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Chlamydia pneumoniae Inhibits Apoptosis in Human Peripheral Blood Mononuclear Cells Through Induction of IL-10[1]

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***Chlamydia pneumoniae* is a common cause of pulmonary infection, with serum positivity in at least 50% of the general population. In this study, we report that human PBMCs exposed to *C. pneumoniae* are resistant to apoptosis induced by the potent photoactivated chemotherapeutic agents 8-methoxypsoralen and hypericin. In contrast, PBMCs treated with a heat-inactivated inoculum exhibit normal susceptibility to apoptosis. We also observed that human PBMCs are responsive to *C. pneumoniae* infection by secretion of key immune regulatory cytokines, including IL-12 and IL-10. While IL-12 may play an important role in limiting *C. pneumoniae* proliferation within cells, IL-10 serves an anti-inflammatory function by down-regulating proinflammatory cytokines such as IL-12 and TNF-α. Depletion of endogenous IL-10, but not of IL-12, abolished the apoptosis resistance of *C. pneumoniae*-infected PBMCs. Furthermore, addition of exogenous IL-10, but not IL-12, significantly increased the resistance of control inoculum-treated PBMCs to photoactivated 8-methoxypsoralen- and hypericin-induced apoptosis. Therefore, we conclude that *C. pneumoniae* possesses an antiapoptotic mechanism. The resistance to apoptosis observed in PBMCs exposed to *C. pneumoniae* is due, at least partially, to the IL-10 induced during *C. pneumoniae* infection. *The Journal of Immunology*, 2000, 164: 5522–5529.**

Apoptosis is an active process of cell death that is important for cell development and tissue homeostasis. Moreover, other biological processes such as host defense against viral (1–3) and bacterial (4, 5) infections may depend critically upon apoptotic events for the normal processing of microbes (6, 7). Many intracellular organisms rely on host cells to survive and propagate; therefore, it is advantageous for them to develop strategies to inhibit host cell apoptosis. A number of virus and viral factors have been described as antiapoptotic (8–10), including caspase inhibitor CrmA in the cowpox virus and p35 in baculovirus, viral Bcl-2 homologues, viral products that can modulate p53 activity, viral homologues of mammalian death receptors, and viral Fas-associated death domain-like IL-1-converting enzyme-inhibitory proteins. Reports of bacterial antiapoptotic activity, however, have been scarce. Recently, Fan et al. (10) reported that host cells infected with *Chlamydia trachomatis* are profoundly resistant to apoptosis induced by a wide spectrum of proapoptotic stimuli, including the kinase inhibitor staurosporine, the DNA-damaging agent etoposide, and several immunological apoptosis-inducing molecules such as TNF-α, Fas Ab, and granzyme B/perforin, through blockage of mitochondrial cytochrome *c* release and caspase activation. The role that cytokines play in infection-mediated antiapoptosis and whether this antiapoptotic effect is unique to *C. trachomatis*, however, were not addressed. Furthermore, the situation of infected cells treated with other clinically relevant apoptotic inducers such as 8-methoxypsoralen (8-MOP)[3] (11) and hypericin (12), two light-activated photochemotherapeutic reagents that have been used in the treatment of lymphoproliferative diseases and that cause a high level of apoptosis, is also unknown.

Chlamydia pneumoniae, the newest member of the chlamydial family, has been established as a common cause of acute and chronic respiratory disease worldwide and implicated in the pathogenesis of coronary artery disease (13, 14). Monocytes/PBMCs have been postulated to serve as a vehicle for systemic dissemination of the infection (15, 16). Like *C. trachomatis*, *C. pneumoniae* has a unique intracellular biphasic life cycle. The spore-like elementary bodies facilitate transit between cells. The metabolically active reticulate bodies are responsible for intracellular replication (13, 14). During chlamydial intracellular growth, maintenance of the host cell's integrity is essential not only for supplying nutrients, but also for shielding the intracellular organisms from host phagocytosis. Therefore, *C. pneumoniae* may also have evolved mechanisms that allow it to actively interrupt host apoptotic process.

Researchers have shown that *C. pneumoniae* is a potent inducer of the proinflammatory cytokines TNF-α, IL-1β, and IL-6 in human monocytic cells as well as freshly isolated human PBMCs (17, 18). However, the ability of *C. pneumoniae* to induce PBMC secretion of key regulatory factors in the immune response such as IL-12 and IL-10 has never been addressed. In this communication, we report that *C. pneumoniae* is able to infect human PBMCs and that human PBMCs respond to infection by secreting the critical immunoregulatory cytokines IL-12 and IL-10. Furthermore, we demonstrate that PBMCs treated with *C. pneumoniae* have

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[3] Abbreviations used in this paper: 8-MOP, 8-methoxypsoralen; HI, heat-inactivated; IF, immunofluorescence; ifu, inclusion-forming unit; RPA, RNase protection assay.

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increased resistance to the high level of apoptosis induced by the photoactivated DNA intercalator, 8-MOP, or hypericin, a naturally occurring photodynamic compound, as well as to soluble Fas ligand-mediated apoptosis. Importantly, *C. pneumoniae* infection-induced IL-10, but not IL-12, is at least partially responsible for this increased resistance to apoptosis.

