CCL5 (or RANTES (regulated upon activation, normal T cell expressed and secreted)) recruits T lymphocytes and monocytes. The source and regulation of CCL5 in pulmonary tuberculosis are unclear. Infection of the human alveolar epithelial cell line (A549) by Mycobacterium tuberculosis caused no CCL5 secretion and little monocyte secretion. Conditioned medium from tuberculosis-infected human monocytes (CoMTB) stimulated significant CCL5 secretion from A549 cells and from primary alveolar, but not upper airway, epithelial cells. Differential responsiveness of small airway and normal human bronchial epithelial cells to CoMTB but not to conditioned medium from unstimulated human monocytes was specific to CCL5 and not to CXCL8. CoMTB induced CCL5 mRNA accumulation in A549 cells and induced nuclear translocation of nuclear factor-κB (NFκB) subunits p50, p65, and c-rel at 1 h; nuclear binding of activator protein (AP)-1 (c-Fos, FosB, and c-Jun) at 4–8 h; and binding of NF-interleukin (IL)-6 at 24 h. CCL5 promoter-reporter analysis using deletion and site-specific mutagenesis constructs demonstrated a key role for AP-1, NF-IL-6, and NFκB in driving CoMTB-induced promoter activity. The IL-1 receptor antagonist inhibited A549 and small airway epithelial cell CCL5 secretion, gene expression, and promoter activity. CoMTB contained IL-1β, and recombinant IL-1β reproduced CoMTB effects. Monocyte alveolar, but not upper airway, epithelial cell networks in pulmonary tuberculosis cause AP-1, NF-IL-6, and NFκB-dependent CCL5 secretion. IL-1β is the critical regulator of tuberculosis-stimulated CCL5 secretion in the lung.

CCL5 is a member of the CC chemokine subfamily and a chemoattractant for CD4+ memory T lymphocytes, monocytes, and eosinophils (1, 2). Originally described as restricted to activated T lymphocytes, CCL5 is expressed by many cell types including fibroblasts, renal and pulmonary epithelium, endothelium, and airway smooth muscle (3–6). Tuberculosis is principally a pulmonary disease causing 3 million deaths each year (7), yet the regulation of cellular influx to pulmonary granuloma is poorly defined. Tuberculous granulomas contain cell types potentially recruited by CCL5, such as antigen-specific T lymphocytes and cells of the monocyte/macrophage lineage. Macrophages from human tuberculosis lymph node granulomas express both CCL5 protein and gene (8). Furthermore, anti-CCL5 antibodies decrease pulmonary granuloma lesion size in Mycobacterium bovis BCG strain-infected mice, suggesting a functional role for CCL5 in murine mycobacterial granulomas (9). CCL5 concentrations in BALF from infected patients rise acutely, correlating with BALF CD4+ T lymphocyte counts, and fall during convalescence (10, 11). However, in vitro studies have demonstrated that human alveolar macrophages and monocytes infected with Mycobacterium bovis BCG secrete only low concentrations of CCL5 (11).

The pulmonary epithelium is the initial barrier to respiratory infection by M. tuberculosis. Epithelial cells, covering the entire alveolar surface area of the lung of ~70 m² (12), may contribute to host defense by chemokine production (13), by adhesion molecule expression (14), and possibly by antigen presentation via HLA-DR expression (15). CCL5 may be secreted by pulmonary epithelial cells in response to proinflammatory cytokines (5, 13, 16, 17), hyperosmolarity (18), or pathogens such as RSV (19, 20). However, virulent M. tuberculosis, strain H37-Rv, which stimulates epithelial cell CXCL8 (IL-8) secretion (21), does not cause CCL5 release (22) despite invasion and replication of the pathogen within alveolar epithelial cells (23). Thus, direct infection of alveolar macrophages, monocytes, or epithelial cells by tuberculosis cannot explain the source of CCL5 detected in the BALF of patients with tuberculosis. We hypothesized that cellular networks between lung epithelium and monocytes involving proinflammatory cytokines were central to CCL5 secretion in pulmonary tuberculosis. Monocyte-derived IL-1β may be critical in such networks as polymorphisms in the IL-1 locus can affect relative IL-1β and IL-1ra monocyte responses to M. tuberculosis (24), and IL-1β and IL-1ra imbalances may affect outcomes of chronic diseases (25).

The mechanisms regulating CCL5 secretion in lung epithelium are not completely understood but may be cell type- and stimulus-specific. Transcriptional regulation of the CCL5 gene is critical in T lymphocytes (1, 2, 26, 27). The CCL5 promoter contains response elements for the transcriptional activators NFκB, AP-1, and NF-IL-6 (26, 27) and for late acting factors restricted to T cells (28). NFκB comprises a family of Rel-related proteins retained in the cytoplasm bound to the inhib-
Mycobacterial Culture—

Human monocytes were prepared from pooled buffy coats (North Thames Blood Transfusion Service, Colindale, UK) by density gradient centrifugation on a Ficoll-Paque (Amersham Biosciences). The monocytes were purified by adherence to tissue culture plastic for 2 h and maintained in RPMI 1640.

Mycobacterial Culture—The virulent strain of M. tuberculosis, H37-Rv (National Collection of Type Cultures, Colindale, UK), was maintained in enriched Dubos medium (Difco). Before use, 1 ml of a Mycobacterium tuberculosis (MOI 100) was left unstimulated for 24 h and in the conditioned medium, CoMTB or CoMCtrolled, respectively, was harvested and stored at -70 °C. Cultures were exposed to M. tuberculosis (MOI 10), CoMTB (at dilutions of 1:10, 1:100, and 1:1000 in appropriate serum-free medium), CoMCtrolled, or TNFα (20 ng/ml) (Peprotech, Rocky Hill, NJ). In experiments involving IL-1α (37) (Peprotech) and IL-6 (38), epithelial cells were incubated for 2 h at 37 °C prior to use, and in those involving neutralizing anti-TNF antibody (Peprotech), CoMTB was preincubated with the antibody for 1 h at 37 °C prior to use. We confirmed previously that 50 μg/ml anti-TNF neutralizes activity of 10 ng/ml TNFα (21).

