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# *Orientia tsutsugamushi* Infection Induces CD4$^+$ T Cell Activation *via* Human Dendritic Cell Activity

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*Orientia tsutsugamushi*, a gram-negative bacterium, causes severe acute febrile illness in humans. Despite this danger, the route of infection, infectivity, and protective mechanisms of the host's immune response to *O. tsutsugamushi* are unclear. Dendritic cells (DCs) are one of the most important cell types in bridging the innate and adaptive immune responses. In this study, we observed that *O. tsutsugamushi* infects and replicates in monocyte-derived DCs (MODCs). During infection and replication, the expressions of the cytokines IL-12 and TNF-α, as well as the co-stimulatory molecules CD80, CD83, CD86, and CD40, were increased in MODCs. When *O. tsutsugamushi*-treated MODCs were co-cultured with autologous CD4$^+$ T cells, they enhanced production of IFN-γ, a major Th1 cytokine. Collectively, our results show that *O. tsutsugamushi* can replicate in MODCs and can simultaneously induce MODC maturation and increase proinflammatory cytokine levels in MODCs that subsequently activate CD4$^+$ T cells.

**Keywords:** *Orientia tsutsugamushi*, dendritic cells, T cell, proinflammatory cytokines

## Introduction

*Orientia tsutsugamushi* is an obligate intracellular bacterium that is the causative agent of scrub typhus, which is an acute febrile and fatal disease characterized by fever, rash, eschar, pneumonitis, and meningitis in humans [17, 35]. It has been reported that a virulent strain of *O. tsutsugamushi* can be accompanied by hemorrhage and intravascular coagulation, which, in untreated cases, can lead to severe multi-organ failure [14].

Scrub typhus is a skin infection that is initiated when the host is bitten by an *O. tsutsugamushi*-infected trombiculid mite [15]. After infection, it is likely that dendritic cells (DCs) in the skin play an important sentinel role, because they are responsible for initial antigen-specific immune responses. Although several studies have investigated *O. tsutsugamushi* infections in macrophages and endothelial cells [20, 30], little is known about the effect of *O. tsutsugamushi* in DCs, particularly in the human immune system.

DCs are the major antigen-presenting cells (APCs) and play an important role in bridging innate and adaptive immune responses by presenting specific antigenic molecules to T cells [7, 28]. DC maturation occurs through multiple

steps, including antigen uptake, migratory activity, expression of co-stimulatory molecules, and cytokine and chemokine secretion; through this pathway, DCs eventually gain the ability to activate T cells [12, 23].

It is possible that *O. tsutsugamushi* could evade the action of DCs during infection, as has been documented in other cell types [27]. Although a large number of pathogens can persist inside DCs, the mechanisms by which *O. tsutsugamushi* is internalized and survives in human DCs are not clearly understood. The induction of immune responses against *O. tsutsugamushi* in human DCs has not been thoroughly investigated. Thus, the main purpose of the present study was to examine the induction of innate immune responses in human DCs treated with *O. tsutsugamushi* and the ability of those DCs to activate CD4$^+$ T cells.

## Materials and Methods

### Cell Culture

L929 (a mouse fibroblast cell line) and ECV304 (an immortalized human umbilical vein endothelial cell line) were obtained from the American Type Culture Collection (Rockville, MD, USA) and cultured in DMEM and Medium 199 (Gibco BRL, USA), respectively. The media were supplemented with 10% fetal bovine serum (FBS; Gibco BRL), 5 mM L-glutamine, penicillin (100 U/ml), and streptomycin (100 μg/ml), and the cells were cultured in an incubator with a humidified atmosphere containing 5% $CO_2$.

