A deletion defining a common Asian lineage of *Mycobacterium tuberculosis* associates with immune subversion

Sandra M. Newton*, Rebecca J. Smith†, Katalin A. Wilkinson*‡, Mark P. Nicol‡, Natalie J. Garton†, Karl J. Staples†, Graham R. Stewart*, John R. Wain*, Adrian R. Martineau*‡, Sarah Fandrich†, Timothy Smallie*, Brian Foxwell*, Ahmed Al-Obaidi†, Jamila Shafi†, Kumar Rajakumar†, Beate Kampmann*‡, Peter W. Andrew†, Loems Ziegler-Heitbrock†, Michael R. Barer†§, and Robert J. Wilkinson*‡§

*Wellcome Trust Center for Research in Clinical Tropical Medicine, Center for Molecular Microbiology and Infection, and Kennedy Institute of Rheumatology, Faculty of Medicine, Imperial College London, London W2 1PG, United Kingdom; †Department of Infection, Immunity, and Inflammation, University of Leicester Medical School, Maurice Shock Building, Leicester LE1 9HN, United Kingdom; and ‡Institute of Infectious Diseases and Molecular Medicine, Faculty of Health Sciences, University of Cape Town, Observatory 7925, South Africa

Edited by Barry R. Bloom, Harvard School of Public Health, Boston, MA, and approved August 24, 2006 (received for review May 24, 2006)

Six major lineages of *Mycobacterium tuberculosis* appear preferentially transmitted amongst distinct ethnic groups. We identified a deletion affecting *Rv1519* in CH, a strain isolated from a large outbreak in Leicester U.K., that coincidentally defines the East African-Indian lineage matching a major ethnic group in this city. In broth media, CH grew less rapidly and was less acidic and H_2O_2-tolerant than reference sequenced strains (CDC1551 and H37Rv). Nevertheless, CH was not impaired in its ability to grow in human monocyte-derived macrophages. When compared with CDC1551 and H37Rv, CH induced less protective IL-12p40 and more antiinflammatory IL-10 and IL-6 gene transcription and secretion from monocyte-derived macrophages. It thus appears that CH compensates microbiological attenuation by skewing the innate response toward phagocyte deactivation. Complementation of *Rv1519*, but none of nine additional genes absent from CH compared with the type strain, H37Rv, reversed the capacity of CH to elicit antiinflammatory IL-10 production by macrophages. The *Rv1519* polymorphism in *M. tuberculosis* confers an immune subverting phenotype that contributes to the persistence and outbreak potential of this lineage.

immunity | innate | polymorphism | virulence

Large sequence polymorphisms (annotated as regions of difference, RD) are common in *Mycobacterium tuberculosis* (MTB), and result in >5% of genes being variably present in clinical isolates (1, 2). Recently, such polymorphisms have been shown to define five of six major lineages of MTB (3), a pathogen that is responsible annually for >2 million deaths. With the exception of RD1, which partially accounts for the attenuation of the vaccine strain *Mycobacterium bovis*-bacillus Calmette–Guérin (4–6), there is no information on the functional or clinical significance of large sequence polymorphisms. If these polymorphisms mark key points in population-specific adaptations of the pathogen, this feature implies they also may have important phenotypic effects. A precedent is provided by a smaller 7-bp polymorphism in the *pks*15/1 gene: The presence of this sequence has been related to the propensity of MTB strain HN878 to produce an immunosuppressive phenolic glycolipid (7).

Outbreaks of tuberculosis present natural "experiments" in which the genotype of the strain responsible may be related to its propensity to cause clinical disease (8–10). In 2001, a large school-associated outbreak of tuberculosis occurred in Leicester, U.K., and both the genotype of the outbreak strain, CH, and the acquired immune responses of the exposed schoolchildren were characterized in unusual detail (10–12). CH was transmitted extensively from a single index case, leading to 254 cases of latent infection by tuberculin skin testing (TST) amongst 1,128 other pupils tested. This degree of transmission was confirmed and extended by a parallel laboratory investigation of transmission based on enzyme-linked immunospot assay analysis (11). Furthermore, 77 cases of active primary tuberculosis were notified within 1 year, 23.3% of the total identified as infected by the TST and, thus, progression to symptomatic disease in this outbreak appeared considerably greater than the usually quoted 5–10% lifetime risk. Of these cases, 17 were culture-positive with the remainder being diagnosed as primary disease by standard clinical criteria (13).