**RNA Extraction and Northern Blotting**—Epithelial cells were homogenized in RNA extraction buffer (4 m guanidinium thiocyanate, 25 mM Tris, pH 7.0, 0.5% N-lauroylsarcosine, and 0.1 ml 2-mercaptoethanol), and RNA extraction was performed using a modified guanidium thiocyanate/phenol/chloroform extraction method (38, 39). 20-μg RNA aliquots were run on denaturing formaldehyde-1% agarose gels, transferred by capillary blotting to Hybond-N, and UV-fixed (UV Stratalink, Stratagene, La Jolla, CA). Blots were hybridized and probed with a γ-32P-end-labeled oligonucleotide probe mixture for CCL5 (BPR246, R&D Systems, Minneapolis, MN) and later a β-actin 42-mer probe (40). Blots were autoradiographed, and images were digitized (Umax, Power Look II) and analyzed with NIH Image 1.52 (from National Institutes of Health Research Services Branch, Bethesda, MD). CCL5 signal densitometry was normalized for total RNA loading using β-actin mRNA densitometry.

**Cytokine Assays**—CCL5 or IL-1β (R&D Systems) was assayed in cell culture supernatants by ELISA. The lower limit of sensitivity was 15 pg/ml for the CCL5 assay and 7.8 pg/ml for the IL-1β assay. CCL5 concentrations are expressed in pg/106 cells, and IL-1β concentrations are expressed as ng/ml.

**Electrophoretic Mobility Shift Assays**—Nuclear extracts were prepared using a modified version of an established protocol (41). Following stimulation, cells were washed with cytoplasmic extraction buffer (10 mM Tris-HCl, 60 mM KCl, 1 mM EDTA, 1 mM diithiorthreitol) containing protease inhibitors and with 0.15% Nonidet P-40 added. After centrifugation (500 × g), the nuclear pellet was resuspended in nuclear extract buffer (20 mM Tris-HCl, pH 8.0, 400 mM NaCl, 1.5 mM MgCl2, 1.5 mM EDTA, 25% glycerol, 1 mM diithiorthreitol, and protease inhibitors), incubated on ice, and recentrifuged. The protein was quantitated by Bradford assay (42). 5 μg of nuclear extract was mixed with 0.7 μg of γ-32P-end-labeled standard DNA probe with specificity greater than 1 × 106 cpm/μg DNA binding buffer (10 mM Tris, pH 7.5, 50 mM NaCl, 1 mM EDTA, 1 mM diithiorthreitol, 0.25 mg/ml bovine serum albumin, 3 mg of poly(dl-dC), and 5% glycerol) and was resolved on 4% polyacrylamide gels. The oligonucleotides contained the consensus sequence corresponding to the NFκB (5'-AGTTGAGGGGACTTCCCCAGG-3'), AP-1 (5'-CTAGTGATGTCATCAACCTCCACC-3'), or NF-IL-6 (5'-TGAGTCA-3') binding sites (43). Consensus sequences allow concurrent investigation of the four different NFκB binding sites and the four TPA-response elements. Dried gels were autoradiographed at ~70 °C overnight. Binding specificity was demonstrated by competition assays using a 10-fold excess of unlabeled probe and by failure to detect the signal with an excess of unlabeled probe. For supershift assays, 1 μg of antibodies either to the NFκB subunits p50, p52, p65, Rel B, and c-rel or to c-Fos (a subunit of the unrelated transcription factor AP-1) (Santa Cruz Biotechnology, Santa Cruz, CA) was added to the binding mix before electrophoresis.

**ELISA-based Transcription Factor Assay**—To investigate the complex binding to the multiple subunits of AP-1, a transcribed ELISA-based assay (TransAMTM, Active Motif North America, Carlsbad, CA), which is 5-fold more sensitive than EMSA, was performed. Nuclear extracts were added to a 96-well plate containing immobilized oligonucleotides including a TPA-responsive element (TRF, 5'-TGGAGCTCA-3'), AP-1 dimers in the nuclear extract bind to this TPA-responsive element and were detected using antibodies c-Fos, c-Jun, NFκB, p50, p52, Rel B, and c-rel or to c-Fos (a subunit of the unrelated transcription factor AP-1) (Santa Cruz Biotechnology, Santa Cruz, CA) was added to the binding mix before electrophoresis.

**Nuclear protein extraction**—Nuclear extracts were prepared using a modified version of an established protocol (41). Following stimulation, cells were washed with cytoplasmic extraction buffer (10 mM Tris-HCl, 60 mM KCl, 1 mM EDTA, 1 mM diithiorthreitol) containing protease inhibitors and with 0.15% Nonidet P-40 added. After centrifugation (500 × g), the nuclear pellet was resuspended in nuclear extract buffer (20 mM Tris-HCl, pH 8.0, 400 mM NaCl, 1.5 mM MgCl2, 1.5 mM EDTA, 25% glycerol, 1 mM diithiorthreitol, and protease inhibitors), incubated on ice, and recentrifuged. The protein was quantitated by Bradford assay (42). 5 μg of nuclear extract was mixed with 0.7 μg of γ-32P-end-labeled standard DNA probe with specificity greater than 1 × 106 cpm/μg DNA binding buffer (10 mM Tris, pH 7.5, 50 mM NaCl, 1 mM EDTA, 1 mM diithiorthreitol, 0.25 mg/ml bovine serum albumin, 3 mg of poly(dl-dC), and 5% glycerol) and was resolved on 4% polyacrylamide gels. The oligonucleotides contained the consensus sequence corresponding to the NFκB (5'-AGTTGAGGGGACTTCCCCAGG-3'), AP-1 (5'-CTAGTGATGTCATCAACCTCCACC-3'), or NF-IL-6 (5'-TGAGTCA-3') binding sites (43). Consensus sequences allow concurrent investigation of the four different NFκB binding sites and the four TPA-response elements. Dried gels were autoradiographed at ~70 °C overnight. Binding specificity was demonstrated by competition assays using a 10-fold excess of unlabeled probe and by failure to detect the signal with an excess of unlabeled probe. For supershift assays, 1 μg of antibodies either to the NFκB subunits p50, p52, p65, Rel B, and c-rel or to c-Fos (a subunit of the unrelated transcription factor AP-1) (Santa Cruz Biotechnology, Santa Cruz, CA) was added to the binding mix before electrophoresis.
before assaying with the c-Fos antibody.

