MAJOR ARTICLE

Streptococcus pneumoniae–Associated Human Macrophage Apoptosis after Bacterial Internalization via Complement and Fcγ Receptors Correlates with Intracellular Bacterial Load

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Opsonization enhances *Streptococcus pneumoniae*–induced human monocyte–derived macrophage (MDM) apoptosis. Both depletion of complement and immunoglobulin from opsonizing serum and blockade of the macrophages CR1, CR3, FcγRII, and FcγRIII partially decreased MDM apoptosis after *S. pneumoniae* phagocytosis, and these effects correlated with reduced numbers of internalized bacteria. Chloramphenicol inhibition of protein synthesis by opsonized *S. pneumoniae* down-regulated subsequent MDM apoptosis. Phagocytosis of an unencapsulated mutant of *S. pneumoniae* resulted in increased MDM apoptosis, in association with enhanced internalization. Caspase inhibition was associated with decreased killing of bacteria. Enhanced induction of apoptosis by opsonized *S. pneumoniae* is the result of increased intracellular burden of bacteria, rather than of a specific pattern of engagement of complement receptor or FcγR. A dynamic interaction between live intracellular bacteria and the host cell is necessary for induction of apoptosis in MDMs, and induction of apoptosis contributes to the host defense against *S. pneumoniae*.

Resident macrophages represent a critical component of the innate immune response and provide a link between innate and adaptive immunity [1]. Macrophages phagocytose a broad range of microbial pathogens by using both innate immune recognition of structures, such as pathogen-associated molecular patterns, and receptors that recognize bacteria coated with serum opsonins [1, 2]. Phagocytosis of a specific bacterium activates a number of different cell-surface receptors and associated signal transduction pathways [1]. These pathways differ with respect to their activation of downstream events, including microbial killing, cytokine production, antigen presentation, and induction of apoptosis.

Streptococcus pneumoniae is the most common cause of community-acquired pneumonia and causes significant morbidity worldwide in all ages [3, 4]. Alveolar macrophages contribute to the clearance of bacteria that reach the distal airway, but failure to clear bacteria and the influx of large numbers of neutrophils results in pneumonia. *S. pneumoniae* has developed specific adaptations to enhance survival and limit bacterial killing, in the host organism. The efficiency of phagocytosis is impeded by the presence of the polysaccharide capsule, and opsonization of bacteria is required to facilitate phagocytosis [5]. During host defense, iC3b is a critical component of the opsonization of *S. pneumoniae,* and

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serotypic variation in iC3b generation has been described elsewhere [6]. Type 1 *S. pneumoniae* activates both C3b and iC3b [7–9]. However, *S. pneumoniae* limits surface complement deposition by a variety of adaptations, including the following: expression of pneumococcal surface protein A [10]; binding of factor H, an inhibitor of complement activation; by the pneumococcal surface protein C family member factor H-binding inhibitor of complement [11, 12]; pneumolysin-induced complement depletion [13]; and C3 degradation [14]. In the presence of these adaptations, the contribution to complement activation, opsonization, and phagocytosis by immunoglobulin and other serum proteins, such as collectins and CRP, increases in importance [15, 16]. Phagocytosis therefore requires the concerted involvement of a number of receptors, including both Fcγ and complement receptors (CR).

The importance of the phagocytosis of opsonized *S. pneumoniae* is confirmed by animal models of pneumonia [17]. In addition, the in vitro opsonization of *S. pneumoniae* with both IgG and complement contributes to phagocytosis and killing of bacteria [18]. Type-specific, protective anticapsular antibody is produced after natural infection or immunization, and naturally occurring antiphosphocholine antibodies contribute to innate immunity [15]. Antibodies, complement, and other serum proteins (including C-reactive protein, mannose binding lectin, and collectins) act in concert to enhance opsonization of bacteria [19–21]. Despite these observations, the consequences to the macrophage of phagocytosis and killing of opsonized *S. pneumoniae* are comparatively poorly understood.

Induction of apoptosis in macrophages is one of the potential consequences of microbial phagocytosis and is also a critical determinant in down-regulation of the inflammatory response [22, 23]. In vivo models of *S. pneumoniae*–induced meningitis demonstrate that induction of apoptosis contributes to disease pathogenesis [24, 25]. We have previously demonstrated that macrophages that phagocytose *S. pneumoniae* in vitro undergo apoptosis, and this is linked to microbial killing [26]. In the present study, which uses an in vitro model of *S. pneumoniae* infection of human monocyte-derived macrophages (MDM), we have explored the relationship between phagocytosis of opsonized bacteria and the induction of apoptosis. We have investigated whether induction of apoptosis is a function of bacterial internalization or whether engagement of specific surface receptors or cytoskeletal rearrangements associated with phagocytosis is sufficient to initiate apoptosis.

MATERIALS AND METHODS

Bacteria. Aliquots of type 1 *S. pneumoniae* (World Health Organization reference laboratory strain SSISP; Statens Seruminstitut) were grown to exponential phase in brain-heart infusion broth (Oxoid Unipath) with 20% heat-inactivated fetal calf serum (FCS) (Bioclear) and were stored at −70°C. Freshly thawed aliquots were spun at 2000 *g,* and the bacterial pellet was washed 3 times in 1× PBS. In certain experiments, type 2 strain D39 (NCTC7466), type 3 (4538, Sheffield strain previously serotyped, provided by Lisa Ridgway), or type 4 (TIGR4) was used under identical conditions.

An unencapsulated derivative of TIGR4, the type 4 strain of *S. pneumoniae,* of which the genome has been sequenced [27], was constructed by deleting the whole capsule locus. A genetic cassette, capable of integrating into the capsule locus of all *S. pneumoniae* and containing a kanamycin-resistance marker (GenBank accession no. AF160759) was obtained by polymerase chain reaction (PCR) on strain FP33 and used to transform TIGR4, as described elsewhere [28]. A representative transformant, FP23, was chosen and analyzed by DNA sequencing and by Neufeld's Quellung Reaction. As expected, integration of the cassette produced a 22,242-bp deletion in the TIGR4 chromosome (nucleotides 316944–339186; GenBank accession no. AE007507), and FP23 no longer reacted with type 4–specific antibodies.

In addition, experiments were also performed on mutant bacteria that lacked the major autolytic protein *LytA*$^{-}$ (AL2) [29], neuraminidase (Δ*NA1*) [30], or hyaluronidase (Δ*HY1*), or the parental D39 strain. Δ*HY1* was constructed by insertion-duplication mutagenesis. PCR was used to amplify a 1378-bp fragment of DNA from D39 chromosomal DNA, by use of primers HY1 (GCCTATACGGGTGCTTATGG) and HY2 (CGAATAGTATAGACTCCACG). The PCR product was digested with *Mbo*I and was cloned into the *Bam*HI site of pG^{+}host5 [31]. This construct was introduced into D39 by transformation and integrants selected by their ability to grow at 37°C. Disruption of the hyaluronidase gene was confirmed by Southern blot test and hyaluronidase assay (data not shown). Pneumococcal cell-wall extraction was performed exactly as described elsewhere [32], except bacteria were grown in brain-heart infusion broth, as above.

Opsonization. Bacteria were opsonized in RPMI 1640 (Sigma) supplemented with 10% antipneumococcal immune serum (IS) [18] or antipneumococcal IS, after immunoglobulin depletion (ID), immunoglobulin reconstitution (RS), or heat inactivation (HI), and were incubated on a rotating device for 30 min at 37°C. A 1-mL Protein A column (HiTrap Affinity Column; Amersham Pharmacia Biotech) was used to prepare ID and antipneumococcal immunoglobulin (IP) from IS, in accordance with the manufacturer's instructions. ID serum was collected after passage through the column, and IP was eluted with 0.1 mol/L citric acid (BDH Laboratory Supplies). Reconstituted serum (RS) was prepared by adding eluted IP from the column to ID serum. Heat-inactivated serum (HI) was prepared by heating IS to 56°C for 30 min. Nonopsonized bacteria were

treated under identical conditions, with PBS substituted for serum.

