Increased pathology in lungs of mice after infection with an α -crystallin mutant of *Mycobacterium tuberculosis*: changes in cathepsin proteases and certain cytokines

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Latency and reactivation are a significant problem that contributes to the incidence, transmission and pathogenesis of tuberculosis. The mechanisms involved in these processes, at the level of both the bacillus and the host, are poorly understood. In *Mycobacterium tuberculosis* the α -crystallin (*acr*) gene has been linked to latency, because it is highly expressed during hypoxic growth conditions. Deletion of the *acr* gene in *M. tuberculosis* H37Rv (Δ *acr* strain) was previously shown to reduce the intracellular growth of bacilli in macrophages; however, its impact on pathogenesis *in vivo* was unknown. This study demonstrated that infection of C57BL6 mice with Δ *acr* results in lung bacillary loads 1–2 log units higher in comparison to parental H37Rv. Haematoxylin/eosin staining of lungs revealed exacerbated pathology characterized by extensive obliteration of alveolar air spaces by granulomatous inflammation. RT-PCR analysis and immunostaining of lungs showed that infection with either H37Rv or Δ *acr* results in the differential expression of lysosomal cathepsin proteases. A slight increase in the expression of the matrix-degrading acidic-type cathepsins B, D and H was noted in Δ *acr*-infected mice and was associated with clusters of macrophages within lung granulomas. Δ *acr*-infected mice also showed high serum levels of TNF- α , IFN- γ and G-CSF, suggesting that *Acr* may play a role in modulating the host response to infection.

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INTRODUCTION

Tuberculosis (TB) is the leading cause of death worldwide due to infectious disease, with an estimated 3 million deaths per year (Bloom & Murray, 1992; Dye *et al.*, 1999). *Mycobacterium tuberculosis* infection occurs when aerosol-droplet nuclei containing a small number of bacilli are deposited in the alveoli of the lung and subsequently phagocytosed by alveolar macrophages. The bacteria replicate within macrophages, inducing the release of pro-inflammatory cytokines, which can lead to the formation of caseating granulomas, tissue destruction, liquefaction and cavity formation (Dannenberg & Rook, 1994). A major concern during TB pathogenesis is the potential of the bacillus to persist in human tissues for long periods in a clinically latent or dormant state without causing any overt disease symptoms. Live bacilli have been isolated from granulomas or tubercles in the lungs of patients with clinically inactive TB (Opie &

Aronson, 1927; Robertson, 1933). It is generally thought that most *M. tuberculosis*-infected individuals have clinically latent infections, since they give a positive tuberculin skin test, but do not present with clinical symptoms and are not contagious (Flynn & Chan, 2001). It is estimated that in 5–10 % of latently infected persons the infection will reactivate and cause active TB disease (Selwyn *et al.*, 1989). Therefore, understanding latency and reactivation, at the level of both the bacillus and the host, is extremely important in the control of this disease.

Some evidence suggests that latency, or the ability of bacilli to remain dormant, is due to reduced growth and/or metabolism, and can be induced by growth under hypoxic conditions within the host. *M. tuberculosis* infections are known to occur at the most oxygen-rich sites of the body (e.g. the upper lobes of the lung; Adler & Rose, 1996), while inhibition of bacillary growth *in vivo* is associated with the formation of hypoxic fibrous granulomas (Dannenberg, 1993). *In vitro* growth of bacilli under a variety of stress conditions such as by ageing growing cultures, or with

Abbreviations: GAPDH, glyceraldehyde-3-phosphate dehydrogenase; TB, tuberculosis.

limited oxygen, results in decreased metabolic activity and growth (Wayne & Diaz, 1967; Wayne & Hayes, 1996). Based on these observations, investigators have utilized hypoxic culture conditions to generate non-replicating but persistent mycobacteria as an *in vitro* model of latency (Imboden & Schoolnik, 1998; Wayne & Diaz, 1967; Wayne & Hayes, 1996; Yuan *et al.*, 1998). These studies have facilitated the identification of mycobacterial factors that may confer *in vivo* growth and persistence advantages upon the pathogen. However, it has been difficult to establish the significance of these factors to latency and reactivation in the host.

In vitro growth of *M. tuberculosis* under hypoxic conditions results in the upregulation of a 16 kDa α -crystallin (Acr) homologue, encoded by the *acr* gene (*hspX*, Rv2031). Acr protein is almost undetectable during exponential growth of *M. tuberculosis*, but is strongly induced in old and stationary-phase cultures (Yuan *et al.*, 1996). It is considered a dominant antigen since antibodies are present in sera from most patients with pulmonary TB examined (Lee *et al.*, 1992; Verbon *et al.*, 1992). Acr belongs to a family of small heat-shock proteins that act as ATP-independent chaperones, and localize to the inner side of the cell membrane (Cunningham & Spreadbury, 1998). In vertebrates, Acr plays an important role in maintaining the transparency of the eye (Groenen *et al.*, 1994; Horwitz, 1992); however, its role in *M. tuberculosis* has not been defined. Disruption of the *acr* gene in H37Rv was shown to not affect infectivity or survival in macrophages during early infection, but growth of the mutant was significantly impaired in both mouse bone-marrow-derived macrophages and THP-1 monocytes (Yuan *et al.*, 1998). However, there is little information on the role of Acr *in vivo*. The present study was designed to determine the infectivity and pathogenicity of the Δ acr mutant in the C57BL6 mouse infection model. We demonstrate that, in comparison to the parental wild-type strain H37Rv, infection of mice with Δ acr results in higher bacillary burdens in the lung, exacerbated lung pathology, elevated expression of pro-inflammatory cytokines, and a slightly increased expression of lysosomal cathepsin proteases. We postulate that Acr in *M. tuberculosis* bacilli is an important modulator of the host response to infection.

