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Mycoplasma fermentans and TNF-β interact to amplify immune-modulating cytokines in human lung fibroblasts

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Abstract

Mycoplasma can establish latent infections and are associated with arthritis, leukemia, and chronic lung disease. We developed an experimental model in which lung cells are deliberately infected with *Mycoplasma fermentans*. Human lung fibroblasts (HLF) were exposed to live *M. fermentans* and immune-modulating cytokine release was assessed with and without known inducers of cytokine production. *M. fermentans* increased IL-6, IL-8/CXCL8, MCP-1/CCL2, and Gro-α/CXCL1 production. *M. fermentans* interacted with TNF-β to release more IL-6, CXCL8, and CXCL1 than predicted by the responses to either stimulus alone. The effects of live infection were recapitulated by exposure to *M. fermentans*-derived macrophage-activating lipopeptide-2 (MALP-2), a Toll-like receptor-2- and receptor-6-specific ligand. The synergistic effect of combined stimuli was more pronounced with prolonged incubations. Preexposure to TNF-β sensitized the cells to subsequent MALP-2 challenge, but preexposure to MALP-2 did not alter the IL-6 response to TNF-β. Exposure to *M. fermentans* or MALP-2 did not enhance nuclear localization, DNA binding, or transcriptional activity of NF-κB and did not modulate early NF-κB activation in response to TNF-β. Application of specific inhibitors of various MAPKs suggested that p38 and JNK/stress-activated protein kinase were involved in early IL-6 release after exposure to TNF-β and *M. fermentans*, respectively. The combined response to *M. fermentans* and TNF-β, however, was uniquely sensitive to delayed application of SP-600125, suggesting that JNK/stress-activated protein kinase contributes to the amplification of IL-6 release. Thus *M. fermentans* interacts with stimuli such as TNF-β to amplify lung cell production of immune-modulating cytokines. The mechanisms accounting for this interaction can now be dissected with the use of this in vitro model.

Keywords

interleukin 6; chemokines; host defense; innate immunity; Toll-like receptors

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MYCOPLASMA (class *Mollicute*) are a class of microorganisms that need to be considered as a potential factor in the genesis of chronic inflammatory disease. These ubiquitous cell wall-free bacteria represent the simplest known self-replicating organisms and have adapted to a strict commensal/parasitic lifestyle in an intimate relationship with a variety of animal and human hosts. *M. pneumoniae* was identified as a causative agent for human tracheobronchitis and atypical pneumonia in 1962 (9); however, this and other species are now reemerging in their importance as covert and chronic infectious agents with potential to act as cofactors in the progression of chronic inflammatory disease (5,7). It was recently shown that *M. pneumoniae* and other mollicute species could be detected in the airways of subjects in the absence of symptoms of acute infection and that the incidence was greater in individuals with asthma (23).

M. fermentans has been previously isolated from the human genitourinary (37) and respiratory tracts (33) but was considered a commensal not causing overt disease. At one time, it was characterized as an “AIDS-associated” mycoplasma (25,39); however, high incidences of PCR positivity in biological fluids, including blood of normal asymptomatic individuals, suggest a high potential for latent, subclinical systemic infection (1,40,47), even in nonimmunocompromised individuals. *M. fermentans* has also been found in joints of patients with active rheumatoid arthritis (20) and in leukemic bone marrow (28). In vitro studies have demonstrated the malignant transforming ability (44) and the immune-activating properties of *M. fermentans* (18,27,34).

Tissue structural cells are of interest because *1*) they serve as important sources of immune-modulating cytokines and growth factors and *2*) they represent a potential site of chronic microbial infection. Our group (15) has previously identified the presence of *M. fermentans* infection in several early-passage fibroblast cell lines derived from lung transplant recipients. The presence of this organism was associated with heightened production of IL-6 and granulocyte-macrophage colony-stimulating factor, as well as synergistic activation of cytokine production by known inducers, such as TNF-β. To partially fulfill Koch's postulate and to facilitate the study of the cellular and molecular mechanisms of host cell response to mycoplasma infection, we developed an experimental model in which cells are deliberately infected in vitro with *M. fermentans*. *M. fermentans* was isolated by standard microbiological culture from several infected human lung fibroblast (HLF) cell lines. Isolated organisms were then used to deliberately inoculate uninfected cell lines. Time- and concentration-dependent production levels of IL-6 and granulocyte-macrophage colony-stimulating factor and various chemokines, such as CXCL8, CCL2, and others, were compared between infected and uninfected cells. The ability of *M. fermentans* to synergize the cytokine-stimulating actions of TNF-β and IL-β was also assessed. Our data clearly establish *M. fermentans* as a stimulus for proinflammatory cytokine production in normal HLF. Importantly, *M. fermentans* interacts synergistically with some, but not all, known inducers of cytokines and therefore may contribute to chronic inflammatory disease by modulating the inflammatory response to specific initiating stimuli. The experimental model described here will provide a powerful tool with which to further explore the mechanisms and significance of mycoplasma-dependent modulation of host cell inflammatory response.

MATERIALS AND METHODS

Materials

Culture medium and components and FBS were from Invitrogen (Gaithersburg, MD). Tissue culture flasks and dishes were from Falcon. Mycoplasma broth base, peptone, tryptone, Yeastolate, yeast extract (all from Becton-Dickenson), Phenol red (Invitrogen), and CMRL-1066 medium and Penicillin G (both from Sigma, St. Louis, MO) were used to formulate SP-4 mycoplasma growth medium according to Tully et al. (45). Calf thymus DNA

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and Hoescht 33258 were also from Sigma. Low-endotoxin BSA was obtained from Intergen (Purchase, NY). TNF-β and IL-1β were from R&D Systems (Minneapolis, MN), and *M. fermentans*-derived macrophage-activated lipopeptide-2 (MALP-2) was from Alexis Biochemicals (San Diego, CA). All other chemicals were certified analytical or molecular biological grade. Bradford reagent for protein assay was from Bio-Rad (Hercules, CA).

Cell culture

HLFs were isolated as outgrowths from explanted surplus transbronchial biopsy tissue obtained during routine follow-up bronchoscopy of lung transplant recipients as previously described (15). Biopsy tissue was obtained during flexible fiberoptic bronchoscopy of lung allograft recipients in accordance with a protocol approved by the University of Pittsburgh Institutional Review Board. The individual cell lines used here were recovered from frozen stocks prepared at *passage 3* and used for experiments over no more than eight additional subcultures. Greater than 95% purity of fibroblasts was determined by positive immunohistochemical staining for vimentin and negative staining for cytokeratin A3 or factor VIII. Cells were maintained in MEM supplemented with FBS (10%, final concentration), glutamine (2 mM), penicillin (100 U/ml), streptomycin (100 μg/ml), and fungizone (1.25 μg/ml) and placed in a humidified incubator at 37°C with 5% CO_2-95% air. All cultures used here were negative for mycoplasma, determined as previously described (15) by fluorescent microscopy using Hoescht 33258 dye before the deliberate introduction of *M. fermentans*.

Isolation and culture of M. fermentans

Several previously obtained HLF cell lines were found to be infected with a mycoplasma-like organism that was subsequently identified as *M. fermentans* (15). To develop an experimental system to investigate the host cell response to infection, it was necessary to obtain and propagate the *M. fermentans* organism in a eukaryotic cell-free system. For this, SP-4 mycoplasma growth medium (50 ml) (45) was inoculated with 10 ml of spent tissue culture medium obtained from mycoplasma-infected fibroblasts and incubated in a tightly closed T75 flask at 37°C. Cultures were examined daily for red to yellow color changes, indicating microbial growth. At the first sign of change toward acidic pH, the cultures were either diluted 1:50 for continued propagation or cryopreserved by addition of 0.8-ml aliquots to 0.2 ml glycerol and freezing at −80°C.

To determine the total amount of mycoplasma present in cultures, we measured DNA content using a modification of the Hoescht 33258 assay described by Cesarone et al. (8). Mycoplasma culture (10 ml) was centrifuged at 10,000 *g* for 20 min, and the resulting pellet was lysed by addition of 15 μl of 1% SDS and 585 μl SSC (0.015 M sodium citrate, 0.154 M NaCl, pH 7.4). Samples (5–15 μl) were adjusted to a final volume of 200 μl (with SSC) in 96-well plates, followed by addition of 100 μl of Hoescht 33258 (1.5 μM). Fluorescence was then read with the use of a Hewlett-Packard Fusion multifunctional plate reader, with excitation at 365 nm and emission at 460 nm. DNA content was determined from a standard curve constructed from known concentrations of calf thymus DNA. The amount of viable organisms introduced was determined by performing serial 1:10 dilutions of the washed suspension made in 1.8 ml of SP-4 medium and observing the highest dilution that showed a subsequent color change after culture (36).

In vitro infection of HLF with M. fermentans

Deliberate infections of HLF with *M. fermentans* were carried out by two separate protocols. In early studies (*protocol 1*), uninfected fibroblasts were seeded onto six-well dishes (1×10^5 cells/well) in 3 ml of complete growth medium and grown for 5 days. At the time of infection, frozen vials of mycoplasma were rapidly thawed and centrifuged at 10,000 *g* for 20 min. The mycoplasma pellet was washed by resuspension in 0.25 M NaCl and recentrifuged.

