

# Induction of colony-stimulating factors by a 30-kDa secretory protein of *Mycobacterium tuberculosis* H37Rv

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Accepted for publication September 1, 2004

**ABSTRACT.** Colony-stimulating factors (CSFs)-induced increased hematopoietic activity is known to occur in various microbial diseases; however, not much is known during tuberculosis (TB). We investigated the CSF-inducing capability of a *Mycobacterium tuberculosis* H37Rv component. Swiss mice intravenously injected with purified 30-kDa secretory protein of *M. tuberculosis* H37Rv (Mtb30; 0.1-10 mg/kg) showed enhanced levels of serum CSFs; maximum response ( $142 \pm 16$  colonies) occurred at 1 mg/kg. *In vitro*, Mtb30 (1-50  $\mu\text{g/mL}$ ) induced mouse peritoneal macrophages (PMs) to elaborate CSFs in the conditioned medium (CM); 25  $\mu\text{g/mL}$  appeared optimal ( $97 \pm 11$  colonies). Both *in vivo* and *in vitro*, peak CSF production occurred 24 h after stimulation which levelled-off to background levels by 72 h. Rabbit anti-Mtb30 antibody significantly ( $p < 0.05$ ) reduced CSF production by both Mtb30-stimulated and *M. tuberculosis*-infected PMs, *in vitro*. The induced CSFs, both in the serum and CM, appeared to be functionally similar, as they supported the formation of granulocyte (G), monocyte (M) and GM colonies, in similar proportions; the GM colonies were maximum ( $>79\%$ ). Neutralizing (100%) rabbit anti-mouse interleukin-1 (IL-1) polyclonal antibody did not affect the Mtb30-induced CSF production, indicating it to be IL-1-independent; whereas, CSF production was partly dependent on tumour necrosis factor- $\alpha$  (TNF- $\alpha$ ), as goat anti-mouse TNF- $\alpha$  immunoglobulin G only partly inhibited it. Mtb30-induced PM production of CSFs was *de novo* as it was completely blocked by cycloheximide (50  $\mu\text{g/mL}$ ). The CSF-inducing capability of Mtb30 appeared to be proteinaceous in nature as it was heat (70  $^{\circ}\text{C}$ ; 1 h)-labile, was destroyed by proteases (pronase E and trypsin) and was unaffected by sodium periodate treatment. Further, compared to the controls, Mtb30 induced significantly ( $p < 0.05$ ) high levels of immunoreactive GM-CSF ( $9 \pm 1$  and  $7.5 \pm 0.8$  ng/mL) and M-CSF ( $4.3 \pm 0.5$  and  $3.9 \pm 0.4$  ng/mL) in serum and CM, respectively; G-CSF levels did not increase significantly ( $p > 0.05$ ). Mtb30-treated mice showed a maximum of 2.23- and 2.36-fold increase, in the splenic and femur colony forming unit-GM counts, respectively, as compared to the controls. This is the first report which demonstrates Mtb30-induced production of CSFs that is up-regulated both posttranscriptionally and functionally, and thus adds to our understanding of the molecular pathogenetic mechanisms of TB.

**Keywords:** colony-stimulating factors, *Mycobacterium tuberculosis*, macrophages, 30-kDa secretory protein

## INTRODUCTION

Tuberculosis (TB) is a worldwide health problem and the leading cause of deaths due to infectious diseases (5% of all deaths worldwide) [1]. Presently, about one-third of world's population is infected with *Mycobacterium tuberculosis* (the main etiological agent of human TB), and every year nearly 54 million new cases including 6.8 million clinical cases, and about three million deaths occur [2]. The emergence of multidrug resistant (MDR) strains of *M. tuberculosis* has further compounded the problem, and in many countries  $>15\%$  MDR TB cases occur [3]. However, despite the severity of TB, the molecular mechanisms of pathogenesis and protective immunity continue to remain elusive.

*M. tuberculosis* secretory antigens interact with the host immune system early in the infection, and thus constitute important potential targets of protective immunity in hu-

man TB [4, 5]. The 30-kDa antigen of *M. tuberculosis* (Mtb30) is a major secretory protein [6], and has also been designated independently as  $\alpha$ -antigen [7], antigen  $a_2$  [8], antigen 6 [9], *M. tuberculosis* purified MPT-59 [10], the one having crossed-immuno electrophoresis No. 85B [11], and *M. bovis* purified MPB-59 [12]. In guinea pig [4] and mouse [13] models of TB, Mtb30 has been reported to be the most immunoprotective protein. Therefore, it is considered as one of the candidate, for subunit [5] and DNA [14] anti-TB vaccines. Recombinant BCG vaccine expressing Mtb30 induced greater protection than conventional BCG vaccine [15], and the immunogenicity of BCG vaccine has been reported to be improved by using granulocyte (G)-macrophage (M;  $M\emptyset$ ) colony-stimulating factor (GM-CSF) transgene-based adjunct formulations [16]. The Mtb30-induced production of Th1 cytokines such as tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) [17], interleukin-12 (IL-12) [18] and interferon- $\gamma$  (IFN- $\gamma$ ) [13], and of Th2 cytok-

ines transforming growth factor- $\beta$  (TGF- $\beta$ ) [19] and IL-10 [20] is now well known. However, the Mtb30-induced production of CSFs, an important group of proinflammatory and regulatory cytokines, has apparently not been reported.

The CSFs (mol. wt. 18-90 kDa) are glycoproteins that are characterized by their ability to induce the proliferation and differentiation of the myeloid hemopoietic progenitor cells, *in vitro* [21, 22]; *in vivo* they stimulate hematopoiesis [23]. The lineage specific CSFs i. e. G-CSF and M-CSF, stimulate G and M colony formation, respectively. GM-CSF, on the other hand, supports the formation of colonies consisting mainly of G, M and eosinophils, whereas multi-CSF (IL-3) induces colonies containing cells of different lineages. The genes for human and mouse CSFs have been cloned [24, 25], and large quantities of recombinant CSFs (rCSFs) can now be produced. Structurally, M-CSF is a homodimer, whereas G-, GM- and multi-CSFs consist of a single polypeptide chain. The CSFs are active at picomolar concentrations, and their constitutive levels are very low; however, during infections, the concentrations of CSFs are rapidly elevated [26]. Functionally, CSFs can enhance the effector functions of mature cells of myeloid lineage for example; human monocyte cytotoxicity can be activated by GM-CSF and IL-3 [27]. CSFs are also known to induce the synthesis and secretion of various cytokines such as IFN- $\gamma$ , TNF- $\alpha$  [28] and IL-1 [29], and can indirectly augment the release of IL-2 by stimulating antigen-presenting cells [30]. Because of their immunopotentiating properties, CSFs are known to boost host defence against infections [31-33]. Treatment of human M $\phi$ s with GM-CSF has been shown to enhance their antimycobacterial activity, *in vitro* [34, 35], and rGM-CSF has been reported to protect patients infected with *M. kansasii* [36]. GM-CSF therapy has also been observed to enhance the uptake and mycobactericidal activity of monocytes from AIDS patients with *M. avium* bacteremia [37, 38]. In another study, *M. avium-M. intracellulare*, an opportunistic pathogen, has been shown to stimulate human monocytes and large

granular lymphocytes to produce CSFs [39]. Clinically, augmented production of monocytes and their efflux from bone marrow (BM) has also been observed in TB [40]. There is, however, apparently no report of the *M. tuberculosis*- or its component(s)-induced production of CSFs. Because CSFs play important role(s) in the pathogenesis and protection from TB [32], we considered it expedient to determine the CSF induction potential of Mtb30. Our results, apparently for the first time, demonstrate that purified Mtb30 can induce the synthesis and secretion of CSFs, both *in vivo* and *in vitro*.

