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***Chlamydomophila pneumoniae* in human immortal Jurkat cells and primary  
lymphocytes uncontrolled by interferon- $\gamma$**

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## Abstract

Lymphocytes are a potential host cell for *Chlamydomphila pneumoniae*, although why the bacteria must hide in lymphocytes remains unknown. Meanwhile, interferon (IFN)- $\gamma$  is a crucial factor for eliminating chlamydiae from infected cells through indoleamine 2,3-dioxygenase (IDO) expression, resulting in depletion of tryptophan. We therefore assessed if lymphocytes could work as a shelter for the bacteria to escape IFN- $\gamma$ . *C. pneumoniae* grew normally in human lymphoid Jurkat cells, even in the presence of IFN- $\gamma$  or under stimulation with phorbol myristate acetate plus ionomycin. Although Jurkat cells expressed IFN- $\gamma$  receptor CD119, their lack of IDO expression was confirmed by RT-PCR and western blotting. Also, *C. pneumoniae* survived in enriched human peripheral blood lymphocytes, even in the presence of IFN- $\gamma$ . Furthermore, *C. pneumoniae* in spleen cells obtained from IFN- $\gamma$  knockout mice with C57BL/6 background was maintained in a similar way to wild-type mice, supporting a minimal role of IFN- $\gamma$ -related response for eliminating *C. pneumoniae* from lymphocytes. Thus, we concluded that IFN- $\gamma$  did not remove *C. pneumoniae* from lymphocytes, possibly providing a shelter for *C. pneumoniae* to escape from the innate immune response, which has direct clinical significance.

**Keywords:** *Chlamydomphila pneumoniae*; indoleamine 2,3-dioxygenase; interferon- $\gamma$ ; lymphocytes

## 1. Introduction

Pathogenic chlamydiae are obligate intercellular bacteria with a recurrent developmental cycle between the elementary (EB) and reticulate body (RB) forms in inclusion bodies surrounded by the membrane vesicle [1]. They consist of two major groups, including two important human pathogens, *Chlamydophila pneumoniae* and *Chlamydia trachomatis*. *C. pneumoniae* is an important cause of community-acquired pneumonia [2,3], and has been implicated in several chronic diseases, including atherosclerosis [2,4,5] and central nervous system diseases, such as Alzheimer's disease and multiple sclerosis [2,6]. Also, *Ch. trachomatis* is responsible for medically important human diseases, such as blinding trachoma, which affects about 84 million people, of whom about 8 million are visually impaired as a consequence [7], and sexually transmitted infection, with 5 million new cases per year [8]. Therefore, during the past decade, antichlamydial cellular events have been investigated to elucidate the exact mechanisms directly associated with prevention of these infections.

Interferon (IFN)- $\gamma$  inhibits the replication of pathogenic chlamydiae in human cells by inducing the production of the enzyme indoleamine 2,3-dioxygenase (IDO), resulting in depletion of tryptophan in infected cells, which is an essential amino acid for intracellular growth of pathogenic chlamydiae [9–11]. Therefore, induction of IDO is the most important antichlamydial factor in humans, induced by IFN- $\gamma$ , and moreover, human-adapted chlamydiae have specifically coevolved with their host to circumvent the effect of IFN- $\gamma$  [9–11]. In fact, IFN- $\gamma$  induces *C. pneumoniae* persistent infection, characterized by an absence of culturability [12–14], altered bacterial RNA and protein levels [6], and morphologically altered chlamydial bodies [12–14], with an uncompleted developmental cycle and inhibition of elementary body maturation, followed by

bacterial elimination [12–14]. The other pathogenic chlamydiae, human genital chlamydiae (*Ch. trachomatis* D–L serotypes), in contrast to ocular chlamydiae (*Ch. trachomatis* A–C serotypes), encode a functional tryptophan synthetase enzyme (*trpAB*), which possibly uses exogenous indole supplied from microbes that colonize the human genital tract [9]. Therefore, they can survive in genital cells, even in the presence of IFN- $\gamma$ , which is directly related to recurrent infection by *Ch. trachomatis* and of clinical significance. Meanwhile, *C. pneumoniae* also sometimes causes recurrent infection, even though no bacterial flora is seen in the lungs [15]. Thus, these findings suggest the presence of a distinct IFN- $\gamma$ -dependent evolutionary pathway in these pathogenic chlamydiae. It is possible that *C. pneumoniae*, an inhabitant of aseptic lung, has evolved an unrecognized strategy for escaping IFN- $\gamma$ , independent of other microbes, or persistent infection followed by reactivation, for instance, the presence of an unknown shelter cells to escape from IFN- $\gamma$ .

We have already demonstrated that both *C. pneumoniae* TW183 and *Ch. trachomatis* 434/Bu serovar L2 can infect and multiply in Jurkat human lymphocytes with a complete developmental cycle [16,17], and survive in human peripheral blood cells or mouse spleen cells [18–20], indicating that lymphocytes are a potential host cell for pathogenic chlamydiae. In the present study, we attempted to assess if lymphocytes could work as a shelter for *C. pneumoniae* to escape IFN- $\gamma$ , which is a crucial factor for elimination of chlamydiae.

## **2. Materials and methods**

### *2.1. Bacteria and estimation of bacterial numbers using inclusion forming unit (IFU)*

assay

*C. pneumoniae* TW183 strain was kindly provided by G. Byrne, University of Tennessee (Memphis, TN, USA). *Ch. trachomatis* 434/Bu (LGV: serovar L2) strain was purchased from ATCC (Manassas, VA, USA). Both bacteria were propagated in a HEp-2 cell culture system as described previously [16]. The infected cells were harvested on day 2 and disrupted by freezing–thawing and ultrasonication. After centrifugation to remove cell debris, bacteria were concentrated by high-speed centrifugation. Bacterial pellets were resuspended in sucrose–phosphate–glutamic acid buffer (0.2 M sucrose, 3.8 mM KH<sub>2</sub>PO<sub>4</sub>, 6.7 mM Na<sub>2</sub>HPO<sub>4</sub>, 5 mM L-glutamic acid, pH 7.4) and stored at –80°C for later use. Numbers of infectious progeny were determined as IFUs by counting chlamydial inclusions formed in HEp-2 cells, using fluorescein isothiocyanate (FITC)-conjugated monoclonal antibody specific to *Chlamydia* lipopolysaccharide (with Evans Blue) (Denka Seiken Co. Ltd., Tokyo, Japan) [16].