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Final pellet was resuspended in complete tissue culture medium to a final concentration of 1 μg of mycoplasma DNA per milliliter. Before infection, spent fibroblast medium was changed to fresh MEM with 10% FBS with the exception of the *day 0–2* collection group, which was changed to serum-free MEM containing 0.1% BSA. Mycoplasma were then added to achieve final microbial DNA levels ranging from 10 to 500 ng DNA per well and final volume of 2.5 ml. Cells were then placed in the incubator for the indicated times. Medium was changed to serum-free MEM containing 0.1% BSA either 3 or 5 days later. Forty-eight hours after the addition of serum-free condition, medium was collected, centrifuged at 400 *g* for 10 min to remove any nonadherent cells, aliquoted, and stored at −80°C until assay. Cell monolayers were lysed in 3 ml of 0.1% citric acid-0.1% crystal violet, and nuclei were counted with a hemacytometer to normalize cytokine content of conditioned medium to the producing cell number.

From results of these early experiments that revealed the kinetics of cytokine responses after live infection, the protocol was modified to achieve a uniform infection of a larger pool of cells before experiments to eliminate potential sample-to-sample variability in organism load (*protocol 2*). In addition, we also chose to normalize cytokine levels to DNA content of the monolayer as a surrogate to cell number. This maneuver eliminated the time-consuming and laborious step of cell counting and substantially enhanced the throughput capacity of the assays. Uninfected HLFs were seeded into T75 flasks under normal growth conditions (6×10^5 cells/flask). One day later, *M. fermentans* organisms, prepared as described above, were added. Unless indicated, each flask received 450 ng of mycoplasma DNA corresponding to the intermediate organism load in *protocol 1* when corrected for the differing plate surface areas. Cells were further cultured for 4 days to establish infections and then trypsinized, counted, and reseeded into appropriate plates for experiments. For cytokine analysis, infected cells and uninfected controls were seeded into 6-well plates ($3–4.5 \times 10^5$ cells·3 ml^{-1}·$well^{-1}$) or 24-well plates ($0.6–1 \times 10^5$ cells cells·1 ml^{-1}·$well^{-1}$) and cultured for 2 days. Medium was then removed and replaced with the same volume of serum-free medium containing 0.1% BSA with or without additional stimuli. At indicated times postexposure, the conditioned medium was collected and processed as described above. The producing cell number was directly measured by nuclei counts obtained after lysis in 0.1% citric acid-0.1% crystal violet. We further amended this approach using 24-well plates and subsequent normalization of cytokine data to DNA content. After conditioned medium collection, cells were lysed in 585 μl of SSC and 15 μl of 1% SDS, and DNA content was measured by Hoescht 33358 fluorescence according to Cesarone et al. (8). Control studies showed that DNA fluorescence was linear with cell number, as determined by parallel nuclei counts, and that the addition of small amounts of mycoplasma DNA contained in infected cultures did not significantly contribute to the total amount of cellular DNA measured.

Cytokine analysis

IL-6 was measured in conditioned media by a specific ELISA kit, according to the manufacturer's instructions (R&D Systems). The quantity of CXCL8, CCL2, CCL5, CXCL1 present in conditioned media was determined by specific ELISA, using a modification of the double ligand method as previously described (6). Briefly, flat-bottomed 96-well microtiter plates were coated with 50 μl/well of specific polyclonal anti-human CXCL8, CCL2, CCL5, or CXCL1 (R&D Systems) (1 μg/ml in 0.6 M NaCl, 0.26 M H_3BO_3, 0.08 N NaOH, pH 9.6) for 24 h at 4°C and then washed with PBS pH 7.5 plus 0.05% Tween 20 (wash buffer). Plates were blocked with 2% BSA in PBS for 1 h at 37°C and then washed three times with wash buffer. Fifty microliters of sample (1:10 and neat) was added, and the plates were incubated at 37°C for 1 h. Plates were washed three times; 50 μl of biotinylated polyclonal anti-human CXCL8, CCL2, CCL5, or CXCL1 (R&D Systems) (3.5 ng/μl in PBS, pH 7.5, 0.05% Tween 20 and 2% FBS) were added; and plates were incubated at 37°C for 45 min. Plates were washed

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three times, streptavidin-peroxidase conjugate was added, and the plates were incubated for 30 min at 37°C. Plates were washed again, and 100 μl of 3,3′,5,5′-tetramethylbenzidine chromogenic substrate were added. Plates were incubated at room temperature to the desired extinction, and the reactions were terminated by the addition of 100 μl/well of 1 M H_3PO_4. Plates were read at 450 nm in an automated microtiter plate reader, and the amount of human CXCL8, CCL2, CCL5, or CXCL1 present was determined by interpolation of a standard curve generated by known amounts of recombinant human CXCL8, CCL2, CCL5, or CXCL1 (R&D Systems), respectively. The sensitivity for the human CXCL8, CCL2, CCL5, or CXCL1 ELISAs was >50 pg/ml, and this assay failed to cross-react with a panel of other known cytokines and chemokines.

Immunohistochemistry

Control and *M. fermentans*-infected HLFs were plated onto sterile glass coverslips pretreated with Cell-Tak (BD Biosciences, Bedford, MA) to promote cell adhesion and cultured in wells of a 24-well plate. Four days later, the medium was removed and replaced with 1 ml fresh media with or without TNF-β (25 ng/ml) for 45 min. Cells were then washed with PBS and fixed with 4% paraformaldehyde in PBS at 4°C for 20 min. Fixative was removed, and coverslips were washed three times in PBS and then permeabilized with 0.1% Triton X-100 in PBS for 20 min. Cells were then washed in PBS containing 0.5% BSA and 0.15% glycine, pH 7.4 (*buffer A*). After a 20-min incubation with purified goat IgG (50 μg/ml) at 25°C and three more washes in *buffer A*, coverslips were incubated with 5 μg/ml rabbit anti-human NF-κB (p65) antibody (Serotec, Raleigh, NC) for 1 h. Cells were washed three times with *buffer A* followed by a 60-min incubation with Alexa 488-tagged goat anti-rabbit IgG (5 μg/ml) (Molecular Probes, Eugene, OR). After extensive washing in *buffer A*, coverslips were mounted in Gelvatol and observed by fluorescent microscopy with an AX70 fluorescence microscope with an FITC filter. Image was acquired digitally using a Magnafire charge-coupled device camera and acquisition software (Olympus America, Melville, NY).

Electrophoretic mobility shift assay

Nuclear extracts from treated and control cells were obtained with modifications to the method of Dignam et al. (12). Cells from duplicate wells of a six-well dish were washed with ice-cold PBS and scraped into 0.4 ml of *buffer A* [10 mM HEPES (pH 7.9), 10 mM KCl, 1.5 mM $MgCl_2$, 0.5 M DTT, 0.2 mM PMSF, and 0.5% NP-40]. Cells were incubated on ice for 15 min, vortexed vigorously for 10 s, and centrifuged at 12,000 *g* for 5 min. Nuclear proteins were extracted by resuspending the pellet in an appropriate volume (usually 50 μl) of *buffer C* [20 mM HEPES (pH 7.9), 1.5 mM $MgCl_2$, 10 mM KCl, 0.2 mM EDTA, 0.5 mM DTT, 0.2 mM PMSF], followed by *buffer D* (same as *buffer C* except with 1.6 M KCl) added dropwise. The ratio of *buffer C* to *buffer D* was 3:1. Nuclei were incubated on ice for 30 min, and then supernatant was collected by centrifugation at 12,000 *g* for 15 min. Nuclear extracts were aliquoted and stored frozen (−80°C) until use. Protein content was determined by Bradford assay.

EMSAs for NF-κB were conducted with a NF-κB family NuShift kit (Geneka, Montreal, Canada) using a specific double-stranded NF-κB oligonucleotide probe (5′-AGT-TGA-GGG-GAC-TTT-CCCAGG-C-3′) (Promega, Madison, WI). Probe was end-labeled with [γ-^{32}P]ATP using T_4 polynucleotide kinase (Promega) and purified using G-50 Sephadex spin columns. Radiolabeled probes (0.5 ng) were incubated with nuclear protein extracts (3 μg protein) on ice for 20 min according to the kit instructions in a final volume of 24 μl. Samples were then loaded on 4% Tris-glycine-EDTA gels and resolved electrophoretically for 4 h at 10°C using a chilled recirculating cooler. Gels were then dried and exposed to X-ray film or placed over a developing screen for analysis using a Packard Cyclone Phosphorimager system.

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>NF-κB promoter activity

The NF-κB-dependent reporter vector pNF-κB-TAL-Luc (Clonetech, Palo Alto, CA) contains the luciferase reporter gene driven by a basic promoter element (TATA box) joined to tandem repeats of an NF-κB enhancer element. HLFs were seeded into 24-well dishes and cultured overnight. Cells were then transferred to 200 μl serum- and antibiotic-free medium (with 0.1% BSA) and transfected with 0.4 μg pNF-κB-TAL-Luc DNA using Lipofectamine Plus (Invitrogen) along with 0.2 μg pCMV-SPORT-β-gal (Invitrogen) to normalize differences in transfection efficiency. Transfections were performed by addition of 25 μl serum-free DMEM containing DNA precomplexed for 15 min with Plus reagent, which was followed by addition of 25 μl medium containing 3 μg lipofectamine. Transfections were allowed to proceed for 3 h after which media were removed and replaced with fresh serum-containing medium. Cells were then allowed to recover overnight before stimulations. For experiments, cells were again changed to serum-free medium containing 0.1% BSA and exposed to TNF-β and/or MALP-2. Four hours after exposure, medium was removed and cell monolayer was lysed in 135 μl Glo lysis buffer (Promega) and stored at −20°C until assay. Luciferase activity was measured in triplicate using the Bright-Glo luciferase assay system (Promega) and 96-well opaque microtiter plates. Luminescent signal was measured in a TopCount-NXT scintillation/ luminescence counter (Perkin-Elmer, Boston, MA). β-Galactosidase activity was measured in aliquots of the same extracts using a high-sensitivity kit that employs chlorphenol-β-D galactopyranoside as a substrate (Stratagene, LaJolla, CA).

