福岡大医紀（*Med. Bull. Fukuoka Univ.*）：36(4), 205–214, 2009

Characterization of a Persistent Chlamydial Infection and the Role of Toll–like Receptors in the IL–6 Secretion in Chlamydia Trachomatis–Infected Human Synovial Fibroblasts

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Abstract：*Chlamydia trachomatis* a urogenital pathogen is a trigger of reactive arthritis（ReA), which is able to induce interleukin–6（IL–6）production in human fibroblast–like synovial cells（HFLS）*in vitro*, and the persistence of *chlamydia* might thus play an essential role in stimulating the synthesis of IL–6 in HFLS. In addition, a persistent infection has been established *in vitro* by treatment with gamma interferon(IFN–γ)or penicillin and by the deprivation of nutrients. This paper compared the mechanism of chlamydial persistence in *C. trachomatis*–infected HFLS to that of IFN–γ induced persistent infection in HeLa 229 cells. The secretion of IL–6 increased only slightly（～1,000 pg/ml）when HeLa 229 cells were treated with IFN–γ regardless of the degree of *C. trachomatis* infection or tryptophan depletion. On the other hand, pretreatment with an antagonistic inhibitor of indoleamine 2,3–dioxygenase, 1–methyltryptophan, had no inhibitory effect on the production of IL–6, and a large amount of IL–6 secretion（～45,000 pg/ml）was observed with the suppression of chlamydial growth in *C. trachomatis*–infected HFLS, i.e., abnormal inclusions could not return to their normal shape by the pretreatment with 1–methyltryptophan. These results indicated that IFN–γ–induced persistence might be different from the persistence of *chlamydia* in HLFS. The role of Toll–like receptors（TLRs）in IL–6 production in *C. trachomatis*–infected HFLS was also investigated. The blockade of TLR2 antibody diminished the infectivity but augmented the IL–6 production in *C. trachomatis*–infected HFLS ; however, TLR4 did not show any correlation with infectivity and IL–6 production. These results suggest that TLR2 is involved in both the process of chlamydial infection and the IL–6 production in HFLS.

Key words：**Reactive arthritis, Human fibroblast–like synovial cells(HFLS), Persistent chlamydial infection, Toll–Like Receptors**

Introduction

Chlamydia trachomatis is an obligate intracellular gram–negative bacterium with a unique biphasic growth cycle. After entering host cells, metabolically inert elementary bodies（EBs）rapidly transform into metabolically active reticulate bodies（RBs）that replicate by binary fission within a membrane–bound vesicle, and this process is known as inclusion. After logarithmic bacterial cell division, the RB reorganizes into infectious EBs, which adapt themselves to survive in the extracellular environment of the host. *C. trachomatis* causing disease in humans is classified as a trachoma or sexually transmitted disease（STD). Although infection with *C. trachomatis* can be successfully treated with antibiotics, asymptomatic and persistent infections are common and can result in scarring sequelae such as blinding trachoma and pelvic inflammatory disease. Moreover, chronic or recurrent chlamydial infections have been associated with salpingitis[1] or reactive arthritis（ReA).[2)3)4)]

The exposure of *in vitro* chlamydial infections to cytokines, particularly gamma interferon（IFN–γ), produces persistence that can plausibly reflect *in vivo* events.[5)6)] IFN–γ inhibits intracellular chla-

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mydial growth by the induction of cellular indoleamine 2,3-dioxygenase (IDO) which causes a depletion of intracellular tryptophan.[7][8] IDO-mediated tryptophan catabolism can lead to a persistent chlamydial infection in cell culture, that is characterized by the production of a few EBs during the developmental cycle and by the presence of atypical enlarged RBs.[9][10] In addition, other mechanisms such as the inducible nitric oxide synthase (iNOS) pathway and iron deprivation could also be attributable to IFN-γ-mediated persistence.[11][12] Furthermore, the complexity of the *in vivo* situation may also be a problem.