### Propagation and Preparation of *O. tsutsugamushi*

*O. tsutsugamushi* (Boryong strain) was propagated in the monolayers of L929 cells as previously described [4]. Briefly, when more than 90% of the cells were infected (as measured by an indirect immunofluorescence antibody assay (IFA)), the cells were collected, homogenized using a glass Dounce homogenizer (Wheaton Inc., USA), and centrifuged at 500 ×*g* for 5 min. The supernatant was stored in liquid nitrogen until use. The infectivity titer of the inoculum was determined using an IFA. Briefly, the bacterial stock was serially diluted and inoculated onto a L929 cell layer on a 12 mm glass coverslip in a 24-well tissue culture plate. After the cells were infected with *O. tsutsugamushi* for 6 h at 34°C, the culture medium was removed. The cells were washed with phosphate-buffered saline (PBS), fixed in cold 100% acetone for 15 min at -20°C, stained, and measured by IFA.

For the infection study, heat-killed *O. tsutsugamushi* were prepared by treating *O. tsutsugamushi* at 56°C for 30 min in complete cell culture media. Four groups were used: MODCs treated with live and high-dose *O. tsutsugamushi* (LH), live and low-dose *O. tsutsugamushi* (LL), heat-killed and high-dose *O. tsutsugamushi* (HH), and heat-killed and low-dose *O. tsutsugamushi* (HL). For the high-dose groups, the cells were treated with $4 \times 10^5$ cell count units of *O. tsutsugamushi*; the low-dose groups were treated with $4 \times 10^4$ cell count units of *O. tsutsugamushi.*

### Infection of Human Monocyte-Derived DCs (MODCs) and Resulting *O. tsutsugamushi* Count

Sera from patients with scrub typhus were used to detect *O. tsutsugamushi* infection. Fluorescein isothiocyanate (FITC)-conjugated goat anti-human IgG (Santa Cruz Biotechnology, USA) was used as the secondary antibody. IFAs were performed as described previously [21]. To examine the infectivity of *O. tsutsugamushi*, 100 cells were counted for each experimental treatment set. The results were expressed as the mean number of infected cell count units (ICU) per cells ± standard deviation.

### Preparation and Culture of Human MODCs

All experiments using human blood were performed under the approval of the Institutional Review Board of Seoul National University, Korea (IRB No. 0612/001-004). Peripheral blood mononuclear cells (PBMCs) were isolated from the blood of healthy donors by density gradient centrifugation using Ficoll-Paque Plus (Amersham Bioscience, UK). CD14$^+$ monocytes were isolated from the PBMCs using the IMag anti-human CD14 antibody kit (BD Biosciences, USA). To generate MODCs, CD14$^+$ monocytes were seeded in a 6-well plate at a density of $3 \times 10^6$ cells per well in RPMI-1640 (Invitrogen, USA) supplemented with 10% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, 25 mM HEPES, 2 mM L-glutamine, 1 mM sodium pyruvate (all from Invitrogen), and 0.05 mM 2-mecaptoethanol (Sigma-Aldrich, USA) in the presence of 50 ng/ml human recombinant granulocyte-macrophage colony-stimulating factor and 1,000 U/ml of human recombinant interleukin (IL)-4 (R&D Systems, USA) for 5 days. The cells were maintained in an atmosphere of 5% $CO_2$ at 37°C, and the media were changed every 3 days.

### Maturation of Human MODCs Treated with *O. tsutsugamushi*

MODCs were treated with a high or low dose of *O. tsutsugamushi* for 24 h. The cells were then washed three times with PBS and stained with the following maturation markers: anti-human CD80-PE, anti-human CD83-FITC, anti-human CD86-APC, anti-human CD40-FITC, and anti-human HLA-A.B.C-PE monoclonal antibodies (BD Biosciences). After staining for 15 min on ice, the cells were washed, and the expression of maturation markers was measured using a FACSCalibur with CellQuest software (BD Biosciences). All cytometric data were analyzed with FlowJo software (Tree Star, USA).