**Western Blotting and SDS-PAGE**—Cells were lysed with phosphate-buffered saline containing 0.1% Nonidet P-40, 0.5% deoxycholate, 10 mM NaF, 1 mM Na3VO4, 170 μg/ml phenylmethylsulfonyl fluoride, and protease inhibitors as described previously (19). Lysates were centrifuged (8000 × g), the supernatant was removed, and loading buffer (10% glycerol, 5% 2-mercaptoethanol, 2% SDS, and bromphenol blue) was added. Samples were boiled, separated by SDS-PAGE, and electroblotted to nitrocellulose membranes. Western blots were blocked and incubated at 4°C with 1 μg/ml rabbit anti-human IκBα (Santa Cruz Biotechnology). After incubation with peroxidase-conjugated goat anti-rabbit IgG, bands were detected using enhanced chemiluminescence reagents and film.

**Transfections and Luciferase Assays**—Promoter-reporter constructs of the 5′-flanking region of the CCL5 gene were used (generous gift of Dr. William Reed, Environmental Protection Agency, Chapel Hill, NC). One construct contained the full-length (–961) WT CCL5 promoter containing four NFκB, four AP-1 (TRE), and one NF-IL-6 binding site(s) (Fig. 5). The remaining constructs had been truncated by serial deletions (at -737, -421, -181, and -79). The -421 construct deleted the 60-bp CD28/NFκB site. The -181 construct deleted all TPA-response elements and a NFκB binding region of low affinity (–231); the -79 construct deleted the NF-IL-6 site but retained two NFκB binding sites at positions -30 and -44 (19, 20). Constructs containing site-specific mutations were the very generous gift of Professor Hiroaki Moriuchi, Nagasaki University Graduate School of Biomedical Sciences, Nagasaki, Japan. These constructs contained mutations of the -κB1 (–44), -κB2 (–30), NF-IL-6 (–92), TRE1/2 (–345), TRE3/4 (–327), TRE1/2/3/4, and CD28/NFκB (–579, –44, –30) sites as described previously (27). Constructs had been inserted into the firefly luciferase expression plasmid PGL2-basic. A549 cells were co-transfected using FuGENE™ 6 (Roche Applied Science) with 4 μg of a CCL5 reporter plasmid and 0.08 μg of a control reporter plasmid, PRL-TK, constitutively expressing low level Renilla luciferase. Following cell stimulation, luciferase activity of extracts was measured using the Dual-Luciferase™ reporter assay system (Promega, Madison, WI) with a luminometer (Bio-Orbit 1253, Labtech International, East Sussex, UK). Renilla luciferase activity was used to normalize firefly activity for control transfection efficiency.

**RESULTS**

**Effect of CoMTB on CCL5 Secretion from Pulmonary Epithelial Cells Compared with Effects of Direct Infection by M. tuberculosis**—CoMTB was used to stimulate A549 cells over 24 h at dilutions of 1:10, 1:100, and 1:1000 in serum-free Dulbecco’s modified Eagle’s medium. A549 cells stimulated with CoMTB (1:10) secreted 20,342 ± 2142 pg of CCL5/10⁶ cells within 4 h and 53,250 ± 2696 pg of CCL5/10⁶ cells after 24 h (Fig. 1A). Such CoMTB-induced CCL5 secretion at 24 h was 12-fold greater than from tuberculosis-infected human monocytes, which as expected secreted only 4310 ± 816 pg of CCL5/10⁶ cells at 24 h (Fig. 1B). A549 cells directly infected by M. tuberculosis (MOI of 10, causing no significant cytotoxicity (44)) did not release CCL5 unlike the positive control TNFα (Fig. 1C). To confirm our findings with CoMTB in primary cells, NHBE were stimulated with CoMTB, but no CCL5 release was detected. Because tuberculosis is primarily an infection of the alveolar airway, we investigated the effect of CoMTB on SAEC, which are harvested from terminal bronchi of 2-mm diameter and below including alveoli consisting of type I and II pneumocytes. In contrast to NHBE, SAEC stimulated with CoMTB did secrete significant CCL5 at concentrations of 809 ± 141 pg/10⁶ cells (Fig. 1D), suggesting a differential responsiveness of upper and lower airway epithelium to CoMTB. For this reason, further experiments focused on distal airway cells.

The finding that lower but not upper airway epithelial cells respond to CoMTB by secreting CCL5 appears specific. Thus, both NHBE and SAEC stimulated with CoMTB secrete CXCL8, although the magnitude of the response in the two cell types is different (Fig. 1E). CXCL8 secretion after CoMTB stimulation increased from background levels (because of monocyte-derived CXCL8 in CoMTB) to 16,911 ± 3381 pg/10⁶ NHBE. This was over 3.5-fold greater than that found in NHBE after stimulation for 24 h with CoMControl. The absolute concentration of CoMTB-induced CXCL8 secretion from lower airway epithelial cells was higher at 57,004 ± 325 pg/10⁶ SAEC, although this represented just a 4.5-fold increase compared with CoMControl at 24 h.

