

Two Distinct Mechanisms for Induction of Dendritic Cell Apoptosis in Response to Intact *Streptococcus pneumoniae*¹

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Apoptotic dendritic cells (DCs) are ineffective at inducing immunity. Thus, parameters that regulate DC viability during a primary infection will help to determine the outcome of the subsequent immune response. In this regard, pathogens have developed strategies to promote DC apoptosis to counterbalance the nascent primary immune response. We demonstrate, using cultured bone marrow-derived DCs, that *Streptococcus pneumoniae* can induce DC apoptosis through two distinct mechanisms: 1) a rapid, caspase-independent mechanism of apoptosis induction, critically dependent on bacterial expression of pneumolysin, and 2) a delayed-onset, caspase-dependent mechanism of apoptosis induction associated with terminal DC maturation. Delayed-onset apoptosis does not require bacterial internalization, but rather is triggered by the interaction of bacterial subcapsular components and bone marrow-derived DC (likely Toll-like) receptors acting in a myeloid differentiation factor 88-dependent manner. In this regard, heavy polysaccharide encapsulation interferes with both DC maturation and apoptosis induction. In contrast, neither CD95/CD95 ligand interactions nor TNF- α appear to play a role in the delayed onset of apoptosis. These data are the first to define two mechanistically distinct pathways of DC apoptosis induction in response to an extracellular bacterium that likely have important consequences for the establishment of antibacterial immunity. *The Journal of Immunology*, 2003, 171: 2354–2365.

Programmed cell death or apoptosis plays a major regulatory role in immune responses (1). TNF- α released early in response to bacterial infections (2) and, specifically, several bacterial pathogens can directly trigger apoptosis in cells of the monocyte/macrophage lineage (3–10). However, the molecular mechanisms of apoptosis induced by bacteria are incompletely delineated, particularly regarding the involvement of death receptors. In the few cases investigated, they have been found to be either not involved or to be CD95 (Fas)-mediated (3, 11). A direct interaction between bacterial components and cell surface receptors as a trigger for macrophage apoptosis has been reported only for lipoproteins released by *Shigella flexneri* (12) and, more recently, for the 19-kDa protein of *Mycobacterium tuberculosis* (13), which activate apoptosis through interaction with Toll-like receptor-2 (TLR-2)³ (12).

Induction of macrophage apoptosis by phagocytosed pathogens is largely regarded as a microbial strategy to evade the immune response, but in some instances it could be an effective host mechanism for pathogen clearance, limiting intracellular replication. Moreover, apoptotic bodies containing killed bacteria could be taken up by dendritic cells (DCs) and used as a source of Ag to initiate a primary immune response (8). Thus, macrophages infected with *M. tuberculosis* exhibit NO-dependent killing of my-

cobacteria, which leads to apoptosis (4), but this is correlated with a relative resistance to the experimental infection (14). Macrophage intracellular killing of opsonized *Streptococcus pneumoniae* has been associated with the induction of a caspase-dependent but Fas-independent apoptosis (11). However, *S. pneumoniae* pneumolysin, which induces NO production in macrophages (15), is ineffective at inducing macrophage apoptosis (11). In contrast, pneumolysin induces in neurons and microglia a caspase-independent apoptosis associated with the release of apoptosis-inducing factor from mitochondria (16), which contributes to enhanced morbidity (17). Thus, various stimuli acting on particular cell types induce apoptosis through distinct pathways leading to different functional or pathologic consequences.

The DC is widely regarded as a critical link between the innate and adaptive immune response. Immature DCs, as well as macrophages, detect microbial infections by recognition, via TLRs, of pathogen-associated molecular patterns (18, 19). Signaling through all TLR members is dependent upon a common adaptor molecule (20), the myeloid differentiation factor 88 (MyD88), which mediates the production of proinflammatory cytokines and, in part, the maturation of the DC. DC maturation is characterized by the loss of phagocytic capacity and the up-regulation of MHC and membrane costimulatory molecules. These DC responses to pathogens are key elements in the induction of innate immunity, the effective priming of naive T cells, and the initiation of adaptive responses (21, 22).

Despite the critical importance of the DC in the response to primary infections, very little is known regarding the ability of bacteria to induce apoptosis in DCs. Only *Listeria monocytogenes* has been demonstrated to induce apoptosis in DCs via the action of hemolysin (23). DCs, however, appear to exhibit mechanisms that counterbalance apoptotic stimuli that otherwise efficiently induce apoptosis in macrophages. Thus, mature DCs are relatively resistant to the proapoptotic action of TNF- α (24, 25) and CD95-mediated apoptosis (26), and this resistance is associated with the up-regulation of FLIP (26) and Bcl-x_L (24). A relative resistance of DCs for apoptosis induction in response to bacteria would potentially play an important role in optimizing their ability to act as the key APC during a primary immune response. In this report, we

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Received for publication April 21, 2003. Accepted for publication June 25, 2003.

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¹ This work was supported by National Institutes of Health Grants 1R01 AI49192 and 1R01 AI46551.

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³ Abbreviations used in this paper: TLR, Toll-like receptor; DC, dendritic cell; MyD88, myeloid differentiation factor 88; BMDC, bone marrow-derived dendritic cell; PLN, pneumolysin-deficient mutant; PG, peptidoglycan; PI, propidium iodide; FMK, fluoromethylketone; CD95L, CD95 ligand; CM-DiI, chloromethylbenzamido derivative of DiI; Ct, cytochalasin; MFI, mean fluorescence intensity.

Report Documentation Page				Form Approved OMB No. 0704-0188	
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1. REPORT DATE APR 2003		2. REPORT TYPE		3. DATES COVERED 00-00-2003 to 00-00-2003	
4. TITLE AND SUBTITLE Two Distinct Mechanisms for Induction of Dendritic Cell Apoptosis in Response to Intact Streptococcus pneumoniae				5a. CONTRACT NUMBER	
				5b. GRANT NUMBER	
				5c. PROGRAM ELEMENT NUMBER	
6. AUTHOR(S)				5d. PROJECT NUMBER	
				5e. TASK NUMBER	
				5f. WORK UNIT NUMBER	
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) Uniformed Services University of the Health Sciences, Department of Pathology, Bethesda, MD, 20814				8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)				10. SPONSOR/MONITOR'S ACRONYM(S)	
				11. SPONSOR/MONITOR'S REPORT NUMBER(S)	
12. DISTRIBUTION/AVAILABILITY STATEMENT Approved for public release; distribution unlimited					
13. SUPPLEMENTARY NOTES					
14. ABSTRACT					
15. SUBJECT TERMS					
16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT Same as Report (SAR)	18. NUMBER OF PAGES 12	19a. NAME OF RESPONSIBLE PERSON
a. REPORT unclassified	b. ABSTRACT unclassified	c. THIS PAGE unclassified			

wished to determine the susceptibility of DCs to apoptosis in response to the Gram-positive extracellular bacterium *S. pneumoniae*. Using bone marrow-derived DCs (BMDCs), we show for the first time that bacteria can induce DC apoptosis through two distinct mechanisms: 1) a rapid, caspase-independent mechanism of apoptosis induction, critically dependent on bacterial expression of pneumolysin, and 2) a delayed-onset, caspase-dependent mechanism of apoptosis induction associated with terminal DC maturation. Delayed-onset apoptosis was independent of Fas/Fas ligand interactions and TNF- α and did not require bacterial internalization, but instead was dependent upon interaction between bacterial subcapsular components with a cell surface BMDC receptor (likely a TLR) acting in a MyD88-dependent manner.

Materials and Methods

Mice

C57BL/6N mice were obtained from the National Cancer Institute (Gaithersburg, MD). MyD88^{-/-} mice (27) were kindly provided by Dr. Douglas Golenbock (Department of Medicine and Microbiology, Boston University School of Medicine, Boston, MA) with permission from Dr. S. Akira (Research Institute for Microbial Diseases, Osaka University, Osaka, Japan) and were bred and genotyped in our facility. C3.MRL-Fas^{lpr} (MRL/lpr) mice, genetically deficient in CD95 expression, C3H/HeJ-Fas^{gld} (C3H/gld) mice, genetically deficient in CD95L expression, and their corresponding background controls, C3H/HeJ mice, were purchased from The Jackson Laboratory (Bar Harbor, ME). Mice were used at 8–10 wk of age and were maintained in a pathogen-free environment at the Uniformed Services University of the Health Sciences (Bethesda, MD). The experiments in this study were conducted according to the principles set forth in the Guide for the Care and Use of Laboratory Animals (Institute of Animal Resources, National Research Council, Department of Health, Education and Welfare, National Institutes of Health, 78-23).

S. pneumoniae strains and bacterial Ags

The *S. pneumoniae* capsular type 14 was generously provided by Sam Wilson (Uniformed Services University of the Health Sciences, Bethesda, MD). The capsular type 3 (strain WU-2) and the stable nonencapsulated variant JD6/11 of this strain (28) were kindly provided by Dr. J. Yother (University of Alabama, Birmingham). The capsular type 2 (strain D39), its isogenic pneumolysin-negative mutant (29), and the nonencapsulated variant of D39 (strain R36A) were kindly provided by Dr. D. E. Briles (University of Alabama). LPS-free, purified peptidoglycan from *Staphylococcus aureus* was provided by Dr. R. Schuman (Biosynex, Rockville, MD).

