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Induction of TNF in Human Alveolar Macrophages As a Potential Evasion Mechanism of Virulent *Mycobacterium tuberculosis*[1]

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The ability of macrophages to release cytokines is crucial to the host response to intracellular infection. In particular, macrophage-derived TNF plays an important role in the host response to infection with the intracellular pathogen *Mycobacterium tuberculosis*. In mice, TNF is indispensable for the formation of tuberculous granulomas, which serve to demarcate the virulent bacterium. TNF is also implicated in many of the immunopathological features of tuberculosis. To investigate the role of TNF in the local immune response, we infected human alveolar macrophages with virulent and attenuated mycobacteria. Infection with virulent strains induced the secretion of significantly higher levels of bioactive TNF than attenuated strains correlating with their ability to multiply intracellularly. Treatment of infected macrophages with neutralizing anti-TNF Abs reduced the growth rate of intracellular bacteria, whereas bacterial replication was augmented by addition of exogenous TNF. Infected and uninfected macrophages contributed to cytokine production as determined by double-staining of *M. tuberculosis* and intracellular TNF. The induction of TNF by human alveolar macrophages at the site of infection permits the multiplication of intracellular bacteria and may therefore present an evasion mechanism of human pathogens.   *The Journal of Immunology*, 2002, 168: 1328–1337.

T he course of infection with many intracellular pathogens is influenced by the systemic and local cytokine environment. TNF is a pleiotropic cytokine produced primarily by monocytes and macrophages, but also by lymphocytes and NK cells. TNF plays a central part in the host immune response to viral, parasitic, fungal, and bacterial infections (1–4). For example, TNF is essential for protection against murine tuberculosis (5–7). Although TNF is crucial to the protective immune response, it also takes part in the pathogenesis of both infectious and autoimmune diseases. Increased levels of TNF trigger the lethal effects of septic shock syndrome and are a critical mediator of joint inflammation in rheumatoid arthritis (8). TNF has also been linked to the clinical symptoms of the reversal reaction in leprosy patients (9) and cachexia in tuberculosis patients (10). It is still a matter of debate whether TNF directly contributes to the elimination of microorganisms in human infectious diseases.

We sought to clarify how TNF participates in the immunopathogenesis of human infection using tuberculosis as a model. *Mycobacterium tuberculosis*, the causative agent of tuberculosis, is a facultative intracellular bacterium that is capable of surviving and persisting within host mononuclear cells. Infection with *M. tuberculosis* occurs via the respiratory route by the inhalation of bacilli-containing droplets. The bacilli are taken up by alveolar macrophages (AM),[4] which represent the source of a primary tuberculous focus in which the pathogen either replicates or is contained. Depletion of AM protected mice against tuberculosis, indicating that the primary host cell for inhaled bacilli may cause more harm than protection in vivo (11). One of the most intriguing questions in tuberculosis concerns the fate of phagocytosed bacteria. Nonactivated AM are not equipped with effector mechanisms sufficient to clear the microbial invader. Ag-specific T cells are attracted to the site of disease by the action of locally released chemokines (12–18). Consequently, T cells secrete macrophage-activating cytokines (e.g., TNF and IFN-γ) (15) or directly lyse infected cells and contribute to the containment of mycobacterial infection (19). Less than 10% of the infected individuals will develop clinically overt disease due to the efficient interplay of the specific and nonspecific effector mechanisms of the immune system (20). Despite successful containment of mycobacterial spread and prevention of tissue destruction, the pathogen manages to persist in the host for decades.

Detailed studies of murine models of tuberculosis have succeeded in unraveling a sequence of immunological events, involving a complex network of cytokines (e.g., IFN-γ and TNF) and effector molecules (oxygen and nitrogen radicals) (21). TNF contributes to immunity against mycobacteria by synergizing with IFN-γ to activate infected macrophages (22) and in recruiting macrophages and lymphocytes to seal up infectious foci by forming granulomas (5–7). Protection is mediated primarily by CD4-positive T cells, which are supported by MHC class I-restricted CD8-positive T cells and T cells expressing the γδ TCR (23).

In contrast, the immune response against *M. tuberculosis* in humans remains poorly defined. Naturally occurring genetic deficiencies in humans have pointed to a critical role of IFN-γ (24) and IL-12 (25) in the protective immune response. Whether TNF is beneficial or detrimental for the clinical course of disease remains to be determined. TNF is involved in the development of tissue damage and can support bacterial multiplication in the lung (26, 27). In addition, high levels of TNF were implicated in clinical worsening of symptoms shortly after the initiation of tuberculostatic therapy in tuberculosis patients (28). The in vivo effects of TNF appear to be dose dependent, with low levels of the cytokine mediating protection against tuberculosis, whereas high concentrations provoke tissue damage (26). A critical role for TNF in maintaining the latent state of dormant bacilli is suggested by the reactivation of tuberculosis in rheumatoid arthritis patients treated with anti-TNF Abs (29–31).

The effect of TNF on human cells infected with mycobacteria varies widely depending on the source of the cells, the mycobacterial strain, and the experimental setting (32, 33). TNF and IFN-γ treatment does not activate antibacterial effects in human monocytes, which is in striking contrast to the effect on murine cells (34). To better understand the functional role of TNF at the site of human infection, we analyzed its expression and impact on mycobacterial growth in human AM.

Materials and Methods

Cell culture reagents

Cells were cultured in RPMI 1640 (Biochrom, Berlin, Germany) supplemented with 10% heat-inactivated FCS (Sigma-Aldrich, St. Louis, MO), 2 mM glutamine (Sigma-Aldrich), 10 mM HEPES, 13 mM NaHCO$_3$ (Biochrom), 5.6 μg/ml amphotericin B (Sigma-Aldrich), and 60 μg/ml penicillin (Biochrom). In experiments involving the infection of cells with *M. tuberculosis*, FCS was replaced by pooled human serum (generated from the blood of healthy volunteers) to optimize the phagocytosis of the bacteria.

AM and monocytes

AM were obtained from the bronchoalveolar lavage fluid of patients who underwent bronchoscopy for diagnostic purposes. AM from patients given a diagnosis of an infectious lung disease or a disease afflicting the alveolar space were discarded. Lavage fluid was filtered through a cell strainer (40 μm; BD Biosciences, Heidelberg, Germany) and centrifuged (1200 rpm for 10 min) at 4°C. The pellet was resuspended in RPMI 1640 supplemented with amphotericin B (5.6 μg/ml; Sigma-Aldrich), penicillin (60 μg/ml), and 10% human serum. Cells were plated in six-well plates (Costar, Cambridge, MA) at a density of 1×10^6/ml. AM were cultured at 37°C overnight to allow adherence of the cells and to minimize the background production of TNF induced by the adherence to plastic. Nonadherent cells were removed by vigorous washing with PBS. AM were harvested by gentle scraping with a cell scraper (Costar), which did not affect the viability of the cells. Purity of the cells was confirmed by α-naphthyl-acetate esterase staining (Sigma-Aldrich) and was found to be >95% in all experiments. Flow cytometric analysis confirmed the purity of the population (CD3 < 1%, CD19, CD56, CD66, CD1 negative). Viability of the cells was examined in representative samples by trypan blue dye exclusion.