METHODS

Bacterial cultures and growth conditions. The *M. tuberculosis* mutant strain Δ acr::hpt (denoted as Δ acr) was obtained from Dr Clifton E. Barry, III (Tuberculosis Research Section, NIH, Rockville, MD, USA). Δ acr was generated by insertion of a hygromycin-resistance cassette by allelic exchange in the H37Rv strain that replaced the 1 kb *acr* gene (Yuan *et al.*, 1998). The parental strain H37Rv (ATCC 27294) was obtained from The American Type Culture Collection (Rockville, MD, USA). Mycobacteria were grown in Middlebrook 7H9 broth supplemented with OADC enrichment (Difco) and containing 1% (v/v) glycerol. Δ acr was grown in 7H9/OADC broth plus hygromycin (50 μ g ml⁻¹). Cultures were grown at 37 °C with slow shaking to mid-exponential growth phase (7–10 days) and bacterial clumps disrupted by repeated passage through syringes with 21, 25 and 27 gauge needles. Numbers of bacilli in the inoculum were determined by measuring OD₆₀₀ and

using a linear regression equation generated from an OD₆₀₀ vs c.f.u. curve previously generated. Bacterial counts were determined by serial dilution of cultures in 7H9 medium, plating in triplicate on 7H11/OADC agar plates, and enumeration of c.f.u. after 3 weeks incubation at 37 °C.

Mouse infections and necropsies. Eight-week-old female C57BL6 mice (Charles River Labs) free of common pathogens were used for these experiments. Mice (nine per group) were infected by inoculation in the tail vein with 0.2 ml (1 × 10⁶ bacilli) of a freshly grown suspension of *M. tuberculosis* H37Rv or the Δ acr strain. Mice were humanely killed at weeks 2, 4 and 6 post-infection (three infected and two normal per time point). Blood was obtained by cardiac puncture and serum was separated. The lungs were removed, rinsed in sterile PBS, and the five lobes (one left, four right) divided as follows: (1) upper left lobe for bacillary load determinations, (2) lower left lobe for histopathology, and (3) all right lobes for RNA isolation and RT-PCR. All mice were kept in microisolator cages in a BSL3 facility and their health status monitored daily. Mice were humanely killed if they showed signs of pain or distress before the end point. All of the protocols were approved by the Emory University and Atlanta VA Institutional Animal Care and Use Committee.

Cytokine analysis. Cytokine analysis of serum samples was done by three different methods: (1) Bioplex multiplex bead immunoassay (Bio-Rad), (2) BD Cytometric Bead Array (BD Biosciences), and (3) Quantikine ELISA (R&D Systems).

(1) Multiplex bead immunoassays were done in filter-bottom ELISA plates using the Bio-Rad Mouse 18-Plex panel kit and protocols for cytokines: IL-1 α , IL-1 β , IL-2, IL-3, IL-4, IL-5, IL-6, IL-10, IL-12(p40), IL-12(p70), IL-17, G-CSF, IFN- γ , GM-CSF, KC, MIP-1 α , RANTES and TNF- α . Briefly, 50 μ l of a mixture of the 18 anti-cytokine-conjugated bead families was added to a 96-well filter plate prewet with Assay Buffer A. Sera from control and *M. tuberculosis*-infected mice were diluted 1:4 in Mouse Diluent. To the filter plate containing the beads was added 50 μ l diluted serum or serially diluted cytokine standards, followed by 30 min mixing at room temperature, and then at 4 °C overnight. The cytokine-bound beads were washed twice with Wash Buffer A using a filter manifold, and incubated for 1 h at room temperature with 50 μ l biotin-conjugated detection antibodies. Bead-cytokine-antibody complexes were washed twice in Buffer A and incubated for 30 min at room temperature with phycoerythrin-conjugated streptavidin. Complexes were washed twice in Buffer A, resuspended in 125 μ l Assay Buffer A, and cytokine levels were measured in a Bioplex instrument using Bioplex Manager Software (Bio-Rad). Assays were performed in duplicate and cytokine concentrations were reported in pg ml⁻¹.

(2) Determination of cytokines IL-2, IL-4, IL-5, IFN- γ and TNF- α in serum samples was further carried out using the Mouse Th1/Th2 Cytokine Cytometric Bead Array kit and protocols from BD Biosciences. Serum samples (in triplicate) were tested undiluted and diluted at 1:10 and 1:100 in assay buffer, along with serially diluted cytokine standards (20–5000 pg ml⁻¹). In glass test tubes, 50 μ l of the mixed cytokine capture beads was mixed with 50 μ l test sample or standard, then 50 μ l PE Detection reagent (phycoerythrin-labelled anti-mouse IgG) was added and incubated for 2 h at room temperature in the dark. After incubation, the beads were washed in 1 ml PBS wash buffer by centrifugation, resuspended in 300 μ l wash buffer, and analysed in a BD FACS Caliber Flow cytometer after calibration with BD Calibrite beads. Data acquisition and analysis were done with BD CBA software.