Bacterial culture and preparation of the bacterial inoculum

Isolated colonies were selected from Columbia blood agar plates and were grown in Todd Hewitt broth to mid-log phase, collected, heat killed by incubation at 60°C for 1 h, and extensively washed in PBS. Sterility was confirmed by subculture on blood agar plates. The pneumolysin-deficient mutant (PLN) was grown in the same medium, but supplemented with 0.3 μ g/ml erythromycin. The bacterial suspension of the type 14 strain was adjusted with PBS to 10⁹ CFU/ml by an absorbance reading of 0.6 at 650 nm. This bacterial suspension was used as a standard to normalize, by FACS analysis, the density of the other heat-killed *S. pneumoniae* strains. Specifically, we determined the number of events acquired in the appropriate forward side scatter gate during 1 min using serial 10-fold dilutions of the bacterial suspensions. This approach avoids errors in the estimation of bacterial density by photometric turbidity due to heavy encapsulation. Bacteria were then aliquoted at 10¹⁰ CFU/ml and frozen at -80°C until their use to pulse BMDCs in culture. When live bacteria were used, cultures in mid-log phase were collected, washed in PBS, and, after adjustment to the same absorbance as the corresponding heat-killed suspension, were used directly in the experiment.

Cell culture medium

The cell culture medium consisted of RPMI 1640 supplemented with 5% FCS (BioWhittaker, Walkersville, MD), 50 μ M 2-ME, 20 μ g/ml gentamicin, 1 mM sodium pyruvate, 2 mM L-glutamine, 0.1 mM nonessential amino acids, and 25 mM HEPES.

Bone marrow cell culture

DCs were cultured from bone marrow, as previously described (30), with slight modifications (22). Briefly, bone marrow cell suspensions were de-

pleted of T cells, B cells, and other MHC class II-expressing cells by incubation for 30 min at 4°C with a mAb cocktail composed of AF6-120.1 (mouse IgG2a anti-I-A^b), RA3-6B2 (rat IgG2a anti-CD45R/B220), 53-5.8 (rat IgG1 anti-CD8b.2), and RB6-8C5 (rat IgG2b anti-Ly-6G) and were negatively selected by magnetic bead cell sorting using a mixture of affinity-purified polyclonal goat anti-rat IgG and goat anti-mouse IgG conjugated beads (BioSpheres; Biosource, Camarillo, CA). All of the mAbs were purchased from BD PharMingen (San Diego, CA). The bone marrow cells obtained were cultured at 1.25 $\times$ 10⁶ cells/ml in cell culture medium supplemented with 10 ng/ml murine recombinant GM-CSF, kindly provided by Dr. L. Grinberg (Biosynex). On days 3 and 5 the supernatant was removed and replaced with fresh medium containing GM-CSF. Experiments were performed with the nonadherent and loosely adherent cell population from cultures at days 6–8.

Bacterial pulsing and stimulation of BMDCs for in vitro experiments

BMDCs collected after 6–8 days of culture were washed two times in cell culture medium and plated at 10⁶ BMDCs/ml of medium without GM-CSF in 24- or 48-well cell culture plates (Costar, Corning, NY). After 30 min, to allow cells to settle, peptidoglycan (PG) (100–0.1 μ g/ml) or a bacterial suspension in cell culture medium containing the desired amount of inactivated *S. pneumoniae* was added to the cultures and maintained during the pulse. Unless indicated otherwise, *S. pneumoniae* type 14 was used at a ratio of 800 bacteria per BMDC throughout the experiments. In some experiments, BMDCs were stimulated with 10 μ g/ml of low endotoxin anti-CD95 proapoptotic mAb (clone Jo2, hamster IgG; BD PharMingen) in the absence of bacteria or 1 h before bacteria were added to the culture to test BMDC susceptibility to CD95-mediated apoptosis. Alternatively, for pulse-chase experiments, BMDCs were collected after the pulse, the excess of bacteria were extensively washed, and the BMDCs were replated in medium without GM-CSF and cultured for the desired chase period. For experiments with live bacteria, the pulse was conducted in cell culture medium without antibiotics and was chased in culture medium containing 20 μ g/ml gentamicin.

Propidium iodide (PI) uptake assay and trypan blue exclusion assay

Cell membrane permeability and viability were tested by PI uptake assay or trypan blue exclusion assay. PI uptake for BMDCs was analyzed by placing 1 $\times$ 10⁶ cells/ml in cold PBS containing 5 μ g/ml PI (Sigma-Aldrich, St. Louis, MO). After 5 min of incubation at room temperature in the dark, the red fluorescence emitted by the PI intercalated in the DNA of cells that have lost membrane integrity was analyzed on a flow cytometer (EPICS XL-MCL; Beckman Coulter, Miami, FL). The trypan blue exclusion assay was conducted by diluting a cell suspension by 1/5 v/v with 0.4% trypan blue (Sigma-Aldrich). Viable cells were counted using a hemocytometer.

Annexin V-FITC staining

Bacteria-pulsed BMDCs and non-pulsed BMDCs were collected at different time points, washed, and adjusted to 10⁶ BMDCs/ml in cold 10 mM HEPES/NaOH (pH 7.4) containing 140 mM NaCl, 2.5 mM CaCl₂, and 5 μ g/ml PI and Annexin V-FITC (BD PharMingen). BMDCs were incubated for 15 min at room temperature in the dark and were immediately analyzed by flow cytometry. BMDCs treated for 4 h in culture with 6 μ M camptothecin (Sigma-Aldrich), a potent inhibitor of topoisomerase I, were used as a positive control for induction of apoptosis. BMDCs treated with 0.2% saponin (Sigma-Aldrich) were used as a positive control for induction of necrosis. Annexin V avidly binds to cells expressing cell surface phosphatidylserine, a phospholipid translocated from the inner leaflet of the plasma membrane to the outer leaflet very early during the apoptosis program, when cells are still nonpermeable to PI. Cells positive for Annexin V and negative for PI are undergoing apoptosis, whereas cells in the end stage of apoptosis are positive for both Annexin V and PI (31).

Determination of hypodiploid nuclei by flow cytometry

The percentage of apoptotic nuclei (i.e., hypodiploid) was measured after PI staining in hypotonic buffer by flow cytometry, essentially as described by Nicoletti et al. (32). Briefly, BMDCs were collected at different time

points during the pulse with bacteria or drug treatment and washed extensively, and the cell pellet containing 1×10^6 BMDCs was gently resuspended in 1 ml of hypotonic fluorochrome solution (50 $\mu\text{g/ml}$ PI in 0.1% sodium citrate plus 0.1% Triton X-100). The tubes were placed at 4°C in the dark overnight, and the PI fluorescence of individual nuclei was measured by flow cytometric analysis (32). BMDCs treated in culture during 12–24 h with 1 $\mu\text{g/ml}$ actinomycin D (Sigma-Aldrich) were used as positive controls of apoptosis. BMDCs treated with 0.2% saponin were used as positive controls of necrosis.

Determination of cellular caspase activity

Caspase-8 and caspase-3 activity in BMDC extracts obtained at different time points during the pulse with bacteria or drug treatment were determined using the corresponding colorimetric assay kits, following the instructions of the manufacturer (Calbiochem, San Diego, CA). The caspase-3 kit uses Ac-DEVD-*p*-nitroaniline as substrate, and the caspase-8 kit uses IETD-*p*-nitroaniline. The specific inhibitors and purified enzymes provided accurate and specific quantitation of the caspase activity.

Inhibition of caspase activity

Caspase activity was irreversibly inhibited by treatment of BMDCs with 50 μM of the corresponding cell-permeable caspase inhibitor (R&D Systems, Minneapolis, MN). The caspase-specific inhibitors used were Z-WEHD-fluoromethylketone (FMK) (caspase-1 inhibitor), Z-DEVD-FMK (caspase-3 inhibitor), Z-YVAD-FMK (caspase-4 inhibitor), Z-YVAD-FMK (caspase-6 inhibitor), Z-IETD (caspase-8 inhibitor), Z-LEHD (caspase-9 inhibitor), Z-AEVD (caspase 10 inhibitor), and Z-LEED-FMK (caspase-13 inhibitor). The inhibitor was added to the cultures 30 min before the pulse with bacteria and was maintained in the culture medium during the pulse. The peptide Z-F-A-FMK, was added to the cultures at the same concentration as the caspase inhibitors to control for the effects of the fluoromethyl-ketone derivatives on caspase activity. BMDC cultures incubated with DMSO were used to monitor any potential cytotoxic effects of the diluents.

Quantitation of soluble CD95 ligand (CD95L)

The concentration of soluble CD95L released into the culture medium, during the pulse with bacteria, was quantified by ELISA (Quantikine M), following the instructions of the manufacturer (R&D Systems).

Flow cytometric analysis of BMDC surface Ag expression

All steps were performed on ice. For staining BMDCs for flow cytometry, the Fc receptors were specifically blocked with 2.5 $\mu\text{g/ml}/10^6$ BMDCs of anti-CD16/CD32 mAb (clone 2.4G2; BD PharMingen) in PBS containing 1% FCS (staining buffer) 45 min before and during the staining. Cells were stained by incubation for 30 min with biotinylated or PE-conjugated mAbs (BD PharMingen) specific for CD11c (clone HL3), CD8 (clone 53-6.7), CD11b (M1/70), I-A^b (clone AF6-120.1), I-A^d (clone AMS-32.1), H2-K^b (AF6-88.5), CD40 (clone 3/23), CD80 (16-10A1), CD86 (clone GL1), CD25 (clone PC61), CD54 (clone 3E2), CD95 (clone Jo2), and CD95L (clone Kay-10). The incubation with biotinylated mAbs was followed by staining with PE-streptavidin conjugate for 15 min. For detection of CD16/CD32 expression with the specific Ab (clone 2.4G2), the Fc blocking step was omitted. Irrelevant isotype and species-matched mAbs were used as staining controls. Cells were analyzed on an EPICS XL-MCL (Beckman Coulter). Dead cells and debris were excluded from analysis by gating on the appropriate forward and side scatter profile.