## 2. 2. Immortal cells

The epithelial cell lines (HEp-2 and HeLa) and lymphocyte (Jurkat) and monocyte (THP-1) cell lines were cultured at 37°C in 5% CO<sub>2</sub> in Dulbecco's Modified Eagle's Medium (DMEM) and RPMI 1640 medium, respectively, each containing 10% heat-inactivated fetal calf serum (FCS) with antibiotics (10 µg/mL gentamicin, 10 µg/mL vancomycin and 1 µg/mL amphotericin B) (Sigma, St. Louis, MO). HEp-2 cells were also used to propagate bacteria as described above.

## 2. 3. Human peripheral lymphocytes and ethical review

Human peripheral blood mononuclear cells were isolated from whole blood

provided by healthy volunteers, by density gradient centrifugation with Histopaque (Sigma). The resultant peripheral blood mononuclear cells (PBMCs) were washed three times with Hank's Balanced Salt Solution (HBSS) and suspended in RPMI 1640 medium containing 20% FCS. The PBMC suspensions were then dispensed in tissue culture flasks and incubated for 2 h at 37°C in 5% CO<sub>2</sub> to allow adherence of the monocytes. After incubation, nonadherent cells (enriched human blood lymphocytes) were collected, washed with HBSS, and resuspended in RPMI 1640 medium containing 20% FCS with antibiotics. Lymphocyte fractions stained with Giemsa showed >95% lymphocytes by morphology. Written informed consent was obtained from all volunteers in this study, and the study was approved by the ethics committee of the Faculty of Health Sciences, Hokkaido University.

#### *2. 4. Mouse spleen cells and ethical review*

C57BL/6 wild-type and IFN- $\gamma$ <sup>-/-</sup> female mice (7–10 weeks old) were purchased from SLC (Hamamatsu, Shizuoka, Japan) and Jackson Laboratory (Bar Harbor, ME, USA), respectively. The mice were housed under specific pathogen free conditions in isolated cabinets to prevent environmental contamination, in accordance with the NIH Guide for Care and Use of Laboratory Animals, and the Animal Care and Use Committee of Hokkaido University also approved this experiment. After feeding for 1 week, the mice were killed, and spleen cells were used as representative mouse lymphocytes for *in vitro* experiments.

#### *2.5. Infection and assessments for infectious progeny, inclusion formation, and gene expression*

Cells were adjusted to a concentration of  $2 \times 10^5$  cells/well and infected with bacteria at an MOI of 1 or 50 by centrifugation ( $800\times g$ ) for 1 h at room temperature. After washing to remove noninfectious bacteria with HBSS (Sigma), infected and uninfected cells at a concentration of  $2 \times 10^5$  cells/well were incubated in either DMEM (for epithelial cells) or RPMI 1640 medium (for lymphatic cells) containing 20% FCS with antibiotics for up to 5 days, in the presence or absence of IFN- $\gamma$  (5 or 20 ng/mL); each of the culture volumes was adjusted at 1mL per well. Also, some experiments performed in the presence of either phorbol myristate acetate (PMA; 2–10 nM; Sigma) with ionomycin (25–125 nM; Sigma) to activate lymphocytes. Cells were pretreated with IFN- $\gamma$  before a day with the same concentration as used for the infection. We confirmed that all drugs used at each of the working concentrations did not have any cytotoxic effect on the cells (Supplementary Fig. S1). The cells were collected immediately after infection (~1 h) and then daily for the entire culture period for IFU assay [16], evaluation of inclusion formation with immunofluorescence microscopy [16], gene expression assessment using RT-PCR [IDO, IMPACT, chlamydial *omcB* (OmcB), chlamydial 16S rRNA, *glyceraldehyde 3-phosphate dehydrogenase* (GAPDH)], and protein expression assessment using western blotting [IDO,  $\alpha$ -tubulin], as described below.

## *2. 6. Assessment of inclusion formations using immunofluorescence microscopy and transmission electron microscopy (TEM)*

For up to 5 days after infection, cells were collected to determine the morphology of chlamydial inclusions, as described previously [16]. After the cells were fixed onto a slide with ethanol, they were stained with FITC-conjugated monoclonal antibody

specific to *Chlamydia* LPS (with Evans Blue) (Denka Seiken Co. Ltd.). Cells stained with specific antibodies were observed using a conventional or confocal laser microscope (LSM510; Carl Zeiss Japan Group, Tokyo, Japan). TEM was also performed in accordance with a method described previously [17]. In brief, bacterial cultures were immersed in a fixative containing 3% glutaraldehyde in 0.1 M phosphate-buffered saline (PBS) pH 7.4, for 24 h at 4 °C. After briefly washing with PBS, cells were processed for alcohol dehydration and embedded in Epon 812. Ultra-thin sections of cells were stained with lead citrate and uranium acetate before viewing by TEM (Hitachi H7100; Hitachi, Tokyo, Japan).

