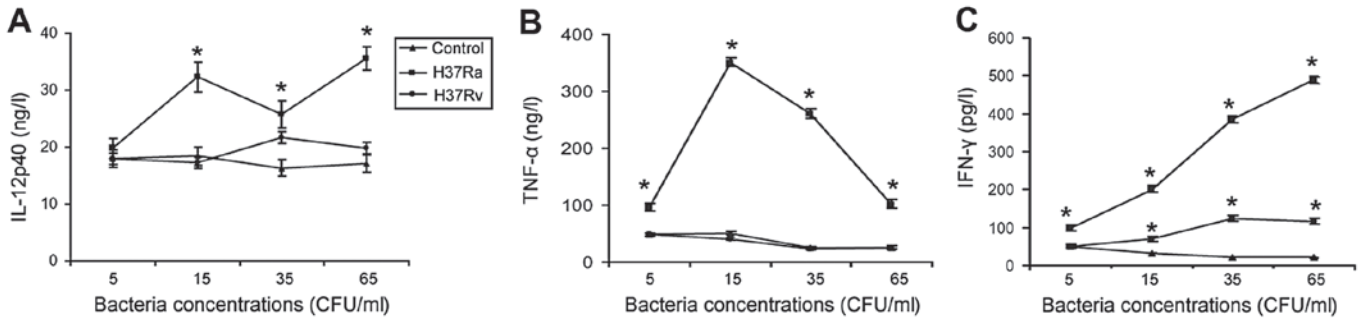

Figure 1. Analysis of the levels of (A) IL-12p40, (B) TNF-α and (C) IFN-γ released from mouse peritoneal macrophages stimulated with the viable H37Ra strain or the heat-inactivated H37Rv strain by ELISA. Each assay was repeated at least 3 times. *P<0.05 compared to the non-stimulated control cells.

rated on a 1.2% agarose gel, stained with ethidium bromide, and photographed.

Flow cytometry. Peritoneal macrophages isolated from immunized mice were incubated with recombinant mouse IFN-γ (10 ng/ml) for 4 h. The macrophages were detached, incubated with fluorescein isothiocyanate (FITC)-labeled anti-mouse CD40L (eBioscinence) at 4˚C for 30 min, and immediately examined for the surface expression of CD40L by flow cytometry (FACSCalibur; Becton-Dickinson, San Jose, CA, USA).

Immunofluorescent staining and confocal microscopy. Macrophages were stimulated with IFN-γ (10 ng/ml) for 4 h as described above, seeded onto coversplips, and cultured for an additional 24-36 h in fresh culture medium. Following washing, the cells were fixed with 4% paraformaldehyde for 15 min, blocked in bovine serum albumin (BSA) for 1 h, and incubated with goat anti-mouse CD40L (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) at 4˚C overnight, followed by incubation with FITC-labeled secondary antibodies for 1 h. The cells were then mounted in 50% glycerol and analyzed using a LEICA TCS SP2 confocal microscope (Leica Microsystems GmbH, Wetzlar, Germany).

Statistical analysis. All statistical analyses were performed using SPSS.11 software (SPSS, Inc., Chicago, IL, USA). Data are expressed as the means ± standard deviation (SD). Significant differences between 2 groups were determined using the Student's t-test. The difference among the means of multiple groups was analyzed by one-way analysis of variance (ANOVA) followed by Tukey's test. P<0.05 was considered to indicate a statistically significant difference.

Results

Ingestion of viable H37Ra and heat-inactivated H37Rv by macrophages. *In vitro* phagocytosis assay demonstrated that mouse peritoneal macrophages had a higher capacity to engulf the viable H37Ra strain compared to the heat-inactivated H37Rv strain, as evidenced by the increased percentage of phagocytosis and the phagocytic index (51.7±20.0% vs. 29.6±3.9% and 0.72±0.31 vs. 0.53±0.15, respectively; Table II).

Cytokine release by H37Ra- and H37Rv-stimulated macrophages. Cytokine measurements using ELISA indicated

Table II. Phagocytic activity of macrophages exposed to the viable H37Ra or heat-inactivated H37Rv strain.