Statistics

For analysis of experiments with multiple experimental groups, data were first analyzed by one-way repeated-measures ANOVA. Because experiments were performed with multiple cell lines derived from different donors, the data obtained from each individual cell line were matched for the repeated-measures design to control for variability between cell lines. The n for each experiment (at least 3 or more as indicated) represents the number of different cell lines tested. Each cell line was tested once per experiment, with the results for each data point taken as a mean value obtained from duplicate or triplicate wells. The final mean ± SE values presented in the figures represent those obtained after summation of data from individual cell lines. Significant differences between specific groups were then assessed by an appropriate comparative posttest when indicated by a significant ANOVA result. Dunnett's multiple comparison test was used for comparing groups to untreated control cells, and comparisons between any other two groups were performed with Bonferroni multiple comparisons test. The ANOVA approach was only appropriate for comparing groups that were totally independent of each other and when the variance of the data contained in different groups was relatively equivalent between groups. In cases where these criteria were obviously not met, statistical comparisons between specific groups were made with multiple paired Student's *t*-tests. For dose-response data containing more than two different concentrations of a single agent (Fig. 4), a linear test for trend was also used to assess the significance of the effect of that stimulus. The ability of infected and uninfected cells to respond to a single stimulus such as TNF-β and IL-1β was assessed by paired Student's *t*-test. The level of statistical significance for all analyses was set at $P \leq 0.05$.

RESULTS

Isolation and propagation of M. fermentans

To study the molecular mechanisms of the host cell response to *M. fermentans* infection, it was necessary to develop an experimental model in which uninfected cells could be deliberately exposed to mycoplasma under controlled and defined conditions. This required the isolation and culture of mycoplasma in the absence of host cells. We successfully isolated *M. fermentans* strains previously documented to be present in several of our early-passage human

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biopsy fibroblast cultures (15). Inoculation of SP-4 medium with spent tissue culture medium from our infected cell lines gave rise to mycoplasma isolates as shown by an acidic change in the media. This isolated strain of *M. fermentans* showed classical "fried-egg" morphology typical of mycoplasma when grown on solid medium and PCR positivity using *M. fermentans* sequence-specific primers (48) (data not shown). In addition, *M. fermentans* species was verified with the use of monoclonal antibodies specific for *M. fermentans* (personal communication, Dr. Shyh-Ching Lo, Armed Forces Institute of Pathology, Washington, D.C.). The particular isolates prepared from these cells were resistant to clonal propagation using triple-filter cloning techniques and thus represent a polyclonal-derived "strain" of *M. fermentans* that we refer to as *M. fermentans tpx*. Although several similar *M. fermentans* strains have been isolated from several of our cell lines, all experiments here utilized a single isolate that was maintained and propagated through three passages.

Effects of M. fermentans on basal and TNF-β-induced cytokine release

We used this preparation of *M. fermentans* to assess its effects on cytokine production in HLF. Figure 1*A* shows the upregulation of IL-6 production by HLF deliberately exposed to varying amounts of *M. fermentans*. Cells were infected on *day 0* and then exposed to conditioning media for 48-h intervals at various times postinfection. Within the immediate 2 days after exposure, the cells that were exposed to the greatest amount of organisms (500 ng of *M. fermentans* DNA/well) showed a 10-fold induction in IL-6, whereas only a small increment in IL-6 could be observed with lower mycoplasma titers. As the time after infection was increased, however, release of IL-6 became robust at all concentrations of organisms, reaching maximum at the 5- to 7-day collection time point. By this later time, the response was no longer dependent on the initial organism load, and all mycoplasma exposures resulted in a 15-fold increase in IL-6 release. One possibility is that mycoplasma present at these later time points have reached a static phase of maximal growth and, as such, no longer display a dose-response based on original organism load.

This cytokine-inducing effect of *M. fermentans* was not specific for IL-6 alone. We also measured the release of two chemotactic factors, namely, CXCL8 (IL-8) (Fig. 1*B*) and CCL2 (macrophage chemotactic peptide) (Fig. 1*C*). Similar to IL-6, several days of infection were necessary to achieve a maximal release of CXCL8 and CCL2. The dependence of the magnitude of cytokine release on original organism concentration was again lost by the later time point, in which CXCL8 and CCL2 were increased by ~10-fold and 6-fold, respectively, in the presence of *M. fermentans*. A similar pattern of CXCL2 (Gro-α) release was observed in response to infection (data not shown), in which cells exposed to high-dose mycoplasma released 10.2 ± 7.1 ng/10^5 cells from *days 0–2*, 26.1 ± 14 ng/ml on *days 3–5*, and 22.1 ± 9.7 on *days 5–7* compared to undetectable levels in uninfected cells (limit of detection >0.05 ng/10^5 cells). The amount of CXCL2 produced during the first 2 days was dependent on the dose of mycoplasma, but all levels of infection produced essentially equivalent amounts at the later time points. In contrast, CCL5 (RANTES) could not be detected in any of the samples regardless of infection status.

We next sought to determine whether *M. fermentans* infection modulated the effect of known inducers of fibroblast-derived cytokines. IL-1β and TNF-β represent two prototypic multifunctional inflammatory cell-derived cytokines known to initiate complex cascades of immune-modulating cytokines from target cells. IL-1β is a secreted macrophage-derived proinflammatory cytokine released in response to a plethora of inflammatory challenges, whereas TNF-β (also known as lymphotoxin-α) is derived from activated lymphocytes. Figure 2*A* shows the IL-6 response to TNF-β and IL-1β over a 24-h period in control HLFs and cells deliberately infected with *M. fermentans*. The basal release of IL-6 in the absence of either cytokine is relatively small, although the significant enhancement by infection alone can be

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seen. TNF-β treatment proved a significant stimulus for cytokine release and increased IL-6 production ~30-fold in uninfected controls over the 24-h time period. Notably, the TNF-β response in mycoplasma-infected cells was triple that seen in uninfected cells and nearly 1,000-fold greater than basal secretion in uninfected cells. The combined effect of TNF-β and *M. fermentans* was clearly synergistic, being greater than that predicted by the sum of the responses to each stimulus alone. IL-1β was a much more efficacious stimulant, with IL-6 levels reaching ~10 times those observed over a similar duration with TNF-β alone. In contrast to that seen with TNF-β, no difference in the effect of IL-1β was observed between mycoplasma-infected and uninfected control cells. A dose-response study using concentrations of IL-1β (down to 10 ng/ml) similarly revealed that mycoplasma infection failed to modulate the IL-6-inducing effects of submaximal concentrations of IL-1β (data not shown). Thus *M. fermentans* infection potentiated the IL-6-inducing effect of TNF-β but not that of IL-1β.

The time course of IL-6 release was also determined by collecting and analyzing medium conditioned by infected and uninfected cells for 10, 24, and 48 h in the presence and absence of TNF-β (Fig. 2*B*). Although it cannot be readily appreciated from Fig. 2*B* because of the magnitude of the TNF-induced responses, the basal rate of IL-6 release in mycoplasma-infected cells was greater than that in uninfected controls (41 ± 33 pg/μg DNA in uninfected cells at 48 h vs. 320 ± 77 pg/μg DNA with mycoplasma alone). TNF-β induced an approximately linear accumulation of IL-6 in the medium from uninfected cells over the 48-h period. TNF-β-induced release of IL-6 from mycoplasma-infected cells, however, was nonlinear and much greater than that seen in TNF-β-treated uninfected cells. Although the rate of IL-6 release in mycoplasma-infected cells approximated that seen in uninfected controls during the first 10 h of incubation, the rate of IL-6 production in infected cells increased exponentially over time and substantially more IL-6 was released in the second half of the incubation compared to that shown in the early time points.

To determine whether the synergistic interaction of TNF-β and *M. fermentans* was specific to IL-6 or was generalized on other proinflammatory cytokines, conditioned medium obtained from mycoplasma-infected and uninfected fibroblasts treated with TNF-β and IL-1β for 48 h were analyzed for several chemotactic factors such CXCL8, CXCL1, CCL2, and CCL5 (Fig. 3). Note that the different scales of the *y*-axes are depicted logarithmically to more easily compare the effects of TNF-β and IL-1β. Three classes of cytokines emerged in regard to their relative inducibility by the two stimulating cytokines. IL-6, CXCL8, and CXCL1 were all induced over an order of magnitude more by IL-1β compared to TNF-β. In contrast, CCL5 showed the opposite pattern, with TNF-β being more efficacious than IL-1β. CCL2 was induced equivalently by both TNF-β and IL-1β. The effect of TNF-β on CXCL8 and CXCL1 was significantly augmented in the presence of *M. fermentans* infection, where the levels of CXCL8 and CXCL1 were both approximately fivefold greater than that shown in uninfected cells. In contrast, the presence of *M. fermentans* did not modulate TNF-β's ability to induce CCL2 or CCL5 despite the fact that TNF-β clearly possessed stimulatory activity for these cytokines. Basal release of CCL2 in uninfected cells was 1.4 ± 1.0 ng·100,000 cells^{-1}·48 h^{-1} compared to 21.9 ± 2.3 ng·100,000 cells^{-1}·48 h^{-1} in the presence of TNF-β. In these same cells, basal release of CCL5 was below the limits of detection by our ELISA (<50 pg/ml) but reached 49 ± 16 ng/ml (equivalent to 19 ± 9 ng/100,000 cells) after 48-h exposure to TNF-β. Although facilitation of the IL-1β-induced CCL2 response by *M. fermentans* was statistically significant compared to IL-1β-treated uninfected cells, this difference was quite small (17% increase) and likely does not share the same biological significance as facilitation of TNF-β-induced IL-6, CXCL8, and CXCL1 responses by mycoplasma.

