INFECTION AND IMMUNITY, Sept. 2009, p. 3826–3837 Vol. 77, No. 9
0019-9567/09/$08.00+0 doi:10.1128/IAI.00290-09

Streptococcus pneumoniae Autolysis Prevents Phagocytosis and Production of Phagocyte-Activating Cytokines▽†

Anna Martner,[1]* Susann Skovbjerg,[1] James C. Paton,[2] and Agnes E. Wold[1]

Department of Infectious Medicine/Clinical Bacteriology, Göteborg University, Guldhedsgatan 10A, 413 46 Göteborg, Sweden,[1] and Research Centre for Infectious Diseases, School of Molecular and Biomedical Science, University of Adelaide, S.A. 5005, Australia[2]

Received 12 March 2009/Returned for modification 15 May 2009/Accepted 9 June 2009

***Streptococcus pneumoniae* is a major pathogen in humans. The pathogenicity of this organism is related to its many virulence factors, the most important of which is the thick pneumococcal capsule that minimizes phagocytosis. Another virulence-associated trait is the tendency of this bacterium to undergo autolysis in stationary phase through activation of the cell wall-bound amidase LytA, which breaks down peptidoglycan. The exact function of autolysis in pneumococcal pathogenesis is, however, unclear. Here, we show the selective and specific inefficiency of wild-type *S. pneumoniae* for inducing production of phagocyte-activating cytokines in human peripheral blood mononuclear cells (PBMC). Indeed, clinical pneumococcal strains induced production of 30-fold less tumor necrosis factor (TNF), 15-fold less gamma interferon (IFN-γ), and only negligible amounts of interleukin-12 (IL-12) compared with other closely related *Streptococcus* species, whereas the levels of induction of IL-6, IL-8, and IL-10 production were similar. If pneumococcal LytA was inactivated by mutation or by culture in a medium containing excess choline, the pneumococci induced production of significantly more TNF, IFN-γ, and IL-12 in PBMC, whereas the production of IL-6, IL-8, and IL-10 was unaffected. Further, adding autolyzed pneumococci to intact bacteria inhibited production of TNF, IFN-γ, and IL-12 in a dose-dependent manner but did not inhibit production of IL-6, IL-8, and IL-10 in response to the intact bacteria. Fragments from autolyzed bacteria inhibited phagocytosis of intact bacteria and reduced the in vitro elimination of pneumococci from human blood. Our results suggest that fragments generated by autolysis of bacteria with reduced viability interfere with phagocyte-mediated elimination of live pneumococci.**

The pneumococcus *Streptococcus pneumoniae* is a leading cause of community-acquired pneumonia, meningitis, otitis media, and sinusitis and is a common cause of infection-related mortality in children and elderly people (28, 37).

There is a large number of streptococcal species whose taxonomic classification is debated (14, 31). A number of streptococci, including alpha-hemolytic and nonhemolytic variants, constitute the viridans group, which can be further subdivided into the mitis, sanguinis, anginosus, salivarius, and mutans groups based on biochemical tests (14). Phenotypic and genetic tests consistently show that *S. pneumoniae* is closely related to and may be placed in the mitis subgroup (14, 30). Although the other members of the mitis group can cause sepsis and endocarditis (53), they are considerably less virulent than *S. pneumoniae*.

Pneumococci are considered strictly extracellular pathogens, whose elimination depends on ingestion and killing by phagocytes (i.e., alveolar and tissue-resident macrophages and neutrophils recruited during the inflammatory process). Accordingly, an important determinant of pneumococcal pathogenicity is the thick, hydrophilic polysaccharide capsule, which impedes elimination by phagocytes in the absence of capsule-specific antibodies.

The ability of phagocytes to kill microbes is augmented by the phagocyte-activating cytokines gamma interferon (IFN-γ) and tumor necrosis factor (TNF), which boost the bactericidal machinery and enhance killing and digestion of bacteria present within the phagosome (4, 39, 47). TNF is produced by monocytes/macrophages and activated T cells, while IFN-γ is produced by NK cells and T cells in response to interleukin-12 (IL-12) from macrophages. Thus, production of TNF, IFN-γ, and IL-12 is necessary for host defense against intracellular bacteria (8, 11, 21, 34, 48). More recently, these phagocyte-activating cytokines have also been shown to be essential for controlling extracellular gram-positive bacteria, including *S. pneumoniae* (36, 42, 50, 52, 54). Thus, a patient with an IL-12 deficiency was shown to suffer from recurrent episodes of pneumococcal infection (20). Phagocyte activation by TNF and/or IFN-γ might be required for decomposition of the thick, sturdy peptidoglycan (PG) layer of gram-positive bacteria after phagocytosis, while gram-negative bacteria may be more easily digested. Thus, human leukocytes produce more TNF, IFN-γ, and IL-12 when they are stimulated with gram-positive bacteria than when they are stimulated with gram-negative bacteria (23, 24).

A peculiar property of *S. pneumoniae* is its tendency to undergo autolysis when it reaches the stationary phase of growth. This process is mediated by enzymes called autolysins (ALs), which, when activated, degrade cell wall PG. The major AL is an *N*-acetyl-muramyl-L-alanine amidase called LytA (27). Other pneumococcal ALs include LytB and LytC, which are believed to be involved mainly in modification of the cell wall during growth and division (16, 17). ALs are anchored to the cell wall via interactions with choline moieties on teichoic

* Corresponding author. Mailing address: Department of Infectious Medicine/Clinical Bacteriology, Göteborg University, Guldhedsgatan 10 A, 413 46 Göteborg, Sweden. Phone: 46 31 342 4623. Fax: 46 31 342 4975. E-mail: anna.martner@microbio.gu.se.

† Supplemental material for this article may be found at http://iai.asm.org/.

▽ Published ahead of print on 15 June 2009.

TABLE 1. Serotype and MLST of seven clinical *S. pneumoniae* isolates, wild-type strain D39, and autolysin- and pneumolysin-deficient mutants of this strain

Strain	Serotype	MLST	Source or reference
A6	19F	ND[a]	Otitis media patient
A7	9V	ND	Otitis media patient
A17	14	124	Otitis media patient
A22	6A	481	Otitis media patient
A24	14	ND	Otitis media patient
CCUG 23261	11A	62	CCUG
CCUG 33774	5	ND	CCUG
D39 WT	2	128	51
D39 AL$^-$	2	128	2
D39 PLY$^-$	2	128	3

[a] ND, not determined.

acid and lipoteichoic acid (LTA). Choline is necessary for pneumococcal growth, but culture in the presence of high concentrations of choline renders the bacteria incapable of undergoing autolysis (6, 19).

Studies with mice have shown that *S. pneumoniae* with mutated LytA is less virulent than wild-type pneumococci (2, 7, 25). The reason for this is not clear, but two main hypotheses have been put forward. First, autolysis promotes the release of the intracellular toxin pneumolysin (Ply) (5, 33). Ply is an important determinant of virulence (3, 41) and interferes with several defense systems, including inhibition of ciliary beating (15), complement activation (38), and induction of intracellular oxygen radical production (33). Second, cell wall degradation products, such as soluble PG fragments and LTA released upon autolysis, have been suggested to augment the inflammatory response (9, 10, 44, 49).

Here we examine a third possibility, that autolysis interferes with the generation of phagocyte-activating cytokines. We have previously shown that intact gram-positive bacteria provide a very efficient stimulus for IL-12 production by human monocytes, regardless of whether they are dead or alive (1, 23, 24), but that decomposed bacteria are inactive in this process and soluble components of the gram-positive cell wall, such as PG and LTA, even downregulate the production of IL-12 in response to intact bacteria in a dose-dependent manner (1). These observations led us to speculate that autolysis may promote virulence by generating bacterial cell wall fragments that block IL-12 production and thereby reduce IFN-γ production and phagocyte activation. Indeed, our data demonstrate that AL-mediated disintegration of pneumococci inhibits production of IFN-γ and also TNF in response to intact bacteria. Further, the presence of autolyzed bacteria reduced elimination of live pneumococci by blood cells in vitro.

MATERIALS AND METHODS

Bacterial strains. The pneumococcal strains used in the present study are listed in Table 1. The virulent strain *S. pneumoniae* D39 (D39 WT) and an AL-deficient mutant (D39 AL$^-$) and pneumolysin-deficient mutant (D39 PLY$^-$) of this strain have been described previously (2, 3). Seven clinical virulent wild-type isolates were included, five of which were isolated from children with acute otitis media causing spontaneous rupture of the tympanic membrane (A6, A7, A17, A22, and A24) and two of which were from the Culture Collection of the University of Gothenburg (CCUG 23261 and CCUG 33774). For assays of phagocytosis and intracellular killing the nonencapsulated wild-type strain *S. pneumoniae* CCUG 10708 was utilized.

The pneumococcal isolates were serotyped by gel diffusion using 46 type- or group-specific antisera obtained from the World Health Organization Collaborating Center for Reference and Research on Pneumococci (Statens Seruminstitut, Copenhagen, Denmark). Isolates belonging to groups with several strains were further characterized to determine their capsular types by gel diffusion and/or the Quelling test.

Selected strains were also analyzed by multilocus sequence typing (MLST) as previously described (12). In brief, internal fragments of the *aroE*, *gdh*, *gki*, *recP*, *spi*, *xpt*, and *ddl* genes were amplified by PCR by use of the primer pairs indicated at http://spneumoniae.mlst.net/misc/info.asp#experimental. Sequences were obtained for both DNA strands by use of an ABI 3730xl DNA analyzer. Alleles from the MLST website (http://spneumoniae.mlst.net) were downloaded for alignment analysis and sequence type determination.

The following seven strains representing species in the *Streptococcus* viridans group were obtained from Virginia Polytechnic Institute and State University (VPI), the National Collection of Type Cultures (NCTC), the American Type Culture Collection (ATCC), or the Culture Collection of the University of Gothenburg (CCUG): *S. mutans* VPI 8290, *S. salivarius* VPI 13484, *S. anginosus* NCTC 10713, *S. intermedius* ATCC 27335, *S. sanguinis* ATCC 10556, *S. oralis* ATCC 35037, and *S. mitis* CCUG 31611.