Results and Discussion

We first investigated whether CH possessed any traits demonstrable *in vitro* that might explain its apparent exceptional capacity to cause clinical disease. The growth rate, resistance to H_2O_2, NO, and decreased pH of CH, CAS2 (a second isolate from the outbreak, indistinguishable from CH by microarray and PCR genotyping; ref. 10), and H37Rv (the sequenced type strain) were investigated. CH and CAS2 grew more slowly and showed greater susceptibility to H_2O_2 and decreased pH (Fig. 1); no differences were observed in susceptibility to NO (data not shown).

We next investigated interactions between the above strains and human monocytes and monocyte-derived macrophages (MDMs). Despite the growth-rate differences observed in axenic bacterial culture, CH and CAS2 were shown to replicate as well as H37Rv in both monocyte and MDM cultures (Fig. 2). There was no significant difference in cfu between strains at any time point in either MDMs or monocytes.

Because strain CH was less resistant to H_2O_2 and acid stress in broth media yet exhibited no growth deficit in mononuclear phagocytes, we were interested by the hypothesis that some strains of MTB can skew the innate cytokine response toward a nonprotective phenotype (7). MDMs therefore were cocultured with H37Rv, CH, and CAS2 for 72 h, and the cytokine content of supernatants was determined by ELISA (Fig. 3). The CH and CAS2 strains induced less protective IL-12p40 (CH $P \leq 0.001$ by comparison with H37Rv). By contrast, CH and CAS2 strains

Author contributions: S.M.N., R.J.S., M.P.N., G.R.S., J.R.W., J.S., P.W.A., L.Z.-H., M.R.B., and R.J.W. designed research; S.M.N., R.J.S., K.A.W., M.P.N., N.J.G., K.J.S., J.R.W., A.R.M., S.F., A.A.-O., B.K., and R.J.W. performed research; R.J.S., N.J.G., G.R.S., T.S., B.F., L.Z.-H., and R.J.W. contributed new reagents/analytic tools; S.M.N., M.P.N., K.J.S., L.Z.-H., M.R.B., and R.J.W. analyzed data; and K.R., L.Z.-H., M.R.B., and R.J.W. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS direct submission.

Abbreviations: MDM, monocyte-derived macrophage; MTB, *Mycobacterium tuberculosis*.

§To whom correspondence may be addressed. E-mail: mrb19@le.ac.uk or r.j.wilkinson@imperial.ac.uk.

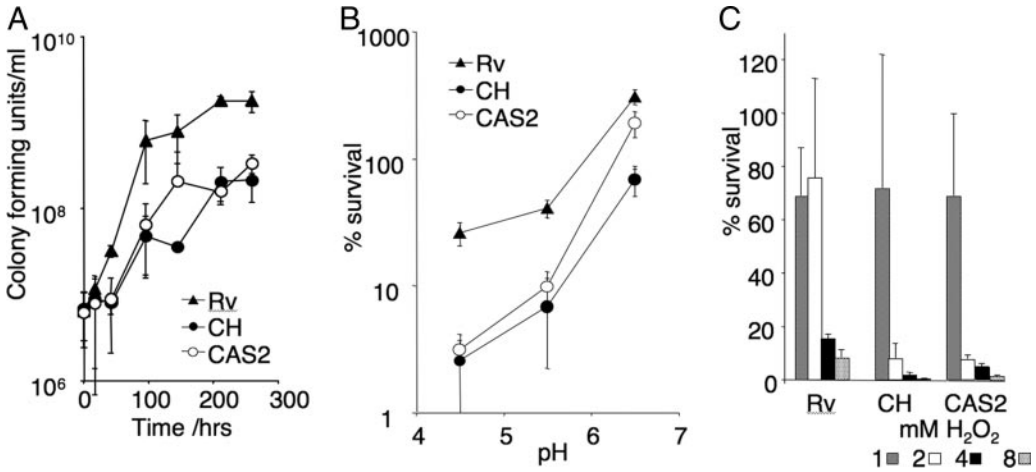

Fig. 1. Phenotype of strains in broth media. (*A*) Multiple replicates of 5×10^6 bacilli were set up in 7H9 media and cultured with shaking for 260 h. Aliquots were withdrawn at intervals and plated for cfu analysis. The growth rate of strains CH and CAS2 and H37Rv was significantly lower by 42 h ($P \leq 0.004$) and remained significantly so at all subsequent time points. Plateau cfu were lower for both CH (2.14×10^8 per ml) and CAS2 (3.36×10^8 per ml) when compared with both H37Rv (1.79×10^9 per ml, $P \leq 0.001$). (*B*) Equal inocula of bacilli were set up in Sauton's media adjusted to pH 4.5, 5.5, and 6.5. Bacilli were grown with shaking for 96 h and then plated for cfu analysis. The average results of three separate experiments conducted in duplicate are shown, with data adjusted to show percentage survival of the inoculum. Strains CH and CAS2 were significantly less able to resist acidic conditions than H37Rv ($P \leq 0.005$). (*C*) Replicates of 1×10^6 bacilli were set up in 7H9 media without catalase in the presence of 0, 1, 2, 4, or 8 mM H_2O_2. Cultures were incubated for 90 min followed by plating for cfu analysis. Results were normalized to percentage survival by comparison with the culture that did not contain H_2O_2. Strains CH and CAS2 were significantly less resistant in a dose-dependent manner to 2, 4, and 8 mM H_2O_2 than H37Rv ($P \leq 0.03$).

