Tumour necrosis factor and interferon-γ are required in host resistance against virulent *Rhodococcus equi* infection in mice: cytokine production depends on the virulence levels of *R. equi*

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SUMMARY

Rhodococcus equi is a facultative intracellular bacterial pathogen that causes pneumonia in foals and immunosuppressed humans. There are at least three virulence levels of *R. equi* and these pathogenicities are associated, in mice, with the presence of virulence plasmids. This study focused on cytokine secretion, in mice, in the course of a primary infection with sublethal doses of *R. equi* strains of different virulence levels (virulent, intermediately virulent and avirulent). Tumour necrosis factor (TNF) and interferon-γ (IFN-γ), but not interleukin-4 (IL-4) and interleukin-10 (IL-10), were induced endogenously in mice in relation to the multiplication and clearance of virulent and intermediately virulent strains of *R. equi*. These cytokines were not detected in mice infected with avirulent *R. equi*. Deaths occurred among mice treated with monoclonal antibodies (mAbs) against either TNF or IFN-γ prior to sublethal dose infection with virulent and intermediately virulent strains of *R. equi*, but not with avirulent *R. equi*. These results suggested that cytokine production depended largely on the virulence levels of *R. equi*: TNF and IFN-γ were required early during infection with virulent *R. equi* to limit replication and clearance of bacteria within the organs, but they were not necessary for limiting infection with avirulent *R. equi*.

INTRODUCTION

Rhodococcus equi is a facultative, intracellular, Gram-positive coccobacillus, which causes suppurative pneumonia and ulcerative enteritis in foals aged 1–3 months.[1] Recently, *R. equi* has been recognized as an emerging opportunistic pathogen of immunocompromised hosts, such as human immunodeficiency virus (HIV)-infected patients, and the number of *R. equi* pneumonia case reports in patients with acquired immune deficiency syndrome (AIDS) has increased.[2–4]

The discovery of virulence-associated antigens and virulence plasmids has clarified some aspects of the virulence of *R. equi*.[5–9] At least three virulence levels of *R. equi* have been identified: virulent *R. equi*, which has 15 000 to 17 000 MW antigens and a virulence plasmid of 85–90 kb, and causes suppurative pneumonia in foals (mouse $LD_{50} = 10^6$); *R. equi* strains of intermediate virulence, which have a 20 000 MW antigen and a virulence plasmid of 79–100 kb, and reside in the submaxillary lymph nodes of pigs (mouse $LD_{50} = 10^7$); and avirulent *R. equi*, which has no virulence-associated antigens or plasmid (mouse $LD_{50} > 10^8$).[10,11] Epidemiological studies have revealed that these virulent *R. equi* live separately in horses and pigs, and that avirulent *R. equi* is distributed widely in our environment.[12] In humans, the majority of isolates from patients with AIDS were either virulent or of intermediate virulence,[9] and most isolates from immunocompromised patients without AIDS were avirulent.[13]

Studies on *R. equi* infection in foal and mouse models have contributed some understanding on the aspects of pathogenesis and immunology.[1,4,14,15] However, the relative contributions of cell-mediated immunity and humoral immunity to resistance against *R. equi* infections remain unclear and paradoxical.[1,4,14] Passive transfer of hyperimmune equine plasma was demonstrated to have a protective effect in foals and to decrease the incidence and severity of *R. equi* pneumonia on farms where the infection was endemic;[16,17] however, vaccination of mares and foals with *R. equi* virulence-associated protein did not protect foals.[18,19] On the other hand, cell-mediated immunity has been shown to be crucial in host defence against *R. equi* in mouse models.[14,15] Live virulent *R. equi*, rather than killed or avirulent organisms, elicited protective immunity to *R. equi* infection in mice, and studies using monoclonal antibodies (mAbs) and transgenic knockout mice indicated that CD4+ T cells participated in the clearance of *R. equi*.[20–24]

The virulence mechanism in *R. equi* is still unknown, but at least two important virulence determinants are involved in the pathogenesis of the disease: one is the virulence plasmid, which is required for *R. equi* to grow within host cells; and the other is the granulomagenic activity that is related to the lipids and nature of the cell wall of the species, which induces the characteristic pathological changes.[25,26] The immune mechanism that mediates resistance during primary infection with *R. equi* strains, which have three different virulence levels, also remains unresolved.[12] To determine which components of the immune system confer protection to primary *R. equi* infection in mice, we evaluated the host immune response to infection with various strains of *R. equi* in one strain of mice. In the present study, the role of cytokines in the development of infection in mice infected with three different virulence types of *R. equi* was examined. Our results showed that cytokine production depended largely on the virulence types of *R. equi*.