Phagocytic capacity of BMDCs

The time course of bacterial internalization was tracked by pulsing the BMDCs with *S. pneumoniae* labeled with the fluorescent lipophilic cell tracker chloromethylbenzamide derivative of DiI (CM-DiI; Molecular Probes, Eugene, OR), as previously reported (22). Briefly, at different time points of the pulse, BMDCs were collected and washed in PBS, and viable BMDCs were analyzed by flow cytometry, gated so as to exclude free bacteria on the basis of cell size. BMDCs pulsed with unlabeled bacteria at the same ratios were used to determine nonspecific fluorescence.

Cytochalasin (Ct) treatment

To block particle internalization, BMDCs were treated for 2–3 h with CtA (5 $\mu\text{g/ml}$) or CtD (5 $\mu\text{g/ml}$) before the pulse with bacteria. For pulse times shorter than 5 h, the Ct was maintained during the pulse, otherwise Ct was washed out and 1 $\mu\text{g/ml}$ fresh Ct was added and maintained during the pulse. Unpulsed BMDCs treated under these conditions remain viable (>82%) after 24 h of culture, undergoing minimal Ct-induced apoptosis (5% in BMDCs treated with CtA; 12% in BMDCs treated with CtD). The

treatment markedly inhibited the internalization of CM-DiI-labeled bacteria (<4% of the mean fluorescence intensity (MFI) uptake by untreated cells). DMSO was used as diluent for the Ct, but its concentration in the culture was <0.2%. Identical volumes of DMSO, as found in Ct-treated cultures, were added as a control.

Cytokine ELISA

The concentrations of specific cytokines released into the medium of BMDC cultures were measured using optimized standard sandwich ELISA. Recombinant cytokines used as standards, as well as the capture mAbs, biotinylated mAbs used for detection, and streptavidin-alkaline phosphatase were purchased from BD PharMingen. Streptavidin-alkaline phosphatase was used in combination with *p*-nitrophenyl phosphate disodium (Sigma-Aldrich) as substrate to detect the specific binding. Standards were included in every plate and the samples were tested in duplicate. The detection limits of the respective ELISAs were 4 pg/ml for IL-6, 70 pg/ml for IL-10, 6 pg/ml for IL-12 (p40/p70), 125 pg/ml for IL-12 (p70), and 12 pg/ml for TNF- α .

Statistics

Data were expressed as arithmetic mean $\pm$ SEM of the individual values. Levels of significance of the differences between groups were determined by the Student *t* test. Any *p* values <0.05 were considered statistically significant.

Results

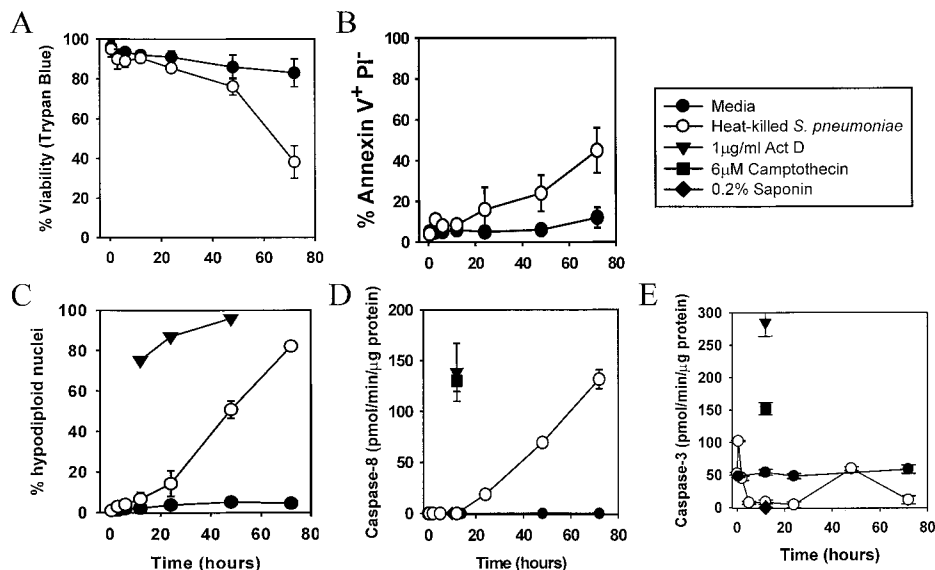
Heat-killed *S. pneumoniae* induce apoptosis of BMDCs

The length of time during which DCs maintain their viability after contact with a bacterial pathogen likely plays a critical role in their ability to promote anti-bacterial Ig responses (22). Thus, initial studies tested the ability of heat-killed *S. pneumoniae* to induce apoptosis in immature in vitro-derived bone marrow dendritic cells. The inability of BMDCs to exclude trypan blue, which reflects their loss of cell membrane integrity and which occurs relatively late during the process of apoptosis, was observed largely between 48 and 72 h after addition of heat-killed bacteria to culture (Fig. 1A). Thus, by 72 h, <35% of BMDCs excluded trypan blue compared with $\geq 90\%$ at initiation of culture or after 72 h in medium alone. The kinetics and percentages of BMDCs showing PI uptake after culture with *S. pneumoniae* were similar to those observed using trypan blue (data not shown).

To determine whether loss of trypan blue exclusion after *S. pneumoniae* activation reflected induction of BMDC apoptosis, we measured both the percentage of cells staining positive for Annexin V (which results from translocation of phosphatidylserine to the outer leaflet of the plasma membrane) and the percentage of hypodiploid nuclei, as determined by PI staining (which reflects nuclear fragmentation) during the 72 h culture period with *S. pneumoniae*. A progressive increase in the percentage of BMDCs either staining for Annexin V (Fig. 1B) or showing hypodiploid nuclei (Fig. 1C) was observed beginning 12 h after addition of *S. pneumoniae* and continuing until 72 h, by which time >80% apoptotic cells were observed. In contrast, <10% of BMDCs cultured in medium alone were apoptotic after 72 h. Induction of BMDC apoptosis after *S. pneumoniae* addition was also closely correlated, beginning after 12 h, with a progressive increase in the enzymatic activity of the apoptosis-initiator caspase-8 (Fig. 1D).

Activity of the effector caspase, caspase-3, although rapidly spiking initially, declined thereafter during the first 24 h to significantly below the levels found in unstimulated BMDCs (Fig. 1D). Caspase-3 activity returned to normal levels by 48 h and was again found at reduced levels at 72 h. BMDCs treated with saponin, an inducer of necrosis, did not up-regulate either caspase-8 (Fig. 1D) or

FIGURE 1. Heat-killed *S. pneumoniae* induced BMDC apoptosis. BMDCs pulsed with heat-killed *S. pneumoniae* were harvested at different time points, and the progression of apoptosis was determined by measuring the percentage of cells permeable to trypan blue (A), the percentage of PI-negative BMDCs binding a conjugate of Annexin V-FITC (B), the percentage of hypodiploid nuclei determined by PI staining (C), and the caspase-8 (D) and caspase-3 (E) activity present within the cell extracts. BMDCs treated with 1 μ g/ml actinomycin D or 6 μ M camptothecin were used as a positive control of apoptosis. BMDCs cultured in 0.2% saponin were used as positive controls of necrosis. The data shown are representative of two independent experiments in duplicate.



caspase-3 (Fig. 1E) activity, as expected. Bacterial interference in the enzymatic assay was excluded by demonstrating that high bacterial densities did not inhibit the enzymatic activity of purified caspase-3 and that caspase-3 activity was not detected in the bacterial preparation itself (data not shown).

BMDCs exhibited minimal apoptosis within the first 20 h after bacterial exposure. In contrast, treatment with actinomycin D or camptothecin, which are primary inducers of apoptosis, led to a rapid increase in percentages of hypodiploid nuclei and both caspase-3 and caspase-8 activity in BMDCs. Thus, >70% of BMDCs were apoptotic 10 h after treatment, a level of apoptosis observed only 72 h after bacterial exposure (Fig. 1). Collectively, these data suggest that heat-killed *S. pneumoniae* induce a delayed-onset, caspase-dependent apoptosis of BMDCs that is likely not secondary to the direct action of an apoptosis inducer, such as a bacterial toxin.

Continuous exposure to heat-killed bacteria is not critical for the induction of apoptosis

We next performed bacterial pulse-chase experiments to determine the time required for BMDCs to be in contact with bacteria to elicit apoptosis. As shown in Fig. 2A (open symbols), BMDCs exposed for either 3 or 6 h to heat-killed bacteria showed a similar time of

onset of apoptosis (>24 h) compared with BMDCs that were exposed continuously to bacteria. No increase in apoptosis was observed when BMDCs were in contact with bacteria for only 30 min. Nevertheless, a progressive increase in the percentage of hypodiploid nuclei at a given time point (Fig. 2A) and extension of DNA fragmentation (data not shown) were observed as the duration of the initial bacterial pulse was increased from 3 to 6 h and again from 6 h to continuous exposure. These results indicate that the signals required to induce delayed apoptosis are provided to the BMDCs within the first hours of bacterial contact, with enhanced levels of apoptosis occurring as bacterial exposure is prolonged. We observed that a similar time course of exposure to bacteria was required for the subsequent induction of BMDC phenotypic maturation (22).