Monocytes were isolated by adhering PBMCs for 2 h in a cell culture flask. Nonadherent cells were removed by rinsing the flask at least five times with PBS. The adherent cells were detached by treatment with EDTA (1 mM for 10 min) or using a cell scraper. The resulting cell population contained at least 95% CD14-positive cells as determined by flow cytometry.

Cytokines and Abs

The following cytokines and Abs were used in this study: recombinant human (rhu)TNF-α (Endogen, Woburn, MA), rhuIL-1β, rhuIL-10, rhuIL-12p40, anti-TNF, goat IgG (all from R&D Systems, Minneapolis, MN), anti-CD56 (clone C218; Immunotech, Marseilles, France), anti-CD3-PerCP (clone SK 7), anti-CD19-allophycocyanin (HIB 19), anti-CD66b-FITC (clone MOPC 315-43X), anti-TNF-allophycocyanin (MAB11; BD Biosciences), anti-CD14-PE (UCHM-1), anti-CD1a-FITC (clone NA1/34-HLK; Serotec, Oxford, U.K.), and goat anti-mouse-FITC (Jackson ImmunoResearch Laboratories, West

Grove, PA). Isotype controls were all purchased from Cymbus Biotechnology (Chandlers Ford, U.K.).

Growth of *M. tuberculosis*

M. tuberculosis (virulent strain H37Rv) was grown in suspension with constant, gentle rotation in roller bottles (Corning, Cambridge, MA) containing Middlebrook 7H9 broth (BD Biosciences) supplemented with 1% glycerol (Roth, Karlsruhe, Germany), 0.05% Tween 80 (Sigma-Aldrich), and 10% Middlebrook OADC enrichment (BD Biosciences). Aliquots from logarithmically growing cultures were frozen in PBS containing 10% glycerol, and representative vials were thawed and enumerated for viable CFU on Middlebrook 7H11 plates. Staining of bacterial suspensions with fluorochromic substrates differentiating between live and dead bacteria (BacLight; Molecular Probes, Leiden, Netherlands) revealed a viability of the bacteria above 90%. Because clumping of mycobacteria is a common problem which can influence the validity and reproducibility of the experiments, we undertook several precautions to minimize clumps: 1) culture conditions (rotation, Tween) were chosen to support the growth of single cell suspensions; 2) before in vitro infection *M. tuberculosis* bacilli were sonicated to disrupt small aggregates of bacteria; and 3) the multiplicity of infection (MOI) was selected such that there were only 1–2 bacilli per AM.

Infection of AM and monocytes

AM or monocytes were infected with single cell suspensions of *M. tuberculosis* in six-well culture plates at 1×10^6 cells/ml in a final volume of 3 ml. In selected experiments mycobacteria were killed by exposure to 80°C for 30 min in a water bath. After 4 h of incubation at 37°C extracellular bacteria were removed by intensive rinsing with PBS. In experiments designed to quantitate mycobacterial growth, the adherent cells were harvested by gentle scraping with a cell scraper and replated at a concentration of 1×10^6 cells/ml in a 24-well plate (final volume 500 μl) in complete medium without antibiotics plus 10% human serum. The efficiency of infection, as quantified by staining of control cultures on Permanox chamber slides (Nunc, Naperville, IL) in every experiment was dependent on the MOI. In selected experiments AMs were incubated with FITC-conjugated latex beads (FluoSpheres; Molecular Probes) under identical conditions. The microscopic evaluation of infected macrophages under the fluorescence microscope confirmed the absence of any mycobacterial aggregates. Cell viability of infected AMs was determined by trypan blue exclusion and was >99% in all experiments.

FACS staining of *M. tuberculosis*

The bacterial suspension was incubated with an equal volume of BacLight viability staining kit (Molecular Probes). Bacteria were incubated for 15 min at room temperature in the dark. Cells were washed seven times in 0.5 ml of PBS, centrifuged at 3200 rpm for 20 min, and sonicated in a preheated water bath for 5 min. The stained mycobacteria were then used to infect AM. Labeled bacteria did not differ in terms of viability, infectivity, intracellular growth, or viability of eukaryotic cells as compared with unstained bacteria (data not shown). This procedure allowed the detection of extracellular bacteria by flow cytometry using the 488-nm laser.

Intracellular FACS staining

AM were infected with stained or unstained mycobacteria (MOI 2.5) for 6 h. The final 2 h of incubation were performed in the presence of brefeldin A (10 μg/ml; Sigma-Aldrich). Infected macrophages were harvested by gentle scraping with a cell scraper. Cells were centrifuged in aerosol-tight tubes and the supernatant was collected and stored at −20°C until further analysis. The pellet was resuspended in 500 μl of PBS/2% FCS/4% paraformaldehyde. An aliquot of the cell suspension was given onto a glass slide and allowed to air dry for auramine-rhodamine staining. After 20 min of incubation cells were washed twice and resuspended in 150 μl of PBS/2% FCS/0.5% saponin (Sigma-Aldrich). Ten minutes later anti-TNF-allophycocyanin (1/150 dilution) was added and incubated for 30 min at 4°C. Acquisition was performed in a FACSCalibur flow cytometer (BD Biosciences) and data were analyzed using CellQuest software (BD Biosciences) or WinMDI.

Measurement of cytokine release

TNF bioactivity was assessed by measuring the lytic effect on the fibrosarcoma cell line WEHI 164 (35). WEHI cells were pretreated with actinomycin D (1 μg/ml; Sigma-Aldrich) for 2 h. After washing, cells were plated in a 96-well plate (5×10^4/well) and the supernatants were added in cell culture medium supplemented with actinomycin D (2 μg/ml). After overnight incubation methylthiazoltetrazolium (final concentration, 500 μg/ml; Sigma-Aldrich) was added for 4 h. To solubilize the formazan

crystals, the cells were treated with 100 μl of acidic sodium-dodecyl-sulfate (Sigma-Aldrich) overnight and the optical density was determined in an ELISA plate reader at a wavelength of 550 nm. Estimates of the concentrations of bioactive TNF in the supernatants were obtained by comparison with calibration curves established with a rhuTNF standard. TNF bioactivity in selected AM supernatant samples was inhibited by anti-TNF (10 μg/ml) but not by control rabbit IgG. All wells were set up in triplicates. The sensitivity of the assays was >50 pg/ml in all experiments. For measurement of cytokine concentrations in the supernatants, a sandwich ELISA was used and performed using Ab pairs as suggested by the supplier (TNF and IL-10, Endogen; IL-12 and IL1β, R&D Systems). The sensitivity was 15 pg/ml (TNF and IL-1β), 60 pg/ml (IL-10), and 125 pg/ml (IL-12).

Transwell experiment

AM were infected (MOI 2.5) and harvested as described above. Infected cells (1 $\times$ 10^6) were plated in the upper chamber of a transfer system (six-well plates; Costar), which is separated from the lower chamber by a membrane permeable only for particles smaller than than 0.4 μm. The diameter of the pores allows cytokines and secreted proteins, but not bacteria, to pass. In the lower chamber 1 $\times$ 10^6 uninfected AM were cultured. After 18 h the supernatants were harvested and examined for TNF.