ReA can be regarded as a subgroup within infection-associated arthritides. It occurs after an infection of the urogenital tract with *C. trachomatis* or of the gut with such enterobacteria as *Yersinia, Salmonella, Shigella, and Campylobacter jejuni. Chlamydia* has been found in the genitourinary tract together with joints of ReA patients, and its involvement in ReA has been further supported by the observation of chlamydial antigens in synovial cells by immunoelectron microscopy,[13] and the identification of chlamydial nucleic acids by the polymerase chain reaction (PCR),[14] and ribosomal RNA hybridization techniques.[15] In addition, abnormal RBs have been found *in vivo* in the synovial membranes of *Chlamydia*-associated ReA, where infectious EBs can hardly be isolated from the site of infection. These abnormal forms might be involved in non-permissive infections with chronic inflammation. Therefore, a recent study showed that *chlamydia* can infect fibroblast-like synovial cells *in vitro* and thereafter multiply to a small degree, thus leading to either persistent or latent infection and to the production of interleukin-6 (IL-6) in human synovial fibroblasts.[3] Other studies have reported that *C. trachomatis* infection activates the expression of interferon regulatory factor and IDO in human synovial fibroblasts.[16]

The Toll-like receptor (TLR)4 is a potentially interesting candidate for study in *C. trachomatis* infections, because signaling through TLR4 is activated by both lipopolysaccharide and heat shock protein 60 of mammalian and microbial origin.[17][18][19][20] In addition, recent papers have noted TLR4-independent cytokine production from inflammatory cells exposed to live chlamydial EBs,[21][22] and a dominant role for TLR2 versus TLR4 in the recognition process of *Chlamydophila pneumoniae*.[23] In another paper, an antibody to TLR4 inhibited *Chlamydia*-induced cytokine secretion in human dendritic cells.[24] These findings suggest that TLR2 and/or TLR4 play important roles in the inflammatory signaling in *Chlamydia*-induced diseases.

The present study studied the characteristics of a persistent chlamydial infection in a human synovial fibroblast cell line, to determine whether the mechanism of persistence in human synovial fibroblasts is similar to that of IFN-γ induced persistence with IL-6 production. In addition, the role of TLR2 and TLR4 in the *in vitro* response to *C. trachomatis*-induced IL-6 production was determined.

Materials and methods

1. *Cell cultures and media*

Human fibroblast-like synovial cells (HFLS) were obtained from Cell Applications Inc. (San Diego, CA). These primary cells were derived from the inflamed synovial tissue of a rheumatoid arthritis patient. The method used to generate HFLS has been described before.[3][16] The HeLa 229 human epithelial cell line (CCL 2.1) and McCoy cell mouse fibroblast cell line (CRL 1696) were used in these experiments. All cell lines were maintained in Dulbecco's modified Eagle Medium (DMEM ; Invitrogen, Carlsbad, CA) supplemented with 10% heat-inactivated fetal calf serum (FCS) and 100 μg/ml streptomycin. Proliferating HFLS were grown in a synoviocyte growth medium (Cell Application Inc.).

2. *Infection of HFLS or HeLa 229 cells*

The chlamydial strain *C. trachomatis* serovar E (E/UW-5/Cx) was propagated and prepared as previously described.[25] HFLS and HeLa 229 cells were seeded into 24-well flat-bottomed culture plates with or without 13-mm glass cover slips. Stocks of chlamydial strains were diluted with sucrose-phosphate-glutamate medium (75.0 g sucrose, 0.52 g KH_2PO_4, 1.22 g Na_2HPO_4, 0.72 g glutamic acid, 1,000 ml distilled water ; pH 7.2)[25] and inoculated onto the monolayer cultures of each cell (1×10^4 cells/well of HFLS and 1×10^5 cells/well of HeLa

229 cells, respectively). The cells were infected with a multiplicity of infection (MOI) of 1 to HFLS and 0.1 to HeLa 229 cells by centrifugation at 1,000 × g for 60 min. After the inoculum was decanted, the cells were washed in medium to remove the non-adsorbed *chlamydia* and further incubated in 1 ml DMEM containing 1% FCS (maintenance medium) without cycloheximide. To examine the effects of IFN-γ on either chlamydial growth or IL-6 production, infected cells were cultured with the indicated concentration of IFN-γ (2.0 and 20 ng/ml). For UV inactivation, chlamydial suspensions in a 60-mm dish were irradiated for 30 min with a germicidal lamp (Westinghouse G30T8) set at a distance of 15-cm from the dish. For mock infection, uninfected McCoy cells were frozen and thawed once, and then were used in the same fashion as the infected McCoy cells.

