Tumor Necrosis Factor α (TNFα) Promotes Growth of Virulent *Mycobacterium tuberculosis* in Human Monocytes

Iron-mediated Growth Suppression Is Correlated with Decreased Release of TNFα from Iron-treated Infected Monocytes

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Abstract

The human immune response to *Mycobacterium tuberculosis* is not well characterized. To better understand the cellular immune response to tuberculosis, a human mononuclear phagocyte culture system using a low-infecting inoculum of *M. tuberculosis* to mimic in vivo conditions was developed. Using this system, monocytes treated with IFNγ/TNFα/calcitriol (CytD) were permissive for the growth of virulent *M. tuberculosis.* In the presence of iron, however, these monocytes suppressed the growth of *M. tuberculosis.* The enhanced permissiveness of CytD-preincubated monocytes was found to be due to TNFα, however, the ability of iron to suppress *M. tuberculosis* growth also required preincubation with TNFα. Iron-mediated growth suppression was correlated with selective suppression of TNFα release from infected monocytes. In addition, removal of TNFα from CytD-treated monocytes 2 d after infection mimicked the suppressive effect of iron, suggesting that iron may also be decreasing monocyte sensitivity to exogenously added TNFα. In the absence of iron, permissive, CytD-treated monocytes formed large infected cellular aggregates. With iron treatment, aggregation was suppressed, suggesting that the iron-suppressive effect on *M. tuberculosis* growth may be related to suppression of monocyte aggregation and diminished cell-to-cell spread of *M. tuberculosis.* The results of this study indicate that TNFα preincubation is required for human monocytes to exert an iron-mediated suppressive effect on *M. tuberculosis* growth. In the absence of iron, however, the continued presence of TNFα has a growth-promoting effect on *M. tuberculosis* in human monocytes. Iron may be an important early modulator of *M. tuberculosis* growth via its effects on TNFα. (*J. Clin. Invest.* 1997. 99:2518–2529.)

Key words: transferrin • lactoferrin • polymorphonuclear leukocyte • interferon gamma • calcitriol

Introduction

Relatively little is known about the effector mechanisms whereby the human immune system controls *Mycobacterium tuberculosis* infection and the influence of specific cytokines in this process. Iron has been shown to enhance, and in some cases suppress, the growth of various intracellular bacterial pathogens (1–8). The availability of intracellular iron to these pathogens can be influenced by cytokines (1, 7, 9). Thus, cytokine/iron interactions may be an important determinant in the outcome of infection caused by these pathogens.

Monocyte-activating agents involved in the pathogenesis of mycobacterial infections include IFNγ, calcitriol, and TNFα (10, 11). TNFα is important in mycobacterial granuloma formation (12). TNFα is also an important modulator of human iron metabolism through its effect on the reticuloendothelial system, which may account for its role in the anemia of chronic disease, and the associated acute hypoferremic response (13–15). Among its functions on cellular iron metabolism, TNFα induces synthesis of ferritin in a variety of cell types (16), and induces degranulation of PMN with release of the iron-binding protein lactoferrin (17). The iron-binding proteins ferritin, lactoferrin, and transferrin have been specifically identified in epithelioid and multinucleated giant cells comprising the mycobacterial granuloma (18). In addition, PMN are present early at sites of mycobacterial infection in humans (19), and are the likely source of lactoferrin found in granulomas.

In this study we have examined the interaction of virulent *M. tuberculosis* (Erdman strain) with human monocytes, focusing on the role of TNFα and iron. This study will demonstrate that (*a*) in the absence of iron, IFNγ/TNFα/calcitriol (CytD)[1]-treated monocytes are permissive for the growth of virulent *M. tuberculosis* in human monocyte monolayers; (*b*) the permissiveness of CytD-treated monocytes for growth of *M. tuberculosis* is due to TNFα; (*c*) iron enables CytD-treated monocytes to suppress virulent *M. tuberculosis* growth; (*d*) iron downregulates TNFα release from infected monocytes; (*e*) iron-mediated growth suppression requires that monocytes be preincubated with TNFα; and (*f*) preincubation of monocytes with TNFα followed by its removal after infection mimics

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1. *Abbreviations used in this paper:* CytD, IFNγ/TNFα/calcitriol; FAC, ferric ammonium citrate; NHS, normal human serum.

the restrictive effect of iron. Since TNFα is a permissive factor for growth of virulent *M. tuberculosis* in human monocytes, the ability of iron to enable TNFα-treated monocytes to restrict *M. tuberculosis* growth may in part be through suppression of TNFα release by infected monocytes, and through decreasing the responsiveness of monocytes to the TNFα signal. TNFα exposure before infection, however, is necessary for monocytes to develop the capacity to restrict *M. tuberculosis* growth.

Methods

Tissue culture media. Iscove's modified Dulbecco's medium was used in tissue culture experiments (Gibco Laboratories, Grand Island, NY).

Iron compounds. Ferric ammonium citrate (FAC) (Sigma Chemical Co., St. Louis, MO) was dissolved in Iscove's medium and filtered through 0.22-μm filter units. Iron-saturated transferrin (Sigma Chemical Co.) and iron-saturated lactoferrin (Calbiochem Corp., La Jolla, CA) were dissolved in Iscove's medium, and were then filtered through 0.22-μm filter units (Spin-X; Costar Corp., Cambridge, MA).

Cytokines/antibodies. Human recombinant IFNγ and human recombinant TNFα (Upstate Biotechnology, Inc., Lake Placid, NY) were reconstituted in PBS and diluted in Iscove's medium. Calcitriol was the generous gift of Dr. Milan Uskokovic (Hoffmann-La Roche, Inc., Nutley, NJ) and was prepared as described (20). IFNγ was used at a concentration of 100 U/ml, TNFα at 1,000 U/ml, and calcitriol at 10^{-8} M. Rabbit anti–human TNFα neutralizing polyclonal antibody (Genzyme Corp., Cambridge, MA) was diluted in Iscove's medium.

Human blood mononuclear cells. For the majority of experiments, mononuclear cells were obtained from buffy coats purchased through the American Red Cross. In some experiments, mononuclear cells were obtained from the blood of healthy, adult volunteers who were tuberculin skin test negative. The blood mononuclear cell fraction was obtained by centrifugation over a Ficoll-sodium diatrizoate solution (Pharmacia LKB Technology, Inc., Piscataway, NJ) as previously described (1).

Serum. Venous blood was obtained from healthy adult volunteers with no history of tuberculosis or positive tuberculin skin test. Serum was separated and stored at −70°C. Autologous or heterologous serum was used in experiments.

Bacterial culture media. Middlebrook 7H9 broth (Difco Laboratories, Detroit, MI) was used for dilution of culture supernates before plating. Middlebrook 7H11 agar (Difco) plates (100 × 15-mm bacteriologic Petri dishes) were used for plating CFU from infected monolayers and supernates.

Bacteria. M. tuberculosis Erdman strain (ATCC 35801), was obtained from the American Type Culture Collection (ATCC, Rockville, MD) as a lyophilized culture. Bacteria were reconstituted as recommended, and plated on 7H11 agar. In preliminary experiments, bacteria were harvested after 4 wk, flash frozen, aliquoted, and stored at −70°C. These aliquots were then used to start new cultures (on 7H11 plates), which were harvested after 2 wk of growth and prepared for infection as described (21). In subsequent experiments, a bacterial stock of the Erdman strain was prepared by culturing reconstituted bacteria on 7H11 agar for 4 wk at 37°C/10% CO_2. The bacteria were then scraped from plates into Iscove's medium containing 50% normal human serum (NHS) and incubated for 30 min at 37°C to allow for complement deposition to occur (21). The suspension was then sonicated using an ultrasonic cell disrupter (Microson XL; Heat Systems, Farmingdale, NY) to disperse clumped bacteria. The bacteria were washed three times by centrifugation at 15,000 *g* for 5 min and were resuspended in Iscove's medium. The bacterial suspension was subsequently divided into 500-μl aliquots, flash frozen, and stored at −70°C. The number, viability, and degree of clumping of the bacterial preparation was then determined by thawing an aliquot and examining *M. tuberculosis* (Petroff Hauser counter; Hansser Scientific, Horsham, PA), and plating bacteria on 7H11 plates to determine CFU and viability. Bacteria were ~ 25% viable, and 95% of bacteria were in single-cell suspension. The endotoxin content of this preparation was below the limit of detection in the Limulus amebocyte assay used (E-Toxate; Sigma Chemical Co.). Bacterial suspensions were then prepared from these stock cultures for each experiment. In several experiments, a frozen stock of guinea pig passaged Erdman strain provided by Esther Byung Lee and Marcus Horwitz (UCLA) was used to start cultures on 7H11 plates which were then used in experiments as described above.