## 2. 7. RT-PCR

Total RNA was extracted from *C. pneumoniae*-infected cells, using an RNeasy Mini Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's protocol. Extracted RNA was treated with DNase I (DNA-free; Ambion, Austin, TX) to eliminate contaminating DNA. The resulting RNA preparations were confirmed to be DNA-free when a negative result was produced using PCR without the reverse transcription step. Reverse transcription of 2 µg total RNA by avian myeloblastosis virus reverse transcriptase was performed with random primers in a commercial reaction mixture (Reverse Transcription System; Promega, Madison, WI, USA). Synthesized cDNA was used for PCR amplification with primers specific for IDO (sense, 5'-CCT GAG GAG CTA CCA TCT GC-3'; antisense, 5'-TCA GTG CCT CCA GTT CCT TT-3') [21], IMPACT (sense, 5'-ACC AGA GGC AGA ATG AGG AA-3'; antisense, 5'-CCA AGG AGC ATT CAA CTG GT-3') [22], OmcB (sense, 5'-GGA TCT GCT ATT GCC CGT

AA-3'; antisense, 5'-CAG AAC ATT TGT GTC CAC CG-3') [23], chlamydial 16S *rRNA* (sense, 5'-GGA CCT TAG CTG GAC TTG ACA TGT-3'; antisense, 5'-CCA TGC AGC ACC TGT GTA TCT G-3') [16] and GAPDH (sense, 5'-AAC GGG AAG CTC ACT GGC ATG-3'; antisense, 5'-TCC ACC AAC CTG TTG CTG TAG-3') [16]. The PCR cycle consisted of 10 min denaturation at 94°C followed by 30–45 cycles, each of 30 s denaturation at 94°C; 30 s annealing at 52–58°C; and 45 s extension at 72°C. The amplified products were separated by 1.2% agarose gel electrophoresis and visualized by ethidium bromide staining. Each PCR was performed at least two times, for confirmation of PCR specificity and reproducibility. In addition, to prevent contamination, the preparation of the PCR mixture was performed in a separate room.

## 2. 8. Western blotting

*C. pneumoniae*-infected cells were collected and freeze–thawed cells were boiled for 5 min in a reducing sample buffer including 2-mercaptoethanol at 100°C. Approximately 10 µg protein was loaded and separated by 10% SDS-PAGE. The separated proteins were transferred to a polyvinylidene difluoride membrane by semi-dry electroblotting. The membrane was blocked with 3% skimmed milk in PBS for 1 h at room temperature and then incubated with human IDO (Sigma) or  $\alpha$ -tubulin specific monoclonal antibody (Sigma) for 1 h at room temperature. Horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (Sigma) was used as a secondary antibody for 1 h at room temperature. The reaction was visualized with an enhanced HRP-ECL color kit (Sigma).

## *2. 9. Detection of IFN- $\gamma$ receptor (CD119) on Jurkat cells by using immunofluorescence microscopy and flow cytometry*

Jurkat and THP-1 cells (control) were separately grown on a cover slip. Cells were washed in cold PBS containing 5% (w/v) bovine serum albumin (BSA) and then fixed in PBS containing 1% (w/v) paraformaldehyde. Fixed cells were incubated with either FITC-labeled CD119 (BioLegend, San Diego, CA, USA) or FITC-labeled isotype control monoclonal antibody (BioLegend). After staining, CD119 was detected by using conventional fluorescence microscopy. We also confirmed the expression on cell surface by using a FACS Calibur (Becton Dickinson Biosciences, Franklin Lakes, NJ). Data were analyzed using CellQuest software (Becton Dickinson Biosciences).

## *2. 10. Statistical analysis*

Statistical software (StatView, version J4.5; Abacus Concept, Berkeley, CA) was used for all statistical analysis. Comparisons between the IFU values were assessed by an unpaired *t* test. A *p* value < 0.05 was considered significant.

# **3. Results**

## *3. 1. C. pneumoniae inclusion formation and growth in Jurkat cells in the presence of IFN- $\gamma$*

We first compared under fluorescence microscopy the traits of inclusions formed by *C. pneumoniae* infection in HEp-2 and Jurkat cells in the presence or absence of IFN- $\gamma$ . We confirmed that the bacteria formed typical inclusions in both cell lines at 3 days after infection, reflecting appropriate culture conditions (Fig. 1A). As expected, when IFN- $\gamma$  was added to the culture, inclusion formation in HEp-2 cells was

significantly impaired (Fig. 1B, Upper-right panel). However, surprisingly, inclusion formation was not impaired in Jurkat cells by IFN- $\gamma$  (Fig. 1B, Lower-right panel), suggesting a minimal effect of IFN- $\gamma$  on the bacteria in lymphocytes. To confirm this, we assessed whether the number of infectious progeny changed in the presence or absence of IFN- $\gamma$ . As a result, while the number of infectious progeny significantly decreased in HEp-2 cells cultured with IFN- $\gamma$  (Fig. 1C), the bacteria in Jurkat cells grew normally even in the presence of IFN- $\gamma$  (Fig. 1D). Collectively, these data clearly indicated that IFN- $\gamma$  did not work against *C. pneumoniae* infection in Jurkat cells. We also confirmed that IFN- $\gamma$  had no effect on *Ch. trachomatis* growth in Jurkat cells in a similar way (Supplementary Fig. S2), suggesting that the phenomenon is not specific for *C. pneumoniae*. We also confirmed through TEM analysis that the *C. pneumoniae* infected in Jurkat cells possess a complete developmental cycle consisting of EB and RB, showing the chlamydial RB to EB re-differentiation in the infected Jurkat cells (Fig. 1E).

### 3. 2. IFN- $\gamma$ did not stimulate IDO induction in lymphoid cells

The effect of IFN- $\gamma$  effect on chlamydial elimination in human epithelial cells is responsible for IDO induction [9–14]; therefore, we next assessed whether gene expression of IDO could be induced by IFN- $\gamma$  in Jurkat cells. As shown in Fig. 2A, although IDO was strongly expressed in HEp-2 cells by stimulation with IFN- $\gamma$ , gene expression in Jurkat cells was not seen, even in the presence of IFN- $\gamma$  stimulation. Also, in Jurkat cells, there was no expression of the IDO-related gene IMPACT, which rescues cellular stress such as depletion of nutrients or apoptosis [22], even with IFN- $\gamma$  exposure.