3. *Immunofluorescence staining and fluorescence microscopy*

HFLS or HeLa 229 cells in a 24-well culture plate with glass cover slips were infected by centrifugation, and then were incubated with 1 ml of maintenance medium. The infected monolayers were washed with PBS, and the cells were fixed with −20℃ chilled methanol 72 h after infection. After the specimens had been dried, the inclusion bodies were stained with fluorescein isothiocyanate (FITC) labeled monoclonal antibody (mAb) against *C. trachomatis* major outer membrane protein (Syva Microtrak, San Jose, CA). The cells were then rinsed with saline and mounted in a 1 : 1 solution of PBS-glycerol. The formation of inclusions and the existence of chlamydial particles were confirmed using a Zeiss Axiophot fluorescence microscope. Infectivity was identified based on the number of inclusion-forming units (IFUs).

4. *Tissue preparation for electron microscopy*

The monolayers of infected HFLS or HeLa 229 cells were washed with PBS at 72 h after infection. The cells were removed from the culture dishes by gentle scraping with a sterile rubber policeman, pelleted by centrifugation, and fixed with 2% glutaraldehyde in PBS for 2 h at 4℃. After two washes in PBS, the samples were fixed again for 2 h in 2% osmium tetroxide-PBS at 4℃. They were then dehydrated in a graded series of ethanol solution and embedded in Epon 812. Thin sections were cut on a Reichert ultramicrotome, placed on 200-mesh copper grids, stained with uranyl acetate and lead citrate, and then examined by a Hitachi H-7100 transmission electron microscope at an accelerating voltage of 100 kV.

5. *Determination of the cytokine levels in culture supernatants by ELISA*

HFLS or HeLa 229 cells in 24-well culture plates without glass cover slips were infected with 0.25 ml of properly diluted stock chlamydial suspension as described above. The plates were centrifuged, and then the cells were incubated in 1 ml of maintenance medium. At the indicated time, the supernatants of the infected cultures were collected, and stored at −80℃ until the cytokine assays could be performed. IL-6, IL-8, TNF-α (tumor necrosis factor-α), and IFN-α in test supernatants were quantified in duplicate wells by capture enzyme-linked immunosorbent assays (ELISAs ; Biosource, Ratingen, Germany) according to the manufacturer's protocol and calculated from the data using standard cytokines.

6. *Tryptophan deleted medium*

After infection, the maintenance medium was replaced with the tryptophan deleted medium, which contained all the constituents of DMEM without tryptophan.

7. *1-methyltryptophan pretreatment*

Before infection, HFLS were pretreated with 12.5 or 25 mM 1-methyltryptophan (1-MT), the IDO competitive inhibitor, at 37℃ for 1 h.

8. *TLR blocking experiments*

HFLS and HeLa 229 cells were preincubated at 37℃ for 2 h with control human IgG (Southern Biotech, Birmingham, AL) and mAb against TLR4 (Abcam, Cambridge, UK) or TLR2 (Hycult biotechnology, Uden, Netherlands) at a concentration of 20 μg/ml before *C. trachomatis* inoculation. At 24 h after infection, the supernatants were collected and stored at −70℃ until assay. The infectivities were determined at 48 h after infection by immunofluorescence staining.