## MATERIALS AND METHODS

### *Mice and mycobacteria*

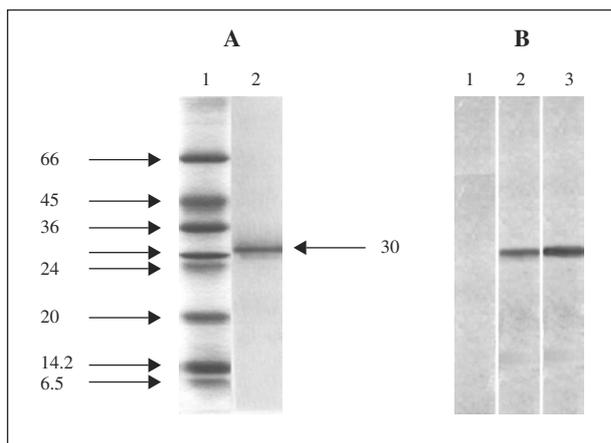
Male Swiss mice (18-20 g), obtained from the Central Animal Facility of the institute, were maintained at 22-24 °C with food and water provided *ad libitum*. All studies were carried out in accordance with the guidelines for Care and Use of Animals in Scientific Research, Indian National Science Academy, New Delhi, India, as adapted and promulgated by the Institutional Animal Ethics Committee. *M. tuberculosis* H37Rv, obtained from Tuberculosis Research Centre, Chennai, India, was cultured at 37 °C in dispersed form in Middlebrook 7H9 broth (Difco, Sparks, MO, USA) supplemented with 1% glycerol, 0.05% Tween 80 (Sigma Aldrich, St. Louis, MO, USA) and 10% Middlebrook OADC enrichment (Difco). Culture aliquots in 1 mL volumes were stored at -70 °C until used.

### *Production of M. tuberculosis culture filtrate proteins and purification of Mtb30*

Culture filtrate proteins were purified from the pooled cultures of *M. tuberculosis* H37Rv grown in 7H9 broth (pH 6.7) prepared with glycerol but without albumin and Tween. The bacteria were cultured at 37 °C for three weeks from an initial OD<sub>540</sub> of 0.05 to a final OD<sub>540</sub> of 0.5. The cells were removed by centrifugation (16,270 g; 20 min; 4 °C) and the supernatant was filtered by first using a 0.45 and then a 0.22  $\mu$ m membrane filter. The sterile culture filtrate was concentrated (x 100) by ultrafiltration using an Amicon PM10 membrane (Amicon, Bedford, MA, USA). The concentrated proteins were brought to 50% ammonium sulphate saturation at 4 °C. The precipitate was dialysed against water, dried by lyophilization and designated culture filtrate, and its protein content was determined by Lowry's method [41] using bovine serum albumin as a standard. The major proteins from the culture filtrate were purified as described [10]. Briefly, the culture filtrate protein concentrate (100 mg) was loaded onto a DEAE-Sepharose CL-6B (Sigma) anion exchange column equilibrated with 30 mM Tris-hydrochloride buffer (pH 8.7) containing 3% (v/v) methyl cellosolve. The column was eluted using a sodium chloride linear gradient from 50 – 300 mM. Each fraction was read at 280 nm and the absorbency was plotted. The resultant fractions obtained were pooled and concentrated separately by ultrafiltration using a YM-3 membrane (Amicon). The concentrated fractions were further purified to homogeneity by applying the pooled fraction serially to columns of DEAE-Sepharose CL-6B (containing 10% ethylene glycol/3M urea), phenyl

### Abbreviations:

AIDS	acquired immunodeficiency
BCG	bacillus Calmette-Guérin a non-virulent strain of <i>Mycobacterium bovis</i>
BM	Bone marrow
CDMEM	Complete DMEM
CFU-GM	Colony forming unit-GM
CM	Conditioned medium
CSF	Colony-stimulating factor
DMEM	Dulbeccos' modified Eagles Medium
FA	Freund's adjuvant
FBS	Fetal bovine serum
G-CSF	Granulocyte-CSF
GM-CSF	Granulocyte macrophage-CSF
HBSS	Hanks' balanced salt solution
HI	Heat-inactivated
IMDM	Iscove's modified Dulbeccos' medium
LAL	<i>Limulus</i> amoebocyte lysate
M-CSF	Macrophage-CSF
MDP	Muramyl dipeptide
MDR	Multidrug resistant
M $\phi$	Macrophage
Mtb30	30-kDa secretory antigen of <i>M. tuberculosis</i>
PM	Peritoneal M $\phi$
RBC	Red blood cell
TB	Tuberculosis



**Figure 1**

A. Coomassie blue-stained SDS-PAGE gel of Mtb30: Lane 1, molecular weight markers in kDa; Lane 2, purified Mtb30. B. Western blot analysis of antiserum for specificity against Mtb30. Lane 1, pre-immune rabbit serum was used as negative control; Lane 2, a positive control with MAb TBc27; Lane 3, antiserum was used at 1:500 dilution on  $1\mu\text{g}$  of Mtb30/lane.

Sepharose CL-4B and sephacryl-S-200 HR (Sigma). Finally, the pooled fractions were analysed by reduced 12% sodium dodecyl sulphate polyacrylamide gel electrophoresis, and stained with Coomassie Blue to determine the apparent molecular mass of proteins (figure 1A). The fractions containing the Mtb30 were pooled, dialysed against water and identified by immunoblotting with the 30-kDa antigen specific monoclonal antibody TB-c-27 [42]. The CSF-inducing capability of Mtb30 was characterized by heat, enzymatic and sodium periodate treatments. For heat stability testing, Mtb30 was heated at  $70^\circ\text{C}$  for 1 h at pH 7.0. The effects of proteases were tested by incubating Mtb30 with pronase E (2.5 mg/mL; pH 7.5; Merck, Darmstadt, Germany) and trypsin (25  $\mu\text{g}/\text{mL}$ ; pH 8.0; Merck). The stability of Mtb30 (10  $\mu\text{L}$ ) in the presence of periodate was determined by treatment with 5  $\mu\text{L}$  of 0.6 M sodium periodate (pH 7.2; Sigma) overnight at room temperature. Excess of periodate was eliminated by the addition of 5  $\mu\text{L}$  of 0.4 M sodium metabisulfite. The endotoxin content of Mtb30 was determined by *Limulus* amoebocyte lysate (LAL) assay and was  $<20$  ng/mg Mtb30. Lipopolysaccharide at this concentration did not induce CSF production by mouse peritoneal M $\phi$ s (PMs). The purified Mtb30 was filter (0.22  $\mu\text{m}$ ) sterilized, and stored at  $-20^\circ\text{C}$  until used. A single preparation of Mtb30 was used in these studies.

#### Generation of rabbit anti-Mtb30 serum

Rabbit anti-Mtb30 serum was generated by injection of 400  $\mu\text{g}$  of Mtb30 in complete Freund's adjuvant (FA; 1:1) at four subcutaneous (s.c.) sites followed by a booster of 200  $\mu\text{g}$  of Mtb30 in incomplete FA, s.c., four weeks later. The rabbit was boosted once again, one week later, with 100  $\mu\text{g}$  of Mtb30, intravenously (i. v.), and was finally bled for antiserum, three days after the last boost. The antiserum was heat-inactivated (HI;  $56^\circ\text{C}$ , 30 min) and stored at  $-20^\circ\text{C}$ . The specificity of the antiserum was determined by Western blot analysis, and was found to be specific (figure 1B). The antiserum had an antibody titre 1:2048 as

determined by enzyme-linked immunosorbent assay (ELISA).