Although a representative bacterial gene *omcB* associated with the late stage of the bacterial developmental cycle in epithelial cells was critically down-expressed in the presence of IFN- $\gamma$ , meaning a shift to persistent infection followed by bacterial elimination, the change in gene expression in Jurkat cells, even after IFN- $\gamma$  exposure, was minimal, indicating bacterial growth with a normal developmental cycle. Furthermore, it was also confirmed by western blotting that Jurkat cells failed to express IDO protein regardless of IFN- $\gamma$  stimulation (Fig. 2B). In addition, we also confirmed using immunofluorescence staining with flow cytometry that the Jurkat cells used for this study absolutely expressed IFN- $\gamma$  receptor (CD119) on their surface with a similar expression level to that in THP-1 cells (control), which normally express CD119 antigen on their surface [Fig. 3A (Representative images of immunofluorescence staining) and B (Representative histograms of flow cytometry)]. This ensured that the cells were equipped with complete response against IFN- $\gamma$ . Thus, the results clearly indicated that IFN- $\gamma$  could not impair the bacterial growth in Jurkat cells, because of the lack of IDO expression.

### *3. 3. Autocrine effect of IFN- $\gamma$ on infected Jurkat cells*

It is obvious that lymphocytes are major IFN- $\gamma$ -producing cells, which could stimulate various cells including themselves. Therefore, we assessed whether bacteria could grow in Jurkat cells stimulated with PMA and ionomycin under autocrine conditions. As shown in Fig. 4, we confirmed that these drugs clearly stimulated IFN- $\gamma$  induction regardless of bacterial infection, suggesting that the infection had no effect on IFN- $\gamma$  induction in Jurkat cells. Furthermore, the number of bacterial infectious progeny

increased to the same extent as in the control cells, regardless of stimulation with IFN- $\gamma$  or PMA and ionomycin (data not shown). The results demonstrated that the autocrine effect of IFN- $\gamma$  on infected Jurkat cells was minimal, indicating that IFN- $\gamma$  did not affect *C. pneumoniae* in Jurkat cells, regardless of autocrine action.

#### 3. 4. *C. pneumoniae* survival in primary human lymphocytes in the presence of IFN- $\gamma$

Immortal cells, such as Jurkat, express incomplete normal traits of primary lymphocytes, therefore, we assessed whether enriched lymphocytes prepared from human blood peripheral mononuclear cells could maintain *C. pneumoniae*, even in the presence of IFN- $\gamma$ . Although the bacterial numbers drastically decreased three logs during 5 days culture, the bacteria obviously survived during the culture period regardless of the presence of IFN- $\gamma$  (Fig. 5A, left panel). Immunofluorescence microscopy showed typical inclusion formation, which supported the hypothesis that bacteria could survive in enriched primary human lymphocytes in the presence of IFN- $\gamma$  (Fig. 5A, right image). Thus, it is likely that human primary lymphocytes also have a sheltering role to help bacteria escape from the effect of IFN- $\gamma$ , similar to immortal Jurkat cells.

#### 3. 5. IFN- $\gamma^{-/-}$ mouse spleen cells maintained *C. pneumoniae* infectious progeny similarly to wild-type cells

To determine the effect of the small amount of IFN- $\gamma$  produced by lymphocytes on *C. pneumoniae* survival, we assessed whether *C. pneumoniae* infectious progeny was maintained in IFN- $\gamma^{-/-}$  mouse spleen cells similarly to wild-type cells. Although the

bacterial numbers decreased by one log during 5 days culture, there was no significant difference in bacterial numbers between wild-type and IFN- $\gamma$ <sup>-/-</sup> mouse spleen cells (Fig. 5B), indicating a minimal effect of IFN- $\gamma$  produced by lymphocytes on *C. pneumoniae* survival. We also performed a similar experiment with *Ch. trachomatis* L2, which is an invasive human pathogen. In contrast to *C. pneumoniae*, *Ch. trachomatis* L2 obviously grew well in IFN- $\gamma$ <sup>-/-</sup> mouse spleen cells when compared to wild-type cells (Fig. 5C). Thus, it is likely that *C. pneumoniae* may be more resistant to IFN- $\gamma$  exposure than *C. trachomatis* L2 in primary mouse lymphocytes. In addition, we also confirmed that under culture conditions with bacteria alone (*C. pneumoniae*), the number of infectious progeny decreased rapidly below the detection limit until 3 days after incubation (Supplementary Fig. S3), supporting our data that *C. pneumoniae* is maintained in primary mouse lymphocytes, as well as human peripheral blood lymphocytes.

#### 4. Discussion

We demonstrated that IFN- $\gamma$  had no effect against *C. pneumoniae* in Jurkat cells, in a similar way to enriched blood lymphocytes or mouse spleen cells, through a lack of IDO expression. It is well known that Jurkat cells normally express IFN- $\gamma$  receptor CD119 on their surface, therefore, presumably providing a complete setting around IFN- $\gamma$  response [24]. This receptor may be lacking in many strain variants that have an abnormal immune response against IFN- $\gamma$  stimulation. However, it was assured the Jurkat cells used for this study absolutely expressing CD119 (Fig. 3A), equipped with a response against IFN- $\gamma$ , as seen in IFN- $\gamma$  induction by stimulation with PMA and ionomycin in Jurkat cells, regardless of *C. pneumoniae* infection (Fig. 4A). Thus, it is

obvious that the Jurkat cells possessed complete IFN- $\gamma$  recognition and production pathways, reflecting traits of primary lymphocytes. So far, we don't have yet any explanation on failure of IDO expression in the Jurkat cells stimulated with IFN- $\gamma$ . Meanwhile, Jurkat cells are well expressing CD45 molecule, leading to negative regulation of JAK/STAT signaling located in downstream of IFN- $\gamma$  stimulation through the recruitment of inhibitory molecule Downstream of Kinase 1 (DOK-1) [25]. Such signal through CD45 molecule expressing on Jurkat cells may contribute failure of IDO expression in the Jurkat cells stimulated with IFN- $\gamma$ , although the signal molecule remains unknown. Alternatively, although IFN- $\gamma$  receptor is formed two chains, IFN- $\gamma$  R1 (IFN- $\gamma$  binding chain) and IFN- $\gamma$  R2 (transducing chain), it is well known that human T cells, but not B cells, including Jurkat cells are poorly expressing IFN- $\gamma$  R2 [26-28], possibly connecting the failure of IDO expression.