Results

1. *Electron microscopic analysis and kinetics of chlamydial growth*

HFLS were less susceptible than HeLa 229 cells to *C. trachomatis* infection. The number of inclusions in *C. trachomatis*–infected HeLa 229 cells were 10–fold greater than that in infected HFLS at an inoculum dose of 1×10^5 IFUs (MOI 1 ; data not shown). Therefore, in the present experiments, HFLS and HeLa 229 cells were infected with MOI 1 and 0.1, respectively, in order to adjust the rate of infection. In HFLS showed inclusions that contained few EBs and abnormally enlarged atypical RBs by electron microscopy (EM) 72 h after infection (Fig. 1A) as reported previously.[3] Atypical RBs in HFLS were localized adjacent to the inclusion membrane and they seem to have been arrested in the process of multiplication. In addition, the abnormal inclusions of HFLS contained a lot of glycogen–like particles. These particles were confirmed to be glycogen granules by staining with Lugol's iodine. In contrast, HeLa 229 cells inoculated with *C. trachomatis* had inclusions that contained a lot of normal EBs and RBs (Fig. 1B).

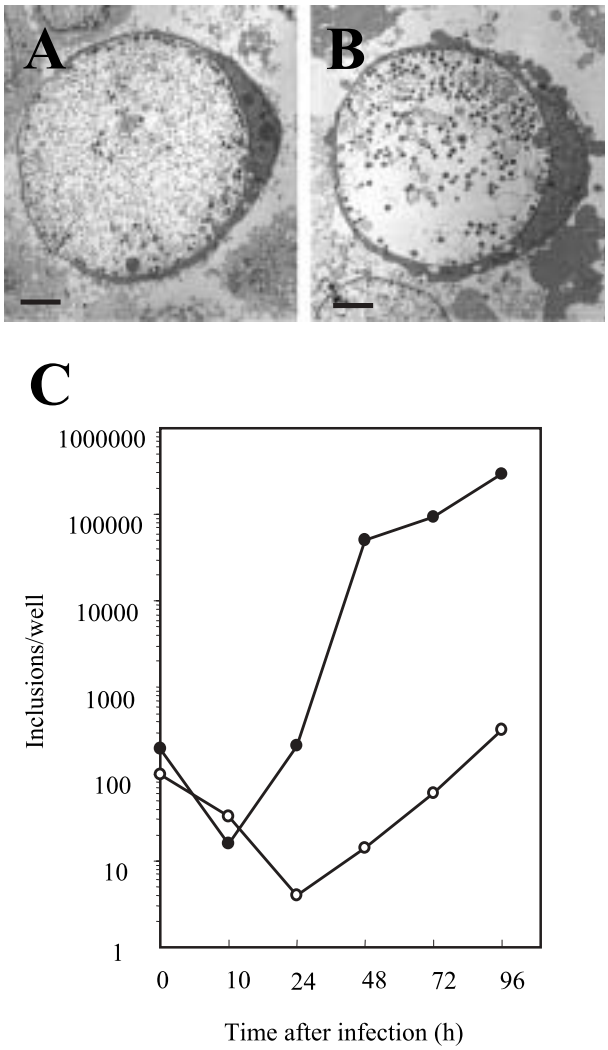

Fig. 1. Electron microscopic analysis of *C. trachomais*infected HFLS or HeLa 229 cells and chlamydial growth curves. Micrographs of (A) *C. trachomais* 72 h after infection of HFLS and (B) of HeLa 229 cells. Bars are 5μm, respectively. Few EBs, abnormal RBs, and a lot of glycogen particles were observed in HFLS. (C) One–step growth of *C. trachomais* in HFLS (○) and HeLa 229 cells (●).

Further experiments were conducted to obtain the growth curves of *C. trachomatis* in HFLS and HeLa 229 cells. The infected cells were collected at 10 h and then every 24 h after infection. After two cycles of freezing and thawing, the lysates were diluted and inoculated into duplicate cultures of McCoy cells in order to estimate the infectivity. The number of infectious EBs decreased at 10 h in HeLa 229 cells and at 10 h and 24 h after infection in HFLS. However, there was a two thousand–fold increase in the number of infective EBs in HeLa 229 cells at 48 h after infection, whereas the number of infective EBs even at 96 h after infection was the same as the initial one in HFLS, i.e., no increase in infectivity was observed (Fig. 1C). It is obvious that HFLS were less susceptible than HeLa 229 cells with regard to the growth of *chlamydia*, and these findings were consistent with the EM observations (Figs. 1A and B).