induced more antiinflammatory IL-6 ($P \leq 0.05$) and IL-10 (CH, $P = 0.09$; CAS2, $P = 0.05$) from MDMs than H37Rv, they also induced more IL-1β ($P \leq 0.002$). We were interested to confirm whether the tendency to greater antiinflammatory IL-10 and lower proinflammatory IL-12p40 secretion induced by CH and CAS2 was reflected at the transcriptional level. The 24-h levels of IL-10 and IL-12p40 mRNA in MDMs from three donors stimulated with live strains (multiplicity of infection 1:1) was investigated by quantitative RT-PCR. The level of IL-10 RNA was higher, and IL-12p40 lower, in cultures stimulated by CH and CAS2 such that the ratio of IL-10/IL-12p40 mRNA was >1 log higher for both CH and CAS2 when compared with H37Rv (Fig. 4*A*). To confirm greater transcriptional activation of the IL-10 promoter by CH, three-day MDMs were infected overnight with the pAdT-IL-10.wt-luc adenovirus carrying the −195 IL-10 promoter in front of a luciferase reporter gene. Cells were stimulated with heat-killed bacteria, and luciferase activity was determined after 6 h. Heat killed CH-induced higher promoter activity compared with H37Rv ($P < 0.05$; Fig. 4*B*). A considerable body of evidence indicates that IL-10 diminishes macrophage microbicidal capacity (14–17). In summary, therefore, infection with CH and CAS2 were associated with a pattern of cytokine secretion that would result in phagocyte deactivation favoring the intracellular survival of bacilli.

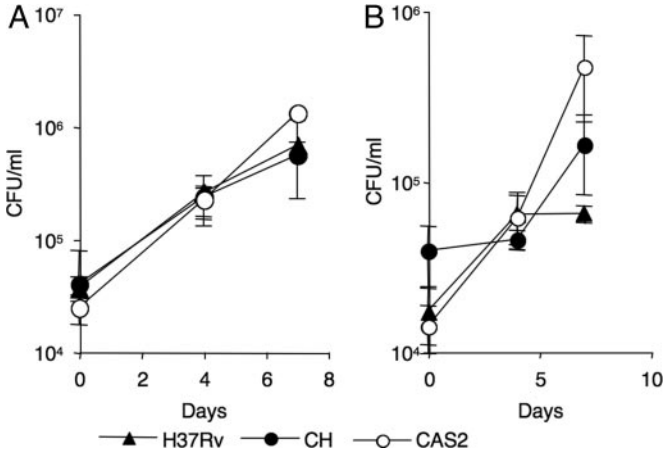

Fig. 2. Growth of strains within human mononuclear phagocytes. (*A*) Monocytes from three donors were infected in duplicate wells at 1:1 (Bacillus:cell). Nonphagocytosed bacilli were washed off after 4 h. Supernatants were discarded, and the cellular layer disrupted at 0, 4, and 7 d and plated for cfu analysis. The strains did not significantly differ in their ability to grow in monocytes. (*B*) MDMs from the same three donors were similarly infected. Although strain CAS2 grew slightly better, this difference was not statistically significant from either H37Rv or CH at 7 d. Error bars show SE.

A different pattern of down-regulation of the human cytokine response has been described for HN878, a Beijing strain (7). Although the behavior of HN878 in human populations has not been fully reported, this strain exhibits hypervirulence in the murine model. This attribute is linked to the presence of an intact *pks*15/1 gene that, by contrast, contains a 7-bp deletion in H37Rv that disrupts its function (18). The presence of an intact *pks*15/1 gene is associated with the production of an immunosuppressive phenolic glycolipid (PGL). Sequencing showed that the *pks*15/1 gene of CH was intact. However, analysis of the lipid content of CH did not reveal PGL (data not shown). It is known that the presence of an intact *pks*15/1 gene is not the only requirement for PGL synthesis and that many genes are involved in complex lipid synthesis (19, 20). Another commonly used method to compare strains relates to their behavior in such immunocompetent, immunocompromised, and transgenic mice. Experiments could be undertaken to gain greater mechanistic insight into the immunopathogenesis of infection by CH.