Quantification of mycobacterial growth

To ensure the reliable quantification of intracellular *M. tuberculosis* we used three independent methods for measuring mycobacterial growth. First, we used acid-fast stain (auramine-rhodamine; Merck, Darmstadt, Germany). Second, for CFU, infected cells were lysed with 0.3% saponin (Sigma-Aldrich) to release intracellular bacteria. At all time points an aliquot of unlysed, infected cells was harvested and counted. This allowed an exact quantification of cells as well as the determination of cellular viability by trypan blue exclusion. Recovery of cells was >80% in all experiments, with cell viability regularly exceeding 90% of total cells. Lysates of infected cells were resuspended vigorously, transferred into screwcaps, and sonicated in a preheated (37°C) water bath sonicator (Elma, Singen, Germany) for 5 min. Aliquots of the sonicate were diluted 5-fold in 7H9 medium. Four dilutions of each sample were plated in duplicates on 7H11 agar plates and incubated at 37°C and 5% CO$_2$ for 21 days. Third, we measured incorporation of tritium-labeled uracil ([^{3}H]uracil). Uptake of [^{3}H]uracil into the mycobacterial RNA was determined following the method published by Rook et al. (36), with several modifications as detailed previously (37). Briefly, 1 $\times$ 10^6 infected AMs were cultured in duplicates as described above. At the end of the incubation period, cells were lysed using 0.3% saponin, resuspended vigorously, and transferred into screwcaps. Lysates were centrifuged in an aerosol-tight microfuge (3000 rpm for 20 min) and resuspended in 100 μl of 7H9 to allow optimal growth of the released mycobacteria. Lysates were then transferred into 96-well round-bottom plates (Nunc) and incubated in the presence of 3 μCi of [^{3}H]uracil (Amersham-Pharmacia, Freiburg, Germany). After 24 h mycobacteria were killed by treatment with paraformaldehyde (final concentration, 4%) for 30 min. The mycobacteria were harvested onto glass fiber filters (Inotech, Dottikon, Switzerland) and [^{3}H]uracil incorporation was measured in a beta counter (Berthold, München, Germany). Background radioactivity in uninfected cells was below 500 cpm in all experiments.

Flow cytometry

A total of 3 $\times$ 10^5 cells were resuspended in 100 μl of staining buffer (2% FCS, 1% NaN$_3$, PBS without Mg^{2+}/Ca^{2+}) and incubated with unconjugated or conjugated Abs for 30 min on ice. Samples were washed twice in staining buffer and, if necessary, incubated for an additional 30 min on ice with goat anti-mouse-FITC Abs (1/500). Cells were then fixed in 2% paraformaldehyde and stored at 4°C until analysis in a FACScan flow cytometer. Data were analyzed using CellQuest software (BD Biosciences).

Measurement of apoptosis

Two independent methods were used to quantify apoptosis. First, negatively charged phospholipid surfaces, which translocate from the inner to the outer leaflet of the plasma membrane early during apoptosis, were stained using annexin V-PE (BD Biosciences) following exactly the protocol supplied by the manufacturer. For quantification, at least 10,000 cells were analyzed by flow cytometry. Second, cytoplasmic, histone-associated DNA fragments (mono- and oligonucleosomes) which occur during apoptosis were identified using the Cell Death Detection ELISA^{Plus} (Boehringer Mannheim, Mannheim, Germany). Briefly, 1 $\times$ 10^4 cells were lysed and centrifuged (1300 rpm for 10 min), and 20 μl of the supernatant were added to the streptavidin-coated microtiter plate. The

detection of nucleosomes was performed according to the protocol supplied by the manufacturer. The conditions for the purification, infection (MOI 0.5), and culture of AM were identical to those used for the quantification of mycobacterial growth (see above).

Statistical analysis

Data are presented as the mean value $\pm$ SEM except where stated otherwise. Student's *t* test was used to determine statistical significance between two differentially treated cultures. Differences were considered significant if $p < 0.05$.

Results

Infection of AM with M. tuberculosis

Human AM were infected with a virulent strain of *M. tuberculosis* (H37Rv) with different bacteria:macrophage ratios for 4 h. The number of infected cells as well as the bacterial load increased with the MOI as determined by acid-fast stain. Both peaked at an MOI of 10, at which 32 $\pm$ 2% of the AM were infected and had engulfed 14 $\pm$ 2 bacteria (data not shown). Further increase of the MOI was not informative, as cells rapidly disintegrated. The majority of AM remained uninfected despite an overwhelming number of bacteria in the culture. Extension of the pulse infection (up to 18 h) had no impact on these results. These data demonstrate that only a limited number of AM have the capacity to phagocytose viable mycobacteria. In contrast, FITC-labeled latex beads were taken up much more efficiently (Fig. 1A) under identical culture conditions. Therefore, AM have a specific inability to phagocytose live *M. tuberculosis* and possibly other bacterial pathogens.

TNF production by AM infected with M. tuberculosis

The secretion of TNF plays an important role in the orchestration of the local immune response to intracellular pathogens. Therefore, we investigated the ability of human AM to secrete TNF in response to infection with virulent *M. tuberculosis*. The concentration of TNF in the supernatant increased with the MOI and the bacterial load of the AM (Fig. 1B). TNF produced by AM is bioactive as determined by lysis of a TNF-sensitive fibrosarcoma cell line (Fig. 1C). The biological activity of TNF was neutralized by an anti-TNF polyclonal Ab (data not shown). Kinetic analysis demonstrated that cytokine release peaked after 18 h and dropped gradually thereafter (Fig. 1D). Phagocytosis alone was not sufficient to mediate release of preformed or membrane-bound TNF, as the amount did not exceed background levels for the first 4 h of incubation (data not shown). In addition, uptake of latex beads did not induce secretion of TNF (data not shown). To investigate how the production of TNF relates to the growth of intracellular bacteria we determined the number of CFU after 1, 18, 28, 48, and 96 h of infection. After an initial lag phase the bacteria multiplied 4-fold during the 96-h incubation period. Therefore, the early TNF burst might be involved in the induction of bacterial growth. These data establish that virulent *M. tuberculosis* induces bioactive TNF in human infection in the critical, early stage of the immune response.