Assay for growth of M. tuberculosis in human monocytes. In contrast to other studies which have used monocyte-derived macrophages (22), monocytes were used in this report. The rationale for the use of monocytes is that in vivo in primary infection, *M. tuberculosis* first encounters resident alveolar macrophages. If initial pulmonary defenses are overcome, successful control of the infection depends upon recruitment of blood monocytes, which are activated by cytokines, and form a granuloma restricting the growth and spread of this pathogen. Granulomatous lesions are in a high state of turnover, with > 90% of the monocytes turning over every 10 d (23). Thus, newly recruited blood monocytes which mature in the cytokine-rich milieu of *M. tuberculosis* infection, play a critical role in containment of *M. tuberculosis* infection.

Freshly explanted monocytes in Iscove's medium containing 2% autologous or heterologous NHS were adhered to flat-bottomed wells (Linbro, 16-mm diameter; Flow Laboratories, McLean, VA), or tissue culture wells (Costar Corp.), at concentrations of ~ 5×10^5 to 1×10^6/well for 90 min in 5% CO_2/95% air at 37°C. The monolayers were washed three times with warm Iscove's medium. Adherent cells were > 95% monocytes by morphology and myeloperoxidase staining. The monocytes were then cultured in Iscove's medium, with or without serum. Monolayers were incubated with medium alone, medium containing cytokines, and/or medium containing iron compounds for 48 h before infection. Serumless conditions were used in some experiments to isolate the effects of cytokines and iron, and to avoid factors found in serum which might interfere with later measurements of cytokines released by infected monocytes into cell supernates. Use of Iscove's medium and serumless conditions has precedent in the study of the intracellular pathogen, *Legionella pneumophila. L. pneumophila* multiplication is inhibited in human monocytes activated with IFNγ—this inhibition is overcome by adding iron in the form of FAC. These effects are observed in Iscove's medium whether or not the monocytes are cultured with human serum (2). Furthermore, changes in monocyte transferrin receptor and ferritin expression induced by IFNγ occur whether or not the monocytes are cultured with serum (9).

Low-inoculum and high-inoculum assays were used in experiments. A low-inoculum assay was used to mimic the natural encounter of *M. tuberculosis* with monocytes. Under these conditions, uninfected monocytes can mature slowly in a milieu of cytokines released by *M. tuberculosis*–infected monocytes in the monolayer. The contribution of uninfected monocytes to containment of *M. tuberculosis* may thus be enhanced. In this assay, preopsonized *M. tuberculosis* was added to monocyte monolayers at a concentration of ~ 5×10^2 bacteria/ml (bacteria/monocyte ratio of ~ 0.0005:1). The bacteria were not removed, and the monolayers were incubated at 37°C in 5% CO_2/95% air. Because of a low multiplicity of infection, the ability of *M. tuberculosis* to multiply in this assay reflects the ability of the bacteria to spread from cell to cell from an initial isolated focus of infection, as well as the ability to multiply in individual monocytes. At various time points, the culture supernates and cell lysates were plated on 7H11 agar as has been described (22), with the exception that bacteria were pulse-vortexed in Eppendorf tubes containing three glass beads before plating. Preliminary studies indicated that there was no difference in CFU between bacteria vortexed with glass beads compared to bacteria plated after sonication with a probe tip sonicating device.

In the high-inoculum assay, preopsonized *M. tuberculosis* were added to monocyte monolayers at a concentration of ~ 5×10^6 bacteria/ml (bacteria to monocyte ratio of 5:1). The monolayers were incu-

bated at 37°C in 5% CO_2/95% for 1–4 h. The bacterial suspension was removed, and infected monocyte monolayers were washed three times with 1.0-ml volumes of warm Iscove's medium. The infected monocyte monolayers were incubated in Iscove's medium with or without serum with cytokines/calcitriol, iron compounds, or control medium. With this assay, ~ 30% of monocytes contain one or more bacteria at the start of infection, which is similar to what others have reported for this type of assay (21). Because of a high multiplicity of infection, the high-inoculum assay is more a reflection of the ability of *M. tuberculosis* to multiply in individual monocytes. At various time points, culture supernates and cell lysates were plated on 7H11 agar.

Assay for growth of M. tuberculosis in monocyte-conditioned tissue culture media. Preliminary studies indicated that *M. tuberculosis* would not grow in Iscove's medium, with or without serum, in the presence or absence of iron. To control for any *M. tuberculosis* growth-enhancing effect of monocyte-conditioned media, monocytes were plated in Costar wells containing Iscove's medium, with or without NHS, and with or without FAC, as described above. Wells also contained transwell inserts with 0.1-μm membranes porous to solutes, but not to bacteria. The porous membrane end of the transwell was inserted into tissue culture media containing monocyte monolayers, with the internal portion of the transwell also containing tissue culture media. Both the transwell insert and the monocyte monolayers were simultaneously inoculated with the same starting inoculum of *M. tuberculosis.* The low-inoculum infection assay as described above was then carried out. At the time that monocyte monolayer CFU were plated, CFU were also plated from inoculated transwells.

Assessment of infection. In each experiment CFU were normalized to 10^5 monocyte nuclei. The number of monocyte nuclei/well was determined as described (24), in replicate infected wells after pretreatment with 10% formalin. Viability was also analyzed in replicate infected wells by Trypan blue exclusion. The purpose of assessing viability was to ensure that various treatments, in conjunction with *M. tuberculosis* infection, did not have toxic effects on the monocyte monolayers (25).

Monocyte monolayer morphology assay. This assay was used to determine the effect of *M. tuberculosis* infection on monocyte monolayer morphology. Monocyte monolayers were plated on chromerge-treated #2 glass coverslips in Linbro tissue culture wells as described above, and infected using the low-inoculum assay. The infected monocyte monolayers were incubated in Iscove's medium with IFNγ/TNFα/calcitriol, with or without iron in the form of FAC. After 7 d, the monolayers were washed to remove any extracellular bacteria, and freshly isolated autologous monocytes were adhered for 90 min to coverslips with infected monocyte monolayers. Monolayers were washed twice with 1.0 ml of Iscove's medium and IFNγ/TNFα/calcitriol, with or without FAC readded. After an additional 7 d the process was repeated. 7-, 14-, and 21-d-old coverslips were fixed and stained with modified Kinyouns AFB stain and/or Diff Quik (Baxter Diagnostics, Inc., McGaw Park, IL), and monolayer morphology was examined.

Assay for determination of cytokine levels. Using the high-inoculum assay, monocytes cultured in the absence of serum, treated with IFNγ, TNFα, and calcitriol, with or without FAC, were infected with *M. tuberculosis.* After washing, Iscove's medium, cytokines (except TNFα which would interfere with measurement of endogenous TNFα), and/or FAC were added back to the wells. At various time points, monocyte number and viability were assessed in replicate infected wells, and cell lysates were plated on 7H11 agar for *M. tuberculosis* CFU. Cell supernates were filtered through 0.22-μm filter units and frozen at −70°C to be used for determination of cytokine levels. For cytokine determinations, supernates from an experiment were thawed, and cytokine levels were determined by ELISA (R & D Systems Inc., Minneapolis, MN) using a microtiter plate reader (Molecular Devices Corp., Menlo Park, CA). The amount of cytokine was corrected to 10^5 monocytes.