**CoMTB Induces Early CCL5 mRNA Accumulation in A549 Cells**—Northern analysis demonstrated CCL5 mRNA accumulation by 2 h, peaking at 8 h and present at 24 h post-CoMTB stimulation of A549 cells (Fig. 2A), with none seen in controls (Fig. 2B). These kinetics are consistent with those for the CCL5 protein secretion. CoMTB exhibited a dose-response effect in stimulating CCL5 mRNA accumulation (Fig. 2C) demonstrating that even at a dilution of 1:100 the CoMTB was capable of inducing significant CCL5 mRNA up-regulation at 24 h. This is consistent with the observed dose-response effect of CoMTB on CCL5 secretion (Fig. 1A).

**Activation of NFκB and Degradation of IκBα in CoMTB-stimulated A549 Cells**—The role of NFκB in the control of CCL5 gene expression in pulmonary epithelial cells was analyzed by EMSA. Cell nuclear extracts of A549 and SAEC were stimulated by CoMTB or CoMControl for over a 24-h period. CoMTB stimulated strong NFκB nuclear binding in A549 cells at 1–2 h, which decreased at 4 h consistent with the kinetics of onset of CCL5 gene expression (Fig. 3A, upper panel). NFκB activation was not observed with CoMControl (Fig. 3A, lower panel). CoMTB-stimulated SAEC demonstrated specific NFκB binding at 1 h post-CoMTB (Fig. 3B, upper panel), which persisted for longer, at least to 8 h, than in A549 cells. Supershift assay showed that the p50 and p65 NFκB subunits were strongly activated and that c-rel was weakly activated in the SAEC stimulated by CoMTB after 1 h. No supershift was seen with an irrelevant antibody c-Fos (Fig. 3C). Rapid degradation of IκBα following A549 cell stimulation by CoMTB was shown by Western analysis (Fig. 3D) with kinetics in keeping with the early but transient nuclear localization of NFκB. IκBα protein disappeared within 5 min, reappeared within 90 min, and was completely resynthesized by 180 min.

**Nuclear Translocation of AP-1 and NF-IL-6 in Response to CoMTB Stimulation of A549 and SAEC**—Nuclear extracts from A549 and SAEC stimulated by CoMTB or CoMControl for 0, 2, 4, 8, and 24 h were analyzed by EMISA using oligonucleotides corresponding to the NF-IL-6 or AP-1 binding sites. To prevent excess constitutive AP-1 expression, cells were prepared in serum-containing medium (45). Although AP-1 binding was constitutively present in A549 cells, increased nuclear translocation was evident 4 h post-CoMTB stimulation, persisting at lower levels between 8 and 24 h (Fig. 4A). A similar pattern of increase in AP-1 binding was demonstrated with CoMTB-stimulated SAEC (Fig. 4B). The kinetics of NF-IL-6 binding was very different compared with AP-1 or NFκB in A549 cells. NF-IL-6 nuclear translocation was seen only after 24 h poststimulation in A549 cells (Fig. 4D) with a relatively low signal intensity. Specificity of binding of each transcription factor was confirmed by competition studies (Figs. 4, A, B, and E). Competition for AP-1 binding was complete in A549 cells; however, in SAEC it appears that there is a small amount of total binding that is nonspecific and is not competed out by a specific probe, although the band intensity is much reduced compared with experiments involving an irrelevant probe (Fig. 4B, 6th and 7th lanes). In terms of specific AP-1 subunits, c-Jun, FosB, and to a lesser extent c-Fos had low level constitutive activity and were activated following the CoMTB but not the CoMControl at 4 h (Fig. 4C). Other members of the Fos/Jun family (Fra-2, JunB) were constitutively activated but were not significantly altered by CoMTB or CoMControl. Signal specificity was shown by an excess of WT AP-1 oligonucleotide

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FIG. 1. CCL5 secretion from pulmonary epithelial cells and monocytes stimulated by CoMTB or M. tuberculosis. Cells were stimulated as described below, and cell culture supernatants were assayed for CCL5 by ELISA. All results are mean ± S.E. of three independent experiments. A, A549 cells were stimulated with CoMTB over 24 h at dilutions of 1:10, 1:100, and 1:1000 and with CoMControl at a dilution of 1:10.
CoMTB induces early CCL5 mRNA accumulation in A549 cells. CCL5 mRNA accumulation from CoMTB-stimulated cells (A) and CoMControl-stimulated cells (B) stimulated at a 1:10 dilution (as described in Fig. 1) was assessed by Northern analysis at 0, 1, 2, 4, 8, and 24 h poststimulation. Shown are a representative autoradiograph and densitometric analysis of CCL5 mRNA normalized for total RNA loading using β-actin densitometry. CCL5 mRNA up-regulation was evident at 2 h and strongly expressed at 8–24 h. C, CCL5 mRNA accumulation from A549 cells stimulated by CoMTB for 24 h at dilutions of 1:10, 1:100, and 1:1000 and from cells stimulated by CoMControl (1:10 dilution) was assessed by Northern analysis as above.

causing a mean 16.65-fold reduction in the c-Fos signal compared with a 1.03-fold increase with mutated AP-1 oligonucleotide.