Culture and preparation of bacteria. For comparison of cytokine release in response to *Streptococcus* viridans group strains and pneumococci, viridans group streptococci and *S. pneumoniae* CCUG 33774 were cultured on horse blood agar overnight. The bacterial colonies were then harvested, washed twice in Dulbecco's endotoxin-free phosphate-buffered saline (PBS) (PAA Laboratories, Linz, Austria), and suspended to an optical density at 580 nm (OD_{580}) of 0.87, which corresponded to 7×10^8 bacteria/ml as determined by counting with a microscope. Suspended bacteria were killed by exposure to UV light for 18 min (which was confirmed by a negative viable count) and stored at −70°C until they were used in the experiments.

For studies of the role of autolysis, the pneumococcal strains and the control strain *S. mitis* CCUG 31661 were cultured to late log phase for 12 to 14 h in Todd-Hewitt broth supplemented with 0.5% yeast extract in the presence or absence of 2% choline chloride (Substrate Department, Clinical Bacteriology Laboratory, Sahlgrenska University Hospital). High concentrations of choline in this broth inhibit the action of LytA and thus prevent bacterial autolysis (6, 19). After two washes, bacteria grown in the presence of choline were suspended in Dulbecco's endotoxin-free PBS with 1% sterile filtered choline chloride, while bacteria grown in the absence of choline were suspended in PBS without choline. Bacteria were counted, the OD_{580} was adjusted to 0.87 (equivalent to 7×10^8 bacterial cells/ml as determined by counting separate cells with a microscope), and then the bacteria were inactivated by UV light and frozen.

UV inactivation of wild-type pneumococci leads to bacterial death followed by autolysis, the latter mediated by ALs. To measure the proportion of autolysis occurring during UV inactivation, freezing, and thawing, the bacterial concentrations before and after this treatment were compared by using OD_{580} measurements and microscopic enumeration. UV inactivation of the *Streptococcus* viridans group strains, the LytA-negative mutant D39 AL$^-$, and the strains grown in the presence of choline had little or no effect on the number of bacterial cells (7×10^8 cells/ml before treatment and 6×10^8 to 7×10^8 cells/ml after treatment). In contrast, when wild-type strains grown in plain broth had been UV inactivated, frozen, and thawed, only 0.5×10^8 to 2×10^8 intact bacterial cells/ml remained in the preparations. Similarly, the OD_{580} values were markedly reduced (from 0.87 to 0.3 to 0.5) in the autolysis-positive preparations but not in the autolysis-negative preparations.

For stimulation of peripheral blood mononuclear cells (PBMC), different dilutions of the thawed bacterial preparations were added directly to PBMC cultures. The concentrations indicated below refer to the numbers of bacteria present before UV inactivation (i.e., before autolysis took place). Thus, in preparations of strains with active ALs, approximately one-fifth of the bacteria were intact, while 80% of the bacteria had lysed and generated cell wall fragments and cytoplasmic constituents that were present in the preparations.

In some experiments, the autolysin-positive preparations were centrifuged at $13,000 \times g$ for 10 min at 4°C to crudely separate the remaining, more or less intact bacteria and soluble fragments. Each supernatant was collected, and the pellet was resuspended in the original volume of PBS with choline to prevent further autolysis.

Purified PG and LTA were also used for stimulation of PBMC. These compounds originated from *S. aureus* and were purchased from Sigma (St. Louis, MO). *N*-Acetylmuramyl-L-alanin-D-isoglutamine (MDP) was also purchased from Sigma.

To generate bacterial fragments of *S. mitis*, UV-inactivated bacteria were sonicated for 10 min with strong cooling at amplitude 70 (Vibra cell; Sonics &

Materials Inc., Danbury, CT). Less than 10% of the bacterial cells remained intact, as determined by enumeration with a microscope.

Preparation and stimulation of blood mononuclear cells. PBMC were prepared from blood donor buffy coats (Blood Bank, Sahlgrenska University Hospital) by density gradient centrifugation (Lymphoprep; Axis-Shield, Norway) for 20 min at 820 × *g* at room temperature. The cells were washed three times in ice-cold endotoxin-free RPMI 1640 medium with 2 mM L-glutamine (PAA Laboratories GmbH, Linz, Austria) and suspended in RPMI 1640 medium with L-glutamine, 5% inactivated fetal calf serum (Invitrogen, San Diego, CA), and 0.1% gentamicin (50 mg/ml; Sigma) to a final concentration of 2×10^6 cells/ml. The cells were stimulated with 3×10^7 or 3×10^6 UV-inactivated bacteria/ml (based on the original numbers of bacteria determined before autolysis took place [see above]) in 96-well flat-bottom plates (Nunc, Roskilde, Denmark) at 37°C in a humidified atmosphere with 5% CO_2. Supernatants were harvested after 24 h and 5 days; the latter time point was used to measure IFN-γ.

The viability of PBMC was assessed after 24 h of bacterial stimulation. The culture plates were placed on ice. Cells were removed using a rubber policeman, washed with PBS, and incubated with the nucleic acid dye 7-amino-actinomycin D (BD Pharmingen), which permeates nonviable cells. The proportion of stained cells was analyzed by flow cytometry (Becton Dickinson FACScan equipped with FlowJoe software).

Cytokine determination. The concentrations of IL-12, TNF, IFN-γ, IL-6, IL-8, and IL-10 were measured by enzyme-linked immunosorbent assays as described in detail elsewhere (29). In brief, Costar plates (Invitrogen) were coated with the following anti-human monoclonal antibodies: anti-IL-12p70 (20C2), anti-TNF-α (MAb1), anti-IFN-γ (NIB42), anti-IL-6 (MQ2-13A5), anti-IL-8 (G265-5), and anti-IL-10 (JES3-9D7), all obtained from BD Pharmingen (San Diego, CA). The plates were blocked with 5% bovine serum albumin (Sigma-Aldrich) in PBS. Standard curves were generated by diluting recombinant human IL-12, TNF, IFN-γ, IL-6, IL-8, and IL-10 (BD Pharmingen) in high-performance enzyme-linked immunosorbent assay dilution buffer (CLB, Amsterdam, The Netherlands). The following biotinylated anti-human monoclonal antibodies were diluted in this buffer and used for detection: anti-IL-12p40/p70 (C8.6), anti-IFN-γ (4S.B3), anti-TNF-α (Mab11), anti-IL-6 (MQ2-39C3), anti-IL-8 (G265-8), and anti-IL-10 (JES3-12G8) (BD Pharmingen). After incubation with streptavidin-horseradish peroxidase (BD Pharmingen), 3,3′5,5′-tetramethylbenzidine liquid substrate (Sigma-Aldrich) was added. The reaction was stopped with 1 M H_2SO_4, and the color reaction was measured by determining the OD_{450} with a spectrophotometer (Molecular Devices Corporation, California).

Examination of interactions between PBMC and bacteria in cytospin preparations. To examine whether bacteria were internalized by monocytes, PBMC were prepared from blood donor buffy coats as described above. Red blood cells were lysed by incubation in distilled H_2O for 30 s. The cells were suspended in RPMI 1640 medium with L-glutamine and 5% inactivated fetal calf serum (Invitrogen) at a concentration of 1×10^6 cells/ml and incubated with 3×10^7 bacteria/ml (enumerated before UV inactivation) in 24-well plates (Nunc) for 30 min at 37°C. The cells were washed, subjected to cytocentrifugation onto glass slides (Cytospin; Shandon Southern, Runcorn, United Kingdom), and then stained with Diff-quick (Dade Behring AG, Düdingen, Switzerland). At least 80 monocytes were examined, and the proportion of monocytes containing surface-associated or internalized bacteria was determined.

Assays for phagocytosis and intracellular killing by whole blood. The capacity of whole blood to eliminate live pneumococci was assessed as described previously, with minor modifications (13). *S. pneumoniae* CCUG 10708 was grown to early exponential phase (A_{600}, 0.15 to 0.2) in Todd-Hewitt broth with yeast extract supplemented with 2% choline chloride, and approximately 10^4 CFU was added to 1 ml of heparinized human blood. After incubation with end-over-end rotation at 37°C for 2 to 3 h, serial dilutions were plated in duplicate on horse blood agar. To include intracellular bacteria in the counts, Triton X-60 was added at a final concentration of 0.1%, and the blood samples were vortexed vigorously before the dilution and plating process was repeated.

To elucidate the effect of intact and autolyzed bacteria on elimination of bacteria by blood, 6×10^7 cells/ml UV-inactivated D39 WT or D39 AL^- (as determined before autolysis) were added to the human blood cells 10 min before addition of 10^4 CFU/ml live *S. pneumoniae* CCUG 10708. Killing of *S. pneumoniae* CCUG 10708 was assessed as described above.

Statistical analysis. The Wilcoxon matched-pair test was used to compare cytokine production induced by AL-positive bacteria and cytokine production induced by AL-negative bacteria. The same test was used to compare cytokine induction by intact bacteria alone and cytokine induction by intact bacteria mixed with autolyzed, sonicated, or soluble or pellet fractions of autolyzed bacteria. The Mann-Whitney test was used to compare the cytokines induced by *S. pneumoniae* with the cytokines induced by the related viridans group streptococci and D39 WT with D39 PLY^-. Further, the Mann-Whitney test was used to compare the mean numbers of phagocytosed pneumococci or pneumococci interacting with monocytes with and without addition of autolyzed pneumococci. The Mann-Whitney test was also used to compare the numbers of viable bacteria remaining after incubation with blood cells for 2 to 3 h with and without addition of UV-inactivated intact or autolyzed pneumococci.

RESULTS

PBMC produce less phagocyte-activating cytokines when they are stimulated with *S. pneumoniae* than when they are stimulated with related *Streptococcus* species. TNF and IFN-γ are the major activators of phagocyte-mediated bacterial killing, while IL-12 is a trigger of IFN-γ production. In general, gram-positive bacteria induce production of large amounts of TNF, IFN-γ, and IL-12 in freshly isolated human PBMC (23, 24).

