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Cytokine profile during latent and slowly progressive primary tuberculosis: a possible role for interleukin-15 in mediating clinical disease

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Summary

Recently, mouse models for latent (LTB) and slowly progressive primary tuberculosis (SPTB) have been established. However, cytokine profiles during the two models are not well established. Using quantitative reverse transcription-polymerase chain reaction (qRT-PCR) we studied the expression levels of interleukin (IL)-2, IL-4, IL-10, IL-12, IL-15, interferon (IFN)-γ and tumour necrosis factor (TNF)-α during the course of LTB and SPTB in the lungs and spleens of B6D2F1Bom mice infected with the H37Rv strain of *Mycobacterium tuberculosis* (*Mtb*). The results show that, except for IL-4, cytokine expression levels were significantly higher during SPTB than LTB in both the lungs and spleens. During LTB, all the cytokines (except IL-2 in the lungs) had higher expression levels during the initial period of infection both in the lungs and spleens. During SPTB, the expression levels of IL-15 increased significantly from phases 1 to 3 in the lungs. The expression levels of IL-10, IL-12 and IFN-γ increased significantly from 2 to 3 in the lungs. IL-10 and IL-15 increased significantly from phases 2 to 3, whereas that of TNF-α decreased significantly and progressively from phases 1 to 3 in the spleens. Over-expression of proinflammatory cytokines during active disease has been well documented, but factor(s) underlying such over-expression is not known. In the present study, there was a progressive and significant increase in the expression levels of IL-15, together with Th1 cytokines (IL-12 and IFN-γ) during SPTB but a significant decrease during LTB. IL-15 is known to up-regulate the production of proinflammatory cytokines, IL-1β, IL-8, IL-12, IL-17, IFN-γ and TNF-α and has an inhibitory effect on activation-induced cell death. IL-15 is known to be involved in many proinflammatory disease states such as rheumatoid arthritis, sarcoidosis, inflammatory bowel diseases, autoimmune diabetes, etc. Our results, together with the above observations, suggest that IL-15 may play an important role in mediating active disease during *Mtb* infection.

Keywords: cytokine, latent, progressive, *Mycobacterium tuberculosis*, RT-PCR

Introduction

An estimated one-third of the world population is believed to be infected with *Mycobacterium tuberculosis* (*Mtb*) globally. However, only 5–10% of immunocompetent humans develop clinical disease during *Mtb* infection [1]. Cytokine-mediated immune responses to *Mtb* infection are important determinants for development of clinical disease/latent infection. It is generally accepted that Th1 cells through production of interferon (IFN)-γ play a protective role during *Mtb* infection, whereas Th2 cytokines induce the development of clinical disease through the production of cytokines such as interleukin (IL)-10 and transforming growth factor (TGF)-β. According to the Th1/Th2 paradigm, failure to resolve infection in susceptible individuals is a consequence of the generation of an inadequate level of Th1-mediated immunity, resulting from the down-regulation of Th1 immunity by Th2 response [2]. On the other hand, it has been shown that Th1 cytokines such as IFN-γ and IL-12 are highly expressed during tuberculosis [3]. Recent studies also show that gene-deleted mice incapable of generating a Th2-response are not more resistant than wild-type mice in

controlling *Mtb* infection [4]. Therefore, immunological parameters that lead to development of latent infection or clinical disease during *Mtb* infection remain unknown. To date, there are a number of newly described cytokines, including IL-15 whose role during *Mtb* is unknown.

IL-15 is a pleiotropic proinflammatory cytokine which is expressed in several inflammatory disorders. It is a 14-kDa glycoprotein which was first described in 1994 as a T cell-activating factor, with structural similarities to IL-2 [5,6]. IL-15 receptor (IL-15R) is composed of three subunits: α, β and γ. The β and γ subunits of the IL-R are shared with IL-2 but the α-chain is unique to IL-15 [5–7].

IL-15 mRNA is expressed in a wide variety of tissues and cells but its protein is produced mainly by monocytes and macrophages [5,8–10]. IL-15Rα mRNA has been observed to have a wide cellular distribution, such as in T cells, macrophages, thymic cells, skeletal muscles, lung and liver [6]. This widespread distribution of the IL-15R system suggests that IL-15 mediates pleiotropic effects on multiple cell types, including cells of non-lymphoid origin [8]. IL-15 induces T cell proliferation and cytokine production, stimulates locomotion and chemotaxis of normal T cells [5,7,11,12] and protects them from apoptosis [13].

Elevated concentrations of IL-15 have been associated with several autoimmune and inflammatory diseases. Increased levels of IL-15 have been detected in the synovial fluids and in the synovium of rheumatoid arthritis patients [14,15]. IL-15 is also highly expressed in inflammatory pulmonary diseases, including tuberculosis, sarcoidosis and chronic bronchitis [16]. IL-15 is also highly expressed in leprosy skin lesions [16].

Recently, animal models for latent tuberculosis (LTB) and slowly progressive primary tuberculosis (SPTB) have been developed and the results show that the two disease models closely mimic human LTB and SPTB [17,18]. However, cytokine profiles, in particular those of IL-15 during LTB and SPTB, have not been well documented.

In the present study, we investigated the expression patterns of IL-2, IL-4, IL-10, IL-12, IL-15, IFN-γ and TNF-α during the course of LTB and SPTB. We show the possible role of IL-15 in mediating active disease during *Mtb* infection by up-regulating the production of proinflammatory cytokines.

Materials and methods

Ethical clearance

The experiments were performed with the permission of the Norwegian Experimental Animal Board, and Norwegian law corresponds with the European Commission on the Protection of Experimental Animals.

Mice

B6D2F1Bom (C57BL/6JBomxDBA/2JBom) mice were purchased from Bomholtgård Breeding and Research Centre Ltd, Denmark. This centre follows the FELASA (Federation of European Laboratory Animal Science Association) recommendations for health control of laboratory animals. The animals were acclimatized for 15 days before being included in the experiments. All mice were aged 17 weeks when they were used in experiments. The average lifespan of these mice is 975 days [19]. Animals were given standard mouse diet (RMI, SDS, Witham, UK) and water *ad libitum*. They were kept in MAKIII macorolon cages. The cages were kept in negative pressure plastic film isolators (Plastic Film Industries, Milton Keynes, UK). The isolators were supplied with temperature (22 ± 1°C) and humidity (50% ± 10% relative humidity (RH)) controlled air. The room lighting was set at 14/10 light/dark.

Mycobacteria

All experiments were conducted with the H37Rv strain of *Mtb*. The mycobacteria were first cultured on Lowenstein–Jensen media for 10 weeks.