Materials and Methods

Bacterial culture and inoculum preparation

C. pneumoniae strain TW 183 was purchased from the American Type Culture Collection (ATCC, Rockville, MD) and was propagated in McCoy cells (ATCC) in supplemented DMEM (Life Technologies, Grand Island, NY) in a 35°C, 5% CO_2-saturated humidified incubator, as described previously (16). Infected cells were harvested on day 3 or 4 and disrupted by two cycles of freezing/thawing and ultrasonification, and different harvests were pooled. After centrifugation at 1000 rpm for 5 min to remove cell debris, bacteria were concentrated by high speed centrifugation at 25,000 $\times$ *g* for 25 min. Pellets were resuspended in PBS, pH 7.4, mixed with an equal volume of sucrose-phosphate-glutamic acid buffer, aliquoted, and frozen at −70°C until use. A control inoculum was prepared according to the same procedure with uninfected McCoy cells.

Chlamydia titers were determined by immunofluorescence (IF) assay. Briefly, McCoy cells were infected with serial dilutions of bacterial stock, incubated for 72 h, fixed with methanol-acetone (1:1), stained with an antimajor outer membrane protein Ab (IgG3 mouse mAb anti-*C. pneumoniae*; Dako, Cambridgeshire, U.K.), and followed by FITC-labeled secondary Ab (goat $F(ab')_2$ anti-mouse IgG; Sigma, St. Louis, MO) to identify chlamydial inclusions. After counting inclusions under a fluorescence microscope and correcting for dilution factors, bacterial titers were expressed as inclusion-forming units per ml (ifu/ml). *C. pneumoniae* infection of human PBMCs/monocytes was also examined by IF assay.

Heat-inactivated (HI) bacteria were prepared by heating a viable bacterial suspension with a known concentration for 45 min at 75°C. Infectivity was abolished, as judged by the undetectable IF titer.

All cultures were free of mycoplasma contamination, as determined by PCR and Hoechst staining (Cell Center, The University of Pennsylvania, Philadelphia, PA).

Human PBMC and monocyte preparations

PBMCs were prepared essentially as previously described (20). First, PBMCs were isolated from blood of healthy human donors through centrifugation on a standard Ficoll gradient (Pharmacia, Piscataway, NJ). The interface containing the mononuclear cell fraction was washed with Dulbecco's PBS through centrifugation and resuspended in complete medium at the indicated cell concentration. Monocytes were enriched within the fraction of mononuclear cells using their adherence to cell culture dishes (incubation for 2 h at 37°C in RPMI 1640 medium, supplemented with 10% HI FCS). After washing off the nonadherent cells, the monocyte-enriched PBMCs were detached from the plastic surface with cell dissociation solution (Sigma). This procedure yielded monocytes of >90% purity, as confirmed by flow-cytometric analysis with a FACScan (Becton Dickinson, Mountain View, CA). All reagents used in this study were free of endotoxin contamination, as determined by the *Limulus* amebocyte assay.

Human PBMC infection and cytokine measurement

PBMCs or monocytes were inoculated with various concentrations of *C. pneumoniae* (viable or HI) and then cultured at 37°C. Supernatants were collected at various times during the culture and stored at −80°C for the cytokine assays. In some experiments, cells were inoculated with the bacteria in the presence or absence of certain cytokine-neutralizing Abs or were treated with certain cytokines before their exposure to the inoculum. In all cases, samples were centrifugated for 1 h at 550 $\times$ *g* immediately after *Chlamydia* inoculation to facilitate contact between cells and the bacteria.

RIAs for human IL-12 p40, IL-12 p70, TNF-α, and IL-10 were performed as previously described (21–24), using mAb pairs C11.79/C8.6, 12H4/C8.6, B154.9/B154.7, and 9D7/12G8, respectively.

RNase protection assay (RPA)

RPA kits were purchased from PharMingen (San Diego, CA). ^{32}P was used for riboprobe labeling. RNAs were extracted with Ultraspect (Biotecx, Houston, TX), and RNA samples were hybridized with an excess amount of [^{32}P]UTP (New England Nuclear-DuPont, Boston, MA)-labeled probes for 12–16 h at 56°C, according to the suggestions of the maufacturer. The protected fragments were fractionated on 5% polyacrylamide/urea-sequencing gels and detected by phosphor imaging.

Cell culture and photoactivation

Purified mononuclear cells were diluted to a concentration of 2 $\times$ 10^6 cells/ml with complete medium and aliquoted into each well of a 24-well plate. Vehicle and predetermined concentrations of hypericin or 8-MOP were added into their respective set of wells in a darkened tissue culture room. Control plates receiving no exposure to photoactivating wavelengths of light were placed immediately in a 37°C incubator. The remaining plates were exposed to either white light from four fluorescent F15T8CW 15 W bulbs under the tissue culture plates or to UVA light with UVA illumination, as described previously (11, 12). Photoactivation with white light lasted for 30 min and delivered the equivalent of 2 J/cm^2; UVA light lasted for 4 min and delivered the equivalent of 2 J/cm^2. The plates were then incubated at 37°C for 24–72 h, depending on the assay to be performed. The quantity of light delivered to the medium-containing wells of a tissue culture plate was measured with an IL-700 A research radiometer (International Light, Newburyport, MA).

Apoptosis assay

Apoptosis was assessed using a modification of the TUNEL method previously described by Sgonc et al. (25), as supplied in kit form by Boehringer Mannheim (Indianapolis, IN). Apoptotic cell death is characterized by the internucleosomal cleavage of DNA into fragments of ~180 bp. The TUNEL assay allows for the incorporation of fluorescein-labeled dUTP (FITC-dUTP) into the free ends of these apoptotic DNA fragments. Briefly, cells cultured as described above were fixed in 4% paraformaldehyde for 30 min at 25°C and permeabilized in 0.1% Triton-containing 0.1% sodium citrate for 2 min on ice. Terminal deoxynucleotide transferase and nucleotide mixture were then added to the cells, and the mixture was incubated for 60 min at 37°C. Then, using a FACScan flow cytometer (Becton Dickinson), the percentage of cells (of the 10,000 total acquired) undergoing apoptosis was determined as: [(number of cells incorporating FITC-dUTP into fragmented DNA)/(total number of cells)] $\times$ 100.