2. *The cytokine levels in culture supernatants in C. trachomatis – infected HFLS or HeLa 229 cells*

Cytokine induction was compared in both HFLS and HeLa 229 cells by *C. trachomatis* infection or mock infection. An infection with *C. trachomatis* in HFLS induced the production of a large amount of IL–6 and IL–8 of up to 45,000 and 30,000 pg/ml, respectively (Figs. 2A and 3A). On the other hand, HeLa 229 cells chlamydial infection never induced the production of IL–6 nor IL–8 in HeLa 229 cells (Figs. 2B and 3B). No TNF–α or IFN–α secretion was detected in supernatants from either in-

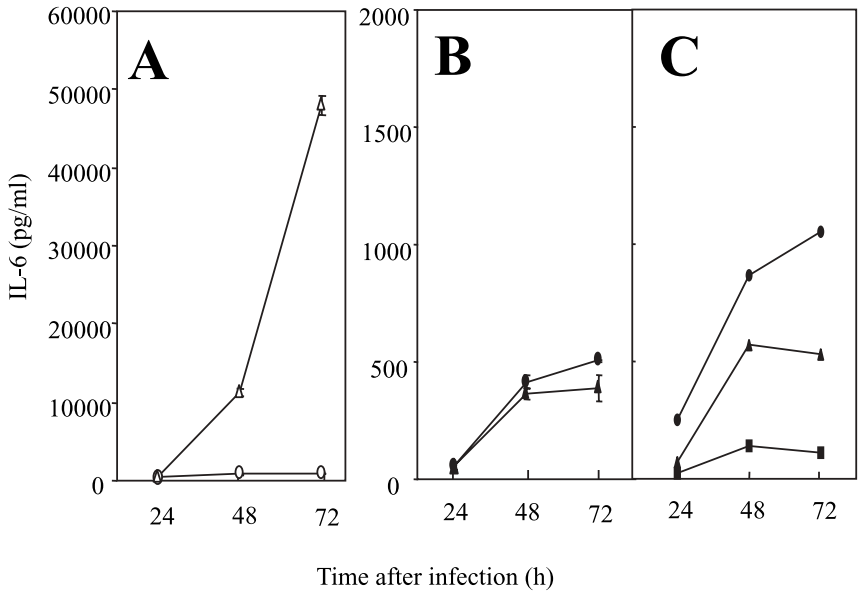

Fig. 2. Kinetics of IL-6 secretion by *C. trachomatis*-infected HFLS and HeLa 229 cells. In (A) HFLS and (B) HeLa 229 cells, *C. trachomatis*-infected cultures (▲ and △), mock infected cultures (● and ○). (C) *C. trachomatis*-infected HeLa 229 cells were cultured with IFN-γ of 2.0 ng/ml (▲) and 20 ng/ml (●), or cultured in deleted tryptophan medium (■). In (A) and (B), data are mean ±SD (n=3).

fected HFLS or HeLa 229 cells (data not shown).