### Proinflammatory Cytokine Production by MODCs Treated with *O. tsutsugamushi*

Proinflammatory cytokine secretion in the supernatants of *O. tsutsugamushi*-treated MODCs was measured by the cytometric bead array assay (BD Biosciences), according to the manufacturer's guidelines. Briefly, beads with distinct fluorescence intensities that were coated with capture antibody proteins were mixed with PE-conjugated detection antibodies and recombinant standards or samples; the beads were then incubated to form sandwich complexes at room temperature. After acquiring data using FACSCalibur,

the cytokine concentrations were calculated using the proprietary FCAP array analysis software (Softflow, USA).

**IFN-γ Production in MODC−T Cell Co-Culture**

To co-culture $CD4^+$ T cells with MODCs, PBMCs were purified by density gradient centrifugation using Ficoll-Paque Plus. $CD4^+$ T cells were sorted from PBMCs using a FACS Aria (BD Biosciences). PBMCs were stained with anti-human CD3-APC and anti-human CD4-FITC monoclonal antibodies. Then, $CD3^+$ and $CD4^+$ double-positive T cells were gated and sorted. The cell purity after sorting was >98%. *O. tsutsugamushi*-infected MODCs were co-cultured with $CD4^+$ T cells for 72 h, and the culture supernatant was collected. The concentration of human IFN-γ in the supernatant was measured using a DuoSet ELISA kit (R&D Systems), according to the manufacturer's instructions.

## Results

***O. tsutsugamushi* Continuously Proliferated After Infecting MODCs**

To evaluate the infectivity and propagation of *O. tsutsugamushi* in MODCs, we treated MODCs with live or heat-killed *O. tsutsugamushi*. ECV304 cells were used as a positive control [4]. We found that live *O. tsutsugamushi* was able to infect MODCs and continued to proliferate in a time-dependent manner (Fig. 1). As expected, a few heat-killed *O. tsutsugamushi* cells were phagocytized and did not replicate in MODCs (data not shown). These results revealed that, similar to *O. tsutsugamushi* infection of ECV304 cells, *O. tsutsugamushi* replicates during the first 48 h after infection in human MODCs. These results revealed a novel feature of *O. tsutsugamushi* invasion and replication in MODCs.

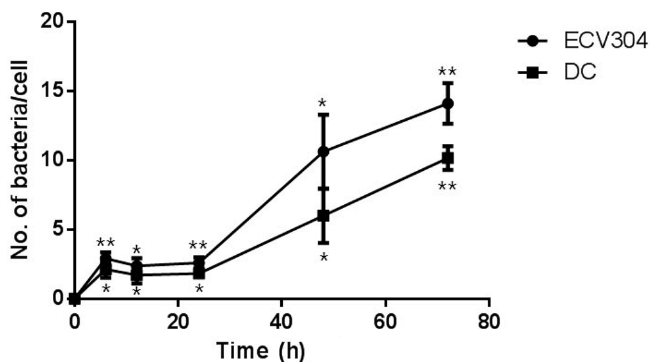


**Fig. 1.** *O. tsutsugamushi* efficiently infected and subsequently replicated in human MODCs.

MODCs and ECV304 cells were infected with $1 \times 10^6$ ICU (infected cell count units) of *O. tsutsugamushi* for 6, 12, 24, 48, and 72 h. The cells were then fixed in cold acetone and labeled primarily with serum from a patient with scrub typhus, followed by goat anti-human IgG conjugated with FITC. Infected bacteria were counted in 100 cells under a fluorescent microscope. Filled squares and filled circles indicate the counts of internalized bacteria at the indicated time points in human MODCs and ECV304, respectively. Results are means ± standard deviation. $^*p < 0.05$, $^{**}p < 0.01$ when compared to 0 h.

**Maturation of Human MODCs Treated with *O. tsutsugamushi***

As DC maturation occurs, the expression of surface markers, including MHC class I and co-stimulatory molecules, increases. To examine the effect of infection on DC maturation, MODCs were treated with four different doses (LL, LH, HL, and HH) of *O. tsutsugamushi*. The surface expressions of CD80, CD83, CD86, MHC class I, and CD40 were enhanced on MODCs from all four treatment groups compared with MODCs that were not exposed to *O. tsutsugamushi* (Fig. 2). The expressions of CD80, CD83, and MHC class I molecules were higher in MODCs treated with a high dose of *O. tsutsugamushi* than in MODCs treated with a low dose. Notably, there were no clear differences in the expressions of these molecules between MODCs treated with live or heat-killed *O. tsutsugamushi*.