	M. tuberculosis strain	
Phagocytic activity	H37Ra	H37Rv
% Phagocytosis	51.7±20.0	29.6±3.9[a]
Phagocytic index	0.72±0.31	0.53±0.15[a]

[a]P<0.05 compared to macrophages exposed to the H37Ra strain. Percentage phagocytosis was defined as the percentage of macrophages containing ≥1 ingested bacterium. Phagocytic index was the mean number of bacteria ingested/macrophage.

that viable H37Ra-stimulated mouse macrophages produced significantly increased concentrations of IL-12p40 and TNF-α compared to the control cells (P<0.05) (Fig. 1). However, there was no significant difference in the release of the 2 cytokines from the H37Rv-stimulated macrophages and the control cells. Additionally, stimulation with either the viable H37Ra strain or the inactivated H37Rv strain yielded a dose-dependent increase in IFN-γ secretion, with more significant changes being observed in the macrophages exposed to H37Ra (Fig. 1).

RT-PCR analysis further demonstrated the viable H37Ra-induced mRNA expression of IL-12p40, TNF-α and IFN-γ in macrophages (Fig. 2). There was also a modest increase in IFN-γ mRNA expression in the macrophages exposed to the inactivated H37Rv strain compared to the control cells. However, IL-12p40 and TNF-α mRNA expression remained stable upon stimulation with inactivated H37Rv.

H37Ra- and H37Rv-stimulated release of NO and H_2O_2 from macrophages. Exposure to the viable H37Ra strain resulted in a dose-dependent increase in the release of NO and H_2O_2 from the macrophages, with 9- and 7-fold changes observed between the highest and lowest concentrations, respectively (Fig. 3). By contrast, there was little to no increase in NO and H_2O_2 production from the macrophages stimulated with the inactivated H37Rv strain.

Expression of surface CD40L in macrophages following exposure to H37Ra and H37Rv. IFN-γ-stimulated peritoneal macrophages from mice immunized with the viable H37Ra

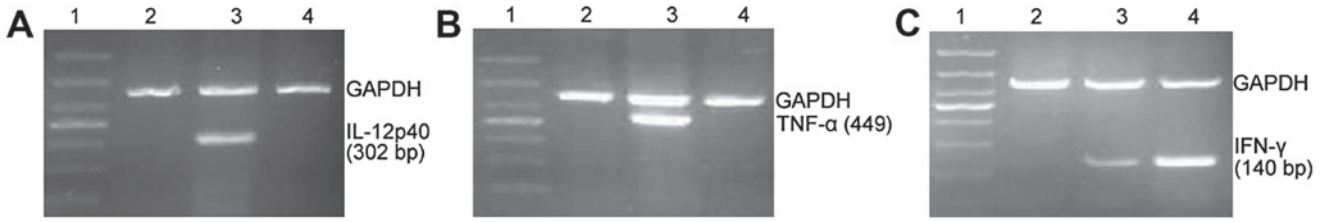

Figure 2. Analysis of the mRNA expression of (A) IL-12p40, (B) TNF-α and (C) IFN-γ in mouse peritoneal macrophages exposed to the viable H37Ra strain or the heat-inactivated H37Rv strain by RT-PCR. Representative gel images of the RT-PCR products of 3 independent experiments with similar results are shown. GAPDH was used as the loading control. Lane 1, molecular marker; lane 2, control; lane 3, stimulation with viable H37Ra (in A and B) or inactivated H37Rv (in C); lane 4, stimulation with inactivated H37Rv (in A and B) or viable H37Ra (in C).