#### Macrophages

For PMs, thioglycollate-injected (4% wt/vol; 0.5 mL/mouse; 96 h) normal mice peritoneal exudates cell suspension was centrifuged (700  $\times g$ ; 7 min;  $4^\circ\text{C}$ ), and the cell pellet was resuspended ( $1 \times 10^6$  cells/mL) in 10 ml antibiotic-free Dulbeccos' modified Eagles Medium (DMEM; Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS, Gibco), 2 mM L-glutamine (Gibco), 0.01 M HEPES (Gibco), and  $1 \times 10^{-4}$  M 2-mercaptoethanol (2-ME, Sigma; CDMEM). The splenic M $\phi$ s were obtained from splenocyte single cell suspension made by using a 20  $\mu\text{m}$  nylon sieve. The cells were then washed by centrifugation (700  $\times g$ ; 12 min;  $4^\circ\text{C}$ ). Erythrocytes were lysed with rbc-lysing buffer (Sigma-Aldrich), and the cells were resuspended in CDMEM. For bone marrow (BM)-derived M $\phi$ s, mouse femurs were flushed with chilled DMEM using a 24G needle, and the BM cells were washed (x2) and suspended in CDMEM. The adherent M $\phi$ s from these three cell suspensions were harvested, separately, by allowing them to attach to the plastic surface of T-25 culture flasks at  $37^\circ\text{C}$  for three hours in 5%  $\text{CO}_2$ -air atmosphere, and were then further incubated for 30 min in an equal volume of CDMEM containing 2  $\mu\text{g}/\text{mL}$  indomethacin (Sigma). The M $\phi$ s were detached using a sterile rubber scraper, washed (x3) and resuspended in 5 mL chilled Hanks' balanced salt solution (HBSS). T-cells from these M $\phi$ s were eliminated by rabbit anti-mouse T-cell serum (1:20) treatment for 1 h at  $4^\circ\text{C}$  followed by a HBSS wash (x1), and incubation with rabbit complement (1 h;  $37^\circ\text{C}$ ). The DMEM and HBSS contained  $<0.1$  ng/mL endotoxin as determined by LAL assay. M $\phi$ s were  $>96\%$  pure as determined by morphologic, phagocytic and non-specific esterase staining criteria, and were  $>98\%$  viable as judged by trypan blue exclusion.

#### Generation of CSFs

For serum CSFs, a single injection of Mtb30 (0.1, 0.5, 1, 5 and 10 mg/kg) was administered to mice (i. v.; 0.1 mL; six mice/dose), and their blood samples were collected aseptically after six, 12, 24, 48 and 72 h. Sera from these blood samples were separated and pooled for each time-point, separately. Pooled sera from mice injected with muramyl dipeptide (MDP; 25  $\mu\text{g}/\text{kg}$ ), HI-Mtb30 ( $70^\circ\text{C}$ ; one h; pH 7.0; 10 mg/kg) or sterile normal saline (vehicle) served as controls. *In vitro*, the cultured M $\phi$ s ( $5 \times 10^5$  cells/mL; 2 mL/well) were exposed to different concentrations of Mtb30 (1, 5, 10, 25 and 50  $\mu\text{g}/\text{mL}$ ) for six, 12, 24, 48 and 72 h. For CSFs induced by whole mycobacteria, the cultured PMs ( $1 \times 10^5$  cells; 1.4 mL) were exposed to live (multiplicity-of-infection, MOI; 1:1 and 1:10) and heat-killed ( $80^\circ\text{C}$ , 20 min; MOI; 1:10) *M. tuberculosis* for 4 h at  $37^\circ\text{C}$  in 5%  $\text{CO}_2$ -air atmosphere, washed (x5) with warm DMEM to remove the extracellular bacteria, and then further cultured for six days in the presence or absence of 100% neutralizing concentration (25  $\mu\text{g}/\text{mL}$ ) of rabbit anti-Mtb30 antibody, and 100  $\mu\text{L}$  of fresh medium containing antibody was added daily for the next six days. The conditioned media (CM) of the M $\phi$ s were then col-

lected aseptically, centrifuged (1000 x g; 10 min; 4 °C) and filter-sterilized (0.2 µ). For controls, CM of MØs treated with MDP (1 µg/mL), HI-Mtb30, normal rabbit serum and CDMEM only were used. All the sera and CM were stored at -20 °C until used.

### Measurement of CSF activity

CSFs were measured in terms of their colony-stimulating activity [43, 44]. Briefly, normal mouse femur BM mononuclear cells were washed (x3) and suspended ( $5 \times 10^6$  cells/mL) in CDMEM. After removal of non-adherent cells by adherence/depletion processes, the adherent cells were resuspended ( $2 \times 10^4$  cells/mL) in CDMEM without FBS but containing 30% HI (56 °C, 30 min)-horse serum (Gibco), 0.9% methylcellulose (Sigma), 0.9% deionized bovine serum albumin (Sigma) and  $1 \times 10^{-4}$  M 2-ME. The test and control serum (5%), and CM (10%) samples were then added to this cell suspension. One mL cultures of this cell suspension were established in 35 mm plastic dishes and incubated at 37 °C in humid 5% CO<sub>2</sub>-air atmosphere for 14 days. The number of colonies with 50 or more cells were then counted under a dark-field inverted microscope (40x magnification) or fixed on the glass slides and stained with May-Grünwald-Giemsa solution for identification.

### Quantification of GM-CSF, M-CSF and G-CSF in serum and CM

Sandwich-ELISAs were performed to quantify mouse GM-CSF, M-CSF and G-CSF secretion in serum and CM. The following pairs of capture and detection antibodies were used to quantify mouse CSFs, respectively: monoclonal antibody (MAb) pairs anti-mouse GM-CSF (clone MP1-22E9) and biotinylated anti-mouse GM-CSF (clone MP1-31G6; PharMingen, San Diego, CA, USA), polyclonal pairs anti-mouse M-CSF and biotinylated anti-mouse M-CSF (R & D Systems Inc.; Minneapolis, MN, USA), and monoclonal anti-mouse G-CSF (clone 67604.111) and polyclonal biotinylated anti-mouse G-CSF (R & D Systems). Capture antibodies were coated on the MaxiSorp (Nunc, GIBCO BRL, Paisely, UK) plates at the concentration of 15 µg/mL and the biotinylated antibodies were added at the final concentration of 10 µg/mL, before adding streptavidin-labelled horseradish peroxidase. CSFs were quantified by extrapolation from a standard curve constructed by determining absorbencies using limiting dilutions of recombinant mouse GM-CSF (rmGM-CSF) and M-CSF (PharMingen), and G-CSF (R & D Systems). The results were expressed as mean ng/mL, and the sensitivities of the assays were 50 pg/mL for GM-CSF, 90 pg/mL for M-CSF and 30 pg/mL for G-CSF.

### GM colony-forming units (CFU-GM) assay

The number of CFU-GM in single cell suspensions prepared from the spleens and BM of Mtb30-treated mice were determined in colony-forming assays, performed in semi-solid cultures [45, 46]. Briefly, spleens were minced and passed through a sterile, nylon mesh (20 µm) to obtain single-cell suspensions which were resuspended in 15 mL of DMEM containing 10% FBS, 2% HEPES, and 40 µg/mL gentamycin, and centrifuged (700 g; 12 min; 4 °C). Erythrocytes were lysed with rbc-lysis buffer, the

cells were washed with DMEM, and erythrocyte ghosts were removed by filtering cell suspensions through sterile gauze. BM cells were flushed out from the mouse femurs with 1 mL of cold Iscove's modified Dulbeccos' medium (IMDM; Gibco) supplemented with 5% FBS, 40 µg/mL gentamycin and 2 mM L-glutamine. The spleen and BM cell suspensions were washed (x3) in IMDM. Total, viable cell counts were obtained by using 0.1% trypan blue exclusion, and finally suspended at a concentration of  $4 \times 10^6$  cells/mL in the same medium. The CFU-GM medium consisted of 0.8% methylcellulose, 30% FBS, 10% pokeweed mitogen-stimulated spleen cell-CM, 2 mM L-glutamine and  $5 \times 10^{-5}$  M 2-ME. The spleen and BM cells were resuspended in CFU-GM medium and plated in 35 mm petri dishes at densities of  $2 \times 10^5$  and  $3 \times 10^5$  cells, respectively. CFU-GM counts were determined according to colony morphology after seven days of incubation in humidified 5% CO<sub>2</sub>-air atmosphere at 37 °C. Based on the total spleen or BM cell counts, the final CFU-GM numbers were expressed/spleen or femur.

### Statistical analysis

All the experiments were run in triplicate, three-times, separately. For statistical analysis, Students' *t*-test was used, and  $p < 0.05$  was considered significant.