Fluorescence microscopic and TEM analysis with growth curves and inclusion formation clearly indicated that although bacterial growth was observed in Jurkat cells, it was probably relatively slow when compared to that in HEp-2 cells. Although the mechanism of slow growth in Jurkat cells remains unknown, bacterial accessibility to multivesicular bodies or vesicles to acquire nutrients may differ among host cell types [29-32]. Alternatively, because IFN- $\gamma$  induction is not sustained in target inflammatory tissues, *C. pneumoniae* in lymphocytes could withstand tryptophan depletion through IDO activation induced by IFN- $\gamma$  stimulation in other cells around lymphocytes, such as epithelial cells, as possible target cells for secondary infection, until local inflammation subsides.

When compared to Jurkat cells, *C. pneumoniae* did not grow well in human

enriched peripheral blood lymphocytes. As supported by our previous studies showing successful inclusion formation and increased bacterial gene expression [16,18], it is obvious that *C. pneumoniae* could survive in human primary lymphocytes, but it is neglected just attaching and maintaining on the cell surface because of cell-free culture showing a rapid decrease of the bacterial infectious progenies below a detection limit. Although the mechanism of growth inhibition is still not clear, it is obvious that IFN- $\gamma$  did not affect the bacteria in the primary lymphocytes as well as in Jurkat cells. Several studies have demonstrated failure of IDO expression in human lymphoid cells such as RPMI1788, Molt-4, CCRF-SB, CCRF-CEM or Raji cells [33–35]. This suggests inactivity of IFN- $\gamma$  against *C. pneumoniae* in human peripheral blood lymphocytes caused by failure of IDO expression, as well as in the Jurkat cells used for this study. Alternatively, since it is well known that *C. pneumoniae* infection could induce TNF $\alpha$  from monocytes/macrophages, which is a critical factor suppressing the bacterial growth in the cells [35, 36]. Therefore, it could not deny the role of TNF $\alpha$  secreted from monocytes/macrophages on lacking of *C. pneumoniae* active replication in the primary lymphocytes.

We observed a difference in growth level between human and mouse primary lymphocytes, regardless of IFN- $\gamma$  was also seen. In mice, the major cause of antichlamydial activity induced by IFN- $\gamma$  stimulation is small-GTPase p47 induction, resulting in autophagy [11,12]. Also, because iNOS<sup>-/-</sup> mice show resistance to pathogenic chlamydial infection, the association of antichlamydial activity with NO induction is minimal [11,12]. Furthermore, IFN- $\gamma$  stimulation cannot induce any IDO expression in mouse cells because of the lack of IFN regulatory factor 1 transcription

factor, coordinated with signal transducer and activator of transcription 1 [37]. In contrast to mice, in humans, inducible NO synthase expression is not induced by IFN- $\gamma$  stimulation, indicating minimal association of NO with antichlamydial activity [12], and no human homolog of small-GTPase p47 induced by IFN- $\gamma$  stimulation has been found [38]. Thus, it is likely that such as small-GTPase p47 or NO induction additional mechanisms may be responsible for bacterial growth inhibition in mouse primary lymphocytes.

Whether lymphocytes play a role in supporting pathogenic chlamydial survival at sites of inflammation, such as the genital tract or lungs, remains a significant question. However, it is apparent that human lymphocytes significantly contribute to the protective effects and the pathogenesis of chlamydial disease at these sites [39]. Also, in primate models, it has been confirmed that, following infection with *Ch. trachomatis*, mononuclear infiltration in the genital tract occurs and is composed of a large number of T cells including CD4<sup>+</sup> and CD8<sup>+</sup> cells, with a capability of producing IFN- $\gamma$  [40]. These findings indicated that, when lymphocytes accumulate at inflammatory sites, they encounter infected epithelial or monocytic cells, presumably occurring bacterial translocation from original host cells to lymphocytes, although this translocation mechanism is unknown.

In conclusion, we demonstrated that *C. pneumoniae* (TW183) grew normally in human lymphoid Jurkat cells and similarly survived in enriched human or mouse primary blood lymphocytes, even in the presence of IFN- $\gamma$ . This suggests that lymphocytes may provide a shelter for the chlamydiae to escape from the effects of IFN- $\gamma$ , which is a novel escape strategy for pathogenic chlamydiae, with possible

clinical significance.

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## Legends to figures

**Fig. 1.** Representative fluorescence microscopy images showing inclusion formation (A, B and E) and one-step growth curves (C and D) for *C. pneumoniae* in Jurkat and HEp-2 cells in the presence or absence of IFN- $\gamma$  for up to 72 h after infection. (A and B) Infected cells were stained with FITC-conjugated anti-*Chlamydia* antibody. See Methods. The images were captured at 72 h after infection. Arrows, inclusion bodies. (A) Conventional microscopy,  $\times 200$ ; (B) confocal laser microscopy,  $\times 1000$ . (C and D) Cell lysates prepared at each time point were inoculated onto HEp-2 cell monolayers to assess the number of infective progeny. Number of inclusions was determined by staining with FITC-conjugated anti-*Chlamydia* antibody. Data shown represent the mean  $\pm$  SD. \* $p < 0.05$  versus the value immediately after infection (day 0). (E) Representative TEM image showing an inclusion body of *C. pneumoniae* formed in Jurkat cells at 3 days after infection in the absence of IFN- $\gamma$ . Dashed squares enlarged to right panels (Upper: representative EB, Lower: representative RB). Bar, 1 $\mu$ m.