Preparation of inocula

Stock inocula were prepared by subculturing colonies in Middlebrook 7H9 liqiud supplemented by Middlebrook ADC (Difco Laboratories, Detroit, MI, USA) (10 : 1) and 0·05% Tween-80. The stock culture was incubated at 37°C for 10 days and shaken briefly on alternate days. Small aliquots of the stock bacillary suspension were maintained at −70°C. The desired number of bacilli for inoculation was prepared from the stock suspension after diluting with phosphate buffered saline (PBS). The bacillary suspension for inoculation was sonicated for 5 s in a water bath sonicator (General-probe^{R}, HF-frequency-35KHz, Type-T460/H, Hanover, Germany) before use.

Assessment of viability of stock culture by colony forming units (CFU)

The stock culture was sonicated for 30 s in the bath sonicator. It was then serially diluted 10-fold with medium and plated onto Middlebrook 7H10. This medium was prepared by mixing agar base Middlebrook 7H10 (Difco) with Middlebrook OADC (Gibco, Life Technologies Ltd, Scotland, UK). The inoculated plates were incubated at 37°C in a CO_2 (5%) incubator. CFU were counted twice weekly after the appearance of pinpoint size colonies.

Experimental design, routes and doses of infection

First, two separate experiments were performed to know which route and dose were suitable for establishing latent and chronic infection [17,18]. To establish LTB and SPTB, the mice were infected intraperitoneally by injecting 10^4 and $1{\cdot}5 \times 10^6$ CFU, respectively, in 0·2 ml PBS. Twelve uninfected mice served as controls for growth and age and were kept in

a separate isolator. Eight uninfected mice were housed with the infected groups as sentinel controls to check the transmission of infection and environmental conditions in the cages. No cross-infection was observed.

Isolation of total RNA and cDNA synthesis

Groups of three mice were sacrificed at each time-point under sterile conditions to collect lungs and spleens. The harvested organs were immediately weighed and partitioned. One part was immediately homogenized in TRIzol™ (Life Technologies, Gaithersburg, MD, USA) using Hybaid Ribolyser and silica matrix (Hybaid Co. Ltd, Teddington, UK). The remaining part was immediately frozen in liquid nitrogen for subsequent use. Total RNA was isolated according the TRIzol procedure supplied by the manufacturer. The amount of total RNA isolated was measured by GENEQUANT spectrophotometer (Pharmacia Biochemicals, La Jolla, CA, USA) and samples were run on agarose gels to monitor RNA integrity. To generate cDNA, 4 μg of total RNA was reverse-transcribed using 1·5 μM 18-mer oligo dT primer and 200 U M-MLV reverse transcriptase (Promega Corp. Madison, WI, USA) at 42°C for 60 min in the presence of 1 U/μl RNasin (Promega Corp. Madison, WI, USA) and one reaction buffer [50 mM Tris HCl (pH 8·3), 3 mM $MgCl_2$, 75 mM KCl, 10 mM DTT] in a 30 μl reaction volume. Reactions were terminated and the enzyme inactivated by brief heating at 90°C for 3–5 min in a water bath. Reactions were then diluted to 100 μl for ease and accuracy in pipetting and stored at −20°C until use. cDNA synthesis was always monitored by performing reverse transcription-polymerase chain reaction (RT-PCR0 on the housekeeping gene hypoxanthine-phospho-ribosyl-transferase (HPRT) and running the products on 2% agarose gels.

Design and sequence of primers and probes used

All probes and primers were designed by the Primer Express Software (Perkin Elmer/Applied Biosystems, Foster City, CA, USA). The probe was synthesized with FAM as reporter dye and Tamra as the quencher dye. The forward primer (FP), reverse primer (RP) and *Taq*man probe (TP) sequences, all in 3′→5′orientation, were the following:

IL-2: forward primer CCTGAGCAGGATGGAGAATTACA, reverse primer TCCAGAACATGCCGCAGAG, *Taq*man-probe CCCAAGCAGGCCACAGAATTGAAAG.
IL-4: forward primer ACAGGAGAAGGGACGCCAT, reverse primer GAAGCCCTACAGACGAGCTCA, *Taq*man-probe TCCTCACAGCAACGAAGAACACCACA.
IL-10: forward primer GGTTGCCAAGCCTTATCGGA, reverse primer ACCTGCTCCACTGCCTTGCT, *Taq*man-probe TGAGGCGCTGTCATCGATTTCTCCC.
IL-12: forward primer GGAAGCACGGCAGCAGAATA, reverse primer AACTTGAGGGAGAAGTAGGAATGG, *Taq*man-probe CATCATCAAACCAGACCCGCCCAA.
IL-15: forward primer CATCCATCTCGTGCTACTTGT GTT, reverse primer CATCTATCCAGTTGGCCTCTGTTT, *Taq*man-probe AGGGAGACCTACACTGACACAGCCCAA AA.
IFN-γ: forward primer TCAAGTGGCATAGATGTGGAAG AA, reverse primer TGGCTCTGCAGGATTTTCATG, *Taq*man-probe TCACCATCCTTTTGCCAGTTCCTCCAG.
TNF-α forward primer CATCTTCTCAAAATTCGAGTGA CAA, reverse primer TGGGAGTAGACAAGGTACAACCC, *Taq*man-probe CACGTCGTAGCAAACCACCAAGTGGA.

PCR amplified cytokine DNA cloned onto a plasmid vector was used to draw standard curves for quantification of cytokines.

qRT-PCR procedure

Quantitative reverse transcription-polymerase chain reaction (qRT-PCR) was performed using the ABI-prism 7700 Sequence Detector Apparatus (Perkin Elmer/Applied Biosystems, Foster City, CA, USA) and the programs were designed on a Power Macintosh connected to the sequence detection appliance. The qRT-PCR was performed for all cytokines and the housekeeping gene HPRT for 40 cycles in a two-temperature profile: a denaturation period of 15 s at 94°C followed by a 1-min annealing/extension period at 60°C. All PCR reactions were carried out in 25 μl final volumes comprising the following components: 5 μl (200 ng) cDNA, 1× reaction buffer [50 mM Tris-HCl (pH 9·0 at 25°C), 50 mM KCl and 0·1% Triton X-100], 9 mM $MgCl_2$, 900 nM primers and 130 nM probes. DEPC-treated sterile H_2O was added to adjust the final volume to 25 μl. PCR of a cloned DNA positive control and an HPRT control were included to control for PCR set up and cDNA synthesis, respectively.

Quantification of cytokine mRNA in mice tissue

The amount of cytokines was calculated by the Sequence Detection Systems (SDS) software which use standard curves generated by serial dilutions of known amounts of cloned counterparts.

