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Production of TNF-α, IL-6 and TGF-β, and expression of receptors for TNF-α and IL-6, during murine *Mycobacterium avium* infection

J. CHAMPSI,*† L. S. YOUNG* & L. E. BERMUDEZ* *Kuzell Institute for Arthritis and Infectious Diseases, Medical Research Institute of San Francisco, at California Pacific Medical Center, San Francisco and †Stanford University, Department of Infectious Diseases and Geographical Medicine, Stanford, California, USA

SUMMARY

The *Mycobacterium avium* complex comprises intracellular bacteria associated with disseminated infection in patients with acquired immune deficiency syndrome (AIDS). Immune defects that lead to infection are unknown but cytokines appear to play an important role in the immunomodulation of host defence mechanisms. We evaluated the cytokine profiles seen temporally after murine *M. avium* infection. Spleen cells were obtained from *M. avium*-infected C57BL/6 mice and uninfected mice at weeks 1, 2, 3, 4 and 5. Cells were cultured *in vitro* and subsequently pulsed with killed *M. avium*. Supernatants were collected from the cultured splenic cells and the concentrations of interleukin-6 (IL-6), transforming growth factor-β_1 (TGF-β_1) and tumour necrosis factor-α (TNF-α) were measured. TGF-β_1 was detected at week 1, followed by IL-6 production at week 2. Elevated TNF-α levels were observed at week 3. The addition of polyclonal anti-TGF-β_1 antibody to *M. avium*-infected peritoneal macrophages in the presence of splenic cell supernatants from weeks 1, 3 and 5 led to decreased bacterial counts compared to controls. Anti-IL-6 antibody did not have any effect on macrophage anti-mycobacterial activity. Concurrently, we observed decreased expression of TNF-α receptors on infected macrophages. We propose that the early elevated levels of TGF-β_1, a known suppressor of macrophage function, in conjunction with down-regulation of TNF-α receptors may help explain the suboptimal macrophage response to TNF-α, leading to impaired anti-mycobacterial activity.

INTRODUCTION

The *Mycobacterium avium* complex comprises facultative intracellular bacteria that cause disseminated infection in acquired immune deficiency syndrome (AIDS) patients.[1] Immune defects that lead to disseminated infection are multifactorial and include impaired cell-mediated immunity and modulation of the pattern of cytokine production in response to the infection.

Cytokines are immunomodulatory proteins that help orchestrate the immune response to many diverse inflammatory processes, including infection. Interleukin-6 (IL-6), tumour necrosis factor-α (TNF-α) and transforming growth factor-β_1 (TGF-β_1) are cytokines that have been shown to play a role in the host immune response against intracellular pathogens in murine and human models.[2–8] For example, TNF-α, produced mainly by macrophages and natural killer (NK) cells, appears to decrease intracellular *Trypanosoma cruzi* replication *in vitro* in the murine model, but this effect has not been seen in *Toxoplasma gondii*.[9] TNF-α stimulates macrophage-dependent mycobacteriostatic/mycobactericidal activity *in vitro* and *in vivo* in both human and murine models.[6,10]

IL-6, produced by macrophages, T cells and NK cells, has many immunomodulatory functions. Blanchard *et al.*[11] showed that IL-6 is produced by macrophages and NK cells when exposed to *M. avium in vitro*, and may have a role in the pathogenesis of infection. Recently, IL-6 has been associated with inhibition of TNF-α-mediated activation of infected macrophages.[5] Denis[12] suggested that IL-6 may be a growth factor for virulent *M. avium*, but other investigators have been unable to show that IL-6 stimulates the growth of intracellular *M. avium*.[13]

Macrophages infected with organisms such as *Toxoplasma gondii*, *Leishmania amazonensis*, *Trypanosoma cruzi* and *M. avium* produce TGF-β_1.[2–4,14] TGF-β_1 can inhibit cytokine-induced macrophage activation and suppress the oxidative response of macrophages.[2,3] Denis & Ghadirian reported that exogenous TGF-β leads to increased mycobacterial counts *in vitro* and *in vivo*.[15]

Immunosuppression observed during *M. avium* infection may be modulated by 'suppressive' cytokines interfering with signal transduction within macrophages. We investigated the influence of *M. avium* infection on the production of TNF-α,

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Correspondence: Dr L. E. Bermudez, Kuzell Institute, 2200 Webster St, Room 305, San Francisco, CA 94115, USA.