***O. tsutsugamushi* Induced Proinflammatory Cytokine Production in MODCs**

The inflammatory cytokines that are produced during the maturation of dendritic cells are critical to initiate the innate and adaptive immune responses [1]. Therefore, we examined cytokine production in *O. tsutsugamushi*-infected MODCs. The results showed that proinflammatory cytokines, including IL-6, IL-8, IL-12p70, and tumor necrosis factor (TNF)-α, all increased in MODCs treated with live *O. tsutsugamushi* for 24 h (Fig. 3). For all cytokines examined, higher cytokine production was found in MODCs treated with live *O. tsutsugamushi* than in MODCs treated with heat-killed *O. tsutsugamushi*. As expected, stimulating MODCs with *E. coli* lipopolysaccharide (LPS) significantly increased the production of IL-6 and IL-8. Notably, the levels of IL-12p70 and TNF-α increased only moderately 24 h after LPS stimulation, whereas the levels were significantly higher in MODCs treated with live *O. tsutsugamushi*.

**MODCs Treated with *O. tsutsugamushi* Promoted IFN-γ Secretion in T Cells**

DC maturation is considered to be one of the key events in T cell priming [33]. To evaluate the T-cell priming ability of MODCs in response to *O. tsutsugamushi* infection, MODCs were treated with *O. tsutsugamushi* and then co-cultured with sorted $CD4^+$ T cells. The results showed that