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Toll-like receptor-2 and -6 agonist MALP-2 recapitulates the effect of live M. fermentans infection

The signaling pathways invoked during exposure to *M. fermentans* could be linked to activation of innate immune responses mediated via Toll-like receptor (TLR) pathways. Mycoplasma lack a characteristic bacterial cell wall and hence do not contain LPS endotoxin. They do, however, contain other novel lipoprotein structures that could serve as ligands for other pathogen-associated pattern-recognition receptors. MALP-2 is a proteolytic fragment derived from a 43-kDa *M. fermentans* membrane lipoprotein (Mg161Ag) that serves as a potent cytokine inducer in several cell types (16,26). The peptide possesses a unique triacylated structure on its NH_2-terminal cysteine and is thought to activate innate immune pathways via specific interaction with TLR-2 and TLR-6 receptors (29).

If the effects of infection with live *M. fermentans* were mediated via TLR-2 and/or TLR-6, then it might be expected that exposure to MALP-2 could recapitulate the effects of live infection. To test this, uninfected HLFs were exposed to TNF-β and MALP-2 alone and in combination and IL-6 release was measured after 48 h. Figure 4 shows that MALP-2 alone produced a small concentration-dependent induction of IL-6 in conditioned medium ranging from 8.6 ± 4.2 pg/mg DNA in the absence of MALP-2 to 312.7 ± 173.6 pg/mg DNA in the presence of 600 pg/ml MALP-2. A one-way ANOVA with a posttest for linear trend confirmed the significant concentration-dependent effect of MALP-2 in control and TNF-β-treated groups. The effect of MALP-2, however, was much more profound when exposed in the presence of TNF-β. MALP-2 increased TNF-β-mediated IL-6 output in a dose-dependent manner. In the presence of 300 and 600 pg/ml MALP-2, TNF-β induced >17-fold and 16-fold more IL-6, respectively, at 48 h vs. that shown with exposure to TNF-β alone. Similar interactions, albeit with lower levels of IL-6, were observed when conditioned medium was collected after 24-h exposures to these same stimuli (data not shown). This represents a clear synergistic interaction between MALP-2 and TNF-β because the response observed with both agents together is greater than that expected by the algebraic sum of the response to each agent alone.

Effects of M. fermentans and TNF-β on NF-κB activation in HLF

The regulation of cytokine gene expression during infection and inflammation fundamentally involves the complex and concerted interaction of multiple signal transduction pathways culminating in the activation of several transcription factors, one of the most notable being NF-κB. The NF-κB family of dimeric transcription factors (p65, p50, p52, c-rel, rel-B) exists sequestered in the cytosol in complex with I-κB proteins, limiting their accessibility to chromatin and transcriptional activity. When stimulated with a host of endogenous and exogenous stimuli, including TNF-β and microbial products, I-κB is rapidly phosphorylated and degraded, permitting translocation of NF-κB to the nucleus, binding to specific DNA enhancer elements in specific gene promoters, and transactivation of transcription of target genes. Therefore, it was important to assess the interactive effects of mycoplasma and TNF-β on nuclear localization, DNA binding, and transcriptional activity of NF-κB. One of the most ubiquitous forms of NF-κB is the p50–p65-containing dimer of which much of the transactivating potential is assigned to p65 (19). We first sought to determine whether TNF-β and infection with *M. fermentans* induced the localization of p65 NF-κB protein into the nucleus of HLF. With an antibody specific to human p65, immunohistochemistry was performed in uninfected and *M. fermentans*-infected HLF before and after a 45-min exposure to TNF-β. The intracellular localization of p65 is shown in Fig. 5 and demonstrates that, in the absence of TNF-β, p65 immunoreactivity appeared to be diffusely spread throughout the cytosol. This pattern was observed regardless of the presence or absence of *M. fermentans*, and no difference could be observed between the two conditions. TNF-β treatment, however, led to the robust and rapid accumulation of p65 into the nucleus after a 45-min exposure to TNF-

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β. Once again, the pattern and intensity of nuclear staining after TNF-β exposure appeared similar in both infected and control cells.

This immunohistochemical approach, however, is difficult to quantify and is limited by its specificity to only a single NF-κB family member. Therefore, we next measured specific basal and TNF-β-induced DNA binding activity of NF-κB by EMSA using nuclear extracts prepared from mycoplasma-infected and uninfected cells. Figure 6*A* shows the short-term time course of NF-κB binding activity after TNF-β stimulation. Very little binding of NF-κB could be detected in uninfected or mycoplasma-infected cells under basal conditions before TNF-β exposure. The addition of TNF-β, however, produced a large and rapid increase in NF-κB DNA binding that was readily apparent at both the 2- and 4-h time points. Quantification of the radioactivity contained within the shifted bands was performed on four separate cell lines (Fig. 6*B*) and revealed essentially no difference in the early induction of NF-κB by TNF-β between uninfected cells and those infected with *M. fermentans*.

To measure the transcriptional activating potential of NF-κB under these conditions, we utilized a NF-κB-driven luciferase reporter plasmid transiently transfected into HLF. To avoid any potential confounding by mycoplasma-dependent effects on transfection efficiency and baseline luciferase activity before TNF-β stimulation, we chose to utilize MALP-2 as a stimulus in place of live mycoplasma because MALP-2 could recapitulate the effects of live infection on IL-6 production. Luciferase activity was measured in HLF cotransfected with pNF-κB-TAL-Luc and pCMV-β-gal treated with TNF-β and/or MALP-2 for 4 h at concentrations known to produce synergistic effects on IL-6 release. Figure 6*C* shows that TNF-β alone produced between a two- and threefold enhancement of NF-κB-dependent luciferase activity. Exposure to MALP-2 alone did not produce a measurable increase in NF-κB-dependent transcription and, most notably, did not further enhance the response to TNF-β alone. The apparent reduction in TNF-β response by MALP-2 was not statistically different from that seen with TNF-β alone. Thus it appears that exposure of HLF to live mycoplasma or its product, MALP-2, does not modulate the early NF-κB signaling events in response to TNF-β despite the fact that TNF-β and *M. fermentans* synergistically interact to amplify the elaboration of immune-modulating cytokines, such as IL-6, by HLF.

Role of MAPKs in effects of MALP-2 and TNF-β

We next sought to probe whether an alternative signaling pathway, such as MAPKs and stress-activated protein kinases (SAPKs), might be operative in these synergistic interactions between TNF-β and *M. fermentans*. To this end, we tested the sensitivity of mycoplasma- and TNF-β-induced IL-6 release to inhibition by various pharmacological inhibitors of specific MAPK isoforms, namely $ERK_{1/2}$ (PD-98059), p38 (SB-203580), and JNK/SAPK (SP-600125). We first measured IL-6 release in response to TNF-β and/or MALP-2 over a 24-h period in the presence or absence of each inhibitor (Fig. 7). Control values denote the response to the stimuli alone in the absence of any inhibitor, and the different scales of the *y*-axes reflect the typical synergistic response. The $ERK_{1/2}$ inhibitor (PD-98059) applied at either concentration (3 and 30 μM) was essentially without effect on IL-6 release in response to mycoplasma and TNF-β alone or in combination. In contrast, SB-203580 significantly and dose-dependently reduced IL-6 whenever TNF-β was involved (Fig. 7, *B* and *C*) with ~70% inhibition of both the TNF-β alone and TNF-β+ MALP-2 responses at the 30 μM concentration. Inhibition of JNK/SAPK with 30 μM SP-600125 appeared to affect IL-6 release in response to all three conditions. Although 30 μM SP-600125 reduced the TNF-β alone responses by ~30%, the inhibitory effect on the combination of MALP-2 and TNF-β appeared more profound and was inhibited by ~70%. Although the effect of MALP-2 alone is very small in this experiment, it also was significantly reduced by SP-600125 to 34% of that seen in the absence of the drug.

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Because the time-course experiment above (Fig. 2*B*) revealed that the synergistic interactions between MALP-2 and TNF-β manifest themselves to a greater extent at later times, we sought to specifically intervene with signaling processes occurring between the 24- and 48-h time points after exposure. For these experiments, we first exposed cells to mycoplasma and/or TNF-β in the absence of inhibitors for 24 h. Without changing the medium, specific MAPK inhibitors were then introduced as 10× concentrated stocks, and the cells were allowed to condition the medium for an additional 24 h. Therefore, ELISA measurement of IL-6 represents the total produced over a 48-h period with the inclusion of drugs only during the second 24-h period. Figure 8 shows that cumulative IL-6 release over the 48-h period in response to TNF-β plus mycoplasma was substantially larger than that seen over the initial 24-h period (compare to Fig. 7). Inhibition of ERK and p38 MAPKs by PD-98059 and SB-203580, respectively, during the second half of the exposure did not attenuate the response to the combination of mycoplasma plus TNF-β. In contrast, JNK/SAPK-dependent processes appeared to be operative at the later time points because SP-600125 reduced the overall IL-6 release by ~50% to a level that approximated that seen after only 24-h exposure (compare to Fig. 7). None of the MAPK inhibitors had a significant effect on the response to TNF-β alone when applied during the second 24 h of exposure (data not shown), and only SP-600125 reduced the response to mycoplasma alone when applied during the second 24 h of exposure (mycoplasma alone = 134.5 ± 32.7 pg/μg DNA vs. mycoplasma alone with 30 μM SP 600125 = 79.9 ± 20.4 pg/μg DNA; $P < 0.05$).