L929 cytotoxicity assay. L929 cells were obtained from the ATCC certified cell line 1 (CCL1). The effect of infected monocyte supernates on L929 cells was determined. L929 cells were cultured in RPMI/20% FBS, and were harvested after monolayers became confluent. Cells were then seeded into microtiter plates. Wells received actinomycin D 1.0 μg/ml (Sigma Chemical Co.) to block further cell replication, and TNFα standards or cell supernates. In addition, since some of the test supernates contained FAC, FAC was added to some groups such that all wells contained the same concentration to correct for the influence of iron on L929 growth and/or viability. After 24 h the cells were processed as described (26), and the absorbance was determined at 540 nm on a microtiter plate reader. Absorbance of test samples was compared to absorbance of a standard curve for TNFα cytotoxicity and the percent decrease of TNFα in supernates from monocyte monolayers treated with FAC relative to monolayers not receiving FAC was determined.

Statistics. Data were compared using the Student's *t* test. Data were considered significant with a $P < 0.05$.

Results

Cytokine/calcitriol (CytD)-treated monocytes are permissive to M. tuberculosis growth, and iron enhances the ability of these monocytes to restrict M. tuberculosis multiplication using a low-inoculum assay. M. tuberculosis intracellular multiplication was similar in the control and CytD-treated monocytes (Fig. 1). The addition of iron to CytD-treated monocytes resulted in statistically significant growth suppression of *M. tuberculosis.* Iron also had a slight, but statistically insignificant effect on *M. tuberculosis* growth in control monocytes. Monocyte adherence as determined by recovery of nuclei was best in the CytD-treated monocytes receiving iron. In this set of experiments, mean viability was 96% in the CytD-treated group receiving FAC, indicating that the decreased growth of *M. tuberculosis* in this group was not due to iron toxicity. An iron-suppressive effect was also observed when the high-inoculum assay, carried out over 10 d, was used. With the high-inoculum assay, the iron suppressive effect developed between days 5 and 10, and was not as pronounced as that seen with the low-inoculum as-

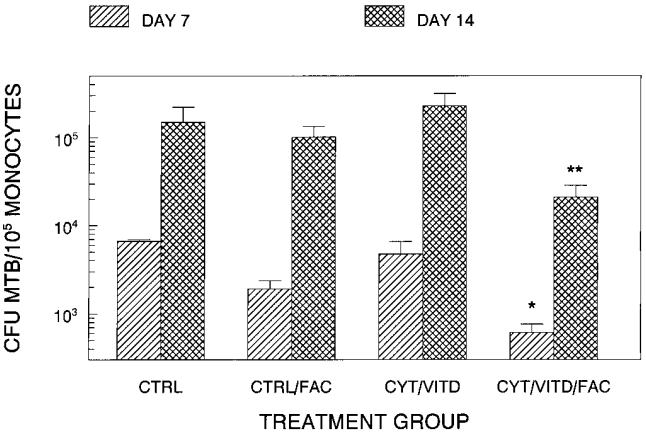

Figure 1. Iron in the form of FAC enhances the ability of CytD-treated human monocytes to restrict the multiplication of *M. tuberculosis.* Control (*CTRL*) and CytD-treated human monocyte monolayers, with or without FAC (50 μg/ml), in the absence of serum, were infected using a low-inoculum assay. There was a statistically significant decrease in CFU in the CytD-treated group with iron relative to the CytD-treated group not receiving iron at 7 and 14 d, *, **$P < 0.01$. Data are mean±SEM for duplicate determinations in five separate experiments using monocytes from different donors.

say (data not shown). Therefore, the low-inoculum assay was used in most experiments.

To assess whether the same iron-suppressive effect could be observed with animal-passaged *M. tuberculosis* which might have enhanced virulence, CytD-treated monocytes were infected using the low-inoculum assay with the Erdman strain which had been passaged in guinea pigs. FAC was able to suppress growth of this organism in the same fashion (data not shown).

The iron suppressive effect was due to iron in FAC, since in a separate experiment, CytD-treated monolayers receiving FAC had 0.9 log less growth at 7 d and 0.8 log less growth at 14 d when compared with activated monolayers receiving an equimolar amount of ammonium citrate without iron. FAC was used as the iron source in most experiments to isolate the effect of iron in this system independent of iron-binding proteins. This compound has a notable lack of toxicity on human monocytes when compared with other nonphysiologic iron chelates such as iron nitrilotriacetate (27). The effect of FAC on human monocyte function has been studied extensively. Viability, phagocytic capacity, and expression of surface markers was not affected by concentrations of FAC up to 100 μg/ml (28). The studies reported here use concentrations in the range of 0.5 to 50 μg/ml (equivalent to 0.6 to 6 mg/ml iron saturated transferrin). These data indicate that iron is able to suppress growth of *M. tuberculosis* apart from any effect on monocyte viability.

Iron enhances CytD-mediated restriction of M. tuberculosis in a dose-dependent fashion. Using the low-inoculum assay, the effect of various concentrations of FAC on the ability of CytD-treated monocytes to restrict *M. tuberculosis* was determined (Fig. 2). FAC demonstrated a dose-dependent inhibitory effect on *M. tuberculosis* growth which was maximal at 50 μg/ml. The physiologic iron-binding proteins iron transferrin and iron lactoferrin were tested for their ability to enhance restriction of *M. tuberculosis* growth in activated monocytes. At the same iron concentrations, both iron transferrin and iron lactoferrin enhanced the ability of CytD-treated monocytes to restrict *M. tuberculosis* in a dose-dependent fashion.

Iron is a catalyst for the Fenton reaction which occurs as a part of the Haber-Weiss reaction (29). To evaluate the possibility that iron could be catalyzing the Fenton reaction with resultant hydroxyl radical having a bactericidal effect on *M. tuberculosis*, CytD-treated, infected monocyte monolayers with and without iron were incubated with the hydroxyl radical scavenger mannitol. Mannitol at concentrations of 25, 2.5, and 0.25 mM had no effect on the iron-suppressive effect against *M. tuberculosis* (data not shown), indicating that the hydroxyl radical is not playing a role in iron-mediated restriction of *M. tuberculosis.* This result is consistent with the data indicating that iron lactoferrin enhanced the ability of CytD-treated monocytes to restrict *M. tuberculosis* (Fig. 2), since it has been reported that hydroxyl radical formation in human monocytes is inhibited by saturated lactoferrin (30).

Differences in extracellular growth of M. tuberculosis in the conditioned medium of M. tuberculosis–infected monocytes do not account for the suppressive effect of iron. Substances released by *M. tuberculosis*–infected monocytes could potentially influence the extracellular growth of *M. tuberculosis* under low-inoculum culture conditions. To examine this possibility, CytD-treated monocyte monolayers were infected in tissue culture wells containing transwells porous to soluble substances, but too small (0.1 micron) for bacterial penetration (Table I). In each experiment, the same starting inoculum was added to each monocyte monolayer and its accompanying transwell in the presence or absence of NHS. The data indicate that growth of *M. tuberculosis* in the conditioned medium of *M. tuberculosis*–infected human monocytes is minimal with the exception of the group receiving serum and FAC at day 14. In spite of increased growth within the transwell of this group, however, there were relatively few bacteria in the monolayer or supernate, and mean monocyte viability was also greatest in this group. This result indicates that the majority of *M. tuberculosis* in monocytes cultured in serum and incubated with iron, remains within viable, adherent monocytes throughout the course of infection. Thus, as long as adequate numbers of appropriately activated mononuclear phagocytes are present to phagocytize *M. tuberculosis* in a permissive extracellular environment, *M. tuberculosis* growth can be suppressed.