Cytokines, Abs, and other reagents

Chinese hamster ovary cell-derived recombinant NK-stimulatory factor/IL-12 was a gift from Dr. S. Wolf (Genetics Institute, Boston, MA). Anti-IL-10 mAb 12G8 was a gift from Dr. A. O'Garra (DNAX, Palo Alto, CA). rTNF-α was a gift from Dr. H. M. Shepard (Genentech, South San Francisco, CA). rIL-4 was purchased from Genzyme (Cambridge, MA). The mAb, anti-IL-12 (C8.6), was produced and characterized, as previously described (26). The control Abs, rat normal Ig, and mouse IgG1 were purchased from Sigma and R&D Systems (Minneapolis, MN), respectively. Chemically synthesized hypericin was supplied by VIMRX Pharmaceuticals (Wilmington, DE). A stock solution was prepared by dissolving 40 mg hypericin in 40 ml 2% benzyl alcohol (vehicle) to yield a 2 mM solution, then kept in the dark at 4°C. 8-MOP was purchased from Sigma, and a stock solution of 462.5 μM was prepared by dissolving 100 μg 8-MOP in 1 ml 2% benzyl alcohol and kept in the dark at 4°C. Recombinant Fas ligand with Fas ligand enhancer were purchased from Alexis (San Diego, CA).

Statistical analysis

The data on cytokine production are presented as means $\pm$ SD. An unpaired, two-tailed Student's *t* test was performed to determine the statistical significance of the data, and *p* values < 0.05 were considered significant. The data on cell survival are presented as the percentage of cells that survived under various experimental conditions. Percentages are not normally distributed. Therefore, the percentage data were transformed by the arcsin of the square root of the percentage. The transformed data were then analyzed using an analysis of variance. To ensure that the overall probability of detecting a significant difference between two pairs was maintained at a constant type I error of 0.05, comparisons between treatments were made using the Tukey-Kramer multiple comparison test.

Results

C. pneumoniae*-treated human PBMCs have increased resistance to apoptosis induced by photoactivated 8-MOP or hypericin*

It has been reported that certain *C. trachomatis*-infected host cells (HeLa, L929, and U937) are profoundly resistant to apoptosis induced by proapoptotic stimuli such as the kinase inhibitor staurosporine, the DNA-damaging agent etoposide, and immunological

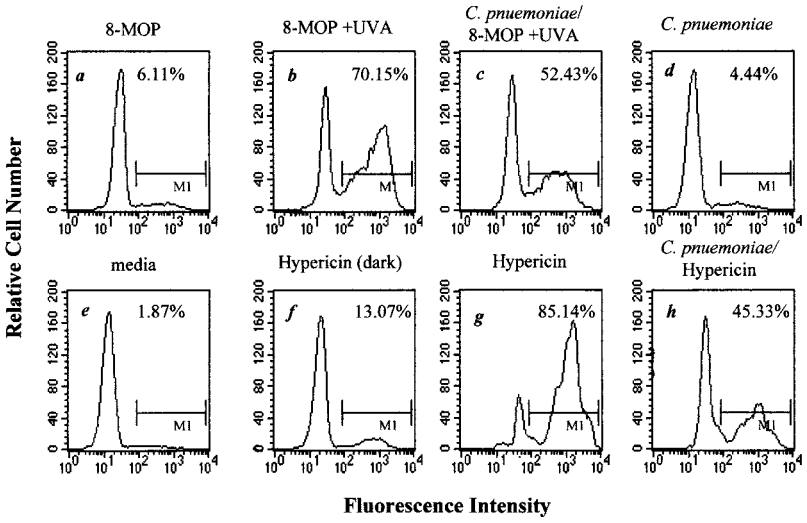

FIGURE 1. Apoptotic resistance of *C. pneumoniae*-infected human PBMCs. PBMCs (2×10^6/ml) were exposed to photoactivated 8-MOP or hypericin in the presence or absence of *C. pneumoniae*, as described in *Materials and Methods*. Sixty hours later, cells were harvested for TUNEL assay. Data are representative histograms (one of four independent experiments) of FITC-dUTP incorporation, and the percentages of FITC-dUTP-positive cells are the numbers above histograms. The percentage of cell death was lower in *C. pneumoniae*-infected cells than noninfected cells following exposure to photoactivated 8-MOP or hypericin.

molecules including TNF-α, Fas Ab, and granzyme B/perforin (10). To understand whether this antiapoptotic effect is unique to *C. trachomatis* and to explore how *Chlamydia*-exposed and nonexposed human PBMCs responded differently following clinically relevant apoptotic inducer treatment, we tested another species of *Chlamydia*, *C. pneumoniae*, using photoactivated 8-MOP or hypericin (18). With IF assays, we observed that *C. pneumoniae* is able to infect PBMCs, as evidenced by the positive immunofluorescent signals detected in bacteria-inoculated cells (data not shown). We also observed that predetermined suboptimal doses of 8-MOP (100 ng/ml and 2 J/cm2 UVA) or hypericin (1 μM and 2 J/cm^2 white light) induce 70–80% apoptosis of PBMCs 60 h following initial photoactivation (Fig. 1, *b* and *g*). But cells treated with *C. pneumoniae* (2000–4000 ifu/5 $\times$ 10^6 host cells) before photoexposure had 18% and 40% reduction in apoptosis (Fig. 1, *c* and *h*). Furthermore, there is a dose-dependent relationship between the amount of *C. pneumoniae* inoculated and the magnitude of apoptosis resistance. For instance, with 1 μM of hypericin (2 J/cm^2 white light), 2000 ifu of *C. pneumoniae* caused a 40% reduction in host cell apoptosis, as shown in Fig. 1*h*, while 200 ifu did not cause any significant changes (data not shown). Cells treated with *C. pneumoniae* alone did not significantly affect the rate of apoptosis (Fig. 1*d*).