Analysis of TNF production on the single cell level

To allow the identification of TNF-producing AM on the single cell level we used intracellular flow cytometry using a fluorochrome-conjugated TNF Ab. Infection of AM resulted in 8 (MOI 1), 14% (MOI 5), and 20% (MOI 10) TNF-expressing cells 6 h after infection (Fig. 2). Stimulation of AM with LPS resulted in almost 3-fold higher numbers of TNF-expressing cells (Fig. 2). This experiment shows that infection of AM with *M. tuberculosis* activates only a subset of AM, whereas the majority of cells, despite producing TNF in response to LPS, remain immunologically silent.
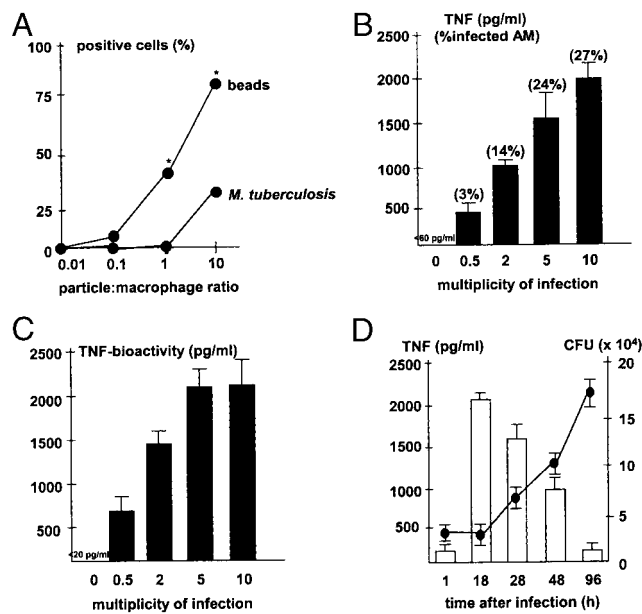
FIGURE 1. TNF production by AM after infection with *M. tuberculosis*. *A*, AM were infected with increasing numbers of mycobacteria or FITC-conjugated inert latex beads. After 4 h cells were detached and the number of *M. tuberculosis*-infected cells was quantified by flow cytometry (50,000 cells were evaluated). The data present the results from one donor of three (∗, $p < 0.01$). *B*, AM were infected with increasing numbers of mycobacteria and supernatants were harvested after 18 h. The concentration of secreted protein was measured by ELISA. The number of infected cells was determined by auramine-rhodamine staining. For each sample at least 500 cells were examined for the presence of intracellular acid-fast rods. *C*, AM were infected as in *A* and the bioactivity in the supernatants were determined by lysis of WEHI cells. The graphs show the average result of 14 independent experiments (±SD) using AM from different donors. *D*, AM were infected with *M. tuberculosis* (MOI 2) for 4 h before extracellular bacteria were removed. Supernatants were collected 1, 18, 28, 48, and 96 h later and the TNF concentration was measured by ELISA. The graph shows the average result of seven independent experiments (±SD) using AM from different donors and are presented as bars. In selected experiments (four), infected AM were lysed after harvesting of the supernatants and the lysates were plated in duplicates on Middlebrook agar. The number of CFU (average of four independent experiments ± SEM) is illustrated by closed circles. Note that the spacing on the *x*-axis is not linear.

Infected and uninfected AM produce TNF

Because the number of TNF-expressing cells was in the same order of magnitude as the number of infected cells, we reasoned that TNF production was limited to infected AM. To address this hypothesis we established a double-staining technique, which permits the simultaneous detection of *M. tuberculosis* and TNF on the single-cell level by flow cytometry. Bacterial DNA was stained with BacLight before the infection of AM. Labeled extracellular bacteria can easily be detected (>40-fold increase in fluorescence intensity) in the FITC channel of the flow cytometer (data not shown). Importantly, infected AM can be clearly distinguished from noninfected cells in the same culture (Fig. 3*A*). The number of infected cells detected by this method were confirmed by microscopical evaluation (Table I). Because the vast majority of infected cells had engulfed only one or two bacteria, differences in the staining intensity are unlikely due to the differences in the bacterial burden but reflect the difference between infected and uninfected cells. The discrete increase of background staining of AM at a high MOI is probably due to the translocation of fluoro-
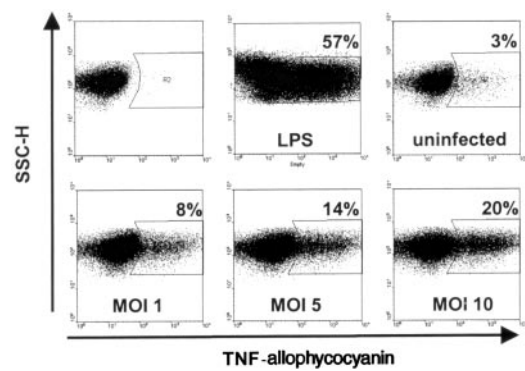
FIGURE 2. Intracellular TNF-staining in *M. tuberculosis*-infected AM. Cells were infected with *M. tuberculosis* for 6 h. Brefeldin A was present during the final 2 h of incubation. Uninfected cells and LPS (20 ng/ml) served as controls. Cells were detached and stained for intracellular TNF. A total of 1×10^5 cells were acquired and analyzed by flow cytometry. The graphs show 5×10^4 cells and are representative of three independent experiments using AM from different donors.

chrome from the prokaryotic to the eukaryotic DNA. To identify infected cells, which simultaneously produce TNF, we performed double-staining 6 h after the infection. Confirming our earlier results, only a subset of AM was infected (23%) with *M. tuberculosis*. Similarly, only 8% of the total cell population produced TNF at an MOI of 2.5 (Fig. 3*B*). These experiments revealed that not only infected (4 of 26% infected cells; Fig. 3*B*, *upper right quadrant*) but also a fraction of uninfected AM (4 of 74% uninfected cells; Fig. 3*B*, *upper left quadrant*) expressed TNF. Therefore, uptake of bacteria promotes, but is not mandatory for, the production of TNF by AM.

TNF production by uninfected AM is not induced by a soluble factor

Because uninfected AM produced TNF we asked whether cytokine production was mediated by a soluble factor released from infected cells. We plated infected AM in the upper chamber of a transwell system, allowing soluble mediators and secreted proteins (smaller than 0.4 μm) but not whole bacteria to pass. Uninfected AM were added to the lower chamber and intracellular TNF staining was performed after 18 h (Table II). Uninfected AM in the lower chamber did not up-regulate TNF (2 ± 0.6%) in response to a soluble factor secreted by the infected cells in the upper chamber. In contrast, 16 ± 3% of the AM in infected control cultures expressed TNF. This suggests that TNF production by noninfected AM is mediated by cell to cell contact or by bacterial metabolites too large to pass the membrane of the transwell system.

Virulent mycobacteria induce more TNF in AM than attenuated bacteria

Next we sought to determine whether mycobacterial strains differ in their capacity to induce TNF production, thereby modulating the outcome of disease early after infection. We investigated two pairs of genetically closely related mycobacterial strains: 1) *M. tuberculosis* H37Rv (virulent) and H37Ra (attenuated strain); and 2) *M. bovis* (virulent) and *M. bovis* bacillus Calmette-Guérin (BCG; attenuated strain). Virulent strains activated more AM for TNF production than their attenuated counterparts as determined by intracellular staining (Fig. 4*A*). Phagocytosis by AM did not differ significantly between the different mycobacterial strains (data not shown). The correlation between virulence and TNF production was confirmed by ELISA for six donors (Fig. 4*B*). On average,
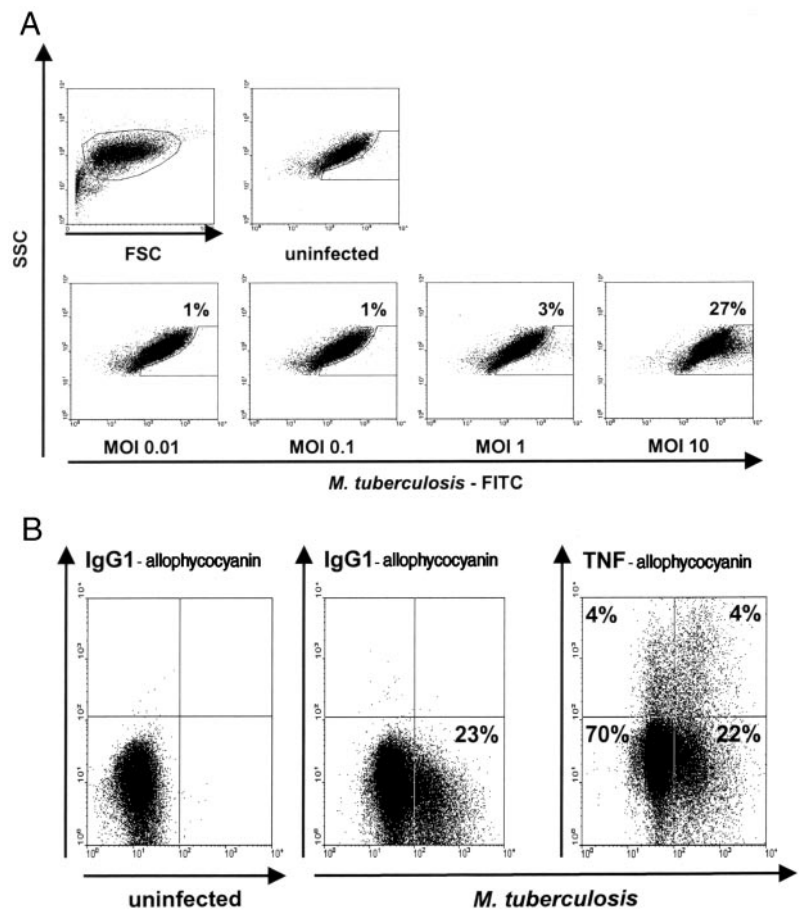
FIGURE 3. TNF production by AM on the single cell level. *A*, AM were infected with BacLight-labeled mycobacteria and incubated for 4 h. Nonphagocytosed bacteria were removed by washing and flow cytometry was performed after 4 h. *B*, AM were infected with BacLight-labeled mycobacteria (MOI 5). After 4 h brefeldin A was added and FACS analysis was performed after an additional 2 h of incubation. Labeled bacteria are detected in the FITC channel (*x*-axis); TNF-allophycocyanin-labeled AM are shown on the *y*-axis. The experiment is representative of 11 with similar results.