**CoMTB Activates the CCL5 Promoter and Requires Intact NFκB-, AP-1-, and NF-IL-6-response Elements**—Nuclear binding of transcription factors provides indirect evidence of their involvement in regulating CCL5 gene activation. To investigate the functional effects of CoMTB, promoter-reporter gene analysis was performed (Fig. 5). Kinetic studies with the WT (−96) CCL5 promoter demonstrated that CoMTB-induced activation in A549 cells occurred by 2 h (2.5 ± 0.6-fold increase over CoMControl) with maximal activity at 8 h (21.7 ± 5.0-fold increase) (Fig. 5B) and continued until 24 h (10.2 ± 0.8-fold increase). These data are consistent with EMSAs showing binding of first NFκB, then AP-1 at 4 h, and finally NF-IL-6 at 24 h. To identify which transcription factor binding sites within the promoter were functionally important, deletion constructs were used (Fig. 5, A and C). Deletion −737 reduced CCL5 reporter activity significantly (to 37.48 ± 7.49% of wild type activity). Few consensus binding sites have been identified in this area; some are T cell-specific, whereas others, such as a CCAAT/enhancer-binding protein (C/EBP) site, are ubiquitous (26). A greater deletion at −579, did not reduce promoter activity more than the −737 deletion (41.9 ± 15.7% versus 37.5 ± 7.5% of wild type, respectively) (Fig. 5C). With the −181 deletion construct, which has also lost the AP-1-response elements, a further reduction in reporter activity to 20.9 ± 2.9% of WT occurred. With the smallest construct, having 79 bp upstream of the transcriptional start site intact and no longer containing the NF-IL-6 binding site but containing two intact NFκB sites, there was a reduction to 5.5 ± 1.9% maximal luciferase activity values, similar to those obtained with the CoMControl stimulus (5.9 ± 1.8% of maximal luciferase activity). These data indicate that AP-1 and NF-IL-6 binding sites may be required for CoMTB-induced CCL5 promoter activity.

Site-directed mutagenesis, however, showed that proximal NFκB binding sites (κB1 and κB2) were important in CCL5 promoter activity with activity decreasing, respectively, to 49.5 ± 7.8% and 20.4 ± 1.72% of WT (Fig. 6). This was not evident from the analysis with deletion constructs presumably because of the significant loss of upstream transcription factor binding sites. ΔCD28RE/κB2/κB1 reduced activity to 19.0 ± 4.2%, which is no different from the effect of the ΔκB2 mutation alone and is consistent with the data using the deletion reporter constructs. ΔNF-IL-6 and ΔTRE3/4 (−327) reduced promoter-reporter activity to 49.4 ± 5.9% and 30.8 ± 16.4% of WT, respectively, whereas TRE1/2 had no effect (108.0 ± 32.2%). Mutation of all four TRE sites did not have any additional effect over that of ΔTRE3/4.

**Monocyte-derived IL-1 Is Essential for CCL5 Secretion by CoMTB-stimulated Pulmonary Epithelial Cells**—Polyclonal anti-human TNFα antibody or IL-1ra (46) was used to identify the active constituents of CoMTB (Fig. 7). IL-1ra completely blocked CCL5 secretion from CoMTB-stimulated A549 cells (71,500 ± 10,259 pg of CCL5/10⁶ cells reduced to 3255 ± 943 pg of CCL5/10⁶ cells), whereas anti-TNFα had no effect (Fig. 7A). Similarly, IL-1ra reduced CCL5 mRNA accumulation from 98.8 ± 10.1% to 24.5 ± 11.7% (mean-normalized densitometry).

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in serum-free Dulbecco’s modified Eagle’s medium. CoMTB induced concentration-dependent CCL5 secretion at 8 and 24 h. B, monocytes were infected with M. tuberculosis (strain H37-Rv, MOI 10) (7B) for 24 h or left unstimulated. M. tuberculosis-stimulated monocytes secreted low level CCL5 at 24 h. C, A549 cells were infected with M. tuberculosis (strain H37-Rv, MOI 10) or with 20 ng/ml TNFα or were exposed to medium alone over 24 h. M. tuberculosis failed to induce significant CCL5 secretion from A549. D, NHBE and SAEC were stimulated with CoMTB and CoMControl at dilutions of 1:10 for 24 h. CCL5 (D) or CXCL8 (E) measured at 7 – 0 represents monocyte-derived chemokine present in CoMTB, SAEC secreted significant levels of CCL5 following stimulation by CoMTB, but NHBE, derived from the upper airway, did not (D). In contrast, SAEC and NHBE both secreted CXCL8 when stimulated by CoMTB (E). RANTES, regulated upon activation, normal T cell expressed and secreted.
try ± S.E.), whereas anti-TNFα had no effect (Fig. 7B). In combination anti-TNFα and IL-1ra decreased CCL5 gene expression to 5.7 ± 1.9%. The inhibitory effect of IL-1ra is thus mediated at the level of gene transcription and consistent with this IL-1ra reduced CCL5 WT (−961) promoter activity to 29.1 ± 1.4% (Fig. 6C). These findings were reproduced in the primary SAEC (Fig. 6D). Monocytes infected by *M. tuberculosis* secrete low levels of CCL5. Therefore, CoMTB contains CCL5, and this is represented by CCL5 at T = 0 (300 ± 46 pg/10⁶ cells). After 24-h stimulation of SAEC by CoMTB, CCL5 levels are 1109 ± 95 pg/10⁶ cells, and after IL-1ra pretreatment of SAEC, this is inhibited to almost base-line values (340 ± 56 pg/10⁶ cells). Anti-TNFα only had a very small inhibitory effect (reduction to 843 ± 47 pg/10⁶ cells).

Next CoMTB IL-1β concentrations were measured and found to be 2.05 ± 0.50 ng/ml (Fig. 8A). As CoMTB was used in experiments at dilutions of 1:10, the effective IL-1β stimulus to epithelial cells will be 0.2 ng/ml. Recombinant IL-1β at 0.2–2 ng/ml induced levels of CCL5 secretion from SAEC (Fig. 8B) comparable with levels of secretion from CoMTB at a 1:10 dilution (Fig. 1D). This confirms IL-1α as the critical mediator in the monocyte epithelial cell network resulting in CCL5 secretion from pulmonary epithelial cells.