Initially focusing on its outbreak potential, we hypothesized that the immunomodulatory phenotype of CH might be a consequence of interruption of one of the 10 ORFs affected by large sequence polymorphisms in this strain (10). We therefore complemented these lesions in trans by cloning the complete sequence of the deleted genes into pSMT3 to bring the genes under the control of the constitutive *hsp60* promoter. Genes were cloned individually with the exception of the tandem genes *Rv3019c-3020c*, *Rv3516–3517*, and *Rv3738c-3739c*, which were reintroduced into CH as pairs. Seven clones were selected

MICROBIOLOGY

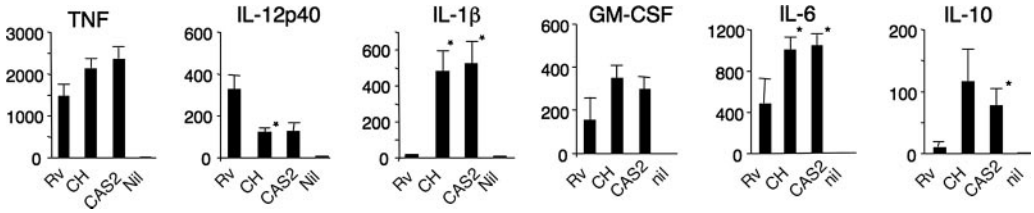

Fig. 3. The cytokine response of MDMs to strains of MTB. MDMs were matured from seven donors and cocultured at a multiplicity of infection of 1:1 with strains for 72 h. At the end of the culture, supernatants were aspirated, and cytokine content was determined by ELISA. The CH and CAS2 strains induced less protective IL-12p40 than H37Rv (CH, $P = 0.009$; CAS2, $P = 0.007$). By contrast, CH and CAS2 strains induced more antiinflammatory IL-6 ($P \leq 0.05$) and IL-10 (CH, $P = 0.09$; CAS2, $P = 0.05$) from MDMs than H37Rv, and they also induced more IL-1β ($P \leq 0.002$). *, significantly different from H37Rv ($P \leq 0.05$). *x* axis units are picograms per milliliter in all cases. Error bars show SE.

in which expression of the introduced genes in CH equaled or exceeded that of H37Rv (Table 1, which is published as supporting information on the PNAS web site).

MDMs from seven new donors were cocultured with the recombinant and WT strains. Strain CH again induced significantly more IL-10 ($P = 0.016$) than H37Rv. The introduction of *Rv1519* led to a complete reversal of the IL-10 stimulatory phenotype to that of H37Rv in all donors ($P = 0.016$ by comparison with CH) (Fig. 4*C*) but had no significant effect on the secretion of IL-12p40 or IL-1β (data not shown). We therefore concluded the reduction of IL-10 secretion attendant on the introduction of *Rv1519* was not mediated via increased IL-12p40. To confirm the effect of introducing *Rv1519* on transcription, 3-day MDMs infected with the pAdT-IL-10.wt-luc adenovirus were treated with heat-killed MTB samples as described above. IL-10 promoter activity was greater in cultures stimulated with heat-killed CH than in those stimulated with CH::Rv1519 ($P < 0.05$) (Fig. 4*D*).