Cytokine production and regulation during C. pneumoniae *infection*

To understand the mechanism of *C. pneumoniae* antiapoptotic activity, we investigated whether PBMCs are able to respond to *C. pneumoniae* infection by inducing cytokine production. Fig. 2 shows that the first cytokine detected following *Chlamydia* infection, among cytokines measured, was TNF-α. TNF-α peaked at about 8 h postinoculation (Fig. 2). IL-12 p40 (Fig. 2) and IL-10 (Fig. 2) peaked between 15–48 and 24–48 h after inoculation, respectively. Optimal cytokine production was measured at 10,000 ifu of *C. pneumoniae* per ml, a maximum dose used in our study. Below 100 ifu/ml, *C. pneumoniae* was unable to induce any statistically significant cytokine production (Table I).

Replication of *C. pneumoniae* appeared to be necessary for the optimal induction of cytokine production. Table I shows that compared with the viable *C. pneumoniae*, HI bacteria are not a strong inducer of the cytokines measured. With 10,000 ifu, viable *C. pneumoniae* induced the production of 10.7 $\pm$ 1.2 ng of IL-12 p40, 18.8 $\pm$ 4.5 ng of IL-10, and 22.9 $\pm$ 4.1 ng of TNF-α; HI bacteria, however, induced only 0.3 $\pm$ 0.1 ng of IL-12-p40, 1.7 $\pm$ 0.5 of IL-10, and 2.8 $\pm$ 0.4 ng of TNF-α.

To examine the relationship between different cytokines and to facilitate our understanding about the functions of those cytokines produced during the infection, a number of cultures were set up in the presence or absence of certain cytokines or cytokine-neutralizing Abs. Fig. 3*A* demonstrates the results from a representative RPA experiment in which PBMCs had been optimally precultured for 16 h in the presence or absence of IFN-γ, then stimulated with *C. pneumoniae* for 8 h in the presence or absence of IL-10-neutralizing Ab. In Fig. 3*B*, *lane 1* shows that *C. pneumoniae* was able

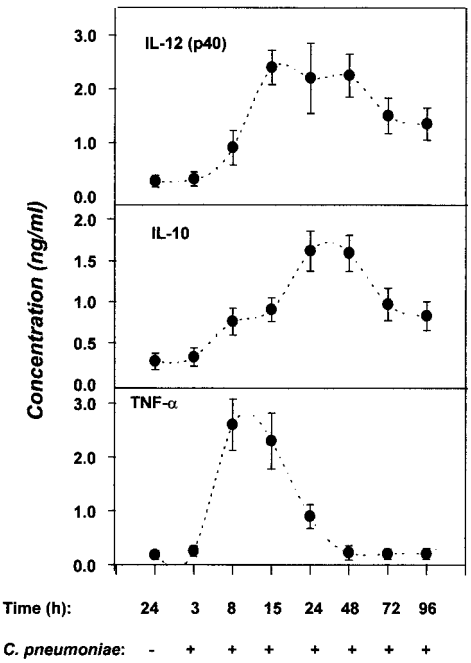

FIGURE 2. Kinetics of IL-12 p40, TNF-α, and IL-10 production in PBMCs stimulated with *C. pneumoniae*. PBMCs (2×10^6) were isolated and stimulated with *C. pneumoniae* (2000 ifu) for 3, 8, 15, 24, 48, 72, or 96 h, as designated. Culture supernatant was collected, and the cytokine protein level was measured as described in *Materials and Methods*. The result is representative of three independent experiments with two donors in each experiment.

Table I. *IL-12 p40, IL-10, and TNF-α production by human PBMCs following stimulation with viable or HI* C. pneumoniae[a]

Stimulus	Cytokine (pg/ml)		
	IL-12 p40	IL-10	TNF-α
Viable *C. pneumoniae* (ifu)			
Media	138 ± 44	159 ± 56	150 ± 56
10,000	10,680 ± 1,236	18,828 ± 4,500	22,890 ± 4,145
1,000	3,958 ± 251	1,327 ± 375	1,311 ± 351
100	1,178 ± 179	987 ± 279	800 ± 268
Heat-inactivated (organism equivalent)			
Media	145 ± 31	95 ± 34	106 ± 29
10,000	302 ± 101	1,701 ± 456	2,872 ± 441
1,000	212 ± 98	299 ± 223	1,615 ± 231
100	103 ± 61	179 ± 91	301 ± 99

[a] Viable but not the heat-inactivated *C. pneumoniae* is a potent inducer of IL-12 p40, TNF-α, and IL-10. Cells (5×10^6) were isolated and stimulated for 48 h (IL-12 p40), 24 h (IL-10), or 15 h (TNF-α) with viable *C. pneumoniae* (10,000, 1,000, or 100 ifu) or equivalent amount of heat-killed organisms. Cytokines were measured by RIA as described in *Materials and Methods.* Results are presented as mean ± SD of three separate experiments with three donors in each experiment.

to induce IL12 p40 and IL-10 mRNA expression. If cells had been pretreated with IFN-γ, *Chlamydia*-induced IL-12 p40 mRNA expression was enhanced (Fig. 3*B*, *lane 2*). *Chlamydia* were also able to induce IFN-γ-pretreated cells to express IL-12 p35 mRNA (Fig. 3*B*, *lane 2*). *Chlamydia*-induced IL-10 mRNA expression, however, was decreased by such a treatment (Fig. 3*B*, *lane 2*). Interestingly, the presence of IL-10-neutralizing Ab in the culture greatly enhanced IL-12 p40 mRNA expression and induced the expression of IL-12 p35 mRNA (Fig. 3*B*, *lane 3*). Furthermore, the presence of IL-10-neutralizing Ab enhanced the expression of IL-10 mRNA (Fig. 3*B*, *lane 3*).

