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**Chlamydia pneumoniae** Inhibits Activated Human T Lymphocyte Proliferation by the Induction of Apoptotic and Pyroptotic Pathways

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*Chlamydia pneumoniae* is an omnipresent obligate intracellular bacterial pathogen that infects numerous host species. *C. pneumoniae* infections of humans are a common cause of community acquired pneumonia but have also been linked to chronic diseases such as atherosclerosis, Alzheimer’s disease, and asthma. Persistent infection and immune avoidance are believed to play important roles in the pathophysiology of *C. pneumoniae* disease. We found that *C. pneumoniae* organisms inhibited activated but not nonactivated human T cell proliferation. Inhibition of proliferation was pathogen specific, heat sensitive, and multiplicity of infection dependent and required chlamydial entry but not de novo protein synthesis. Activated CD4⁺ and CD8⁺ T cells were equally sensitive to *C. pneumoniae* antiproliferative effectors. The *C. pneumoniae* antiproliferative effect was linked to T cell death associated with caspase 1, 8, 9, and IL-1β production, indicating that both apoptotic and pyroptotic cellular death pathways were activated after pathogen–T cell interactions. Collectively, these findings are consistent with the conclusion that *C. pneumoniae* could induce a local T cell immunosuppression and inflammatory response revealing a possible host–pathogen scenario that would support both persistence and inflammation. *The Journal of Immunology*, 2011, 186: 7120–7126.

**Chlamydia pneumoniae** is an obligate intracellular bacterial pathogen that is a major cause of community acquired pneumonia. *C. pneumoniae* infection has also been linked to several chronic diseases such as atherosclerosis, asthma, Alzheimer’s disease, and multiple sclerosis (1–6). A precise causative relation to these important chronic human diseases has yet to be shown definitively and in fact remains controversial.

Among chlamydiae, *C. pneumoniae* is unique as it is capable of infecting a broad spectrum of host species and replicates in various host cell types (7). In humans, target cells of respiratory infection include both epithelial cells and alveolar macrophages. *C. pneumoniae* can disseminate from the lung to extrapulmonary tissues where infection has been found in PBMCs and endothelial and smooth muscle cells (8–10). A pathogenic feature of *C. pneumoniae* is the organism’s propensity to cause cryptic persistent infection of alveolar macrophages, monocyte-derived dendritic cells, and endothelial cells elicits the production of numerous proinflammatory cytokines and chemokines. These include IL-1β, TNF-α, IL-8, IL-12/IL-23, MCP-1, MIG, RANTES, and the cell adhesion molecule ICAM-1 (10, 12–14). Thus, a combination of immunosuppressive and inflammatory biological activities could potentially generate an infection environment that links persistent infection and chronic inflammatory stimulation.

*Chlamydia pneumoniae* interaction with host cells can both inhibit and induce apoptosis (15–21). The bacterial elements involved in the subversion or induction of the cellular death process are unknown, and the general relationship of these cell death pathways to the pathophysiology of *C. pneumoniae* infection and disease is not completely understood. Little is known about *C. pneumoniae* interaction with human T cells. To gain a better understanding of how *C. pneumoniae* might affect host immunity, we have studied this pathogen–host interaction. We report that *C. pneumoniae* specifically kills activated human T cells by inducing apoptotic and inflammasome cell death pathways. These findings are consistent with the conclusion that *C. pneumoniae* T cell interactions could simultaneously suppress cellular immunity and bring about damaging tissue inflammation.

**Materials and Methods**

**Bacteria**

*C. pneumoniae* (AR-39) and *Chlamydia trachomatis* strains D/UW-3 and A2497 were propagated in HeLa 229 cells, and elementary bodies (EBs) were purified by density gradient centrifugation and stored at −80°C as previously described (22).

**Fluorescein labeling of primary human T cells**

Peripheral blood was obtained from healthy adult donors in accordance with the approved protocol of the Institutional Review Board for Human
Subjects, National Institute of Allergy and Infectious Diseases. The PBMC purification was conducted as described by Kobayashi et al. (23). Briefly, leukocytes were separated from erythrocytes by sedimentation of fresh heparinized blood and mixed v/v with 0.9% sodium chloride (Injection USP; Baxter Healthcare, Deerfield, IL) containing 3% Dextran T-500 (Amersham Pharmacia) and incubated for 20 min at room temperature. The enriched supernatant of leukocytes was centrifuged at 550 × g for 10 min, and the pellet was resuspended in 0.9% sodium chloride solution. The cellular suspension was Stratified on Ficoll-Paque PLUS (1.077 g/ml; Amersham Pharmacia) and centrifuged for 20–30 min at room temperature. The PBMC layer was aspirated and washed twice in PBS. Total T cells were purified from fresh human PBMCs by using a human pan T cell separation kit (Miltenyi Biotech, Auburn, CA). The purified cells were 95% CD3+ as assessed by staining and flow cytometry analysis. For monitoring cellular proliferation, the cells were labeled with 5 μM CFSE (Invitrogen Molecular Probes, Eugene, OR) following the manufacturer’s instructions. Cells were labeled prior to the addition of activation stimuli.