After opsonization, bacteria were washed 3 times and were resuspended in 1 mL of 10% FCS–RPMI 1640, at 4°C at the required concentration. Mock infections involved the same treatments in the absence of bacteria. In certain experiments, 1 h before opsonization, *S. pneumoniae* were treated with 4 μg/mL of chloramphenicol (Kemicetine; Pharmacia and Upjohn) (sub-MIC concentration) to inhibit bacterial protein synthesis [33]. The MIC for the strain was determined by tube dilution, in accordance with the National Committee for Clinical Laboratory Standards recommendations, and absence of bacterial regrowth was confirmed by bacterial viability testing.

Cells. Peripheral blood mononuclear cells were isolated by Ficoll-Paque (Pharmacia-Amersham) density centrifugation from whole blood donated by healthy volunteers after informed consent was obtained. For all blood donations, informed consent was provided in accordance with local ethics committee guidelines. Cells were plated, at 2×10^6 cells/mL in RPMI 1640 media containing 2 mmol/L L-glutamine (Gibco BRL) and 10% human AB serum (First Link), in 24-well plates (Costar) containing glass coverslips (BDH). Nonadherent cells were removed after 24 h, and adherent cells were cultured in RPMI 1640 containing 10% FCS, in 5% CO_2 at 37°C. THP-1 cells (1.5×10^6 cells/mL) were cultured under identical conditions to those used for MDM.

Infection of macrophages. Three- or 14-day MDMs were washed in RPMI 1640 and were either infected with *S. pneumoniae* (multiplicity of infection [MOI], 10 [unless otherwise indicated]) or mock infected. Macrophages were incubated for 1 h at 4°C and then for 3 h at 37°C. Cultures were washed in RPMI 1640 3 times, to remove nonadherent bacteria, and were incubated in 10% FCS–RPMI 1640 until fixation with 2% paraformaldehyde (Sigma). In certain experiments, MDM were incubated with opsonized 0.5-μm amine-modified (L-1155) or carboxylate-modified (L-4030) latex beads (Sigma), under identical conditions, or with cell-wall extracts at concentrations equivalent to the number of bacteria required to infect MDM at an MOI of 10 [32]. Viability of bacteria in culture supernatants was determined as described elsewhere [26].

Antibodies and reagents. Fcγ receptor blockade was performed by use of blocking IV.3 $F(ab')_2$ anti-FcγRII or 3G8 $F(ab')_2$ anti-FcγRIII, with nonblocking 22 $F(ab')_2$ anti-FcγRI as control (all Medarex), at 10 μg/mL. CR blockade was performed by use of To5 anti-CR1, 2LPM19C anti-CR3 monoclonal antibodies, or murine IgG1 isotype (all Dako), at 10 μg/mL. Because anti-CR $F(ab')_2$ was unavailable, these experiments were repeated after preblocking FcγR with 100 μg/mL of anti–human IgG_1 (Sigma). Phosphatidylinositol 3-kinase (PI3K) was inhibited with 50 μmol/L LY249002 (Sigma). The pancaspase inhibitor z-Val-Ala-D-Asp-fluoromethylketone (zVADfmk) (Enzyme Systems Products) or the control z-Phe-Ala-fluoromethylketone (zFAfmk) was added at 50 μmol/L to certain cultures. All reagents were added 1 h before and 4 h after MDM infection, unless otherwise indicated. Phorbol myrisate acetate (Sigma) was added at 5 nmol/L to certain cultures of THP-1.

Fluorescence microscopy. To quantify cell-associated and internalized bacteria, fixed cells were incubated sequentially with rabbit antipneumococcal (serotype 1) serum (Statens Seruminstitut) and a fluorescein isothiocyanate (FITC)—conjugated goat anti–rabbit IgG Fc (Dako), as described elsewhere, followed by incubation with 4′6′-diamidino-2-phenylindole (DAPI; Molecular Probes) [18]. The number of internalized bacteria was calculated by subtracting the number of adherent *S. pneumoniae* (FITC positive) from the total number of DAPI-positive cell-associated bacteria when cells were viewed by fluorescent microscopy (Leica DMRB 1000). Apoptosis was detected by nuclear morphology after DAPI staining. Cells demonstrating condensed and fragmented nuclei were scored as positive. In certain experiments, cells were stained with TUNEL reagents, in accordance with the manufacturer's (Oncor) instructions, to verify the findings by DAPI staining [26]. A total of 2.5 μg/mL of propidium iodide (Sigma) was added with the Vectashield, and the coverslips were then mounted and viewed by a reviewer who was blinded to the treatment and who was experienced in the detection of apoptosis. At least 300 cells were counted in duplicate preparations for each treatment.

Flow cytometry. After opsonization treatments, *S. pneumoniae* were washed in 1× PBS and 10^6 bacteria resuspended in 100 μL of 0.1% PBS-azide. Bacteria were incubated with 1 μg of FITC-conjugated goat anti–human IgG Fc (Dako) for 15 min at 20°C. Samples were then spun at 2500 *g* for 2 min, then the pellet was washed and resuspended in 400 μL of 0.5% filtered paraformaldehyde. Bacteria were analyzed by flow cytometry (FACSCalibur; Becton Dickinson). The main bacterial population was gated, and 10,000 events were analyzed.

Statistical analysis. All results are recorded as medians and interquartile ranges (IQRs). Percentage specific reduction or inhibition of apoptosis was calculated as the following: (percentage IS-MDM apoptosis − percentage of MDM apoptosis after infection with *S. pneumoniae* opsonized with specific treatment)/(percentage of IS-MDM − percentage of C-MDM) × 100. Specific inhibition of bacterial internalization was calculated as the following: (number of internalized bacteria per IS-MDM − number of internalized bacteria per IS-MDM in presence of specific blocking antibody)/(number of internalized bacteria per IS-MDM). Differences between groups of treatments were calculated by nonparametric (Mann-Whitney) testing or nonparametric 1-way analysis of variance (Friedman's test with Dunn's posttest) if stated, and nonparametric correlations were calculated by Spearman's ρ correlation coefficient by use of Minitab 11 software. Significance was defined as $P \leq .05$.