Pneumococci are closely related to streptococci in the viridans group, but they have a range of unique virulence factors, including the peculiar ability to undergo spontaneous autolysis in stationary culture. One strain each of *S. pneumoniae* (CCUG 33774) and seven related *Streptococcus* species (*S. mutans*, *S. salivarius*, *S. anginosus*, *S. intermedius*, *S. sanguinis*, *S. oralis*, and *S. mitis*) were added to human blood donor PBMC. Whereas all viridans group streptococci induced production of massive quantities of TNF and IFN-γ (8 to 38 ng/ml and 50 to 160 ng/ml, respectively), the amounts induced by the *S. pneumoniae* strain were 10 to 50 times lower (Fig. 1). TNF is produced by monocytes after direct contact with bacteria, while IFN-γ is produced by T cells and NK cells in response to IL-12 from monocytes and related cells. In accord with this, we observed no IL-12 in *S. pneumoniae*-stimulated cultures, while all cultures stimulated with viridans streptococci contained very large amounts of IL-12 after 24 h (1 to 5 ng/ml) (Fig. 1). In sharp contrast, pneumococci induced the production of levels of IL-6, IL-8, and IL-10 similar to the levels obtained with the other *Streptococcus* species tested (Fig. 1).

To investigate whether these findings could be generalized, the cytokine production induced by six additional *S. pneumoniae* strains belonging to various sero- and genotypes (A6, A7, A17, A22, A24, and CCUG 23261 [Table 1]) was examined. All of the pneumococcal strains tested induced production of little TNF and IFN-γ (0.3 to 1.2 ng/ml and 1 to 20 ng/ml, respectively), and none of them induced production of detectable IL-12 (≥20 pg/ml). Thus, on average, the pneumococcal strains induced production of 30 times less TNF, 15 times less IFN-γ, and at least 50 times less IL-12 than related streptococcal species. On the other hand, the levels of IL-6, IL-8, and IL-10 were comparable to the levels induced by the other *Streptococcus* species (data not shown). Thus, the pattern of cytokines generated by human PBMC in response to pneumococci was characterized by conspicuous reductions in the levels of cytokines which promote bacterial killing by macrophages (39, 40) and neutrophils (4).

Chemical inhibition of autolysis restores the ability of pneumococci to induce production of phagocyte-activating cytokines. A fraction of *S. pneumoniae* cells spontaneously lyse when a culture reaches stationary phase due to activation of PG-degrading enzymes called ALs, and the dominant AL is LytA (27). We have previously shown that soluble components of the gram-positive cell wall, such as PG and LTA, readily

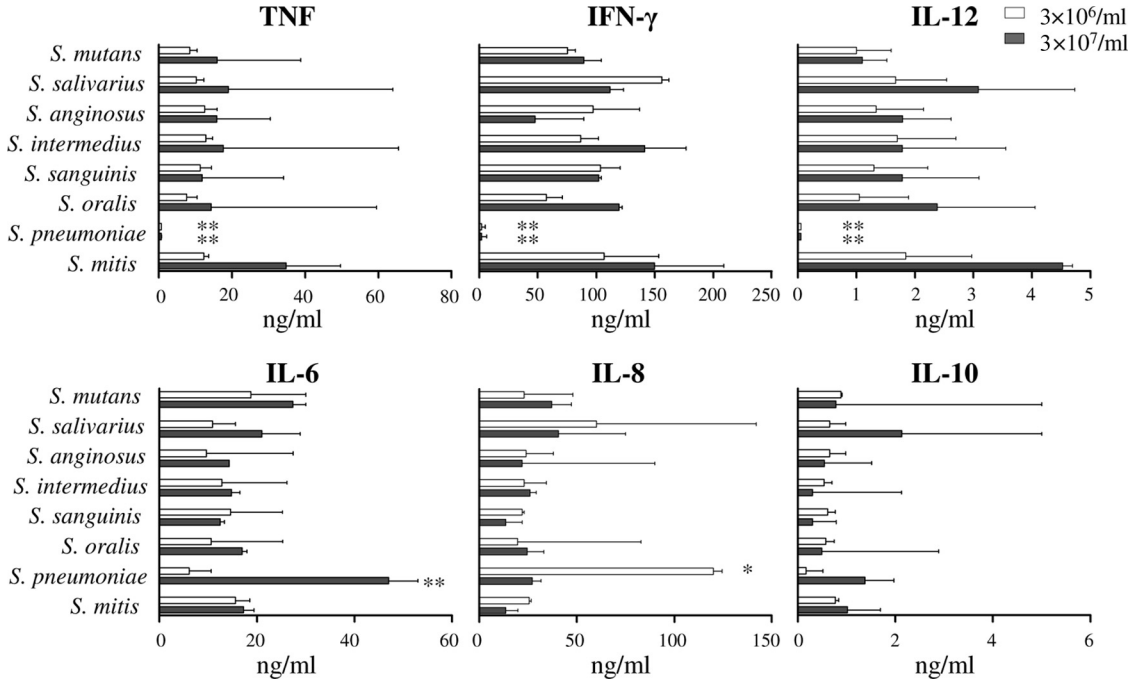

FIG. 1. *S. pneumoniae* induces production of less TNF, IFN-γ, and IL-12 from PBMC than other *Streptococcus* viridans group bacteria. PBMC (2×10^6 cells/ml) from three blood donors were stimulated with eight *Streptococcus* strains, including *S. pneumoniae* A17, using concentrations of 3×10^7 and 3×10^6 bacteria/ml. Cytokine production was measured using the supernatant after 24 h (TNF, IL-12, IL-6, IL-8, IL-10) or 5 days (IFN-γ). The bars and error bars indicate the median cytokine concentrations produced and interquartile ranges. The concentrations of cytokines produced in response to *S. pneumoniae* compared to the concentrations produced in response to other streptococcal strains were analyzed using the Mann-Whitney test. *, $P < 0.05$; **, $P < 0.01$.

induce IL-6 and IL-8 production in monocytes but induce production of much less TNF and IL-12 than the corresponding amount of intact gram-positive bacteria (1, 23). We therefore speculated that the specific failure of pneumococci to induce production of TNF, IFN-γ, and IL-12 may be related to the capacity of pneumococci to autolyze.

To determine if spontaneous autolysis was responsible for the unique cytokine pattern induced by pneumococci, the seven *S. pneumoniae* isolates were grown to stationary phase in normal broth or broth supplemented with 2% choline chloride. Growth in medium with excess choline is known to inhibit the function of ALs (6, 19). The bacteria were washed, and the concentration was adjusted to 7×10^8 cells/ml (in choline-containing PBS for bacteria cultured with an excess of choline), after which the preparations were UV inactivated and frozen at −70°C. After thawing, preparations of pneumococci cultured in the presence of high levels of choline (CC^+) contained almost the original number of intact bacteria, as determined by counting with a microscope, while for bacteria cultured and prepared without added choline (CC^-), <30% of the original number of bacteria were in the intact form and the rest of the bacteria had been degraded (Fig. 2A). Preparations containing variable proportions of intact and fragmented bacteria were added to human blood donor cells at 1/20 and 1/200 dilutions (corresponding to 3×10^7 and 3×10^6 bacteria/ml, respectively, in the original preparations before autolysis occurred).

Pneumococcal strains prevented from autolysis by culture and preparation in the presence of high concentrations of choline (CC^+) induced the production of significantly more TNF, IFN-γ, and IL-12 than the same strains cultured and prepared under standard conditions (CC^-) (Fig. 2). In contrast, the induction of production of IL-6, IL-8, and IL-10 was the same regardless of the culture conditions. The same pattern was seen for all seven pneumococcal strains, but the strongest induction of IL-12, TNF, and IFN-γ production among the CC^+ bacteria and the largest difference related to culture conditions (CC^+/CC^-) were seen for strain A17, which is a serotype 14 and MLST 124 strain (Fig. 2B). To exclude the possibility that choline had an effect on PBMC function, *S. mitis*, which is incapable of autolysis, was grown and prepared in media with and without choline and used to stimulate PBMC. The presence of choline did not affect the production of any of the cytokines in response to *S. mitis* (data not shown).

We compared the cytokine responses to intact pneumococci and to the soluble cell wall components PG and LTA, as well as MDP, which is the smallest basic unit of PG interacting with NOD2 (18). We assumed that PG accounts for 50% of the gram-positive bacterial cell mass and that the bacteria are spherical with a diameter of 1 μm. Using these assumptions, 3×10^7 bacteria would contain 10 μg of PG and probably less LTA. Stimulation of PBMC with 10 μg/ml of PG or LTA induced production of levels of IL-6, IL-8, and IL-10 that were similar to or higher than the levels produced when 3×10^7 cells/ml of *S. pneumoniae* or *S. mitis* were used for induction, while intact bacteria were much more potent inducers of TNF, IFN-γ, and IL-12 production than corresponding amounts of