These results were further corroborated by data from protein studies: pretreatment of cells with IFN-γ enhanced the *C. pneumoniae*-induced production of IL-12 p40, p70, and TNF-α, but inhibited the production of IL-10 protein (Fig. 4*A*). The presence of IL-10-neutralizing Ab along with *Chlamydia* also enhanced the production of IL-12 p40, p70, and TNF-α (Fig. 4*B*). Addition of exogenous IL-10, on the other hand, caused a significant reduction in this infection-induced production of IL-12 and TNF-α (Fig. 4*B*). *C. pneumoniae* infection mediated PBMC production of IFN-γ as well. But under no circumstance was IL-4 production detectable (data not shown).

IL-10, but not IL-12, contributes to the resistance of C. pneumoniae*-infected PBMCs to apoptosis*

To explore the role of cytokines in the antiapoptotic activity of *C. pneumoniae*- treated PBMCs, we performed depletion experiments. As shown in Fig. 5, in comparison with control Ab treatment, anti-IL-10 treatment before *C. pneumoniae* inoculation significantly decreased the resistance of the cells to photoactivated 8-MOP- or hypericin-induced apoptosis (Fig. 5, *Ae*, *Ak*, and *B*). The presence of IL-12-neutralizing Ab, on the other hand, did not significantly affect the apoptotic activity of these cells (Fig. 5, *Ad*, *Aj*, and *B*). Furthermore, supplement of exogenous IL-10, but not IL-12, in the absence of *C. pneumoniae*, significantly decreased 8-MOP- or hypericin-induced apoptosis (Fig. 5, *Af*, *Al*, and *B*). Similar results were obtained in the system of soluble Fas ligand-mediated cell death (Fig. 6). Following culture of *C. pneumoniae*-infected cells with soluble recombinant Fas ligand, the level of apoptosis was markedly augmented by addition of anti-IL-10-neutralizing Ab to the culture system (Fig. 6). Together, these results strongly indicated that *C. pneumoniae* infection-mediated secretion of IL-10, but not IL-12, is at least partially responsible for the antiapoptotic activity of *C. pneumoniae*. Similar

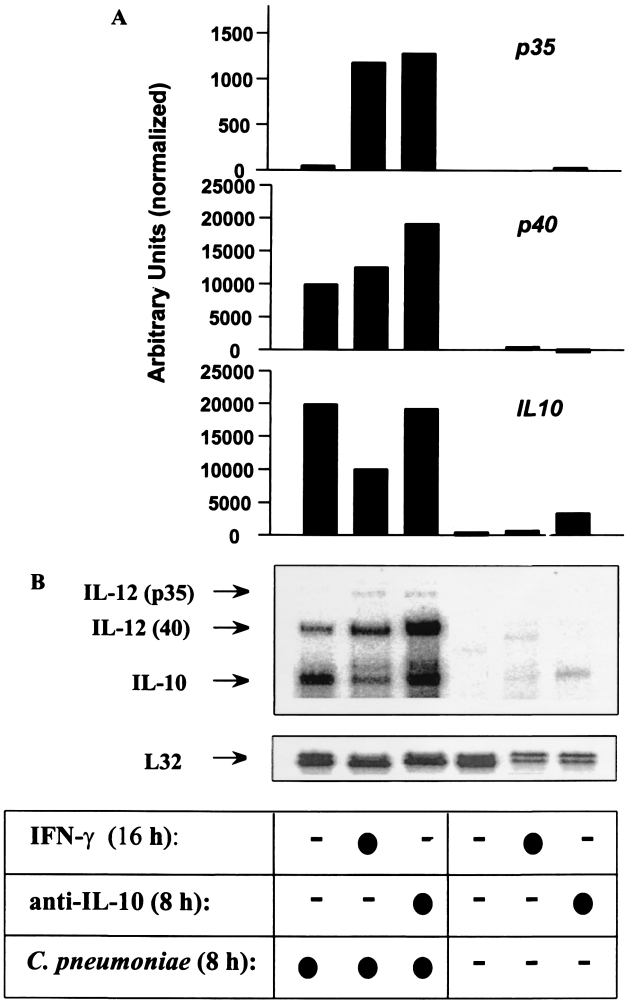

FIGURE 3. Effects of IFN-γ and anti-IL-10 on *C. pneumoniae* infection-induced IL-12 (p35 and p40) and IL-10 mRNA expression. Cells were precultured for 16 h in the presence or absence of IFN-γ (100 ng/ml), then stimulated with *C. pneumoniae* in the presence of anti-IL-10 or control Ab (10 μg/ml). Eight hours later, cells were harvested and RNAs were extracted, and RPA was conducted to detect the expression of IL-12 p35, IL-12 p40, and IL-10 mRNA, as described in *Materials and Methods*. *A*, Normalized arbitrary units of detected mRNA. *B*, The mRNA detected on a 5% polyacrylamide/urea sequencing gel. This result is representative of three independent experiments with two donors in each experiment.