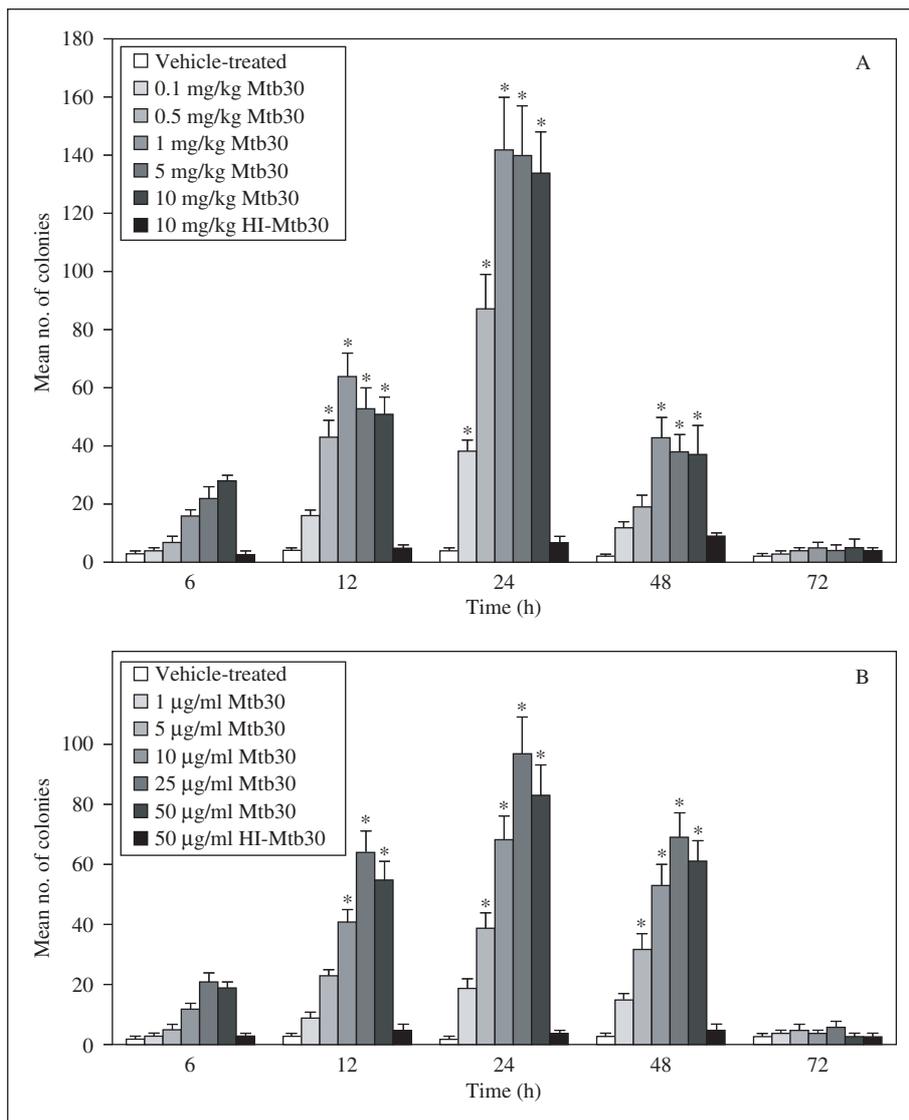
## RESULTS

### Induction of serum CSFs by Mtb30

Mtb30 (0.1–10 mg/kg) induced dose-dependent production of serum CSFs in mice (*figure 2a*). Whereas, as low as 0.1 mg/kg Mtb30 could induce detectable increase in serum CSF activity, a 1 mg/kg dose induced maximum response ( $142 \pm 16$  colonies); at further higher doses, the response was a plateau. The Mtb30-induced increased serum CSF production was observed as early as six hours after stimulation, reached its maximum after 24 h, and then gradually returned to normal levels by 72 h. No increased CSF production was observed in mice treated with HI-Mtb30 or vehicle.

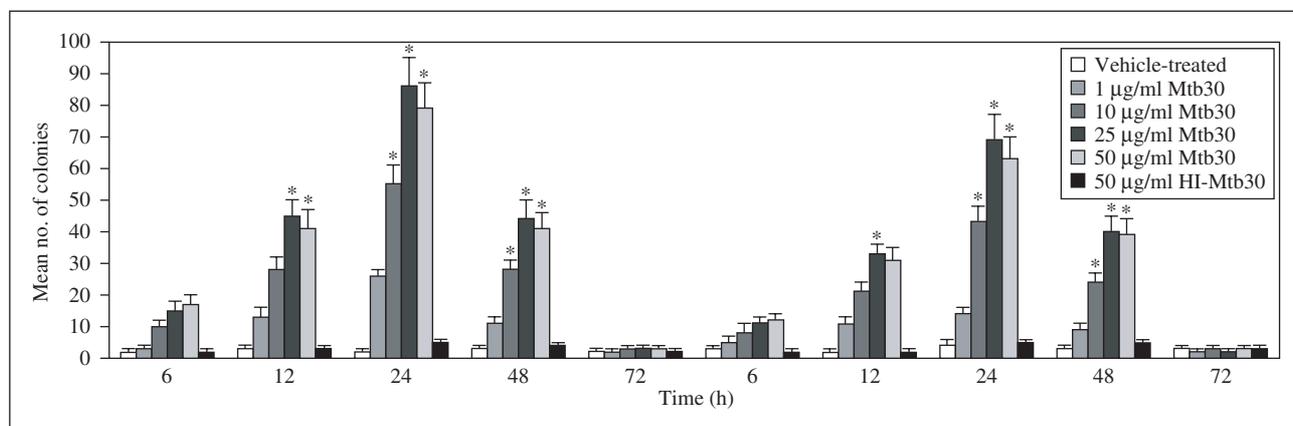
### Mtb30-induced production of CSFs by MØs, in vitro

PMs ( $5 \times 10^5$  cells/well; 2 mL) incubated with Mtb30 (1–50 µg/mL), in a concentration-dependent manner, elaborated CSFs in the CM (*figure 2b*). Although as low as 1 µg/mL of Mtb30 induced detectable amounts of CSFs in the CM, maximum CSF induction ( $97 \pm 11$  colonies) occurred at 25 µg/mL; at higher concentration the response was a plateau. CSFs were detectable in the CM six h after stimulation, peaked at 24 h and levelled-off to background levels by 72 h. HI-Mtb30 (50 µg/mL) did not induce the elaboration of CSFs, over and above the background levels. Similar results were obtained using splenic and BM-derived MØs (*figure 3*). Furthermore, Mtb30 (1–50 µg/mL) did not directly induce the colony formation (data not shown). Polyclonal rabbit anti-Mtb30 antibody neutralization of Mtb30 significantly ( $p < 0.05$ ) diminished its CSF-inducing capability, *in vitro* (*figure 4*). The Mtb30-induced CSFs in the serum and CM were tested for their functional/molecular similarities by examining the colony



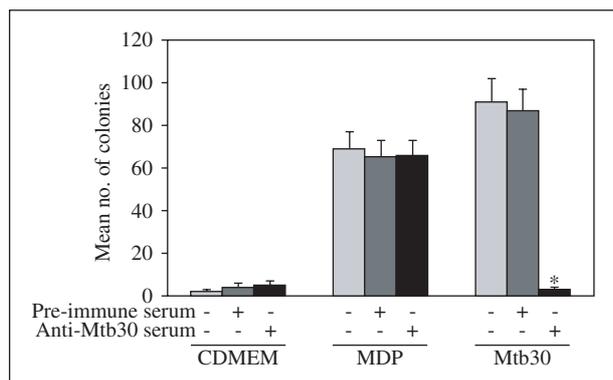
**Figure 2**

Mtb30-induced CSF production in mice serum, and in the CM of PMs, *in vitro*. A. Mice (six/group) were injected with Mtb30 (0.1–10 mg/kg), *i.v.*, on day 0. Blood samples from each mouse were collected aseptically at the time indicated, pooled and the sera were separated. The serum samples were heat-inactivated (HI; 56 °C, 30 min). B. *In vitro*, PMs ( $5 \times 10^5$  cells/mL; 2 mL cultures) were incubated with Mtb30 (1–50 µg/mL) at 37 °C in a 5% CO<sub>2</sub>-air atmosphere for the durations indicated. For the generation of control serum and CM, vehicle and HI-Mtb30 were used. The CSF activity was determined in the serum and CM. Data are the mean number of colonies ± SD of three separate experiments, run in triplicate. \*Significantly ( $p < 0.05$ ) different from vehicle- and HI-Mtb30-treated groups.