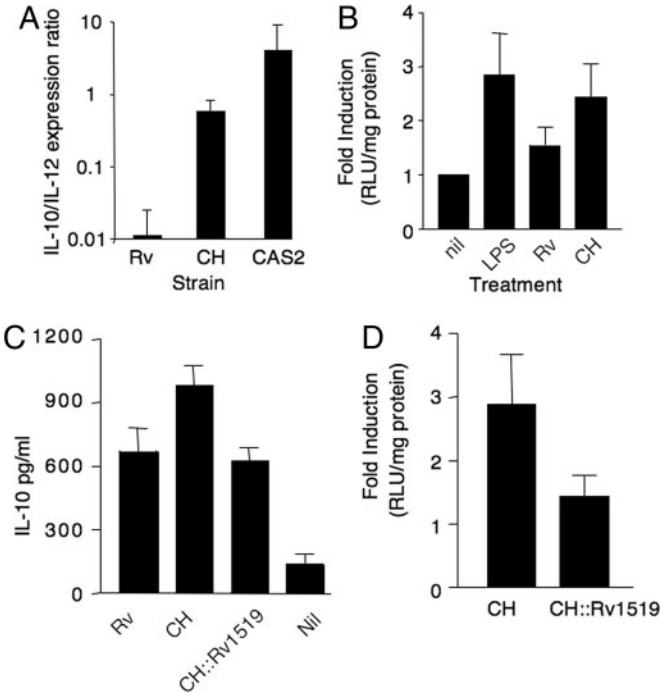

Fig. 4. Transcription of the IL-10 and IL-12p40 genes and effect of introduction of deleted genes into CH on IL-10 secretion and promoter activity. (*A*) The 24-h expression of IL-10 and IL-12p40 in MDMs expressed as a ratio of IL-10 to IL-12p40 mRNA from three donors. The level of IL-10 mRNA was higher and IL-12p40 lower in cultures stimulated by CH and CAS2 such that the ratio was >1 log higher for both strains when compared with H37Rv or CDC1551. (*B*) Three-day MDMs infected with the pAdT-IL-10.wt-luc adenovirus were treated with heat-killed MTB samples. LPS (100 ng/ml) was used as a positive control. Data were normalized to protein concentration and are expressed as means ± SE of six independent experiments. IL-10 promoter activity was greater in cultures stimulated with heat-killed CH than in those stimulated with H37Rv ($P < 0.05$). (*C*) MDMs from seven donors were cocultured with strains. Strain CH induced significantly more IL-10 ($P = 0.0156$) than H37Rv. Introduction of *Rv1519* reversed this phenotype to that of H37Rv in all donors ($P = 0.016$ by comparison with CH). (*D*) Three-day MDMs infected with the pAdT-IL-10.wt-luc adenovirus were treated with heat-killed MTB samples. Data were normalized to protein concentration and are expressed as means ± SE of six independent experiments. IL-10 promoter activity was greater in cultures stimulated with heat-killed CH than in those stimulated with CH::*Rv1519* ($P < 0.05$)

In attempting to explain the clinical properties of an outbreak strain, we appear to have identified a polymorphism in the MTB genome that is significant in marking the emergence of a separate lineage and causing an immunologically significant change in phenotype. Given that the East African-Indian (other synonyms being Delhi or Central Asian) lineage to which CH belongs are distinct from Beijing in phylogenies (3, 22), this finding also raises the intriguing possibility of convergent evolution toward similar phenotypes in the MTB complex. The noteworthy and important difference in this case is that a deletion appears to have increased, rather than decreased, the capacity of this lineage of MTB to cause immune deviation and contribute to its persistence and outbreak potential in human populations.

Materials and Methods

Bacterial Strains and Growth Conditions. All strains of MTB (clinical isolates and recombinants) were grown at 37°C in a shaking

incubator to mid-log phase in Middlebrook 7H9 broth (Difco, Detroit, MI) containing 0.2% glycerol, 0.05% Tween 80, and 10% ADC enrichment. Multiple vials were prepared in 15% glycerol and stored at −80°C until use. A new vial was defrosted before each experimental procedure. The cfu content of vials was determined by serial dilution and plating on Middlebrook 7H11 agar containing 0.5% glycerol and 10% OADC enrichment. Media were supplemented with 50 μg/ml hygromycin (Roche, Indianapolis, IN) where necessary. *Escherichia coli* TOP10 (Invitrogen, Carlsbad, CA) was grown at 37°C in LB broth and agar containing 50 μg/ml hygromycin and 50 μg/ml kanamycin as appropriate.

Broth Phenotype Assays. ***Growth curve.*** To establish cultures in log phase, aliquots of each strain were thawed and inoculated into an equal volume of 7H9 broth and incubated overnight at 37°C in a shaking incubator (150 rpm). To determine the growth rate, multiple replicates in broth were set up, and cfu enumeration was determined at intervals by serial dilution and plating onto 7H11 agar.

Resistance to reactive oxygen intermediates. Each strain was inoculated into 1 ml of catalase-free 7H9 broth containing 0, 1, 2, 4, and 8 mM H_2O_2 to give a final inoculum of 10^6 cfu/ml. Cultures were incubated at 37°C for 90 min, then serially diluted and plated for cfu quantification onto 7H11 agar. Results were calculated as percentage survival by comparison with cultures that contained no H_2O_2.

Resistance to NO. Strains were inoculated in triplicate into 1 ml of catalase-free 7H9 broth containing 0, 4, or 8 mM acidified $NaNO_2$ (pH 5.4) to give a final inoculum of 10^6 cfu/ml. Cultures were incubated at 37°C for 24 h, followed by serial dilution, and plated on to 7H11 agar for cfu quantification. Percentage survival was determined by reference to acidified cultures that did not contain $NaNO_2$.