virulent *M. tuberculosis* induced 1954 ± 235 pg/ml TNF, whereas the attenuated strain induced only 623 ± 135 pg/ml. Similarly, *M. bovis*-treated AM secreted 2156 ± 303 pg/ml TNF, whereas *M. bovis* BCG-infected AM secreted only 498 ± 218 pg/ml (Fig. 4*B*). To determine whether the release of additional monokines was also dependent on the virulence, we compared the concentrations of IL-1β, IL-12, and IL-10 in the supernatants of infected AM. Virulent strains induced higher amounts of all three monokines (Fig. 4*C*) than attenuated strains. The absolute amounts of IL-12 and IL-10 were low as compared with TNF and IL-1β for both strains. Heat-killed mycobacteria only induced IL-10 (1076 ± 34 pg/ml). TNF, IL-1β, or IL-12 were not detected, indicating the requirement of a heat labile component or metabolic activity of the pathogen for the secretion of these monokines. This cytokine secretion pattern of AM in response to mycobacterial infection is reminiscent of other human cell populations, including dendritic cells (37, 38) and macrophages (39).

*TNF supports the growth of virulent* M. tuberculosis *in human AM*

Because virulent mycobacteria induce high TNF production, we hypothesized that TNF supports the intracellular replication of the bacteria. To address this issue, we initially compared the growth of virulent and attenuated mycobacteria in human AM. While virulent *M. tuberculosis* and *M. bovis* grew more than one order of magnitude within 7 days (*M. tuberculosis*, 11.8-fold increase; *M. bovis*, 13.2-fold increase), both attenuated strains failed to demonstrate significant proliferation at all time points investigated (Fig. 5). These data demonstrate a positive correlation among the virulence of mycobacteria, the ability to mediate the release of high amounts of TNF, and the growth rate in human AM.

We reasoned that TNF might be responsible for the accelerated growth of virulent mycobacteria in AM. We investigated this possibility by measuring intracellular growth of *M. tuberculosis* and

Table I. *Correlation between flow cytometric and microscopical detection of* M. tuberculosis[a]

Donor	Flow Cytometry (%)	Acid-Fast Stain (%)
1	11	14
2	13	10
3	35	26
4	8	11
5	20	17
6	29	19

[a] For each donor AM were infected (MOI 2.5) with BacLight-labeled or unlabeled mycobacteria in a six-well plate. After 4 h, cells were harvested and either applied to flow cytometry or given on a microscopy slide (1 × 10⁵ in 250 μl). After fixation and air drying auramine-rhodamine staining was performed. At least 400 cells were scored for each donor. The majority of infected AM contained one to two bacilli.

Table II. *TNF in uninfected AM is not elicited by a soluble factor[a]*

Upper Chamber	Lower Chamber	TNF-Positive Cells (%) (lower chamber)
No cells	Uninfected AM	2 ± 0.4%
No cells	Infected AM	16 ± 3%
Infected AM	Uninfected AM	2 ± 0.6%

[a] AM were pulse-infected with *M. tuberculosis* (MOI 2.5) in a six-well plate or remained untreated. Cells were then detached and plated in the upper or lower chamber of a transwell system as indicated. After 18 h cells in the lower chamber were harvested by gentle scraping and intracellular TNF staining was performed. The table shows the average ± SEM of three independent experiments using AM derived from different donors.
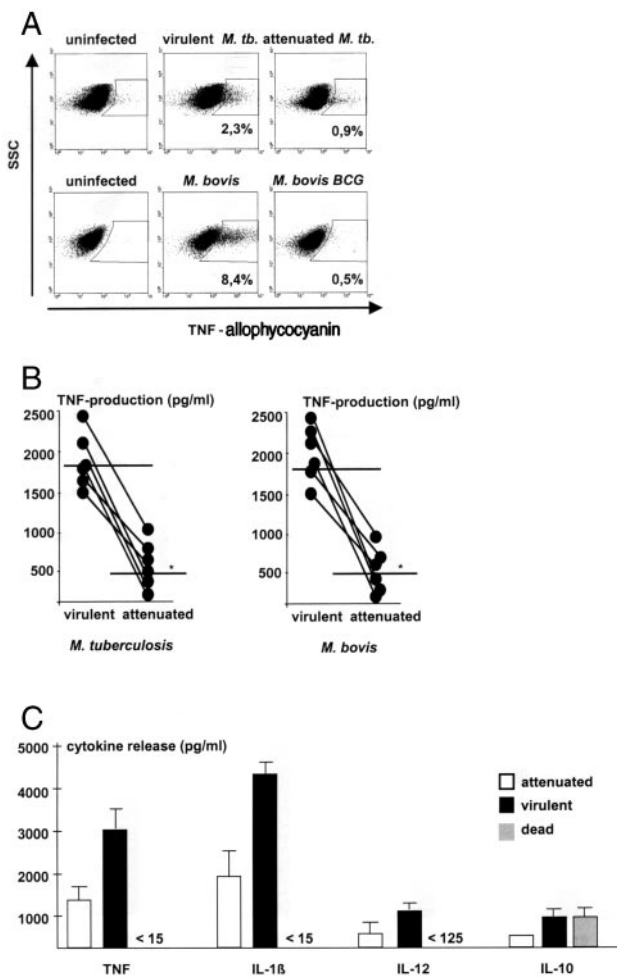
FIGURE 4. Comparison of monokine induction of virulent and attenuated mycobacteria. AM were infected with *M. tuberculosis* or *M. bovis* (virulent or attenuated strains) at a MOI of 2.5. The low level of TNF-positive cells for this particular donor is within the normal range of interindividual variability. *A*, 4 h after infection brefeldin A (1 μg/ml) was added and the culture was continued for 2 h. Cells were then harvested and intracellular TNF staining was performed. For each donor 1 × 10^5 cells were acquired and analyzed by flow cytometry. The data present one representative experiment of four. *B*, Supernatants were collected after 18 h and TNF was measured by ELISA. The graph summarizes the results of AM derived from six different donors. The asterisk indicates statistical significance between the average TNF production of virulent and attenuated strains (*, $p < 0.01$). *C*, AM were infected with live or heat-killed *M. tuberculosis* for 48 h. Cytokine secretion of uninfected cells was below the detection limit (TNF, 15 pg/ml; IL-1β, 15 pg/ml; IL-12, 125 pg/ml; IL-10, 60 pg/ml). The figure shows data from one representative donor of 12.