Phases of infection

SPTB was divided into three distinct phases based on lung weight, which is roughly proportional to granuloma index. Granuloma index, on the other hand, is proportional to gross pathological changes (granuloma type and size) observed in the lungs of infected mice during the course of infection [18]. During phase 1 (weeks 1–7) an increase in the bacillary number are noted in the lungs; lesions are absent or small but the mice do not show any sign of sickness. During phase 2 (weeks 8–17) granulomas increase in size and cellular composition of the granulomas mature into separate

Table 1. Differences in cytokine expression levels (mean total mRNA/0·5 μg total RNA) in the lungs of mice during the two disease models (***$P < 0{\cdot}0001$). The expression levels of all cytokines increased at least by twofold [interleukin (IL)-4 and interferon (IFN)-γ] during slowly progressive primary tuberculosis (SPTB) compared to latent tuberculosis (LTB). IL-15 and IL-10 increased by 10-fold and 20-fold, respectively.

Cytokine	Control mice	LTB	SPTB	Differences in cytokine expression levels between LTB and SPTB	Level of significance (*P*-value) between LTB and SPTB
IL-2	254	334	971	Threefold	***
IL-4	278	418	667	Twofold	***
IL-10	968	96	1 785	20-fold	***
IL-12	5 015	1 778	4 520	Threefold	***
IL-15	2 086	566	5 548	10-fold	***
IFN-γ	1 014	6 788	14 434	Twofold	***
TNF-a	3 366	9 106	36 644	Fourfold	***

aggregates of vacuolated macrophages and lymphocytes. The bacillary numbers are stabilized in the lungs and spleens. Some mice start to show signs of disease but mortality is low. During phase 3 (weeks 21–33) the lesions increase in size and there is an increase in mortality. The lung weight increases significantly from phases 1 to 2 and from 2 to 3, in a manner proportional to the granuloma index (data not shown). No distinct phases comparable to that of SPTB were observed during LTB.

Statistical analysis

Differences in cytokine expression levels in the lungs and spleens and between LTB and SPTB were compared using the Mann–Whitney *U*-test. The SPSS computer program was used for data analysis and *P*-values < 0·05 were considered statistically significant.

Results

The results of cytokine expression levels (mean mRNA/0·5 μg total RNA) during LTB and SPTB are presented in Figs 1–4 and Tables 1 and 2. As indicated in the Materials and methods, SPTB was divided into distinct phases based on disease progression. Although no such distinct phases were observed during LTB, results are presented in three phases to make comparisons between the two models easier. Cytokine expression levels, except for IL-4, were higher during SPTB than LTB in both the lungs and spleens. The differences between LTB and SPTB were pronounced in the lungs than in the spleens (Tables 1 and 2) and ranged from twofold (IL-4) to 20-fold (IL-10) in the lungs and two-fold (IL-2) to fourfold (IL-15) in the spleens. In the lungs, the expression levels of IL-10, IL-12 and IL-15 were significantly ($P < 0{\cdot}0001$) higher in controls than in latently infected mice. Moreover, control mice had significantly higher ($P < 0{\cdot}0001$) expression levels of IL-12 than latently infected mice in the spleen (Tables 1 and 2).

During LTB (Figs 1 and 3), all the cytokines with the exception of IL-2 (in the lungs) had generally high expression levels 1–3 weeks post-infection, followed by a decline in both the lungs and the spleens. Some of the cytokines showed an intermittent increase both in the lungs and spleens; for instance, IL-2 and IL-4 (in the lungs) and TNF-α (in the spleens) had their peaks 8 weeks post-infection (data not shown).

Table 2. Differences in cytokine expression levels (mean total mRNA/0·5 μg total RNA) in the spleens of mice during the two disease models (***$P < 0{\cdot}0001$). The expression levels of all cytokines [except interleukin (IL)-2 and IL-4] increased at least by twofold during slowly progressive primary tuberculosis (SPTB) compared to latent tuberculosis (LTB). IL-15 increased by fourfold.

Cytokine	Control mice	LTB	SPTB	Differences in cytokine expression levels between LTB and SPTB	Level of significance (*P*-value) between LTB and SPTB
IL-2	357	1 013	1 396	NS	n.s.
IL-4	376	984	923	NS	n.s.
IL-10	971	2 296	3 914	Twofold	***
IL-12	6 486	2 434	4 517	Twofold	***
IL-15	2 531	3 582	13 126	Fourfold	***
IFN-γ	1 012	8 554	18 843	Twofold	***
TNF-α	3 647	12 184	29 376	Twofold	***

n.s. = No significant difference.