Previous studies have demonstrated the inhibitory effects of IFN-γ on *C. trachomatis* replication, thus resulting in the development of inclusions containing enlarged atypical RBs.[5)6)9)10)] This study examined whether *C. trachomatis*-infected HeLa 229 cells secreted IL-6 during a persistent infection in response to IFN-γ. Although IFN-γ at 2.0 or 20 ng/ml completely inhibited the chlamydial growth in HeLa 229 cells, the secretion of IL-6 was only 1,000 pg/ml regardless of *C. trachomatis* infection (Fig. 2C). The IL-6 levels of 1,000 pg/ml in IFN-γ treated HeLa 229 cells were almost the same as those in uninfected or mock infected HFLS (Figs. 2A and C). IFN-γ affects the host cells *in vitro* by inducing IDO, which catalyzes the initial step in degradation of tryptophan, and correlates with the growth inhibition of Chlamydia.[26)] This process is recovered by the addition of an excessive amount of tryptophan, and the depletion of exogenous tryptophan induces growth inhibition even further. Therefore, the effect of exogenous tryptophan on IL-6 secretion in infected or mock infected HeLa 229 cells was analyzed. The maintenance medium without tryptophan did not increase IL-6 secretion in infected HeLa 229 cells (Fig. 2C). Taken together, these observations demonstrated that IFN-γ induced persistent infection caused a slight increase of IL-6 secretion, which showed no correlation with either *C. trachomatis* infection or the absence of tryptophan. Furthermore, the exogenous IL-6 (0.01～100 ng/ml) did not effect the morphology of inclusions and chlamydial growth in HeLa 229 cells (data not shown).

3. *Effect of* 1-*methyltryptophan pretreatment*

Abnormal inclusions induced by IFN-γ could return to a normal shape after the addition of a competitive inhibitor of IDO, 1-MT, in *Chlamydophila pneumoniae*-infected HEp-2 cells.[27)28)] Therefore, *C. trachomatis*-induced IL-6 production and chlamydial growth were measured in HFLS following exposure to 1-MT. Pretreatment with 12.5 mM 1-MT for 1 h allowed a large amount of IL-6 secretion and suppressed chlamydial growth (Figs. 4A and B). In addition, a similar result was observed after the pretreatment with 25 mM 1-MT (data not shown).

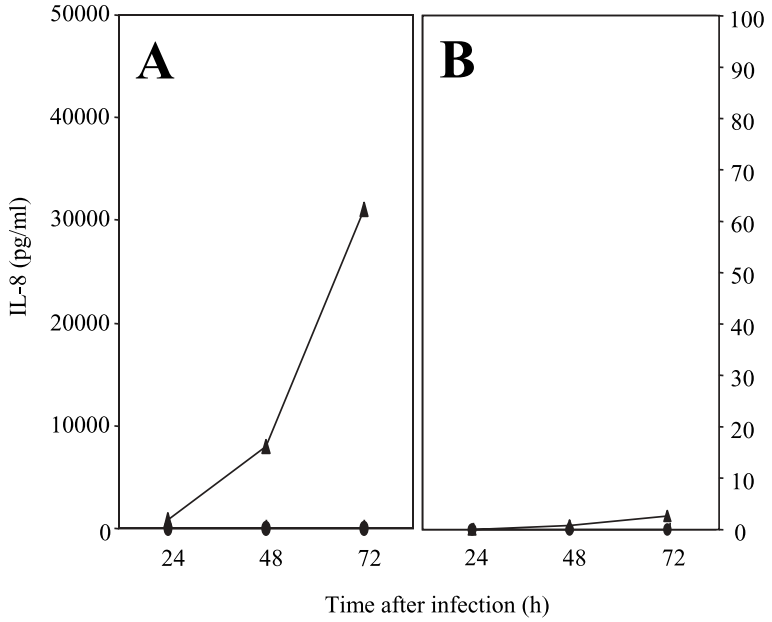

Fig. 3. IL-8 production in *C. trachomatis*-infected HFLS or HeLa 229 cells. In (A) HFLS and (B) HeLa 229 cells, *C. trachomatis*-infected cultures (▲), and mock infected cultures (●).