The suppressive effect of iron was apparent whether CFU from both the monolayer and supernate, or the monolayer alone were quantified, indicating that selective loss of heavily in-

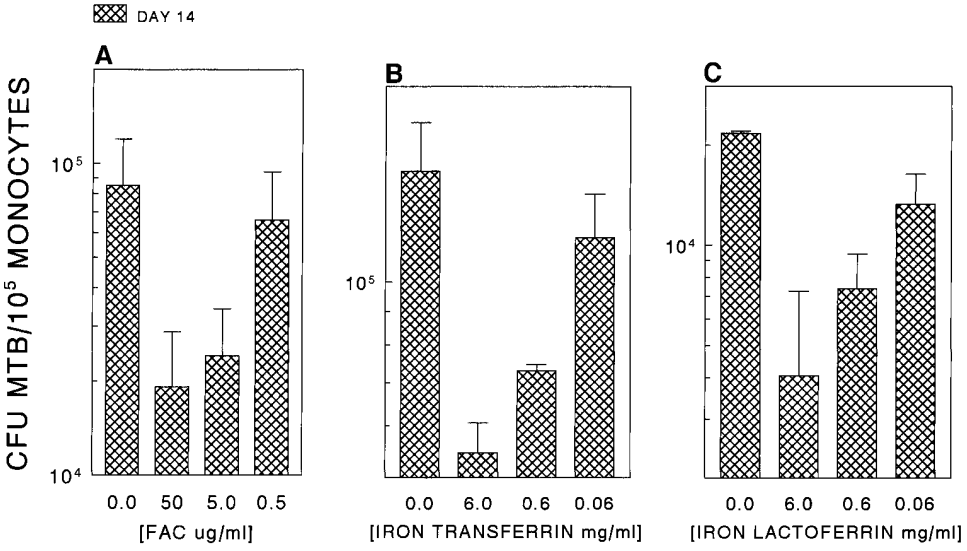

Figure 2. Iron enables CytD-treated monocytes to restrict *M. tuberculosis* in a dose-dependent fashion. CytD-treated human monocyte monolayers in the absence of serum were incubated with various forms of iron at varying concentrations, and were infected using the low-inoculum assay. (*A*) FAC. (*B*) Iron transferrin. (*C*) Iron lactoferrin. Data are the mean±SD for duplicate determinations.

Table I. Mycobacterium tuberculosis Low-inoculum Infection Assay

Time	CFU (10^3)			Total CFU (10^3) (excluding transwell)	MN No. (10^5)	Monolayer CFU/10^5 MN (10^3)	% Change with iron
	Monolayer	Supernate	Transwell				
Day 0							
−serum		0.25 (0.18)	0.25 (0.18)		7.2 (0.61)		
−serum/FAC		0.25 (0.18)	0.25 (0.18)		7.4 (1.33)		
+serum		0.25 (0.18)	0.25 (0.18)		7.1 (0.19)		
+serum/FAC		0.25 (0.18)	0.25 (0.18)		6.4 (0.56)		
Day 2							
−serum	0.26 (0.14)	< 0.02	0.28 (0.11)	0.26	4.5 (0.71)	0.06	−10
−serum/FAC	0.24 (0.13)	< 0.02	0.19 (0.07)	0.24	4.6 (1.10)	0.05	
+serum	0.22 (0.11)	< 0.02	0.22 (0.09)	0.22	5.7 (0.10)	0.04	+35
+serum/FAC	0.29 (0.15)	< 0.02	0.15 (0.04)	0.29	5.6 (0.05)	0.05	
Day 7							
−serum	13.0 (2.3)	1.9 (1.1)	1.4 (0.6)	14.9	1.3 (0.68)	10.4	−56
−serum/FAC	10.0 (2.6)	0.4 (0.2)	1.2 (0.4)	10.4	2.2 (0.26)	4.5	
+serum	12.0 (4.0)	2.7 (1.4)	0.9 (0.5)	14.7	2.2 (0.21)	5.5	−42
+serum/FAC	8.2 (2.9)	0.4 (0.1)	1.7 (0.3)	8.6	2.6 (0.39)	3.2	
Day 14							
−serum	411 (66)	42.0 (2.3)	6.2 (1.4)	453	0.8 (0.26)	520	−85
−serum/FAC	110 (26)	22.0 (2.6)	4.8 (1.5)	132	1.4 (0.40)	78	
+serum	470 (150)	125 (4.0)	3.9 (0.008)	595	0.5 (0.34)	970	−92
+serum/FAC	170 (70)	28 (2.9)	14 (7.8)	198	2.1 (0.12)	81	

Using a low-inoculum assay, IFNγ/TNFα/calcitriol-treated monocyte monolayers, with and without FAC, in tissue culture wells containing porous transwell inserts, were infected with *M. tuberculosis*. The identical inoculum was added to transwell inserts porous to solutes but not to bacteria. This initial inoculum in the supernate and the transwell was recorded as CFU at day 0. At days 2, 7, and 14, CFU from the monocyte monolayer, supernate, and transwell from each group were assessed, in addition to monocyte number in the monolayers, and monocyte viability. Data are expressed as total CFU (monolayer and supernate), and monolayer CFU corrected to 10^5 monocytes. The percent change in monolayer CFU/10^5 monocytes with and without iron is also recorded. Data are the mean±SEM of duplicate determinations of three separate experiments. *MN*, monocyte. Mean viability D0: −serum 98%, −serum/FAC 94%, +serum 98%, +serum/FAC 96%; mean viability D2: −serum 96%, −serum/FAC 93%, +serum 97%, +serum/FAC 98%; mean viability D7: −serum 96%, −serum/FAC 94%, +serum 95%, +serum/FAC 98%; mean viability D14: −serum 58%, −serum/FAC 86%, +serum 70%, +serum/FAC 97%.

fected monocytes into the supernate did not account for the decrease in *M. tuberculosis* CFU seen with iron treatment (Table I).

Uptake of M. tuberculosis by CytD-treated monocyte monolayers is similar in the presence or absence of iron. To ensure that the observed differences in *M. tuberculosis* growth between CytD-treated monolayers with and without iron were not due to differences in bacterial uptake between these two groups, the number of monocyte-associated bacteria after a standard inoculum was assessed by two methods. Using the low-inoculum assay (Table I), 2 d after infection, the majority of the starting inoculum had become associated with CytD-treated monocyte monolayers in the presence or absence of iron, with minimal loss of viable CFU. In addition, an assay was performed in which a high inoculum of bacteria was added to CytD-treated monocyte monolayers in the presence or absence of iron, and washed away after 1 h (Table II). Again, there was no significant difference in uptake as assessed by CFU or the percentage of infected monocytes. This result indicates that the iron-suppressive effect on *M. tuberculosis* growth is not due to differences in initial uptake of bacteria. In addition, the findings from both these assays argue against the difference between these groups being due to oxidative metabolites released by iron-treated, activated monocytes, which would be expected to have a bactericidal effect upon first encountering the bacteria.

The suppressive effect of iron on M. tuberculosis growth in CytD-treated monocytes requires preincubation with TNFα. We next sought to determine whether the permissive effect of CytD on *M. tuberculosis* growth was due to IFNγ, TNFα, or calcitriol, and whether IFNγ, TNFα, or calcitriol individually could enable monocytes to restrict *M. tuberculosis* in the presence of iron (Fig. 3). Both IFNγ and calcitriol individually had

Table II. Uptake of M. tuberculosis by Untreated and Iron-treated Monocytes Is Identical

Treatment	CFU (10^3)	MN No. (10^4)	CFU (10^3)/10^5 MN	% MN with MTB
−FAC	9.95 (1.2)	5.6 (0.6)	17	31 (7)
+FAC	12.0 (1.1)	5.9 (4.6)	20	31 (6)

Using a high-inoculum assay, IFNγ/TNFα/calcitriol-treated monocyte monolayers, with and without FAC, were cultured on glass coverslips. After incubation with *M. tuberculosis* for 1 h, monolayers were washed, fixed, and stained for acid fast bacilli (AFB). Replicate, infected monolayers were assessed for viability, monocyte number, and *M. tuberculosis* CFU. Shown are the CFU and monocyte number for each treatment group, the number of CFU corrected to 10^5 monocytes, and the percentage of monocytes containing one or more bacillus. *MN*, monocyte; MTB, M. tuberculosis.