**Figure 3**

CSF production by Mtb30-treated mouse splenic and BM-derived MØs, *in vitro*. Splenic and BM-derived MØs ( $5 \times 10^5$  cells/mL; 2 mL cultures) were incubated with Mtb30 (1–50 µg/mL) at 37 °C in a 5% CO<sub>2</sub>-air atmosphere for the durations indicated. The CSF activity was determined in the CM collected at the time indicated. Other details as for figure 2.



**Figure 4**

Effect of rabbit anti-Mtb30 antibody on the Mtb30-induced production of CSFs by PMs, *in vitro*. PMs ( $5 \times 10^5$  cells/mL; 2 mL cultures) were incubated with MDP (1  $\mu$ g/mL) or Mtb30 (25  $\mu$ g/mL) at 37 °C in a 5% CO<sub>2</sub>-air atmosphere for 24 h. Neutralizing (100%) concentrations of rabbit Mtb30 polyclonal antibody were mixed with MDP or Mtb30 just before PM treatment. Pre-immune rabbit serum was used as negative control. \*Significantly ( $p < 0.05$ ) different from that mixed with pre-immune serum. Other details as for *figure 2*.

types they induced. The data (*figure 5*) show that CSFs from both sources formed G, M and GM colonies in similar proportions; the GM colonies were maximum (>79%). IL-1 is known to induce the production of CSFs, *in vitro*. Therefore, we determined the role of IL-1 in Mtb30-induced CSF production, *in vitro*. Incubation of PMs with Mtb30 along with neutralizing (100%) rabbit anti-mouse IL-1 polyclonal antibody (Endogen Inc., Boston, MA, USA) did not block Mtb30-induced production of CSFs (*figure 6*), thereby, excluding the role of IL-1 in CSF production, *in vitro*. To exclude the role of TNF- $\alpha$  that may have been produced by Mtb30-treated PMs [17], in CSF production, PMs were stimulated with Mtb30 in the presence of 100% neutralizing rabbit anti-mouse TNF- $\alpha$  polyclonal IgG (R & D Systems). Data in *figure 6* show that anti-mouse TNF- $\alpha$  IgG only partially blocked the production of CSFs. Polymyxin B (25  $\mu$ g/mL), an antibiotic that can neutralize the biological activities of LPS, did not inhibit the Mtb30-induced CSF production (data not

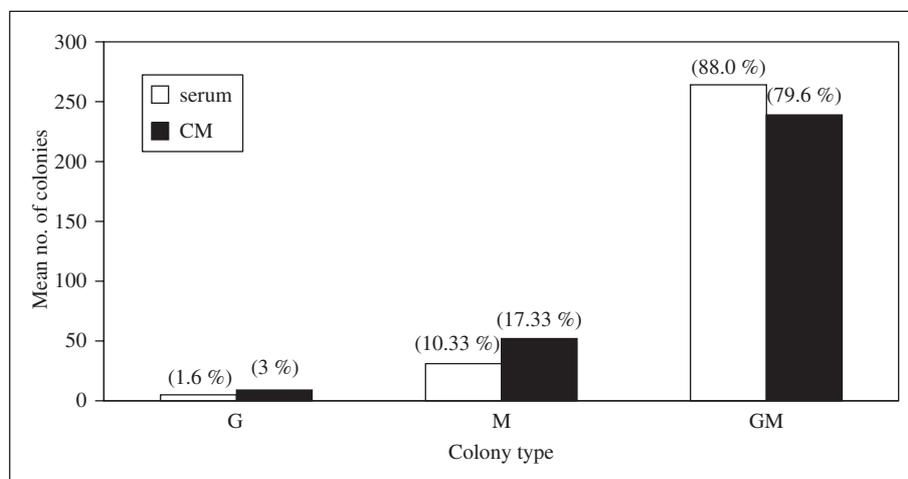
shown). Further, polymyxin B had no effect on the basal production of CSFs by unstimulated PMs or on the responsiveness of the committed progenitor BM cells to CSFs (data not shown). The Mtb30-induced CSF production was *de novo* as it was completely inhibited by cycloheximide (50  $\mu$ g/mL; *figure 7*). The CSF-inducing capability of Mtb30 was destroyed by proteases (pronase E and trypsin), and was unaffected by periodate treatment (*figure 8*).

#### **Effect of rabbit anti-Mtb30 polyclonal antibody on the CSF elaboration by *M. tuberculosis*-infected PMs**

To demonstrate whether whole, mycobacteria-induced CSF elaboration was through an Mtb30-dependent process, PMs infected with live and killed mycobacteria were cultured in the presence or absence of rabbit anti-Mtb30 polyclonal antibody, and the CSFs elaborated in the CM were measured. Infected-PMs cultured without rabbit anti-Mtb30 antibody elaborated CSFs that could be detected as early as on day one, peaked on day +3 ( $182 \pm 18$  colonies) and then plateaued thereafter (*figure 9*). Contrastingly, incubation of infected-PMs with rabbit anti-Mtb30 polyclonal antibody significantly ( $p < 0.05$ ) reduced the peak CSF ( $42 \pm 6$  colonies) elaboration on day +3 and at all the other time-points. Heat-killed mycobacteria induced significantly ( $p < 0.05$ ) less CSF production on day +2, which declined thereafter.

#### **Mtb30-induced GM-CSF, M-CSF and G-CSF production in serum and CM**

Mtb30-treated mice (1 mg/kg) and PMs (25  $\mu$ g/mL) showed significantly ( $p < 0.05$ ) high GM-CSF levels in serum ( $9 \pm 1$  ng/mL) and CM ( $7.5 \pm 0.8$  ng/mL), respectively, as compared to the controls, 24 h later (*figure 10*). Similarly, the levels of M-CSF in both serum ( $4.3 \pm 0.5$  ng/mL) and CM ( $3.9 \pm 0.4$  ng/mL) were also significantly ( $p < 0.05$ ) higher than the controls, after 24 h of Mtb30-stimulation (*figure 11*). However, Mtb30-treatment did not induce significant ( $p > 0.05$ ) G-CSF production in either serum or CM (data not shown).



**Figure 5**

Composition of the colonies formed in response to Mtb30-induced CSFs. Serum and CM CSF-supported colonies were fixed on glass slides and stained with May-Grünwald-Geimsa solution for identification. G, granulocyte; M, macrophage; GM, granulocyte-macrophage. The data, based on an examination of 300 colonies, are representative of one of the three experiments with similar results.

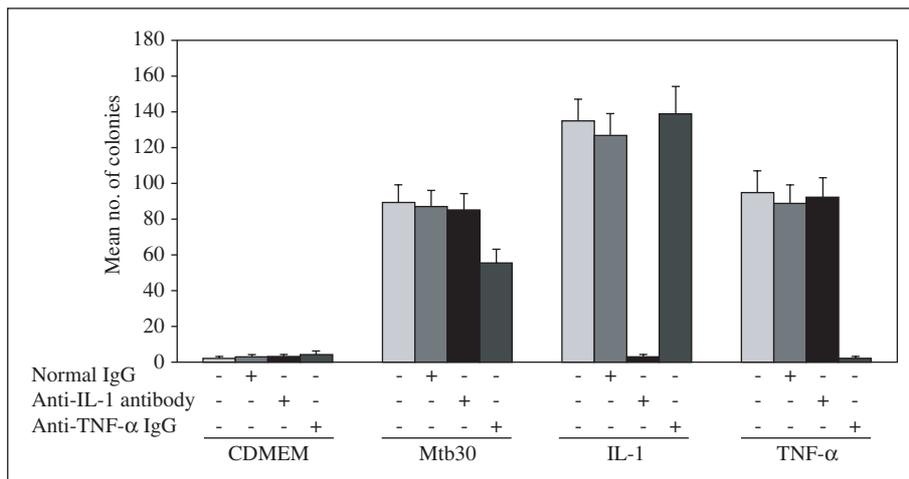


Figure 6

Effect of rabbit anti-mouse IL-1 polyclonal antibody and goat anti-mouse TNF- $\alpha$  polyclonal IgG on the Mtb30-induced production of CSFs by mouse PMs, *in vitro*. PMs ( $5 \times 10^5$  cells/mL; 2 mL cultures) were incubated with Mtb30, rmIL-1 $\beta$  (5  $\mu$ g/mL; R & D Systems) or rmTNF- $\alpha$  (10  $\mu$ g/mL; R & D Systems) at 37 °C in a 5% CO<sub>2</sub>-air atmosphere for 24 h. Neutralizing (100%) concentrations of rabbit anti-mouse IL-1 polyclonal antibody (50  $\mu$ g/mL) or goat anti-mouse TNF- $\alpha$  polyclonal IgG (2  $\mu$ g/mL) were added to Mtb30, IL-1 or TNF- $\alpha$  just before PM treatment. Normal rabbit IgG was used as negative control. Other details as for figure 2.