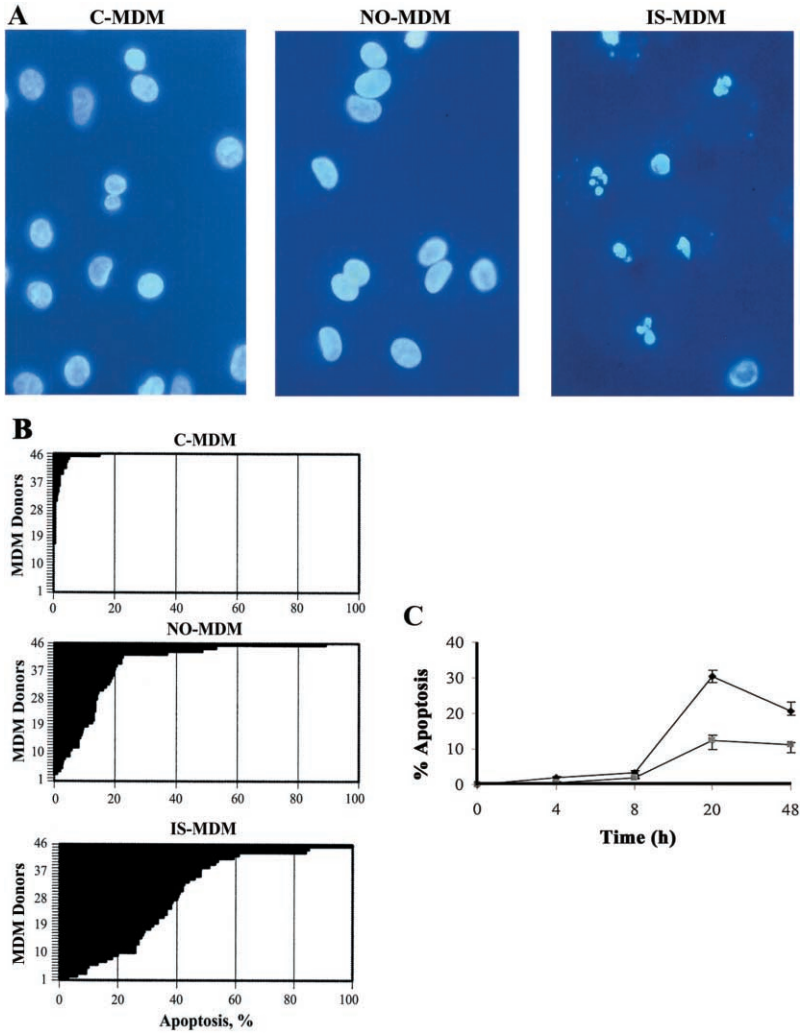

Figure 1. Opsonization of *Streptococcus pneumoniae* enhances monocyte-derived macrophage (MDM) apoptosis. *A,* Fourteen-day-old MDM were analyzed 20 h after infection with *Streptococcus pneumoniae* by fluorescence microscopy after 4′6′-diamidino-2-phenylindole (DAPI) staining. Shown are control MDM (C-MDM) *(left),* MDM infected with nonopsonized bacteria (NO-MDM) *(middle),* and MDM infected with opsonized pneumococci (IS-MDM) *(right).* Typical morphological features of apoptosis were more apparent with IS-MDM *B,* MDM (rank order 1–46) were analyzed by TUNEL (terminal UTP nick-end labeling) 20 h after infection with *S. pneumoniae* and the level of apoptosis for each donor recorded. Median percentage apoptosis was 0.5% (0%–1.5%) (C-MDM), 13.3% (8%–18.9%) (NO-MDM), and 36.5% (26.3%–45.5%) (IS-MDM). C-MDM versus NO-MDM, $P <$ .001; C-MDM versus IS-MDM, $P <$ .001; and NO-MDM versus IS-MDM, $P <$ .001 *C,* Progression of apoptosis over time in IS-MDM *(solid diamonds)* and NO-MDM *(solid squares)* apoptosis detected by DAPI staining and viewed by fluorescence microscopy, $n = 3$.

RESULTS

Opsonization of* S. pneumoniae *enhances the induction of MDM apoptosis. Macrophages phagocytose and kill opsonized *S. pneumoniae,* but little is known about the fate of the macrophages after these events [18]. We have previously demonstrated that MDM apoptosis is associated with in vitro infection with *S. pneumoniae* [26]. To examine whether opsonization has any effect on the induction of MDM apoptosis, MDM were incubated with *S. pneumoniae* opsonized in IS or incubated in PBS alone. As shown in figure 1*A*, the nuclei of mock-infected MDM (C-MDM) rarely demonstrated features of apoptosis after DAPI staining, but apoptotic nuclei were occasionally seen after incubation with nonopsonized *S. pneumoniae* (NO-MDM) and were frequently seen after incubation with *S. pneumoniae* opsonized in IS (IS-MDM). Levels of IS-MDM apoptosis were similar for types 1, 2, 3, and 4 *S. pneumoniae* (data not shown). The enhancement of *S. pneumoniae*–induced apoptosis, by opsonization, was confirmed by examining a large number of donors at 20 h after infection ($n = 46$). Levels of IS-MDM apoptosis (median, 36.5%; IQR, 26.3%–45.5%) were greater than NO-MDM (median, 13.3%; IQR, 8%–18.9%) or C-MDM (median, 0.5%; IQR, 0%–1.5%).

The results are shown in rank order in figure 1*B*. Statistically significant differences for C-MDM versus NO-MDM ($P <$ .001), C-MDM versus IS-MDM ($P <$ .001), and NO-MDM versus IS-MDM ($P <$ .001) were observed. Induction of apoptosis was not merely delayed in NO-MDM: as time increased to 4–48 h after exposure to *S. pneumoniae,* there was a steady increase in the level of apoptotic cells at 4–20 h, an increase that decreased by 48 h and that never reached the levels seen in IS-MDM (figure 1*C*).

Induction of apoptosis requires differentiated macrophages. As MDM differentiate in vitro from peripheral blood monocytes to macrophages, they undergo a variety of phenotypic changes [34, 35]. The 14-day MDM demonstrates a differentiated phenotype that closely matches that of mature tissue macrophages such as alveolar macrophages [35]. In matched donors after infection with *S. pneumoniae*, 14-day MDM exhibited greater levels of apoptosis than did 3-day MDM (figure 2). The levels of 3-day apoptosis were 3.5% (IQR, 3.3%–7.3%) for IS-MDM, 1.5% (IQR, 1%–4.3%) for NO-MDM, and 0.5% (IQR, 0.5%–1%) for C-MDM; the levels for 14-day MDM were 32% (IQR, 30%–40%) for IS-MDM, 13.5% (IQR, 12.3%–18.5%) for NO-MDM, and 0.5% (IQR, 0.5%–0.5%) for C-MDM. There was no significant difference between NO-MDM and IS-MDM at 3 days, but there were statistically significant differences in 3-day versus 14-day MDM, for IS-MDM ($P = .004$) and NO-MDM ($P = .004$). This corresponded to greater median numbers of internalized bacteria per MDM at 14 days versus at 3 days: 1 (IQR, 1–1.5) versus 0.4 (IQR, 0.25–0.7). These results were confirmed in other monocyte/macrophage cell types.

For the monocytic cell line THP-1, apoptosis was 4.9% (IQR, 4.8%–5.3%) after infection with opsonized *S. pneumoniae,* compared with 0% for control THP-1 cells ($P <$ .01). Differentiation of THP-1 cells with phorbol myrisate acetate increased levels of apoptosis, whereas other differentiated macrophage cell types, including murine bone marrow–derived macrophages and alveolar macrophages, showed comparable levels of apoptosis to human MDM (data not shown). This confirmed that apoptosis was enhanced in differentiated macrophages.

***Efficiency of internalization of bacteria contributes to enhanced MDM apoptosis after infection with opsonized* S. pneumoniae.** Differences in apoptosis between MDM exposed to opsonized and nonopsonized bacteria could be a function of differing cell-surface receptor interactions or the increased internalization of opsonized bacteria. As shown in figure 3*A,* despite altering the MOI from 1 to 50, overall numbers of cell-associated bacteria were similar at each MOI, for IS-MDM and NO-MDM. Differences, however, were apparent in the numbers of internalized bacteria (figure 3*B*). Increasing the MOI also increased the level of MDM apoptosis (figure 3*C*). To further explore the relationship between the number