M. bovis in the presence of neutralizing Abs to TNF. After 5 days the number of bacilli in untreated cultures had increased from 16 ± 2 × 10^4 to 78 ± 4 × 10^4 for *M. tuberculosis* (Fig. 6*A*). In the presence of anti-TNF the number of bacteria only increased to 43 ± 2 × 10^4 (45% growth reduction). Addition of anti-TNF to *M. bovis*-infected AM decreased the bacterial proliferation even more strikingly (67%). To strengthen this observation we supplemented the infected cultures with exogenous TNF and monitored the bacterial load for 5 days. TNF accelerated the multiplication of both mycobacterial strains by 35 ± 3% for *M. tuberculosis* and 38 ± 4% for *M. bovis* as compared with untreated controls (Fig. 6*A*). Because freshly recruited monocytes may also participate in the local protection against *M. tuberculosis*, we investigated mycobacterial growth in monocytes in the presence or absence of TNF and
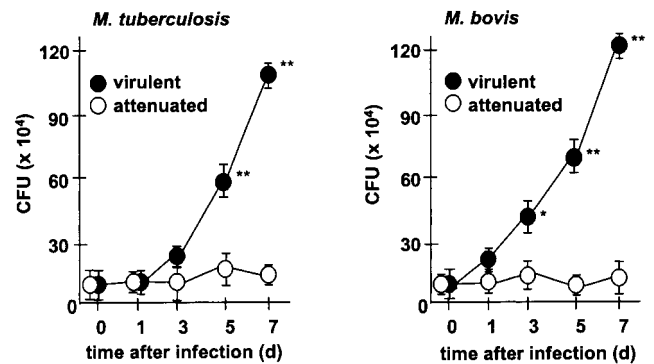
FIGURE 5. Comparison of the growth of virulent and attenuated mycobacteria. AM were allowed to adhere overnight, washed thoroughly, and infected with virulent or attenuated *M. tuberculosis* (*A*) and virulent or attenuated *M. bovis* (*B*). At indicated time points AM were lysed and 5-fold dilutions were plated on Middlebrook agar. All samples were plated in four dilutions, each in duplicate. The graphs show one representative experiment of six. Results are expressed as CFU ± SD. The asterisks indicate statistical significance between the growth of virulent and attenuated strains (*, $p < 0.05$; **, $p < 0.01$).

anti-TNF. Exogenous TNF increased the intracellular growth, whereas anti-TNF had the opposite effect (Fig. 6*B*). Finally, TNF also increased the proliferation of *M. tuberculosis* in immature dendritic cells (M. Büttner, M. Röllinghoff, and S. Stenger, manuscript in preparation). Therefore, TNF alone is insufficient to activate antibacterial effector mechanisms against virulent mycobacteria in infected human phagocytes.

To take into account the technical obstacles in measuring the mycobacterial growth in human cells, we confirmed these results using two additional independent methods (Table III). First, we determined the metabolic activity of the bacteria by measuring the uptake of [^{3}H]uracil. In the presence of anti-TNF the uptake was reduced from 9487 ± 423 to 4730 ± 301 cpm (50% reduction). In contrast, treatment with TNF enhanced the metabolic activity of the bacterial inoculum by 38%. Second, we used acid-fast staining, which does not discriminate between live or dead bacilli, but allows quantitation of the number of infected cells. Culture in the presence of anti-TNF clearly decreased the number of infected cells as well as the bacterial burden of the individual AM (Table III). TNF-treated AM harbored significantly more bacilli than control cultures (12.9 ± 2.7 vs 8.2 ± 2). The number of infected cells was also significantly increased (68 ± 3% vs 49 ± 5%).

In striking contrast to its effect on the growth of virulent mycobacteria, TNF limits the proliferation of the attenuated mycobacterial strains *M. tuberculosis* H37Ra (Fig. 6*C*) (32) and *M. bovis* BCG (Fig. 6*C*), suggesting that the biological activity of this cytokine varies depending on the virulence of the intracellular invader.

Because mycobacteria induce apoptosis in human macrophages in a TNF-dependent manner (40–42), we considered the possibility that apoptosis is the underlying mechanism of accelerated bacterial growth. As a measure for apoptotic cell death we compared the expression of annexin V (Fig. 7*A*) and the enrichment of cytoplasmic nucleosomes (Fig. 7*B*) in uninfected and infected AM. In our low-dose model of infection, mycobacteria did not induce apoptosis after 5 days of incubation. UV-irradiated Jurkat cells, which we used as a positive control, were readily labeled (Fig. 7). Therefore, apoptosis is not the mechanism by which TNF supports the growth of *M. tuberculosis* in human AM.

These findings establish that virulent mycobacteria induce the production of high amounts of TNF early after infection, which
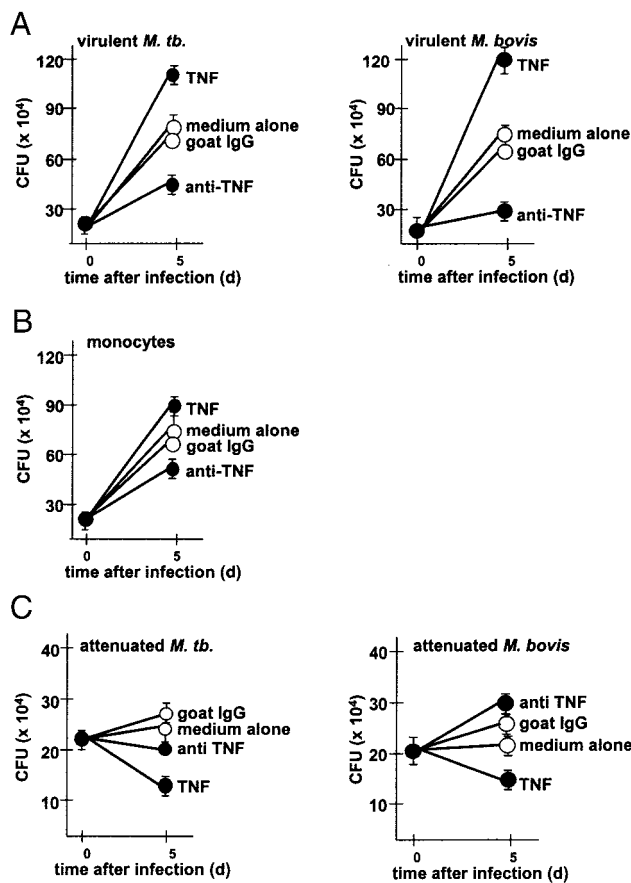
FIGURE 6. TNF supports the intracellular growth of virulent, but not attenuated, mycobacteria. *A–C*, AM or freshly isolated monocytes were pulse-infected with mycobacteria (MOI 0.5, 4 h). After removing extracellular bacteria, TNF (20 ng/ml), anti-TNF (20 μg/ml), or goat IgG (20 μg/ml) was added to the cultures. After 5 days of infection cells were lysed and 5-fold dilutions were plated on Middlebrook agar. The graphs shows the average ± SEM of eight (*A* and *B*) or five (*C*) independent experiments. The asterisk indicates statistical significance between the growth of treated and untreated cultures (∗, $p < 0.05$; ∗∗, $p < 0.01$).

acts as a stimulus of bacillary growth. The induction of TNF by intracellular bacteria may therefore represent a bacterial virulence factor that has evolved to support survival and multiplication at the site of primary disease.