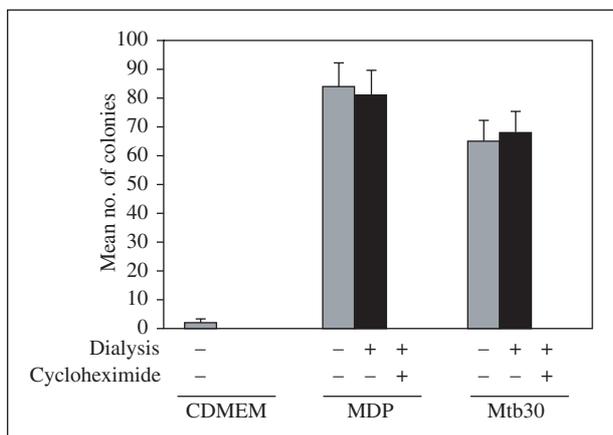


Figure 7

Effect of cycloheximide on the Mtb30-induced CSF production by PMs, *in vitro*. PMs ( $5 \times 10^5$  cells/mL; 2 mL cultures) were cultured with or without Mtb30 (25  $\mu$ g/mL) at 37 °C in a 5% CO<sub>2</sub>-air atmosphere for 24 h. PMs were cultured with or without cycloheximide at 50  $\mu$ g/mL. The CM were dialyzed or not dialyzed against DMEM. Other details as for figure 2.

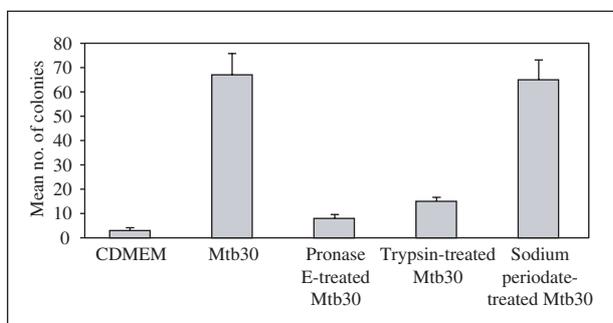


Figure 8

Characterization of the CSF-inducing capability of Mtb30. Mtb30 was treated with pronase E (2.5 mg/mL; pH 7.5), trypsin (25  $\mu$ g/mL; pH 8.0) or 0.6 M sodium periodate (pH 7.2) followed by incubation with PMs ( $5 \times 10^5$  cells/mL; 2 mL cultures) for 24 h at 37 °C in a 5% CO<sub>2</sub>-air atmosphere. CDMEM and untreated Mtb30 were used as controls. Other details as for figure 2.

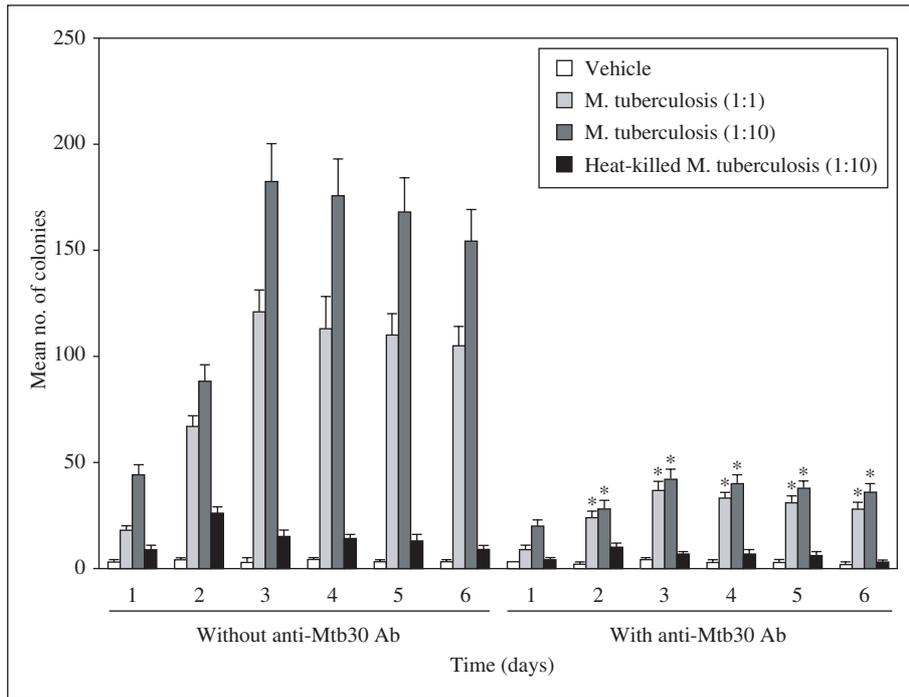
### Hematopoietic activity in the spleen and BM of Mtb30-treated mice

The spleen and BM of mice, 24 hours after Mtb30 (0.1–10 mg/kg) administration, showed a maximum of 2.23- and 2.36-fold increases in CFU-GM counts, respectively, with 1 mg/kg Mtb30, compared to those given vehicle only or HI-Mtb30 (table 1).

### DISCUSSION

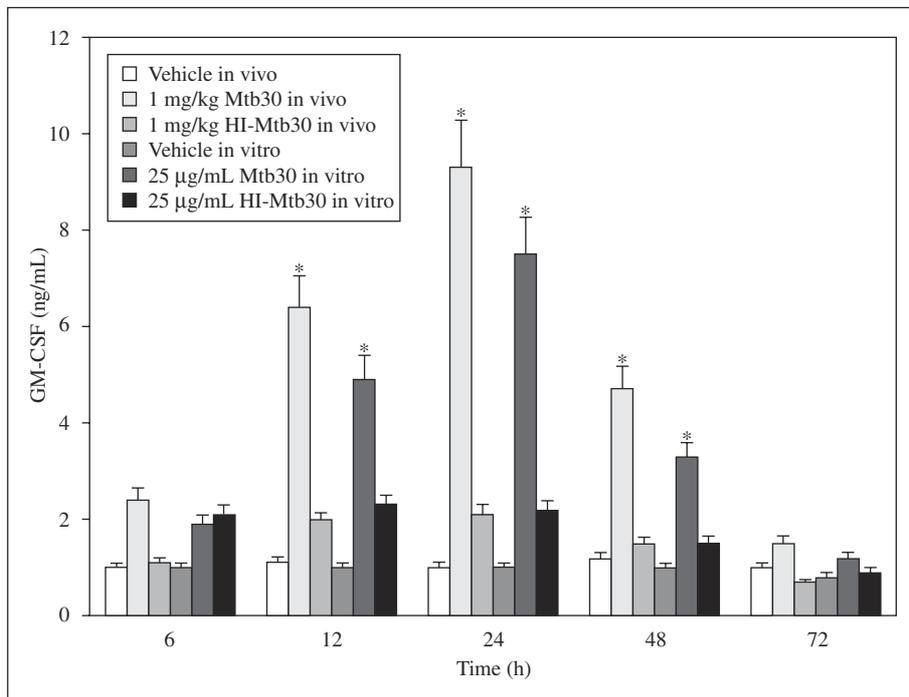
The most important observation of this study was that Mtb30 can induce the synthesis and secretion of serum CSFs in mice, and can induce M $\phi$ s to elaborate CSFs, *in vitro*. Both *in vivo* and *in vitro*, elaboration of GM-CSF appeared to be the major activity. Additionally, the Mtb30-treated mice showed increased hematopoietic activity both in the spleen and BM. Our observations, therefore, appear to be in line with earlier reports suggesting the induction of GM-CSF in mycobacteria-infected monocytes and large granular lymphocytes [39, 47]. Interestingly, our earlier reports, which demonstrate the plasmodial [44] and leishmanial [48] antigen-induced production of CSFs are also in consonance with our present observations. Because CSFs are an important group of pro-inflammatory cytokines, and are known to play definitive role(s) in host defense [49, 50], their production by *M. tuberculosis* secretory component(s) may have considerable bearing on the outcome of infection.

