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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 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

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Abbreviations used in this article: 7-AAD, 7-aminoactinomycin D; EB, elementary body; MOI, multiplicity of infection; PS, phosphatidylserine.
Subjects, National Institute of Allergy and Infectious Diseases. The PBMC purification was conducted as described by Kobyashki et al. (23). Briefly, leukocytes were separated from erythrocytes by sedimentation of fresh heparinized blood and mixed 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 x 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/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 nonactivated T cells, PBMCs were infected with chlamydiae at an MOI of 100 or with SPG buffer.

Flow cytometry assays were performed using an LSRII flow cytometer (BD Biosciences Pharmingen, San Jose, CA). Briefly, after 2, 4, and 6 h of incubation with chlamydial EBs, the cells were collected by centrifugation 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). Upon chlamydial–T lymphocytes interaction, cells were washed and T cells were activated by using BD Biocoat 96-well plates coated with anti-human CD3 Ab (BD Biosciences, Bedford, MA) and anti-human CD28 Ab (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 (20 U/ml; BD Biosciences) in a round-bottom 96-well plate. For chlamydia–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 (2 U/ml; BD Biosciences) and washed, and surface receptor staining was performed using the following Abs: anti-human CD3-Alexa 700, anti-human CD4-phycoerythrin (eBioscience). The cells were incubated with chlamydial EBs or SPG buffer. When indicated, the experiments were performed in the presence of inhibitors.

**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 x 10⁶ T cells per lane was separated by 4–15% gradient SDS-PAGE (Criterion 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 20) 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, Beverly, MA), anti-human caspase 8 mAb (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 20 containing anti-rabbit or anti-mouse IgG secondary Abs conjugated to HRP (Cell Signaling Technology). The membranes were developed using chemiluminescence using ECL Pico reagent (Pierce, Rockford, IL).

**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, washed three times in PBS, and incubated for 30 min at room temperature with 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-phycocerythin or CD8-allophycocyanin (eBioscience).

**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. 1 A) but not nonactivated (Fig. 1 B) 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. 1 B).

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 C. pneumoniae organisms. We next investigated the biological properties of these C. pneumoniae-specific T cell virulence factor(s).

**Direct contact of 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 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 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 C. pneumoniae organisms and T cells was required to induce cellular death.

The 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 C. pneumoniae to kill activated T cells. 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. 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 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.
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

**FIGURE 4.** Killing of T cells by *C. pneumoniae* is dependent on live organisms, independent of de novo mRNA synthesis, and requires pathogen entry. A, Chlamydial organisms were heat killed (56°C, 30 min) or untreated (live) and incubated with activated T cells at an MOI of 100. B, Similar experiments were performed using activated T cells and live *Chlamydia* EBs at an MOI of 100 in the presence of DMSO, rifampicin, or cytochalasin D. After 6 h of incubation, the percentage of cellular death was assessed using Aqua Live/Dead stain, followed by flow cytometry analysis. The graphs represent the average of three independent experiments in duplicate, and data are expressed as mean ± SD.

**FIGURE 5.** Analysis of PS exposure and nuclear fragmentation in human T lymphocytes. A, Activated T cells were incubated with *C. trachomatis* or *C. pneumoniae* at a MOI of 100, and at 2, 4, or 6 h postinfection, the cells were washed and double stained with annexin V and 7-AAD and analyzed by flow cytometry. FACS analysis of a representative experiment (left) shows the cellular phenotype. The graph (right) represents the average of early apoptotic cells (PS + 7-AAD−) at each time point from three independent experiments. B, At 12 h postinfection, the cells were fixed and subjected to the TUNEL assay. The graphs represent the average of three independent experiments each one done in duplicate using different donors and expressed as mean ± SD.
are expressed as mean 

were collected, and the presence of IL-1 

of cells positive for each activated caspase was determined. Flow cytometry analysis was performed, and the percentage positive controls, respectively. 

untreated or etoposide-treated lymphocytes were included as negative and control conditions, respectively. 

are shown by the appearance of appropriate cleaved polypeptide fragments (asterisks) at 8 and 12 h postinfection with C. pneumoniae. The figure is representative of two independent experiments. Lysates from untreated or etoposide-treated lymphocytes were included as negative and positive controls, respectively.

T cell lysates were harvested at the indicated times and subjected to Western blot analysis with anti-caspase 8-, anti-Bid-, or anti-caspase 9-specific Abs. Evidence for proteolytic cleavage of caspase 8, Bid, and caspase 9 are shown by the appearance of appropriate cleaved polypeptide fragments (asterisks) at 8 and 12 h postinfection with C. pneumoniae. The figure is representative of two independent experiments. Lysates from untreated or etoposide-treated lymphocytes were included as negative and positive controls, respectively.

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 chlamydial, 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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