IL-6 and TGF-β_1 by splenic cells, the effect of blocking these cytokines on macrophage anti-mycobacterial activity, and the expression of TNF-α and IL-6 receptors on *M. avium*-infected splenic macrophages.

MATERIALS AND METHODS

Organisms

Mycobacterium avium, strain 101 (serovar 1), a clinical isolate,[6] was cultured on Middlebrook 7H10 agar medium (Difco Laboratory, Detroit, MI) for 10 days, and transparent colonies were resuspended in Hanks' balanced salt solution (HBSS; Applied Scientific, San Francisco, CA). The concentration of the suspension was determined using McFarland turbidity standards Remel, Lenexa, KS. An aliquot of the bacterial suspension was plated to confirm the bacterial count.

Mice

C57BL/6 bg^-/bg^+ pathogen-free female mice, aged 6–7 weeks, were obtained from Jackson Laboratories (Bar Harbor, ME). Mice remained in quarantine for 2 weeks before use in experiments.

Infection

Mice were infected intravenously (i.v.) via the tail vein with 1×10^8 *M. avium* strain 101 in 0·1 ml of HBSS. Control mice were injected with 0·1 ml HBSS i.v.

Antigen

Mycobacterium avium was cultured as described, and transparent colonies were transferred to Middlebrook 7H9 broth for 5 days. Then bacteria were washed three times with HBSS and incubated in 10% formalin for 2 hr. Dead bacteria were washed three times in phosphate-buffered saline (PBS) and resuspended to a concentration of 1×10^8 bacteria/ml.

Purification of splenic cells

Mycobacterium avium-infected mice and controls (four animals/group) were killed weekly. The spleen was fragmented and reduced to a fine cell suspension with the use of a sterile needle. The cell suspension obtained was washed and resuspended to a final concentration of 1×10^5 cells/ml in RPMI-1640 (Gibco, Grand Island, NY) supplemented with 5% heat-inactivated fetal bovine serum (FBS; Sigma Chemicals Co., St Louis, MO).

Splenic cells (2×10^5) were incubated with 1×10^7 formalin-fixed *M. avium* in 24-well tissue culture plates (Costar, Cambridge, MA), overnight at 37° in 5% CO_2 moist air. Collected supernants were filtered using a 0·22 μm Acrodise filter (Gelman Science, Ann Arbor, MI) and stored at −70°. After all time-points were collected, supernatants were assayed in duplicate for the presence of specific cytokines. Media were tested for the presence of endotoxin using a Limulus Amebocyte assay (Sigma Chemical Co., St Louis, MO) and found to contain less than 0·01 endotoxin U/ml.

Isolation of splenic macrophages

Infected and control mice (five animals/group) were killed at weeks 0, 1, 2, 3, 4 and 5; splenic cell suspension was obtained as described above. The cells were pelleted by centrifugation at 500 *g* for 10 min and washed twice in HBSS. Macrophage suspensions (5×10^5 cells/well) were allowed to adhere to plastic for 2 hr at 37° in 5% CO_2 in 24-well tissue culture plates in RPMI-1640 supplemented with 5% heat-inactivated FBS and 2 mM L-glutamine. Macrophage viability was determined by the trypan blue exclusion test, and plates with more than 95% of viable cells were used for the experiments.

Isolation and in vitro *infection of murine peritoneal macrophages*

Peritoneal macrophages were obtained from uninfected C57BL/6 mice as described previously.[6] Briefly, saline was injected and reaspirated from the peritoneal cavity. Cells obtained were washed and resuspended in RPMI-1640 supplemented with 5% heat-inactivated FBS and 2 mM L-glutamine, and plated (10^6 cells/well) into a 24-well tissue culture plate. After 2 hr of incubation at 37° in 5% CO_2, non-adherent cells were removed by washing with HBSS. Macrophage viability was determined as described above. Macrophage monolayers were incubated with a suspension of *M. avium* 101 (10^7/well) in RPMI-1640 supplemented with 5% heat-inactivated FBS and 2 mM L-glutamine. After 4 hr, supernatant containing extracellular bacteria were removed and the wells were washed exhaustively with HBSS. The number of intracellular *M. avium* was determined by lysing the monolayers with sterile water and 0·025% sodium dodecyl sulphate (SDS) in HBSS. The macrophage lysate was serially diluted and plated onto Middlebrook 7H10 nutrient agar for quantification of the initial load of bacteria. Infected macrophage monolayers were cultured in RPMI-1640 supplemented with 5% FBS and 2 mM glutamine at 37° and 5% CO_2. After 4 days, macrophage monolayers were lysed as described and the number of viable *M. avium* was determined. The number of macrophages in the monolayers was determined at day 4 by the method of Nakagawara & Nathan[16] in order to rule out preferential detachment of cells. Approximately 20 ± 3% of the macrophages detached from the wells in all experimental groups and controls.