**DISCUSSION**

In this study we have demonstrated that the large alveolar epithelial cell surface area is a significant contributor to production of the chemokine CCL5 during the pulmonary immune response to *M. tuberculosis*. Epithelial cell CCL5 secretion occurs as a result of monocyte-derived IL-1 released following phagocytosis of *M. tuberculosis*. We confirmed that epithelial cells infected directly by *M. tuberculosis* do not secrete significant concentrations of CCL5 (22). In contrast, when the alveolar epithelial cell line A549 is stimulated by CoMTB, high level CCL5 secretion occurs. A similar response of lower magnitude in human primary alveolar epithelial cells was observed. Alveolar macrophages and monocytes are an important source of immunoregulatory mediators in pulmonary tuberculosis (8, 47, 48). Although there are relatively few macrophages/alveolus, monocytes are recruited early to sites of tuberculous infection in significant numbers. We found that monocytes infected with *M. tuberculosis* at an MOI of 10:1 secrete CCL5 at only slightly
**Fig. 4. Kinetics of activation of AP-1 and NF-IL-6 in CoMTB-stimulated A549 cells and SAEC.** Nuclear extracts from A549 or SAEC stimulated by CoMTB or CoMControl over 24 h were analyzed by EMSA for AP-1 (A and B) or NF-IL-6 (D and E) binding. A, for A549 cells, AP-1 binding was maximal at 4 h and continued until 24 h. Binding specificity was shown by a 10-fold excess of unlabeled probe, but not unlabeled irrelevant NFκB probe, competing out the 4 h AP-1 binding signal. B, nuclear extracts from SAEC showed a strong AP-1 binding signal at 4 h, which persisted at a low level up to 24 h. Binding specificity of the 4 h AP-1 binding signal was demonstrated by competition studies as described above. C, specific AP-1 subunit binding was assessed by an AP-1 transcription factor assay in A549 cells stimulated with CoMTB or CoMControl at T = 0 h and T = 4 h. Subunit activation measured as optical density units is expressed as fold difference over time. c-Fos, FosB, and c-Jun were activated. D, NF-IL-6 binding was seen late and only at 24 h. A 10-fold excess of unlabeled NF-IL-6 probe (E, 3rd lane), but not of unlabeled AP-1 probe (E, 4th lane), competed out the NF-IL-6 binding.
higher concentrations than from CoMTB-stimulated SAEC. Even lower concentrations of monocyte-derived CCL5 secretion occurs in response to lower MOIs of *M. tuberculosis* such as are likely to be present in infected patients (11). The very large surface area of alveolar epithelium suggests therefore that the predominant source of CCL5 in the BALF during pulmonary tuberculosis will be epithelial cells exposed to secreted mediators from infected monocytes. The CCL5 secreted by pulmonary epithelium will stimulate the influx of T lymphocytes and further monocytes, critical for mycobacterial elimination and granuloma formation. CCL5 secretion from CoMTB-stimulated epithelial cells was high at 24 h consistent with the kinetics of CCL5 protein expression in pulmonary granulomas in murine models of mycobacterial inflammation (9).

We found that CCL5 secretion by CoMTB-stimulated pulmonary epithelium requires new gene transcription. In contrast, studies have shown that CCL5 secretion from tuberculosis-infected monocytes involved release of preformed, stored chemokine (11). In addition, posttranscriptional regulation of CCL5 gene expression, which we have not studied, may be important in RSV-induced CCL5 activation (49, 50). Differential control of CCL5 gene activation depends on transcriptional regulators and may occur in a cell type- and stimulus-specific fashion. The transcription factor NFκB comprises a family of at least five Rel-related subunits within the cytoplasm: p65, p50, p52, c-rel, and Rel B (29). Differing subunit combinations have stimulatory and inhibitory effects on gene promoter regions. CoMTB induced rapid nuclear translocation of NFκB in A549 cells and primary alveolar epithelium with activation of p65, p50, and c-rel subunits. In contrast, using adenoviral vectors overexpressing IkBα we have shown that RSV-induced CCL5 expression from pulmonary epithelium is NFκB-dependent and
involves the p50/p65 heterodimer of NFκB but not the c-rel subunit (19). The kinetics of CCL5 gene expression production differed from those of NFκB nuclear translocation in A549 cells. NFκB binding to the CCL5 promoter peaked at 1 h or earlier consistent with rapid IκBα degradation and the early generation of CCL5 mRNA transcripts at 2 h. CCL5 mRNA was still detected at 8–24 h even though NFκB binding was virtually absent by 4 h in A549 cells. Late detection of mRNA may be because of accumulation of mRNA that has undergone post-transcriptional stabilization rather than because of new gene transcription. RSV increases CCL5 mRNA half-life from 0.8 to 6.8 h in airway epithelial cells (49). Other transcription factors may assume greater importance in late promoter activation, and we have shown that AP-1 and NF-IL-6 are translocated at later time points than NFκB. Interestingly, in SAEC, NFκB nuclear translocation may be more critical in late CCL5 gene expression because NFκB translocation persisted until at least 8 h. Relatively few studies have directly investigated transcriptional regulation of CCL5 in lung epithelium in response to cytokine stimulation. CCL5 gene expression was shown to be NFκB-dependent in TNFα-stimulated A549 cells (16, 51) and in CD40-stimulated airway epithelial cells (52). However, NFκB may be necessary but not sufficient for CCL5 gene activation as indicated by the studies with our maximally truncated promoter containing the two proximal NFκB binding sites. IL-1β has been shown previously to activate p65 and p50 subunits in epithelial cell lines (53), and the fact that it drives IL-8 secretion, which is usually NFκB-dependent, suggests that this transcription factor is activated in NHBE. In addition, in another study involving primary epithelial cells in which the source is not clearly specified and may be a mixture of upper and lower airway cells, CCL5 gene expression and NFκB nuclear binding were demonstrated, although no CCL5 secretion was reported (35).