**Fig. 2.** IFN- $\gamma$ -inducible IDO expression regardless of *C. pneumoniae* infection was absent in Jurkat human lymphoid cells. (A) Representative RT-PCR band images showing IDO, IMPACT, OmcB, 16S rRNA and GAPDH gene expression levels. We compared IFN- $\gamma$ -inducible gene expression (IDO and IMPACT) and bacterial major gene expression (OmcB and 16SrRNA) between HEp-2 and Jurkat cells in the presence or absence of 5 ng/mL IFN- $\gamma$ . (B) Representative western blot images showing IDO and  $\alpha$ -tubulin protein expression levels. We compared IFN- $\gamma$ -inducible protein expression

(IDO) between HEp-2 and Jurkat cells in the presence or absence of 5 ng/mL IFN- $\gamma$ .

**Fig. 3.** Expression of IFN- $\gamma$  receptor (CD119) on Jurkat cells. (A) Representative immunofluorescence microscopic images of CD119 on Jurkat and THP-1 (positive control) cells. Green color, CD119 expression. Magnification,  $\times 1000$ . (B) Representative histograms of flow cytometry showing CD119 expressions on Jurkat and THP-1 (positive control) cells.

**Fig. 4.** Representative RT-PCR image showing IFN- $\gamma$ , GAPDH and bacterial 16S rRNA gene expression in the presence or absence of 5 nM PMA and 25 nM ionomycin or 5 ng/mL IFN- $\gamma$ . Cp, *C. pneumoniae* infection. P/I, stimulation with PMA and ionomycin.

**Fig. 5.** Changes in infectious progeny for chlamydiae in either primary enriched human blood lymphocytes obtained from three healthy donors [A (with *C. pneumoniae* infection)] or IFN- $\gamma^{-/-}$  mouse spleen cells [B (with *C. pneumoniae* infection) and C (with *C. trachomatis* infection)]. The cells were infected with bacteria at an MOI 10 (for *Ch. trachomatis*) or 50 (for *C. pneumoniae*) by centrifugation, and incubated in the presence of IFN- $\gamma$  (20 $\mu$ g/ml) for up to 5 days. Number of infectious progeny in cultures post-infection was estimated by the IFU assay (See Fig. 2 legend). Plots and bars show average values and SD, respectively. Arrows in image show specific inclusion formation at 3 days after infection. NS, not significant. \* $p < 0.05$  versus the value at the same time after infection.

## Legends to supplementary figures

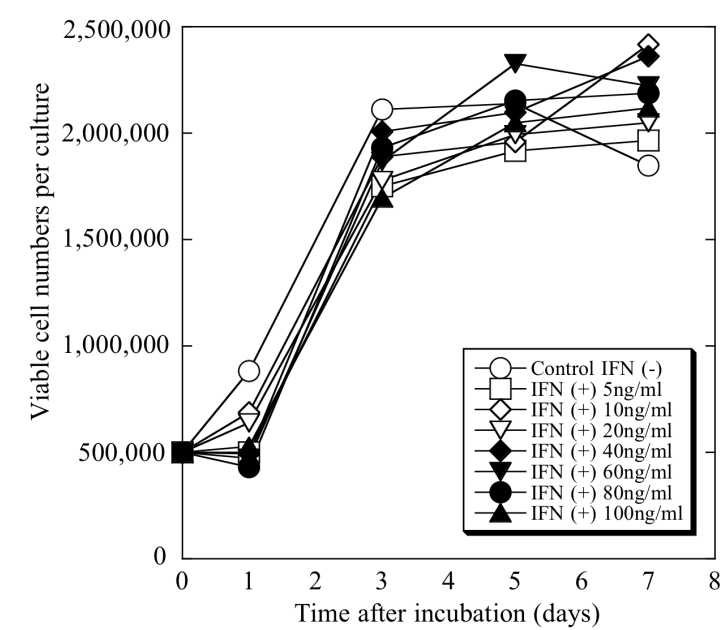
**Fig. S1.** Cytotoxic effect of drugs (IFN- $\gamma$  and PMA with ionomycin) on growth of Jurkat cells. Cell growth was monitored in the presence of various concentrations of IFN- $\gamma$  (5–100 ng/mL) (A) or PMA (2–10 nM) with ionomycin (25–125 nM) (B) for up to 7 days. The number of cells was determined by using trypan blue exclusion assay.

**Fig. 2S.** *Ch. trachomatis* growth in human immortal Jurkat lymphoid cells was not controlled by IFN- $\gamma$ , similar to *C. pneumoniae*. (A) Representative fluorescence microscopy images showing inclusion formation in *Ch. trachomatis*-infected Jurkat and HeLa-2 cells in the presence or absence of IFN- $\gamma$  (5 ng/mL) at 5 days after infection. Infected cells were stained with FITC-conjugated anti-*Chlamydia* antibody. Magnification,  $\times 400$ . (B and C) One-step growth curves for *Ch. trachomatis* in HeLa cells (B) and Jurkat cells (C) showing number of infectious progeny post-infection in the presence or absence of IFN- $\gamma$  (5 or 20 ng/mL). See Fig. 2 legend. Data shown represent the mean  $\pm$  SD. \* $p < 0.05$  versus the value immediately after infection (day 0).