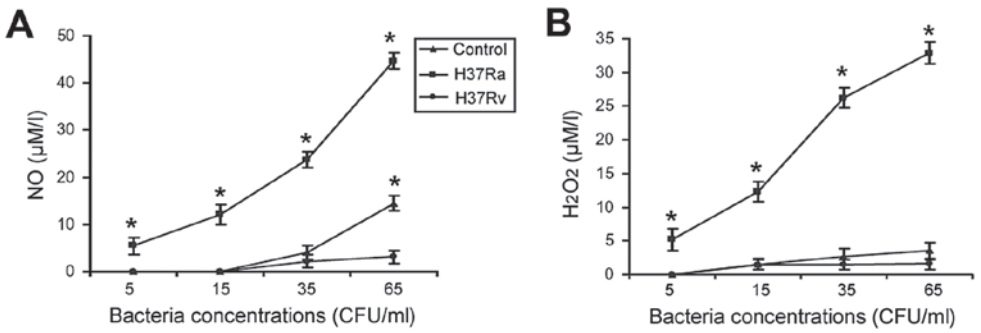

Figure 3. Measurement of the release of (A) nictric oxide (NO) and (B) hydrogen peroxide (H_2O_2) from mouse peritoneal macrophages stimulated with the viable H37Ra or heat-inactivated H37Rv strain. Each assay was repeated at least 3 times. *P<0.05 compared to non-stimulated control cells.

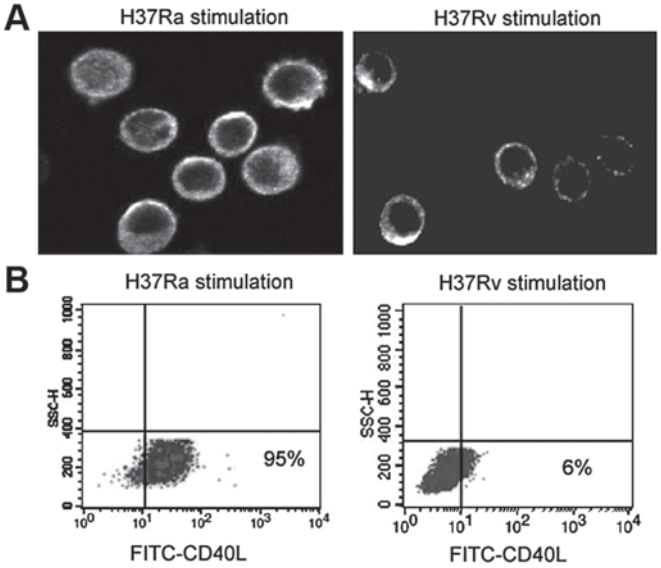

Figure 4. Examination of surface CD40L expression in IFN-γ-stimulated peritoneal macrophages from mice immunized with the viable H37Ra or heat-inactivated H37Rv strain. (A) Confocal microscopy (magnification, x800) and (B) flow cytometric analysis consistently indicated an enhanced surface expression of CD40L in macrophages from viable H37Ra-immunized mice compared to inactivated H37Rv-immunized mice. The percentage of surface CD40L-positive macrophages is indicated.

strain showed an enhanced surface expression of CD40L compared to those from mice exposed to the inactivated H37Rv strain (Fig. 4A). Flow cytometric analysis further demonstrated that the majority (95%) of the IFN-γ-stimulated macrophages exposed to the H37Ra strain displayed a strong surface expression of CD40L, while only 6% of the IFN-γ-stimulated macrophages exposed to the H37Rv strain displayed CD40L surface expression (Fig. 4B).

Discussion

Toll-like receptors (TLRs), as a family of pattern-recognition receptors, are pivotal mediators of the recognition of pathogens by the innate immune system (16). They discriminate between chemically diverse classes of microbial components. Our data demonstrated that the live H37Ra strain was more readily recognized and internalized by mouse peritoneal macrophages compared to the heat-inactivated H37Rv strain. This finding may reflect genetic differences between the H37Ra and H37Rv strains (12). Alternatively, the specific TLR recognition patterns for *M. tuberculosis* may be altered by heating stimuli, thus affecting the ability of the macrophages to sense and internalize the H37Rv strain. This is indirectly supported by a previous study according to which heat-inactivated and live group B streptococcus strains initiate distinct response pathways in macrophages to activate antibacterial host defense (17).