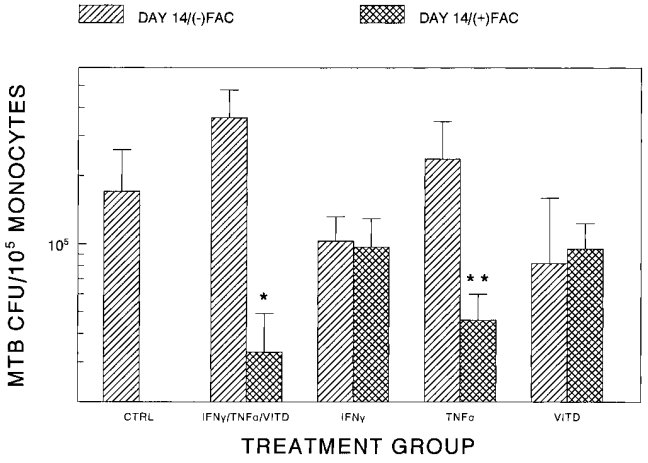

Figure 3. The restrictive effect of iron on *M. tuberculosis* growth in CytD-treated monocytes requires preincubation with TNFα. Untreated monocytes (*CTRL*), CytD-treated monocytes, IFNγ-treated monocytes, TNFα-treated monocytes, and calcitriol-treated monocytes, in the presence of 1% NHS, with and without iron, were infected with *M. tuberculosis* using a low-inoculum assay. There was a statistically significant difference ($P < 0.05$) in CFU with and without iron in the CytD-treated group*, and in the TNFα-treated group**. Data are the mean±SEM of duplicate determinations from three separate experiments using monocytes from different donors.

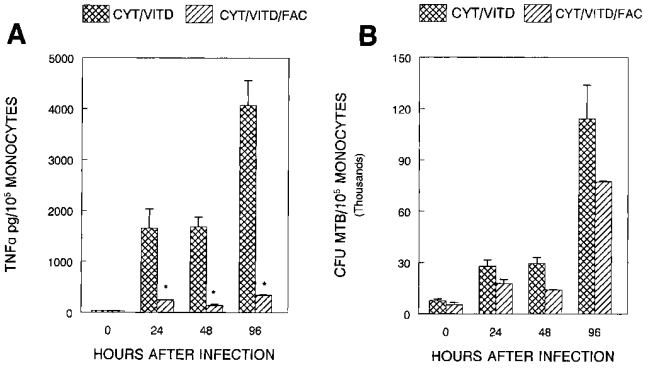

Figure 4. TNFα in the supernate of activated monocytes is markedly decreased in the presence of iron, and this decrease is not accounted for by differences in *M. tuberculosis* uptake. (*A*) Using a high-inoculum assay, CytD-treated monocytes, in the absence of serum, with or without iron in the form of FAC (50 μg/ml), were infected with *M. tuberculosis.* TNFα levels in supernates were determined by ELISA immediately after infection, and at 24, 48, and 96 h. There was a statistically significant decrease in TNFα supernate levels in the group receiving iron at 24, 48, and 96 h, $P < 0.01$ (*B*) In the same experiment, monocyte-associated CFU were determined at the same time that supernates were collected for ELISA determination. There was no statistically significant difference in CFU between the two groups. Data are the mean±SD for duplicate determinations.

modest inhibitory effects on *M. tuberculosis* growth in monocytes relative to control monocytes, a result which was unaffected by iron. In contrast, *M. tuberculosis* growth in CytD-treated and TNFα-treated monocytes was similar to control monocytes, and iron resulted in marked restriction of bacterial growth. These results indicate that TNFα has a permissive effect on *M. tuberculosis* growth, however, preincubation with TNFα is required for the iron-suppressive effect to occur.

TNFα secretion by monocytes in response to M. tuberculosis is markedly downregulated by iron, and this difference is not accounted for by differences in M. tuberculosis uptake. Since TNFα has a permissive effect on *M. tuberculosis* growth, iron might be influencing TNFα release from infected monocytes. To examine this potential interaction, the effect of iron on TNFα levels in infected, CytD-treated monocyte supernates was determined (Fig. 4). Monocytes were preincubated with IFNγ, TNFα, and calcitriol, and infected with *M. tuberculosis.* 24 h later, the monolayers were washed, the supernates were harvested (time 0), and IFNγ and calcitriol were added back. After an additional 24 h, the supernates were harvested and assayed for TNFα. TNFα levels paralleled CFU over 96 h in monocytes not receiving iron. Iron treatment resulted in a marked reduction in TNFα levels at 24, 48, and 96 h relative to monocyte monolayers not receiving iron (Fig. 4 *A*). The difference in TNFα levels between the two groups was statistically significant, and was not accounted for by differences in CFU (Fig. 4 *B*). Monocytes in both groups were 95–98% viable at all time points during the experiment. In subsequent experiments, 48 h was chosen as the time point for measurement of TNFα in cell supernates. Duplicate determinations of monocyte supernates at 48 h from four subjects showed nonactivated supernates to have qualitatively similar results as in Fig. 4 *A* (data not shown). When activated monocytes were treated with varying amounts of iron and incubated with *M. tuberculosis*, a TNFα dose–response to iron was noted in supernates collected at 48 h (Fig. 5). To confirm that the reduction in TNFα determined by ELISA was in a functionally active form of the molecule, an L929 cytotoxicity assay was performed. With this assay there was a 94% reduction in cytolytic activity of infected monocyte supernates containing 50 μg/ml FAC, an 81% reduction with 5 μg/ml FAC, and a 43% reduction with 0.5 μg/ml FAC, suggesting that these differences were in functionally active TNFα.

The results of these experiments suggest that the suppressive effect of iron on *M. tuberculosis* multiplication in CytD-

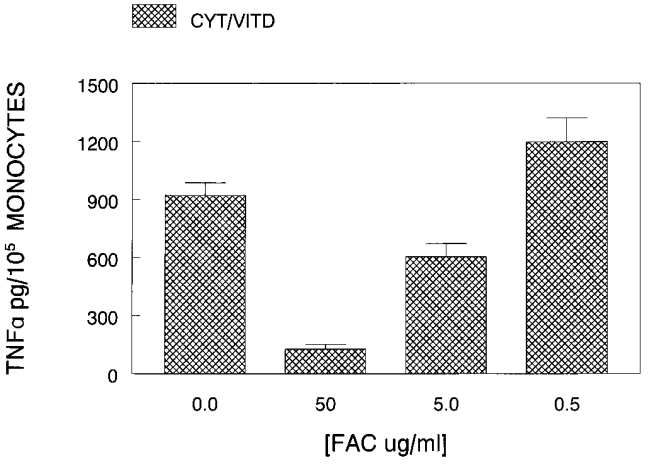

Figure 5. Iron decreases TNFα levels in supernates of *M. tuberculosis*-infected, activated human monocytes in a dose-dependent fashion. CytD-treated human monocytes, in the absence of serum, were incubated with varying concentrations of FAC, and were infected with *M. tuberculosis* using a high-inoculum assay. TNFα levels in supernates were determined by ELISA at 48 h after infection. Data are the mean±SD for duplicate determinations.

activated monocytes may be in part due to suppression of TNFα release from infected monocytes. Since nonactivated monocytes, however, do not significantly restrict *M. tuberculosis* growth when treated with iron (Fig. 1), but do release significant amounts of TNFα which is suppressed by iron treatment, TNFα preincubation is also required.

Iron selectively suppresses TNFα release from monocytes. To determine whether iron-mediated suppression of TNFα is specific for TNFα, supernates collected from *M. tuberculosis*–infected monocytes 48 h after infection were assayed for monocyte proinflammatory cytokines (Fig. 6). TNFα levels were increased in control and CytD-treated monocytes infected with *M. tuberculosis*, and TNFα was reduced in these supernates with iron treatment. Reductions in GM- CSF, IL-8, IL-6, and MIP-1α were minimal in response to iron treatment when compared to the reduction in TNFα seen with iron treatment. TNFα levels in supernates of CytD-treated monocytes treated with 50 μg/ml of FAC were significantly decreased by 87% compared to CytD-treated monocytes not receiving iron. Iron treatment also resulted in decreased IL-1β in monocyte supernates (74% in CytD-treated monocytes receiving iron relative to CytD-treated monocytes without iron). It is noteworthy that IL-1β has many overlapping functions with TNFα, including regulation of cellular iron metabolism (14, 31).