Discussion

The local secretion of cytokines is a double-edged sword, contributing either to protective immunity or to the immunopathology of disease. TNF is expressed at the site of human tuberculosis (43–46) and is considered to be pivotal for determining the course of
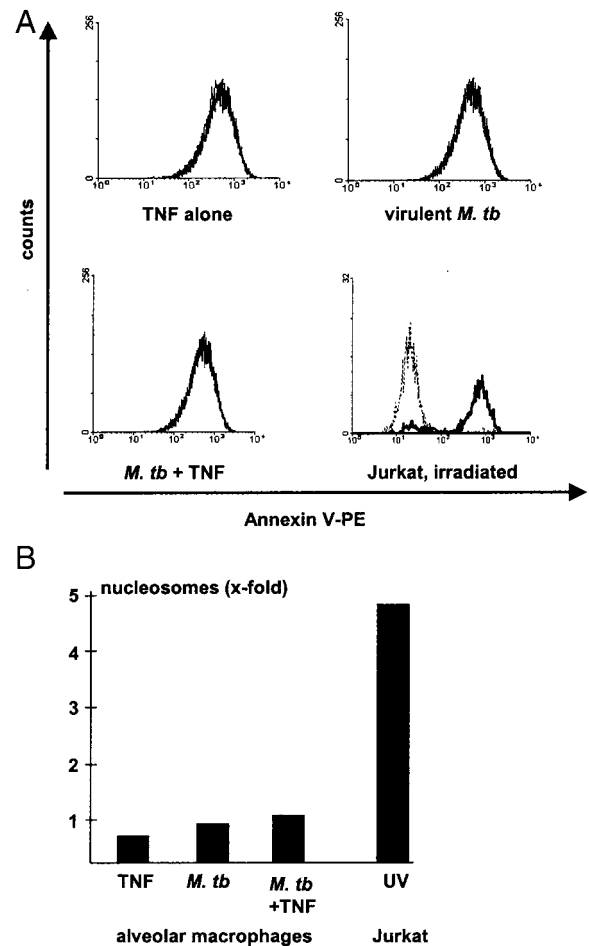
FIGURE 7. TNF does not induce apoptosis in infected AM. AM were infected with virulent *M. tuberculosis* (MOI 0.5) and cultured in the presence or absence of TNF (20 ng/ml) for 5 days. *A*, AM were detached using EDTA and stained with annexin V PE (5 μl) for 15 min and analyzed by flow cytometry. The graphs show an overlay of treated cells and untreated AM (dashed line). *B*, Cells were lysed and the content of cytoplasmic nucleosomes was measured by ELISA. The data present the x-fold enrichment of nucleosomes in treated cells as compared with untreated AM calculated by the formula OD_{405} (treated cells)/OD_{405} (untreated cells). The experiment shown is representative of four with similar results. Jurkat cells, which were UV-irradiated for 60 s and incubated for 4 h, served as a positive control (solid line in *A*).

disease. We investigated AM from the human lung in terms of their capacity to secrete TNF in response to mycobacterial infection and, more importantly, to determine the functional role of TNF on the growth of *M. tuberculosis*. Our results demonstrate that 1) virulent mycobacteria induce higher amounts of TNF in

Table III.   *TNF supports the growth of* M. tuberculosis *in AM*[a]

	Medium Only	Anti-TNF	TNF
[³H]uracil uptake (cpm)	9487 ± 423	4730 ± 301∗∗	15256 ± 496∗∗
infected cells (%)	49 ± 5	35 ± 3∗	68 ± 3∗
bacteria/infected cell	8 ± 2	4 ± 1∗	14 ± 3∗

[a] AM were pulse-infected with a virulent strain of *M. tuberculosis* (MOI 1, 4 h) in a Permanox chamber slide (auramine-rhodamine stain) or a 24-well plate ([³H]uracil uptake). Extracellular bacteria were removed and TNF (20 ng/ml) or anti-TNF (20 μg/ml) were added. After 1 (data not shown) and 5 days mycobacterial quantification was performed. After 1 day 27 ± 7% of the AM were infected harboring 2 ± 0.4 bacteria. [³H]uracil uptake was 740 ± 42 cpm. The data give a representative experiment of four, all done in duplicates (acid-fast stain, at least 400 cells counted per well) or triplicates (uracil uptake). An isotype control (goat IgG, 20 μg/ml) had no significant effect on the [³H]uracil uptake. The asterisk indicates statistical significance between treated and untreated cultures (∗, $p < 0.05$; ∗∗, $p < 0.01$).

AM than attenuated strains, 2) infected and noninfected AM contribute to TNF production, and 3) TNF supports the growth of virulent, but not attenuated, *M. tuberculosis*. In this manner the ability of intracellular pathogens to induce TNF release may contribute to virulence and the progression of infection.

Our initial experiments revealed that only a fraction of AM phagocytosed *M. tuberculosis*. Binding of mycobacteria to AM is mediated by complement receptors binding to complement components fixed to mycobacteria (32, 47) and by mannose receptors (48) under the participation of surfactant protein A (48, 49), CD14 (50), and sialoglycoprotein CD43 (51). These molecules serve as pattern recognition receptors that bind conserved bacterial structures (50, 52). Therefore, the interaction between mycobacteria and AM is highly dependent on the expression pattern of cell surface receptors on the phagocyte. The healthy or diseased lung contains a heterogenous population of cells, comprising resident AM, elicited macrophages, and possibly immune-activated macrophages (53, 54). This heterogeneity of AM will most likely pertain to the expression of pattern recognition receptors, thereby influencing the ability to take up airborne pathogens. Ongoing studies are designed to use the double-staining technique described above to identify and characterize subpopulations capable of taking up viable mycobacteria.

The failure of a majority of primary AM to take up mycobacteria does not correlate with global immunological silence. We show that uninfected AM contribute to TNF production (Fig. 3*B*). Similarly, the attenuated vaccination strain *M. bovis* BCG has recently been shown to induce TNF production in uninfected and infected monocyte-derived macrophages (55). Our initial hypothesis that mediators released by infected cells (e.g., IL-1, IL-6, IL-10) stimulate noninfected cells was confuted by transwell experiments (Table II). Alternatively, TNF production from bystander cells could be induced by digested and regurgitated bacterial products of larger size. Despite being incapable of taking up whole microbes, AM may phagocytose degraded bacterial material by receptor-independent macropinocytosis. Bacterial products could also be shuttled into APCs by apoptotic bodies that represent cells that have undergone apoptosis due to an overwhelming bacterial burden. This mechanism has been shown to permit Ag presentation to cytolytic T cells (56) and may also induce the secretion of immunomodulatory cytokines. In our low-dose model of infection, AM did not undergo apoptosis (Fig. 7), suggesting that this mechanism is not involved in TNF induction.