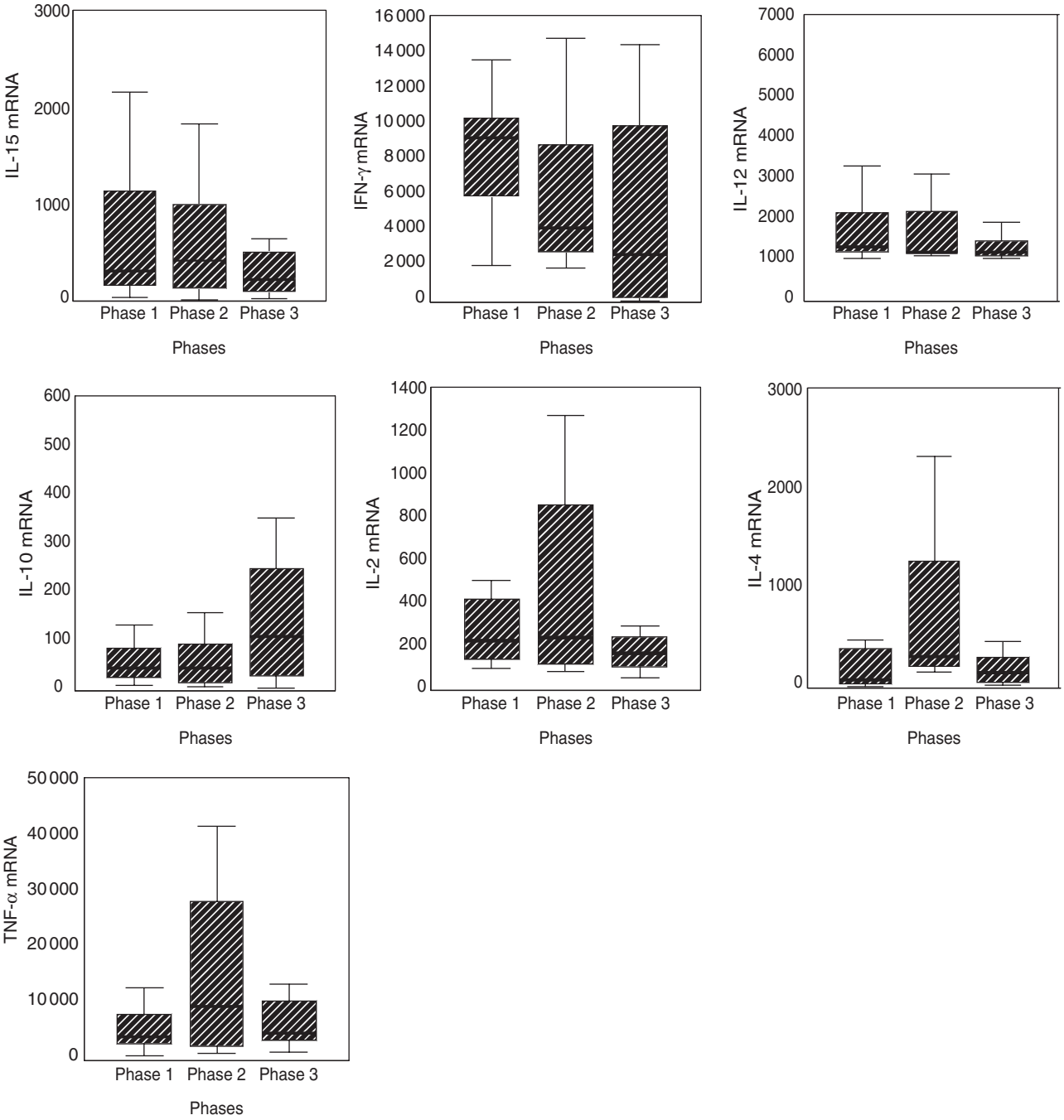

Fig. 1. Box plots (10th, 25th, 50th, 75th and 90th percentiles) showing cytokine expression levels (mean mRNA/0·5 μg total RNA) during latent tuberculosis (LTB) in the lungs of infected mice. The expression levels of interleukin (IL)-15, interferon (IFN)-γ and IL-12 showed a decreasing trend from phases 1 to 3. However, IL-10 levels showed an increasing trend. IL-2 and tumour necrosis factor (TNF)-α levels showed an increasing trend from phases 1 to 2 but a decreasing trend from 2 to 3. However, none of the differences was significant. IL-4 expression levels increased significantly ($P = 0{\cdot}02$) from phases 1 to 2 but decreased significantly ($P = 0{\cdot}02$) from phases 2 to 3 in the lungs of infected mice.

The expression levels of cytokines during SPTB are given in Figs 2 and 4. In the lungs, the expression levels of IL-15 increased significantly from phases 1 to 2 ($P < 0{\cdot}0001$) and 2 to 3 ($P = 0{\cdot}01$). The expression levels of IL-10, IL-12 and IFN-γ increased significantly from phase 2–3 ($P = 0{\cdot}003$, $P = 0{\cdot}006$, $P = 0{\cdot}007$, respectively). However, no significant changes were observed in the expression levels of IL-2, IL-4 and TNF-α in the lungs of infected mice.

In the spleens, the expression levels of IL-2 decreased significantly from phases 1 to 2 ($P = 0{\cdot}049$); that of IL-12 decreased from phases 1 to 2 ($P = 0{\cdot}03$) but increased again from phases 2 to 3 ($P = 0{\cdot}02$). The expression levels of