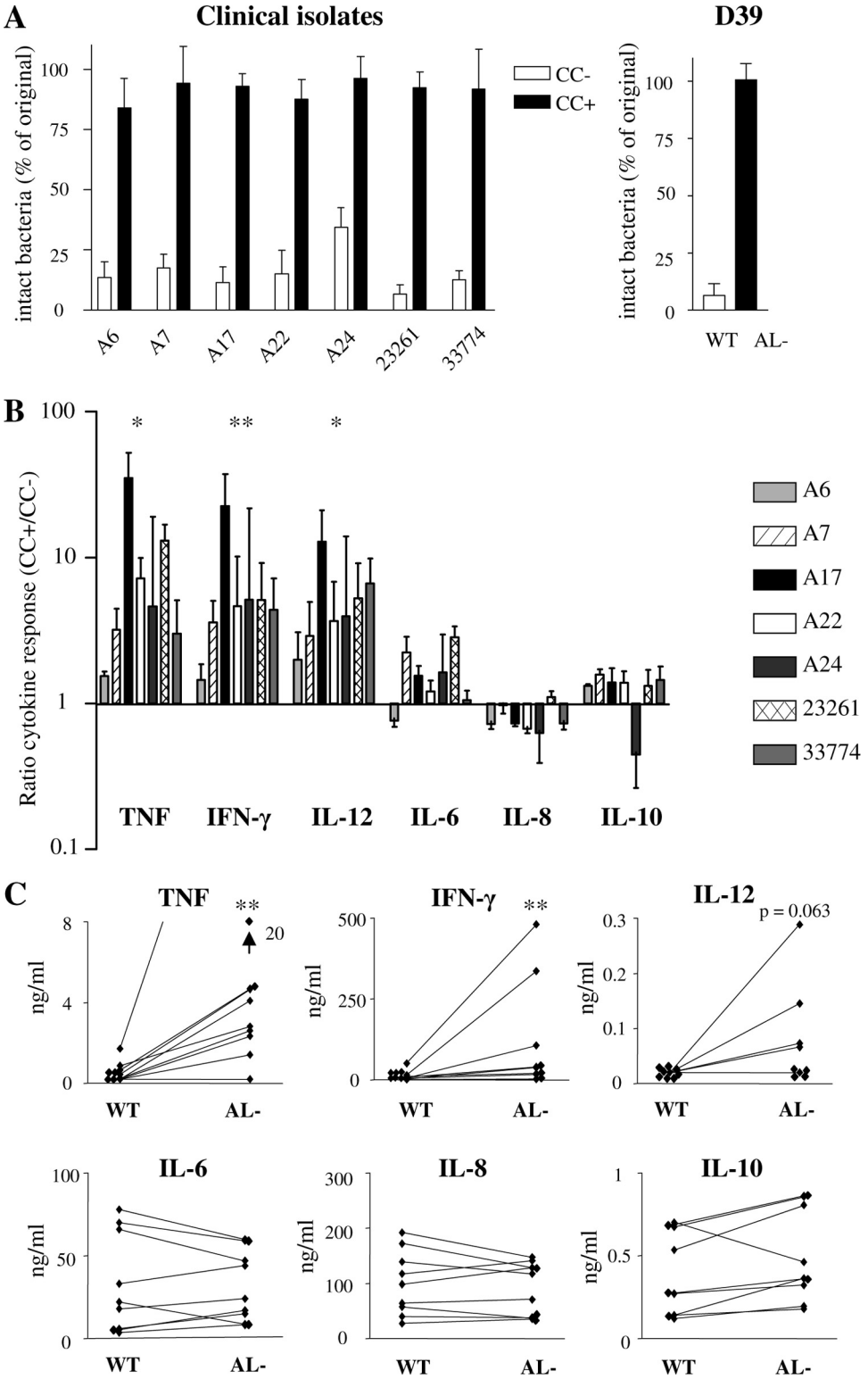

FIG. 2. Pneumococci with inhibited ALs induce production of higher concentrations of TNF, IFN-γ, and IL-12 from PBMC than their AL-positive counterparts. Seven clinical isolates of *S. pneumoniae* (A6, A7, A17, A22, A24, CCUG 23261, and CCUG 33774 [Table 1]) were cultured, washed, and prepared in normal broth (CC−) or in broth containing high levels of the autolysis inhibitor choline chloride (CC+). The AL-negative mutant D39 AL$^-$ and the wild-type control D39 WT (WT) were cultured and prepared in normal broth. (A) The amounts of autolysis that occurred in the bacterial preparations during the process that included UV inactivation, freezing, and thawing were determined by counting the remaining intact cells with a microscope and comparing the results to the original concentrations. Each cell in bacterial chains was counted separately, and the percentages of intact cells (based on the 7×10^8 cells/ml which were present in the preparations before UV inactivation; mean ± standard deviation for three individual counts) are shown. (B) PBMC from six blood donors were stimulated with the seven clinical isolates

TABLE 2. Cytokine production in human PBMC in response to bacteria and doses of bacterial products[a]

Stimulus	Cytokine concn (ng/ml)					
	TNF	IFN-γ	IL-12	IL-6	IL-8	IL-10
S. pneumoniae A17 CC^+ (3×10^7 bacteria/ml)	11 ± 9.7	147 ± 180	0.15 ± 0.2	45 ± 29	66 ± 32	0.57 ± 0.33
S. pneumoniae CCUG 10708 CC^+ (3×10^7 bacteria/ml)	30 ± 12	372 ± 207	0.43 ± 0.3	41 ± 14	70 ± 17	1.4 ± 0.75
S. mitis CCUG 31661 (3×10^7 bacteria/ml)	30 ± 14	310 ± 170	1.05 ± 0.85	30 ± 10	41 ± 14	1.3 ± 0.65
PG (*S. aureus*) (10 μg/ml)	1.9 ± 1	4 ± 3.9	0.04 ± 0.03	51 ± 33	327 ± 91	1.5 ± 1
MDP (10 μg/ml)	0.18 ± 0.12	1.2 ± 1.3	0.06 ± 0.08	<1	<5	<0.2
LTA (*S. aureus*) (10 μg/ml)	1.2 ± 1.2	3.8 ± 3	0.034 ± 0.04	41.5 ± 3.5	318 ± 71	1.0 ± 0.2

[a] PBMC were incubated with various stimuli, and the production of cytokines was measured using the supernatant after 24 h (TNF, IL-12, IL-6, IL-8, and IL-10) or after 5 days (IFN-γ) by an enzyme-linked immunosorbent assay. CC^+ indicates that the organism was grown and prepared in the presence of high levels of choline. The data are the means ± standard deviations for three to six blood donors.

isolated bacterial components (Table 2). MDP was a poor inducer of cytokine production at doses up to 10 μg/ml (Table 2).

Although the AL-inhibited pneumococci induced the production of levels of the phagocyte-activating cytokines that were higher than the levels induced by strains allowed to autolyze, they were still inferior inducers of production of these cytokines compared to related viridans group streptococci. This finding was most pronounced for IL-12 and may be related to the poor phagocytosis of encapsulated *S. pneumoniae*, as we have previously shown that phagocytosis is an absolute requirement for IL-12 production by human monocytes in response to gram-positive bacteria (1). Indeed, when the nonencapsulated strain *S. pneumoniae* CCUG 10708 was grown in a manner that inhibited autolysis, it induced the production of levels of TNF and IFN-γ similar to the levels induced by *S. mitis* and the production of only two to three times less IL-12 (Table 2).

The ability to stimulate production of phagocyte-activating cytokines is increased in a LytA-deficient strain of *S. pneumoniae*. Autolysis is achieved mainly by activation of the LytA enzyme (27). We compared the cytokine patterns induced by the LytA-negative mutant D39 AL^- and the wild-type control strain D39 WT. Stationary-phase wild-type and AL^- cultures were adjusted so their concentrations were the same and then UV inactivated and frozen. After the preparations were thawed, 94% of the wild-type bacteria had lysed, compared to only 5% of the AL^- bacteria (Fig. 2A). Indeed, *S. pneumoniae* D39 AL^- induced the production of significantly more TNF and IFN-γ than D39 WT (Fig. 2C). Again, IL-6 production, IL-8 production, and IL-10 production were induced equally well by lysed and intact pneumococci (Fig. 2C). IL-12 was produced by some of the blood donors in response to the mutant but by none of the donors in response to the wild-type strain (Fig. 2C). The pattern was similar when a 10-fold-lower dose of pneumococci was used (data not shown).

Release of pneumolysin is not responsible for the modulated cytokine pattern induced by autolyzed pneumococci. Pneumolysin (Ply) is an intracellular toxin known to be released during autolysis of pneumococci (5). To investigate if released Ply was responsible for the "pneumococcus-specific" cytokine pattern, we examined the cytokines produced in response to a pneumolysin mutant strain, *S. pneumoniae* D39 PLY^-, and the wild-type control strain D39 WT. D39 PLY^- and D39 WT both have the capacity to autolyze and induced practically identical cytokine patterns (see Fig. S1 in the supplemental material). Release of Ply into the supernatant of a wild-type strain but not of a PLY^- mutant strain was previously confirmed by Western blotting (33). We concluded that release of Ply during autolysis was not responsible for the reduction in the production of phagocyte-activating cytokines induced by pneumococci capable of autolysis.

Reduced PBMC viability cannot explain the altered cytokine production. The viability of PBMC from six blood donors was assessed after culture for 24 h with D39 WT, D39 AL^-, D39 PLY^-, and clinical isolates A17 and CCUG 23261 cultured with or without added choline. The viability of PBMC was assessed by determining the resistance to uptake of the DNA-binding dye 7-amino-actinomycin D and was analyzed by flow cytometry. Cultures stimulated with bacteria contained slightly more dead cells than nonstimulated cultures contained (6.4% ± 2.1% versus 2.9% ± 0.6%; $P < 0.01$). However, cultures stimulated with pneumococci capable of autolysis contained no more dead cells than cultures stimulated with bacteria prevented from autolyzing contained (data not shown). Thus, cell death could not account for the selectively reduced production of phagocyte-activating cytokines in response to autolyzed pneumococci.

Autolyzed pneumococci induce the production of less phagocyte-activating cytokines than intact pneumococci at all time points tested. To exclude the possibility that our findings were due to differences in the kinetics of cytokine production in

of *S. pneumoniae* grown under normal conditions or in the presence of excess choline using 3×10^7 bacteria/ml (measured before autolysis took place; 3×10^6 bacteria/ml for IL-12). The cytokine production was measured using the supernatant after 24 h (TNF, IL-12, IL-6, IL-8, and IL-10) or 5 days (IFN-γ). The cytokine responses to each *S. pneumoniae* strain when cells were grown in normal broth or in broth with added choline are expressed as the cytokine response ratio (CC^+/CC^-) (mean and standard deviation) and were compared using the Wilcoxon matched-pair test. *, $P < 0.05$; **, $P < 0.01$. (C) PBMC were stimulated with 3×10^7 bacteria/ml (before autolysis) of *S. pneumoniae* D39 WT or the LytA-deficient mutant D39 AL^-, and the cytokine production was measured using the supernatant after 24 h (TNF, IL-12, IL-6, IL-8, and IL-10) or 5 days (IFN-γ). Each symbol indicates the response of one of nine blood donors. The cytokine responses to wild-type and mutant bacteria in the same donor were compared using the Wilcoxon matched-pair test. **, $P < 0.01$.

response to intact and autolyzed pneumococci, we measured cytokines in supernatants at 4, 8, 12, 18, and 24 h and 3, 5, and 7 days after stimulation with pneumococci capable (D39 WT and A17 CC^-) or not capable (D39 AL^- and A17 CC^+) of autolysis. Confirming our findings for the 24-h and 5-day time points, larger amounts of TNF and IFN-γ were produced in response to intact bacteria at all time points tested. IL-12 was not always detected, but when it was present, the level was maximal after 24 h and the cytokine was produced only in response to bacteria prevented from autolysis (D39 AL^- or A17 CC^+). Production of equal amounts of IL-6, IL-8, and IL-10 was induced in response to autolyzed and intact bacteria at all time points examined. Similar results were obtained using high and low doses of bacteria (3×10^7 and 3×10^6 bacteria/ml before autolysis) (see Fig. S2 in the supplemental material; data not shown). We concluded that a shift in kinetics in response to intact and autolyzed bacteria could not account for the low level of TNF at 24 h or the low level of IFN-γ at 5 days after stimulation with autolyzed pneumococci. Furthermore, the absence of IL-12 or production of a very low level of IL-12 in response to pneumococci, particularly pneumococci capable of autolysis, was confirmed at all time points.