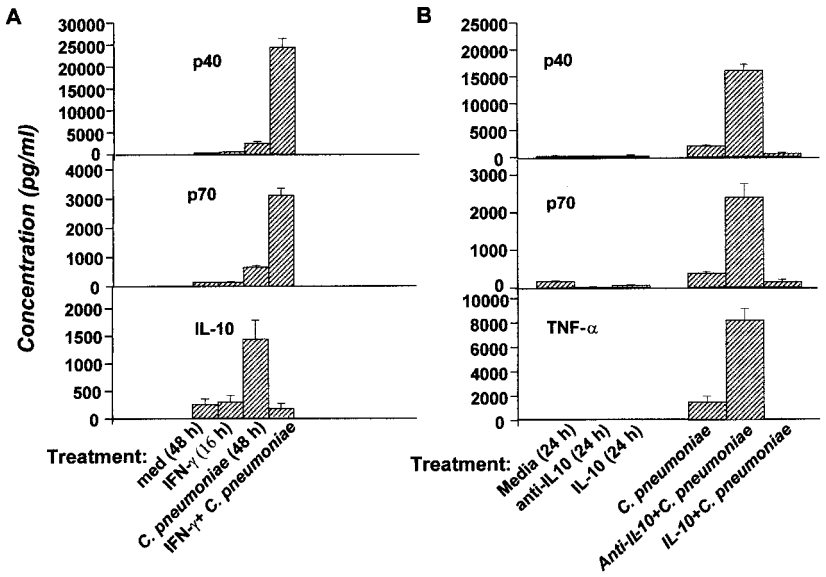

FIGURE 4. *A*, Effects of IFN-γ on *C. pneumoniae*-induced IL-12 and IL-10 production. Cells (2×10^6/ml) were isolated and cultured overnight (12–16 h) in the presence of IFN-γ (100 ng/ml), followed by *C. pneumoniae* stimulation (48 h). Culture supernatants were collected, and IL-12 p40, p70, and IL-10 productions were measured, as described in *Materials and Methods*. The result here is representative of three experiments with two donors in each experiment. *B*, Effects of IL-10 on IL-12 and TNF-α production. Freshly isolated PBMCs (2×10^6) were inoculated with *C. pneumoniae* for 24 h in the presence of IL-10 or anti-IL-10. Culture supernatants were collected, and IL-12 p40, p70, and TNF-α productions were measured. The result is representative of three independent experiments with two donors in each experiment.

experiments were also conducted to investigate the role of IFN-γ in the antiapoptotic function of *C. pneumoniae*-treated cells. We did not observe any significant effect of IFN-γ in our system (data not shown).

Discussion

It is known that host cells are able to respond to intracellular bacterial invasion with apoptosis (4, 5). Organisms that induce this response include *Shigella fexeneri* (27), *Listeria monocytogenes* (28), *Mycobacterium tuberculosis* (29), and *Salmonella typhimurium* (30). Because host apototic responses can facilitate the killing of intracellular bacteria, it is advantageous for intracellular organisms to evolve strategies against apoptosis to ensure the continuous supply of nutrients and protection from the host. There has been no report, however, of bacterial antiapoptotic activity until very recently. Fan et al. (10) reported for the first time that *C. trachomatis*-infected host cells (HeLa, L929, and U937) become resistant to a number of apoptotic stimuli. But issues such as whether this antiapototic effect is unique to *C. trachomatis* and what is the role of cytokines in this infection-mediated resistance to apoptosis were not addressed. Furthermore, responses of infected cells to potent photochemotherapeutic reagents such as 8-MOP and hypericin, which have been used in the clinic to treat skin-related lymphoproliferative disorders such as cutaneous T cell lymphoma and Sezary syndrome, were not studied. In this communication, we demonstrated for the first time that human PBMCs from *C. pneumoniae*-inoculated cell cultures are profoundly resistant to apoptosis induced by 8-MOP and hypericin. Furthermore, IL-10 produced during *C. pneumoniae* treatment contributes, at least partially, to the infection-mediated resistance to apoptosis. A similar protective effect against apoptosis of *C. pneumoniae*-induced IL-10 was also demonstrated using Fas ligand-treated PBMC.

The ability of *C. pneumoniae* to infect human PBMCs and monocytes has been reported previously. In our study, we observed that there are only a small percentage of PBMCs or purified monocytes that by IF assay appear infected. This is consistent with partial resistance to 8-MOP- or hypericin-induced apoptosis. These results may be due to the small amount of inoculum used in our studies. It is also possible, however, that *C. pneumoniae* infects and protects only specific subpopulations of PBMCs. As to whether those cells that were positive for *C. pneumoniae* by IF staining are the same cells that have increased resistance to apoptosis induction is an issue currently under investigation.

Photochemotherapy is a unique type of antitumor therapy. It has been used in the treatment of cutaneous T cell lymphoma and Sezary syndrome. The therapeutic efficacy of psoralen in combination with UVA has been linked to the potent induction of apoptosis (11). Hypericin, although still in clinical trial, has also been reported to induce a high rate of apoptotic death of normal, transformed, and malignant T lymphocytes and, thus, has promise for the treatment of cutaneous lymphoproliferative and inflammatory disorders (12). Recently, Abrams et al. (31) described a *C. pneumoniae*-associated peptide identified in the blood of Sezary syndrome patients that is able to sustain the growth of malignant Sezary T cells and to prevent apoptotic death. This observation is relevant to our in vitro findings. Clearly, understanding the mechanisms of apoptotic resistance induced by *C. pneumoniae* infection is relevant to enhancing our immunotherapeutic approaches to lymphoproliferative disorders in general and to the use of photochemotherapeutic agents in particular.