Polyclonal rabbit anti-TGF-β and anti-IL-6 sera

Recombinant IL-6, purchased from Genzyme (lot no. B8320; Cambridge, MA), and recombinant TGF-β, purchased from R & D Systems (lot no. AV053041; Minneapolis, MN) were injected subcutaneously (TGF-β 100 ng/injection; IL-6 50 ng/injection), with Freund's adjuvant (Difco Laboratory), into rabbits, followed by injections of antigen with Freund's incomplete adjuvant weekly for 3 weeks. Animals were bled and the sera was tested for the presence of anti-IL-6 by enzyme-linked immunosorbent assay (ELISA), and anti-TGF-β using a biological assay, as described below. The polyclonal antibodies were partially purified by ammonium sulphate precipitation and had neutralization titres of 10^5 U/ml for IL-6 and 10^3 U/ml for TGF-β. This anti-TGFβ, polyclonal antibody was shown to have specific neutralizing activity against TGF-β, using human as well as mouse serum and macrophage culture supernatants.

Incubation of infected peritoneal macrophage monolayers in the presence of splenic cell supernatant and anti-IL-6 or anti-TGF-β antibody

Infected peritoneal macrophage monolayers were incubated in the presence of splenic cell supernatant (0·1 ml/well) from mice infected with *M. avium*, and control mice, at weeks 1, 3, and 5 that had been previously treated for 30 min at 37° with rabbit anti-IL-6 (2 μg/ml) or rabbit anti-TGF-β (3 μg/ml).

Supernatants and antibodies were replenished daily. After 4 days, macrophage monolayers were lysed as described above and plated to determine the number of viable bacteria. Samples were run in duplicate. Controls included incubation with rabbit sera alone and incubation without splenic cell supernatant.

TGF-β assay

The MV1Lu mink lung cell line (American Type Culture Collection, Rockville, MD) was used to measure TGF-β activity, as described previously.[3] Supernatants were assayed both untreated and after treatment with 0·12 N HCl for 15 min at room temperature, followed by neutralization with 0·1 M HEPES buffer containing 0·144 M NaOH. Samples were added to approximately 5×10^3 MV1Lu cells/well in 96-well flat-bottomed microtitre plates (Costar). Plates were incubated at 37° with 5% CO_2 for 24 hr and pulsed during the last 6 hr with 1 μCi of [^{3}H]thymidine (6·7 Ci/mol; [^{3}H]TdR; ICN, Costa Mesa, CA). Cells were lysed with Triton X-100 and the lysate was used to determine [^{3}H]TdR incorporation using a LKB liquid scintillation counter (Pharmacia, Uppsala, Sweden). Results were calculated based on the percentage decrease in [^{3}H]TdR incorporation. A control assay using recombinant TGF-β (R & D Systems) was run in parallel. Anti-TGF-β antibody was used in some wells to assure the specificity of the assay. The lower detection limit of the bioassay was 20 pg/ml TGF-β.

The specificity of the assay was demonstrated by inhibiting activity using anti-TGF-β antibody (rabbit or chicken anti-human TGF-β; R & D Systems).