Human T cell activation and chlamydial infection

The biological effect of C. pneumoniae–T cell interactions was done using nonactivated and activated T cells. The assays performed with nonactivated cells were conducted as follows: unlabeled or CFSE-labeled purified nonactivated T cells (2 × 10^5 cells/well) in an 0.2 ml volume of RPMI 1640 containing 10% FCS, 2 mM L-glutamine, nonessential amino acids, 10 mM HEPES, and 55 mM l-mercaptopethanol were incubated 6 to 8 h with chlamydial EBs at various multiplicity of infection (MOI) in a round-bottom 96-well plate (Corning, Corning, NY) or in SPG buffer only (250 mM sucrose, 10 mM sodium phosphate, 5 mM L-glutamic acid pH 7.2). After chlamydial–T lymphocytes interaction, cells were washed and T cells were activated by using BD Bio coat 96-well plates coated with anti-human CD3 Ab (BD Biosciences, Bedford, MA) and soluble anti-human CD28 Abs (1 μg/ml; BD Biosciences) (hereafter termed TCR/CD28 stimulation). After 48 h of incubation at 37°C/5% CO2, the cells were removed from activation signals, washed, and incubated for 5 d in media supplemented with human rIL-2 (2 U/ml; BD Biosciences) in a round-bottom 96-well plate.

For chlamydial-interaction studies with activated T cells, unlabeled or CFSE-labeled cells were first stimulated with TCR/CD28 and incubated for 48 h in media supplemented with human rIL-2. Subsequently, the cells were removed from stimulus and incubated with chlamydial EBs or SPG buffer. Transwell assays were performed using activated T cells. T cells were stimulated with TCR/CD28 and 4 × 10^5 cells placed in each chamber of Transwell permeable supports with 0.4 μM polyester membrane and 24-well culture plates (Costar, Corning, NY). T cells in the upper chamber were infected with chlamydiae at an MOI of 100 or with SPG buffer. Cultures were incubated for 12 h at 37°C and T cells from both chambers subjected to flow cytometry analysis.

When indicated, the experiments were performed in the presence of rifampicin (1 μg/ml) or cyclohexalin D (1 μg/ml) for 30 min prior to chlamydial infection. At the end of each experimental protocol, flow cytometry assays were performed using an LSRII flow cytometer (BD Biosciences, San Jose, CA) and FlowJo software version 8.7.3 (Tree Star, Ashland, OR). Proliferation was measured by CFSE dilution.

Cellular viability assessment

Differential between live and dead cells was assessed using the single-color Aqua Live/Dead Fixable Cell Stain Kit (Invitrogen, Molecular Probes, Eugene, OR). Briefly, after 6 h of incubation with chlamydial EBs, the cells were collected, washed three times in PBS, and incubated for 30 min at room temperature with the fluorescent reactive dye. After staining, the cells were washed, and surface receptor staining was performed using the following Abs: anti-human CD3–Alexa 700, anti-human CD4–phycoerythrin or CD8–allophycocyanin (eBioscience).

Phosphatidylserine staining

To discriminate apoptotic versus necrotic cells, a double staining was assayed using annexin V and 7-aminoactinomycin D (7-AAD) (BD Biosciences Pharmingen, San Jose, CA). Briefly, after 2, 4, and 6 h of incubation with chlamydial EBs, the cells were collected by centrifugation followed by 15 min staining with FITC-conjugated annexin V and 7-AAD following the manufacturer’s instructions. Cells were then analyzed by flow cytometry within 1 h of labeling

TUNEL assay

After 12-h incubation with chlamydial EBs, the cells were fixed in 1% paraformaldehyde and placed on ice for 15 min. The cells were then washed twice in PBS, reconstituted in ice-cold 70% ethanol, and stored in a –20°C freezer for 12–18 h prior to the staining. The TUNEL reaction was performed using an APO-BrdU TUNEL assay kit according to the recommendations of the manufacturer (Invitrogen, Molecular Probes).