Live and heat-killed *S. pneumoniae* induce apoptosis through different mechanisms

The induction of BMDC apoptosis by heat-killed *S. pneumoniae* indicated that heat-sensitive metabolic products, including toxins, were not necessary for apoptosis induction, but it did not rule out an additional role for these products when BMDCs are exposed to live bacteria. To determine this, we directly compared the efficiency of apoptosis induction in response to live vs heat-killed

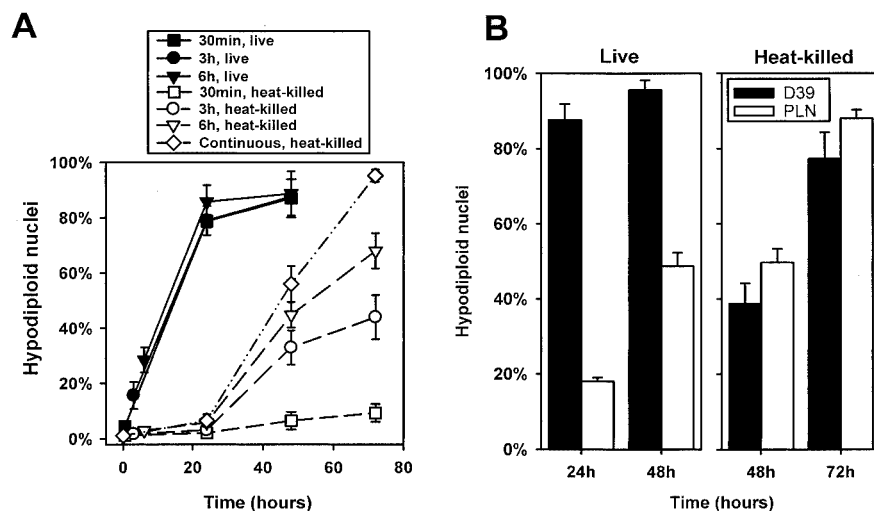


FIGURE 2. Kinetics of DNA fragmentation induced in BMDCs by live or heat-killed wild-type and pneumolysin-defective mutants of *S. pneumoniae*. A, BMDCs were pulsed with heat-killed or live *S. pneumoniae* capsular type 14 at a ratio of 800 bacteria per BMDC. During the periods of pulse indicated, free bacteria were removed by washing, and BMDCs were recultured for the indicated periods of chase. The percentages of hypodiploid nuclei were determined after the pulse and after 20, 48, or 72 h of chase. B, Comparison of the percentage of BMDC hypodiploid nuclei after pulse with live or heat-killed *S. pneumoniae* capsular type 2 (strain D39) and its isogenic pneumolysin-defective mutant (strain PLN). The percentage of hypodiploid nuclei was determined at 24, 48, and/or 72 h. One experiment representative of two is shown.

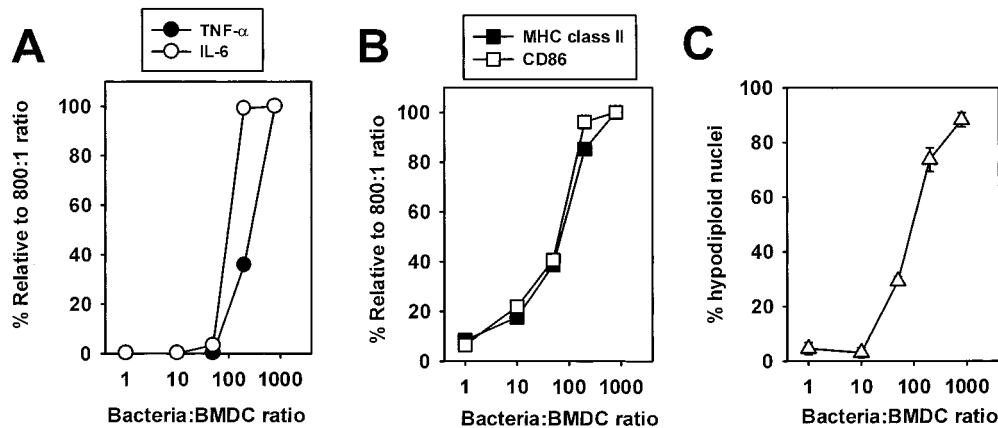


FIGURE 3. Bacterial dose dependence of cytokine secretion, phenotypic maturation, and DNA fragmentation induced by heat-killed *S. pneumoniae*. Concentrations of TNF- α and IL-6 secreted into the culture medium (A) and MFI of BMDCs stained with mAbs specific for MHC class II and CD86 after 12 and 24 h, respectively, after pulse with bacteria at variable bacteria:BMDC ratios (B), expressed as the percentage of the concentration or MFI obtained after pulse with bacteria at a 1:800 ratio (100%). C, The percentage of hypodiploid nuclei was determined after a 72-h pulse with the indicated bacteria:BMDC ratios. One experiment representative of three is shown.

bacteria. Live *S. pneumoniae* induced BMDC apoptosis after a much shorter exposure time and with significantly faster kinetics than was observed in response to heat-killed bacteria (Fig. 2A). The presence of live, but not heat-killed, bacteria in BMDC cultures for as little as 30 min was sufficient to induce a significant increase in apoptosis by 3 h (>15% hypodiploid nuclei) with >80% apoptosis by 24 h. No further changes occurred in the kinetics or extent of apoptosis induction with greater bacterial exposure times of 3 or 6 h. To eliminate any continued proliferation of residual bacteria, possibly remaining after washing and reculture of BMDCs in medium alone, we added either gentamicin (which inactivate extracellular bacteria) or an antibiotic cocktail of gentamicin, penicillin, and streptomycin (which likely also inactivate intracellular bacteria). Supernatants from fresh cultures of *S. pneumoniae* grown in medium (RPMI 1640 supplemented with 5% FCS without antibiotics), added to BMDC cultures supplemented with antibiotics, induced BMDC apoptosis at similar levels and following similar kinetics as in BMDC cultures pulsed with live *S. pneumoniae* (data not shown).

The rapid kinetics of induction of apoptosis in response to live bacteria were roughly similar to those observed using the direct inducers of apoptosis, actinomycin D or camptothecin. This, and the ability of *S. pneumoniae* culture supernatants to induce apoptosis, suggested the possibility that live, but not heat-killed, *S. pneumoniae* produce a toxin that directly induces BMDC apoptosis. In this regard, the hemolysin activity of Group B *Streptococcus* has been shown to induce apoptosis in human monocytes (7), and the pneumolysin of *S. pneumoniae* induces neuronal and microglial apoptosis (16). Thus, we compared the ability of live *S. pneumoniae* capsular type 2 (strain D39) and pneumolysin-deficient mutant (PLN) to induce apoptosis in BMDCs. As shown in Fig. 2B, at 24 h >80% of BMDCs treated with live D39 exhibited hypodiploid nuclei vs <20% for BMDCs treated with PLN, demonstrating a critical role for pneumolysin in the induction of rapid onset apoptosis by live bacteria. In contrast, both D39 and PLN, when heat-killed, and live PLN were equally efficient at inducing the delayed onset of apoptosis seen in Fig. 2, demonstrating the existence of a second mechanism of apoptosis induction that was unrelated to bacterial viability or pneumolysin activity. Pneumolysin is heat-sensitive, very unstable in culture, and requires metabolic activity for its continued release (33). These data indicate that *S. pneumoniae* can induce DC apoptosis through two different mechanisms. Live bacteria trigger rapid BMDC

apoptosis through release of a toxin (pneumolysin), whereas nonviable (or live pneumolysin-deficient) bacteria are capable of stimulating BMDCs to undergo a primary process that leads to a delayed onset of apoptosis.

*The extent of apoptosis induced by heat-killed *S. pneumoniae* is directly correlated with the level of BMDC activation and maturation*

Apoptosis induced by heat-killed *S. pneumoniae* begins to occur soon after the phenotypic maturation of BMDCs is complete (i.e., ~24 h), suggesting that delayed onset apoptosis is a direct reflection of terminal maturation. As shown in Fig. 3, both the level of secretion of IL-6 and TNF- α (Fig. 3A) and especially the magnitude of phenotypic maturation (i.e., up-regulation of MHC class II and CD86; Fig. 3B) in response to varying ratios of bacteria to BMDC directly correlated with the peak percentages of hypodiploid nuclei observed at 72 h (Fig. 3C). Bacteria to BMDCs ratios under 50:1, which were ineffective at inducing apoptosis, also failed to induce significant secretion of cytokines and phenotypic maturation. These data suggest a cause-and-effect relationship between BMDC phenotypic maturation and apoptosis in response to bacterial activation.

To further explore the potential relationship between BMDC maturation and apoptosis, we determined the potential role of GM-CSF in comodulating these processes. GM-CSF is released early after bacterial infection and has been shown to inhibit apoptosis of neutrophils (34), in addition to stimulating the generation of DC precursors from the bone marrow. Thus, we wished to determine whether GM-CSF could likewise modulate BMDC apoptosis in response to heat-killed bacteria and whether this correlated with a change in the level of phenotypic maturation. Addition of GM-CSF to BMDC cultures at the time of addition of bacteria significantly reduced the percentage of hypodiploid nuclei observed at 48 h (Fig. 4A). The difference observed in apoptosis induction at 72 h between GM-CSF-treated and untreated groups was much less, suggesting that GM-CSF delays, but does not prevent, the onset of apoptosis. To be effective in reducing BMDC apoptosis, GM-CSF was required during the first 24 h after bacterial exposure (Fig. 4A) and needed to be present at high concentrations (≤ 10 pg/ml of GM-CSF was ineffective). Furthermore, when varying doses of GM-CSF were tested, a direct relationship was observed between the ability of GM-CSF to reduce apoptosis and an