The Mtb30-induced CSF production, both *in vivo* and *in vitro*, appeared to be dose/concentration-dependent within the dose/concentration limits studied. Whereas, a single Mtb30 stimulus of as low as 0.1 mg/kg (*in vivo*) and 1  $\mu$ g/mL (*in vitro*) could trigger the onset of the induction of CSF production activity, a maximum production occurred only at 1 mg/kg and 25  $\mu$ g/mL, respectively. At higher doses/concentrations the response became static suggesting the saturation of the induction mechanism(s). Furthermore, the *in vivo* and *in vitro* kinetics of Mtb30-induced CSF production also appeared to be similar, with peak production occurring after 24 h and which



**Figure 9**

Effect of rabbit anti-Mtb30 polyclonal antibody on the CSF elaboration by *M. tuberculosis*-infected PMs. PMs ( $1 \times 10^5$ ; 1.4 mL) were exposed to *M. tuberculosis* H37Rv for 4 h at 37 °C in 5% CO<sub>2</sub>-air atmosphere, and then washed (x5) to remove the extracellular bacteria. The infected-PMs were then again cultured up to six days in the presence or absence of rabbit anti-Mtb30 polyclonal antibody. Only CDMEM was used for controls. \*Significantly ( $p < 0.05$ ) different from the cultures without antibody.



**Figure 10**

GM-CSF levels in Mtb30-treated mice serum and CM. Specific capture ELISAs were performed to quantify GM-CSF production. \*Significantly ( $p < 0.05$ ) different from other groups. Other details as for figure 2.

levelled-off to background levels by 72 h, possibly due to the “turning-off” of the stimulatory signal through decay and/or the possible initiation of the IL-10-mediated “negative feed-back loop”, resulting in the cytokine inhibitory functions. Nevertheless, after another 48 h in culture in fresh medium, these seemingly unresponsive MØs were

fully responsive to a fresh Mtb30 stimulus (data not shown). Further, MØs from three different anatomical sites were used in this study. Elicited PMs were used because TB is known to cause chronic inflammation that results in the activation of MØs. Because in TB the bulk of MØs that infiltrate the infected-foci are recently derived from the

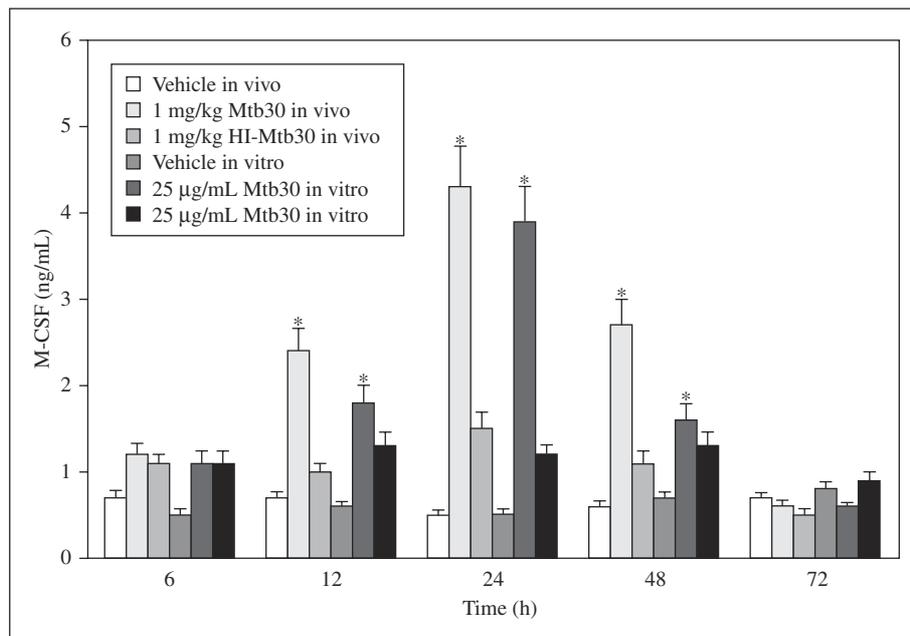


Figure 11

M-CSF levels in Mtb30-treated mice serum and CM. Specific capture ELISAs were performed to quantify M-CSF production. \*Significantly (p<0.05) different from other groups. Other details as for figure 2.

Table 1  
CFU-GM counts in the spleen and BM of Mtb30-treated mice <sup>a</sup>

Mtb30 (mg/kg)	CFU-GM/spleen <sup>b</sup> (no. x 10 <sup>3</sup> )			CFU-GM/femur <sup>b</sup> (no. x 10 <sup>3</sup> )		
	0 h	24 h	48 h	0 h	24 h	48 h
Vehicle	1.2 ± 0.3	1.3 ± 0.2	1.1 ± 0.2	1.0 ± 0.2	1.1 ± 0.1	1.2 ± 0.1
0.1	1.1 ± 0.2	1.8 ± 0.6	1.3 ± 0.1	1.1 ± 0.2	1.5 ± 0.3	1.2 ± 0.2
0.5	1.2 ± 0.2	2.2 ± 0.4	1.4 ± 0.1	1.1 ± 0.3	2.0 ± 0.3	1.3 ± 0.1
1.0	1.3 ± 0.3	2.9 ± 0.6*	1.4 ± 0.1	1.1 ± 0.1	2.6 ± 0.5*	1.4 ± 0.4
5.0	1.0 ± 0.2	2.4 ± 0.8*	1.2 ± 0.2	1.0 ± 0.1	2.3 ± 0.8*	1.1 ± 0.1
10.0	1.0 ± 0.1	2.3 ± 0.6*	1.2 ± 0.1	1.1 ± 0.3	2.0 ± 0.6*	1.2 ± 0.2
HI-Mtb30 (10 mg/kg)	1.0 ± 0.2	1.1 ± 0.1	1.0 ± 0.1	1.0 ± 0.3	1.0 ± 0.1	1.1 ± 0.1

\*Significantly (p<0.05) different from vehicle- and HI-Mtb30-treated groups.

<sup>a</sup> Spleen and BM cells (2x10<sup>5</sup> and 3x10<sup>5</sup> cells/dish, respectively) from Mtb30-treated mice were cultured in CFU-GM assay medium (1 mL) for 48 h at 37 °C in a 5% CO<sub>2</sub>-air atmosphere. For controls, mice were treated with vehicle only or HI-Mtb30.

<sup>b</sup> After seven days, CFU-GM colonies were counted. Data are mean number of colonies ± SD of three separate experiments, run in triplicate.

bone-marrow [40], BM-derived MØs were tested for their responsiveness to Mtb30. The spleen, on the other hand, is a highly immunocompetent organ in the body and a site where *M. tuberculosis* grows and multiplies, and hence the splenic MØs were also tested for their potential to elaborate Mtb30-induced CSFs. Curiously, all three types of MØs, irrespective of their anatomical origin and activation status, responded to Mtb30 similarly. The MØs used in this study were depleted of T-cells. Therefore, the CSFs elaborated in the CM, to a large extent, must have been released from the Mtb30-stimulated MØs only. On the other hand, purified T-cells from the spleen and blood of mice, following *in vitro* stimulation with Mtb30, did not elaborate CSFs (data not shown).