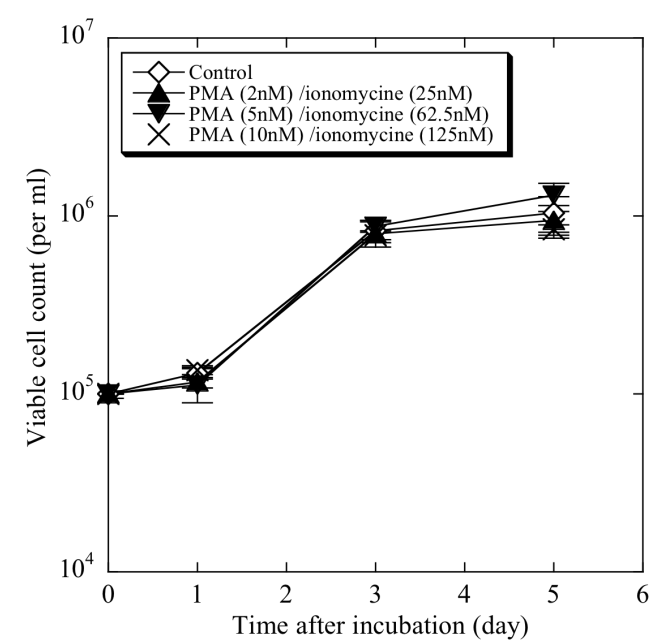
**Fig. 3S.** Changes in *C. pneumoniae* infective progeny in cultures under cell-free conditions. The bacteria [ $5 \times 10^3$  (low) or  $5 \times 10^2$  (high) IFU] were solely incubated without any cells under conventional cell-culture conditions for up to 5 days. The number of bacteria was assessed by the IFU assay. Each of the plots shows the average IFU count.