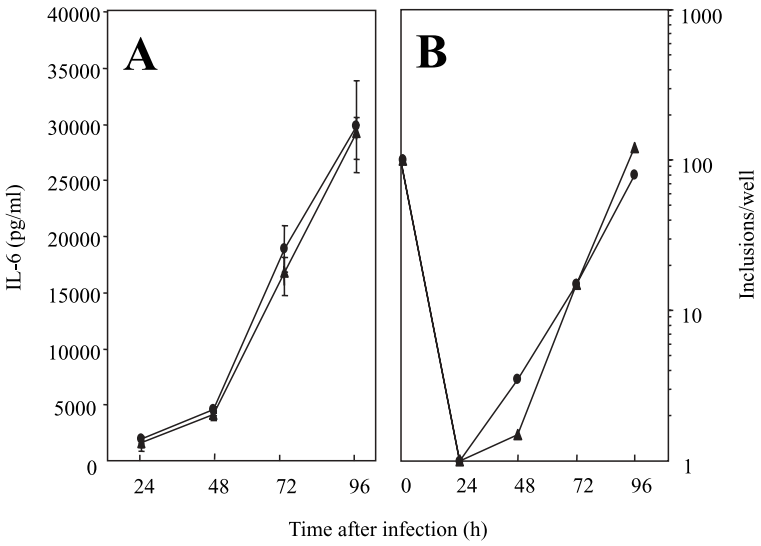

Fig. 4. Effect of 1-MT on IL-6 production and chlamydial growth of *C. trachomatis* infected HFLS. In (A) IL-6 production and (B) chlamydial growth, *C. trachomatis*-infected cultures (●), *C. trachomatis*-infected cultures pretreated with 12.5 mM α-MT (▲). In B, number of infectious EBs were determined on McCoy cells, and shown as number of inclusions. Data are mean±SD (n=3).

4. *Effect of neutralizing TLR2 or TLR4 mAb on infectivity and IL-6 production*

TLR blocking experiments were carried out using a TLR neutralizing mAb. The HFLS or HeLa 229 cells were incubated with anti TLR2 or anti TLR4 neutralizing mAb for 2 h before and during *C. trachomatis* infection. As shown in Figures 5A and B, the TLR2 mAb blocked approximately 90% of the infectivity ; however, the neutralization of TLR2 mAb produced a five-fold increase in IL-6 production in comparison to the control in HFLS. The TLR4 mAb blocked neither the infec-

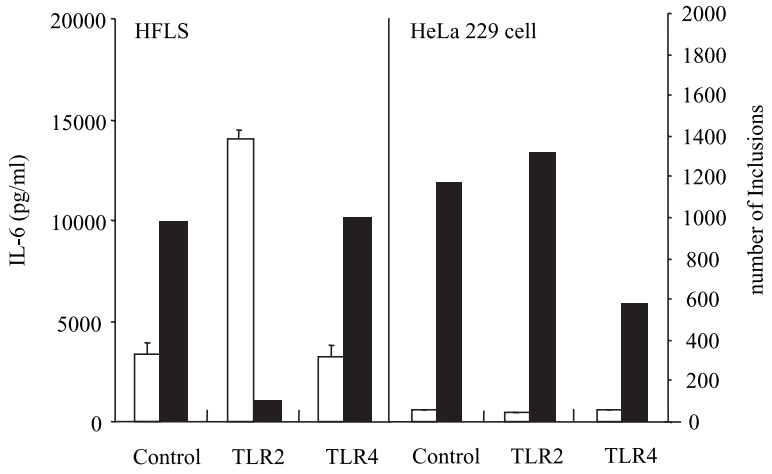

Fig. 5. Effects on IL-6 production and formation of inclusions with pretreatment of anti-TLR 2 or anti-TLR4 antibody in *C. trachomatis*-infected HFLS or HeLa 229 cells. IL-6 production (□), formation of inclusions (■). In graphs of IL-6 production, data are mean ±SD (n=3).

tivity nor the IL-6 production in *C. trachomatis*-infected HFLS. On the other hand, the TLR4 mAb blocked 50% of the infectivity in HeLa 229 cells (Fig. 5). The TLR2 mAb blocked neither the infectivity nor IL-6 production in *C. trachomatis*-infected HeLa 229 cells.