Removal of TNFα from cytokine/calcitriol-treated monocytes 2 d after infection with M. tuberculosis mimics the suppressive effect of iron. TNFα preincubation is required for iron-mediated suppression of *M. tuberculosis* growth (Fig. 3). TNFα, however, is also a permissive factor for growth of *M. tuberculosis.* In Fig. 1, exogenously added TNFα is present throughout the experiments. Thus, iron-mediated restriction of *M. tuberculosis* growth in this assay must involve factors in addition to iron-mediated suppression of TNFα release. One potential explanation for these findings is that iron may decrease monocyte responsiveness to the TNFα signal (exogenously added TNFα). To further examine the role of TNFα in monocyte permissiveness for *M. tuberculosis* growth, and the role of TNFα in iron-mediated suppression of *M. tuberculosis*, monocytes were preincubated for 2 d with IFNγ/TNFα/calcitriol, and were then infected with *M. tuberculosis* using the low-inoculum assay (Fig. 7). After 2 d, the monocyte monolayers were washed. Wells then received (*a*) IFNγ/TNFα/calcitriol, (*b*) IFNγ/calcitriol, or (*c*) IFNγ/TNFα/calcitriol with an amount of anti-TNFα polyclonal antibody to neutralize exogenously added TNFα. Wells which received IFNγ/TNFα/calcitriol (*a*) throughout, remained permissive to *M. tuberculosis* growth, whereas monolayers from which TNFα was later removed (*b*), and monolayers to which TNFα was added back along with a neutralizing amount of anti-TNFα polyclonal antibody (*c*), restricted growth of *M. tuberculosis.* This difference was statistically significant, and was of a similar magnitude to that seen with iron treatment. These data provide additional

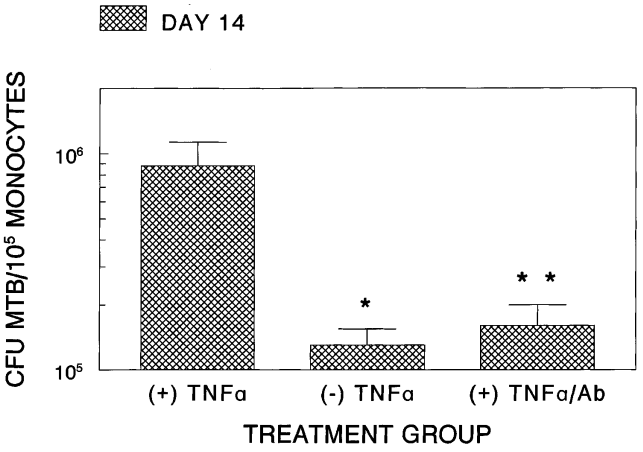

Figure 7. Removal of TNFα from CytD-treated monocytes 2 d after infection with *M. tuberculosis* mimics the restrictive effect of iron. Monocytes were preincubated with IFNγ/TNFα/calcitriol in 1% NHS followed by infection with *M. tuberculosis* using a low-inoculum assay. After 2 d, the monolayers were washed, and IFNγ/TNFα/calcitriol was added to one group, IFNγ/calcitriol to another, and IFNγ/TNFα/calcitriol with polyclonal anti-TNFα antibody to another, all in the presence of 1% NHS. There was a statistically significant difference between the IFNγ/TNFα/calcitriol group compared to the IFNγ/calcitriol group *, and the group receiving IFNγ/TNFα/calcitriol with polyclonal anti-TNFα antibody ** ($P < 0.05$). Data are the mean±SEM of duplicate determinations from three separate experiments using monocytes from different donors.

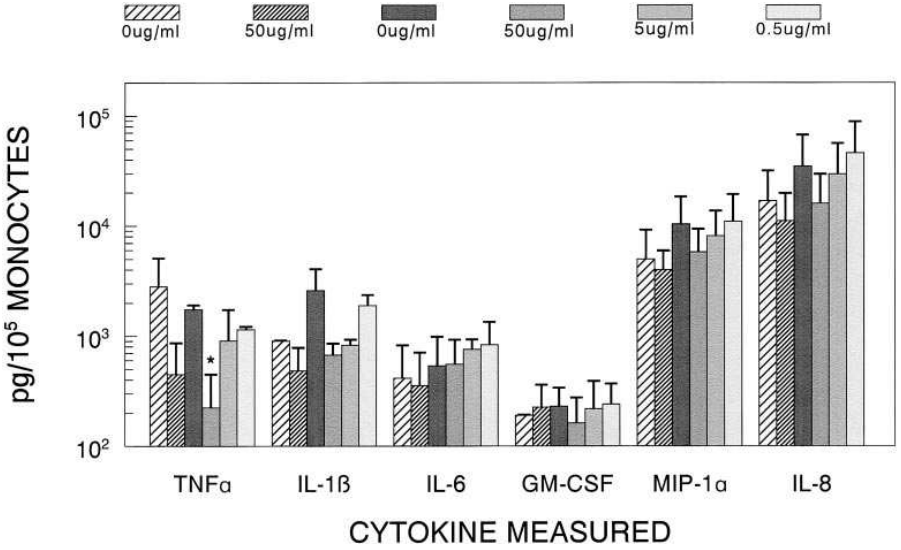

Figure 6. Iron selectively decreases TNFα levels in supernates of *M. tuberculosis*–infected human monocytes. Control monocytes (*hatched bars*), and CytD-treated monocytes (*shaded bars*), in the absence of serum, were incubated with varying concentrations of FAC, and were infected using a high-inoculum assay. Levels of TNFα, IL-1β, GM-CSF, IL-8, MIP-1α, and IL-6 in supernates were determined by ELISA 48 h after infection. There was a statistically significant difference ($P < 0.05$) in TNFα levels between the CytD group treated with 50 μg/ml of FAC compared to the CytD group receiving no FAC. Data are the mean±SEM of duplicate determinations from two separate experiments using monocytes from different donors.

evidence that TNFα, interacting with monocytes throughout the course of *M. tuberculosis* infection, promotes the growth of this pathogen in human monocyte monolayers. The finding that removal of exogenously added TNFα after infection results in *M. tuberculosis* growth suppression comparable to that seen with iron treatment suggests that in addition to suppressing *M. tuberculosis* growth by downregulating TNFα release, iron may be acting to decrease monocyte responsiveness to TNFα.

CytD-treated monocytes form large cellular aggregates and aggregation is suppressed by iron. It was noted that inhibition of growth of *M. tuberculosis* in CytD-treated monocyte monolayers using the low-inoculum assay was accompanied by striking changes in monocyte monolayer morphology (Fig. 8). In the monocyte morphology assay, 14-d-old CytD-treated monocyte monolayers without iron showed numerous infected monocyte aggregates (Fig. 8 *A*). These aggregates developed between 7 and 14 d after infection. Uninfected monocytes near aggregates were oriented with their long axes in the direction of the aggregates, suggesting a chemotactic gradient. Acid fast staining demonstrated cords of *M. tuberculosis* in the aggregates, and in cells at points of contact with the aggregates (Fig. 8, *B* and *C*). This raised the possibility that cell-to-cell spread of virulent *M. tuberculosis* might be facilitated by monocyte aggregation. In contrast, CytD-treated monocytes in the presence of iron showed uniform cell morphology with only rare, small aggregates (Fig. 8 *D*). In addition, the morphology of CytD-treated monolayers from which TNFα was removed after infection (Fig. 7) also showed markedly diminished monocyte aggregation along with less growth, supporting the possibility that the iron-restrictive effect is due to decreased monocyte aggregation. Since TNFα promotes aggregation of monocytes (32) and has monocyte chemotactic effects (33), iron-mediated suppression of TNFα release along with decreased monocyte sensitivity to exogenous TNFα may be the cause of decreased monocyte aggregation with resultant decreased cell-to-cell spread of *M. tuberculosis.* Thus, the suppressive effect of iron on *M. tuberculosis* growth may be related to suppression of monocyte aggregation.

Discussion

In this study, human monocytes were treated with IFNγ, TNFα, and calcitriol (CytD) because of the important role each of these agents is felt to play in the pathogenesis of *M. tuberculosis* infection in vivo (10, 11). Since these monocyte-activating agents also play an important role in granuloma formation (34), the culture conditions in this study may approximate conditions present in vivo within granulomas developing in response to *M. tuberculosis* infection. From this perspective, important effects of IFNγ, TNFα, and calcitriol on mononuclear phagocytes relevant to this model include upregulation of intercellular adhesion molecules (35) and increased cellular aggregation/fusion (32, 36). In addition, each of these agents has been reported to have effects on mononuclear phagocyte iron metabolism (1, 13, 14, 37), a finding consistent with data suggesting that iron plays a role in granuloma formation (18).