The mechanism(s) of Mtb30 interaction with MØs remains unclear. However, our results, which demonstrate that the Mtb30-induced CSF production was saturated by both dose/concentration and time, and was specifically inhibited by rabbit anti-Mtb30 polyclonal antibody suggest it to be a ligand-receptor interaction. Additionally, these observations clearly demonstrate that Mtb30 was a potent inducer of CSF production, and thus provide un-

equivocal evidence that this purified secretory component of *M. tuberculosis* can induce CSF production. This contention is further supported by our observations showing the rabbit anti-Mtb30 polyclonal antibody inhibition of the elaboration of CSFs by *M. tuberculosis*-infected PMs, *in vitro*, and thus demonstrate that mycobacteria induced CSF elaboration through a Mtb30-dependent process. The CSF-inducing capability of Mtb30 appeared to be proteinaceous in nature. Firstly, it was heat-labile (70 °C; 1 h). Secondly, proteolytic enzymes (pronase E and trypsin) abrogated CSF induction. Finally, sodium periodate treatment did not affect the production of CSFs. Nevertheless, besides Mtb30, several other proteins of *M. tuberculosis* may also induce the production of CSFs.

The functional and molecular properties of the CSFs are mirrored by the formation of the colony types they support. Thus, our data, which demonstrate the similar, if not identical, proportion of G, M, and GM colony formation under the influence of Mtb30-induced CSFs present both in the serum and CM, suggest that they were functionally and molecularly similar. Additionally, the GM-CSF appeared to be the major activity in CSFs from both of these sources

as indicated by the maximum proportion of GM colonies formed *in vitro*, as well as by the increased GM-CSF production as determined by ELISA. Furthermore, our data also indicated that quantitatively, the extent of colony formation induced by CSFs from both serum and CM appeared to be same. Taken together, our results demonstrate a commonality in the underlying molecular mechanism(s) of CSF induction both *in vivo* and *in vitro*. Curiously, the serum from *M. tuberculosis* H37Rv-infected mice also induced the increased formation of colonies in similar proportions (data not shown).

The Mtb30-treated mice, as compared to the controls, showed up to 2.23- and 2.36-fold increases in the CFU-GM counts, in the spleen and BM, respectively. The *M. tuberculosis* secretory component(s)-induced local production of CSFs by MØs at these sites might be a strategy employed by this highly adapted pathogen to maintain a continuous supply of myeloid cells ("safe sanctuaries") for its own survival and persistence. Additionally, the locally produced CSFs may also increase the terminal differentiation/maturation and activation of the infiltrating MØs.

Bronchoalveolar lavage cells of TB patients synthesize increased quantities of IL-1 $\beta$  and TNF- $\alpha$  [51]. The mycobacterial proteins have been reported to induce IL-1 and TNF in monocytes [52], and Mtb30 has been demonstrated to induce production of TNF- $\alpha$  by monocytes [17]. Furthermore, both *M. bovis* BCG-infected and uninfected bystander MØs have also been shown to elaborate TNF- $\alpha$  in their CM [53]. Because both IL-1 and TNF- $\alpha$  are known to induce the production of CSFs [54, 55], the CSF induction observed in this study can be attributed to them. Therefore, to gain insight into the possibility that IL-1 and TNF- $\alpha$  may mediate the Mtb30-induced CSF production, we generated evidence that demonstrates the lack of the effect of neutralizing (100%) concentrations of anti-mouse IL-1 antibody on the Mtb30-induced production of CSFs by MØs, *in vitro*; whereas, neutralizing (100%) concentrations of anti-mouse TNF- $\alpha$  IgG, only partially blocked it. Our results therefore, suggest that the Mtb30-induced production of CSFs was IL-1-independent, but was only partly attributable to TNF- $\alpha$ .

The physiological relevance of highly immunoprotective protein Mtb30 [4], is incompletely understood, and appears very complex. Mtb30 may modulate cytokine production by binding to fibronectin on the mycobacterial surface and thus play role in the immunopathogenesis of TB [56]. Mtb30 induces mononuclear phagocytes to secrete TNF- $\alpha$  [56], and this, in turn, is known to increase Mtb30 expression [57]. This reciprocal production of TNF- $\alpha$ -induced Mtb30 may be further enhanced by the infiltration by the T-cells at the sites of infection, as Mtb30 is a strong inducer of IFN- $\gamma$  [58] that is known to increase TNF- $\alpha$  production [57]. Furthermore, in mycobacteria-infected mice, TNF- $\alpha$  is involved in microbicidal granuloma formation [59], and has been reported to both suppress [60, 61] and enhance the growth of mycobacteria [62]. In latent *M. tuberculosis* infection, neutralizing TNF- $\alpha$  therapy has been associated with reactivation [63]. The TNF- $\alpha$ -dependent apoptosis in *M. tuberculosis*-infected phagocytes is thought to be a protective mechanism [64]. Thus, whether TNF- $\alpha$  plays a protective or pathological role in TB, remains unclear. *In vivo*, TNF- $\alpha$  functions as a dose-dependent, double-edged sword, by

mediating protection at low levels, whereas at high concentrations it provokes necrosis [65]. It should be noted here that, as compared to the less-virulent *M. tuberculosis* H37Ra strain, the highly virulent H37Rv strain induced greater expression of both Mtb30 and TNF- $\alpha$  mRNA [66]. Recently, Mtb30 has also been shown to induce macrophage deactivating cytokines TGF- $\beta$  [19] and IL-10 [20]. Thus Mtb30 appears to influence the *in vivo* cytokine milieu, and the interaction and the final balance of the macrophage-activating and immunoenhancing, and macrophage deactivating and immunosuppressive cytokines most likely determines the final outcome of the infection. Because CSFs function as proinflammatory and regulatory cytokines, the results of this study, which unequivocally demonstrate Mtb30-induced production of CSFs, apparently support this contention. Thus, Mtb30 appears to play extended central role(s) in the immunoregulation and immunopathogenesis of TB, in addition to several other, yet-to-be defined functions.

The precise biological role(s) of CSFs in TB is not completely understood. GM-CSF has been reported to activate MØs to kill mycobacteria, *in vitro* [34, 35]. Treatment of *M. avium*-infected mice with rmG-CSF has been reported to exert a protective effect [67]. In beige mice, rmGM-CSF has also been reported to enhance the effects of antibiotics against *M. avium* complex infection [68]. GM-CSF therapy enhances the uptake and the mycobactericidal activity of monocytes from AIDS patients with *M. avium* bacteremia [37, 38]. The results of the first ever clinical trial of recombinant human GM-CSF in pulmonary TB patients indicate its promise as adjuvant therapy [69]. Our results, which demonstrate Mtb30-induced production of CSFs, may have two potential implications: that the induced CSFs may merely serve to increase the number of phagocytes, which are the host cells for the *M. tuberculosis* expansion, as well as for increasing the phagocytic uptake of the pathogen, and that the CSFs produced may increase the mycobactericidal activity of the MØs. Overall, it is conceivable that the control of TB infection is determined by the maintenance of a balance between these two seemingly paradoxical functions of CSFs. Therefore, CSFs appear to play important role(s) in the pathogenesis of TB, wherein the immune competence of the host is integral to disease state. Detailed studies along these lines should provide greater insights into the molecular mechanism(s) of the pathogenesis of this highly successful, ancient pathogen of humanity.

**Acknowledgements.** We are grateful to Dr. C. L. Kaul, Director, National Institute of Pharmaceutical Education and Research, for his help and continued encouragement. Mrs. Sukhraj Kaur is grateful to the Council of Scientific and Industrial Research, New Delhi, India, for the award of a Senior Research Fellowship (National Entrance Test). This work was partly funded by a research grant to Dr. Prati Pal Singh, by the Indian Council of Medical Research, New Delhi, India, out of which Ms. Harsimrat Kaur received a Junior Research Fellowship. The technical assistance provided by Mr. Vijay Kumar Misra is greatly acknowledged. "Communication No. 305"

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