TNF-α assay

An ELISA for TNF-α has been described previously.[17] Briefly, polyclonal rabbit IgG was obtained by immunizing rabbits with recombinant murine TNF-α. Antibody was purified by passage through a protein A column. Purified antibody was diluted in PBS, pH 7·2, to a protein concentration of 10–20 μg/ml and 0·1 ml of the solution added to each well of a 96-well ELISA plate (Costar). Plates were incubated overnight at 4° with 1% gelatin (Difco Laboratory). The blocking solution was removed and plates rinsed in PBS. Plates were then incubated with the experimental culture supernatants for 1 hr at room temperature. After washing the plates with PBS three times, 50 μl TNF-α monoclonal antibody was added and incubated for 1 hr at room temperature. Plates were rinsed four times with PBS and treated with goat anti-mouse IgG conjugated with peroxidase for 1 hr, followed by four rinses in PBS. A substrate solution containing 2,2′ azino-bis-[3-ethyl benzithiazoline-6-sulphonic acid] (Boehringer, Mannheim, Germany) and 5 μl of 30% H_2O_2 (Sigma Chemical Co.) was added and the plates were incubated for 10 min at 37° in an incubator. The enzyme reaction was stopped by the addition of 4 M H_2SO_4 (50 μl/well). Plates were read spectrophotometrically at 450 nm with an enzyme immunoassay (EIA) autoreader (model GL 310, Biotech Instruments, Burlington, CA). Samples were run in duplicate. Negative and positive controls were included with each assay.

IL-6 assay

The IL-6 concentration in the culture supernatant was determined with an ELISA kit purchased from R & D systems. All samples were run in duplicate. Negative and positive controls were included with each assay.

Expression of TNF-α and IL-6 receptors on splenic macrophages

TNF-α was iodinated with Na^{125}I by the Chloramine-T method (ICN), to yield a specific activity of 40 Ci/ml. The protein concentration of this preparation, estimated by BioRad Assay I (Hercules, CA), exhibited full biological activity, as evidenced by the L929 assay as described previously.[4]

A binding assay was performed as follows: at weekly time intervals, adherent macrophages (10^5 cells/ml) from infected and uninfected control mice were plated and acid-washed to remove any adherent TNF-α, and expression of TNF-α receptors was determined by incubation with ^{125}I-labelled TNF-α (10^3 U/ml) for 2 hr at 4°. Monolayers were washed three times with HBSS to remove unbound ^{125}I-labelled TNF-α. Macrophage monolayers were treated with 0·05% EDTA for 30 min to detach the cells from the plastic. Harvested macrophages were centrifuged at 800 *g* for 5 min, resuspended in HBSS, and the amount of radioactive material associated with the cells was read in a Beckman gamma-counter (High Wycombe, UK).

Macrophage viability was monitored and only preparations containing at least 95% of the viable cells were considered for the study. Control wells for non-specific binding of the ligand were performed in the presence of a 200-fold excess of unlabelled TNF-α. Equilibrium was achieved at 4°, and specific binding peaked in 2 hr and declined thereafter. Measurements were performed in triplicate; background was always < 20% of the total counts.

Murine IL-6 was iodinated with Na^{125}I by the Chloramine-T method to yield a specific activity of approximately 40 Ci/ml). The binding assay was performed as described for TNF-α.

Statistics

Experiments were repeated at least three times. Data were obtained in duplicate and the mean ± SD was calculated. Significance between experimental and control groups at the same time-points was determined using the Student's *t*-test.

RESULTS

Production of cytokines by spleen cells obtained from infected mice

Inoculation of C57BL/6 mice with 1×10^8 *M. avium* 101 leads to systemic infection. Splenic bacterial counts averaged $1·7 \times 10^8$ colony-forming units (CFU)/g at week 1, $2·1 \times 10^8$ at week 2, $4·7 \times 10^8$ at week 3, $8·9 \times 10^8$ at week 4 and $1·6 \times 10^9$ by week 5. We studied the temporary relationship of cytokine production and secretion in spleen cells, cultured *in vitro*, obtained from *M. avium*-infected mice. Analysis of splenic cell supernatants at weekly time intervals showed an initial rise in TGF-β production at week 1 after infection (Fig. 1a). Levels of TGF-β remained elevated during the 5 weeks of infection. A rise in IL-6 production was observed at week 1 (Fig. 1b), and peaked at 2 weeks after infection. TNF-α was elevated during the third and fourth week of infection, with a rapid drop in the fifth week of infection (Fig. 1c). Levels of IL-6 and TGF-β remained elevated during the 5 weeks of the study. Spleen cells from control uninfected mice did not produce detectable levels of TGF-β or IL-6 in response to *M. avium* antigen, hence ruling out the possibility of the *in vitro* presence of endotoxin as a cause of the observed cytokine response.