The presence of bacteria that have undergone autolysis reduces the production of phagocyte-activating cytokines in response to intact bacteria. Although the preparations of AL-positive strains contained up to 20% intact bacterial cells when they were examined with a microscope, a high dose of AL-positive pneumococci (3×10^7 bacteria/ml before autolysis) still induced production of significantly less TNF and IFN-γ than a 10-fold-lower dose of intact pneumococci (data not shown). Thus, the absence of intact bacteria might not be the only explanation for the low levels of phagocyte-activating cytokines produced. We hypothesized that bacterial degradation products released during autolysis inhibited the cytokine production which otherwise would be induced by intact bacteria.

To test this hypothesis, PBMC from six blood donors were stimulated with two intact clinical pneumococcal isolates (CCUG 23261 and the otitis media strain A17 cultured and prepared with choline) at a concentration of 3×10^7 or 3×10^6 bacteria/ml in the presence of various concentrations of the corresponding autolyzed bacteria (3×10^7, 3×10^6, and 3×10^5 cells/ml before autolysis). Figure 3 shows that addition of preparations containing autolyzed bacteria reduced the amounts of TNF, IFN-γ, and IL-12 induced by intact bacteria in a dose-dependent manner. Compared to intact bacteria, significant inhibition of the TNF ($P < 0.05$) and IFN-γ ($P < 0.01$) responses was obtained by adding a 10-fold-lower dose of autolyzed bacteria (Fig. 3). Thus, if only 1 bacterium in 10 autolyzes, the TNF and IFN-γ responses to the remaining intact bacteria are significantly reduced. The IL-12 responses were optimal when 3×10^6 intact bacteria/ml were used, and addition of an equal amount of autolyzed bacteria caused significant inhibition ($P < 0.05$) (Fig. 3). Production of IL-6, IL-8, and IL-10 was not affected or was slightly increased when autolyzed bacteria were added to intact pneumococci (Fig. 3), which was expected, as both fragmented and intact bacteria could induce production of these cytokines.

The same pattern of strong and dose-dependent inhibition of the phagocyte-activating cytokines was seen when D39 AL^- was mixed with graded concentrations of D39 WT. Addition of a 10-times-lower dose of autolyzed wild-type bacteria significantly reduced the production of TNF in response to D39 AL^-, and adding a 100-fold-lower dose of wild-type bacteria significantly inhibited the IFN-γ response to D39 AL^- (see Fig. S3 in the supplemental material). Most donors did not produce IL-12 in response to this strain, but when IL-12 was detected in response to intact bacteria, the amount was reduced by adding bacteria capable of autolysis (data not shown). The levels of IL-6, IL-8, and IL-10 were the same or increased when autolyzed bacteria were added to the intact bacteria (see Fig. S3 in the supplemental material).

The soluble fraction of autolyzed bacteria inhibits induction of phagocyte-activating cytokines by intact bacteria. The preparations of wild-type bacteria grown in normal broth contained both intact bacteria and presumably both larger and smaller fragments of bacterial cell walls. Autolyzed *S. pneumoniae* D39 WT and A17 were separated into soluble and pellet fractions by centrifugation ($13{,}000 \times g$, 10 min). Various amounts of the soluble or pellet fractions were mixed with intact D39 AL^- or A17 CC^+ and added to PBMC, after which cytokine production was measured. The inhibition of TNF and IFN-γ production was most pronounced when the supernatant fraction was added. Thus, supernatant originating from only 3×10^6 autolyzed bacteria/ml significantly inhibited TNF production induced by 3×10^7 intact bacteria/ml, while the pellet from 3×10^7 autolyzed bacteria was required for a similar inhibitory effect (Fig. 4A). The level of IL-12 produced was low or undetectable, but, when IL-12 was present, its production was inhibited by both the pellet and the supernatant fraction (data not shown).

Sonicated bacteria and soluble bacterial components inhibit production of phagocyte-activating cytokines in response to intact bacteria. While phagocytes may have to be activated in order to efficiently disrupt microbial cell walls, such activation is not necessary once the bacteria have been degraded. Thus, it would be logical for immune cells to turn off production of TNF and IFN-γ in response to bacterial fragments. Sonicated gram-positive bacteria induce less TNF, IFN-γ, and IL-12 production than the same amount of intact bacteria (1; unpublished data). We tested whether fragments of streptococci generated by sonication also downregulated these cytokines in response to intact bacteria. *S. mitis*, which is not capable of autolysis, was subjected to sonication, and PBMC from six blood donors were stimulated with 3×10^7 bacteria/ml of intact UV-inactivated *S. mitis* in the presence or absence of various doses of sonicated *S. mitis*. Dose-dependent inhibition of production of TNF, IFN-γ, and IL-12 (i.e., cytokines yielding phagocyte activation) but not of IL-6, IL-8 or IL-10 was seen when intact and sonicated *S. mitis* cells were mixed (see Fig. S4 in the supplemental material).

We also determined whether purified PG, MDP, and LTA (i.e., components of the gram-positive cell wall) could inhibit TNF, IFN-γ, and IL-12 production induced by intact pneumococci. PBMC from six blood donors were stimulated with *S. pneumoniae* A17 CC^+ in the presence or absence of various amounts of PG, MDP, and LTA. Indeed, both PG and MDP significantly inhibited production of TNF and IFN-γ but not production of IL-6, IL-8, and IL-10 in response to intact *S. pneumoniae* A17. LTA significantly inhibited IFN-γ produc-

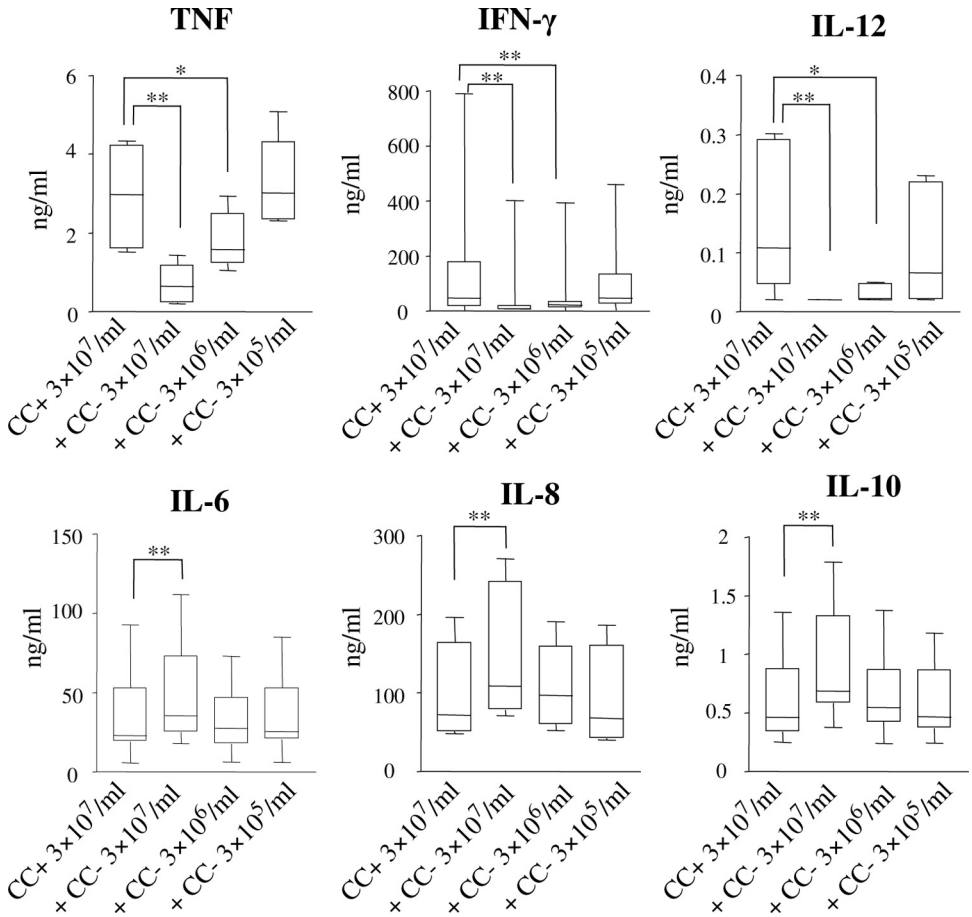

FIG. 3. Autolyzed bacteria selectively inhibit the production of TNF, IFN-γ, and IL-12 in response to intact pneumococci. PBMC from six blood donors were stimulated with 3×10^7 bacteria/ml (or 3×10^6 bacteria/ml for measurements of IL-12) of clinical isolates A17 and CCUG 23226 cultured in the presence of excess choline chloride (CC+). Graded concentrations of the corresponding bacteria cultured in normal broth (CC−) were added, and the cytokines produced were measured using the supernatant after 24 h or after 5 days (for IFN-γ). The medians and quartiles are indicated by boxes, and the error bars indicate the 10th and 90th percentiles. The responses to intact bacteria (CC+) with and without addition of autolyzed bacteria (CC−) in the same donor were compared using the Wilcoxon matched-pair test. *, $P < 0.05$; **, $P < 0.01$.

tion but not production of the other cytokines in response to A17 CC^+ (Fig. 4B). Since the level of IL-12 was low or production of IL-12 was undetectable in response to A17 CC^+, PBMC were also stimulated with *S. mitis* together with PG, MDP, and LTA. The *S. mitis*-induced IL-12 production was inhibited by PG, MDP, and LTA strongly and in a dose-dependent manner (data not shown). These data confirm and extend our previous findings that IL-12 production by monocytes in response to bifidobacteria is downregulated by bacterial sonicates, as well as by soluble cell wall components, such as PG and LTA (1).