Because of the temporal nature of the synergistic response and the differential and time-dependent effects of specific MAPK inhibitors on each response, we next wondered whether sequential application of each stimuli alone could mimic the synergistic response and whether the order of addition was an important determinant for these effects. In other words, could one of the stimuli (mycoplasma infection or TNF-β) in a sense "prime" the cell to enhance the response to the other? To avoid the time lag needed to achieve maximal response to live infection and to be able to easily terminate the exposure, we used MALP-2 as a surrogate for live infection. For these experiments, we first exposed three groups of cells to either MALP-2 alone, TNF-β alone, or no stimuli for 24 h. The medium was then removed, and cells were extensively washed and replenished with fresh serum-free medium containing additional test stimuli. Cells from each of the initially defined groups were again divided into four groups and exposed to either no stimulus, MALP-2 alone, TNF-β alone, or TNF-β plus MALP-2 combined. Conditioned medium was collected after 24 h of the second exposure and analyzed for IL-6 by ELISA. Figure 9 shows the IL-6 response in HLF to each single stimulus after the various pretreatment regimens. With no pretreatment, HLF released ~250 pg/μg DNA in response to 24-h exposure to TNF-β and the response to MALP-2 was ~25% of that seen with TNF-β. There was essentially no change in responsiveness to TNF-β or MALP-2 if cells were pretreated with MALP-2. In contrast, if cells were pretreated with TNF-β, the release of IL-6 in response to MALP-2 was greatly augmented and much greater than that seen without pretreatment or pretreatment with MALP-2. This appeared specific for MALP-2 because preexposure to TNF-β did not alter the response to the second TNF-β stimulus and because TNF-β pretreatment only marginally increased the "basal" release when TNF-β was removed and cells remained untreated.

DISCUSSION

Our data are the first to demonstrate the application of deliberate infection of HLF with *M. fermentans* as an in vitro model to study the cellular and molecular basis for the effects on this important lung cell type during microbial infection. *M. fermentans* alone produced significant cell activation, as measured by elaboration of several immune-modulating cytokines, but also selectively amplified the effects of known inducers of cytokines such as TNF-β. We also begin

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to shed some light on the roles of NF-κB and MAPK signaling pathways in mediating these synergistic interactions.

Despite their ubiquity, numerous reports have associated various *Mycoplasma spp*. (including *M. fermentans*) and similar organisms with multiple chronic inflammatory diseases, including arthritis (43), urethritis/pelvic inflammatory disease (42), chronic fatigue syndrome/ fibromyalgia (47), and atherosclerosis (38), among others. The lung is no exception to this emerging trend. Recently, Kraft et al. (23) detected *M. pneumoniae* and other *Mycoplasma spp*. in the airways of human subjects in the absence of symptoms of acute infection, and the incidence was considerably greater in patients with asthma. This observation has led to the reported successful treatment of some adult patients with asthma with administration of antibiotics (24).

Previous studies have shown the ability of several species of mycoplasma to upregulate immune effector mechanisms in vitro (53). A high-molecular-weight extract of *M. fermentans* induced IL-6 (34), TNF-β, and cell-associated IL-1 (27) in monocytes and macrophages. Because mycoplasma lack a characteristic cell wall, they must activate innate immunity via mechanisms different from gram-negative endotoxin-containing bacteria. The cytokine-stimulating activity of *M. fermentans* has been ascribed to specific lipid-associated membrane lipoproteins (35). MALP-2 and its precursor, the 43-kDa membrane lipoprotein M161Ag, both contain a unique NH_2-terminal lipoamino acid (D-diacylglycerol cysteine) (26) that is recognized as a pathogen-associated molecular pattern by TLR-2 (29). Because the acylated cysteinyl residues are a common feature to all known bacterial membrane lipoproteins, the results observed with our experimental model here are likely relevant to many other infectious agents as well.

Our data are significant in that they extend the repertoire of cells with capacity to respond to mycoplasma-derived products to include HLF. Tissue structural cells themselves cannot be ignored as important sources of immune-modulating cytokines and growth factors (13,14, 46). Several studies indicate that HLF are refractory to the effects of bacterial LPS (30,52), although it is unclear whether they fail to express the requisite TLR-4 receptor or lack some other component of the signaling pathway. It appears that HLF express functional signal transduction pathways that allow for recognition of certain microbial-derived products with consequent induction of an innate immune response. Thus mycoplasma within the lung may stimulate these and other cell types to elaborate immune-modulating cytokines and promote inflammation.

We have focused on IL-6 as a prototypic cytokine whose regulation is shared with many other cytokines. The effects of IL-6 are pleiotropic and may exert both inflammatory (31) or protective (49,51) effects, as well as, produce systemic manifestations characteristic of inflammation. IL-6 may also serve to promote Th_2 differentiation during T-cell activation (11), which may, in part, explain the association of mycoplasma and similar infections with asthma. We have also observed the elaboration of several chemokines that could promote the accumulation of neutrophils, monocytes, and other immune effector cells within the lung. The overall regulation of inflammation undoubtedly results from the concerted action of a plethora of immune-modulating cytokines produced by a wide variety of cell types. Because we limited these studies of a single cell type and a relatively few select cytokines, it is difficult to fully predict the effects of *M. fermentans* in vivo. Because many cytokines, however, share common regulatory pathways in diverse cell types, it is likely that *M. fermentans* similarly affects multiple cytokines and other cell types.

The prevalence of *M. fermentans* infections even in apparently healthy individuals supports the notion that this organism can establish chronic covert subclinical infection and produces

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disease only in the presence of additional, but as yet undefined, cofactors. Shibata et al. (40) found an overall incidence of >50% of *M. fermentans* in saliva of normal adult subjects and 28% of preschool aged children, suggesting that *M. fermentans* colonizes the upper respiratory tract very early in life. Similarly, significant incidences of *M. fermentans* in blood (8–11% positivity) (10,22) and urine (17%) (1) have been detected in apparently normal healthy subjects. In our studies mycoplasma (and MALP-2) alone was capable of stimulating a significant increment in cytokine production. The most notable increases in IL-6, however, were observed primarily in the presence of additional stimuli such as TNF-β. Thus the most significant effect of mycoplasma in vivo may be fully realized only in the presence of other injurious or stressful stimuli. We have recently reported similar synergistic interactions between *M. fermentans* and metal-containing atmospheric particulate matter (17). It should be noted that not all HLF-derived cytokines were equally potentiated by *M. fermentans*. For example, *M. fermentans* potentiated the TNF-β-dependent release of the CXC class of chemokines (CXCL8, CXCL1) but not those of the CC class (CCL2 and CCL5). Such a differential response could lead to varying compositions of the inflammatory cell influx since CXC chemokines are specific for neutrophils and CC chemokines preferentially recruit mononuclear cells (41). Thus mycoplasma might alter the qualitative nature of inflammatory responses by modulating the relative composition and function of the activated cell population.

The reasons for the selective augmentation of some cytokines (i.e., IL-6, CXCL8, CXCL1) but not others (CCL2, CCL5) by the combination of TNF-β and *M. fermentans* remain unknown and will require future mechanistic experiments. Although regulation of cytokine expression shares many common features, such as dependence on NF-κB activation, subtle differences in how multiple pathways converge to integrate gene expression of individual factors serve to orchestrate specific responses. Our data indicate that cytokines derived from HLF possess differing sensitivities to induction by IL-1β and TNF-β. Only those that are induced to a greater extent by IL-1β compared to TNF-β show synergy between TNF-β and *M. fermentans*. This may reflect the fact that IL-1β-dependent signaling shares considerable overlap with the TLR-dependent signaling pathway (32), and thus those genes relatively unresponsive to IL-1β might also be expected to be less responsive to TLR-2 agonists. Barchowsky et al. (4) demonstrated a dichotomy in IL-1β and TNF-α-signaling in which both agents equally induced NF-κB activations but IL-1β preferentially induced MAPK activity, c-Jun phosphorylation, AP-1 activation, and collagenase-1 expression compared to TNF-α. Other differences between the regulation of individual cytokines have been noted. For example, IL-17 selectively downregulates TNF-α-induced RANTES gene expression in human fibroblasts but amplifies IL-8 and IL-6 release by the same agonist (3). In addition, IL-6 and RANTES gene promoters both contain cAMP response element binding protein sites, although elevations of cAMP with β-agonists lead to inhibition of RANTES but enhancement of IL-6 (2).