During infection with *M. tuberculosis*, host macrophages provide the preferred environment for mycobacterial growth and also serve as pivotal effector cells responsible for the clearance of the pathogen or killing the bacteria. IFN-γ and TNF-α represent the key macrophage-activating cytokines in *M. tuberculosis* infection. IL-12 plays a key role in cell-mediated immune responses and stimulates the production of IFN-γ from T cells (18). Our data demonstrate that exposure to the viable H37Ra strain markedly promotes the secretion of IL-12p40, TNF-α

and IFN-γ from mouse peritoneal macrophages. Moreover, the mRNA levels of the 3 cytokines were increased in the viable H37Ra-stimulated macrophages, indicating regulation at the transcriptional level. The regulation of gene expression in response to *M. tuberculosis* stimulation has also been described in a previous study (19). By contrast, stimulation with the heat-inactivated H37Rv strain caused little to no increase in the levels of these cytokines in the macrophages. The poor cytokine induction by heat-inactivated H37Rv is consistent with the lower phagocytic capacity of the macrophages to ingest this *M. tuberculosis* strain. These findings demonstrate that the live H37Ra strain is a stronger inducer of macrophage activation compared to the inactivated H37Rv strain.

It is noteworthy that unlike the other 2 cytokines examined, the amount of TNF-α released from macrophages peaked when $15x10^7$ CFU/ml *M. tuberculosis* was used, followed by a gradual decline at higher concentrations of mycobacteria. This may reflect a negative feedback mechanism on TNF-α secretion. TNF-α is clearly essential in fighting against pathogen infection. TNF-α blockade is associated with the reduced response to vaccination (20). However, the excessive production of TNF-α by macrophages has potentially adverse effects, since it may initiate cell apoptosis (21,22). Keane *et al* (23) demonstrated that at low multiplicities of infection, attenuated *M. tuberculosis* strains induce the apoptosis of human alveolar macrophages by promoting TNF-α release and activating the extrinsic apoptotic pathway. Therefore, an optimal TNF-α level plays an important role in host defense against pathogen invasion.

NO and H_2O_2 are 2 primary antimicrobial effectors produced by activated macrophages. In a murine model of *M. tuberculosis* infection, RNI including NO were established to be responsible for macrophage-mediated killing and the growth inhibitory effect of virulent *M. tuberculosis* (24). Jackett *et al* (25) suggested that H_2O_2 production by macrophages is involved in killing *M. tuberculosis in vivo*. They found that the exposure of macrophage monolayers to phorbol myristate acetate and opsonized H37Ra increased the release of H_2O_2. In line with these previous studies, our data demonstrated that exposure to the viable H37Ra strain stimulated the release of NO and H_2O_2 from the peritoneal macrophages in a dose-dependent manner. By contrast, exposure to the inactivated H37Rv strain caused little to no production of either NO or H_2O_2 from macrophages. These results further confirm the potential induction of macrophage activation by the viable H37Ra strain as opposed to the inactivated H37Rv. It has been demonstrated that IFN-γ stimulates macrophages to produce NO and H_2O_2 (26,27). In agreement with these previous studies, we noted that there were similar alteration patterns for the levels of IFN-γ, NO and H_2O_2 following exposure to the H37Ra strain.

Compelling evidence points toward the importance of CD40 signaling in the development of protective immunity. It has been reported that CD40 deficiency predisposes mice to *Mycobacterium avium* infection, which is associated with the impaired production of IL-12p40 and IFN-γ (28). Lazarevic *et al* (29) demonstrated that CD40-deficient mice succumbed to low-dose aerosol infection with *M. tuberculosis* due to deficient IL-12 production, leading to impaired T cell IFN-γ responses. Depressed CD40L expression has been found to contribute to reduced IFN-γ production in human tuberculosis (30). Our data indicated an enhanced surface expression of CD40L in macrophages exposed to the viable H37Ra strain, which provided an explanation for the increased secretion of IL-12p40 and IFN-γ from the activated macrophages. In addition to the classical IFN-γ-dependent pathway, CD40 ligation may directly induce the antimicrobial activity of macrophages against an intracellular pathogen (31,32). These results suggest that the increased surface expression of CD40L and the activation of the CD40 pathway are involved in the live H37Ra-induced macrophage activation. However, the exact mechanism for the H37Ra-induced upregulation of CD40L remains to be fully ellucidated in future studies.

In conclusion, our data demonstrate that exposure to the viable H37Ra strain as opposed to the heat-inactivated H37Rv strain induces a potent macrophage response, which is associated with the enhanced surface expression of CD40L in activated macrophages. These findings warrant further investigation of the prophylactic potential of a H37Ra-based vaccine in the treatment of tuberculosis.

Acknowledgements

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