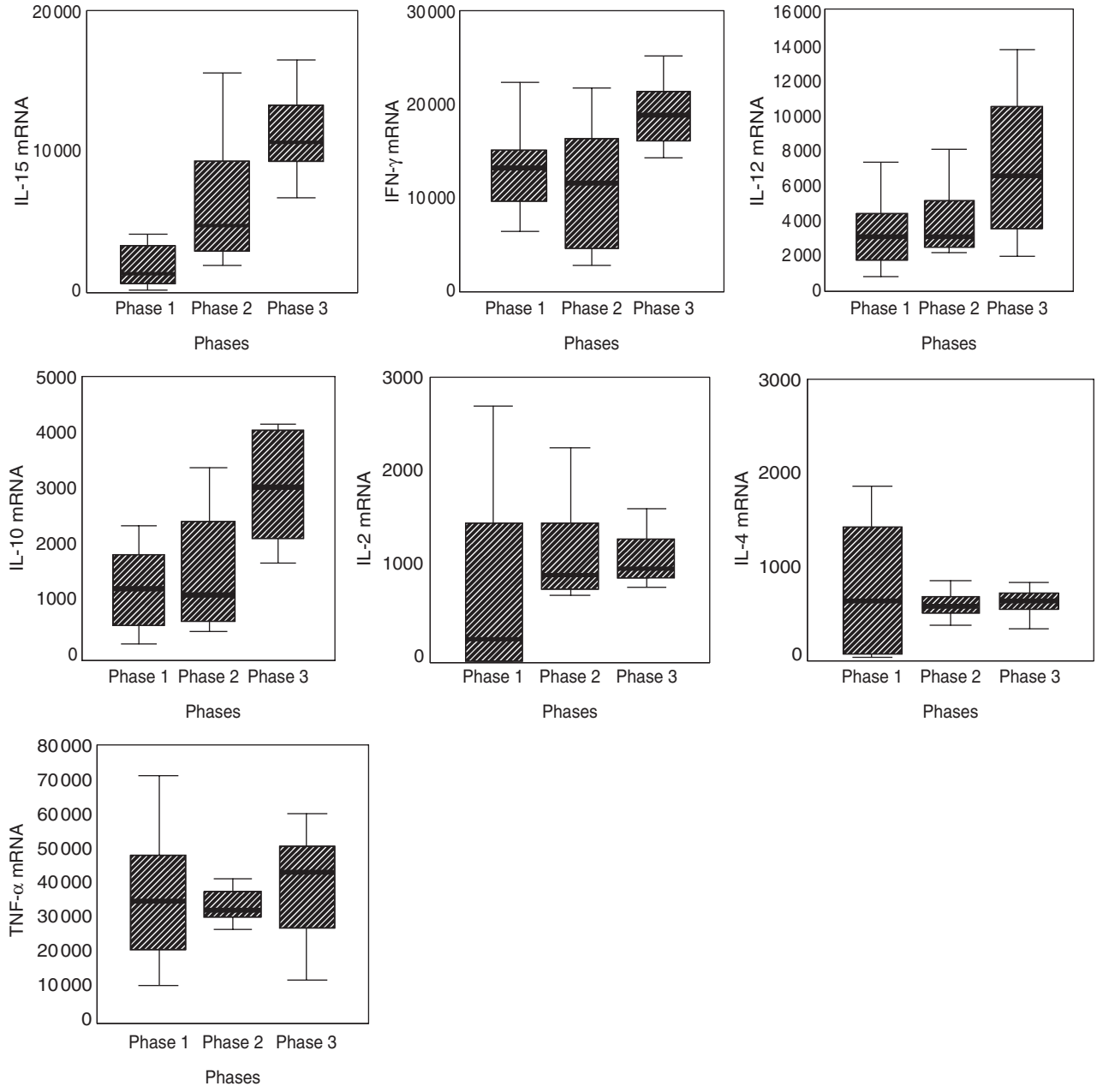

Fig. 2. Box plots showing cytokine expression levels (mean mRNA/0·5 μg total RNA) in the lungs of infected mice during slowly progressive primary tuberculosis (SPTB). The expression levels of interleukin (IL)-15 increased significantly ($P < 0{\cdot}0001$) from phases 1 to 2 and from 2 to 3 ($P = 0{\cdot}01$). The expression levels of interferon (IFN)-γ, IL-12 and IL-10 increased significantly from phases 2 to 3 ($P = 0{\cdot}003$, $P = 0{\cdot}006$ and $P = 0{\cdot}007$, respectively). However, no significant changes were observed in the expression levels of IL-2, IL-4 and tumour necrosis factor (TNF)-α in the lungs of infected mice.

TNF-α decreased significantly from phases 1 to 3 ($P = 0{\cdot}01$). The expression levels of IL-10 increased from phases 2 to 3 ($P = 0{\cdot}005$) and IL-15 increased from phases 2 to 3 ($P = 0{\cdot}03$). No significant changes were seen in the levels of IL-4 and IFN-γ.

Quantitative relationships between cytokines during LTB and SPTB in the lungs and spleens are given in Tables 3–6. During LTB, IL-15 was significantly positively correlated with IL-2, IL-4, IL-10 and IFN-γ (phase 1), IL-10 and IL-12 (phase 2) and IL-10 and IFN-γ (phase 3) in the lungs of infected mice. In the spleens, IL-15 was significantly positively associated with IL-10 and IL-12 (phase 1), TNF-α (phase 2) and IL-2, IL-10, IL-12 and IFN-γ (phase 3).

During SPTB, IL-2, IL-4, IL-10, IL-12 and IL-15 were significantly positively correlated with each other in the lungs of infected mice during phase 1. IL-15 had a highly significant

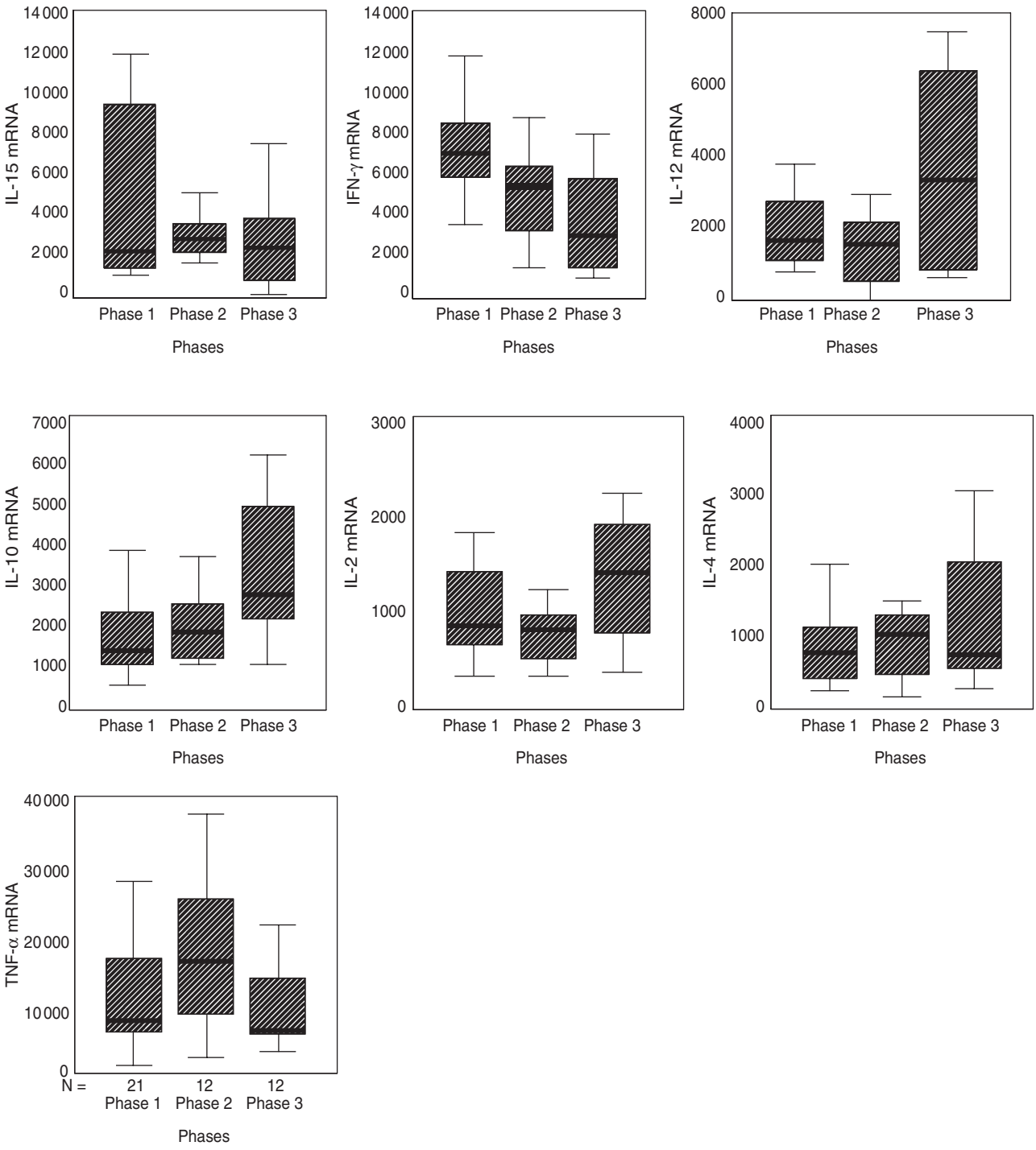

Fig. 3. Box plots showing cytokine expression levels (mean RNA/0·5 μg total RNA) in the spleens of mice during latent tuberculosis (LTB). Similar to the lungs, cytokine expression was depressed during LTB in the spleens. Levels of interleukin (IL)-15 showed a decreasing trend from phases 1 to 3 but the differences were not statistically significant. Interferon (IFN)-γ levels decreased significantly ($P = 0{\cdot}01$, $P = 0{\cdot}02$) from phases 1 to 2. Levels of IL-10 increased significantly ($P = 0{\cdot}02$) from phases 2 to 3.

positive correlation with IL-2, IL-4, IL-10 and IL-12 (phase 1), with IL-10 (phases 2 and 3).