Determination of caspase 1, caspase 8, and caspase 9 activity

Active caspases were detected with FLICA Apoptosis Detection Kit (Immunochemistry Technologies, Bloomington, MN). After 5 h, chlamydia-infected T cells were incubated with a fluorescent inhibitor peptide specific to either caspase 1 (FM-YVAD-FMK), caspase 8 (FM-LETD-FMK), or caspase 9 (FM-LEHD-FMK) for 60 min at 37°C/5% CO2. Inhibitors were removed by rinsing; the cells were fixed and then analyzed by flow cytometry.

Western blotting

Chlamydia-infected T lymphocytes were washed and incubated with a buffer containing 20 mM Tris-HCl (pH 7.4), 1% (v/v) Nonidet P-40, 10% (v/v) glycerol, 137 mM NaCl, 1 mM EDTA, and a protease inhibitor mixture. The protein equivalent to 4 × 10^6 T cells per lane was separated by 4–15% gradient SDS-PAGE (Cramer gels; Bio-Rad, Hercules, CA) and transferred to a polyvinylidene difluoride membrane (BioRad). The membranes were blocked for 1 h at room temperature in TBS (0.012 M Tris pH 7.4, 0.14 M NaCl, 3.0 mM KCl) containing 0.05% Tween 20 (TBS–Tween α20 and 5% nonfat milk). The membranes were incubated overnight at 4°C in TBS–TWEEN 20 containing the following primary Abs: rabbit anti-human caspase 9, rabbit anti-human Bid (Cell Signaling Technology, Beverley, MA), anti-human caspase 8 α20 (clone 12F5; Apotech), or anti-β–actin mAb (clone AC-74; Sigma, St. Louis, MO). After incubation, the membranes were washed and incubated at room temperature for 1 h in TBS–TWEEN containing anti-rabbit- or anti-mouse IgG secondary Abs conjugated to HRP (Cell Signaling Technology). The membranes were developed via chemiluminescence using ECL Pico reagent (Pierce, Rockford, IL).

IL-1β detection

Culture supernatants from mock or chlamydia-infected T cells were harvested at 0.5, 2, 4, and 6 h postinfection, clarified, and stored at –80°C until use. IL-1β was detected and quantified using Quantikine ELISA kit (R&D Systems, Minneapolis, MN) following vendor specifications.

Statistical analysis

Differences in the means for the groups were compared by the two-tailed Student t test, and a p value <0.05 was considered significant.

Results

C. pneumoniae specifically abrogates the proliferation of activated but not nonactivated T lymphocytes in an MOI-dependent manner

C. pneumoniae infection resulted in a near complete inhibition of proliferation of activated (Fig. 1B) but not nonactivated (Fig. 1A) T cells. Inhibition of activated T cells was MOI dependent. The inhibitory effect was specific to C. pneumoniae as related C. trachomatis organisms failed to suppress proliferation of activated T cells. Moreover, C. pneumoniae was equally inhibitory against both activated CD4+ and CD8+ T cell subpopulations (Fig. 1B). The C. pneumoniae antiproliferative effect is the result of T cell death

Purified activated T cells were infected with different MOI of C. pneumoniae or C. trachomatis and analyzed by flow cytometry for cell death. These analyses showed that C. pneumoniae infection resulted in significant cell killing (65%) of both activated CD4+ and CD8+ T cells (Fig. 2). Similar to the antiproliferation results shown in Fig. 1, T cell killing was C. pneumoniae specific and MOI dependent. Collectively, these findings support the conclusion that the inhibition of activated human CD4+ and CD8+ T cells by C. pneumoniae was the result of cell death, that the putative virulence factors that mediate T cell killing required a high infectious
dose, and that the biological effect on T cells was unique to \textit{C. pneumoniae} organisms. We next investigated the biological properties of these \textit{C. pneumoniae}-specific T cell virulence factor(s).

Direct contact of \textit{C. pneumoniae} with T cells is required to induce T cell death

We performed Transwell studies to determine whether T cell killing required direct interactions of \textit{C. pneumoniae} organisms or was due to a bystander effect resulting from the secretion of soluble T cell molecules on uninfected T cell populations. Activated T cells were placed in both the upper and lower chambers of Transwell plates. The cells in the upper chamber were infected with \textit{C. pneumoniae} (MOI 100) and the cells in the lower chamber left uninfected. The viability of T cells in both chambers was monitored after incubation with chlamydiae. We observed T cell death only in the upper chamber of the Transwell plates (Fig. 3) arguing that lymphocyte-secreted components were not responsible for T cell killing. The findings indicate that direct contact between \textit{C. pneumoniae} organisms and T cells was required to induce cellular death.