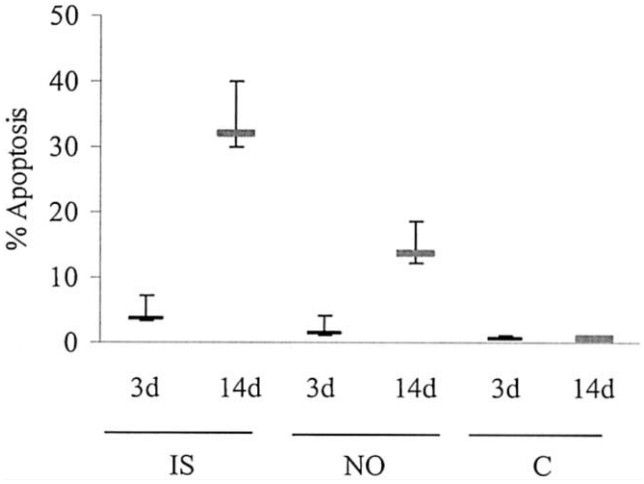

Figure 2. Induction of apoptosis by *Streptococcus pneumoniae* is enhanced by macrophage differentiation. Three-day (3d; *solid squares*) and 14-day (14d; *gray squares*) monocyte-derived macrophages (MDM) were stained with 4′6′-diamidino-2-phenylindole (DAPI) 20 h after infection with opsonized *Streptococcus pneumoniae* (IS-MDM), nonopsonized *S. pneumoniae* (NO-MDM), or mock infection (C-MDM) and analyzed by fluorescence microscopy for morphological features of apoptosis. Data presented represents median and interquartile range for apoptosis. 3d versus 14d: IS-MDM $P = .004$ and NO-MDM $P = .004$, $n = 3$.

of internalized bacteria per cell and the percentage apoptosis, we calculated correlation coefficients from these experiments, as follows: nonopsonized bacteria: $r = 0.83$; $P = .001$; opsonized bacteria: $r = 0.81$; $P <$ .001; and all bacteria regardless of opsonization status: $r = 0.86$; $P <$ .001 (figure 3*D*). When the number of internalized bacteria was similar, there was no difference in the level of MDM apoptosis after infection with opsonized bacteria, compared with that after infection with nonopsonized bacteria. This finding suggests the enhanced apoptosis of IS-MDM, at a specific MOI, is a consequence of the greater efficiency of bacterial internalization.

Internalization via both complement and Fcγ receptors contributes to the induction of MDM apoptosis. To determine the role of factors that contribute to the opsonization of *S. pneumoniae* in human serum, experiments were performed in which groups of these proteins were depleted. In these experiments, IS was either heat-inactivated (HI) to remove heat-labile complement factors, or IS was passed through protein A columns to remove immunoglobulin. Immunoglobulin eluted from protein A columns was added to PBS or was added back to immunoglobulin-depleted serum, to reconstitute IS. Heat-inactivated serum, IP, and ID serum were used to opsonize *S. pneumoniae* and the level of MDM apoptosis used to determine the percentage specific reduction of apoptosis. As shown in figure 4*A,* there was a partial and significant reduction of apoptosis for each of these treatments, compared with that for IS: HI, 52% (47%–56%; $P <$ .001); IP, 46% (44%–49%; $P <$

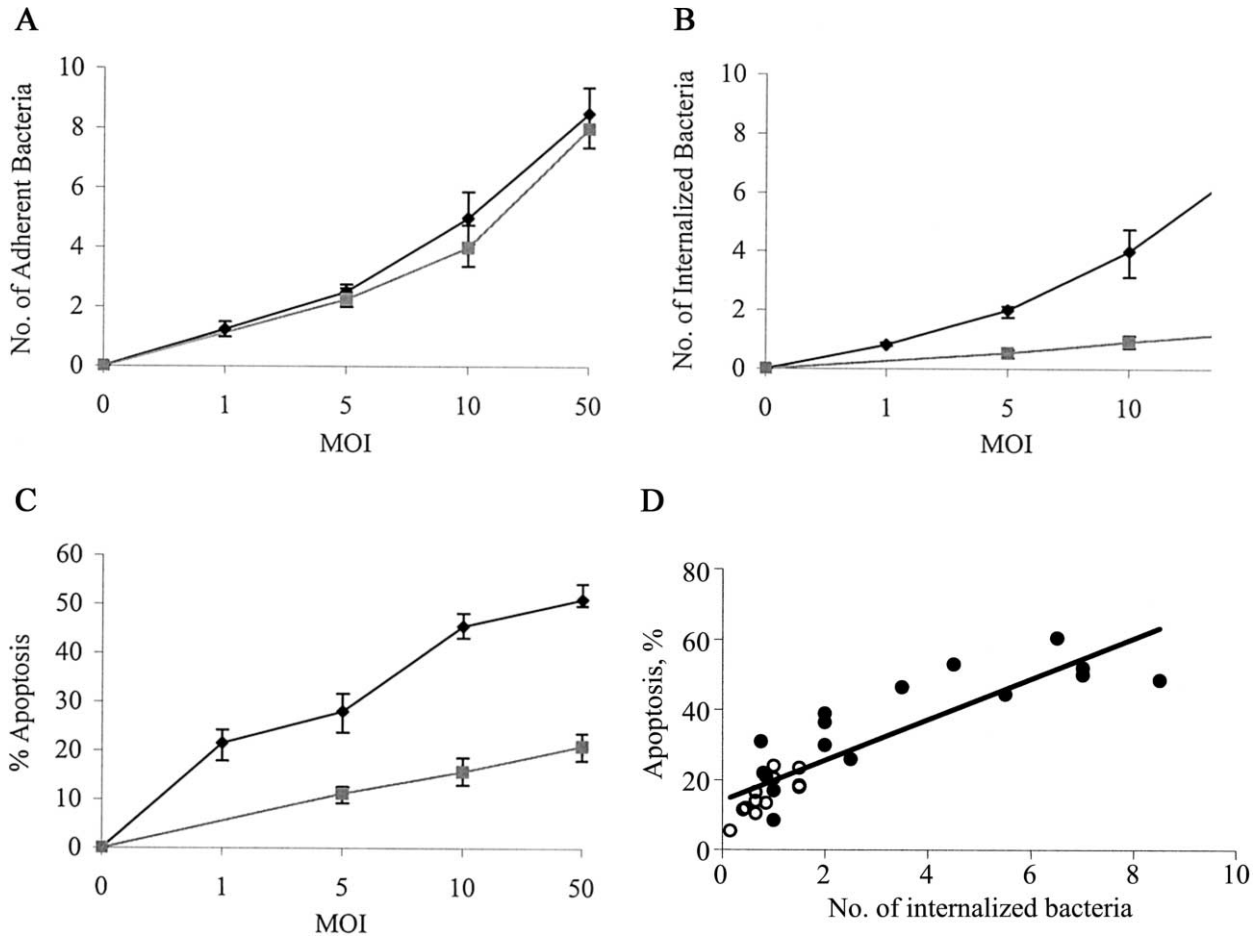

Figure 3. Effect of dose response of *Streptococcus pneumoniae* on the level of adherence, internalization, and monocyte-derived macrophage (MDM) apoptosis. *A,* Adherence per MDM of opsonized (IS-MDM; *solid diamonds*) and nonopsonized (NO-MDM; *gray squares*) *Streptococcus pneumoniae* to 14-day MDM 4 h after infection assessed by fluorescence microscopy *B,* internalization of IS-MDM *(solid diamonds)* and NO-MDM (*gray squares*) *S. pneumoniae* to 14-day MDM 4 h after infection viewed by fluorescence microscopy *C,* Percentage MDM apoptosis detected by TUNEL (terminal UTP nick-end labeling) 20 h after infection with opsonized *S. pneumoniae* (IS-MDM; *solid squares*) or nonopsonized *S. pneumoniae* (NO-MDM; *gray squares*) was assessed by fluorescence microscopy ($n = 4$). Data presented represents median and interquartile range. *D,* The relationship between percentage MDM apoptosis and the number of internalized opsonized *(solid circles)* or nonopsonized *(*) S. pneumoniae* with an MOI ranging from 1 to 50 was analyzed by linear regression. Spearman correlation coefficient $r = 0.86$, $P < .001$, $n = 28$.

.001); and ID, 62% (59%–65%, $P < .001$). Bacteria opsonized with reconstituted serum showed no specific reduction of apoptosis.