In the spleens, IL-15 had a significant positive correlation with IL-10, IL-12, IFN-γ and TNF-α but a significant negative correlation with IL-4 during phase 1. During phases 2 and 3, IFN-γ had a significant positive correlation with IL-2 and IL-15. IL-10 had a significant positive correlation with IL-15 and IFN-γ but a significant negative correlation with TNF-α.

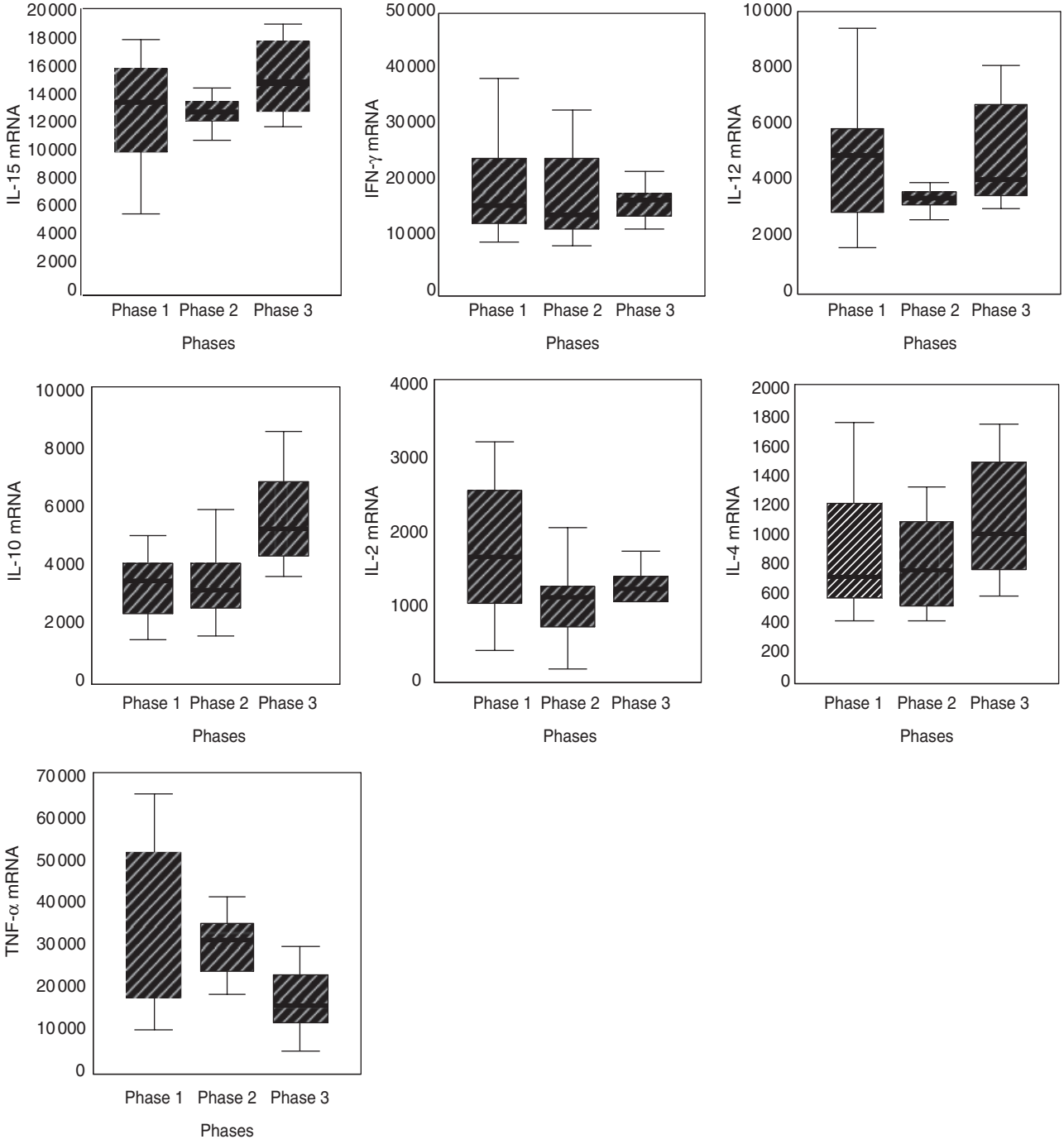

Fig. 4. Box plots (10th, 25th, 50th, 75th and 90th percentiles) showing cytokine expression levels (mean mRNA/0·5 μg total RNA) in the spleens of mice during slowly progressive primary tuberculosis (SPTB). The expression levels of interleukin (IL)-15 increased significantly ($P = 0{\cdot}03$) from phases 2 to 3. IL-12 decreased significantly ($P = 0{\cdot}03$) from phases 1 to 2 but increased again from phases 2 to 3 ($P = 0{\cdot}02$). IL-10 increased significantly ($P = 0{\cdot}005$) from phases 2 to 3. IL-2 decreased significantly ($P = 0{\cdot}049$) from phases 1 to 2. Tumour necrosis factor (TNF)-α decreased significantly ($P = 0{\cdot}001$) from phases 2 to 3.

Discussion

In the present study, the expression levels of IL-15 increased significantly and progressively from phase 1–3, whereas those of IL-10, IL-12 and IFN-γ increased significantly from phases 2 to 3 in the lungs of infected mice. Increased expression of these cytokines was associated with progression of active disease, whereas decreased expression of IL-10, IL-15 and IFN-γ were associated with containment of *Mtb* infection, leading to LTB in the lungs of infected mice. Similarly,

Table 3. Quantitative relationships between cytokines during latent tuberculosis (LTB) in the lungs of infected mice based on Spearman's rank correlation. There are no distinct phases during LTB; however, it is divided into three phases to make comparison with slowly progressive primary tuberculosis (SPTB) easier. Significant correlations (*P*-values) are given as $^{*}P < 0{\cdot}05$, $^{**}P < 0{\cdot}01$ and $^{***}P < 0{\cdot}0001$.