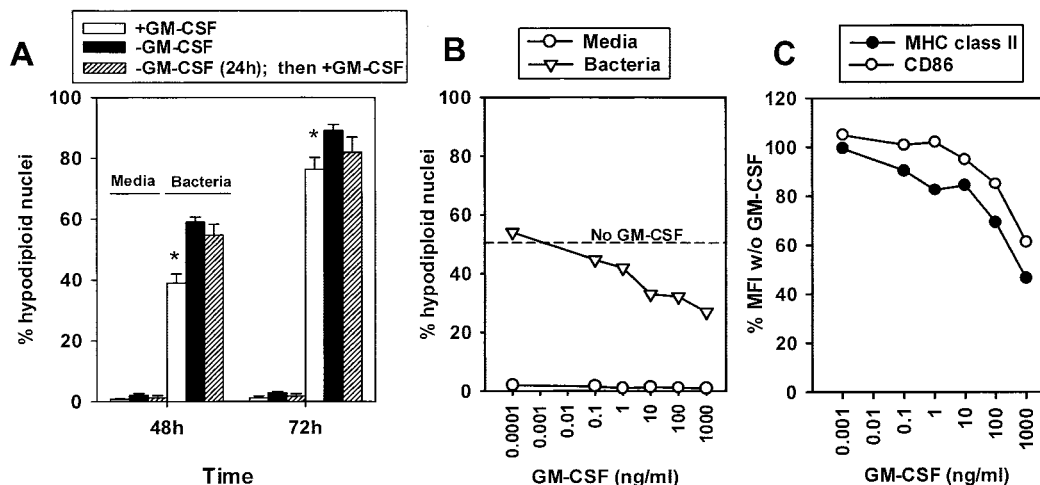


FIGURE 4. Inhibition of bacteria induced BMDC apoptosis by GM-CSF. **A**, BMDCs were cultured in the absence (medium) or presence of heat-killed *S. pneumoniae* in medium containing 10 ng/ml GM-CSF (+GM-CSF) or in medium without GM-CSF (–GM-CSF) during the entire period of pulse with bacteria or only during the first 24 h of pulse. After 48 or 72 h, the percentages of hypodiploid nuclei were determined. Values are expressed as the arithmetic mean of triplicate cultures $\pm$ SEM. **B**, Titration of the GM-CSF effect. BMDCs were pulsed with bacteria during 48 h in the presence of increasing concentrations of GM-CSF, and the percentages of hypodiploid nuclei were determined. **C**, Expression of CD86 and MHC class II by flow cytometry after 24 h of pulse with bacteria in the same GM-CSF titration experiment depicted in **B**.

inhibitory effect on phenotypic maturation (Fig. 4, **B** and **C**), reinforcing the view that delayed apoptosis in response to bacterial stimulation is a reflection of terminal maturation.

Although addition of GM-CSF allowed for the generation of immature DCs from bone marrow precursors, all experiments were conducted in the absence of GM-CSF. As illustrated in Fig. 4, **A**

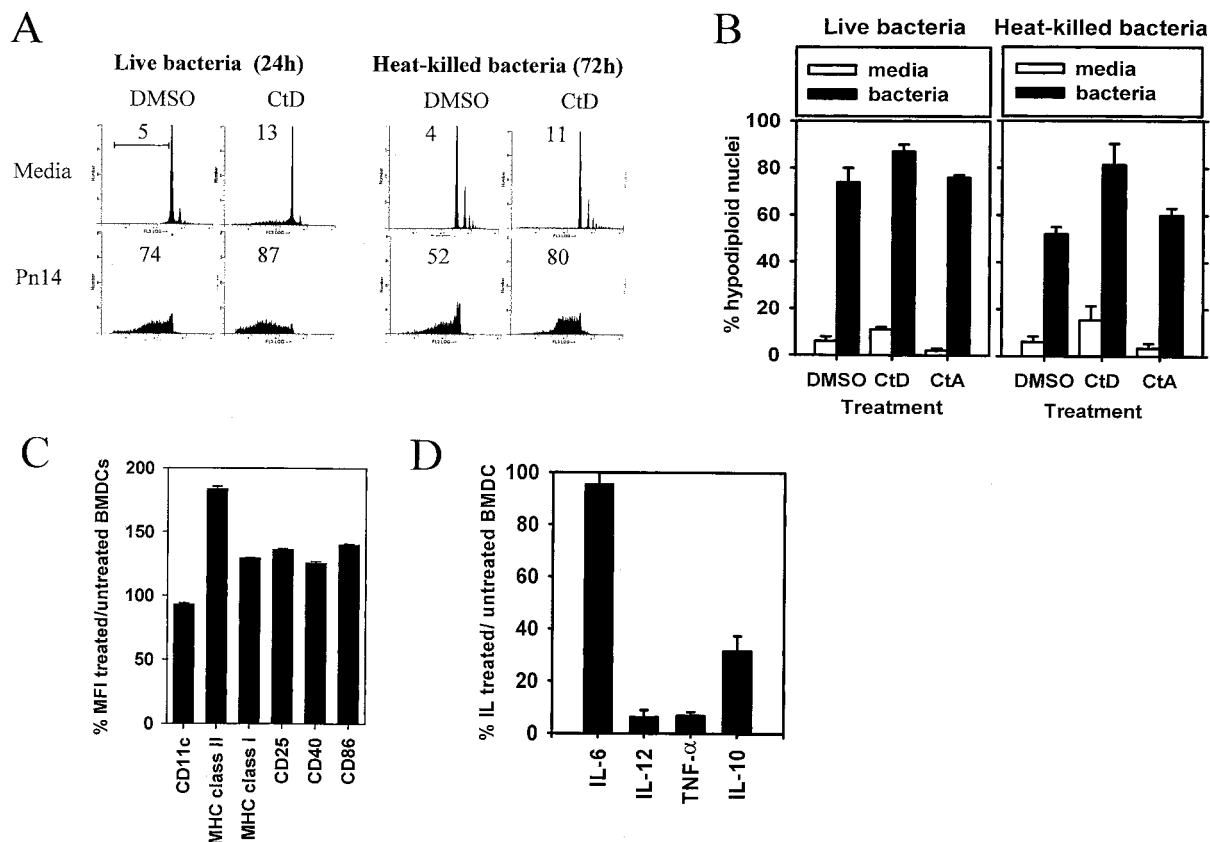


FIGURE 5. DNA fragmentation induced by *S. pneumoniae* does not require bacterial internalization. **A**, Flow cytometric analyses of PI-stained BMDC nuclei to determine percentages of hypodiploid nuclei after 24-h pulse with live (left) or 72-h pulse with heat-killed (right) *S. pneumoniae* capsular type 14 with or without pretreatment with CtD. **B**, Percentages of hypodiploid nuclei in a different set of similar experiments in which BMDCs were pretreated with either CtA or CtD. **C**, MFI ratio (%) of cell surface marker expression at 24 h on BMDCs pulsed with heat-killed *S. pneumoniae* treated or untreated with CtD. **D**, Cytokines secreted as ratio (%) of treated or untreated with CtD as in **C**. Untreated cells were incubated with the same volume of the diluent DMSO. One experiment representative of five is shown.

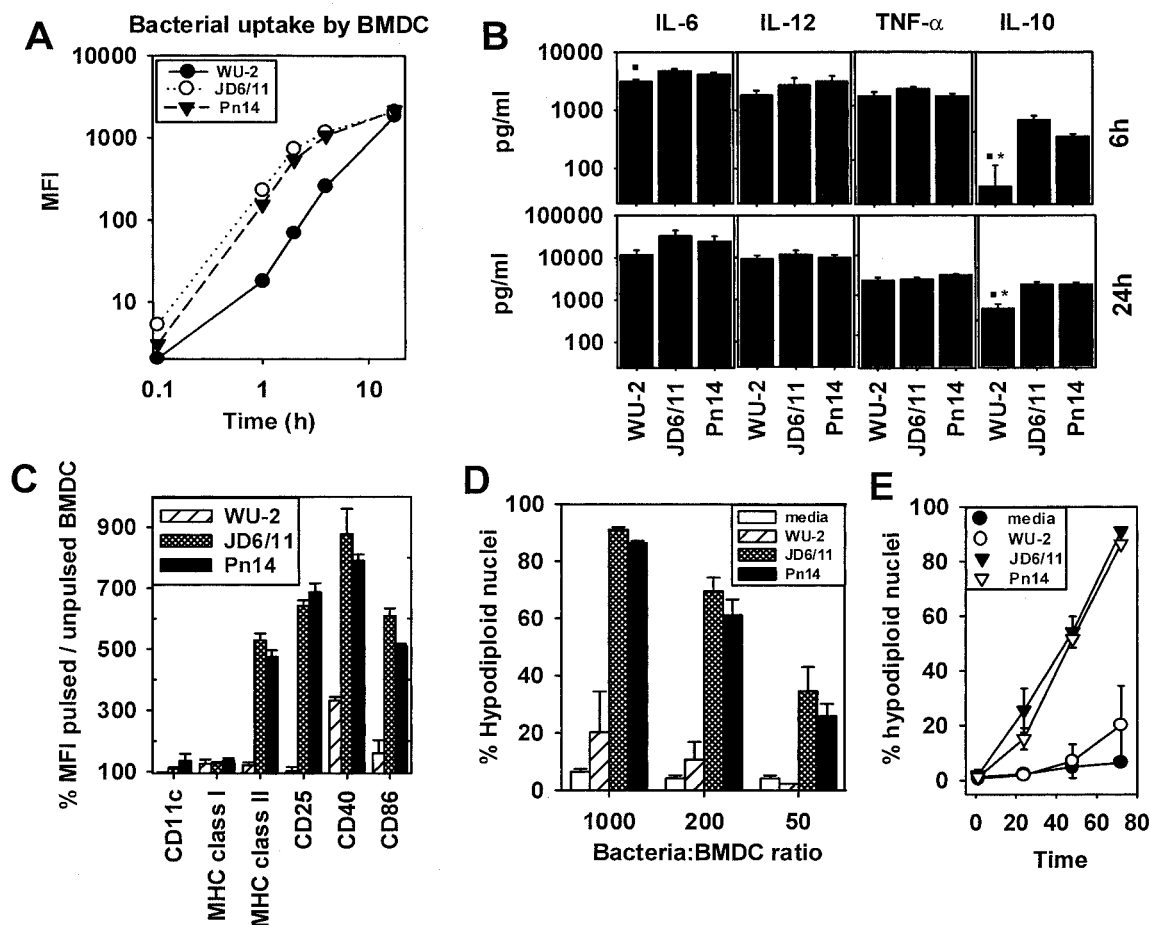


FIGURE 6. Heavily encapsulated *S. pneumoniae* are not effective inducers of BMDC maturation or apoptosis. **A**, Kinetics of BMDC internalization of heat-killed *S. pneumoniae* of a heavily encapsulated type 3 strain (WU-2), its nonencapsulated defective mutant (JD6/11), or the moderately encapsulated type 14 strain (Pn14) labeled with the fluorescent tracer CM-DiI when used at bacteria: BMDC 200:1 ratio. **B**, Concentrations of cytokines secreted into the medium after 6 h (upper panel) and 24 h (lower panel) of pulse with the same 200:1 bacteria to BMDC ratio. Statistically significant differences between the concentrations of cytokines in the supernatants of BMDCs pulsed with WU-2 and JD6/11 are indicated by dots (•) and for the comparison between WU-2 and Pn14 by asterisks (*). **C**, Percentages of increase in the expression of several phenotypic markers of BMDC maturation and CD11c, after 24 h of pulse at a 500:1 bacteria to BMDC ratio. **D**, Percentage of hypodiploid nuclei obtained after 72 h of pulse with different strains of *S. pneumoniae* at different ratios. **E**, Time course of DNA fragmentation at an 800:1 bacteria to BMDC ratio.

and B, GM-CSF was not required for the survival of BMDCs over a 72 h culture period, following their generation after 6–8 days of in vitro culture in the presence of GM-CSF.