(3) Quantification of G-CSF in mouse serum was done by ELISA using the Quantikine kit and protocol from R&D Systems. Serum samples in triplicate (50 μ l, undiluted or diluted 1:10 in diluent buffer), and

the G-CSF standard (serially diluted $14.1\text{--}900\text{ pg ml}^{-1}$ in diluent buffer), were added to microtitre wells precoated with anti-G-CSF antibody, containing $50\text{ }\mu\text{l}$ assay diluent, mixed and incubated at room temperature for 2 h. After incubation, the wells were aspirated, washed ($5\times 400\text{ }\mu\text{l}$ wash buffer) and $100\text{ }\mu\text{l}$ anti-mouse G-CSF-horseradish peroxidase conjugate added for 2 h. After aspiration and washing, $100\text{ }\mu\text{l}$ substrate solution was added and incubated for 30 min in the dark; the reactions were stopped by addition of $100\text{ }\mu\text{l}$ stop solution, and A_{450} was measured in a Molecular Devices ThermoMax reader. G-CSF concentrations were determined based on the standard curve generated by a four parameter logistic (4-PL) curve fit.

C.f.u. determinations. Lung tissue samples ($0.05\text{--}0.1\text{ g}$) were homogenized in 1.0 ml sterile PBS until no tissue clumps were visible. After a brief sonication (10 s pulse), serial dilutions ($1:100$, $1:500$, $1:1000$) were prepared in PBS, and $100\text{ }\mu\text{l}$ aliquots plated in triplicate on 7H10/OADC plates. The plates were sealed and incubated for 3 weeks at 37°C . Colonies were counted and c.f.u. ml^{-1} per g tissue determined. Two colonies from each plate were tested for acid-fast bacilli by Ziehl-Neelsen staining with TB stain kit ZN (Becton Dickinson).

Histopathology and immunohistochemical analysis. The dissected lower left lobes were placed in 4% paraformaldehyde for 2 h, transferred to 10% buffered formalin and stored at 4°C . The tissues were dehydrated, paraffin-embedded, and sectioned in $5\text{ }\mu\text{m}$ increments starting at the pleural surface. Sections were stained with haematoxylin/eosin for histopathological examination. Several sections were stained for acid-fast bacilli by the Ziehl-Neelsen technique.

Morphometric image analysis was done as described by Schacker *et al.* (2002), with multiple digital photomicrographs (Olympus) of sections (three mice per group) at $\times 200$ magnification. Photomicrographs were imported into Photoshop 7.0 (Adobe Systems) and a colour sampler tool was used to gate shades of white, which represent unaffected alveolar spaces. The remaining non-selected areas of the field were removed, the resulting image was loaded into Scion Image Beta 4.0.2 software (Scion Corporation), and the number of occupying pixels was quantified. The mean density and standard deviation were calculated for each section.

Immunostaining of paraffin-embedded lung sections was performed using goat and rabbit polyclonal antibodies directed against mouse CatG, CatB, CatD, CatH and G-CSF at a $1:100$ dilution (San Cruz Biotechnology). Anti-Mac-3 (Biosciences) monoclonal antibody at $1:50$ dilution was used to identify macrophages and monocytes. Biotin-conjugated rabbit anti-goat and goat anti-rabbit secondary antibodies (Vector Labs) were used at $1:200$ dilution. The primary and secondary antibody concentrations were optimized for each application. Immunostaining reactions were visualized by the avidin-biotin complex method employing a Vectastain ABC alkaline phosphatase kit (Vector Labs), and 3,3'-diaminobenzidine as the substrate. The sections were counterstained with haematoxylin and mounted. The specificity of immunostaining was tested by substitution of the primary antibody with normal goat IgG and by preincubation of antibodies with blocking peptides. Whole sections were examined using a conventional microscope at $\times 200\text{--}400$ magnification and digitally photographed.

RT-PCR. Analysis of gene expression levels was done by RT-PCR using manufacturer's reagents and protocols (Promega). RNA was isolated from mouse lungs by extraction in RNeasy and reverse transcribed with gene-specific reverse primers for mouse genes encoding CatG, B, D, H, cystatin C (CysC) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) control. Primers designed to amplify a 300 bp fragment based on GenBank-published sequences were as follows: *catG* (F, ACCCCTACATGGCATTCTTC; R,