It is possible that HLF were relatively refractory to the NF-κB-activating properties of *M. fermentans*. It is important to point out that the levels of IL-6 released after infection or MALP-2 alone are substantially below those maximally achievable with other stimuli such as IL-1β, for example (see Fig. 2*A*). Thus, although HLF presumably express some level of TLR-2 receptors, they may lack optimal expression of this or other components of the TLR-2 signaling pathway necessary for a fully competent response. Because TNF-β pretreatment effectively enhanced a subsequent challenge with MALP-2, it is tempting to speculate that TNF-β may have specifically induced or otherwise facilitated various components of TLR-2 signaling. In contrast, the presence of live *M. fermentans* did not appear to augment TNF-β-induced NF-κB DNA activation, which suggests that the early TNF-β signaling pathway was essentially unaltered by the presence of *M. fermentans*. Another topic of future experiments will be to address whether mycoplasma and TNF-β interact at the posttranscriptional level to affect alterations in such parameters as mRNA stability (21,50)

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Our results do not mean that NF-κB is not required for the overall combined effects of *M. fermentans* and TNF-β but rather that the synergistic interactions arise at times later than the early signaling events induced by TNF-β and/or through the concerted action of multiple distinct signaling pathways and transcription factors besides NF-κB. At least part of the interactive response appears dependent on specific MAPK pathways, most notably JNK/SAPK and p38. At early times, it appears that TNF-β and *M. fermentans* induction of IL-6 each selectively involved p38 and JNK/SAPK-dependent pathways, respectively. Thus it is not surprising that both the p38 (SB-203508) and JNK/SAPK (SP-600125) inhibitors significantly inhibited the response to combined stimuli. It was also apparent that MAPK pathways remained operative even after 24 h of TNF-β stimulation of infected cells, whereas delayed exposure during the second 24-h period to the JNK/SAPK inhibitor reduced IL-6 release by over 50%. This finding is also consistent with the hypothesis above, which states that TNF-β treatment facilitated subsequent TLR-2 signaling and JNK/SAPK activation.

In summary, our results are the first to characterize an experimental model using deliberate infection of HLF with *M. fermentans* to characterize the cellular and molecular regulation of this cell type in response to infection with mycoplasma-like organisms. We show that *M. fermentans* alone serves as a stimulus for the production of several immune-modulating cytokines and can synergistically interact with other endogenous stimuli such as TNF-β. Thus the presence of mycoplasma alone may stimulate components of an inflammatory response by these cells or may amplify the cellular response initiated by other factors. Therefore, mycoplasma may modulate inflammatory and immune processes within the lung and ultimately contribute to chronic inflammatory/fibrotic disease, as well as potentiate T-cell-dependent immunopathologies such as asthma. Precise analysis of the cellular and molecular basis for these effects will be aided by application of this in vitro experimental model.

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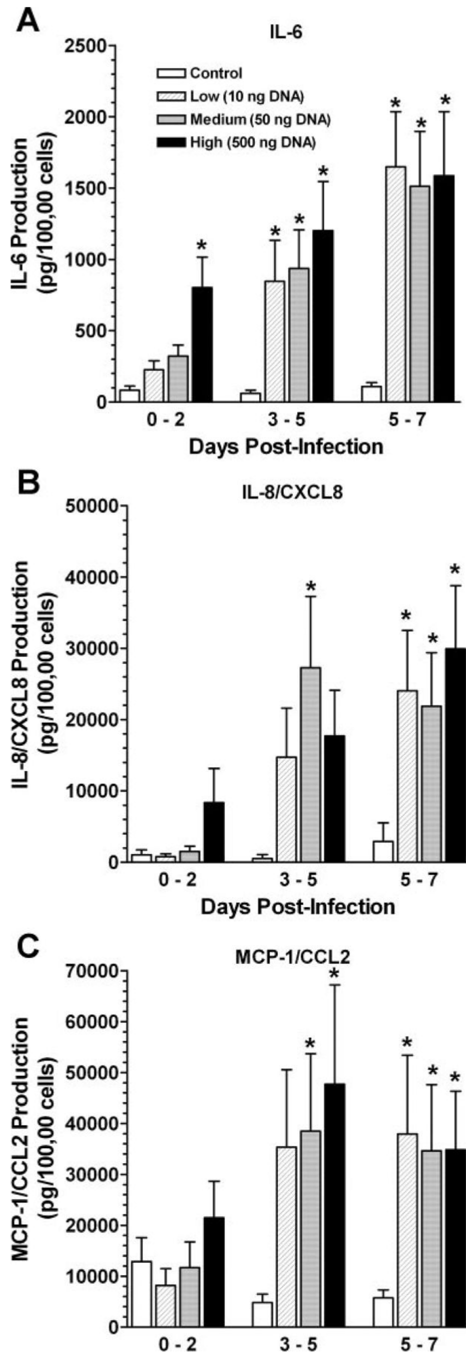

Fig. 1.

Deliberate infection of human lung fibroblasts (HLF) with live *Myco-plasma fermentans* upregulates IL-6 and chemokine release. HLF were seeded into 6-well plates and infected with varying amounts of viable *M. fermentans* (10–500 ng DNA/well). Cells were then allowed to condition in 3 ml of serum-free MEM containing 0.1% BSA for 48 h immediately or on *days 3* or *5* postinfection. Medium was collected and analyzed for IL-6 (*A*), CXCL8 (*B*), and CCL2 (*C*) by ELISA. Cytokine levels were normalized to the producing cell number as determined by nuclei counts after lysis in 0.1% crystal violet-0.1% citric acid. Data represent means ± SE; $n = 6$. *$P < 0.05$ vs. uninfected control cells from the same time points by one-way repeated-measures ANOVA and Dunnett's multiple comparisons to control.

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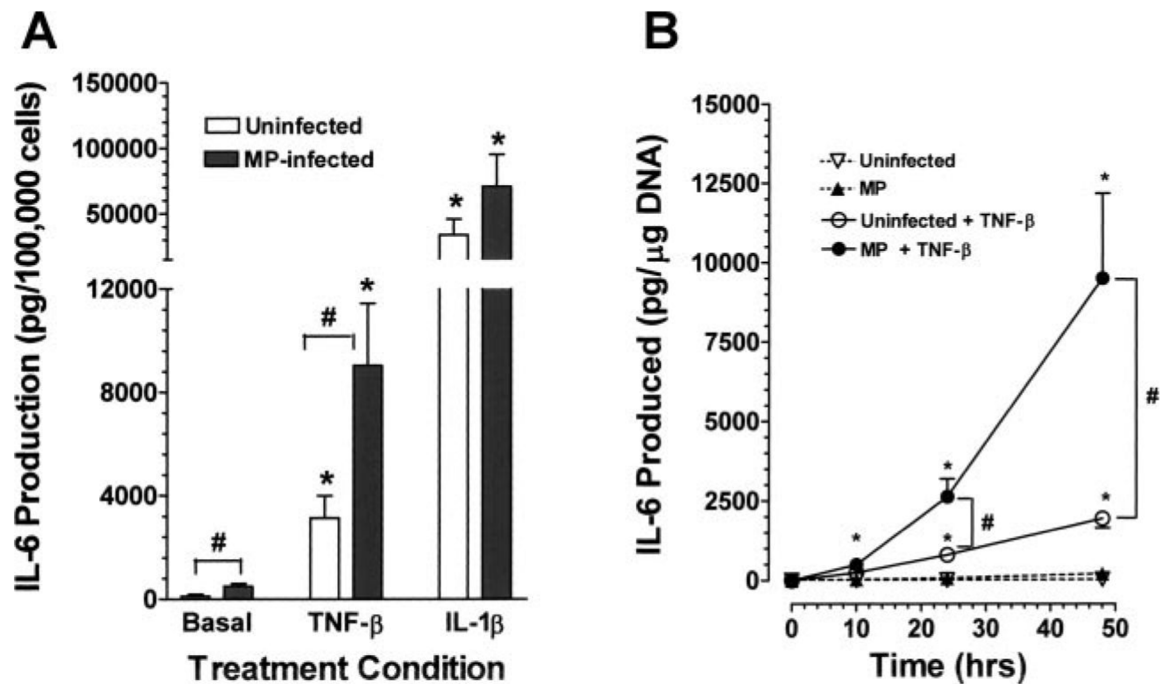

Fig. 2.

M. fermentans potentiates the IL-6-inducing effect of TNF-β but not IL-1β on HLF. HLF were infected according to *protocol 2* by inoculation with 450 ng *M. fermentans* DNA in T75 flask in complete medium and grown for 4 days. Infected cells and uninfected controls were seeded in parallel into 6-well (*A*) or 24-well plates (*B*) and allowed to attach for 24 h. Medium was then changed to MEM ± 0.1% BSA with TNF-β (25 ng/ml), IL-1β (1 ng/ml), or no addition. Medium was collected at the indicated times after stimulation, and IL-6 was measured by specific ELISA. *A*: amount of IL-6 appearing in conditioned medium of uninfected and infected cells treated with TNF-β or IL-1β for 24 h and normalized to the producing cell number. *B*: time course of IL-6 release by mycoplasma (MP)-infected and uninfected cells treated with 25 ng/ml TNF-β for up to 48 h. IL-6 content in *B* was normalized to the DNA content of the monolayer. Data are means ± SE; $n = 5$ for *A* and $n = 3$ for *B*. *Significant difference ($P < 0.05$) by Student's *t*-test between each cytokine treatment and its respective uninfected or MP-infected basal level. #Significant difference between uninfected and MP-infected cells at the equivalent level of cytokine treatment.