The results of this study specifically shed light on the role of TNFα and iron in human tuberculosis. The data indicate that CytD-treated monocytes are permissive for the growth of

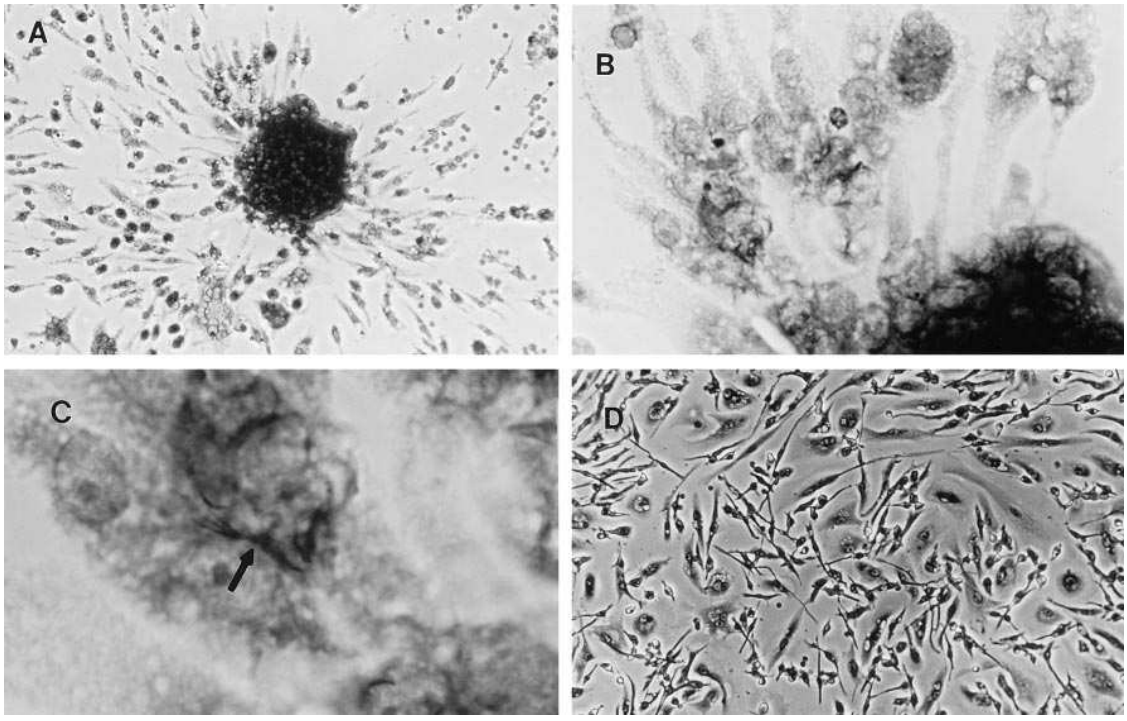

Figure 8. M. tuberculosis–infected, CytD-treated human monocytes form aggregates, and aggregation is suppressed by iron. CytD-treated monolayers were infected using the monocyte morphology assay. (*A*) Day 14 aggregate of CytD-treated monocytes. ×100. (*B*) Higher magnification of *A* showing *M. tuberculosis* within monocytes at points of contact with central aggregate. ×400. (*C*) Higher magnification of *B* showing *M. tuberculosis* cord in monocyte contiguous with central aggregate. ×1,000. (*D*) Day 14 monolayer of CytD-treated monocytes in the presence of FAC (50 μg/ml). ×100.

virulent *M. tuberculosis* in human monocyte monolayers, that this permissiveness is due to TNFα, and that iron enables TNFα-pretreated monocytes to restrict virulent *M. tuberculosis* growth. The accumulation of iron in mononuclear phagocytes decreases TNFα release from infected monocytes, thereby modulating the *M. tuberculosis* growth-promoting effect of TNFα. In addition, iron may be acting to decrease monocyte responsiveness to TNFα. In support of this idea, even though TNFα is present throughout the course of infection in our assays, in the presence of iron, *M. tuberculosis* growth is restricted. Furthermore, the iron-suppressive effect can be duplicated without iron by removing TNFα shortly after infection. This finding suggests that as iron accumulates in monocytes, sensitivity to the TNFα signal decreases. Downregulation of cellular responsiveness to cytokine signaling with iron treatment has been described with IFNγ (38). The effect of iron on monocyte responsiveness to TNFα is currently under investigation.

This study demonstrates that iron selectively suppresses TNFα release from CytD-treated, *M. tuberculosis*–infected human monocytes. In addition to secreting large amounts of functionally active TNFα, activated monocytes infected with *M. tuberculosis* also secrete the proinflammatory cytokines IL-6, IL-8, MIP-1α, and GM-CSF. The amount of TNFα released by iron-treated, CytD-activated monocytes in response to *M. tuberculosis* was significantly decreased by a mean of 87% relative to CytD-activated monocytes not receiving iron. Of the other inflammatory cytokines measured, there was a comparable decrease in IL-1β secretion in CytD-treated monocytes receiving iron relative to those without iron. Iron had relatively little effect on the secretion of other cytokines by *M. tuberculosis*–infected monocytes. These results suggest a heretofore unreported negative feedback loop for regulation of TNFα secretion; release of TNFα promotes uptake and sequestration of iron by mononuclear phagocytes, which then downregulates further TNFα secretion. The relatively selective downregulation of TNFα secretion by monocytes treated with iron might also be accompanied by decreased expression of TNFα receptors. Such a phenomenon would make sense from the point of view of the monocyte which is intimately involved in iron homeostasis. TNFα increases iron storage within cells. If large amounts of iron begin to accumulate in monocytes, these cells would need to decrease iron storage to avoid toxic consequences; downregulating the ability to sense TNFα stimulation would be one such mechanism. Recent clinical data indirectly support our finding that intracellular iron can influence TNFα release by human mononuclear phagocytes. It has been found that monocytes from patients with iron deficiency anemia secrete more TNFα in response to lipopolysaccharide than monocytes from normal control patients (39).

The finding of this paper—that iron downregulates TNFα release from *M. tuberculosis*–infected monocytes—has important implications for cellular iron metabolism and infectious diseases in general. Iron, specifically iron lactoferrin released by PMN at sites of infection, may be an important regulator of TNFα release, and hence of *M. tuberculosis* growth in human monocytes. PMN have been found at sites of *M. tuberculosis* infection early in the course of human disease (19). In addition to having direct effects on *M. tuberculosis* (40), our study suggests that PMN may indirectly influence *M. tuberculosis* infection by providing iron lactoferrin and suppressing TNFα release, thereby modulating *M. tuberculosis* growth.

The findings of our paper are consistent with previously reported results indicating a minimal effect of cytokine activation on the ability of human monocytes to restrict growth of virulent *M. tuberculosis* (41, 42). In contrast to our results which show a permissive effect of these cytokines with calcitriol against *M. tuberculosis* in the presence or absence of serum, one study has reported that IFNγ, TNFα, and calcitriol promoted a rapid bactericidal effect against *M. tuberculosis* in a monocyte tissue culture assay (43). Similar to our results, another laboratory was unable to duplicate this finding (44). The discrepancy may be due to methodologic differences. In the study showing a bactericidal effect, viability of the monocyte monolayers was not determined in spite of an extremely heavy initial inoculum which remained in contact with the monolayers for an extended period of time.

In contrast to our findings correlating elevated TNFα levels with growth of virulent *M. tuberculosis* in monocyte monolayers, one study has shown that TNFα release by human alveolar macrophages is correlated with restriction of growth of the attenuated *M. tuberculosis* strain H37Ra (45). Although this difference may be due to inherent differences between human monocytes and alveolar macrophages, this discrepancy may also be due to differences between the virulent *M. tuberculosis* Erdman strain used in our study, and the attenuated *M. tuberculosis* H37Ra strain used by these investigators.