MATERIALS AND METHODS

Experimental animals
Female ddY mice were obtained from S. L. C. Hamamatsu (Shizuoka, Japan) at 5 weeks of age and were housed under standard conditions.

Preparation of bacteria
R. equi ATCC 33701 (virulent), its plasmid-cured derivative, ATCC 33701P⁻ (avirulent) and A5 (intermediately virulent) were used. Virulence of the strains has been reported elsewhere.[6,9] The 50% lethal doses (LD$_{50}$) of ATCC 33701, ATCC 33701P⁻ and A5 were $2·6 \times 10^6$, $>10^8$ and $1·0 \times 10^7$, respectively. The strains were grown in Brain–Heart Infusion broth (BHI; Difco Laboratories, Detroit, MI). Cultures of these strains were incubated in a rotary shaker at 100 r.p.m. for 48 hr at 30°, and the cultures were stored as suspensions of cells in 20% glycerol at −80°. Aliquots (1 ml) were periodically thawed, and viable colony-forming units (CFU) were quantified by plating serial dilutions on nutrient agar plates. For inoculation of mice, bacterial cultures were thawed and washed with 0·01 M phosphate-buffered saline (PBS; pH 7·4) immediately before use and then diluted to a predetermined number in PBS; actual numbers of bacteria inoculated were confirmed by plate counts at the time of injection.

Determination of viable R. equi bacteria in the organs
The number of bacteria in the liver, spleen and lungs were estimated at various time intervals following intravenous inoculation. Mice were killed by cervical dislocation and their organs were removed aseptically and homogenized in 2 ml of sterile PBS, as described previously.[26] Results were expressed as mean CFU ± standard error (SE) per gram of organ for each group of three mice, and transformed by log$_{10}$.

Preparation of organs for cytokine assays
The liver, spleen and lung homogenates used for interferon-γ (IFN-γ), tumour necrosis factor (TNF), interleukin (IL)-4 and IL-10 assays were prepared as described previously.[27,28] Half of each organ, aseptically removed from mice as described previously, was suspended in RPMI-1640 medium (Gibco Laboratories, Grand Island, NY) containing 1% (wt/vol) 3 (cholamidopropyl)   dimethylammonio-)-1-propanesulfonate

(CHAPS; Wako Pure Chemical Co., Kyoto, Japan) and 10% of the homogenates were ground up using a Dounce grinder. After the homogenates were clarified by centrifugation at 2000 **g** for 20 min at 4°, the organ extracts were stored at −80° until required for the cytokine assay.

TNF assay
TNF was quantified by a double-sandwich enzyme-linked immunosorbent assay (ELISA), as described previously.[27] Flat-bottom 96-well plate wells were coated with 200 ng of hamster antirecombinant mouse (rMu) TNF mAb (Genzyme Co., Boston, MA) in 0·1 M carbonate buffer (pH 9·6). Following washing three times with PBS containing 0·05% Tween 20, the plates were blocked with 0·1 M carbonate buffer containing 0·1% bovine serum albumin (BSA; Sigma Chemical Co., St. Louis, MO) and 2% goat serum (Vector Laboratories, Burlingame, CA). The wells were incubated with 100 μl of a sample, in duplicate, at 4° and then exposed to 5 μg rabbit polyclonal antirMuTNF immunoglobulin G (IgG) in PBS containing 0·1% BSA and 2% goat serum. The plate was developed by using peroxidase-labelled goat antirabbit IgG (Jackson Immunoresearch Laboratories, Inc., Avondale, PA) and *o*-phenylendiamine (Wako) substrate. Selected amounts of rMuTNF-α (Genzyme), diluted in 100 μl of PBS containing 0·1% BSA and 2% goat serum, were used to create a standard curve to accompany each experiment. The assay was determined to detect TNF concentrations as low as 25 pg/ml.

IFN-γ assay
The IFN-γ assay was carried out by using a double-sandwich ELISA, as described previously.[27] Purified rat antimouse IFN-γ mAb, produced by hybridoma R4-6 A2 and rabbit antirMu IFN-γ serum, was used for the ELISA. All ELISAs were run with rMu IFN-γ, which was produced and purified by Genetech, Inc. (San Francisco, CA).