The \textit{C. pneumoniae} T cell inhibitory effect requires viable organisms, is independent of de novo chlamydial mRNA synthesis, and is dependent on pathogen entry

We next investigated the biological properties that affected the ability of \textit{C. pneumoniae} to kill activated T cells. \textit{C. pneumoniae} T cell killing assays were performed using live and heat-killed organisms in the presence of rifampicin (a prokaryotic RNA synthesis inhibitor) or cytochalasin D (an actin cytoskeleton inhibitor that prevents chlamydial entry) (24). The results are shown in Fig. 4. \textit{C. pneumoniae} organisms treated at 56°C for 30 min did not kill T cells (Fig. 4A). Chlamydial killing was not inhibited after treatment of T cells with rifampicin but was completely inhibited after treatment of T cells with cytochalasin D (Fig. 4B). These findings show that the \textit{C. pneumoniae} virulence factors that mediate T cell killing require both infectious organisms and their entry into cells; however, once chlamydiae have been internalized, the induction of T cell killing occurs independently of de novo chlamydial protein synthesis. The findings are consistent with the conclusion that the chlamydial virulence factors are present on the infectious organisms either as surface components or secreted.
molecules that rapidly induce T cell death early post-chlamydial infection in a growth-independent manner.

**C. pneumoniae induces phosphatidylserine exposure and nuclear fragmentation in human T lymphocytes**

To characterize further *C. pneumoniae*-induced T cell death mechanism(s), we studied whether infected cells were apoptotic. Infected T cells were double stained with annexin V and 7-AAD to detect early surface exposure of phosphatidylserine (PS) PS^+ 7-AAD^- cells (Fig. 5A) and DNA strand breaks by TUNEL staining (Fig. 5B) as indicators of early and late stages of apoptosis. Highly significant and specific PS^+ staining (Fig. 5A) and TUNEL^+ cells (Fig. 5B) were observed at 2, 4, and 6 h postinfection with *C. pneumoniae*. No significant PS staining or DNA strand breakage was detected after *C. trachomatis* infection. The early surface expression of PS and subsequent DNA fragmentation induced by *C. pneumoniae* indicates that lymphocyte cellular death was due to apoptosis.

**Induction of multiple cell death pathways in T lymphocytes by C. pneumoniae infection**

We used Abs specific to the activated forms of caspase 8 and caspase 9 to probe cellular extracts of *C. pneumoniae*-infected T cells by Western blotting to differentiate between intrinsic and extrinsic apoptotic pathways, respectively (25, 26). Fig. 6 provides evidence that both caspase 8 and caspase 9 were cleaved into their respective proteolytic fragments (asterisks) at 8 and 12 h postinfection. Cleavage was specific to *C. pneumoniae* infection. A similar specific proteolytic cleavage of Bid into its active p15 fragment was observed at 8 and 12 h postinfection. These findings are consistent with the conclusion that caspase 8 activation was linked to the intrinsic mitochondrial death pathway through the generation of t-Bid, which translocates to mitochondria promoting release of cytochrome c. We used an independent approach to confirm that *C. pneumoniae* infection induces multiple apoptotic pathways in T cells. *C. pneumoniae*-infected T cells (MOI 100) were incubated with the fluorescein-labeled inhibitor peptides specific to caspase 1, caspase 8, or caspase 9, *C. pneumoniae*, but not mock or *C. trachomatis*-infected T cells, stained positive with all three fluorescent-labeled inhibitor peptides (Fig. 7A). Staining with caspase 8 and 9 confirmed the Western blot findings indicating that T cell killing was occurring by programmed cell death. Caspase 1 activation induced by *C. pneumoniae* infection suggested that activation of the inflammasome pathway was occurring. Therefore, the secretion of two signature cytokines of this death mechanism, IL-1β and IL-18, was also investigated. We were able to detect the presence of IL-1β but not IL-18 (data not shown) after *C. pneumoniae* or *C. trachomatis* treatment, consistent with the fact that macrophages and monocytes but not lymphocytes are the primary producers of IL-18 (27). *C. trachomatis* induced significantly higher levels of IL-1β than those in the control uninfected cultures; however, the levels detected in *C. pneumoniae* supernatants were significantly greater than those found in *C. trachomatis*-infected lymphocyte supernatants (Fig. 7B).