ACATTTGGTCCATCTGCACTC), *catB* (F, CTTCCCATGTGCGG-CAATCAGAAG; R, AAGACATCTAGAGTACCCCAAG), *catD* (F, CACGTCCTTTGACATCCACTACG; R, CAGCTCCTTCACCTCTT-CCACAG), GAPDH (F, CAGCCGCATCTTCTTGTG; R, AGGGGG-GCTAAGCAGTTG), *catH* (F, TGCCCAAGCCTTCAACAATCATG; R, AAGTACCCATTCTCCCCCAC TG), *cysC* (F, CTGTGAGCGA-GTACAACAAGG; R, GGAGCACAAGTAAGGAACAG). The first-strand cDNA synthesis reaction was carried out at 42°C for 60 min in a $25\text{ }\mu\text{l}$ reaction mixture consisting of $1.0\text{ }\mu\text{g}$ RNA, $1\text{ }\mu\text{l}/20\text{ }\mu\text{M}$ reverse primer, $5\text{ }\mu\text{l}$ $5\times$ M-MLV buffer, $5\text{ }\mu\text{l}$ dNTP 10 mM mix, $1\text{ }\mu\text{l}/200\text{ U}$ M-MLV reverse transcriptase, 25 U rRNasin ribonuclease inhibitor and nuclease-free H_2O . After cDNA synthesis, $5\text{ }\mu\text{l}$ cDNA was added to $44\text{ }\mu\text{l}$ PCR master mix ($3\text{ }\mu\text{l}$ 25 mM MgCl_2 , $5\text{ }\mu\text{l}$ $10\times$ PCR buffer, $0.2\text{ }\mu\text{l}/1.0\text{ U}$ *Taq* DNA polymerase, $4\text{ }\mu\text{l}$ 10 mM dNTP mix and DEPC-treated water), and $1.0\text{ }\mu\text{l}$ forward and reverse primers ($20\text{ }\mu\text{M}$) was added to each reaction tube. The thermal cycling parameters were: 94°C (5 min), 20 or 30 cycles of 94°C (45 s), 55°C (45 s), 72°C (45 s), and a final extension at 72°C (10 min). The RT-PCR products were separated on 1% (w/v) TBE agarose gels, visualized by staining with ethidium bromide, and the intensity of bands was quantified by densitometry scanning.

Statistical analysis. This consisted of simple descriptive statistical methods such as mean, standard deviation and two-tailed Student's *t* test.

RESULTS

Increased virulence and pathogenicity of the Δ acr knockout strain

As shown in Fig. 1, infection with either the parental strain H37Rv or Δ acr resulted in an exponential increase in bacillary burdens in the lung. An increase in recovered c.f.u. of approximately 0.5 log unit was observed after week 4, while a 1 log unit increase was found after week 6 with both strains. Interestingly, infection with the Δ acr strain resulted in significantly higher lung burdens (by approx. 2 log units) than H37Rv at weeks 2, 4 and 6 post-infection. In other experiments, where c.f.u. were determined using whole lung and spleen homogenates at day 1 (24 h) and week 2

Fig. 1. Bacillary burdens in lungs of C57BL6 mice ($n=3$) after 2, 4 and 6 weeks of infection via tail vein inoculation (1×10^6 bacilli) with *M. tuberculosis* H37Rv or Δ acr. Infection with Δ acr results in a significant increase in lung bacillary c.f.u. in comparison to parental strain H37Rv. *, $P<0.05$ (Student's *t* test) in comparison to H37Rv infected mice.

post-infection, it was found that both H37Rv and Δacr strains gave similar c.f.u. at day 1 (2.6×10^4 and 3×10^4 , respectively), showing that mice received an equal-sized inoculum. However, at week 2 the Δacr strain was at least 1 log unit higher (2.6×10^5) in comparison to H37Rv (3.6×10^4), confirming the increased virulence of Δacr .

We then examined the lungs of mice for differences in pathology generated by the two strains. As shown in Fig. 2(a), in comparison to the normal lung architecture observed in control mice, infection with either H37Rv or Δacr resulted in marked infiltration of inflammatory cells into the alveolar walls, extensive obliteration of alveolar air spaces, and the formation of multifocal coalescing diffuse granulomatous lesions. The pathology was more pronounced in mice infected with the Δacr strain, where very few air spaces remained, and more diffuse granulomatous inflammation was evident.

Morphometric image analysis of lung sections was performed to determine quantitative differences in volume density, which would correlate with differences in granulomatous lung inflammation and fibrosis caused by the two strains. As shown in Fig. 2(b), a significant decrease in lung volume density was observed 2 weeks after infection with both H37Rv and Δacr . Further decreases in volume density were observed 4 and 6 weeks post-infection with both strains; however, the decreases were significantly greater (>2 -fold) in the Δacr -infected mice.

The finding that infection with the Δacr mutant, in comparison to parental wild-type H37Rv, results in higher c.f.u. and a more severe pathology in the lung suggests that the Δacr deletion results in hypervirulence.

Infection with H37Rv and Δacr results in increased expression of cathepsins in the lung

We recently demonstrated that *M. tuberculosis* infection of THP-1 monocytes results in the differential expression of lysosomal cathepsin proteases. CatG, a neutral serine protease, was shown to have tuberculocidal activity and to be downregulated after *M. tuberculosis* infection, while the acidic-type cathepsins (CatB and CatD) were upregulated (Rivera-Marrero *et al.*, 2004). Here, we asked if this would also occur in the lungs of infected mice, and if infection with the hypervirulent Δacr strain would result in altered expression of cathepsins. By RT-PCR analysis we found that a 4 week infection with either H37Rv or Δacr (3 mice each) resulted in a significant downregulation of *catG* mRNA expression, when compared to non-infected controls (Fig. 3). However, the expression of *catD*, *catB* and *catH* mRNA was upregulated after infection, and was slightly higher in mice infected with Δacr . As estimated by densitometry, the expression of *catD*, *catB* and *catH* mRNA in the Δacr -infected mice was 1.2-, 1.1- and 3.0-fold greater, respectively, than in the H37Rv-infected mice. No changes in the expression of the housekeeping GAPDH gene were detected. We also tested for the expression of *cysC*, which