Cytokines participate in many physiologic processes, including the regulation of immune and inflammatory responses. Our previous studies (19) with murine infection models (BALB/c and 129 mice) have shown that host immune systems are able to respond to *C. pneumoniae* infection by producing a number of cytokines, including IL-12, TNF-α, and IL-10. Although IL-12 appears to play an important role in early stage host defense against this infection, the function of IL-10 was not addressed. We observed that depletion of IL-10 by addition of IL-10-neutralizing Ab to the cell culture significantly increased the infection-mediated production of IL-12 and TNF-α, and supplementation with exogenous IL-10 at the time of infection decreased such proinflammatory cytokine production. Together, these results strongly suggest an anti-inflammatory role of IL-10 produced during *C. pneumoniae* infection. Interestingly, the presence of IL-10-neutralizing Ab in the culture significantly reduced IL-10 protein levels, yet it increased IL-10 mRNA expression, confirming IL-10 as an autocrine regulatory cytokine, as reported previously by Masood et al. (32).

To further explore the role of IL-12 and IL-10 produced during *C. pneumoniae* infection in host cell resistance to apoptosis, we performed blockade experiments with specific mAbs to IL-12 and

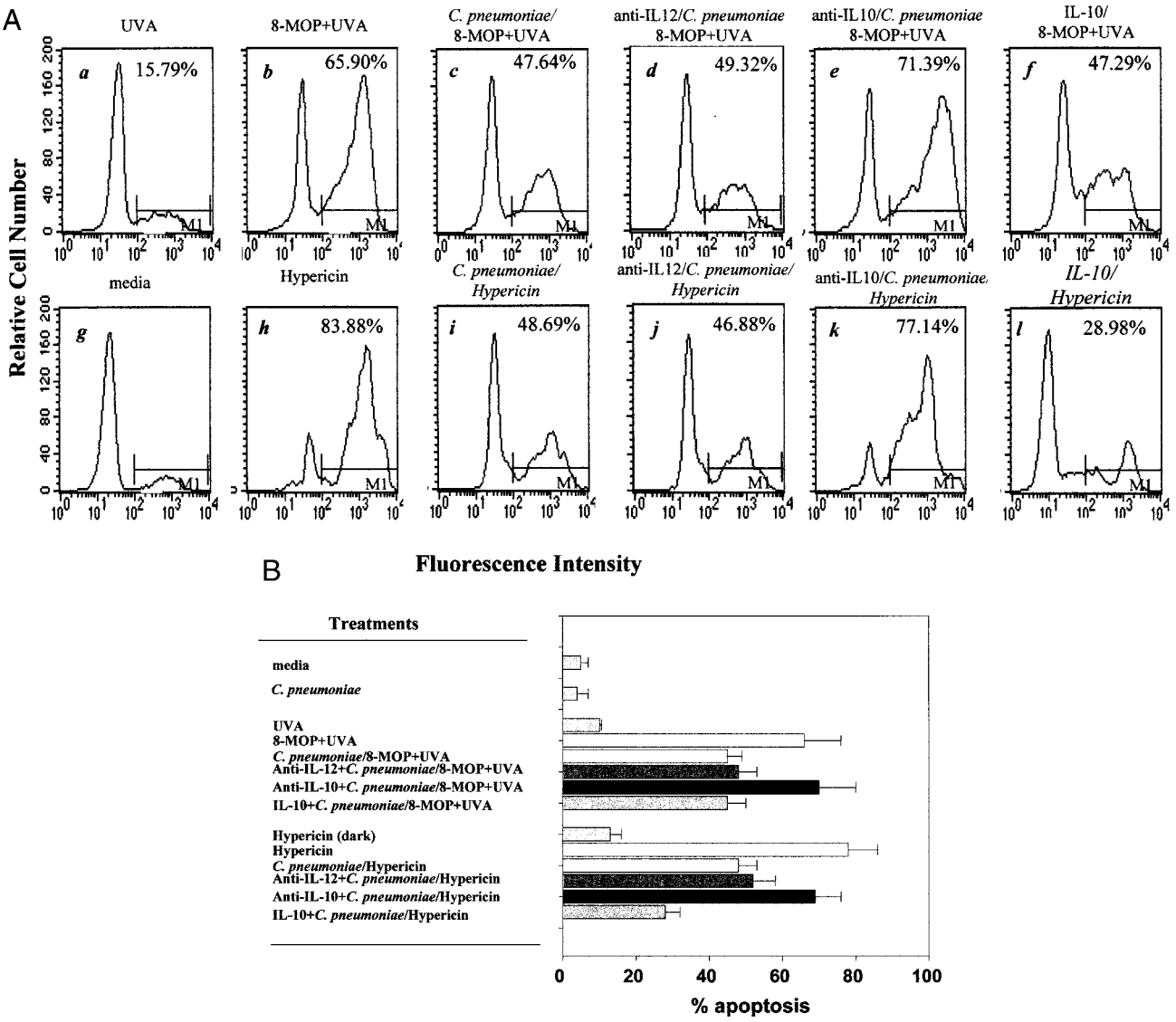

FIGURE 5. Role of IL-10 in apoptotic resistance of *C. pneumoniae*-infected human PBMCs. Cells were cultured for 60 h following exposure to photoactivated 8-MOP or hypericin in the presence of IL-10 (10 ng/ml) or anti-IL-10 (10 μg/ml) or anti-IL-12 (10 μg/ml) and/or *C. pneumoniae* (2000 ifu). Data in *A* are representative histograms of FITC-dUTP incorporation, and the percentages of FITC-dUTP positive cells are the numbers above histograms. Data in *B* are means $\pm$ SD from four independent experiments. The percentage of cell death was significantly ($p < 0.05$, Tukey-Kramer test) higher in cells that have been cultured under IL-10-deprived condition following exposure to 8-MOP or hypericin and *C. pneumoniae* in comparison with infected cells without neutralizing anti-IL-10 added.