Cytokine	IL-2	IL-4	IL-10	IL-12	IFN-γ	TNF-α	IL-15
Phase 1							
IL-2	1·00	0·383	0·540*	0·323	–0·144	–0·351	0·661**
IL-4		1·00	0·496*	0·644**	0·229	0·338	0·512*
IL-10			1·00	0·321	0·634**	0·061	0·340
IL-12				1·00	0·045	0·466	0·662**
IFN-γ					1·00	0·112	0·224
TNF-α						1·00	0·726**
IL-15							1·00
Phase 2							
IL-2	1·00	0·923***	0·231	0·741**	0·860***	–0·308	0·343
IL-4		1·00	0·314	0·790**	0·811**	–0·336	0·413
IL-10			1·00	0·427	0·042	–0·616*	0·925***
IL-12				1·00	0·608*	–0·175	0·629*
IFN-γ					1·00	–0·210	0·175
TNF-α						1·00	–0·441
IL-15							1·00
Phase 3							
IL-2	1·00	0·837**	0·102	0·333	0·655*	–0·788**	0·417
IL-4		1·00	–0·196	0·207	0·441	–0·815***	0·231
IL-10			1·00	0·319	0·734	0·252	0·790**
IL-12				1·00	0·518	–0·441	0·266
IFN-γ					1·00	–0·336	0·888***
TNF-α						1·00	–0·098
IL-15							1·00

the expression levels of IL-10 and IL-15 was increasing during SPTB, whereas the expression levels of IL-2, IL-4, IL-12, IL-15 and IFN-γ was decreasing significantly during LTB in spleens of infected mice. Increased expression of Th1 cytokines (IL-12 and IFN-γ), together with IL-10, in the present study is not consistent with the widely held view of Th1/Th2 paradigm which explains the failure to resolve *Mtb* infection, leading to development of active disease as a consequence of the down-regulation of Th1 cytokines by Th2 cytokines, IL-10 and TGF-β [2,3].

Previously, several studies have implicated IL-10 with development of active disease through multiple proposed immunosuppressive mechanisms, such as inhibition of IFN-γ production [20–22]; down-regulation of macrophage activation [23]; inhibition of TNF-α production [24]; and down-regulation of the expression of co-stimulatory molecules [25]. Both Th1 cytokines (IL-12 and IFN-γ) and a Th2 cytokine (IL-10) increased concurrently in the present study, indicating that IL-10 has no effect on the generation of these cytokines. Our findings are in line with a recent observation that IL-10 gene disrupted and wild-type mice generated similar and vigorous IL-12p40 and IFN-γ synthesis and displayed identical capacity to control *Mtb* infection, as opposed to the views of Th1/Th2 paradigm [4].

One possible explanation for this inconsistent observation is partly because little is known about some of the newly emerging cytokines, such as IL-15 during *Mtb* infection. In the present study, the expression of IL-15 increased progressively and significantly (10-fold in the lungs and fourfold in the spleens) during SPTB, but decreased significantly during LTB in the lungs and spleens of infected mice. IL-15 was associated more significantly with IL-10 than any other cytokine in the lungs during progressive disease (Table 5). Although it is difficult to determine the exact relationships, IL-10 has been shown to up-regulate the production of IL-15 in mouse macrophages [10]. Similarly, IL-15 has also been shown to up-regulate the production of IL-10 [24].

IL-15 was significantly positively correlated with IL-12, IFN-γ and TNF-α during phase 1 and with IFN-γ during phase 2 in the spleen, and with IL-12 during phase 1 in the lungs. Although IL-12 and IFN-γ increased from phases 2 to 3 during disease progression lack of correlation between IL-15 and these cytokines in the lungs during the last two phases could be explained by the fact that the expression of IL-15 rose earlier (during phase 2) compared to the expression of IL-12 and IFN-γ, which increased progressively during the third phase of disease progression. Peak IL-15 expression was observed at around week 17 post-infection, followed by a continued rise until week 30 where it showed a decreasing

Table 4. Quantitative relationships between cytokines during latent tuberculosis (LTB) in the spleens of infected mice based on Spearman's rank correlation. There are no distinct phases during LTB; however, it is divided into three phases to make comparison with latent (LTB) and slowly progressive primary tuberculosis (SPTB) easier. Significant levels (*P*-values) are given as $^{*}P < 0{\cdot}05$, $^{**}P < 0{\cdot}01$ and $^{***}P < 0{\cdot}0001$

Cytokine	IL-2	IL-4	IL-10	IL-12	IFN-γ	TNF-α	IL-15
Phase 1							
IL-2	1·00	0·629**	–0·038	0·473*	–0·247	–0·034	0·105
IL-4		1·00	0·018	0·361	–0·428	0·127	–0·181
IL-10			1·00	0·577**	0·273	0·606**	0·737***
IL-12				1·00	0·136	0·461	0·565**
IFN-γ					1·00	0·067	0·268
TNF-α						1·00	0·248
IL-15							1·00
Phase 2							
IL-2	1·00	0·664*	0·594*	0·797**	0·154	–0·336	–0·427
IL-4		1·00	0·028	0·846**	0·350	0·284	0·147
IL-10			1·00	0·371	0·091	–0·417	–0·594*
IL-12				1·00	0·399	0·004	–0·049
IFN-γ					1·00	0·553	0·217
TNF-α						1·00	0·595*
IL-15							1·00
Phase 3							
IL-2	1·00	0·259	0·755**	0·671*	0·685*	0·161	0·587*
IL-4		1·00	–0·035	–0·343	–0·287	–0·364	–0·280
IL-10			1·00	0·692*	0·678*	0·399	0·685*
IL-12				1·00	0·958***	0·098	0·825**
IFN-γ					1·00	0·000	0·839***
TNF-α						1·00	–0·112
IL-15							1·00

trend. On the other hand, the expression levels of IL-12, IFN-γ and TNF-α increased after week 20 post-infection and the peak for the three cytokines was at week 33 (termination of the study). Moreover, the association between IL-12, on one hand, and IL-15, IFN-γ and TNF-α on the other hand, followed some kind of stage-specific sequence in the lungs during disease progression. IL-12 had a significant positive association with IL-15 during phase 1, with IFN-γ during phase 2 and finally with IFN-γ and TNF-α during phase 3, which suggests the pattern in which these cytokines increased during disease progression. Moreover, IFN-γ was the only cytokine that was significantly positively associated with IL-15 during phase 2 of disease progression in the spleens. Previously it has been shown that during SPTB mice remained healthy until week 16, after which infected mice started to develop pathology [18]. The above results may suggest that the increase in IL-15 expression during the second phase of disease progression may have contributed to increased expression (during phase 3) of IL-12, IFN-γ and TNF-α (not significant), cytokines believed to contribute to disease pathology, particularly in the development of granulomatous lesions [26].