Fig. 2. (a) Haematoxylin/eosin-stained paraffin-embedded lung sections of normal mice and mice infected for 6 weeks with *M. tuberculosis* H37Rv or Δacr . In comparison to the normal lung architecture observed in uninfected mice, infection with either H37Rv or Δacr results in marked infiltration of cells into the alveolar walls, resulting in extensive obliteration of alveolar air spaces and multifocal coalescing diffuse granulomatous lesions. This pathology is more pronounced in Δacr mice. Bar: 100 μ m. (b) Morphometric image analysis of lung sections showing a significant decrease in lung volume density (estimated by pixels) at 2, 4 and 6 weeks post-infection in mice ($n=3$ per group) infected with H37Rv (grey bars) or Δacr (black bars) compared with normal lungs (white bars). The Δacr -infected mice show a 2-fold decrease (weeks 2, 4) in lung volume density in comparison to H37Rv. *, $P < 0.05$ (Student's *t* test) in comparison to normal lung; ^, $P < 0.05$ (Student's *t* test) in comparison to H37Rv-infected lung.

Fig. 3. RT-PCR analysis to determine differences in the expression of cathepsin G, D, B and H genes in the lungs of mice after 4 weeks infection with H37Rv or Δ acr. After infection with either strain, *catG* gene expression is decreased compared with that in normal lungs (N), while the expression of *catD*, *catB* and *catH* is slightly increased, the increase being slightly higher in the mice infected with Δ acr. A small increase in *cysC* expression is seen in the infected lungs with either H37Rv or Δ acr, while no changes were detected in the house-keeping gene GAPDH.

encodes cystatin C, an inhibitor of cathepsin cysteine proteases such as *catB* and *catH*. A small increase (1.2-fold) in *cysC* mRNA expression was seen in the infected lungs, but no differences were detected between H37Rv and Δ acr. These results demonstrate that *M. tuberculosis* infection of mice results in differential gene regulation of cathepsin proteases in lung tissue, with *catG* downregulated and *catB*, *catD* and *catH* upregulated.

We then asked if the differential expression of cathepsin genes observed in total lung tissue was associated with the localization of phagocytic cells in areas of granulomatous inflammation. Lung sections of normal mice vs mice infected with H37Rv or Δ acr were analysed by immunostaining with antibodies directed against mouse CatG, CatB, CatD and CatH. As shown in Fig. 4(a), normal lung tissue showed slight diffuse staining for CatG, mostly in the alveolar epithelium. Lungs infected with H37Rv or Δ acr for

6 weeks showed no CatG staining but only the haematoxylin counterstain. Immunostaining for CatB (Fig. 4a), CatD and CatH (Fig. 4b) was also positive in the normal lung, showing faint staining of the alveolar epithelium. In contrast, very strong staining was observed after infection with either of the *M. tuberculosis* strains. Clusters of cells, presumably macrophages, surrounding areas of granulomatous inflammation showed strong staining for CatB, CatD and CatH, while the control IgG showed no staining. Interestingly, more clusters of cathepsin-expressing cells were observed in lungs from Δ acr-infected mice than those from H37Rv-infected mice.

The identity of these cathepsin-expressing cells was demonstrated by staining for the monocyte and macrophage specific marker Mac-3. Fig. 4(c) shows that the expression of CatD in mouse lung granulomas from Δ acr-infected mice co-localized with macrophages expressing Mac-3. Strong intracellular staining for CatD is associated with macrophages and multinucleate cells within the granulomatous lesions. The expression of CatB and CatH also co-localized with Mac-3-expressing macrophages (not shown).

Taken together, these results demonstrate that *M. tuberculosis* infection results in the differential expression of cathepsin proteases in the lung, particularly in areas of granulomatous inflammation.

Infection with Δ acr results in increased serum levels of TNF- α , IFN- γ and G-CSF

Since the proinflammatory cytokines TNF- α and IFN- γ play such important roles in the pathogenesis of TB, we asked if their expression would be altered in mice infected with the Δ acr strain vs H37Rv. Fig. 5 shows the levels of cytokines TNF- α and IFN- γ in the serum of normal and infected mice after 2, 4 and 6 weeks of infection with H37Rv or Δ acr. An increase in TNF- α (2–3-fold) was detected in mice infected with H37Rv for 2, 4 and 6 weeks, in comparison with control mice. Also, IFN- γ was elevated (3–5-fold) in mice infected for 2 and 4 weeks. Most significantly, infection with the Δ acr strain resulted in a substantial increase in TNF- α and IFN- γ in all mice, in comparison to uninfected and H37Rv-infected mice. The highest levels of TNF- α (120–160 pg ml⁻¹) and IFN- γ (150–170 pg ml⁻¹) were at weeks 2 and 4 post-infection and were at least 2–3-fold (TNF- α) and 3–4-fold (IFN- γ) higher than those in the H37Rv-infected group. These results demonstrate that infection with the Δ acr strain results in an exaggerated induction of proinflammatory cytokines TNF- α and IFN- γ .

Further analysis to differentiate the serum cytokine profile of H37Rv-infected vs Δ acr-infected mice was done by multiplex bead immunoassay. These studies revealed that of the 18 cytokines tested in the assay (see Methods) only G-CSF was significantly different in the Δ acr-infected mice. As shown in Fig. 6, G-CSF was elevated in all of the mice infected with H37Rv, with the most significant increases (1–2-fold) seen in mice infected for 4 and 6 weeks.