So far it remained controversial whether virulent or attenuated mycobacteria induce higher levels of TNF in human macrophages. Results published in the past show higher induction by attenuated strains in macrophages (57), a comparable up-regulation in AM (40), and finally higher TNF induction by virulent strains (58, 59) (Fig. 4). TNF induction in human primary macrophages appears to be highly dependent on the experimental setting and the origin and treatment of the cells under investigation, as well as the bacterial strains. Therefore, when studying TNF production, it is mandatory to use well-defined experimental procedures under stringent control of variables including purity of the cells, efficiency of infection, and culture conditions. In our studies we infected the cultures with a low number of bacteria to exclude loss of cell viability, we adjusted the bacterial inoculum such that different strains infected the AM with nearly equal efficiencies, and we routinely stained an aliquot of the cultures with α-naphthyl-acetate-esterase to document the purity of the population. In addition, we confirmed our results by three independent methods (intracellular flow cytometry, ELISA, and bioactivity) and used two pairs of virulent/attenuated bacteria (*M. tuberculosis* and *M. bovis*).

The key finding of our study is that TNF supports the growth of virulent *M. tuberculosis*. This observation suggests that TNF may not only harm the infected individual by contributing to the immunopathology of tuberculosis (28, 58, 60, 61) but also directly supports the survival of intracellular pathogens. Of note, TNF has opposite effects on the growth of virulent and attenuated strains. While virulent mycobacteria appear to have developed efficient evasion mechanisms for protection against the attack of the host immune system, attenuated strains are susceptible to treatment of the host cell with TNF (32) (Fig. 6). The question remains: how does TNF interfere with the antibacterial effector mechanisms of human AM? Possible mechanisms include 1) the deprivation of intracellular iron, 2) modulation of the maturity of cells, 3) regulation of chemokine/cytokine release, and 4) induction of apoptosis of infected cells.

The deprivation of intracellular iron

TNF is known to modulate the iron metabolism of phagocytes (62). The activity of many mycobacterial enzymes is dependent on the presence of iron (63), and virulent mycobacteria can be attenuated by expression of an iron repressor (64). By inference, it is conceivable that TNF increases the availability of iron for the bacilli, thereby facilitating their growth. This hypothesis is strengthened by a study showing that TNF promotes mycobacterial growth in monocytes by interfering with the iron metabolism (33).

Modulation of the maturity of cells

The AM used in our experiments were enriched from the bronchoalveolar lavage by adherence to plastic. Despite staining positively for the macrophage-specific enzyme α-naphthyl-acetate esterase, the macrophage population is likely to comprise different stages of maturation. One possible effect of TNF could be to modulate the maturity of the macrophages, thereby influencing antibacterial activity. Antimycobacterial activity has been shown to be dependent on the maturity of the host cells; e.g., immature dendritic cells kill *M. tuberculosis* more efficiently than mature dendritic cells (37). Therefore, the growth-promoting effect might be a result of the modulation of the maturity of infected cells rather than the deactivation of antibacterial effector mechanisms.

Regulation of chemokine/cytokine release

TNF could induce the synthesis of immunomodulatory cytokines or chemokines by infected macrophages. Candidates that have been shown to down-regulate macrophage functions in macrophages include IL-10 and TGFβ (65). Both cytokines are expressed in human AM in response to infection with *M. tuberculosis* (41, 66), and their secretion could be promoted by TNF.

Induction of apoptosis of infected cells

Finally, TNF could mediate its growth-promoting effect by the regulation of apoptosis. Infection with *M. tuberculosis* increases the susceptibility of fibroblasts to undergo TNF-induced apoptosis (67). In addition, infection of human AM with attenuated mycobacteria promotes apoptosis of the host cell by the induction of TNF (40). Tuberculosis is a chronic infection, which suggests that macrophage apoptosis might favor the host by depriving the pathogen of its intracellular sanctuary. Apoptosis, as opposed to necrosis, has been shown to induce killing of mycobacteria (68). Alternatively, apoptosis could result in the spread of mycobacteria to uninfected macrophages via the uptake of apoptotic bodies, thereby offering a more favorable environment for the bacilli than highly infected cells (69).

Recent observations suggest that reactivation of tuberculosis is a potential side effect of treatment with anti-TNF (Infliximab) (29–

31). The incidence of tuberculosis in patients undergoing anti-TNF therapy for rheumatoid arthritis or Crohn's disease was higher than that of other infections with opportunistic pathogens (e.g., listeriosis, *Pneumocystis carinii* pneumonia, aspergillosis, histoplasmosis, legionellosis). Because TNF is an inflammatory cytokine that induces a broad spectrum of biological effects, the mechanisms underlying this observation are most likely multifactorial and complex. In mice, the lack of TNF results in the failure to form (5–7) or maintain granulomas (70), possibly due to decreased expression of adhesion molecules and chemokines (71). Alternatively, apoptosis of infected cells could be diminished in the absence of TNF, thereby supporting mycobacterial survival (41). The present (Fig. 6) and earlier studies (22, 33) show that TNF alone does not activate antimycobacterial activity in murine or human macrophages. Therefore, the reactivation of tuberculosis in Infliximab-treated patients is probably not due to a loss of antimycobacterial activity, but reflects the disintegration of granulomas, which allows dormant mycobacteria to convert into metabolically active and destructive pathogens (31).

It is undebatable that TNF is critical for protection in murine tuberculosis. This applies to in vivo models (5–7, 72) and in vitro studies demonstrating antimycobacterial activity in purified macrophage populations stimulated with TNF and IFN-γ (22, 73, 74) but not TNF alone (22). In contrast, TNF and IFN-γ did not induce killing of *M. tuberculosis* in human monocytes (34). In addition, effector molecules in mice and humans are distinct, as illustrated by the recently characterized antibacterial effector pathway initiated by the binding of bacterial lipopeptides to Toll-like receptor (TLR)2. In mouse macrophages, bacterial lipoprotein activation of TLR2 leads to NO-dependent killing of intracellular tubercle bacilli. In human monocytes and AM, bacterial lipoproteins similarly activated TLR2 to kill intracellular *M. tuberculosis*, but by an antimicrobial pathway that is NO independent. This suggests that similar antibacterial effector pathways are conserved among different species but the executing molecules are distinct. Therefore, it is meaningful to investigate human cells to gain comprehensive insight into the interactions of the constituents of the immune system.

This study suggests a scenario in which virulent mycobacteria infect a subset of AM, which secrete TNF and release degraded bacterial products, which activate bystander cells to perpetuate TNF production. This chain of events allows accelerated proliferation of the bacilli and may serve as an evasion mechanism of virulent *M. tuberculosis*. However, in vivo during the course of a protective immune response T cells will be attracted and activated by APCs. T cells will contribute to the protective immune response by secreting stimulatory cytokines (75) or antibacterial molecules like granulysin (19), thereby counterregulating the growth-permissive state of AM.

Acknowledgments

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