The potential contribution of both immunoglobulin and complement components was further examined by receptor blockade. As shown in figure 4*B,* FcγRII blockade resulted in 40% (IQR, 34%–51%) inhibition of apoptosis ($P = .05$) and FcγRIII blockade resulted in 32% (IQR, 28%–42%) inhibition of apoptosis ($P = .05$). In these experiments, the control FcγRI $F(ab')_2$ resulted in a nonsignificant inhibition of apoptosis, and C-MDM apoptosis remained <1% after treatment with $F(ab')_2$. CR1 blockade resulted in 44% (IQR, 44%–53%) inhibition ($P = .03$), and CR3 blockade resulted in 68% (IQR, 63%–70%) inhibition ($P = .03$, figure 4*C*). Results were unaltered by preblocking FcγR with IgG (data not shown). In these experiments, isotype antibody treatment resulted in no inhibition of apoptosis. We determined whether the degree of inhibition of apoptosis was related to the degree of inhibition of bacterial internalization after incubation with each blocking FcγR or CR antibody. As shown in figure 4*D,* a significant relationship was detected ($r = 0.76$, $P = .001$). This further supports the conclusion that enhancement of induction of apoptosis by opsonization is not the result of signaling via any one specific receptor but is a consequence of increased bacterial phagocytosis involving multiple diverse receptors.

Opsonization of inert particles does not induce phagocytosis-related apoptosis. Internalization of opsonized bacteria via FcγR and CR could result in enhanced apoptosis as a result of signal transduction induced by phagocytosis, or could result from the production of factors by the host cell in direct response

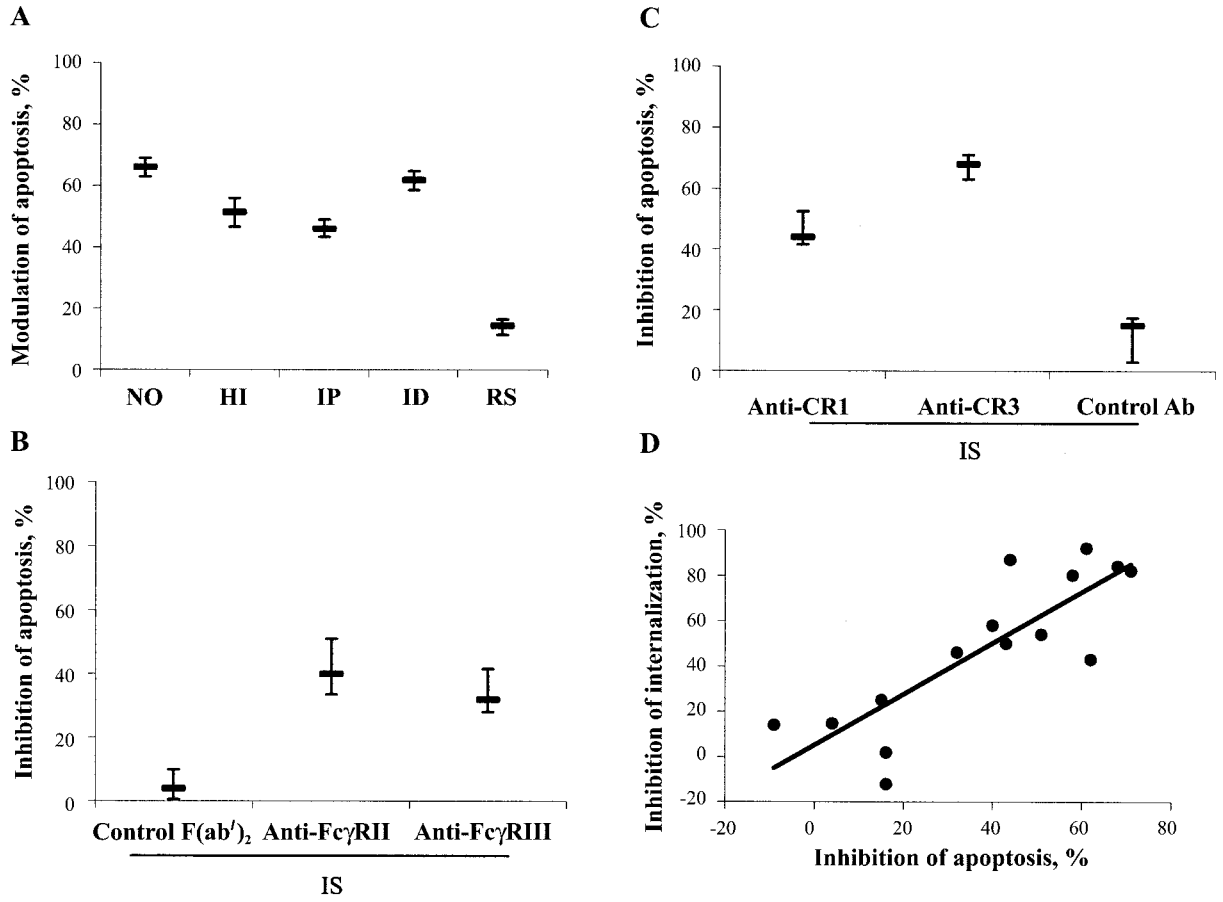

Figure 4. Fcγ and complement receptors both contribute to *Streptococcus pneumoniae*–induced monocyte-derived macrophage (MDM) apoptosis. *A,* Percentage specific reduction (interquartile range) of MDM apoptosis after incubation with bacteria opsonized with modified serum. Apoptosis of 14-day MDM was detected by 4′6′-diamidino-2-phenylindole (DAPI) staining 20 h after infection with *S. pneumoniae* opsonized with whole immune serum (IS), PBS treatment (NO), heat-inactivated serum (HI), pneumococcal immunoglobulin (IP), immunoglobulin-depleted serum (ID), reconstituted serum (RS), or mock infection (C), and percentage specific reduction of apoptosis was calculated. IS versus HI, ID, or IP: $P < .001$, $n = 4$. C-MDM apoptosis was 0.5% (0.4%–0.6%). *B,* Percentage specific inhibition (interquartile range) of MDM apoptosis after FcγR blockade. MDM apoptosis was detected by 4′6′-diamidino-2-phenylindole (DAPI) 20 h after infection with opsonized *S. pneumoniae* (IS) in the presence of blocking FcγRII or FcγRIII $F(ab')_2$ or control $F(ab')_2$ and percentage specific inhibition of apoptosis was calculated ($n = 3$): IS versus IS + anti-FcγRII, $P = .05$; IS versus IS + anti-FcγRII, $P = .05$. *C,* Percentage specific inhibition (interquartile range) of MDM apoptosis after complement receptor blockade. Apoptosis was detected by DAPI 20 h after infection with opsonized *S. pneumoniae* (IS) or mock infection (C-MDM) in the presence of control, anti-CR1, or anti-CR3 blocking monoclonal antibodies, and percentage specific inhibition of apoptosis was calculated. IS + control versus IS + anti-CR1, $P = .03$; IS + control versus IS + anti-CR3, $P = .03$, $n = 3$. C-MDM apoptosis was 0.5% (0.3%–2.0%). *D,* The relationship between percentage internalization inhibition of *S. pneumoniae* and MDM apoptosis inhibition was analyzed by linear regression for each antibody. Spearman correlation coefficient $r = 0.76$, $P = .001$, $n = 15$.

to intracellular microbial products. To investigate this possibility, MDM were treated with opsonized latex beads, of comparable size to *S. pneumoniae*, that contain either a positive or negative surface charge. The level of apoptosis observed was compared with MDM incubated with opsonized *S. pneumoniae*. As shown in figure 5, latex beads were unable to raise levels of apoptosis significantly above those observed for C-MDM, despite being internalized efficiently by MDM to comparable levels to those observed with opsonized *S. pneumoniae* (IS-MDM vs. L1155, $P = .02$; IS-MDM vs. L4030, $P = .021$). This indicates that activation of signal transduction pathways by receptors involved in the phagocytosis of opsonized bacteria is not the principal cause of apoptosis.