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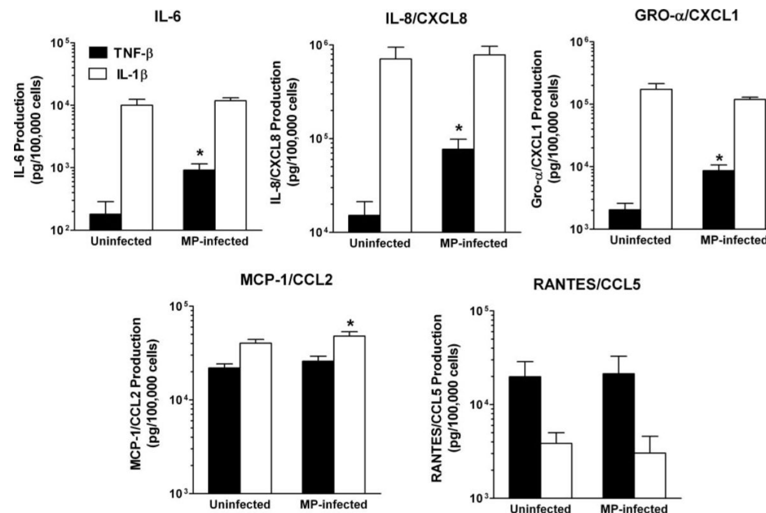

Fig. 3.

Selective synergy between *M. fermentans* and TNF-β for some but not all HLF-derived immune-modulating cytokines. HLFs were seeded into 6-well plates and then infected with live *M. fermentans* (50 ng DNA/well). Control, uninfected cells received vehicle alone without MP. At 3 days postinfection, medium was changed to 3 ml serum-free MEM with 0.1% BSA containing either TNF-β (25 ng/ml), IL-1β (1 ng/ml), or no addition. Forty-eight hours later, conditioned medium was harvested, centrifuged, and analyzed for the indicated cytokines by ELISA. Cell monolayer was lysed in 0.1% crystal violet-0.1% citric acid, and cell number was determined by nuclei counts. Data are mean ± SE; $n = 3$. *Significant difference ($P < 0.05$) by Student's *t*-test compared to uninfected cells exposed to the same inducing cytokine. The ability of TNF-β to induce various cytokines (expressed as fold increase above basal level for uninfected and infected cells, respectively) was as follows: IL-6 (21-fold, 161-fold), IL-8/CXCL8 (7-fold, 55-fold), Gro-α/CXCL1 (16-fold, 22-fold), and MCP-1/CCL2 (47-fold, 14-fold). Similarly, IL-1β induced IL-6 (2,376-fold, 2,907 fold), IL-8/CXCL8 (334-fold, 670-fold), Gro-α/CXCL1 (1,357-fold, 237-fold), and MCP-1/CCL2 (99-fold, 26-fold). The magnitude of RANTES induction was difficult to quantify because this cytokine was undetectable in both uninfected and MP-infected unstimulated cultures, although was substantially elevated above the lower limit of detection (~50 pg/100,000 cells) after TNF-β and IL-1β stimulation.

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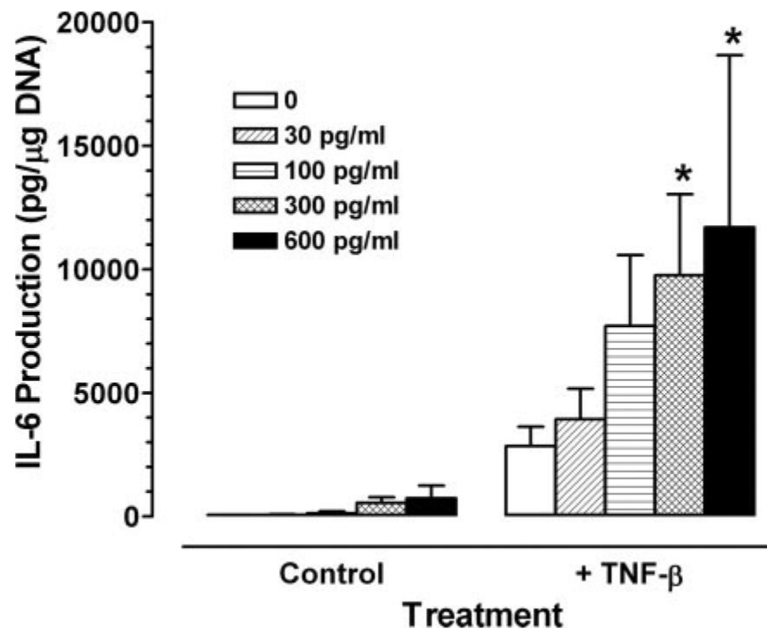

Fig. 4.

Macrophage-activating lipopeptide 2 (MALP-2) derived from *M. fermentans*, a specific Toll-like receptor (TLR)-2/TLR-6 agonist, is sufficient to synergistically interact with TNF-β to induce IL-6 from HLF. HLFs were seeded into 24-well plates and allowed to attach overnight. Cells were then exposed to TNF-β (25 ng/ml) with or without the indicated concentrations of MALP-2. Control cells received MALP-2 alone in the absence of TNF-β. IL-6 content in conditioned medium was measured 48 h after exposure. Cytokine release is expressed per μg cellular DNA in the producing cell monolayer. Data are means ± SE; $n = 4$. *Significant difference relative to the control group exposed to the same concentration of MALP-2 in the absence of TNF-β by one-way ANOVA and Bonferroni multiple comparisons of selected groups. The effect of MALP-2 alone was significant at both conditions by a posttest for linear trend over the range of MALP-2 concentrations applied.

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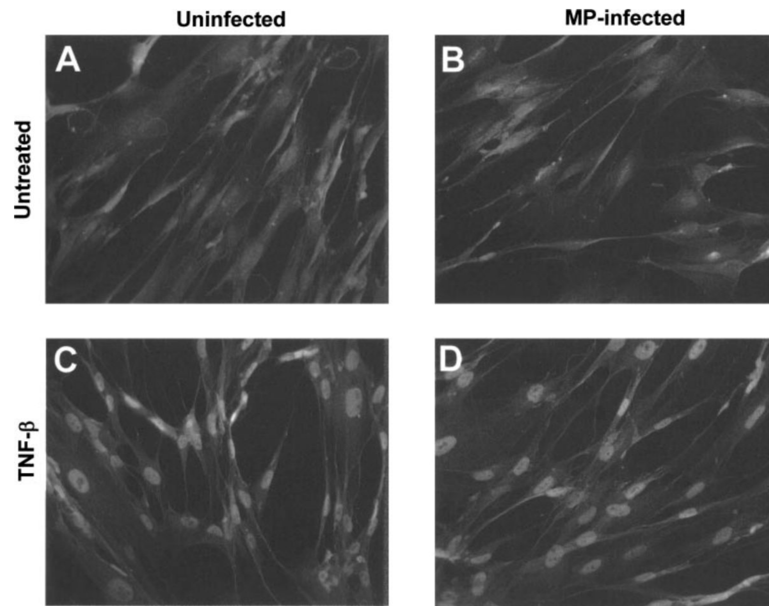

Fig. 5.

TNF-β induces early nuclear localization of p65 NF-κB subunit in both the absence (*A* and *C*) and presence (*B* and *D*)of *M. fermentans*. Control and *M. fermentans*-infected HLFs were seeded onto coverslips pretreated with Cell-Tak and cultured for 4 days. Medium was then changed and replaced with fresh medium with or without TNF-β (25 ng/ml) and incubated for 45 min. Cells were then fixed, permeabilized, and prepared for immunohistochemistry using anti-human NF-κB p65 antibody as described in MATERIALS AND METHODS. Cells were viewed under epifluorescence by ×40 objective.

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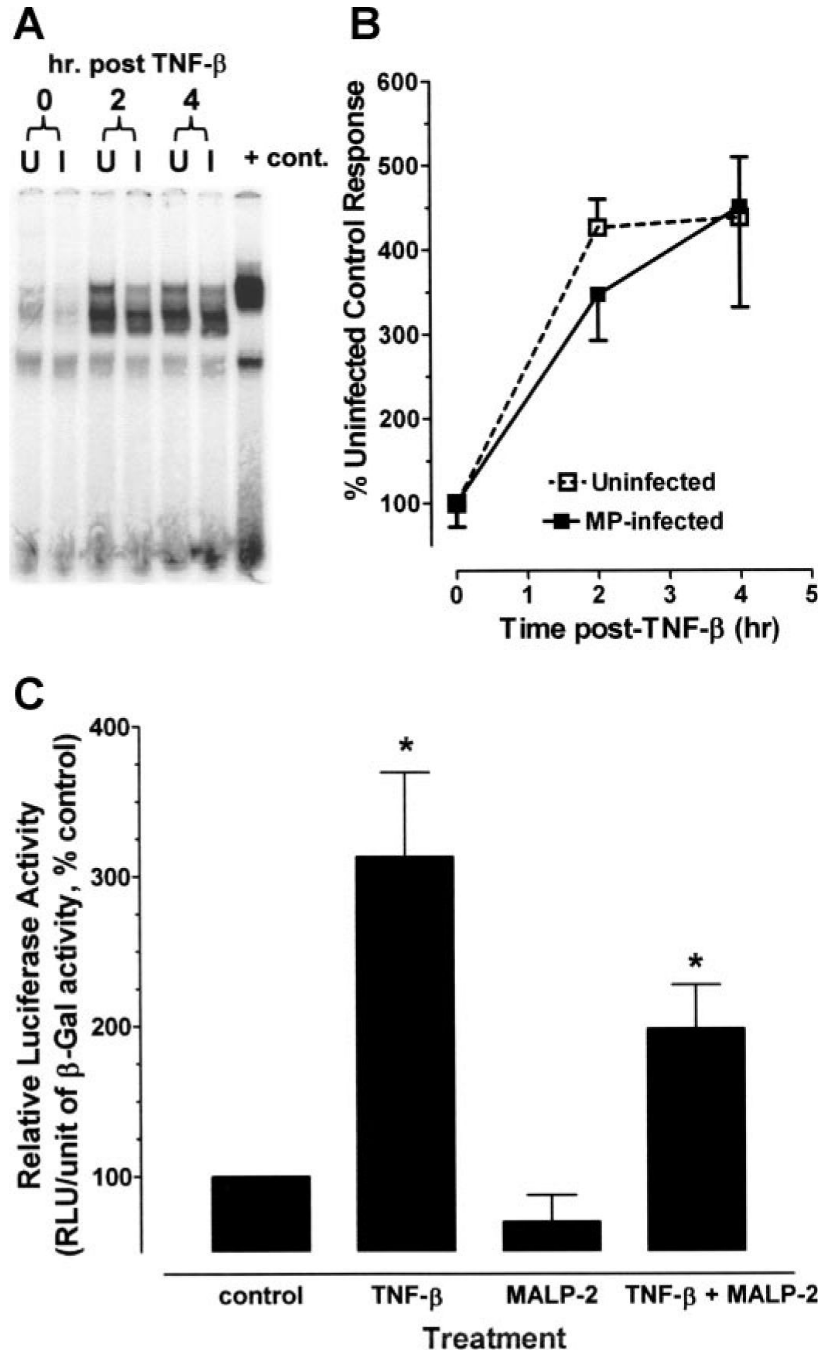

Fig. 6.