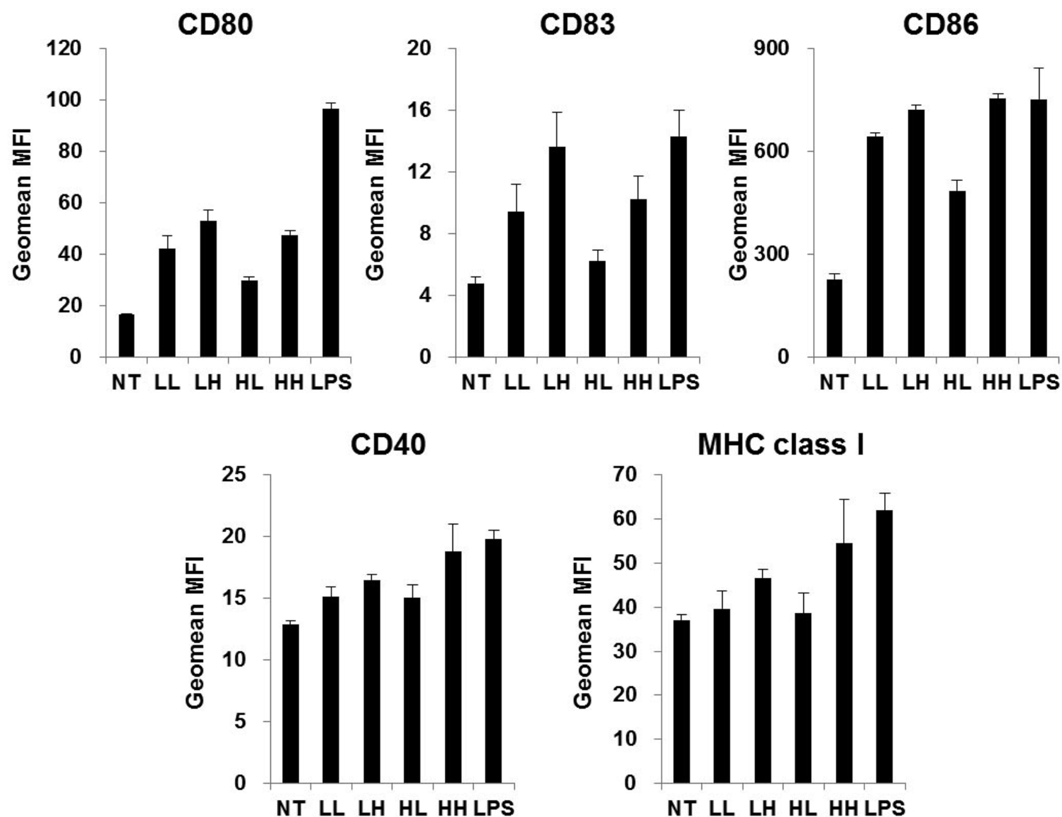


**Fig. 2.** Live and heat-killed *O. tsutsugamushi* induce the maturation of human MODCs.

Immature human MODCs were treated with PBS, different doses of live and heat-killed *O. tsutsugamushi*, or 100 ng/ml of *E. coli* LPS. After 24 h of incubation, human MODCs were stained with an appropriate combination of CD11c-APC, CD40-FITC, CD80-PE, CD83-FITC, CD86-PE, and MHC class I-FITC and analyzed by flow cytometry. All cytometric data are expressed as the mean fluorescence intensity (MFI) and were analyzed using FlowJo software. NT, non-treated; LL, live low-dose; LH, live high-dose; HL, heat-killed low-dose; HH, heat-killed high-dose of *O. tsutsugamushi*. Representative data from three independent experiments are shown

IFN-γ secretion was significantly higher in the T cells co-cultured with the MODCs that were treated with a high dose of live *O. tsutsugamushi* than in the MODCs treated with the other groups of *O. tsutsugamushi* (Fig. 4). Interestingly, the T cells co-cultured with the MODCs that had been treated with a high dose of heat-killed *O. tsutsugamushi* also produced substantial levels of IFN-γ (Fig. 4).

## Discussion

The major findings of this study are as follows: (i) Infected *O. tsutsugamushi* can replicate in MODCs; (ii) *O. tsutsugamushi* induces the maturation of MODCs, shown by the enhanced expressions of cell-surface CD80, CD83, CD86, and MHC class I molecules; (iii) *O. tsutsugamushi* induces IL-12p70 and TNF-α production in MODCs; and (iv) MODCs treated with *O. tsutsugamushi* preferentially induce IFN-γ production in T cells.

As a part of the essential host defense mechanism, bacteria including *Shigella flexneri* [19] and *Listeria monocytogenes* [11] are thought to progress to a degradation pathway (phagolysis) after uptake by MODCs; as a result, an antigenic peptide from the bacteria is presented together with the MHC to activate T cells [39]. However, a recent study reported that some bacteria can resist phagolysis in phagocytic cells [2, 34].

After the internalization of *O. tsutsugamushi* into the skin, the bacteria can spread to other areas of the skin, lungs, brain, and other remaining organs *via* the bloodstream. *O. tsutsugamushi* infections result in rash, pneumonitis, and encephalitis [32, 35]. Despite the danger of *O. tsutsugamushi*, only a few studies have reported that *O. tsutsugamushi* can modulate human immune responses [35, 40]. Therefore, we investigated the activity of MODCs treated with *O. tsutsugamushi*. First, we infected MODCs with live *O. tsutsugamushi* and found that the number of intracellular *O.*