Resistance to varying pH. Sauton's media of varying pH (4.5, 5.5, and 6.5) were prepared and inoculated with mid-log phase strains as above to give a final count of $\approx 10^6$ cfu/ml. Cultures were incubated for 4 d at 37°C before serial dilution and plating onto 7H11 agar. The average of four to six estimations was recorded. Percentage survival was determined by comparison with the inoculum.

Isolation, Infection, and Culture of Monocytes and MDMs. Monocytes and MDMs were prepared from healthy blood donors and isolated, characterized, and infected exactly as described in ref. 23. Cells were infected at a multiplicity of infection of 1:1 (bacillus:cell) for 4 h, followed by washing three times in warm RPMI medium 1640 to remove nonphagocytosed bacteria. On average, this procedure led to infection of between 5% and 10% mononuclear phagocytes (data not shown). We have shown that the washing procedure ensures that $\approx$90% of bacilli subsequently recovered from cultures are in the cell lysate rather than supernatant (24). To determine the rate of MTB growth in cells, cfu were determined at time 0, 4, and 7 d after infection; the supernatant was aspirated, and the cell monolayer was lysed for 12 min with sterile H_2O followed by serial dilution and plating onto 7H11 agar. For the estimation of cytokine content, nonphagocytosed bacilli were not washed away from the cells in an attempt to eliminate differences in cytokine production between cell types that could be accounted for by differing initial uptake. After 72 h of incubation at 37°C, culture supernatants (1 ml) were harvested and sterile-filtered for storage at −80°C. Supernatants were analyzed for the presence of IL-1β, TNF-α, IL-12p40, granulocyte–macrophage colony-stimulating factor, IL-10, and IL-6 by ELISA with antibody pairs from R & D Systems (Abingdon, U.K.).

IL-10 Promoter Activity. Peripheral blood mononuclear cells were isolated from heparinized (10 units/ml) blood from healthy donors by centrifugation on Ficoll-Paque (Amersham Pharmacia, Piscataway, NJ) according to the manufacturer's instructions. Cells were resuspended at a density of 1×10^6 cells per ml in RPMI medium 1640 culture supplemented with 2 nM L-glutamine (Life Technologies, Gaithersburg, MD)/200 units/ml penicillin/200 μg/ml streptomycin (Life Technologies)/1–2× nonessential amino acids (Life Technologies)/10 ml/liter OPI supplement (Sigma, St. Louis, MO). This medium was passed through a Gambro U-2000 ultrafiltration column (Gambro Medizintechnik, Planegg-Martinsried, Germany) to deplete contaminating LPS, and this procedure was followed by addition of low LPS FCS to a final concentration of 10% (vol/vol). Cells were cultured in Costar (Cambridge, MA) ultra low attachment microplates for 3 d before any analyses or manipulation. MDMs derived in this fashion expressed statistically significant greater amounts of CD14 (2-fold), CD16 (5-fold), and CD68 (6-fold) compared with freshly isolated peripheral blood mononuclear cells as determined by FACS.

For analysis of promoter activity in primary MDMs, we cloned the 195-bp fragment of the human IL-10 promoter together with the luciferase reporter gene into the pAdTrack vector (Quantum Appligene, Illkirch, France) to generate pAdT-IL-10.wt-luc. MDMs were infected with this adenovirus at a multiplicity of infection of 100:1 for 2 h in serum-free media. After addition of serum to 10% (vol/vol), peripheral blood mononuclear cells were cultured overnight before being resuspended in fresh, serum-supplemented media. Cells were replated at 2.5×10^5 cells in 1 ml of media onto a 24-well ultralow attachment plate. Heat-killed MTB were diluted by using serum-free cell culture media to 1×10^8 cfu/ml and 10 μl of each MTB sample added to the appropriate wells. *Salmonella abortus equii* LPS (100 ng/ml) was used as a positive control. Cells were incubated at 37°C in 5% CO_2 for 6 h before being harvested and resuspended in 1× reporter lysis buffer (Promega, Madison, WI). Luciferase activity in cell lysates was determined by using a Sirius model luminometer (Berthold, Wildbad, Germany) and the Luciferase Assay System (E1500) from Promega. Protein concentrations were determined by using Bio-Rad (Hemel Hempstead, U.K.) protein assay reagents. Data were normalized to protein concentration and expressed as mean ± SE of six independent experiments.

RNA Isolation and Quantitation of mRNA Expression By Using Real-Time RT-PCR. RNA was extracted by chaotropic lysis and reverse-transcribed to cDNA by established protocols in ref. 23. Quantitative PCR for IL-10, IL-12p40, and β-actin was performed on the ABI Prism 7000 platform. Primers and probes were obtained as predeveloped assay reagents (Applied Biosystems, Foster City, CA). Each reaction was normalized to β-actin content, and fold induction over unstimulated samples was calculated by the $\Delta\Delta C_T$ method as described (user bulletin no. 2, available for download from www.appliedbiosystems.com).