The CCL5 promoter contains four potential NFκB binding sites, –30, –44, –213, and –579 relative to the transcription start site (27). However, the –213 site is a nuclear factor of activated T cells (NFκB/AT) binding site with only a weak NFκB affinity, and the distal (–579) site also serves as a CD28-responsive element (26, 27). Transient transfection assays using a WT CCL5 promoter-reporter expression plasmid confirmed that the CoMTB stimulus activated the CCL5 promoter, evident at 4 h and peaking at 8 h poststimulation of A549 cells. The observation that deletion −737 reduced CCL5 reporter activity and that deletion −431 has no further effect indicated that the most distal NFκB binding site (−579) is of little functional significance in the alveolar epithelial cell response to CoMTB. Site-directed mutagenesis did not demonstrate a key role for this distal site but did confirm that the two proximal κB binding sites are important with promoter activity reduced to 20.4% of WT in studies using ∆κB2 construct. ∆NFκB1 and ∆CD28RE markedly reduced human immunodeficiency virus-induced CCL5 promoter activity (27), whereas ∆κB1/2 reduced both TNFα- (54) and RSV-induced activity (55), demonstrating stimulus specificity in CCL5 transcription factor binding site activation. However, in the distal promoter between −961 and −737, there is a cis-acting element for CCL5 promoter activity in alveolar epithelial cells. Previously, distal negative rather than positive regulatory sites have been identified in T cells (27), but this region has not been extensively investigated. In addition, in other cell types the proximal part of the promoter alone controls activity. For example, in IL-1β-stimulated astrocytoma cells (56) and T cells (26), deletions to 400 base pairs have little effect on CCL5 promoter activity. This was also the situation in alveolar epithelial cells stimulated by either RSV or TNFα, where a −220 deletion of the CCL5 promoter retained near wild type activity (54, 55).

An interesting finding was the involvement and functional significance of AP-1 and NF-IL-6 in the control of pulmonary epithelial cell CCL5 secretion in response to CoMTB. The CCL5 promoter contains four AP-1 TPA-response elements (26, 27). Evidence exists to indicate a role for AP-1, often involving cooperative interactions with NFκB, in the regulation of chemokine genes other than CCL5 such as CXCL8 gene activation in RSV-stimulated A549 cells and CCL2 (monocyte chemotactrant protein-1) gene activation in IL-1β-stimulated endothelial cells (34, 43, 45, 57). We demonstrate for the first time in epithelial cells a functional role for AP-1 in CCL5 gene activation, both in CoMTB-stimulated A549 cells and in human primary alveolar epithelial cells. CoMTB induced the nuclear translocation of AP-1 in both A549 and SAEC, exhibiting delayed kinetics compared with NFκB binding with maximal binding at 4–8 h poststimulation. The AP-1 family members binding to the TRE site were identified as c-Jun, FosB, and c-Fos in contrast with the c-Fos/JunD heterodimer, which binds to AP-1 sites in the CXCL8 gene promoter of A549 cells activated by TNFα (58). RSV infection of alveolar cells causes heterodimers involving c-Jun to bind to a cAMP-response element within the CCL5 promoter (55). These data suggest that there may be stimulus specificity in the profile of AP-1 family members that are activated to bind the CCL5 promoter in different diseases (59). Early promoter reporter studies in an erythroleukemic cell line do suggest a role for AP-1 in CCL5 promoter activation (26), but little data exists on whether AP-1 may functionally activate the CCL5 promoter in pulmonary epithelium. We found a significant amount of reporter activity in experiments using the −421 deletion construct containing all four TREs (41.9 ± 15.7% of WT activity). This was reduced by −50% to (20.9 ± 2.9% of WT activity) by a truncation of the promoter region to remove all AP-1-response elements, suggesting that these sites contribute to CCL5 promoter activity. Site-specific analysis revealed that the ΔTRE3/4 region was the most critical, and independent or concurrent mutation of TRE1/2 did not further reduce promoter-reporter activity. This is a stimulus-specific effect of CoMTB because the minimal CCL5 promoter truncated at −220 base pairs retained activity similar to a full-length promoter of −974 base pairs in cytokine- and RSV-stimulated epithelial cells (54, 55). In addition, there is cell specificity because in an astrocytoma line, a CCL5
promoter with a −278 deletion was activated to a similar extent to a full-length promoter by IL-1β in part as a consequence of translocation of p50/p65 but not c-rel-containing NFκB complexes (56).

NF-IL-6 is a human basic domain leucine zipper-containing transcription factor important in inducible gene expression in acute inflammatory responses and is able to regulate promoter activity of the CXCL8 gene (60). There is a single NF-IL-6 binding site within the CCL5 promoter at position −92 relative to the transcriptional start site. NF-IL-6 protein expression in the lung is unique because in the lung, in contrast to tissues such as liver or macrophages, NF-IL-6 translation is constitutively repressed (33, 61). In normal lung tissue unlike elsewhere there is an abundance of NF-IL-6 mRNA, but protein is almost undetectable, suggesting posttranscriptional modifications are regulating protein expression in a tissue-specific fashion. RSV-induced NF-IL-6 expression in pulmonary epithelium has been shown to involve de novo synthesis of the protein without any increases in NF-IL-6 mRNA expression assessed by Northern analysis (33). The role of NF-IL-6 in cytokine-induced CCL5 promoter activity in pulmonary epithelium is emerging. To our knowledge, we show for the first time that an IL-1-mediated stimulus, CoMTB, induces CCL5 gene activation in pulmonary epithelium by stimulating NF-IL-6 DNA binding. NF-IL-6 nuclear translocation was evident only 24 h poststimulus. This late binding of NF-IL-6 may be of functional significance as CoMTB-induced CCL5 secretion increases over a 24-h period; moreover, WT CCL5 promoter activity was present 24 h poststimulus. Furthermore, the CCL5 promoter deletion construct (−79), which removes the NF-IL-6 binding site at position −92, causes almost a 75% reduction in promoter activity (from 20.9 ± 2.9% to 5.5 ± 1.9% of WT). Site-directed mutation of the NF-IL-6 binding site confirmed a central role for this region with promoter activity falling to 49.4% of WT. This is a stimulus-specific response; in studies using a minimal CCL5 promoter (up to −220 bp), NF-IL-6 had a very limited influence on RSV-induced CCL5 secretion, and the interferon regulatory factor binding site was key (55). NF-IL-6 was more critical in control of epithelial cell-derived CCL5 secretion responses to TNFα,
where NF-IL-6 activity was detected on gel shifts within 1 h, much earlier than we found in response to CoMTB (54). In addition, the effects of NF-IL-6 activation are gene-specific because NF-IL-6 site mutations have no effect on CXCL8 promoter activity (62). Taken together the EMSA and promoter-reporter data suggest that it is possible that NFκB is more important in early activation of CCL5 mRNA accumulation and secretion and that AP-1 is responsible for the increased promoter activity between 4 and 8 h with NF-IL-6 contributing to late stage maintenance of CCL5 gene expression.