Autolyzed bacteria block access of intact bacteria to the monocyte surface and phagocytosis. We speculated that fragments of the pneumococcal cell wall, released during autolysis, block interactions between intact bacteria and monocytes. PBMC were incubated with intact pneumococci (3×10^7 bacteria/ml) in the presence or absence of autolyzed bacteria (originally 3×10^7 bacteria/ml) for 30 min, after which they were spun onto glass slides, stained, and examined microscopically. However, the bacteria were not internalized after 30 min of incubation, reflecting the resistance of encapsulated pneumococci to uptake by phagocytes in the absence of capsule-specific antibodies. *S. pneumoniae* D39 AL^- and clinical isolates of pneumococci prevented from autolysis bound in chains to the monocyte surface (Fig. 5A), but when the same bacteria were cultured under conditions which allowed them to autolyze, they exhibited negligible interactions with monocytes. After 30 min of incubation of PBMC with D39 AL^-, 22% ± 4% (mean ± standard deviation) of the monocytes had D39 AL^- chains bound to their surfaces. Addition of both intact D39 AL^- and autolyzed D39 wild-type bacteria simultaneously reduced the percentage of monocytes interacting with bacteria to 9% ± 3% ($P < 0.05$ compared with the AL mutant alone). Similarly, interactions between monocytes and bacteria were significantly reduced when equal amounts of intact and autolyzed *S. pneumoniae* A17 or CCUG 23261 bacteria were added to monocytes compared to the results for incubation with only intact A17 or CCUG 23261 (data not shown). Thus, fragments released during autolysis appeared to block access of intact pneumococci to the monocyte surface.

As encapsulated virulent pneumococci were not phagocytosed under the conditions employed, we used a capsule-neg-

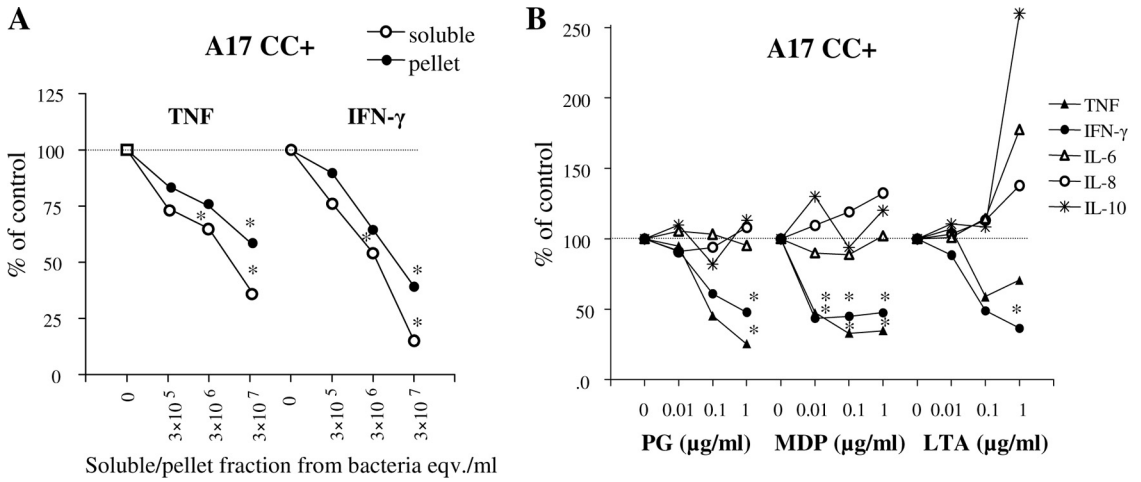

FIG. 4. Production of phagocyte-activating cytokines in response to intact bacteria is selectively inhibited by soluble cell wall fragments (A) The soluble and pellet fractions of autolyzed *S. pneumoniae* A17 were separated by centrifugation (see Materials and Methods). PBMC from six blood donors were stimulated with 3×10^7 bacteria/ml of intact *S. pneumoniae* A17 (A17 CC+) in the presence or absence of the soluble or pellet fraction originating from 3×10^5, 3×10^6, or 3×10^7 autolyzed bacteria/ml (A17 CC^-). Production of TNF and IFN-γ was measured using the 1-day and 5-day supernatants, respectively. The responses to intact bacteria with and without addition of the soluble or pellet fraction of autolyzed bacteria in the same donor were compared using the Wilcoxon matched-pair test. *, $P < 0.05$. (B) PBMC from six blood donors were stimulated with 3×10^7 bacteria/ml of intact *S. pneumoniae* A17 (A17 CC+) in the absence or presence of various doses of PG, MDP, or LTA. Cytokines were measured using the supernatant after 24 h (TNF, IL-6, IL-8, and IL-10) or 5 days (IFN-γ). The responses to *S. pneumoniae* A17 with and without addition of PG, MDP, or LTA in the same donor were compared using the Wilcoxon matched-pair test. *, $P < 0.05$.

ative pneumococcal strain (CCUG 10708) to study the effect of autolyzed bacteria on phagocytosis. PBMC from four blood donors were incubated with UV-inactivated *S. pneumoniae* CCUG 10708 cultured and prepared in the presence of an excess choline. In contrast to the encapsulated strains, this pneumococcal strain was frequently seen inside the monocytes within vacuoles (Fig. 5B), and 18% ± 3% (mean ± standard deviation) of the monocytes had phagocytosed bacteria after 30 min of incubation. When an equal amount of autolyzed *S. pneumoniae* CCUG 10708 grown without excess choline was added, only 11% ± 2% of the monocytes had internalized bacteria after 30 min of incubation ($P < 0.05$ compared to CCUG 10708 CC^+ alone). Also, the presence of autolyzed *S. pneumoniae* D39 and A17 inhibited internalization of *S. pneumoniae* CCUG 10708 to at least a similar degree (data not shown).

Autolyzed pneumococci block the in vitro bactericidal effect of human blood cells. Elimination of live pneumococci by blood cells in the absence and presence of autolyzed pneumococci was assessed by viable counting. The nonencapsulated strain *S. pneumoniae* CCUG 10708 was cultured in the presence of an excess of choline to early exponential phase (A_{600}, ~0.17). Live bacteria (10,000 CFU/ml) and heparinized blood were incubated at 37°C for 2 to 3 h, and the number of remaining bacteria was then assessed by viable counting. Approximately 300 CFU/ml *S. pneumoniae* remained in the blood plasma after 2 to 3 h (Fig. 5C). Addition of UV-inactivated intact D39 AL^- did not affect the rate of bacterial elimination. However, when the same amount of UV-inactivated autolyzed D39 WT was added, approximately 10 times more viable pneumococci remained in the blood plasma (Fig. 5C). Similarly, viable counting after Triton lysis of blood cells (i.e., intra- and extracellular bacteria were measured simultaneously) showed that more viable pneumococci tended to remain in the blood after addition of UV-inactivated autolyzed bacteria but not after addition of UV-inactivated intact bacteria (Fig. 5C). Thus, the presence of autolyzed bacteria, but not the presence of inactivated intact bacteria, decreased the ability of blood cells to eliminate viable pneumococci.

DISCUSSION

Gram-positive bacteria are, in general, very strong inducers of TNF and IL-12 production in human monocytes and IFN-γ production in PBMC (24). Here we show that *S. pneumoniae* is a striking exception to this rule. Eight *S. pneumoniae* wild-type strains, representing various sero- and genotypes, induced, on average, production of 30-fold less TNF and 15-fold less IFN-γ in PBMC than *S. mitis* and other closely related species in the viridans *Streptococcus* group. Furthermore, most pneumococcal strains completely failed to induce IL-12 production. *S. pneumoniae* has previously been reported to induce production of much lower levels of TNF than group B streptococci in murine macrophages (22). As TNF and IFN-γ are the key activators of phagocyte microbicidal mechanisms and IL-12 induces IFN-γ production in T cells and NK cells, we regard the capacity of pneumococci to avoid triggering production of these cytokines as a putative virulence mechanism.

The poor ability of pneumococci to induce production of TNF, IFN-γ, and IL-12 was related, at least in part, to the capacity of these organisms to undergo autolysis in stationary culture. When PBMC were stimulated with pneumococci that were prevented from autolyzing, either by culture in the presence of excess choline or by mutation of the major LytA AL gene, significantly more TNF and IFN-γ were produced than when PBMC were stimulated with the AL-positive counterparts. Although most clinical pneumococcal strains induced production of low levels of IL-12 even when autolysis was