Discussion

A cure for a persistent chlamydial infection in humans is very important ; however, it remains difficult to achieve. Because Chlamydiae respond to a variety of environmental stimuli that alter their growth characteristics, Chlamydia thus has the potential to establish a chronic or persistent relationship with the host. If chronic or persistent infections are established, then those infections may serve as a reservoir for new infections, and contribute to the immunopathological consequences of infection. Therefore, understanding the consequences of persistent chlamydial infection could thus play an important role in the control and prevention of chlamydial disease.

A persistent chlamydial infection causes severe and difficult-to-treat diseases such as ReA, which develop in 1 to 3% of patients after a genital infection.[29] EM observations of synovial membranes from ReA patients show atypical RBs *in vivo*,[30] where infectious EBs cannot be isolated from the site of infection.[31] These morphological observations may correspond to the IFN-γ induced persistent Chlamydiae seen *in vitro*.[5][6] The characterization of the *in vitro* persistent phase and of the *in vivo* evidence suggests that Chlamydiae persist in an altered atypical form during chronic disease. Further study examined whether the mechanism of persistence in HFLS was similar to that of IFN-γ induced persistence. The most important mechanism underlying the effect of IFN-γ on chlamydial growth is thought to be tryptophan depletion through the activation of the host tryptophan-degrading enzyme IDO.[7][8][32] However, *C. trachomatis*-infected HeLa 229 cells cultured in IFN-γ or the tryptophan-deleted medium did not show an increase in the IL-6 production (Fig. 2), despite the loss of chlamydial growth. Pretreatment with 1-MT could have resulted in a decrease of the IL-6 production and chlamydial normal replication if tryptophan depletion occurred in *C. trachomatis*-infected HFLS because of the antagonistic effect of α-MT on IDO.[28] However, the pretreatment with 1-MT could neither restore the abnormal form to a normal shape nor suppress the IL-6 production in *C. trachomatis*-infected HFLS (Fig. 4). Tryptophan depletion may not correlate with persistent infection in synovial fibroblasts. Collectively, the current results demonstrated that a chlamydial persistent infection in HFLS was different from

IFN−γ induced persistence.

TLRs are innate immune receptors involved in the pattern recognition of bacterial antigens, and it is currently believed that their differential activation helps to discriminate between microbial pathogens.[33] It has been suggested that TLR4 is involved in the recognition of gram−negative bacteria through their LPS,[34] and TLR2 mainly recognizes the elements of gram−positive bacteria and yeasts,[35] although this dichotomy is not always sustained. TLR2 is essential for the early cytokine production such as IL−1 and IL−6 in *C. trachomatis* genital infection,[17] and an antibody to TLR4 inhibits *C. trachomatis*−induced IL−1β, IL−6, and TNF−α production by dendritic cells.[24] The recognition of *C. pneumoniae* depends on the TLR2 activation of either dendritic cells or mononuclear cells.[21)23] This study investigated the role of TLR2 and TLR4 in the growth of *chlamydia* and *C. trachomatis*−induced IL−6 production in both HFLS and HeLa 229 cells. Interestingly, the augmentation of IL−6 production inversely correlated with the decrease in the inclusion formation by TLR2 mAb (Fig. 5). TLR4 did not contribute to the IL−6 production nor to the formation of inclusions in *C. trachomatis*−infected HLFS. On the other hand, although the formation of chlamydial inclusions of *C. trachomatis* in HeLa 229 cells depended on TLR4, it did not imply the stimulation of IL−6 production (Fig. 5). These results suggest that TLR2 might affect both the process of chlamydial infection and the IL−6 production in HFLS, and is different from that in HeLa 229 cells.

In conclusion, the present study showed that mechanisms other than IFN−γ induced persistence might contribute to the chlamydial persistence on ReA. It is probable that TLR2 and other mechanisms might therefore be involved in the IL−6 production. Further investigation into the interplay between chlamydia and its environment is thus needed to both understand the host−microbial interactions and provide important insight for the treatment and prevention of ReA.

Acknowledgments

We thank Dr. Ataru Kuroiwa (Faculty of Medicine, Fukuoka University, Japan) for his comments on the manuscript.

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(Received on June 19, 2009,
Accepted on September 9, 2009)