IL-4 and IL-10 assays
IL-4 and IL-10 levels were determined using ELISAs, as described previously.[28] Flat-bottom 96-well plates were coated with 200 ng rat antimouse-IL-4 mAb (11B11; PharMingen, San Diego, CA) or rat antimouse-IL-10 mAb (JES5-2 A5; PharMingen) in 0·05 M carbonate buffer (pH 9·6). The plates were blocked with PBS containing 2·5% BSA. The wells were then incubated with 100 μl of a sample, in duplicate, overnight at 4° and were then exposed to 50 ng of biotinylated rat anti-IL-4 mAb (BVD6-24G2; PharMingen) or biotinylated rat anti-IL-10 mAb (SXC-1; PharMingen) in PBS containing 1% BSA. The plate was developed with avidin-biotinylated peroxidase complex (Vector Laboratories) and *o*-phenylendiamine (Wako) substrate. Selected amounts of rMuIL-4 (Genzyme) or rMuIL-10 (Genzyme), diluted in 100 μl of PBS containing 1% BSA, were used to create a standard curve to accompany each experiment. The assay was sensitive enough to detect concentrations of IL-4 and IL-10 below 15·7 pg/ml and 78·13 pg/ml, respectively.

In vivo depletion of endogenous IFN-γ and TNF
Hybridoma cells secreting mAbs against mouse IFN-γ (R4-6 A2; rat IgG1) and mouse TNF (MP6-XT22·11; rabbit IgG1) were used to study *in vivo* depletion of endogenous IFN-γ and TNF. mAbs found in the ascites fluid were partially

purified by $(NH_4)SO_4$ precipitation. The mice were given a single intravenous injection of 1 mg rat antimouse IFN-γ mAb or rat antimouse TNF mAb (in 0·2 ml of pyrogen-free saline) 30 min before infection with *R. equi*, as described previously.[27,28] Normal rat globulin was injected as a control.

RESULTS

Kinetics of bacterial growth in mice infected with sublethal doses of *R. equi*

After the mice were infected intravenously with $4·0 × 10^5$ of ATCC 33701 (virulent), $1·0 × 10^6$ of A5 (intermediately virulent) or $3·3 × 10^5$ of ATCC 33701P⁻ (avirulent), the courses of infection were compared in the liver, spleen and lungs. Two distinct patterns emerged (Fig. 1). Strains ATCC 33701 and A5 demonstrated viable counts in the liver and spleen that decreased slightly 1 day after infection, followed by five to 10-fold increases over the next 3 days. Thereafter, there was a slow decline in viable counts. In the lungs, the viable counts decreased more rapidly, and without multiplication, than they did in the liver and spleen. By contrast, there was no apparent growth of bacteria in the liver, spleen and lungs of mice infected with ATCC 33701P⁻.

IFN-γ, TNF, IL-4 and IL-10 production in mice infected with sublethal doses of *R. equi*

The titres of IFN-γ, TNF, IL-4 and IL-10 from pooled sera and extracts from the spleens, livers and lungs of mice were monitored at various time-points after infection with the three different strains of *R. equi*. The titres of IFN-γ and TNF in the spleens and livers of mice infected with virulent *R. equi* increased to day 4 after infection, in accordance with the number of bacteria in those organs, and then decreased (Table 1). IFN-γ and TNF were detected also in both the bloodstream and lungs, but with titres lower than those of the liver and spleen (Table 1). By contrast, minimal levels of IFN-γ and TNF were detected in the bloodstream and lungs of the mice infected with avirulent *R. equi*. In the spleens and livers, TNF appeared several hours after infection and the titre decreased gradually thereafter.

Neither IL-4 nor IL-10 were detected in the mice infected with any strain of *R. equi* used in this study (data not shown).

Effect of *in vivo* administration of mAbs against IFN-γ and TNF in mice infected with a sublethal dose of *R. equi* ATCC 33701 or A5