M. fermentans microbial stimulation does not augment early TNF-β-dependent induction of NF-κB DNA binding or transcriptional activity in HLF. *A* and *B*: results of EMSA assays of NF-κB DNA binding ability. Infected (I) and uninfected (U) HLF were treated with TNF-β (25 ng/ml), and nuclear extracts were prepared 2 and 4 h later. NF-κB DNA binding activity was assessed by EMSA as described in MATERIALS AND METHODS. Data shown are a typical Phosphorimager scan of radioactivity from a gel shift experiment (*A*) and quantification of the mean ± SE responses obtained from 4 different cell lines (*B*). To measure NF-κB transcriptional activity (*C*), normal uninfected HLF were cotransfected with pNFκBTAL-Luc and pCMV-β-Gal using Lipofectamine Plus and allowed to recover overnight. Cells were then stimulated

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with TNF-β (25 ng/ml) and/or MALP-2 (600 pg/ml) under serum-free conditions. Cell lysates were prepared at 4 h after stimulation, and luciferase activity was measured. Luciferase activity was normalized to the β-galactosidase activity in each well to control for well-to-well variability in transfection efficiency. Data represent the induction of luciferase activity relative to that seen in untreated control cells processed at the same time point. Data are means ± SE; $n = 3$. RLU, relative light units.

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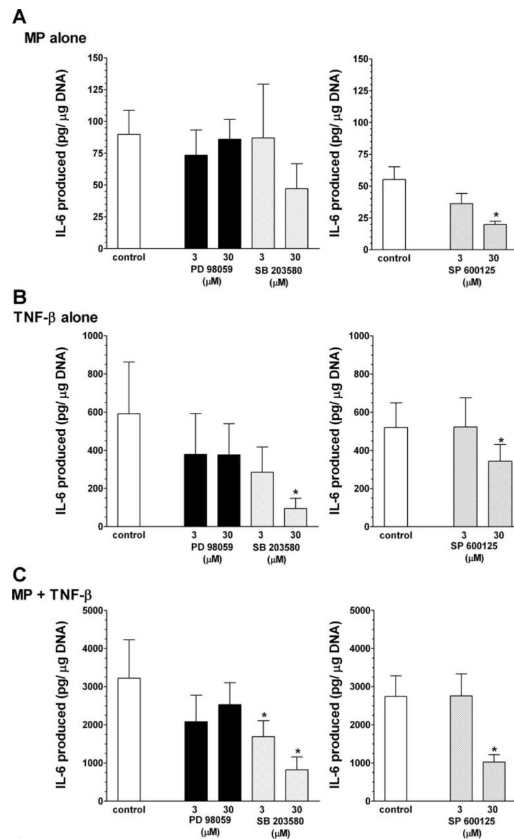

Fig. 7.

Effect of pharmacological inhibitors of specific MAPK pathways on early phase of TNF-β and MP-induced IL-6 release by HLF. Uninfected and MP-infected HLFs were seeded into 24-well plates and cultured for 48 h. Medium was changed to 1 ml MEM plus 0.1% BSA, and the cumulative release of IL-6 over 24 h was measured in MP-infected cells without TNF-β (*A*), control cells treated with 25 ng/ml TNF-β (*B*), or MP-infected cells treated with TNF-β (*C*) in the presence or absence of 3 or 30 μM PD-98059, SB-203508, or SP-600125. Control cells received vehicle alone. IL-6 release is expressed relative to the DNA content of each well. Data represent means ± SE; $n = 3$. *Statistically significant difference compared to control in the absence of drug by one-way repeated-measures ANOVA and Bonferroni multiple comparisons test ($P < 0.05$).

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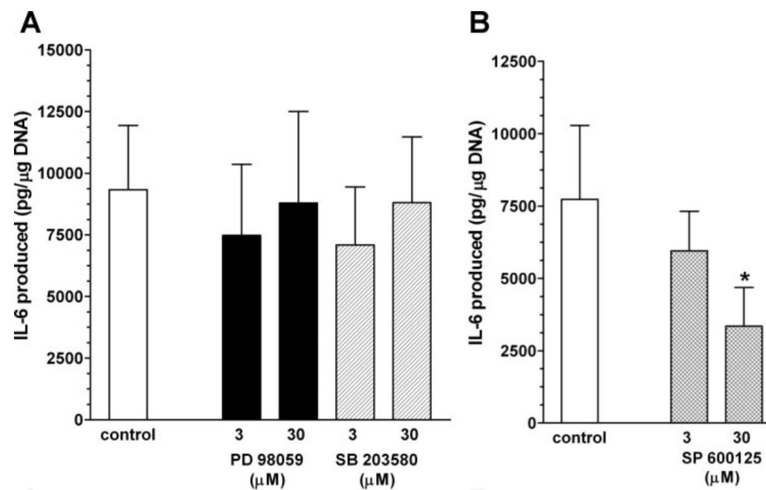

Fig. 8.

Effect of MAPK inhibitors on late phase of HLF IL-6 release in response to MALP-2 and TNF-β. MP-infected fibroblasts were seeded into 24-well plates and cultured for 48 h. Medium was then changed to 1 ml MEM plus 0.1% BSA containing 25 ng/ml TNF-β. After 24-h exposure, MAPK inhibitors were introduced to the indicated final concentrations without changing the medium, and cells were then allowed to continue conditioning the media for another 24 h. Medium was then harvested, and IL-6 was measured by ELISA and normalized to the DNA content of the monolayer. Data represent means ± SE; $n = 4$ for *A* and 3 for *B*. *Statistically significant difference compared to control in the absence of drug by one-way repeated-measures ANOVA and Bonferroni multiple comparisons test ($P < 0.05$).

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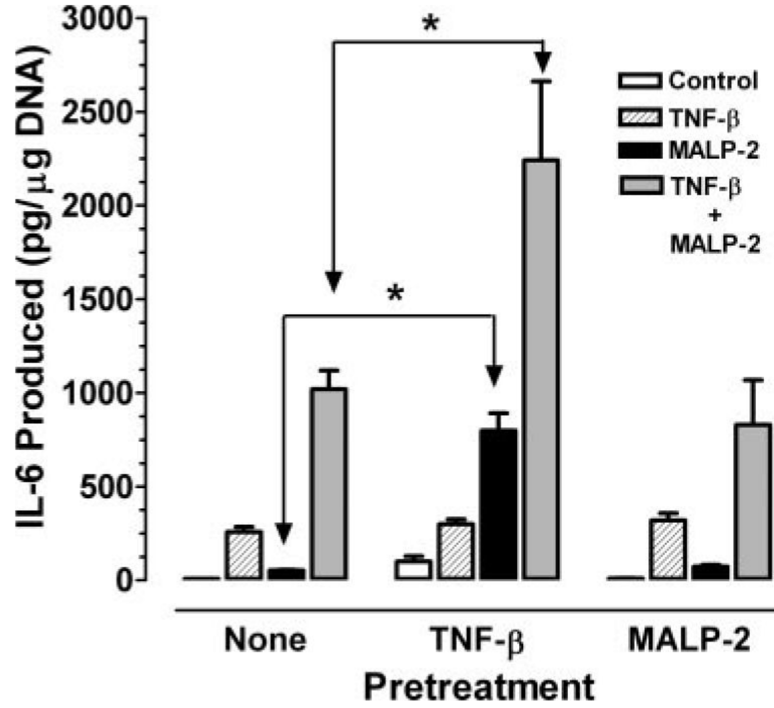

Fig. 9.

TNF-β preexposure enhances sensitivity of HLF to MALP-2, but MALP-2 does not enhance subsequent response to TNF-β. HLFs were seeded into 24-well plates and grown for 48 h. Cells were then preexposed to MALP-2 (600 pg/ml) or TNF-β (25 ng/ml) in serum-free MEM plus 0.1% BSA. Control cells received serum-free medium alone. Medium was then removed 24 h later, and each preexposure condition then received either fresh medium alone (control) or medium containing 600 pg/ml MALP-2 or 25 ng/ml TNF-β alone or in combination for an additional 24 h at which time medium was collected and analyzed for IL-6. Data represent means ± SE; $n = 4$. *Statistically significant difference between indicated groups by one-way ANOVA and Bonferroni multiple comparisons test ($P < 0.05$).