To further confirm this, we examined the role of one of the elements of signal transduction implicated in both phagocytosis and macrophage apoptosis. PI3K is activated during phagocytosis and contributes to the internalization of particles [36]. Recently PI3K has been shown to play a key role in macrophage survival [37]. We therefore investigated whether PI3K inhibition using LY294002 modulated internalization of bacteria or induction of apoptosis. When PI3K inhibition was induced before MDM incubation with bacteria, the median number of internalized bac-

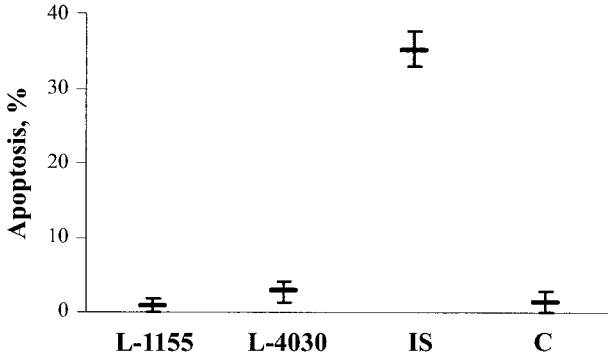

Figure 5. Phagocytosis of opsonized latex beads fails to induce monocyte-derived macrophage (MDM) apoptosis. Percentage 14-day MDM apoptosis detected by 4′6′-diamidino-2-phenylindole (DAPI) 20 h after infection with opsonized *Streptococcus pneumoniae* (IS-MDM), opsonized amine-modified latex beads (L-1155), opsonized carboxylate-modified latex beads (L-4030), or mock infection (C-MDM) was assessed by fluorescence microscopy. Data represent median and interquartile range for apoptosis. IS-MDM versus L-1155, $P = .02$; IS-MDM versus L-4030, $P = .021$, $n = 4$.

teria per MDM was significantly decreased (median [IQR], 2.1 [1.6–2.6] vs. 4.5 [4.2–6.4], $P = .009$), as was apoptosis (median [IQR], 13.5% [10.5%–26%] vs. 41.5% [35%–45%] for MDM in the absence of LY294002, $P = .021$). However, when the PI3K inhibitor was added 4 h after infection, levels of MDM apoptosis were not altered (median [IQR], 37.5% [31.5%–39%] vs. 41.5% [35%–45%], compared with MDM in the absence of LY294002, $P > .05$). This was despite a 2.6-fold increase in C-MDM apoptosis in the presence of LY294002. This suggested that the principal effect of early PI3K inhibition is to decrease the phagocytosis of bacteria and further supports the observation that inhibition of bacterial internalization decreases levels of MDM apoptosis.

Inhibition of protein synthesis by internalized bacteria reduces apoptosis. We next examined whether protein synthesis by opsonized bacteria was required for the maximal induction of IS-MDM apoptosis. *S. pneumoniae* were treated with sub-MIC doses of chloramphenicol and then opsonized in IS (chloramphenicol treated [CT]). As shown in figure 6*A*, treatment with chloramphenicol did not modify opsonization of bacteria, and levels of internalized bacteria 30 min after incubation at 37°C were identical for MDM infected with untreated and CT bacteria after opsonization (data not shown). However, despite similar internalization, the CT-MDM demonstrated significantly lower levels of apoptosis than IS-MDM; 6.8% (5.9%–9.1%) versus 37% (32.6%–39.4%) ($P < .001$, figure 6*B*). This provides evidence of the importance of intracellular bacterial proteins as triggers of induction of apoptosis.

To further investigate the role of some specific pneumococcal proteins in induction of apoptosis, we have screened mutant *S. pneumoniae* to compare the level of apoptosis they induce in comparison with wild-type bacteria. In these experiments, no difference in levels of MDM apoptosis between an autolysin-deficient mutant (*LytA*$^-$) (median, 21.7%; IQR, 14.7%–25.2%), a hyaluronidase-deficient mutant (Δ*HY1*) (median, 16.9%; IQR, 12.8%–20.0%), a neuraminidase-deficient mutant (Δ*NA1*) (median, 18.3%; IQR, 12.5%–21.0%), and the parental D39 strain (median, 20.4%; IQR, 17.0–24.4%; $P > .05$, $n = 6$) was noted. In addition, a concentration of cell wall extract equivalent to the amount of cell wall present in bacteria at an MOI of 10 had no effect on apoptosis (MDM apoptosis with cell wall, median [IQR], 1.9% [1.3%–2.2%] vs. control MDM, median [IQR], 1.1% [0.7%–2.4%]; $n = 5$; $P > .05$), although at higher concentrations, cell wall modestly increased the median level of apoptosis to levels that remained at <5% median apoptosis.

Enhancement of macrophage apoptosis by a polysaccharide capsule deficient mutant. *S. pneumoniae* polysaccharide capsule has multiple biologic effects [38]. Phagocytosis of *S. pneumoniae* by macrophages is impeded by the presence of a polysaccharide capsule [5]. To explore whether a virulence factor linked to bacterial internalization played any role on induction of apoptosis we examined whether an unencapsulated mutant

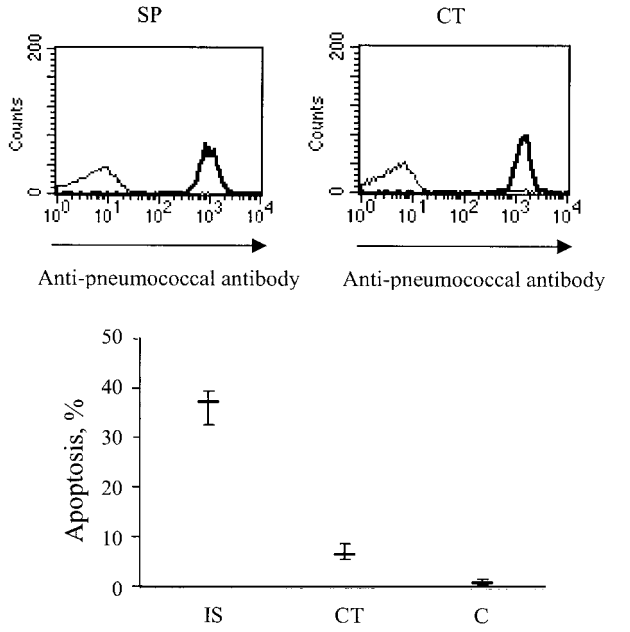

Figure 6. Protein synthesis by *Streptococcus pneumoniae* enhances the levels of monocyte-derived macrophage (MDM) apoptosis. *A,* IgG binding *(bold line)* to the surface of *Streptococcus pneumoniae* (SP; *left panel*) and chloramphenicol-treated pneumococci (CT; *right panel*) versus nonopsonized SP and CT controls *(faint lines)* as detected by flow cytometry. Histograms are typical of 1 of 3 sets of experiments. *B,* Percentage MDM apoptosis detected by 4′6′-diamidino-2-phenylindole (DAPI) 20 h after infection with opsonized *S. pneumoniae* (IS-MDM), chloramphenicol-treated *S. pneumoniae* (CT-MDM), or mock infection (C-MDM) was detected by fluorescence microscopy. Data presented represent median and interquartile range for apoptosis. IS-MDM versus CT-MDM, $P < .001$, $n = 3$.