IL-10. We observed that the presence of anti-IL-10-neutralizing Ab in the infected PBMC culture significantly increased the percentage of apoptotic cells following 8-MOP or hypericin and photoexposure. The presence of anti-IL-12, however, did not change the apoptotic rate. Furthermore, we determined that exogenous IL-10 was able to rescue human PBMCs from photoactivated 8-MOP- or hypericin-induced apoptosis. Exogenous IL-12, however, did not affect the apoptosis-inducing efficacy of those reagents. These findings clearly indicate that the resistance of *C. pneumoniae*-infected human PBMCs to apoptosis is, at least partially, mediated through the IL-10 produced following infection. The ineffectiveness of HI *C. pneumoniae* in inducing production of cytokines has been reported previously with an in vivo system (19). In this current study, we observed that HI *C. pneumoniae* is unable to elicit significant cytokine responses in vitro in comparison with viable organisms. Furthermore, human PBMCs inoculated with HI *C. pneumoniae* did not show resistance to apoptosis (data not shown). These observations indicated a relationship between cytokines produced during *C. pneumoniae* infection and the antiapoptotic activity of these bacterium-exposed cells.

The effect of cytokines on cell survival has been studied previously. It has been shown, in different experimental systems, that signals transduced through TNFR1 (p55) can induce an activation of proteases, including caspases. Caspases mediate apoptosis by proteolytic cleavage of the death substrates (26, 33–38). TNF-α also increases synthesis of NO in different cells, and this molecule has been extensively associated with induction of DNA damage and apoptosis (39–42). Additionally, TNF-α may activate sphingomyelin breakdown into ceramide, which has a recognized role in apoptosis (43). IL-10, on the other hand, has been reported to have variable effects on apoptosis, depending upon the cell type and model systems used (44–46). IL-10 has been observed to prevent lymphocyte activation-induced apoptosis and spontaneous death of germinal center B cells (47, 48). This effect of IL-10 has been

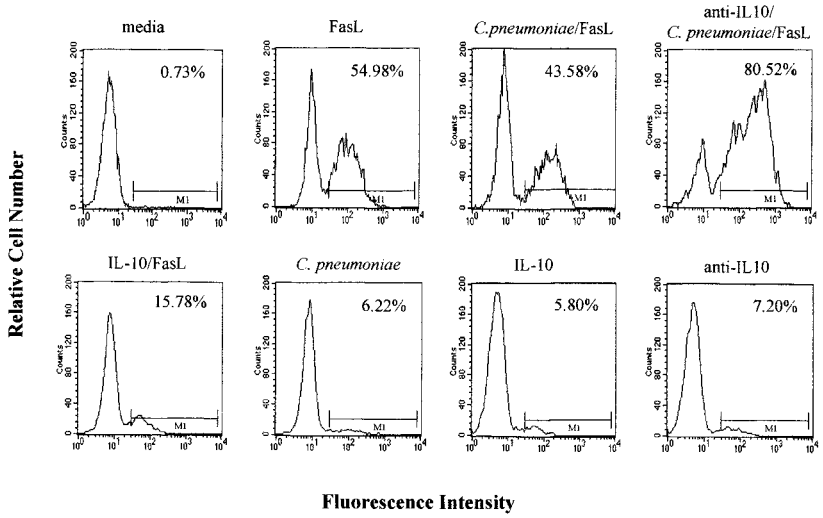

FIGURE 6. Role of IL-10 in resistance to Fas ligand-mediated apoptosis. *C. pneumoniae*-infected or noninfected cells were cultured in the presence of IL-10 (10 ng/ml) or anti-IL-10 (10 μg/ml) for 36 h before exposure to Fas ligand (100 ng/ml) and Fas ligand enhancer (1 μg/ml). Twelve hours later, cells were harvested for TUNEL assay, as described in *Materials and Methods*. Data demonstrate representative histograms of FITC-dUTP incorporation, and the percentages of FITC-dUTP-positive cells are the numbers above histograms.

shown to be associated with increased expression of the antiapoptotic factor Bcl-2. Furthermore, treatment of cells with anti-IL-10 diminished cell viability and Bcl-2 expression, while increasing caspase 1 activation and p53 expression. Balcewicz-Sablinska et al. (49) reported that IL-10 down-regulates apoptosis in human alveolar macrophages infected with *M. tuberculosis* by inducing the release of TNFR2, leading to the formation of nonactive TNF-α-TNFR2 complexes. More recently, Rojas et al. (50) reported that the TNF-α/IL-10 ratio might account for the balance between apoptosis and survival of virulent *M. tuberculosis*-infected murine macrophages. In our system, the time course for optimal IL-10 production following *C. pneumoniae* infection appeared to occur at a point when TNF-α production had fallen to baseline. Thus, induction of IL-10 most likely played a role in the decline of TNF-α levels. As to whether *C. pneumoniae* infection-mediated production of IL-10 up-regulates antiapoptotic factors such as Bcl-2 and Fas-associated death domain-like IL-1-converting enzyme-inhibitory protein expression and decreases caspase activation are questions deserving further study.

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