Notably, the staining of infected T cells by caspase 1 and secretion of IL-1β by *C. pneumoniae*-infected T cells provides evidence for the activation of the inflammasome or pyroptosis pathway. Thus, taken together our findings support the conclusion that *C. pneumoniae* infection induces multiple apoptotic pathways in human T cells. It is worth noting that none of these cell death activation pathways were induced by *C. trachomatis* infection making the potential significance of these observations unique to *C. pneumoniae* pathogenesis.

**Discussion**

In the current study, we have described a novel host–pathogen interaction elicited by *C. pneumoniae*: the suppression of activated but not nonactivated human lymphocytes. The suppressive effect...
was the result of T cell killing. Activated T cell killing was shown to be pathogen specific as similar infections with *C. trachomatis* organisms failed to evoke T cell death. Notably, *C. pneumoniae*-mediated killing did not require de novo protein synthesis, and hence chlamydial growth, but was dependent on chlamydial entry, findings that suggest the virulence factors responsible for *C. pneumoniae* T cell killing are surface components that function during the attachment or entry phase of infection or perhaps secreted molecules that exert toxic effects early after internalization.

The requirements for high MOI suggest that the effectors are of relatively low abundance or require modification or activation to exert their biological effect. We also showed that infection of T lymphocytes induced diverse cell signaling apoptosis and pyroptosis pathways (25, 26); conclusions collectively supported by the findings of caspase 8, 9, and 1 activation and the secretion of IL-1β by infected T cells. Related studies have shown that *C. pneumoniae* can affect T cell function but by different mechanisms than those described herein. *C. pneumoniae* can antagonize...
Are expressed as mean ± SD.

T cells by affecting the expression of the T cell surface-receptors CD25 and CD3 complex (28, 29). Also, cocultivation of *C. pneumoniae*-infected human macrophages with autologous T cells interferes with the intracellular T cell redox environment resulting in T cell death (21).

T lymphocytes are critical to adaptive protective immune responses that function in the control and elimination of intracellular chlamydiae, but paradoxically they also play a role in the establishment of chronic inflammatory disease (30). Notably, our findings demonstrated the potential of *C. pneumoniae* to suppress specifically adaptive T cell-mediated immunity through the induction of apoptotic pathways but also to influence T cells to secret proinflammatory cytokines that exacerbate pathology. This T cell-targeted biological strategy is consistent with the association of *C. pneumoniae* infection in humans to evoke persistent infection and chronic diseases.

The suppression and killing of human T cells was *C. pneumoniae* specific, a fascinating finding that highlights the molecular differences in surface or secreted molecules between *C. pneumoniae* and *C. trachomatis*. Comparative genomics of *C. pneumoniae* and *C. trachomatis* indicate a large degree of genomic synteny, however there are a number of genes that encode surface and potentially secreted proteins that are unique to *C. pneumoniae* organisms. The multiple copy autotransporter gene family present in the chromosome of members of the family Chlamydiaceae encodes multiple surface proteins termed polymorphic membrane proteins (Pmps). The function of these proteins is poorly understood, but it is of interest that chlamydiae with their small genome size (1.03 Mb) have duplicated these genes rather extensively implying a conserved yet potentially functionally distinct role in pathogenesis. Remarkably, *C. pneumoniae* possess an extended family of 21 *pmp* genes compared with nine genes found in the *C. trachomatis* genomes (31). The reason for this selected expansion of genes encoding for polymorphic surface proteins is unclear, but one can speculate that they have evolved to carry out distinct biological functions that are unique to *C. pneumoniae* infection and pathogenesis. Notably, the *Helicobacter pylori* VacA cytotoxin, a type V autotransporter, has been shown to exert similar antiproliferative effects on both human B and T cells, and this immunomodulatory action is thought to contribute to the pathophysiology of *H. pylori* chronic infection of the gastric mucosa (32, 33).

In contrast, chlamydial organisms possess a type III secretion system that could potentially deliver effectors to the cell cytosol early postinfection (34, 35). However, currently characterized type III secretion chlamydial effectors are shared among the majority of chlamydial species (36–38) thus making them unlikely candidates in the human T cell killing activities described in this study. Nevertheless, it is possible *C. pneumoniae* also possess unique type III secretion effectors that have yet to be discovered that might function in T cell killing (39, 40).

In conclusion, it will be an important future goal to identify those *C. pneumoniae*-specific proteins that target human T cell killing and better define how these virulence factors interact with T cells to avoid immune clearance and promote chronic inflammatory disease; findings that would have important implications to *C. pneumoniae* vaccine design and development.
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Disclosures
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