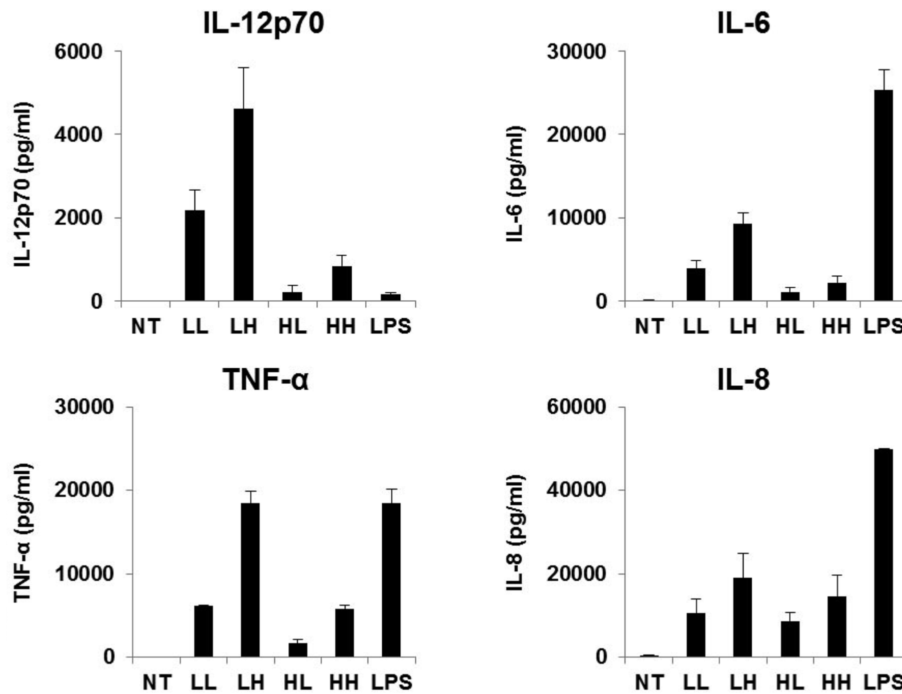


**Fig. 3.** Cytokine secretion in human MODCs infected with live and heat-killed *O. tsutsugamushi*.

Immature human MODCs were treated with PBS, different doses of live and heat-killed *O. tsutsugamushi*, or 100 ng/ml of *E. coli* LPS. Supernatants were harvested after 48 h, and cytokines were measured by using the BD Cytometric Bead array kit. NT, non-treated; LL, live low-dose; LH, live high-dose; HL, heat-killed low-dose; HH, heat-killed high-dose of *O. tsutsugamushi*. Representative data from three independent experiments are shown.

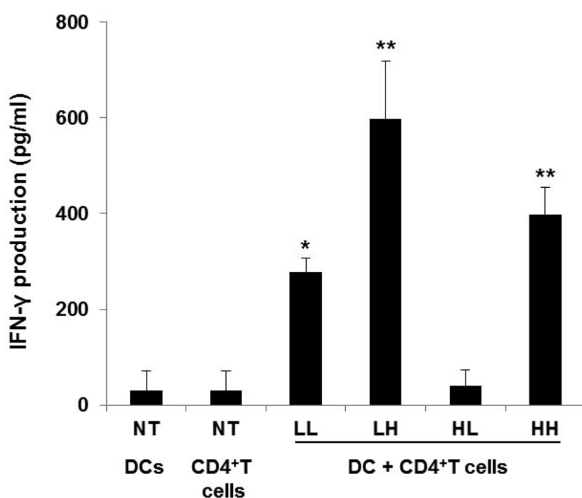


**Fig. 4.** IFN-γ production by CD4$^+$ T cells co-cultured with *O. tsutsugamushi*-infected human MODCs.

Immature human MODCs were stimulated with different doses of live and heat-killed *O. tsutsugamushi*. The cells were washed and co-cultured with CD4$^+$ T cells for 72 h. The supernatants were then harvested, and IFN-γ was measured using ELISA. NT, non-treated; LL, live low-dose; LH, live high-dose; HL, heat-killed low-dose; HH, heat-killed high-dose of *O. tsutsugamushi*. Representative data from at least two independent experiments are shown.

*tsutsugamushi* increased in a time-dependent manner, indicating that *O. tsutsugamushi* cells have the ability to resist phagolysis. Indeed, we previously reported that *Shigella* can replicate after infecting MODCs, using a unique lysosome escape system [19]. Therefore, our data suggested that *O. tsutsugamushi* has likely evolved an evasion mechanism against phagolysis.