Although IFNγ-induced iron restriction by human monocytes has been reported to inhibit the multiplication of the bacterial pathogens *L. pneumophila* (1) and *Ehrlichia chaffeensis* (7), the results of this study indicate that iron is not limiting to *M. tuberculosis* growth in IFNγ-activated monocytes. This result is likely due to the fact that *M. tuberculosis* has a relatively low requirement for iron to grow—1 μM when compared to the 20-μM iron concentration required by *L. pneumophila* (46). In addition, unlike *L. pneumophila*, *M. tuberculosis* synthesizes iron-chelating compounds known as exochelins to aid in iron acquisition (47). Along this line, iron has been found to have paradoxical effects on intracellular growth when comparing a variety of intracellular bacterial pathogens. *L. pneumophila* and *E. chaffeensis* are sensitive to intracellular iron deprivation, and as a consequence, alterations in human monocyte iron metabolism induced by IFNγ will influence their growth (1, 2, 7). In a fashion similar to *L. pneumophila*, intracellular iron dependence in mononuclear phagocytes has been demonstrated for *Francisella tularensis* (3, 8), and iron has been found to have a permissive effect on the intracellular growth of *Mycobacterium avium intracellulare* (5). In contrast, iron has been found to have both positive and negative effects on intracellular growth of *Listeria monocytogenes* depending on intracellular iron concentrations, and a negative effect on intracellular growth of *Brucella abortus.* In the case of *L. monocytogenes*, the mechanism of the negative effect was not elucidated (4). In the case of *B. abortus*, evidence was presented suggesting a role for reactive oxygen intermediates, particularly the hydroxyl radical (6). Although iron restricts *L. monocytogenes* and *B. abortus* growth in mononuclear phagoctyes, both of these studies were done with murine macrophages so that the mechanisms may not be the same as those occurring with *M. tuberculosis* infection of human mononuclear phagocytes. Against a role for the hydroxyl radical in this study is the finding that over the initial 4–5 d after infection, CFU are not significantly different among groups, and that effects become manifest beginning at days 5–7. It would be expected that kill-

ing via oxidative metabolites would be an early event concomitant with the initial encounter and uptake of the bacteria by monocytes. In addition, the hydroxyl radical scavenger mannitol over a range of concentrations had no effect on the ability of iron to restrict the growth of *M. tuberculosis* in activated monocytes. Finally, and perhaps most importantly, the iron-suppressive effect can be duplicated in the absence of iron by removing exogenously added TNFα after infection.

TNFα is an important activator of macrophage functions against a variety of intracellular pathogens, but it is a permissive factor for the replication of HIV 1 (48). Our results suggest that it may be a permissive factor for virulent *M. tuberculosis* as well. This result has particular relevance to disease caused by these pathogens since they often coexist, and, individually, both HIV 1 disease and mycobacterial infection have been associated with elevated serum TNFα levels (49, 50). A recent study using thalidomide, an inhibitor of TNFα production by mononuclear phagocytes (51), indicates that decreasing TNFα levels in patients with HIV 1 infection and tuberculosis is associated with a reversal of tuberculosis-induced weight loss, and a concomitant reduction in plasma HIV levels (52). Thus, blocking TNFα may have beneficial effects for both these disease states. Recent data also indicate that suppression of TNFα release in a murine model of *M. tuberculosis* infection correlates with decreased size of mycobacterial granulomas and a lesser mycobacterial burden associated with improved survival, supporting the concept that unregulated TNFα secretion may promote growth of virulent *M. tuberculosis* (53).

Silicosis, another disease state which has long been associated with *M. tuberculosis* infection, is mediated by mononuclear phagocyte TNFα release. Elevated TNFα levels are felt to explain the associated pulmonary fibrosis (TNFα has chemotactic and growth-promoting effects on fibroblasts [54, 55]), and the increased iron found in silicotic areas (56). One study suggests that silica may promote expression of the TNFα gene, an event which might render normal regulatory mechanisms ineffective (57). Unregulated TNFα secretion at local lung sites involved with silicosis might be one explanation for the association between tuberculosis and silicosis.

Although the results of this study indicate that TNFα is a permissive factor for *M. tuberculosis* growth, these results also suggest that TNFα pretreatment is necessary for monocytes to restrict the growth of virulent *M. tuberculosis* in response to iron. The mechanism for this observation is unclear from the present study. Transient exposure to TNFα before infection may allow monocyte differentiation to occur down a path that allows an antimicrobial effect to be exerted in the presence of iron. Because of varying effects of TNFα on monocytes, the continued presence of the TNFα signal might promote certain effects in infected monocytes that would facilitate growth and/or spread of *M. tuberculosis*, i.e., increased death of infected cells, allowing bacteria to escape and infect adjacent monocytes, or upregulation of certain cell surface adhesion ligands promoting cell-to-cell spread. Indeed, in CytD-treated monocytes, there is extensive monocyte aggregation around foci of infection between 7 and 14 d after inoculation. This is also the time period in which the greatest divergence in CFU between iron-treated and untreated monocyte monolayers occurs. These monocyte aggregates contain cords of *M. tuberculosis*, which appear to be traversing cellular boundaries suggesting that aggregation may facilitate cell-to-cell spread of this pathogen. In support of this result, recent data indicate that the ability to spread from cell to cell may be an important virulence determinant of *M. tuberculosis* (58). Thus, exposure to TNFα before infection may be necessary to promote certain beneficial effects against *M. tuberculosis*, but decreased local concentrations of TNFα through iron-mediated downregulation, and/or an iron-mediated decrease in responsiveness to the TNFα signal during infection may be required to prevent other detrimental effects from occurring.

In vivo data support a varying role for TNFα in infection, depending on the stage of the evolving inflammatory response. Data from animal studies have indicated that containment of certain infections involves precise orchestration of cytokines produced by T cells and macrophages over the course of infection. In one study, TNFα levels were downmodulated as progression to a mature granuloma occurred with IFNγ secretion persisting during this period of observation (59). The results from our study suggest a sequence of events which might occur in vivo in the pathogenesis of *M. tuberculosis* in the human host. Initial TNFα release from mononuclear phagocytes infected with *M. tuberculosis* may be involved in the initial priming of adjacent uninfected cells for antituberculous activity, and for granuloma formation. This event would be enhanced by IFNγ from natural killer cells and/or γ/δ T cells, as well as IL-3 from γ/δ T cells arriving early to the site of infection (60, 61). At the same time the early inflammatory lesion, stimulated by TNFα, enlarges along with growth of *M. tuberculosis* within cells, iron from iron lactoferrin (from PMN) and/or iron transferrin (from serum) would begin to accumulate in response to TNFα, TNFα secretion would diminish, and local concentrations would decline. Consistent with the data from this study, the priming with TNFα followed by a decrease in TNFα brought about by accumulating iron would enable the mononuclear phagocytes within such a lesion to restrict/contain *M. tuberculosis.* In support of this hypothesis, iron has been identified in mycobacterial granulomas in the early stages of granuloma formation, and has been correlated with an increased mycobacterial burden (62). As a consequence of decreased TNFα secretion, there would be a decrease in the influx and aggregation of monocytes, thereby modulating the size of the inflammatory lesion. With the arrival of immune lymphocytes, modulation/suppression of TNFα secretion would be taken over by lymphokines such as IL-10 and IL-4 (which have been found to modulate TNFα secretion) (59, 63), and iron sequestration would decrease. The net result would be a stable lesion or granuloma containing decreased amounts of iron. As the granulomas mature, there is a loss of stainable iron (62) in support of this hypothesis. This phase of mycobacterial granuloma formation might be accompanied by continued and enhanced local secretion of IFNγ, and additional lymphokines such as IL-3, which enhance cellular aggregation around foci of infection and promote formation of multinucleated giant cells (64–66). Thus, monocyte differentiation into nonpermissive epithelioid cells and multinucleated giant cells would be facilitated, forming an effective granuloma.

In summary, priming of human monocytes with TNFα is necessary for iron to enable monocytes to restrict the growth of virulent *M. tuberculosis.* Iron, by decreasing subsequent release of TNFα as shown, and possibly by diminishing the responsiveness of monocytes to the TNFα signal, may allow for differentiation of monocytes to occur such that *M. tuberculosis* infection is restricted. The mechanism of this restriction may involve limitation of cell-to-cell spread of *M. tuberculosis*, and

is under investigation in this laboratory. Finally, this study supports a growing body of work indicating that iron may play a varying role in infection caused by intracellular bacterial pathogens.

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