Induction of BMDC apoptosis does not require the internalization of bacteria

Macrophages have been shown to undergo apoptosis as a result of mediator release consequent to intracellular killing of bacteria (11). By analogy, these data raise the possibility that bacteria need to be internalized to deliver the requisite signaling for BMDC apoptosis. Thus, we used either CtA or CtD to block BMDC internalization of bacteria and measured the subsequent effects on release of cytokines, induction of phenotypic maturation, and development of hypodiploid nuclei (i.e., apoptosis) (Fig. 5). Addition of Ct to BMDC cultures, in the absence of bacteria, induced minimal apoptosis (<12%) relative to BMDCs cultured in DMSO diluent or medium alone (<5%) after 24 h of culture. Treatment with Ct effectively blocked the internalization of CM-DiI-labeled bacteria (MFI of CtA-treated BMDCs was 2.5–3.8% of the MFI of untreated BMDCs; data not shown). Neither CtA nor CtD prevented apoptosis induced by either live or heat-killed bacteria (Fig. 5, A and B). Likewise, Ct did not inhibit phenotypic maturation of

BMDCs and indeed effected a modest enhancement (Fig. 5C). In contrast, CtD strongly suppressed the secretion of both IL-12 and TNF- α and partially inhibited IL-10 release, but had no effect on IL-6 (Fig. 5D). These data strongly suggest that interaction of bacteria with structures expressed on the BMDC surface is sufficient to mediate the cell signaling required for both maturation and induction of apoptosis. Nevertheless, the modest enhancement of phenotypic maturation seen in the presence of Ct might reflect the decrease in release of IL-10.⁴ The data also suggest that neither IL-12 nor TNF- α is critical for apoptosis induction.

Heavy encapsulation can interfere with both phenotypic maturation and apoptosis induced by heat-killed *S. pneumoniae*

Our observation (Fig. 5) that heat-killed *S. pneumoniae* induces phenotypic maturation and apoptosis in the absence of significant internalization strongly suggests that these processes were mediated by an interaction of the bacteria with cell surface BMDC

⁴ J. Colino and C. M. Snapper. Opposing signals from PAMPs and IL-10 are critical for optimal dendritic cell induction of in vivo humoral immunity to *Streptococcus pneumoniae*. Submitted for publication.

receptors. In the next series of experiments, we wished to determine whether the bacterial components involved in this interaction were present within the bacterial capsular and/or subcapsular domain. In light of previous studies demonstrating that polysaccharide encapsulation can in fact mask underlying immunostimulatory bacterial subcapsular structures, we compared phenotypic maturation and apoptosis induction of BMDCs in response to either a heavily encapsulated WU-2 strain of *S. pneumoniae* type 3 or its nonencapsulated isogenic mutant, JD6/11 (28). As a further control, we used a low-encapsulated nonvirulent *S. pneumoniae* type 14 strain (Pn14). Both morphology and size of Pn14 colonies (similar to JD6/11 colonies) confirmed a much lower level of encapsulation than for WU-2.

After 1 h of bacteria-BMDC coculture, encapsulation had no significant effect on the rate of internalization of nonopsonized bacteria by BMDCs in vitro, as suggested by the similar slopes of the kinetic curves of internalization of the three strains (Fig. 6A). The delayed kinetics observed for the heavily encapsulated WU-2 strain may be explained by the greater flotation coefficient and electrostatic repulsion mediated by the thick layer of capsular polysaccharide, which delayed optimal BMDC-bacterial contact after initiation of culture.

Encapsulation also had no significant effect on induction by BMDCs of the cytokines TNF- α and IL-12 (Fig. 6B), whose secretion was inhibited by CtD (Fig. 5). In contrast, IL-10 secretion and, to a lesser extent, IL-6 secretion were significantly lower in response to WU-2. Thus, these observations are consistent with those using Ct (Fig. 5) and strongly suggest that signals originating from BMDC cell surface structures are sufficient for induction of IL-6 and in part IL-10 secretion and that heavy polysaccharide encapsulation can mask the relevant bacterial subcapsular stimuli.

In agreement with the hypothesis that heavy encapsulation interfered with surface signaling, the up-regulation of phenotypic markers of maturation in response to bacteria at 24 h, which does not require internalization (Fig. 5C), was dramatically impaired in

BMDCs stimulated with WU-2 (Fig. 6C). Furthermore, at 72 h, the percentage of hypodiploid nuclei was markedly reduced in BMDCs stimulated with WU-2 at any ratio between 50 and 800 bacteria per BMDC (Fig. 6D). Kinetic studies indicate that heavy encapsulation significantly delayed, but did not eliminate, the onset of BMDC apoptosis (Fig. 6E). These data further reinforce the notion that delayed BMDC apoptosis is a reflection of terminal maturation, both being related to signaling mediated by interactions between BMDC cell membrane receptors and bacterial subcapsular structures, without a requirement for internalization or bacterial processing.

Purified PG or intact *S. pneumoniae* induce BMDC apoptosis in a MyD88-dependent manner

We next wished to directly determine whether subcapsular bacterial structures were capable of mediating BMDC maturation and apoptosis and whether this involved a MyD88 (TLR)-dependent process. To accomplish this, we used an LPS-free preparation of purified PG from *Staphylococcus aureus*, which has previously been shown to be a TLR-2 ligand. PG induced BMDCs to secrete cytokines (IL-6, IL-12, TNF- α , and IL-10) in a dose-dependent manner (Fig. 7A) and to up-regulate cell surface markers of maturation (MHC class I, MHC class II, CD25, CD40, and CD86) (Fig. 7B). Induction of cytokine secretion was completely dependent, whereas phenotypic maturation was partly dependent, on MyD88 signaling, in response to either PG (Fig. 7, A and B) or intact *S. pneumoniae* (Fig. 7B and data not shown).

PG also induced delayed BMDC apoptosis in a dose-dependent manner (Fig. 7C) with kinetics similar to those observed for intact bacteria. As observed for phenotypic maturation, apoptosis induction in response to either PG or intact bacteria was partly MyD88 dependent. These data suggest that intact *S. pneumoniae* can stimulate BMDC maturation and apoptosis, at least in part, through MyD88-dependent signaling via TLR-2 interaction with PG in the bacterial cell wall.

FIGURE 7. Induction of BMDC apoptosis in both wild-type and MyD88^{-/-} mice in response to PG. BMDCs obtained from wild-type (MyD88^{+/+}) or MyD88^{-/-} were stimulated with variable concentrations of PG (A) and, after 20 h of culture, the concentration of cytokines secreted into the medium was determined by ELISA. Concentrations secreted by MyD88^{+/+} are shown with a continuous line, and those secreted by MyD88^{-/-} are shown as broken lines. B, BMDCs were stimulated for 20 h with 100 μ g/ml PG or with 800 bacteria per BMDC, and the expression of different markers of phenotypic maturation were determined and expressed as percentages of the levels expressed in nonstimulated BMDCs. C, Time course of DNA fragmentation induced during the first 72 h of stimulation with different PG concentrations in wild-type MyD88^{+/+} BMDCs. D, The percentages of hypodiploid nuclei at 72 h in MyD88^{+/+} and MyD88^{-/-} BMDCs stimulated with different concentrations of PG or R36A (bacteria).