To determine the roles of IFN-γ and TNF in host defence against virulent *R. equi*, mice were injected with 1 mg anti-IFN-γ mAb, anti-TNF mAb or normal rat globulin 30 min before infection with a sublethal dose of virulent *R. equi* ($2·3 × 10^5$ of ATCC 33701 and $4·0 × 10^5$ of A5). Sixty-seven per cent or 50% of the mice that received anti-TNF mAb died following a sublethal dose infection with ATCC 33701 or A5, respectively (Fig. 2). On the other hand, 17% or 23% of the mice that received anti-IFN-γ mAb, died following a sublethal dose infection with ATCC 33701 or A5, respectively. No normal rat globulin-treated mice died until 14 days postinoculation.
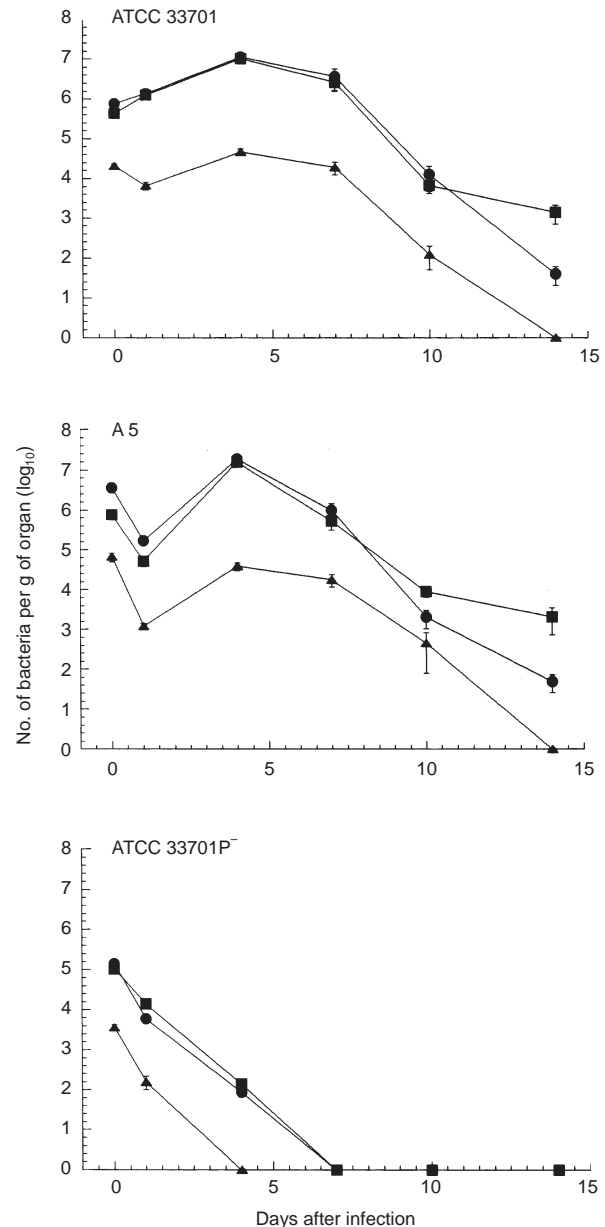
Figure 1. The course of *R. equi* infection in mice infected intravenously with a sublethal dose of virulent strain ATCC 33701, intermediate virulent strain A5 or avirulent strain ATCC 33701P⁻ was followed against time in the spleen (closed circle), liver (closed square) and lungs (closed triangle). Data shown are the mean number of bacterial counts ± SEM ($n = 3$).

Effect of *in vivo* administration of mAbs against IFN-γ and TNF on bacterial growth in mice infected with *R. equi* ATCC 33701, A5 or ATCC 33701P⁻

To confirm the effect of *in vivo* administration of mAbs against IFN-γ and TNF on host defence in mice, mice treated with 1 mg anti-IFN-γ mAb or anti-TNF mAb were infected with a sublethal dose of ATCC 33701, A5 or ATCC 33701P⁻, and the number of bacteria in the livers, spleens and lungs were determined on days 0, 4, 7 and 14 after infection. Suppression of the elimination of bacteria from these organs was observed in anti-IFN-γ and anti-TNF mAb-treated mice infected with

Table 1. Kinetics of production of interferon-γ (IFN-γ) and tumour necrosis factor (TNF) in the spleen, liver, lungs and bloodstream of mice infected intravenously with a sublethal dose of virulent strain ATCC 33701, intermediate virulent strain A5 or avirulent strain ATCC 33701P$^-$

| Strain | Days after infection | Cytokine production (pg/ml) | | | | | | | |
| | | IFN-γ | | | | TNF | | | |
		Spleen	Liver	Lungs	Bloodstream	Spleen	Liver	Lungs	Bloodstream
ATCC	0	<25	<25	<25	<25	885±158	<25	<25	<25
33701	1	995±330	326±130	<25	319±319	1541±641	65±64	<25	<25
	4	1192±41	936±163	171±88	586±133	3073±123	1673±383	108±44	240±160
	7	440±14	74±74	<25	<25	1305±88	439±168	<25	<25
	10	<25	<25	<25	<25	754±202	56±56	<25	<25
	14	<25	<25	<25	<25	246±26	<25	<25	<25
A5	0	<25	<25	<25	<25	488±169	39±23	<25	<25
	1	1078±305	347±111	73±12	570±22	646±54	119±69	<25	<25
	4	1635±305	1537±180	398±191	2363±462	3152±455	495±286	209±32	514±48
	7	1040±239	318±149	127±7	379±71	2651±839	1574±910	245±87	<25
	10	<25	<25	<25	<25	<25	<25	<25	<25
	14	<25	<25	<25	<25	<25	<25	<25	<25
ATCC	0	<25	<25	<25	<25	929±389	211±92	<25	<25
33701P$^-$	1	<25	<25	<25	<25	229±94	<25	<25	<25
	4	<25	<25	<25	<25	185±77	<25	<25	<25
	7	<25	<25	<25	<25	97±40	<25	<25	<25
	10	<25	<25	<25	<25	36±16	<25	<25	<25
	14	<25	<25	<25	<25	89±37	<25	<25	<25