Supplementary Fig. S1

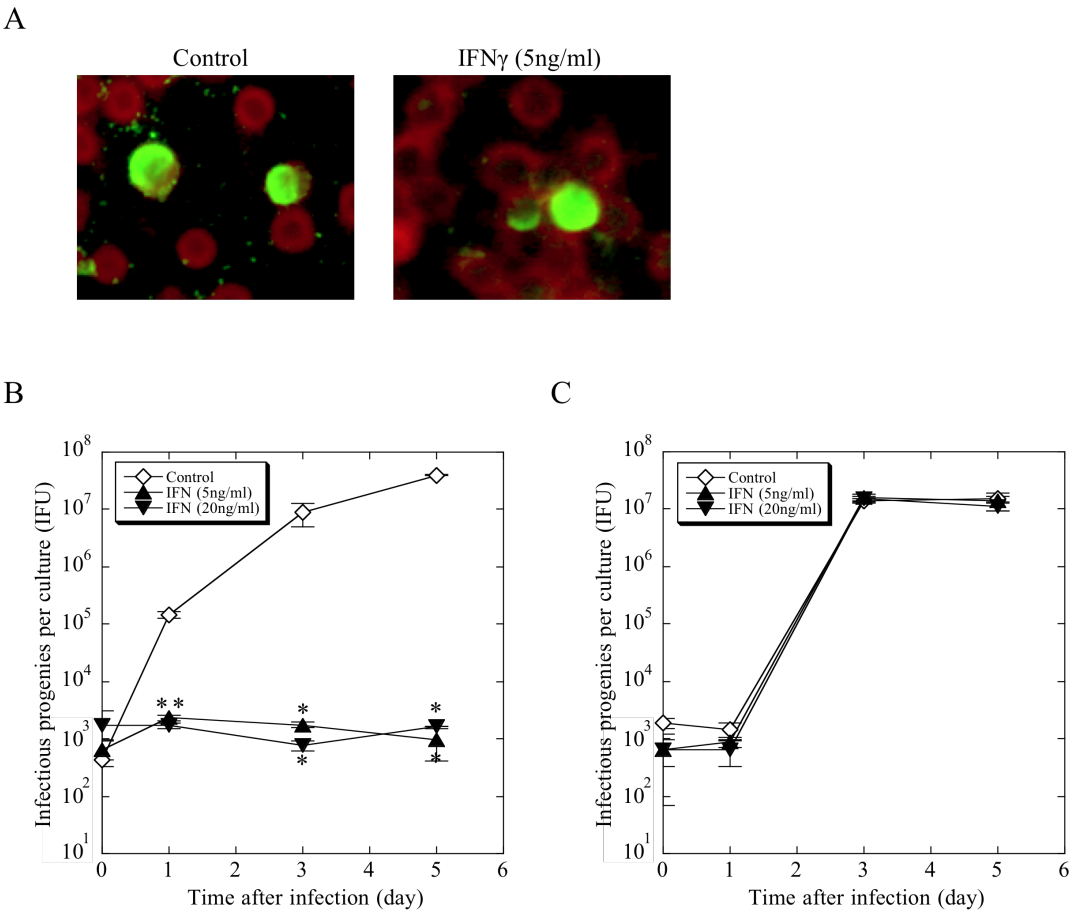
A



B



Supplementary Fig. S2



Supplementary Fig. S3

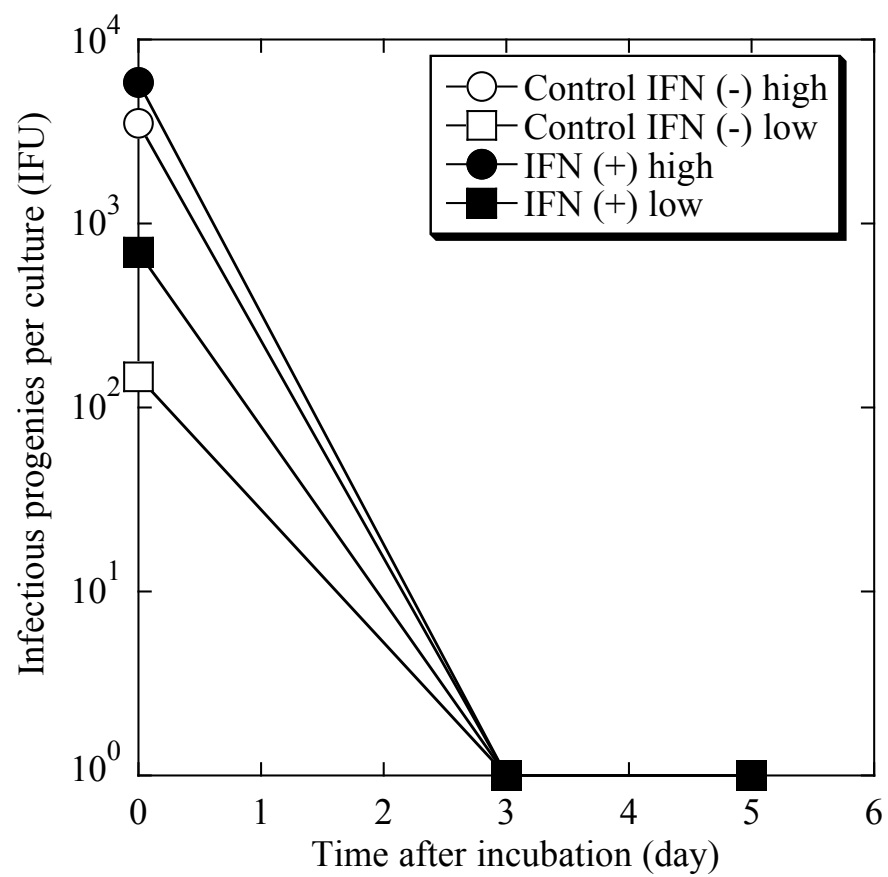


Fig. 3

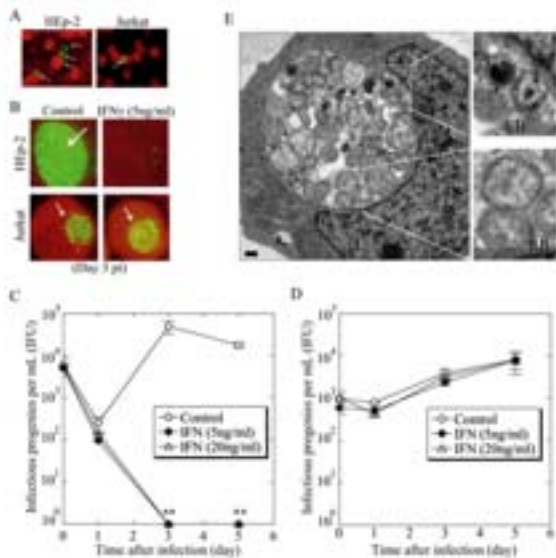


Fig. 2

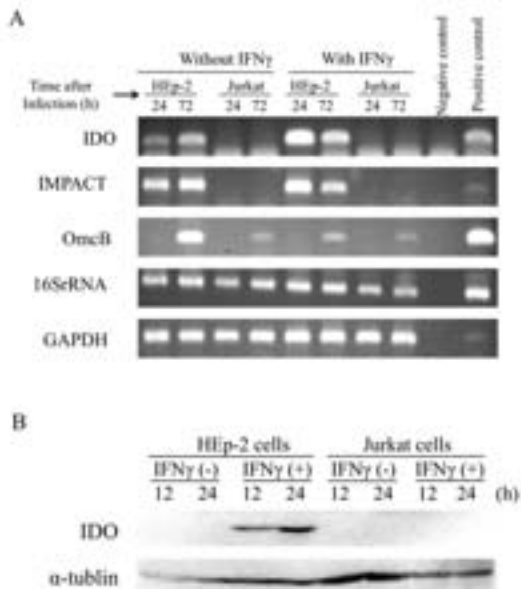


Fig. 3

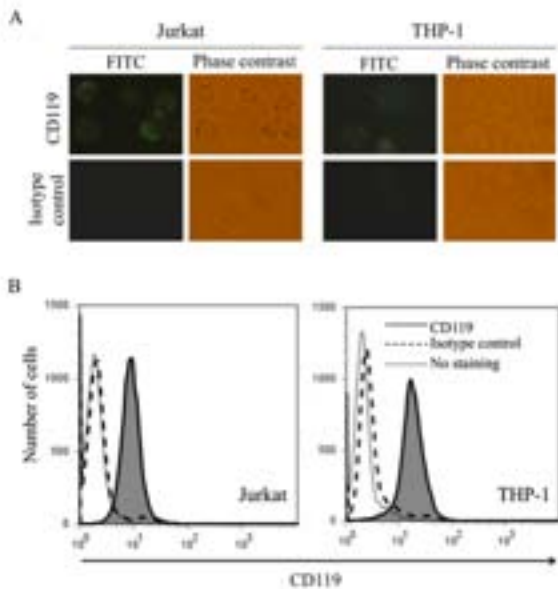


Fig. 4

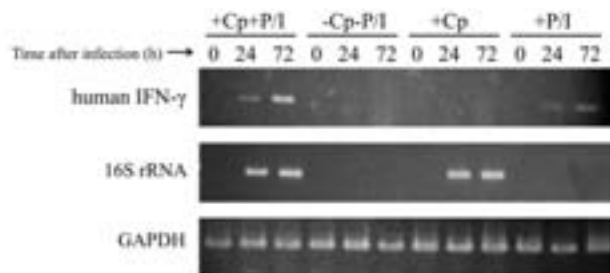
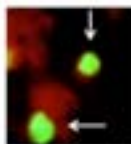
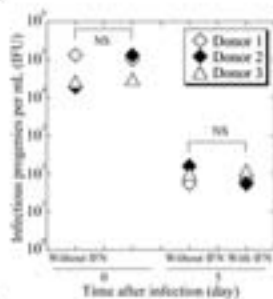
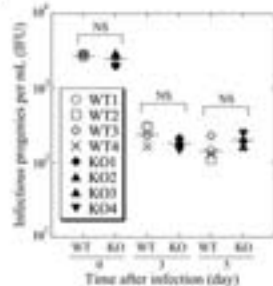


Fig. 5

A



B



C