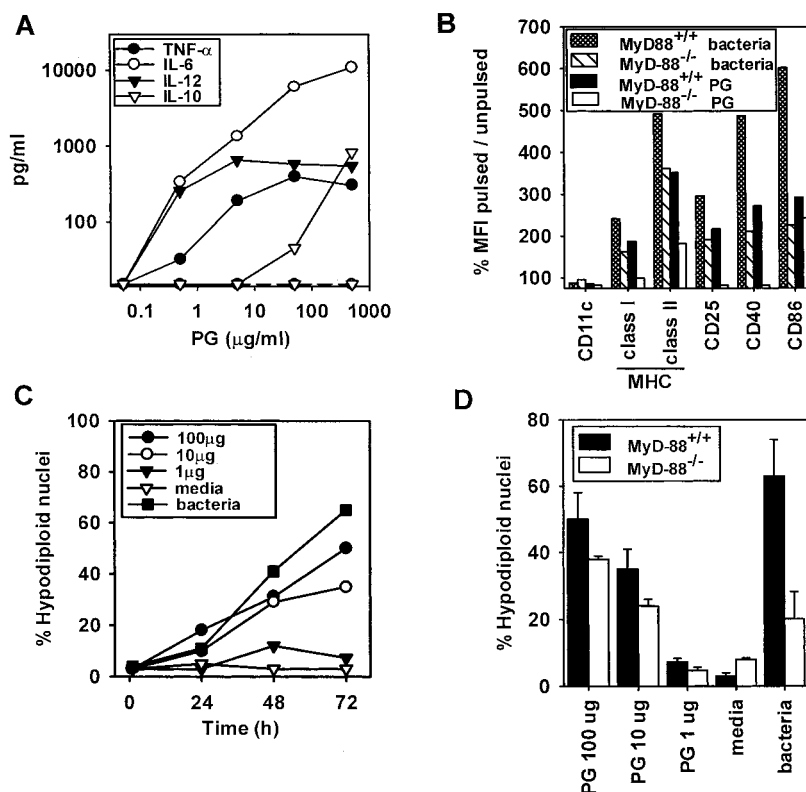
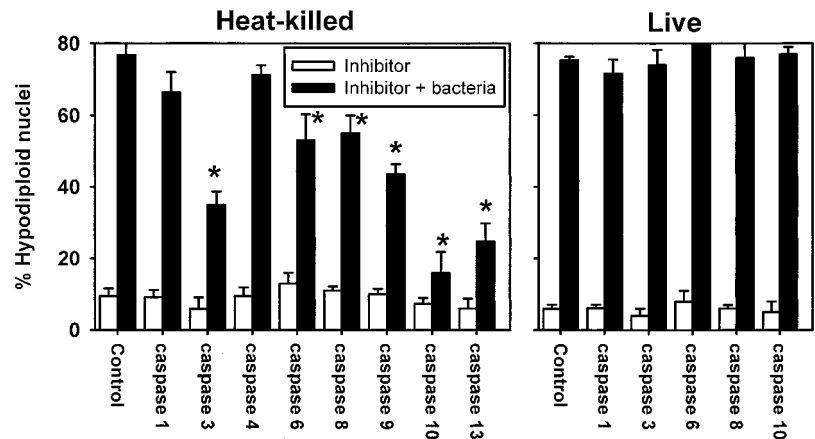


FIGURE 8. Heat-killed *S. pneumoniae*-induced BMDC apoptosis is dependent on caspase activity. Percentages of hypodiploid nuclei in BMDCs treated with 50 μ M of the specific inhibitors of the caspase activities indicated in the absence (inhibitor) or in the presence (inhibitor + bacteria) of heat-killed *S. pneumoniae* for 72 h (left) or live *S. pneumoniae* for 5 h (right). BMDCs pulsed with live bacteria were cultured for an additional 20-h period in the continued presence of caspase inhibitor before analysis of DNA fragmentation.



Inhibition of selected caspases block delayed-onset, but not pneumolysin-dependent, BMDC apoptosis

We detected the induction of caspase-8 activity in BMDCs destined to undergo apoptosis in response to heat-killed *S. pneumoniae* (Fig. 1). To test whether the activity of caspase-8, as well as that of other caspases, was critical for the onset of apoptosis, we irreversibly blocked the activity of specific caspases by treatment with cell-permeable peptide derivatives. As shown in Fig. 8, of the two initiator caspases (caspase-8 and caspase-10) recruited into apoptosis signaling complexes associated with death receptors (35, 36), the inhibition of caspase-10 activity completely abrogated the ability of heat-killed bacteria to induce BMDC apoptosis, whereas inhibition of caspase-8 activity had only a partial, though significant, effect. These results suggest that heat-killed bacteria induced BMDC apoptosis through death cell receptor signaling, where caspase-10 played a critical role as initiator.

The inhibition of caspase activities located downstream of the apoptosis signaling cascade, such as caspase-3 or caspase-9, had only partial inhibitory effects (Fig. 8), suggesting multiple pathways of amplification, and as expected, the inhibition of members of the caspase-1 subfamily, caspase-1 and caspase-4, primarily in-

involved in cytokine processing and inflammation, had no effect. However, inhibition of caspase-13 activity, another member of the caspase-1 subfamily, deeply impaired apoptosis (Fig. 8).

In striking contrast, apoptosis induced by live bacteria was not affected by the inhibition of any of the caspase activities tested, indicating that caspase activation is not a major requirement for induction of pneumolysin-dependent BMDC apoptosis.

*Intact *S. pneumoniae* induces BMDC apoptosis in a CD95/CD95L-independent manner*

We demonstrated that caspase-8 activity is induced in BMDCs destined to undergo apoptosis in response to intact *S. pneumoniae* (Fig. 1) and that caspase 10 and, to a much lesser extent, caspase 8 are critical for induction of apoptosis. Because both caspase 8 and caspase 10 have been implicated in CD95-dependent induction of apoptosis (36, 37), we wished to determine whether CD95/CD95L interactions were involved in *S. pneumoniae*-induced BMDC apoptosis. As shown in Fig. 9A, BMDCs from LPS-defective C3H/HeJ mice constitutively expressed cell surface CD95, with progressive up-regulation in response to bacteria, but not in the presence of medium alone over a 72 h period. However, as

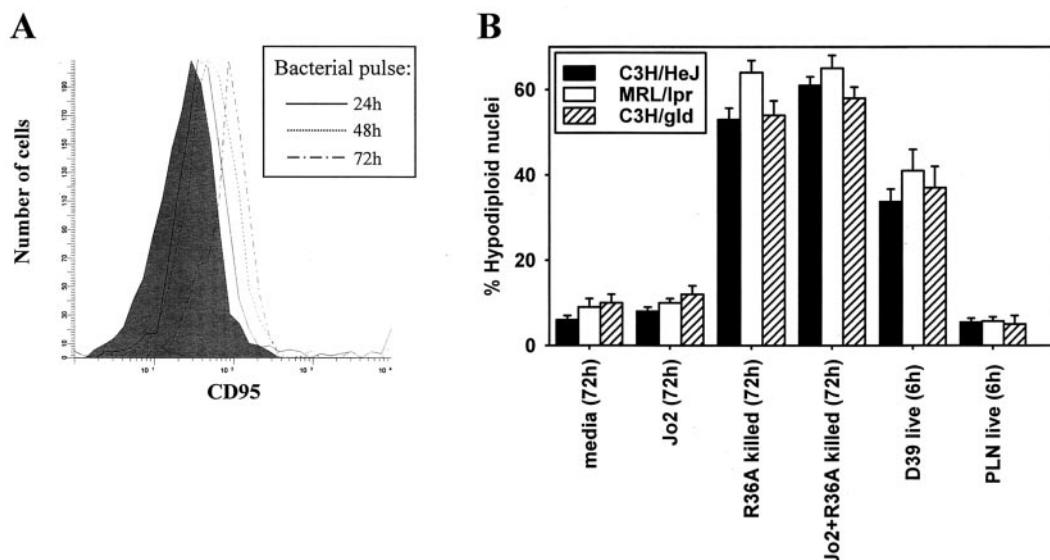


FIGURE 9. *S. pneumoniae*-induced BMDC apoptosis is independent of CD95 or CD95L expression. A, Time course of cell surface expression of CD95 (MFI) during the pulse with R36A strain of *S. pneumoniae*. Filled histogram represents the expression of CD95 in BMDCs derived from C3H/HeJ mice cultured during 72 h in medium alone. B, Percentage of hypodiploid nuclei in BMDCs derived from MRL/lpr, C3H/gld, and control C3H/HeJ mice, cultured by triplicate in the presence or absence of 200:1 bacteria to BMDC during the pulse time indicated.

previously reported by others (26), BMDCs failed to undergo apoptosis in response to the agonist anti-CD95 mAb (Jo-2) (Fig. 9B). Moreover, BMDCs derived from MRL/lpr mice, genetically deficient in the expression of CD95, underwent apoptosis in a similar manner to BMDCs derived from wild-type control C3H/HeJ mice in response to either live or heat-killed bacteria (Fig. 9B). BMDCs from MRL/lpr and C3H/HeJ also exhibited similar levels of cytokine secretion, phenotypic marker up-regulation, and bacterial uptake (data not shown).

Because BMDCs have been reported to express CD95L (38), an alternative but unlikely possibility is that membrane CD95L acts by itself as a death receptor and/or that bacteria could induce the release of soluble CD95L to act on bystander BMDCs. However, we were unable to detect significant expression of membrane CD95L by FACS on the BMDC surface before or during the pulse with bacteria (data not shown), and the concentrations of soluble CD95L in culture supernatants during the bacterial pulse (30 min to 72 h) were undetectable (<75 pg/ml). Moreover, bacteria-pulsed BMDCs derived from C3H/gld mice, genetically deficient in the expression of CD95L, underwent apoptosis to a similar degree as BMDCs derived from C3H/HeJ control mice in response to either live or heat-killed bacteria (Fig. 9B). These results demonstrate that CD95/CD95L interactions are not involved in the induction of BMDC apoptosis induced by either live (pneumolysin-dependent) or heat-killed bacteria in vitro.

Discussion

Programmed cell death or apoptosis plays a major regulatory role in adaptive immune responses, being involved in the control of lymphocyte proliferation, selection of Ag-specific cells, clonal abortion of lymphocytes with autoreactive specificities, and generation of immune memory (1). Additionally, pathogens have developed a broad variety of strategies to trigger apoptosis in key cells of the innate immune system including epithelial cells, macrophages, and DCs (3–11, 15, 23). Apoptosis induced by ingested bacteria in macrophages having high bactericidal capacity could be a host mechanism to limit intracellular replication, favoring the clearance and reprocessing of the killed bacteria for presentation of bacterial Ags by professional APCs such as DCs (8, 14). However, the induction of the same premature apoptosis in DCs could be regarded as pathogenic, because DCs are critical for the induction of primary immune responses and must interact with naive T cells for an extended time to mediate efficient priming. Thus, a decrease in DC numbers at the location of an infection may impair the nascent T cell-dependent response. Furthermore, DCs undergoing apoptosis are not efficient APCs in vivo (39). Indeed, we recently demonstrated that preapoptotic BMDCs loaded with *S. pneumoniae* showed a strong decrease in their ability to induce primary in vivo humoral immune responses specific for protein and polysaccharide Ags⁴ (22).