Fig. 4. Immunohistochemical staining of *M. tuberculosis*-infected lungs to detect differential expression of cathepsins. Paraffin-embedded lung sections of normal versus mice infected with H37Rv and Δ *acr* for 6 weeks were immunostained for (a) CatG and B, or (b) CatD and H, plus normal mouse IgG control. (a) Normal lung positively stained for CatG shows diffused brownish staining of alveolar epithelial cells, while lungs infected with H37Rv and Δ *acr* show no CatG staining, but only the haematoxylin counterstain. Normal lung also stained mildly for CatB, but very strong staining is observed after infection with either *M. tuberculosis* strain. (b) Normal lung shows mild staining for CatD and CatH, whereas very strong staining is seen after infection with H37Rv or Δ *acr*. Clusters of macrophages surrounding areas of granulomatous inflammation (arrows) are seen positively stained for (a) CatB and (b) CatD and CatH, while control IgG showed no cross-reactivity. (c) CatD expression in mouse lung granulomas from Δ *acr*-infected mice co-localizes with macrophages expressing the macrophage-specific marker Mac-3. Bars: 100 μ m (a, b); 50 μ m (c).

Fig. 5. Levels of TNF- α and IFN- γ in the serum of C57BL/6 mice after 2, 4 and 6 weeks of infection with H37Rv or Δ *acr*, as determined by the Cytokine Cytometric Bead Array method (see Methods). As shown, infection with the Δ *acr* strain results in a marked increase in TNF- α and IFN- γ at weeks 2 and 4 in comparison with normal (N) and H37Rv-infected mice. The results shown are the mean of $n=3$ and the bars denote standard deviations. [^], $P<0.05$ (Student's *t* test) in comparison to uninfected control. ^{*}, $P<0.05$ (Student's *t* test) in comparison to H37Rv-infected mice.

Interestingly, all of the mice infected with Δ *acr* showed increased levels of serum G-CSF, with the most significant increase reaching 530 pg ml⁻¹ (5-fold over control and 3-fold over H37Rv-infected mice) at week 2 post-infection. The levels of G-CSF at week 4 and 6 were also higher, but not statistically different from those observed in the H37Rv-infected group. These results were confirmed by ELISA (data not shown) using the G-CSF Quantikine kit and protocol from R&D Systems.

To determine if the increase in serum G-CSF could be attributed to the differential expression of G-CSF in lung cells, lung sections from H37Rv- and Δ *acr*-infected mice at week 2 post-infection were immunostained for G-CSF. Control lungs exhibited mild staining for G-CSF around the epithelium, while lungs from H37Rv and Δ *acr*-infected mice showed clusters of macrophages, strongly stained for G-CSF (Fig. 7). The Δ *acr*-infected lungs had more cells strongly stained for G-CSF than did the H37Rv-infected lungs. At this early stage of the infection no granulomas were seen but there was much cellular infiltration. Large macrophages with cytoplasmic granules densely stained for G-CSF were visible at the periphery of vessels and the surrounding tissue. These results demonstrate that the increase in serum G-CSF observed in the Δ *acr*-infected mice is due to the increased expression of G-CSF by macrophages in the lung

Fig. 6. Serum levels of G-CSF in C57BL/6 mice after 2, 4 and 6 weeks of infection with H37Rv and Δacr , as determined by the multiplex bead immunoassay (see Methods). Infection of mice with Δacr resulted in increased levels of G-CSF, with the most significant increase reaching 530 pg ml⁻¹ (5-fold over normal, 3-fold over H37Rv-infected mice) at week 2 post-infection. G-CSF at weeks 4 and 6 was also higher in the Δacr group but not statistically different from the H37Rv group. Results are the mean of $n=3$ and bars denote standard deviations. ^, $P<0.05$ (Student's t test) in comparison to uninfected control. *, $P<0.05$ (Student's t test) in comparison to H37Rv-infected mice.

early (at 2 weeks) during the inflammatory response to *M. tuberculosis* infection.

Taken together, these results suggest that the increased pathogenicity observed for the Δacr strain could be due to its ability to induce high levels of pro-inflammatory cytokines (TNF- α , IFN- γ) and growth factors (G-CSF).

DISCUSSION

There is little information on the role of the *acr* gene during the pathogenesis of TB and its implications in latency and

reactivation. The purpose of this study was to determine the effects of the *acr* mutation *in vivo*, with particular attention to its role in infectivity, modulation of the host response, and pathogenicity in the mouse model. First, we demonstrated that intravenous tail infection of C57BL/6 mice with Δacr resulted in exponential multiplication of bacilli with at least a 2 log increase in c.f.u. in lungs after 2, 4 and 6 weeks of infection, in comparison to its parental strain H37Rv. However, in this experiment, we noticed low bacillary loads in lung with both strains, since intravenous inoculation with 10⁶ bacilli typically results in at least a 1 log increase by 2 weeks (10⁵ c.f.u. ml⁻¹ g⁻¹ recovered) and a 2–3 log increase by 4–6 weeks (10⁶–10⁷ c.f.u. ml⁻¹ g⁻¹ recovered) (Copenhaver *et al.*, 2004). We attribute the low numbers in Fig. 1 to the fact that only a small portion of the lung was utilized for c.f.u. counts and therefore the calculation of c.f.u. ml⁻¹ g⁻¹ may not be a true estimation of the bacillary load in the entire lung. Using the entire lung and spleen for c.f.u. counts we showed a bacillary load of >10⁴ at day 1 with both strains, a small increase of >10 000 c.f.u. at week 2 with H37Rv, and a 1 log increase (2.6 × 10⁵) with Δacr . The small increase in c.f.u. obtained with wild-type H37Rv at 2 weeks post-infection after such a large inoculum (10⁶) can be misleading and give the impression that the strain is not growing well. However, similar bacillary loads with H37Rv have been reported by another group in this model (Copenhaver *et al.*, 2004).