It has been suggested in other models using intracellular bacterial infection that *Shigella flexneri* induces apoptosis in human monocyte-derived macrophages through a caspase-1 and IpaB-independent mechanism [19, 37]. Although the induction of cell death in DCs infected with live *O. tsutsugamushi* is yet to be further defined, live *O. tsutsugamushi* infection caused apoptosis in endothelial cells after disassembly of focal adhesion [18] and in a J774 murine macrophage-like cell line [3]. Based on these results, it is clear that live *O. tsutsugamushi*, like other intracellular bacteria, induces apoptosis in DCs during the infection. However, further studies are required for verifying the exact mechanism and stage of cell death during the maturation and replication in DCs.

A number of pathogenic bacteria, such as *Staphylococcus aureus* [24] and *E. coli* [5], are well known to be good

inducers of MODC maturation. We have found that, similar to other pathogenic bacteria, *O. tsutsugamushi* causes increased expression of cell-surface CD80, CD83, CD86, and MHC class I molecules. Because mature MODCs produce high levels of proinflammatory cytokines, we then examined cytokine production in MODCs treated with *O. tsutsugamushi* [31]. The results showed that *O. tsutsugamushi* induced an increase in the levels of IL-12p70, TNF-α, IL-6, and IL-8 in MODCs; these increases suggest that live *O. tsutsugamushi*, similar to LPS, can produce TNF-α, which plays an important role in inducing fever and disease severity [13]. Notably, live *O. tsutsugamushi*-infected MODCs, similar to LPS-treated MODCs, also produced high levels of IL-6 and IL-8, compared with levels in MODCs treated with heat-killed *O. tsutsugamushi*. IL-6 and IL-8 are well known for their ability to activate lymphocytes and induce neutrophil migration [10, 16]. Therefore, it is likely that these cytokines that are induced by infected DCs play an important role in controlling the severity and antigen-specific immune responses against *O. tsutsugamushi*. IL-12 is known to induce the differentiation of Th1 cells [38], and our results indicate that MODCs treated with *O. tsutsugamushi* could preferentially induce Th1 cell activation.

We also observed that live *O. tsutsugamushi*-infected MODCs could induce T cell stimulation, as shown by IFN-γ secretion, which is well known to play a critical role in inducing protective immunity against intracellular bacterial infections [26]. IFN-γ is also an important factor in the activation of infected macrophages to produce reactive oxygen species [25] and to directly kill intracellular pathogens, including *L. monocytogenes*[11], *Mycobacterium tuberculosis* [6], and *Leishmania major* [36]. IFN-γ production by activated $CD4^+$ T cells inhibits the growth of *O. tsutsugamushi* in fibroblasts and macrophages *in vitro* [8, 9, 29]. It has been demonstrated that adoptively transferred $CD4^+$ T cells that can produce IFN-γ induced protective immunity against *O. tsutsugamushi* infections *in vivo* [22]. This IFN-γ production could help $CD8^+$ T cells to become cytotoxic T lymphocytes, which play a critical role in eliminating cells with intracellular pathogens [11].

One interesting finding from this study was that IFN-γ was induced in $CD4^+$ T cells that had been co-cultured with MODCs that had been treated with a high dose of heat-killed *O. tsutsugamushi*. Furthermore, whereas MHC class I and co-stimulatory molecules were enhanced in MODCs infected with a high dose of heat-killed *O. tsutsugamushi*, proinflammatory cytokines were induced at lower levels in these cells than in MODCs treated with a high dose of live *O. tsutsugamushi*. However, it is important to note that MODCs treated with high doses of heat-killed *O. tsutsugamushi* were still capable of priming T cells. These results show that heat-killed *O. tsutsugamushi* is a promising candidate for a vaccine that could activate the T cell-mediated immune response.

In conclusion, our study reveals that live *O. tsutsugamushi* has a mechanism for escaping phagolysis and replicating in MODCs. Furthermore, MODCs that are matured by live or heat-killed *O. tsutsugamushi* acquire the ability to activate $CD4^+$ T cells.

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