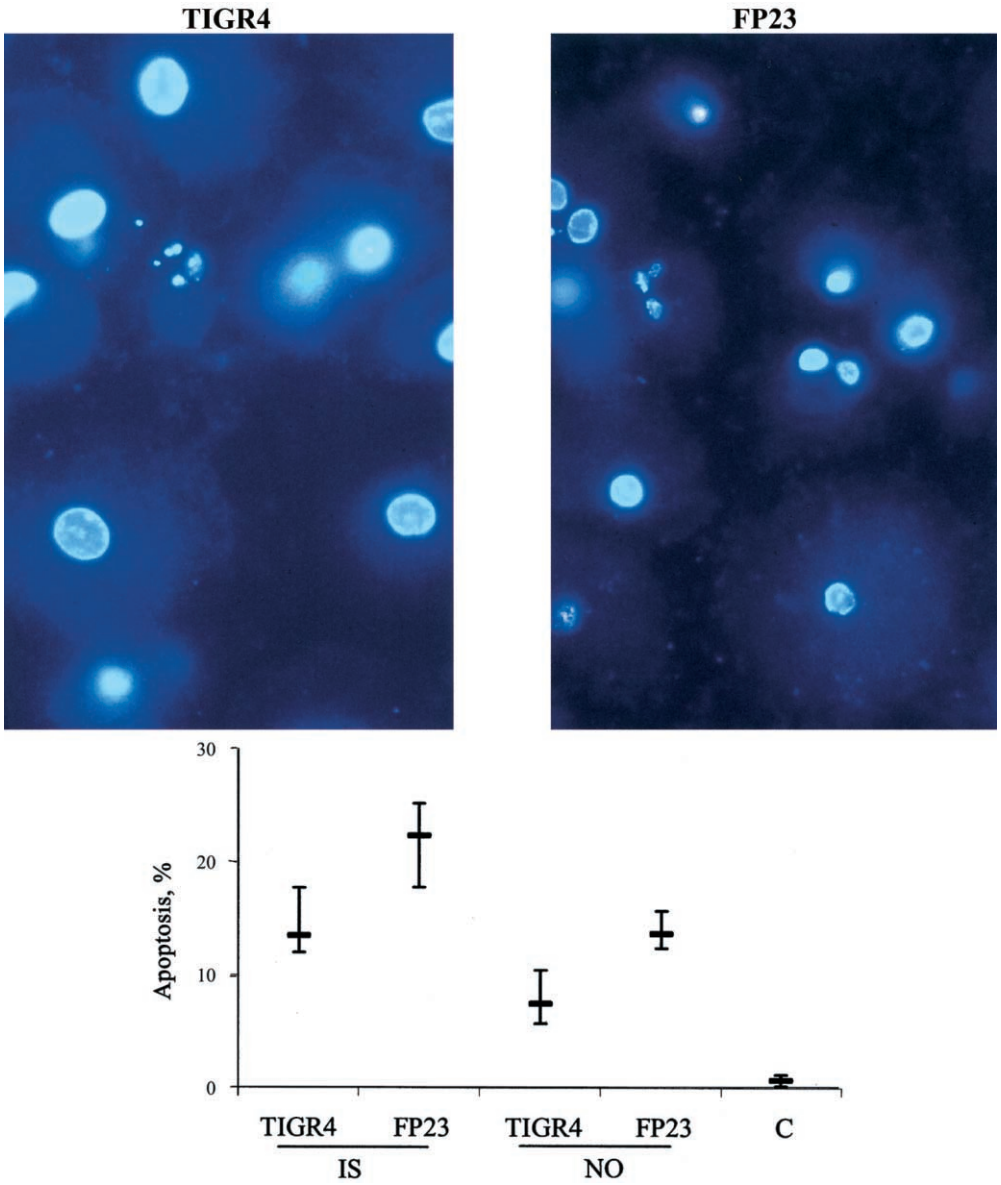

Figure 7. Enhancement of monocyte-derived macrophage (MDM) apoptosis by a polysaccharide capsule deficient mutant of *Streptococcus pneumoniae. A,* Fourteen-day MDM was analyzed by fluorescence microscopy after 4′6′-diamidino-2-phenyl indole (DAPI) staining 20 h after infection with TIGR4 *(left)* or its unencapsulated mutant FP23 *(right). B,* Percentage MDM apoptosis was estimated by DAPI staining 20 h after infection with opsonized (IS) or nonopsonized (NO) TIGR4 or FP23 or mock infection *(C).* Data represent median and interquartile range of duplicate experiments ($n = 6$). IS TIGR4 versus IS FP23, $P = .041$; NO TGR4 versus NO FP23, $P = .009$.

of *S. pneumoniae* that would be more efficiently phagocytosed and killed also resulted in greater levels of MDM apoptosis. As shown in figure 7, the unencapsulated mutant FP23 resulted in greater levels of apoptosis than its wild-type, type 4, parent strain. A significant increase in MDM apoptosis was apparent for opsonized ($P = .04$) bacteria but was most marked for nonopsonized bacteria ($P = .008$). This was associated with a 50% greater level of internalization of opsonized unencapsulated bacteria, and a 75% greater level of internalization for nonopsonized unencapsulated bacteria. Clearly, as a result of the multiple biologic effects of capsule, this observation has many potential explanations, but it is nevertheless still consistent with a relationship between bacterial internalization and MDM apoptosis generation.

Caspase inhibition decreases bacterial killing. We have previously demonstrated that *S. pneumoniae*–associated MDM apoptosis is caspase dependent [26], and we linked clearance of bacteria to induction of apoptosis. To investigate whether the association between internalized bacteria and MDM induction of apoptosis reflected part of the host response to

infection or was an example of pathogen-mediated immune evasion, we examined the effect of caspase inhibition on bacterial killing. As shown in figure 8, zVADfmk significantly reduced killing of bacteria in infected cultures ($P = .028$).

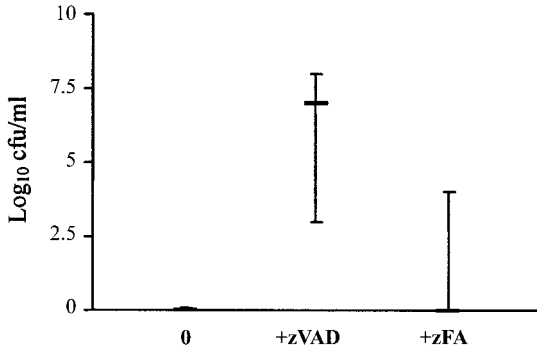

Figure 8. Caspase inhibition decreases bacterial clearance from the supernatants of monocyte-derived macrophage (MDM) cultures. Fourteen-day MDM were infected with opsonized *Streptococcus pneumoniae* and bacterial viability estimated in culture supernatants 20 h after infection in the absence (0) or presence of z-Val-Ala-D-Asp-fluoromethylketone (zVADfmk) (+ZVAD) or z-Phe-Ala-fluoromethylketone (zFAfmk) (+zFA). Log_{10} colony-forming units (cfu) are shown, and results represent medians and interquartile ranges. Friedman's test $P = .028$, $n = 3$.

DISCUSSION

In this study, we demonstrate that opsonization enhances *S. pneumoniae*–induced macrophage apoptosis. Furthermore, we make the novel observation that opsonin enhancement of macrophage apoptosis is a function of the number of internalized bacteria but not specifically the result of an individual FcγR or CR, several of which contribute to bacterial internalization and resultant apoptosis. Apoptosis requires bacterial protein synthesis and is decreased in the presence of polysaccharide capsule.

Our conclusion that opsonization-induced apoptosis is a direct function of the intracellular bacterial burden rather than specific engagement of an individual FcγR or CR is supported by the following observations: (1) the induction of equivalent high levels of MDM apoptosis by nonopsonized bacteria when the infecting inoculum is increased to normalize numbers of internalized bacteria, (2) the partial effect on bacterial internalization and induction of apoptosis by blockade of multiple separate FcγR or CR involved in phagocytosis of opsonized bacteria despite the divergent signal transduction resulting from engagement of these receptors, (3) lack of significant induction of apoptosis by opsonized latex beads, (4) reduction of apoptosis and internalization of bacteria by early PI3K inhibition, (5) the requirement for bacterial protein synthesis by internalized bacteria for maximal induction of apoptosis, and (6) enhancement of the level of apoptosis in association with removal of the polysaccharide capsule that is known to impede phagocytosis.