Evidence for increased virulence of the Δacr strain also comes from an unpublished study by Smith *et al.* (2000). In that study, C57BL/6 mice were infected via aerosol with 25 c.f.u., and lung bacillary c.f.u. were determined at 1, 2, 4, 6 and 12 weeks. That study also showed an increase in c.f.u. of 1.1–1.2 log units after infection with Δacr . Therefore, based on these studies we can conclude that Δacr has increased virulence in mice equivalent to 1–2 log units in comparison to parental H37Rv. Thus, in contrast to the *in vitro* results obtained by Yuan *et al.* (1998), these *in vivo* studies demonstrate that the *acr* mutation does not impair

Fig. 7. G-CSF immunostaining of representative lung sections of normal vs infected mice after 2 weeks infection with H37Rv or Δacr . As shown, G-CSF is expressed by infiltrating macrophages and is more abundant in the Δacr -infected lung. Bar: 50 μ m.

the growth of *M. tuberculosis* bacilli in mouse tissues during an acute infection but, on the contrary, results in a state of hypervirulence. The observation that *acr* expression is upregulated *in vitro* during hypoxic and stationary-phase growth conditions (Schnappinger *et al.*, 2003; Yuan *et al.*, 1996, 1998), and *in vivo* in mouse (Shi *et al.*, 2003; Timm *et al.*, 2003) and human lungs (Timm *et al.*, 2003), suggests that it is an important gene for survival of bacilli under stress and is possibly part of a genetic programme which allows adaptation to hypoxic microenvironments of the host. This adaptation may involve the shutdown of genes necessary for aerobic metabolic pathways, with the ultimate consequence of entering into a state of non-replicative stasis or latency. A study by whole-genome microarray analysis showed that the expression of more than 100 genes is altered *in vitro* by growth under hypoxic conditions and that many of the repressed genes are involved in aerobic metabolism (Sherman *et al.*, 2001). In addition, many genes were induced under hypoxia, including members of the LuxR two-component response regulators such as Rv3133c, which when disrupted, resulted in the elimination of the hypoxic regulation of *acr* (Sherman *et al.*, 2001). Therefore, the disruption of the *acr* gene may also affect the expression of other metabolic genes associated with growth and survival *in vivo*, allowing bacilli to replicate faster and preventing them from going dormant under stress conditions. Further studies are needed to determine the mechanism that affords the Δ *acr* strain increased bacillary multiplication *in vivo*.

Latency is often associated with decreased bacillary burdens in tissues, and lack of disease symptoms and pathology (Flynn & Chan, 2001). The finding that infection with Δ *acr* resulted in exacerbated lung pathology, in comparison with H37Rv, supports the hypothesis that disruption of *acr* can result in increased virulence and pathogenicity; however, its role in the highly complex events leading to latency and reactivation *in vivo* is not known. Since the *acr* gene has been associated with hypoxia-induced dormancy *in vitro*, events that are reminiscent of latency *in vivo*, we wanted to know if its disruption would have an effect on the host response to infection. To test this, we proceeded to determine the effects of Δ *acr* infection on the host immune and tissue remodelling responses.

We demonstrated by RT-PCR and immunostaining of lungs that *M. tuberculosis* infection with both H37Rv and Δ *acr* strains resulted in the differential expression of cathepsin proteases. The neutral serine protease CatG was downregulated, while the acidic-type cathepsins CatB, D and H were upregulated after infection and their expression associated with macrophages within granulomas. CatB, D and H expression was slightly more elevated in the Δ *acr*-infected mice, suggesting that the increased pathogenicity observed with this strain could be the result of increased protease induction in the lung. In previous studies we have shown that tissue matrix proteases, such as metalloproteinases (Rivera-Marrero *et al.*, 2000, 2002) and cathepsins (Rivera-Marrero *et al.*, 2004), are important in *M. tuberculosis*

infection. Cathepsins are a large family of lysosomal proteases that not only function in intralysosomal protein degradation, but participate in tissue remodelling responses by degrading extracellular matrix proteins. CatB and CatH are cysteine proteases, while CatD is an aspartyl protease (Wolters & Chapman, 2000). In particular, CatD has been shown in mature epithelioid macrophages surrounding the caseous and liquefied areas of pulmonary cavities in *M. tuberculosis*-infected rabbits (Converse *et al.*, 1996). Therefore, our finding that cathepsins (B, D and H) are increased in lung after infection with both *M. tuberculosis* strains, but slightly higher with Δ *acr*, suggests that these proteolytic enzymes are involved in the process of granuloma formation and play an important role in the pathogenesis of *M. tuberculosis*.