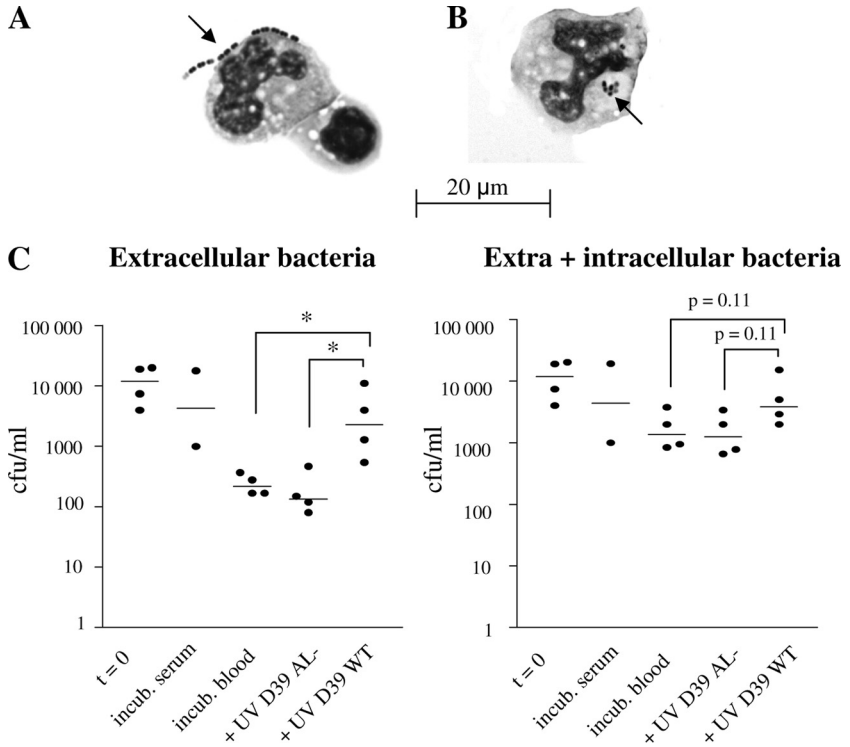

FIG. 5. Fragments of autolyzed pneumococci inhibit phagocytosis. (A) Encapsulated *S. pneumoniae* D39 AL$^-$ bound in chains to the monocyte surface. (B) Nonencapsulated *S. pneumoniae* CCUG 10708 inside a monocyte, within a vacuole. (C) Nonencapsulated *S. pneumoniae* strain CCUG 10708 was grown to early exponential phase (A_{600}, ~0.17) in the presence of 2% choline chloride. UV-killed intact bacteria (D39 AL$^-$) or autolyzed bacteria (D39 WT) (at a concentration of 6×10^7 bacteria/ml before autolysis) were added to 1 ml of heparinized human blood cells, after which live *S. pneumoniae* CCUG 10708 (10^4 CFU/ml) was added and the mixture was rotated end over end at 37°C for 2 to 3 h. Viable counts were determined by quantitative culture of blood samples on horse blood agar before and after lysis of blood cells with Triton. Each symbol indicates the counts obtained for one of four blood donors. Bacterial counts obtained with and without addition of UV-inactivated intact (UV D39 AL$^-$) or autolyzed (UV D39 WT) pneumococci were compared using the Mann-Whitney test. *, $P < 0.05$.

inhibited, slightly higher levels were produced in PBMC in response to intact bacteria than in response to autolyzed bacteria. In contrast, similar amounts of IL-6, IL-8, and IL-10 were produced in response to intact and autolyzed strains, and pneumococci induced production of as much IL-6, IL-8, and IL-10 as related *Streptococcus* species. Further, production of similar amounts of the latter cytokines was induced by both intact bacteria and purified PG or LTA, while production of much larger amounts of TNF, IFN-γ, and IL-12 was induced by intact bacteria than by a corresponding amount of isolated bacterial components (Table 2). Although the PG and LTA utilized in this study were isolated from *S. aureus* and not *S. pneumoniae*, previous studies have shown that LTA from both of these species stimulate Toll-like receptor 2 and that *S. aureus* LTA is an even more potent inducer of TNF production in macrophages than *S. pneumoniae* LTA (44).

Collectively, our data point to a dichotomy between the stimuli inducing production of the two groups of cytokines; production of TNF, IFN-γ, and IL-12 is induced chiefly by intact bacterial particles, and production of IL-6, IL-8, and IL-10 is induced by both intact bacteria and their fragments. This confirms and extends our previous findings that IL-12 production is induced only by intact gram-positive bacteria and not by PG, LTA, or bacterial sonicates, while IL-6 is produced in response to both intact gram-positive bacteria and their fragments and cell wall components (1). TNF and IFN-γ enhance the lytic capacity of phagocytes, and it is logical that their production is induced by intact bacteria but not by their fragments.

Little IL-12 was produced in response to most virulent pneumococcal strains, even when autolysis was prevented. We suggest that the low level of phagocytosis of encapsulated pneumococci could be the reason for their limited capacity to trigger IL-12 production. We have previously shown that cytochalasin inhibits IL-12 production in response to intact gram-positive bacteria (1). The capsule-negative strain *S. pneumoniae* CCUG 10708 was phagocytosed to a greater degree and induced production of higher levels of IL-12, TNF, and IFN-γ than any of the encapsulated pneumococcal strains tested, while production of similar levels of the other cytokines was induced. Accordingly, phagocytosis of lactobacilli has been shown to be necessary for induction of IL-12 production, and strains relatively resistant to lysis within the macrophages were the strongest inducers of IL-12 production (46). Furthermore, phagocytosis of chitin particles induces production of TNF, IFN-γ, and IL-12 but not production of other cytokines, like IL-10, in mouse spleen cells (35, 45).

One possibility is that production of IL-12, as well as production of high levels of TNF, is induced by the presence of bacteria within the phagosome inside the phagocyte. Frag-

mented bacteria or components of the bacterial cell wall were not only inefficient in inducing production of IL-12, TNF, and IFN-γ but also inhibited production of these cytokines in response to intact bacteria in a dose-dependent manner. It is logical that fragments of bacteria turn off production of TNF and IFN-γ, since the purpose of the phagocyte-activating cytokines is to promote killing and decomposition of phagocytosed particles (26, 32, 43, 47). This kind of activation is no longer necessary once the microbe has been decomposed. We cannot distinguish whether this inhibition results from blocking of the necessary interactions between intact bacteria and cytokine-inducing receptors or whether the fragments themselves trigger inhibitory pathways reducing cytokine production.

Autolyzed bacteria reduced the tendency of blood cells to eliminate viable pneumococci in vitro. Further, interactions between monocytes and intact pneumococci were partially prevented by the presence of bacterial fragments. We propose that bacterial fragments released during autolysis have dual synergistic functions, both blocking phagocytosis of bacteria and preventing the normal triggering of phagocyte-activating cytokines aiming to increase the bactericidal mechanisms of phagocytes. We suggest that in pneumococci the autolytic mechanisms that are present in all bacteria, as they are necessary for restructuring the cell wall during growth and fission, are adapted to generate fragments that paralyze phagocyte effector functions in a fraction of bacteria that have reduced viability. As little as 10% of the pneumococcal cells needed to decay to significantly downregulate production of phagocyte-activating cytokines in response to the majority of viable, intact cells. Thus, bacterial cells which die also serve a posthumous purpose in preventing elimination of their living relatives. We propose that this mechanism contributes to the highly pathogenic nature of pneumococci.

ACKNOWLEDGMENTS

This work was supported by the Swedish Research Council (grants K2001-06GX-14072-01 and K2007-58X-08298-20-3), the Medical Faculty of Göteborg, the Swedish government under the ALF agreement, the King Gustaf Vth 80-Year Foundation, and the Adlerberth Research Foundation.

We are most grateful to Jessica Darenberg, Christina Johansson, and Birgitta Henriques-Normark at Smittskydds Institutet (SMI), Stockholm, Sweden, for serotyping and MLST the pneumococcal isolates.