Construction of Recombinant Strains of CH. ORF were amplified from MTB H37Rv DNA by using the oligonucleotide primers shown in Table 2, which is published as supporting information on the PNAS web site. PCR amplification was performed in a total reaction volume of 50 μl containing a 500 nM concentration of each primer (Sigma Genosys, Haverhill, U.K.), a 250 μM concentration of each dNTP (Qiagen), 1× PCR buffer containing 15 mM $MgCl_2$ (Roche), 1× Solution Q (Qiagen), and 3.5 units of High Fidelity *Taq* polymerase (Roche). Reaction conditions consisted of one cycle at 95°C for 5 min, then 30–40 cycles at 94°C for 30 s, 55°C for 30 s, and up to 3 min of elongation at 72°C depending on GC content and product length. Products were separated on 1% (wt/vol)

agarose gels and purified by using a DNAace quick clean kit (Bioline, Randolph, MA). The products encoding Rv3019c/3020c, Rv3516/3517, and Rv3738c/3739c were subcloned into the PCR-blunt II TOPO vector (Invitrogen), and transformants were selected on LB agar. Plasmids were isolated by using the QIAprep Spin Miniprep kit (Qiagen), and the selected ORF were excised from the TOPO cloning vector by restriction digestion at 37°C overnight with BamHI and ScaI (Rv3019/3020 and Rv3738/3739) and ScaI and BgIII (Rv3516/3517). PCR products of Rv0180, Rv1519, Rv1995, and Rv1996 were restriction-digested by using ClaI and HindIII (Rv1995) or BamHI/PstSI (Rv0180, Rv1519, and Rv1996). All digested products were ultimately cloned into BamHI, or BamHI and EcoRV were linearized pSMT3 by using a rapid ligation kit (Roche). The pSMT3 vector carries a pUC19 backbone with a pAL5000-based mycobacterial origin of replication and bears hygromycin resistance. Genes inserted into this vector are downstream of a constitutive mycobacterial promoter (Hsp60 and Rv0440). Approximately 4 μl of each of the seven plasmids was individually electroporated into competent log phase CH cells, and transformants were selected on 7H11 agar containing 50 μg/ml hygromycin.

RNA Extraction and Characterization of Recombinant CH Strains by Quantitative RT-PCR. The extraction of MTB RNA from strains grown in 7H9 broth was performed as described in ref. 25. Reverse transcription was carried out for 1 h at 37°C by using 0.1 μg/μl random primers (Promega), 0.28 unit/μl AMV-RT (ABgene, Epsom, U.K.) in the supplier's reaction buffer, and a 500 μM concentration of each dNTP (Qiagen). A duplicate control sample with no RT enzyme also was included to ensure lack of DNA contamination of the RNA samples. To assess MTB cDNA levels, quantitative PCR was performed by using TaqMan probes. The PCR primer and probe sequences are shown in Table 3, which is published as supporting information on the PNAS web site. With the exception of 16S rRNA (5′ VIC) all probes were labeled with 5 carbofluorescein at the 5′ end and *N*,*N*,*N*′-tetramethyl-6-carborhodamine at the 3′ end. In this way, every sample underwent duplex PCR with 16S rRNA as an internal control. For PCR, 2.5 μl of cDNA was assayed in a total reaction volume of 25 μl containing TaqMan Master Mix (Applied Biosystems). For 16S rRNA, the concentration of forward and reverse primers was 300 nM, and the 16S rRNA probe concentration was 100 nM. The concentration of all other forward and reverse primers was 900 nM, and the probe concentration was 200 nM. Reaction conditions consisted of one cycle of 50°C for 2 min, 1 cycle of 95°C for 10 min, and 40 cycles of 95°C for 20 s, followed by annealing and elongation at 60°C for 60–80 s. The cycle threshold (C_T) for each sample was compared with known amounts of standard mycobacterial genomic DNA to generate a copy number. Results were calculated as ratios normalized to the 16S cDNA content.

Marcela Simsova (Institute of Microbiology of the Czech Academy of Sciences, Prague, Czech Republic) is thanked for producing recombinant Rv1519 protein. This work was supported by grants from the Wellcome Trust, the Medical Research Council (U.K.), and MediSearch (Leicester, U.K.), and by Biotechnology and Biological Sciences Research Council Grant 91/C19230.