Differentiation of macrophages in vitro is associated with increasing resistance to apoptosis in association with a long-lived cell type in vivo [37]. Although numerous bacteria have been demonstrated to induce macrophage apoptosis, this has been viewed as a microbial strategy to facilitate immune evasion [22]. Evidence of macrophage apoptosis in a model of bacterial infection in which intracellular bacteria are efficiently killed has not been provided. However, macrophage apoptosis induced by *Mycobacterium tuberculosis* is associated with killing of bacteria [39–42]. S*treptococcus pneumoniae* is not believed to survive for prolonged periods in the intracellular environment, but the demonstration that macrophage apoptosis is associated with microbial killing in vitro suggests a role in host defense [26]. The demonstration that opsonization, a process that enhances phagocytosis and killing of bacteria, also increases macrophage apoptosis supports this view, as does the demonstration of enhanced apoptosis induced by the less virulent unencapsulated mutant. However, the requirement for protein synthesis by viable bacteria suggests the relationship between host and pathogen derived factors is complex. Microbial proteins could either trigger host factors that simultaneously induce apoptosis and microbial killing or initiate a program of apoptosis that is unrelated to host cell microbicidal factors. Screening of mutant bacteria and cell wall extracts has failed to demonstrate any additional protein that explains the decrease in apoptosis associated with bacterial protein synthesis inhibition. Pneumolysin production contributes to apoptosis [26], and cell wall proteins make a modest contribution. Although we cannot exclude the possibility that an unidentified bacterial protein triggers apoptosis, these results, and other unpublished observations from our group, suggest that it may be the cumulative effect of the interaction between a number of different bacterial proteins and specific macrophage responses to these proteins that triggers induction of apoptosis.

To address whether induction of apoptosis is associated with a particular pattern of cell surface engagement, we have depleted serum factors and used monoclonal blocking antibodies with similar results. Induction of apoptosis could not be linked to any one group of serum factors or receptors. Many of the downstream effects of phagocytosis of opsonized bacteria require simultaneous signals generated through a variety of different receptors [1]. CR1 and CR3 require second activating steps provided by phorbol myrisate acetate–induced protein kinase C activation, microbial products such as lipopolysaccharides, inflammatory cytokines such as TNF-α, and adhesion factors such as fibronectin to enhance phagocytosis [43, 44]. Simultaneous engagement of CR1/CR3 and FcRs has synergistic effects on the internalization of opsonized particles [45, 46]. Killing of *S. pneumoniae* by neutrophils in the presence of capsule polysaccharide specific polymeric IgA requires both Fc$R\alpha$ and CR1/CR3 [47].

Many signal transduction pathways are activated during phagocytosis, and it is possible that a signal transduction pathway associated with phagocytosis and common to a number of receptors could induce apoptosis. FcR and CR activate distinct signal transduction pathways and have different effects in terms of phagocytosis and activation of downstream effects [1]. Even within a class of receptors, quite different effects can result from receptor engagement. We focused on blocking either FcγR or CR, receptors that are known to mediate, either directly or indirectly, particle internalization. Blockade of each partially inhibited apoptosis.

FcγR signaling results in activation of tyrosine kinases [48]. This process is similar to that observed after CD3 ligation, a stimulus that leads to induction of apoptosis under specific conditions. Downstream targets of the Syk family of tyrosine kinases activated during FcγR-mediated macrophage phagocytosis include PI3K and Rho GTPases [49]. PI3K signaling contributes to macrophage survival [37]. PI3K inhibition before exposure of MDM to bacteria decreased bacterial phagocytosis and decreased the level of apoptosis observed at 20 h. Furthermore, when PI3K inhibition was induced after bacterial internalization, there was no alteration in the level of apoptosis. These findings might initially appear to contradict the previous findings that PI3K inhibition enhances macrophage apoptosis. However, PI3K inhibition may already be maximal at these later time points after infection. Alternatively, the balance of survival and death factors may be altered to such an extent that this pathway is no longer the critical determinant of macrophage survival.

It is also possible, however, that at 20 h, the period of maximal induction of apoptosis after internalization of *S. pneumoniae,* it is too early to demonstrate induction of apoptosis via PI3K inhibition, and it should be noted that a previous study demonstrated that apoptosis associated with PI3K inhibition was maximal at 48–72 h [37]. However, the lack of induction of apoptosis by opsonized latex beads, despite internalization, suggests that signal transduction via cell-surface receptors cannot adequately explain the induction of apoptosis, although it should be noted that some receptors such as the C1qR and collectin receptors were not specifically targeted in our studies.

We conclude that the major determinant of induction of apoptosis is the intracellular bacterial load. There have been few reports linking the number of intracellular bacteria with macrophage induction of apoptosis. Gastric epithelial cell apoptosis induced by *Helicobacter pylori* [50] and macrophage apoptosis in a murine model of *Yersinia pseudotuberculosis* in which bacteria are extracellular [51] have been related to the bacterial burden in tissue. A higher intracellular bacterial load could result in a higher concentration of certain bacterial proteins. Bacterial protein synthesis inhibition decreases macrophage apoptosis. Bacterial virulence factors such as pneumolysin contribute to macrophage and neurone apoptosis induced by *S. pneumoniae* [25, 26] and could explain this relationship. However, soluble factors might be expected to exert a significant effect regardless of internalization of bacteria. Overall, however, the relationship between apoptosis and bacterial killing [26] suggests the role of these proteins could be to trigger production of host factors that limit bacterial replication and also induce apoptosis. The demonstration that caspase inhibition decreases bacterial killing and that a less virulent unencapsulated strain enhances apoptosis supports the concept that induction of apoptosis is part of a host defense to infection and that a complex bidirectional relationship exists between killing of bacteria and induction of apoptosis. However, it must be recognized that caspase inhibition is likely to inhibit other processes in addition to apoptosis in MDM.

The current study represents an important advance to previous studies of *S. pneumoniae*–induced apoptosis. In these, induction of apoptosis was either demonstrated to be the consequence of extracellular proteins [25] or related to adherence of bacteria and subsequent killing [26]. By systematically relating apoptosis to internalization of opsonized bacteria in the context of specific opsonic conditions or pathways of receptor-mediated internalization, we have demonstrated for the first time a clear relationship between bacterial intracellular burden and induction of apoptosis in host defense. This contrasts with the apoptosis observed with another streptococcus pathogenic in humans, group B streptococcus [52]. It has been shown that group B streptococci also induce macrophage apoptosis. However, in this case, apoptosis does not require bacterial internalization and is independent of caspase activation, unlike the present model. These differences may reflect the phenotype of macrophages as in the studies with *S. pneumoniae* differentiated macrophages are required, whereas a less differentiated monocyte phenotype was used in the group B streptococcal model. Furthermore, differences in intracellular survival have been described with greater intracellular persistence of group B streptococci [53]. Differences in the bacterial proteins expressed in an intracellular location or differences in microbicidal molecule generation are other potential differences [54].

In conclusion, we demonstrate that opsonization of bacteria enhances the induction of macrophage apoptosis by increasing the intracellular bacterial load of *S. pneumoniae.* Intracellular bacteria capable of protein synthesis, as opposed to a particular receptor/signal transduction pathway, provide the trigger for apoptosis. The observed apoptosis may contribute to limiting proinflammatory responses to infection. Further studies that increase the understanding of the role of macrophage apoptosis in bacterial infection are needed to determine its potential for therapeutic modulation.

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