REFERENCES

1. **Barkman, C., A. Martner, C. Hessle, and A. E. Wold.** 2008. Soluble bacterial constituents down-regulate secretion of IL-12 in response to intact Gram-positive bacteria. Microbes Infect. **10:**1484–1493.
2. **Berry, A. M., R. A. Lock, D. Hansman, and J. C. Paton.** 1989. Contribution of autolysin to virulence of *Streptococcus pneumoniae*. Infect. Immun. **57:** 2324–2330.
3. **Berry, A. M., J. Yother, D. E. Briles, D. Hansman, and J. C. Paton.** 1989. Reduced virulence of a defined pneumolysin-negative mutant of *Streptococcus pneumoniae*. Infect. Immun. **57:**2037–2042.
4. **Blanchard, D. K., H. Friedman, T. W. Klein, and J. Y. Djeu.** 1989. Induction of interferon-gamma and tumor necrosis factor by Legionella pneumophila: augmentation of human neutrophil bactericidal activity. J. Leukoc. Biol. **45:**538–545.
5. **Boulnois, G. J., J. C. Paton, T. J. Mitchell, and P. W. Andrew.** 1991. Structure and function of pneumolysin, the multifunctional, thiol-activated toxin of Streptococcus pneumoniae. Mol. Microbiol. **5:**2611–2616.
6. **Briese, T., and R. Hakenbeck.** 1985. Interaction of the pneumococcal amidase with lipoteichoic acid and choline. Eur. J. Biochem. **146:**417–427.
7. **Canvin, J. R., A. P. Marvin, M. Sivakumaran, J. C. Paton, G. J. Boulnois, P. W. Andrew, and T. J. Mitchell.** 1995. The role of pneumolysin and autolysin in the pathology of pneumonia and septicemia in mice infected with a type 2 pneumococcus. J. Infect. Dis. **172:**119–123.
8. **Chang, H. R., G. E. Grau, and J. C. Pechere.** 1990. Role of TNF and IL-1 in infections with Toxoplasma gondii. Immunology **69:**33–37.
9. **Chetty, C., and A. Kreger.** 1980. Characterization of pneumococcal purpura-producing principle. Infect. Immun. **29:**158–164.
10. **Chetty, C., and A. Kreger.** 1981. Role of autolysin in generating the pneumococcal purpura-producing principle. Infect. Immun. **31:**339–344.
11. **Cooper, A. M., J. Magram, J. Ferrante, and I. M. Orme.** 1997. Interleukin 12 (IL-12) is crucial to the development of protective immunity in mice intravenously infected with mycobacterium tuberculosis. J. Exp. Med. **186:**39–45.
12. **Enright, M. C., and B. G. Spratt.** 1998. A multilocus sequence typing scheme for Streptococcus pneumoniae: identification of clones associated with serious invasive disease. Microbiology **144:**3049–3060.
13. **Eriksson, A., and M. Norgren.** 2003. Cleavage of antigen-bound immunoglobulin G by SpeB contributes to streptococcal persistence in opsonizing blood. Infect. Immun. **71:**211–217.
14. **Facklam, R.** 2002. What happened to the streptococci: overview of taxonomic and nomenclature changes. Clin. Microbiol. Rev. **15:**613–630.
15. **Feldman, C., T. J. Mitchell, P. W. Andrew, G. J. Boulnois, R. C. Read, H. C. Todd, P. J. Cole, and R. Wilson.** 1990. The effect of Streptococcus pneumoniae pneumolysin on human respiratory epithelium in vitro. Microb. Pathog. **9:**275–284.
16. **Garcia, P., M. P. Gonzalez, E. Garcia, R. Lopez, and J. L. Garcia.** 1999. LytB, a novel pneumococcal murein hydrolase essential for cell separation. Mol. Microbiol. **31:**1275–1281.
17. **Garcia, P., M. Paz Gonzalez, E. Garcia, J. L. Garcia, and R. Lopez.** 1999. The molecular characterization of the first autolytic lysozyme of Streptococcus pneumoniae reveals evolutionary mobile domains. Mol. Microbiol. **33:** 128–138.
18. **Girardin, S. E., I. G. Boneca, J. Viala, M. Chamaillard, A. Labigne, G. Thomas, D. J. Philpott, and P. J. Sansonetti.** 2003. Nod2 is a general sensor of peptidoglycan through muramyl dipeptide (MDP) detection. J. Biol. Chem. **278:**8869–8872.
19. **Giudicelli, S., and A. Tomasz.** 1984. Attachment of pneumococcal autolysin to wall teichoic acids, an essential step in enzymatic wall degradation. J. Bacteriol. **158:**1188–1190.
20. **Haraguchi, S., N. K. Day, R. P. Nelson, Jr., P. Emmanuel, J. E. Duplantier, C. S. Christodoulou, and R. A. Good.** 1998. Interleukin 12 deficiency associated with recurrent infections. Proc. Natl. Acad. Sci. USA **95:**13125–13129.
21. **Havell, E. A.** 1989. Evidence that tumor necrosis factor has an important role in antibacterial resistance. J. Immunol. **143:**2894–2899.
22. **Henneke, P., O. Takeuchi, R. Malley, E. Lien, R. R. Ingalls, M. W. Freeman, T. Mayadas, V. Nizet, S. Akira, D. L. Kasper, and D. T. Golenbock.** 2002. Cellular activation, phagocytosis, and bactericidal activity against group B streptococcus involve parallel myeloid differentiation factor 88-dependent and independent signaling pathways. J. Immunol. **169:**3970–3977.
23. **Hessle, C., B. Andersson, and A. E. Wold.** 2000. Gram-positive bacteria are potent inducers of monocytic interleukin-12 (IL-12) while gram-negative bacteria preferentially stimulate IL-10 production. Infect. Immun. **68:**3581–3586.
24. **Hessle, C. C., B. Andersson, and A. E. Wold.** 2005. Gram-positive and Gram-negative bacteria elicit different patterns of pro-inflammatory cytokines in human monocytes. Cytokine **30:**311–318.
25. **Hirst, R. A., B. Gosai, A. Rutman, C. J. Guerin, P. Nicotera, P. W. Andrew, and C. O'Callaghan.** 2008. Streptococcus pneumoniae deficient in pneumolysin or autolysin has reduced virulence in meningitis. J. Infect. Dis. **197:** 744–751.
26. **Hoffman, M., and J. B. Weinberg.** 1987. Tumor necrosis factor-alpha induces increased hydrogen peroxide production and Fc receptor expression, but not increased Ia antigen expression by peritoneal macrophages. J. Leukoc. Biol. **42:**704–707.
27. **Holtje, J. V., and A. Tomasz.** 1976. Purification of the pneumococcal N-acetylmuramyl-L-alanine amidase to biochemical homogeneity. J. Biol. Chem. **251:**4199–4207.
28. **Jacobs, M. R.** 2004. Streptococcus pneumoniae: epidemiology and patterns of resistance. Am. J. Med. **117**(Suppl. 3A)**:**3S–15S.
29. **Karlsson, H., C. Hessle, and A. Rudin.** 2002. Innate immune responses of human neonatal cells to bacteria from the normal gastrointestinal flora. Infect. Immun. **70:**6688–6696.
30. **Kawamura, Y., R. A. Whiley, S. E. Shu, T. Ezaki, and J. M. Hardie.** 1999. Genetic approaches to the identification of the mitis group within the genus Streptococcus. Microbiology **145:**2605–2613.
31. **Kohler, W.** 2007. The present state of species within the genera Streptococcus and Enterococcus. Int. J. Med. Microbiol. **297:**133–150.
32. **Lorsbach, R. B., W. J. Murphy, C. J. Lowenstein, S. H. Snyder, and S. W. Russell.** 1993. Expression of the nitric oxide synthase gene in mouse macrophages activated for tumor cell killing. Molecular basis for the synergy between interferon-gamma and lipopolysaccharide. J. Biol. Chem. **268:**1908–1913.
33. **Martner, A., C. Dahlgren, J. C. Paton, and A. E. Wold.** 2008. Pneumolysin released during *Streptococcus pneumoniae* autolysis is a potent activator of intracellular oxygen radical production in neutrophils. Infect. Immun. **76:** 4079–4087.

34. **Mastroeni, P., A. Arena, G. B. Costa, M. C. Liberto, L. Bonina, and C. E. Hormaeche.** 1991. Serum TNF alpha in mouse typhoid and enhancement of a Salmonella infection by anti-TNF alpha antibodies. Microb. Pathog. **11:** 33–38.
35. **Nishiyama, A., S. Tsuji, M. Yamashita, R. A. Henriksen, Q. N. Myrvik, and Y. Shibata.** 2006. Phagocytosis of N-acetyl-D-glucosamine particles, a Th1 adjuvant, by RAW 264.7 cells results in MAPK activation and TNF-alpha, but not IL-10, production. Cell. Immunol. **239:**103–112.
36. **O'Brien, D. P., D. E. Briles, A. J. Szalai, A. H. Tu, I. Sanz, and M. H. Nahm.** 1999. Tumor necrosis factor alpha receptor I is important for survival from *Streptococcus pneumoniae* infections. Infect. Immun. **67:**595–601.
37. **Ortqvist, A., J. Hedlund, and M. Kalin.** 2005. Streptococcus pneumoniae: epidemiology, risk factors, and clinical features. Semin. Respir. Crit. Care Med. **26:**563–574.
38. **Paton, J. C., B. Rowan-Kelly, and A. Ferrante.** 1984. Activation of human complement by the pneumococcal toxin pneumolysin. Infect. Immun. **43:** 1085–1087.
39. **Peck, R.** 1985. A one-plate assay for macrophage bactericidal activity. J. Immunol. Methods **82:**131–140.
40. **Philip, R., and L. B. Epstein.** 1986. Tumour necrosis factor as immunomodulator and mediator of monocyte cytotoxicity induced by itself, gamma-interferon and interleukin-1. Nature **323:**86–89.
41. **Rubins, J. B., D. Charboneau, J. C. Paton, T. J. Mitchell, P. W. Andrew, and E. N. Janoff.** 1995. Dual function of pneumolysin in the early pathogenesis of murine pneumococcal pneumonia. J. Clin. Investig. **95:**142–150.
42. **Rubins, J. B., and C. Pomeroy.** 1997. Role of gamma interferon in the pathogenesis of bacteremic pneumococcal pneumonia. Infect. Immun. **65:** 2975–2977.
43. **Schaible, U. E., S. Sturgill-Koszycki, P. H. Schlesinger, and D. G. Russell.** 1998. Cytokine activation leads to acidification and increases maturation of Mycobacterium avium-containing phagosomes in murine macrophages. J. Immunol. **160:**1290–1296.
44. **Seo, H. S., S. M. Michalek, and M. H. Nahm.** 2008. Lipoteichoic acid is important in innate immune responses to gram-positive bacteria. Infect. Immun. **76:**206–213.
45. **Shibata, Y., W. J. Metzger, and Q. N. Myrvik.** 1997. Chitin particle-induced cell-mediated immunity is inhibited by soluble mannan: mannose receptor-mediated phagocytosis initiates IL-12 production. J. Immunol. **159:**2462–2467.
46. **Shida, K., J. Kiyoshima-Shibata, M. Nagaoka, K. Watanabe, and M. Nanno.** 2006. Induction of interleukin-12 by lactobacillus strains having a rigid cell wall resistant to intracellular digestion. J. Dairy Sci. **89:**3306–3317.
47. **Silva, J. S., G. N. Vespa, M. A. Cardoso, J. C. Aliberti, and F. Q. Cunha.** 1995. Tumor necrosis factor alpha mediates resistance to *Trypanosoma cruzi* infection in mice by inducing nitric oxide production in infected gamma interferon-activated macrophages. Infect. Immun. **63:**4862–4867.
48. **Tripp, C. S., M. K. Gately, J. Hakimi, P. Ling, and E. R. Unanue.** 1994. Neutralization of IL-12 decreases resistance to Listeria in SCID and C.B-17 mice. Reversal by IFN-gamma. J. Immunol. **152:**1883–1887.
49. **Tuomanen, E., H. Liu, B. Hengstler, O. Zak, and A. Tomasz.** 1985. The induction of meningeal inflammation by components of the pneumococcal cell wall. J. Infect. Dis. **151:**859–868.
50. **van der Poll, T., C. V. Keogh, W. A. Buurman, and S. F. Lowry.** 1997. Passive immunization against tumor necrosis factor-alpha impairs host defense during pneumococcal pneumonia in mice. Am. J. Respir. Crit. Care Med. **155:**603–608.
51. **Walker, J. A., R. L. Allen, P. Falmagne, M. K. Johnson, and G. J. Boulnois.** 1987. Molecular cloning, characterization, and complete nucleotide sequence of the gene for pneumolysin, the sulfhydryl-activated toxin of *Streptococcus pneumoniae*. Infect. Immun. **55:**1184–1189.
52. **Wellmer, A., J. Gerber, J. Ragheb, G. Zysk, T. Kunst, A. Smirnov, W. Bruck, and R. Nau.** 2001. Effect of deficiency of tumor necrosis factor alpha or both of its receptors on *Streptococcus pneumoniae* central nervous system infection and peritonitis. Infect. Immun. **69:**6881–6886.
53. **Westling, K., I. Julander, P. Ljungman, M. Vondracek, B. Wretlind, and S. Jalal.** 2008. Identification of species of viridans group streptococci in clinical blood culture isolates by sequence analysis of the RNase P RNA gene, rnpB. J. Infect. **56:**204–210.
54. **Yamamoto, N., K. Kawakami, Y. Kinjo, K. Miyagi, T. Kinjo, K. Uezu, C. Nakasone, M. Nakamatsu, and A. Saito.** 2004. Essential role for the p40 subunit of interleukin-12 in neutrophil-mediated early host defense against pulmonary infection with Streptococcus pneumoniae: involvement of interferon-gamma. Microbes Infect. **6:**1241–1249.

Editor: J. N. Weiser