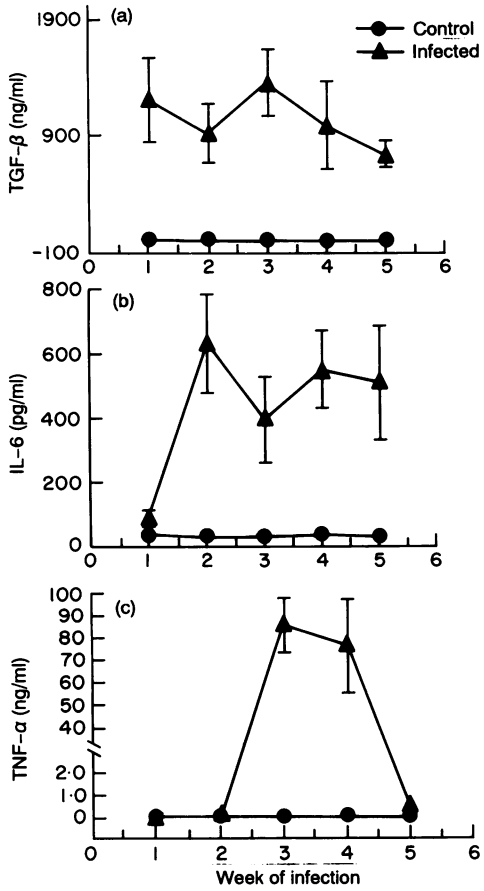

Figure 1. C57/BL6 mice were infected with *M. avium* strain 101. Spleens were obtained at weekly time-points and cultured *in vivo*, in the presence of *M. avium* antigen. (a) Culture supernatant was assayed for TGF-β production. TGF-β levels were elevated from week 1 to week 5 of infection. (b) Culture supernatant was assayed for IL-6 production. IL-6 levels were elevated at week 2 and remained elevated to week 5. (c) Culture supernatant was assayed for TNF-α production. TNF-α levels were initially low during the first 2 weeks of infection. There was a rise in TNF-α levels at week 3 and week 4 of infection, followed by a significant drop in the level of TNF-α at week 5.

Effect of polyclonal anti-IL-6 antibody and anti-TGF-β antibody on infected murine peritoneal macrophages cultured in the presence of splenic cell culture supernatant

In order to elucidate the role of high levels of IL-6 and TGF-β observed in the infected splenic cell supernatant, we incubated infected murine peritoneal macrophages in the presence of splenic cell supernatant from weeks 1, 3 and 5 previously exposed to either anti-IL-6 antibody or anti-TGF-β antibody. Addition of anti-TGF-β to splenic cell supernatant from weeks 1 and 5 led to a significant decrease in the number of viable bacteria within macrophages (Table 1); however, no significant effect was observed when supernatants were treated with anti-IL-6 antibody. Anti-IL-6 by itself did not appear to have any significant effect on mycobacterial growth.

Level of expression of TNF-α and IL-6 receptors on infected macrophages

Infection could alter the expression of cytokine receptors on target cells. Therefore, we investigated the expression of TNF-α and IL-6 receptors on splenic macrophages obtained from infected mice.

The specific binding of ^{125}I-TNF and Scatchard analysis are shown in Fig. 2. The results indicated that the macrophages had a K_d of $1{\cdot}4 \times 10^{-10}$ M and an average of 21 600 ± 1700 receptors/cell. The specific binding of ^{125}I-IL-6 and Scatchard analysis are shown in Fig. 3. Macrophages had a K_d of $2{\cdot}1 \times 10^{-10}$ M and an average of 2600 ± 400 receptors/cell.

The level of TNF-α receptor expression on splenic macrophages from infected mice gradually decreased during infection (Fig. 4a). IL-6 receptor expression remained unchanged during infection (Fig. 4b). TNF-α and IL-6 receptor expression on splenic macrophages from control uninfected mice remained unchanged from baseline during the course of the experiment.