1. Tsolaki AG, Hirsh AE, DeRiemer K, Enciso JA, Wong MZ, Hannan M, Goguet de la Salmoniere YO, Aman K, Kato-Maeda M, Small PM (2004) *Proc Natl Acad Sci USA* 101:4865–4870.
2. Hirsh AE, Tsolaki AG, DeRiemer K, Feldman MW, Small PM (2004) *Proc Natl Acad Sci USA* 101:4871–4876.
3. Gagneux S, Deriemer K, Van T, Kato-Maeda M, de Jong BC, Narayanan S, Nicol M, Niemann S, Kremer K, Gutierrez MC, *et al.* (2006) *Proc Natl Acad Sci USA* 103:2869–2873.
4. Behr MA, Wilson MA, Gill WP, Salamon H, Schoolnik GK, Rane S, Small PM (1999) *Science* 284:1520–1523.
5. Pym AS, Brodin P, Majlessi L, Brosch R, Demangel C, Williams A, Griffiths KE, Marchal G, Leclerc C, Cole ST (2003) *Nat Med* 9:533–539.
6. Hsu T, Hingley-Wilson SM, Chen B, Chen M, Dai AZ, Morin PM, Marks CB, Padiyar J, Goulding C, Gingery M, *et al.* (2003) *Proc Natl Acad Sci USA* 100:12420–12425.
7. Reed MB, Domenech P, Manca C, Su H, Barczak AK, Kreiswirth BN, Kaplan G, Barry CE, III (2004) *Nature* 431:84–87.
8. Valway SE, Sanchez MP, Shinnick TF, Orme I, Agerton T, Hoy D, Jones JS, Westmoreland H, Onorato IM (1998) *N Engl J Med* 338:633–639.
9. Zhang M, Gong J, Yang Z, Samten B, Cave MD, Barnes PF (1999) *J Infect Dis* 179:1213–1217.
10. Rajakumar K, Shafi J, Smith RJ, Stabler RA, Andrew PW, Modha D, Bryant G, Monk P, Hinds J, Butcher PD, Barer MR (2004) *J Clin Microbiol* 42:1890–1896.
11. Ewer K, Deeks J, Alvarez L, Bryant G, Waller S, Andersen P, Monk P, Lalvani A (2003) *Lancet* 361:1168–1173.
12. Yesilkaya H, Forbes KJ, Shafi J, Smith R, Dale JW, Rajakumar K, Barer MR, Andrew PW (2006) *Tuberculosis (Edinb)* 86:357–362.
13. British Thoracic Society (2000) *Thorax* 55:887–901.
14. Flesch IE, Hess JH, Oswald IP, Kaufmann SH (1994) *Int Immunol* 6:693–700.
15. Sieling PA, Abrams JS, Yamamura M, Salgame P, Bloom BR, Rea TH, Modlin RL (1993) *J Immunol* 150:5501–5510.
16. Turner J, Gonzalez-Juarrero M, Ellis DL, Basaraba RJ, Kipnis A, Orme IM, Cooper AM (2002) *J Immunol* 169:6343–6351.
17. Sendide K, Deghmane AE, Pechkovsky D, Av-Gay Y, Talal A, Hmama Z (2005) *J Immunol* 175:5324–5332.
18. Constant P, Perez E, Malaga W, Laneelle MA, Saurel O, Daffe M, Guilhot C (2002) *J Biol Chem* 277:38148–38158.
19. Minnikin DE, Kremer L, Dover LG, Besra GS (2002) *Chem Biol* 9:545–553.
20. Perez E, Constant P, Laval F, Lemassu A, Laneelle MA, Daffe M, Guilhot C (2004) *J Biol Chem* 279:42584–42592.
21. Gascoyne-Binzi DM, Barlow RE, Essex A, Gelletlie R, Khan MA, Hafiz S, Collyns TA, Frizzell R, Hawkey PM (2002) *Int J Tuberc Lung Dis* 6:492–496.
22. Baker L, Brown T, Maiden MC, Drobniewski F (2004) *Emerg Infect Dis* 10:1568–1577.
23. Wilkinson KA, Stewart GR, Newton SM, Vordermeier HM, Wain JR, Murphy HN, Horner K, Young DB, Wilkinson RJ (2005) *J Immunol* 174:4237–4243.
24. Wilkinson RJ, Patel P, Llewelyn M, Hirsch CS, Pasvol G, Snounou G, Davidson RN, Toossi Z (1999) *J Exp Med* 189:1863–1874.
25. Wilkinson RJ, DesJardin LE, Islam N, Gibson BM, Kanost RA, Wilkinson KA, Poelman D, Eisenach KD, Toossi Z (2001) *Mol Microbiol* 39:813–821.