We demonstrated that IL-1β is the predominant active constituent involved in monocyte-epithelial cell networks in pulmonary tuberculosis. CCL5 secretion and gene expression from CoMTB-stimulated A549 and SAEC are inhibited by IL-1ra but not neutralizing anti-TNFα. Furthermore, IL-1ra inhibited CoMTB-induced WT CCL5 promoter activity to 29.0 ± 1.4% suggesting that IL-1ra inhibited IL-1-mediated CCL5 gene activation. We determined that CoMTB contained IL-1β and that recombinant IL-1β at 200–500 pg/ml stimulated SAEC CCL5 secretion similar to that of CoMTB (1:10 dilution). These results suggest the existence of a paracrine cytokine network involving very low concentrations (200 pg/ml) of monocyte-derived IL-1β. This highlights a potential critical role for IL-1β in the immune response to pulmonary tuberculosis and for IL-1ra in modifying these responses. IL-1β is secreted from tuberculosis-infected human monocytes (24, 63) and is found both in tuberculosis granulomas of infected lymph nodes (64) and the BALF of patients with active disease (65). We show that exogenous IL-1ra can inhibit epithelial CCL5 responses during tuberculosis infection. However, airway epithelium can secrete endogenous IL-1ra to modulate IL-1 bioactivity in the airway microenvironment (37). Interestingly polymorphisms at the IL-1 locus have been shown to influence the molar ratios of IL-1ra/IL-1β secreted by tuberculosis-infected monocytes (24), and a proinflammatory haplotype for this locus (higher IL-1β and lower IL-1ra expression) can affect disease phenotype in tuberculosis (24). Such host genetic factors, by influencing net bioactive IL-1β, may influence monocyte epithelial cell networks and subsequent CCL5 secretion. Cytokine networks involving lipopolysaccharide-stimulated monocytes stimulate pulmonary epithelial CXCL8 secretion, but in contrast to the present findings, both TNFα and IL-1 are involved (66). We previously have found that conditioned medium from tuberculosis-infected monocytes may stimulate primary pulmonary epithelial CXCL8 secretion; however, unlike for CCL5, this epithelial response is much less than that from the tuberculosis-infected monocytes themselves (21).

Finally, the data demonstrate differential responsiveness of NHBE and SAEC to monocyte-derived IL-1β. Such differences between similar but distinct cell types within a single tissue are likely to be important in immune responses to different pathogens. Primary alveolar epithelium of the small airways secretes CCL5 in response to the monocyte-derived IL-1β and to recombinant IL-1β, whereas upper airway cells of the trachea and main bronchi did not, consistent with data showing that IL-1β stimulates secretion of only picomolar CCL5 concentrations in these cells (5). Upper airways require both TNFα and interferon γ to stimulate CCL5 secretion (5, 13). There remains one report that demonstrates up-regulation of CCL5 mRNA and NFκB nuclear binding in human bronchial epithelial cell cultures stimulated by IL-1β, although no CCL5 secretion was observed (35). SAEC are a better primary cell correlate than NHBE for the human type II alveolar cell carcinoma line A549 because they are derived from terminal bronchioles (2 mm and below) and include type I and II pneumocytes. We have found that this differential responsiveness to IL-1 is specific for CCL5. CXCL8 is secreted by CoMTB-stimulated NHBE and SAEC, although CoMControl also induces relatively low level CXCL8 secretion from SAEC but not NHBE. Thus, small airway cells may be primed to secrete CXCL8 in the absence of exogenous stimulus, although whether this is found in vivo is not known. The full significance of this differential responsiveness of the upper and lower airway is unclear except that pulmonary tuberculosis is primarily a disease of the lower airway, a site where monocyte epithelial cell networks stimulating CCL5 secretion will be functional.

This study defines a new contextual function for monocyte-derived IL-1β and a new role for the lung epithelium in host response to tuberculosis. The large surface area of pulmonary epithelial cells generates high levels of CCL5 during the pulmonary immune response to M. tuberculosis. Investigation into the mechanism of regulation of the CCL5 gene expression in the pulmonary epithelium has demonstrated that the two proximal NFκB binding sites are required for gene activation and not the −579 CD28RE/NFκB binding site, although the distal area of the promoter has a regulatory role in CoMTB-stimu-
lated respiratory epithelium. This study has also defined functional roles for the transcription factors AP-1 and NF-IL-6 in CCL5 responses in our cellular model of tuberculosis. Very low IL-1 concentrations are identified as mediating this TNF-independent monocyte epithelial cell network in pulmonary tuberculosis. The effects of this proinflammatory cytokine on CCL5 secretion are shown to be specific for lower airway alveolar epithelial cells. Identifying the alveolar epithelium as a significant cellular source of CCL5 and defining the role of IL-1 may help in the design of local immunotherapeutic interventions to regulate T cell and monocyte recruitment to diseased lung infected with tuberculosis, particularly in patients with organisms resistant to conventional therapies.

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