DISCUSSION

Disseminated *M. avium* infection in AIDS patients is associated with a suboptimal immune response to the pathogen. Several laboratories have shown that cytokine interactions play an

Table 1. Effect of polyclonal anti-IL-6 and polyclonal anti-TGF-β antibodies on the anti-mycobacterial activity of murine peritoneal macrophages, incubated in splenic cell supernatants obtained from infected mice†

Week of infection	Supernatant alone*	Rabbit serum 10%	Anti-IL-6 (2 μg/well)*	Anti-TGF-β (3 μg/well)*
1	$4{\cdot}45 \pm 0{\cdot}49 \times 10^6$	$3{\cdot}6 \pm 0{\cdot}3 \times 10^6$	$2{\cdot}8 \pm 0{\cdot}35 \times 10^6$ ($P = 0{\cdot}065$)	$8{\cdot}7 \pm 0{\cdot}42 \times 10^5$ ($P = 0{\cdot}009$)
3	$2{\cdot}15 \pm 1{\cdot}34 \times 10^6$	$3{\cdot}5 \pm 0{\cdot}6 \times 10^6$	$6{\cdot}6 \pm 0{\cdot}71 \times 10^5$ ($P = 0{\cdot}054$)	$2{\cdot}9 \pm 1{\cdot}13 \times 10^5$ ($P = 0{\cdot}19$)
5	$3{\cdot}85 \pm 0{\cdot}07 \times 10^6$	$4{\cdot}1 \pm 0{\cdot}6 \times 10^6$	$3{\cdot}3 \pm 0{\cdot}57 \times 10^6$ ($P = 0{\cdot}36$)	$9{\cdot}4 \pm 0{\cdot}28 \times 10^5$ ($P < 0{\cdot}001$)

* Initial inoculum $9{\cdot}4 \pm 0{\cdot}4 \times 10^4$; infected macrophage monolayers were treated with supernatants for 4 days. Control monolayers cultured with RPMI-1640 and 5% FBS after 4 days had $1{\cdot}1 \pm 0{\cdot}3 \times 10^6$. Data expressed as mean ± SD.

† Experiments were performed in duplicate.

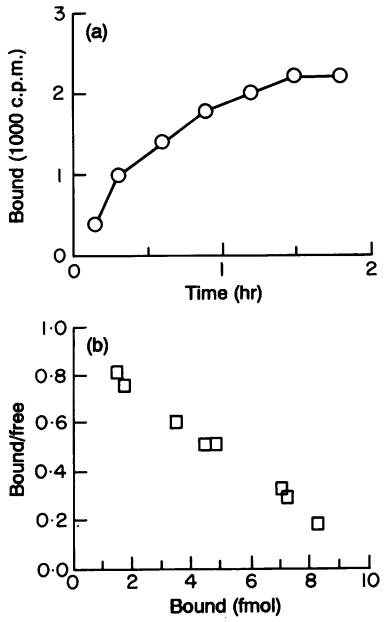

Figure 2. (a) Kinetics of binding of TNF-α to murine splenic macrophages from infected mice; (b) Scatchard plot analysis of the binding of TNF-α to receptors on mouse splenic macrophages (10^5 cells/ml). Cells were incubated with increasing concentrations of ^{125}I-TNF-α at 4° for 2 hr.

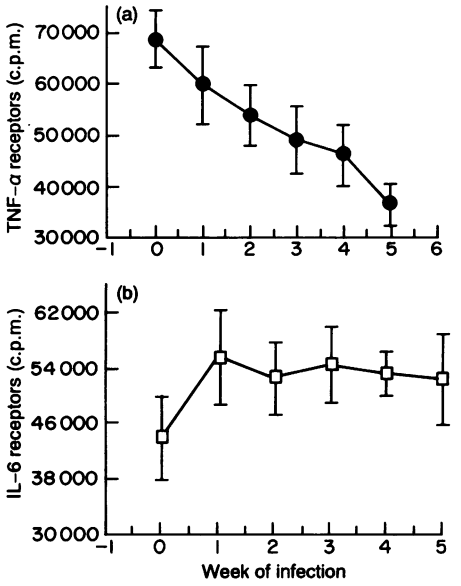

Figure 4. C57BL/6 mice were infected with *M. avium* strain 101. Splenic macrophages (10^5 cells/ml) from infected mice were assayed for (a) the level of TNF-α receptor and (b) the level of IL-6 receptor expression, from weeks 1 to 5. (a) There was a steady decline in TNF-α receptor expression in infected mice in contrast with uninfected controls. (b) There was an increase in receptor expression from week 0 to week 1, but from week 1 to week 5 there was no significant change in the level of IL-6 receptor expression ($P > 0{\cdot}05$).