The downregulation of CatG in lungs after infection with both the H37Rv and Δ *acr* was an interesting finding in this study. CatG is highly abundant in the azurophilic granules of neutrophils and monocytes (Senior & Campbell, 1984; Senior *et al.*, 1982), and is synthesized during the promyelocytic and promonocytic stages of maturation, respectively (Bainton *et al.*, 1971; van der Meer *et al.*, 1981). In U937 monocytes, treatment with the phorbol ester 12-O-tetradecanoylphorbol 13-acetate (TPA) results in transcriptional downregulation of *catG* (Hanson *et al.*, 1990; Ley *et al.*, 1989; Welgus *et al.*, 1986). Since there is no evidence that *catG* is expressed by alveolar epithelial pneumocytes, it is possible that the CatG detected in the normal lung is derived from circulating monocytes and/or neutrophils. Nonetheless, this is an important finding in view of our recent work showing that the downregulation of *catG* in THP-1 human monocytes after *M. tuberculosis* infection coincided with increased bacillary multiplication in cells and that CatG and its cationic peptide CG117-136 have tuberculocidal activity *in vitro* (Rivera-Marrero *et al.*, 2004). The downregulation of *catG* in lung after infection may be advantageous to *M. tuberculosis* bacilli and represent an important mechanism for evasion of host innate immune defences. However, further studies are needed to fully define the role of CatG in TB pathogenesis.

To explore the mechanisms by which the Δ *acr*-infected mice show increased pathology we determined their cytokine profile. We found that Δ *acr*-infected mice had very high levels of IFN- γ and TNF- α at weeks 2 and 4 post-infection, in comparison to H37Rv-infected mice. It is well documented that both IFN- γ and TNF- α play important roles in the control of a persistent TB infection (Flynn & Chan, 2001). IFN- γ is involved in macrophage activation (Dalton *et al.*, 1993; Flynn *et al.*, 1993) and the production of reactive nitrogen intermediates that can kill intracellular bacilli (Chan *et al.*, 1992). Knockout mice for IFN- γ are highly susceptible to *M. tuberculosis* infection, succumbing to disseminated TB infection (Cooper *et al.*, 1993; Flynn *et al.*, 1993). TNF- α is also very important for the control of *M. tuberculosis*, with effects on macrophage activation, production of reactive nitrogen intermediates, granuloma formation and pathology (Bean *et al.*, 1999; Flynn *et al.*,

1995; Kindler *et al.*, 1989). High levels of TNF- α in lung cause extreme pathology, as shown in a murine model by infection with a recombinant BCG strain that secreted TNF- α at the site of infection (Bekker *et al.*, 2000). However, the effects of TNF- α are also dose-dependent and determine whether the cytokine is protective or destructive. Mice functionally deficient in TNF- α develop fatal acute *M. tuberculosis* infections (Bean *et al.*, 1999; Flynn *et al.*, 1995; Roach *et al.*, 2002), characterized by extensive necrosis in the lungs and infected organs, and failure to form functional granulomas. Although it is widely accepted that IFN- γ and TNF- α have a protective role during active TB infection, their roles in latency and reactivation are not completely understood. Our finding that the Δ *acr* strain causes extreme pathology, which is concomitant with increases in IFN- γ and TNF- α , may suggest that these cytokines are elevated during reactivation as a protective mechanism to prevent disseminated infection. However, their high levels in the lung may also contribute to the exacerbated pathology either directly (Bekker *et al.*, 2000) or via the activation of proteolytic pathways involving the action of cathepsins (Wang *et al.*, 2000).

We also showed that mice infected with Δ *acr* had higher levels of G-CSF in serum than those infected with H37Rv and that this could be attributed to the elevated expression by lung macrophages. G-CSF is involved in the proliferation, survival, maturation and functional activation of cells from the neutrophilic granulocyte lineage (Basu *et al.*, 2002). Serum G-CSF levels rapidly increase in response to bacterial infection and cell-mediated immune responses, at times when granulocyte levels become elevated, suggesting that G-CSF is a crucial regulator of an emergency response involving granulocyte production (Cheers *et al.*, 1988; Demetri & Griffin, 1991; Nicola, 1989). Bacterial products such as endotoxin, or inflammatory cytokines induced during infections, such as TNF, interleukin (IL-1) and IFN- γ , are the major stimulators of G-CSF production *in vivo* and result in a rapid but transient elevation in serum G-CSF levels. G-CSF is produced mainly by haematopoietic cells, such as monocytes/macrophages, and lymphocytes (Nicola *et al.*, 1983; Sallerfors, 1994). Other cells, such as fibroblasts (Kaushansky *et al.*, 1988), endothelial cells (Zsebo *et al.*, 1988), astrocytes (Aloisi *et al.*, 1992) and bone marrow stromal cells (Fibbe *et al.*, 1988), can also produce G-CSF following activation by LPS, IL-1 or TNF- α . Therefore, the rapid increase in G-CSF observed in Δ *acr*-infected mice (at week 2) could be caused directly by the bacillus, or indirectly by the action of cytokines TNF- α and IFN- γ . G-CSF could be involved in triggering a rapid mobilization of granulocytes to sites of granulomatous inflammation in the Δ *acr*-infected mice that results in the exacerbated pathogenesis.

In conclusion, this work provides tantalizing new information about the *in vivo* pathogenicity of the Δ *acr* mutant of *M. tuberculosis*. We demonstrate that the Δ *acr* strain is hypervirulent in mice and causes exacerbated lung pathology, and that this effect could be the result of increased

induction of pro-inflammatory cytokines (TNF- α , IFN- γ , G-CSF) and lysosomal cathepsin proteases (CatB, D, H) in the lung. Future studies are designed to define the molecular mechanisms by which *Acr* affects the pathogenesis of *M. tuberculosis* and its role in latency and reactivation.

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