Nevertheless, apoptosis induced in any host cell type by bacteria acting external to the cell is likely to be a pathogenic mechanism for promoting invasiveness. Here, we demonstrate that intact *S. pneumoniae*, acting externally, induce two distinct pathways of apoptosis in DCs. Apoptosis induced by the first pathway is relatively rapid and caspase independent and requires live *S. pneumoniae* that release pneumolysin. All pneumococcal strains tested that expressed pneumolysin, but not the pneumolysin-defective mutant PLN, were able to initiate an apoptotic program in the whole BMDC population after a brief period of coculture (30 min). A significant percentage of BMDCs had begun to undergo DNA fragmentation as early as 3–6 h of culture (15–33%). Because DNA fragmentation is a late event in apoptosis (40), it clearly indicated that pneumolysin exerted its effector mechanism rapidly.

Induction of BMDC apoptosis by pneumolysin was similar to that previously reported for pneumolysin-mediated apoptosis induction in neuronal and glial cells of the hippocampus (16) and for the induction of apoptosis in macrophages by Group B streptococcal hemolysin (7). It is of note that during invasive infections, Group B *Streptococcus* also induces neuronal apoptosis in vivo (41), but an association with hemolysin production has not been established. Apoptosis, in every case, was found to be caspase independent and related to an alteration in cell membrane permeability (Refs. 7 and 16 and this study), consistent with the known pore-forming activity of pneumolysin (42). Although we did not test whether release of apoptosis-inducing factor from the mitochondria was involved in this pneumolysin-mediated apoptosis, it has been reported for neurons (16). This would be anticipated given that caspase-independent pathways of apoptosis are generally associated with mitochondrial damage.

The mechanisms by which live *S. pneumoniae* induce apoptosis in DCs and macrophages appear to be different. Opsonized *S. pneumoniae* has been reported to induce apoptosis in macrophages through a caspase-dependent pathway associated with intracellular killing of bacteria (11). Pneumolysin had only an indirect and marginal effect on macrophage apoptosis, by increasing the rate of bacterial phagocytosis (11). In contrast, we observed that internalization of live bacteria was not a requirement for apoptosis induction in BMDCs. Thus, 1) blocking internalization of live bacteria with CtD did not inhibit DNA fragmentation and 2) live pneumolysin-defective *S. pneumoniae* did not induce significant levels of apoptosis during the first 24 h, despite its being significantly taken up by DCs. Furthermore, these levels of apoptosis did not differ from the levels induced by heat-killed bacteria at this time point. Thus, distinct cell types appear to have differing susceptibilities to apoptosis after alterations in membrane permeability; hemolysin of Group B *Streptococcus* altered membrane permeability of all cell types tested, but it only induced apoptosis in monocyte/macrophages (7).

DCs, unlike macrophages, sample bacteria directly from mucosal surfaces by sending dendrites between the epithelium (43). Thus, pneumolysin-induced apoptosis of such DCs could facilitate *S. pneumoniae* colonization at the mucosal surface by impairing the antibacterial immune responses during the initial colonization. In this regard, pneumolysin is expressed by both highly virulent (WU-2, type 3) and less virulent (Pn14, type 14) *S. pneumoniae* strains, which both induced similar levels of BMDC apoptosis and hence may be equally efficient at colonization. Once virulent strains invade the host, pneumolysin could reduce the numbers of viable APCs by targeting subepithelial and systemic DCs, favoring invasiveness. In this way, pneumolysin might also impair the putative beneficial effects of macrophage apoptosis associated with the killing of ingested bacteria by limiting overall reprocessing of apoptotic bodies containing bacteria, secondary to the reduced DC population. This view agrees with the observation in a mouse model of sepsis that pneumolysin is an exotoxin acting as a critical virulence factor during the first few hours after infection, permitting the continued exponential growth of the bacteria by interfering with the generation of inflammation-induced immunity (29).

In this report, we further demonstrate a second distinct pathway of DC apoptosis in response to intact *S. pneumoniae* that is likely shared by other Gram-positive extracellular bacteria. This pneumolysin-independent mechanism of BMDC apoptosis was delayed (>24 h) and likely reflects terminal maturation, did not require bacterial metabolic activity, and was mediated by the interaction between subcapsular components in the bacteria and death receptors on the DC surface linked to MyD88 and likely a TLR(s). The engagement of MHC class II could be discarded as a trigger of

apoptosis because the experiments were conducted in the absence of T cells or exogenously added class II ligands. Furthermore, MHC class II-mediated apoptosis of mature DCs was reported to be a caspase-independent mechanism (44). This delayed-onset apoptosis was also seen to be independent of CD95/CD95L interactions and TNF- α , but could be inhibited by IL-10, which delays BMDC maturation in response to *S. pneumoniae*.⁴ Similar to this latter observation, we show here that GM-CSF, which also delays BMDC maturation, similarly delays the onset of apoptosis. Pneumolysin production requires metabolic activity, is very unstable in culture, and is rapidly inactivated at 60°C (33). Thus, heat-killed preparations of *S. pneumoniae* are free of pneumolysin activity. Moreover, heat-killed bacteria or the live pneumolysin-defective mutant PLN exhibited a comparable potency to induce delayed apoptosis in BMDCs, indicating that bacterial metabolic activity or heat-shock proteins induced by the heating of the bacteria were not responsible for induction of this delayed-onset apoptosis.

The relevant bacterial stimuli necessary for delayed-onset apoptosis induction, as demonstrated here, are located in the subcapsular domain and likely involve, at least in part, PG, a TLR2 ligand, which is a component of the cell wall. This does not rule out additional participation of other bacterial components. For example, lipoteichoic acid and bacterial lipoproteins, both TLR-2 ligands, and both competent stimuli for DC maturation (18, 45) could also be involved. Nevertheless, we demonstrate that capsular components, including the composition of the capsular polysaccharide, were not determinants involved in apoptosis induction. Thus, nonencapsulated mutants of *S. pneumoniae* and low-encapsulated strains of two different serotypes expressing polyanionic (type 2) or neutral (type 14) capsular polysaccharides were equally effective inducers of apoptosis. Moreover, heavily encapsulated strains showed a diminished ability to induce apoptosis in BMDCs, suggesting that encapsulation might instead mask the subcapsular components involved in the interaction, which is a common role of the subcapsular domain (46). The capsular polysaccharide is a major virulence factor for this pathogen, and virulence correlates with the extent of encapsulation (47). Thus, impaired DC maturation by heavily encapsulated variants may be an *in vivo* mechanism to reinforce virulence by counteracting the nascent host defense during the preimmune phase of the infection.

The profile of relevant caspase activities present in BMDCs undergoing apoptosis induced by heat-killed *S. pneumoniae* further support the hypothesis of a death receptor being involved in this process. The caspase initiators, caspase-10, and to a lesser extent caspase-8 were required for the induction of apoptosis, and an increase in caspase-8 activity was observed during the pulse with bacteria after a kinetic compatible with the development of apoptosis. Caspase-8 and caspase-10 are recruited, through the adaptor Fas-associated death domain protein, to death-inducing signaling complexes of CD95 and TNF-related apoptosis-inducing ligand to initiate apoptosis (36, 37). This is likely a shared mechanism among death receptors. Thus, TLR-2-mediated apoptosis involved the engagement of MyD88 and Fas-associated death domain protein, which can bind and activate caspase-8 (12, 48). Although it is still controversial whether caspases 8 and 10 have redundant or distinctive functions (35, 36), our results suggest that caspase-10 is functionally different from caspase-8 and plays a more critical role in the initiation of heat-killed *S. pneumoniae*-induced DC apoptosis. In this regard, defective caspase-10 has been implicated in the resistance to TNF-related apoptosis-inducing ligand-induced apoptosis of mature DCs from autoimmune lymphoproliferative syndrome II patients (49). Inhibition of downstream effector caspase activities, such as caspase-3 or caspase-9, had only partial inhibitory effects on apop-

sis, suggesting that multiple redundant routes of amplification were involved, including mitochondrial amplification.

Inhibition of the activity of caspase-13, a member of the caspase-1 subfamily, deeply impaired apoptosis. Caspase-13 is directly processed by caspase-8 (50) and, therefore, could have a major regulatory role in the death receptor-mediated signaling pathway. However, the physiological role of caspase-13 is, at present, poorly understood. Thus, because members of the caspase-1 subfamily are primarily involved in cytokine processing and inflammation, we cannot exclude that the inhibition of the apoptosis observed may be a side effect of its primary function.

DCs must exert tight control over signaling processes leading to apoptosis. Thus, mature DCs are relatively resistant to the proapoptotic action of TNF- α (24, 25) and CD95-mediated apoptosis (26), and this resistance has been associated with the up-regulation of FLIP (26) and Bcl-x_L (24). Because BMDCs are sensitive to C2-ceramide, an endogenous mediator of apoptosis, it has been suggested that signals blocking apoptosis could be proximal to the generation of ceramide and likely occur at, or proximal to, caspase-3 (26). In this regard, we show that within the first hour of contact between bacteria and BMDCs, caspase-3 is down-regulated and remains low until 20 h, a period during which BMDCs remain viable. Later, coinciding with BMDC maturation, this regulatory mechanism become inefficient and the BMDCs undergo apoptosis.

Acknowledgments

We thank Drs. Andrew Lees, Luba Grinberg, and Rick Schuman (Biosynex) for providing phosphorylcholine-keyhole limpet hemocyanin, GM-CSF, and purified PG, respectively, and Drs. David Briles and Janet Yother (University of Alabama) for providing various *S. pneumoniae* strains.

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