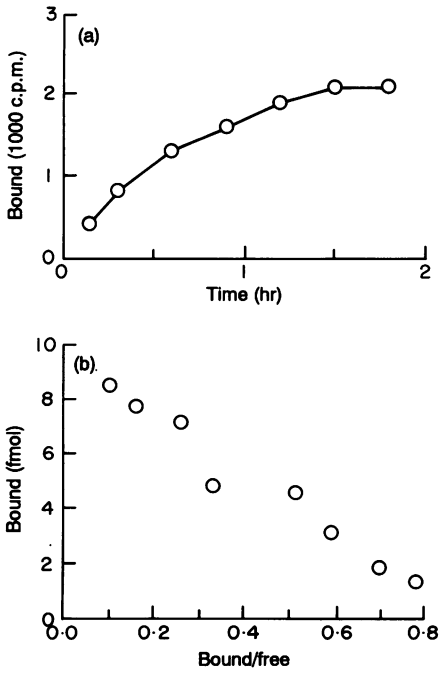

Figure 3. (a) Kinetics of binding of ^{125}I-IL-6 to murine splenic macrophages from infected mice; (b) Scatchard plot analysis of the binding of ^{125}I-IL-6 to receptors on mouse splenic macrophages (10^5 cells/ml). Cells were incubated with increasing concentrations of ^{125}I-IL-6 at 4° for 2 hr.

important role in the modulation of the host's immune response to mycobacterial infection.[3,5,10,11,18,19] In this study, we have described the temporary relationship between the production of three cytokines, which have been described previously as being involved in the immunoregulation of infection by *M. avium*. TNF-α can stimulate human and murine macrophages to inhibit intracellular growth of *M. avium*.[6] IL-6 has been shown to suppress TNF-α release by mononuclear phagocytes, and decrease the ability of *M. avium*-infected human macrophages to respond to TNF-α stimulation and to down-regulate TNF-α receptors on macrophages.[5]

TGF-β is a macrophage suppressor factor that has been shown to be produced by macrophages infected with *M. avium*.[3] The early production of TGF-β, at week 1, followed by increasing levels of IL-6 at week 2, may help explain the muted immune response seen in *M. avium* infection. The observation of increased mycobactericidal activity when infected peritoneal macrophages were incubated with splenic cell supernatant from infected mice in the presence of anti-TGF-β antibody supports the theory that the early induction of TGF-β may be an important mechanism by which mycobacteria evade the host's immune response. Although TNF-α was present in the splenic cell supernatant at week 3 of infection, its immunostimulatory effect was most probably down-regulated in the presence of IL-6 and TGF-β.

Mycobacterium avium strain 101 was chosen as the infectious agent because this strain has been described as being extremely virulent in the beige mouse model.[20] The cytokine profile induced by *M. avium* may be a key factor in the pathogenesis of the infection. Furney *et al.*[21] compared TNF-α production, by murine bone marrow macrophages, in response

to different *M. avium* isolates. Isolates, including *M. avium* 101, that multiplied within the host cell delayed TNF-α production, whereas *M. avium* isolates that did not grow within macrophages triggered early TNF-α production. Therefore delayed production of TNF-α may play an important role in the pathogenesis of infection. However, the cytokine profile shown in our study may pertain only to *M. avium* 101.

The steady decrease in the expression of TNF-α receptors on infected splenic macrophages could be secondary to the production of immunosupressive cytokines such as IL-10 and TGF-β. We have previously shown that high levels of IL-10, which peaked at week 2 of infection, were associated with a suppression of response of infected macrophages to TNF-α and granulocyte–macrophage colony-stimulating factor.[18]

This study strongly implicates the early presence of potent immunosuppressors such as TGF-β and IL-6 as a probable cause of the inadequate immune response observed in clinical *M. avium* infection, despite the elevated serum levels of TNF-α reported in AIDS patients.[22] In addition, our observations give further data on the temporary production of cytokines during infection, and implicate TGF-βas an important immunosuppressor of mycobactericidal activity. Further studies examining cell receptor populations, cell recruitment and interactions with other cytokines could possibly elucidate further the mechanisms of immunosuppression observed during *M. avium* infection.

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