Although it is difficult to determine the exact role of IL-15 during development of clinical disease from the present study, this cytokine has been shown to exhibit broad inflammatory effects on several disease states, including dermatological inflammatory diseases, inflammatory rheumatic diseases, autoimmune diabetes, inflammatory bowel diseases and pulmonary inflammation, reviewed by McInnes and Gracie [27]. As a proinflammatory cytokine, IL-15 has been shown to enhance local T cell activation and proliferation and the production of proinflammatory cytokines, such as IFN-γ and TNF-α by T cells and IL-12 by monocytes in patients with bowel disease [28]; induction of IL-1β production [29]; stimulation of IL-8 and IL-17 production, cytokines known for their promotion of neutrophilia [30,31]; up-regulation of IL-12 and IFN-γ expression in patients with TB [3] and sarcoidosis [32]. IL-15 has been shown to antagonize the biological activities of IL-2 by inhibiting activation-induced cell death and by maintaining $CD4^{+}$ and $CD8^{+}$ memory cells that are important in the maintenance of long-lasting specific immune responses to foreign pathogens [29].

The known proinflammatory effects of IL-15 indicated above, together with its increased expression during the progression of clinical disease in the present study, may suggest that by up-regulating proinflammatory cytokines, IL-15 could determine the course *Mtb* infection. Our results are supported by an earlier study by Taha *et al.* [3], who found increased expression of IL-12 and IFN-γ but found no significant difference between active and inactive TB in the expression of IL-2, IL-4 and TNF-α in the lungs of human patients. More specifically, IL-15 has been shown to

Table 5. Quantitative relationships between cytokines during the three phases of slowly progressive primary tuberculosis (SPTB) in the lungs of infected mice, based on Spearman's rank correlation. Significant levels (*P*-values) are given as $^{*}P < 0{\cdot}05$, $^{**}P < 0{\cdot}01$ and $^{***}P < 0{\cdot}0001$.

Cytokine	IL-2	IL-4	IL-10	IL-12	IFN-γ	TNF-α	IL-15
Phase 1							
IL-2	1·00	0·662**	0·807***	0·830***	0·241	0·501*	0·906***
IL-4		1·00	0·415	0·559**	0·289	0·005	0·766***
IL-10			1·00	0·831***	−0·012	0·684**	0·786***
IL-12				1·00	−0·243	0·639**	0·732
IFN-γ					1·00	−0·495*	0·274
TNF-α						1·00	0·409
IL-15							1·00
Phase 2							
IL-2	1·00	0·105	0·573	0·559	0·615*	−0·259	0·042
IL-4		1·00	−0·420	−0·580	−0·538	−0·025	0·042
IL-10			1·00	0·245	0·266	−0·683*	0·587*
IL-12				1·00	0·937**	−0·004	−0·524
IFN-γ					1·00	0·095	−0·497
TNF-α						1·00	−0·536
IL-15							1·00
Phase 3							
IL-2	1·00	0·144	0·123	0·315	0·466	0·396	0·480
IL-4		1·00	−0·804**	0·671*	0·615*	0·629*	−0·378
IL-10			1·00	−0·699*	−0·720**	−0·741**	0·734**
IL-12				1·00	0·881***	0·937***	−0·392
IFN-γ					1·00	0·979***	−0·392
TNF-α						1·00	−0·427
IL-15							1·00

Table 6. Quantitative relationships between cytokines during the three phases of slowly progressive primary tuberculosis (SPTB) in the spleens of infected mice, based on Spearman's rank correlation. Significant levels (*P*-values) are given as $^{*}P < 0{\cdot}05$, $^{**}P < 0{\cdot}01$ and $^{***}P < 0{\cdot}0001$.

Cytokine	IL-2	IL-4	IL-10	IL-12	IFN-γ	TNF-α	IL-15
Phase 1							
IL-2	1·00	0·575**	0·188	0·353	−0·285	−0·211	−0·110
IL-4		1·00	−0·135	−0·414	−0.257	−0·251	−0·514*
IL-10			1·00	0·534*	0·517*	0·676**	0·734***
IL-12				1·00	0·330	0·195	0·686**
IFN-γ					1·00	0·748***	0·748***
TNF-α						1·00	0·696***
IL-15							1·00
Phase 2							
IL-2	1·00	0·187	−0·446	−0·093	0·709**	−0·005	0·157
IL-4		1·00	0·226	−0·044	−0·319	−0·291	−0·102
IL-10			1·00	−0·157	−0·256	−0·077	0·388
IL-12				1·00	0·038	0·044	0·129
IFN-γ					1·00	0·132	0·608*
TNF-α						1·00	−0·146
IL-15							1·00
Phase 3							
IL-2	1·00	0·345	0·591	0·491	0·679*	−0·309	0·391
IL-4		1·00	0·427	−0·055	0·189	−0.318	0·355
IL-10			1·00	−0·073	0·615*	−0·818**	0·782**
IL-12				1·00	0·510	0·473	−0·164
IFN-γ					1·00	−0·355	0·433
TNF-α						1·00	−0·755**
IL-15							1·00

be an important trigger for IL-17-mediated pulmonary inflammation, reviewed by McInnes and Gracie [27]. A recent study shows that IL-15 participates in pulmonary disease by attracting neutrophils, modulating cell surface expression molecules and neutrophil adhesion onto pulmonary cells, a major event leading to development of inflammation [33].

During LTB, most cytokines showed elevated expression 1–3 weeks post-infection, followed by a decline. The expression levels of IL-10, IL-12 and IL-15 (in the lungs) and IL-12 (in the spleens) were significantly lower than those of control mice (Tables 1 and 2), and this depressed expression was associated with development of latency. Although reasons for such a phenomenon is not clear, it could be related to tight regulation of the expression of IL-15 by complex mechanisms at the transcriptional [8,34–36] and secretion levels [37].

In the present study, we observed high IL-4 expression levels between weeks 5–9 in both disease models, followed by a decline. Our results confirm (in mice) earlier reports that IL-4 is generated in the lungs of human TB patients [37–40]. On the other hand, however, we observed neither an increase in IL-4 expression with disease progression nor the down-regulation of Th1 cytokines, associated with this cytokine, reported by Biedermann *et al.* [41].

We assume that the failure of Th1-mediated immunity to resolve *Mtb* infection in mice is not due to the down-regulation of Th1-mediated immunity by Th2 cytokines (IL-10) but may be due to the over-expression of these cytokines together with other proinflammatory cytokines, mediated by IL-15.

The study has one limitation, in that the intraperitoneal route of infection has been used. Although the method has been successful as evidenced from progression of disease in latently infected mice following corticosterone treatment and during SPTB, it is worth noting that it may not be the best method as aerosol is the natural route of *Mtb* infection.

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