Data shown are the mean of cytokine titres ± SEM ($n=3$).
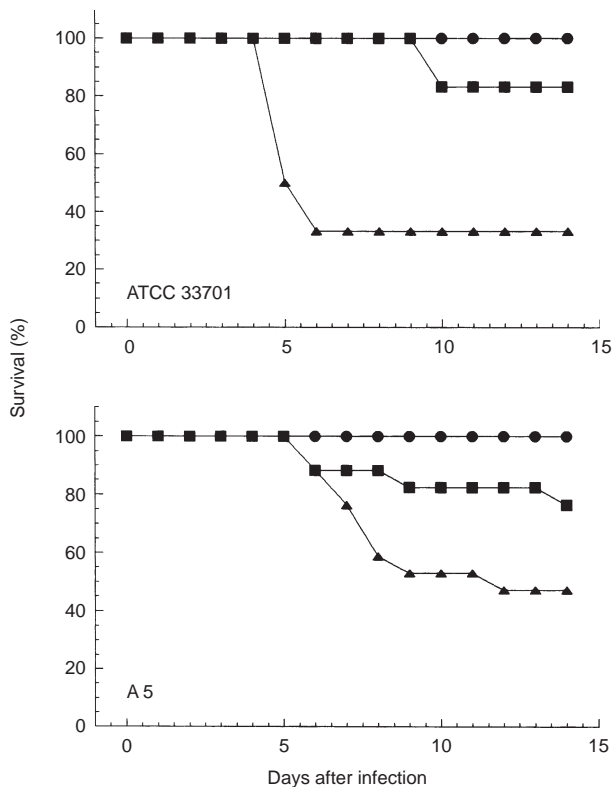
Figure 2. Effect of IFN-γ and TNF neutralization on the survival of *R. equi*-infected mice. Mice were injected with 1 mg anti-IFN-γ mAb (closed square), anti-TNF mAb (closed triangle) or normal rat globulin (closed circle) 30 min before infection with a sublethal dose of virulent *R. equi*.

Table 2. Course of *Rhodococcus equi* infection in anti-interferon-γ (anti-IFN-γ) monoclonal antibody (mAb)-treated, anti-tumour necrosis factor (anti-TNF) mAb-treated or normal rat globulin-treated mice infected intravenously with a sublethal dose of virulent strain ATCC 33701, intermediate virulent strain A5 or avirulent strain ATCC 33701P$^-$ followed against time in the spleen

| Strain | Days after infection | Treatment ($\log_{10}$ no. of bacteria/g of spleen) | | |
		Control	Anti-IFN-γ	Anti-TNF
ATCC	0	5·61±0·06	5·61±0·06	5·61±0·06
33701	4	7·02±0·02	7·49±0·16	7·54±0·06
33701	7	6·42±0·15	6·60±0·17	8·95±0·03*
	14	1·93±0·16	0	9·00±0·01*
A5	0	6·25±0·01	6·25±0·01	6·25±0·01
	4	7·02±0·07	7·14±0·05	7·52±0·14
	7	5·73±0·16	7·20±0·18*	8·79±0·19*
	14	2·06±0·28	4·44±0·31*	9·00±0·01*
ATCC	0	6·23±0·50	6·23±0·50	6·23±0·50
33701P$^-$	4	3·79±0·60	3·74±0·13	4·36±0·13
	7	2·66±0·10	2·87±0·02	3·16±0·06
	14	0	0	0

Data shown are the mean number of bacterial counts ± SEM ($n=$ 3 to 5).

*Significantly different from the value for normal globulin-treated mice at $P<0.05$.

R. equi strains of virulence and intermediate virulence, but not in normal rat globulin-treated mice, on days 7 and 14 (Table 2). Anti-TNF mAb treatment more effectively suppressed elimination of bacteria in mice than did anti-IFN-γ mAb.

DISCUSSION

The present study focused on cytokine secretion, in mice, during the course of primary infection with *R. equi* strains of virulence, intermediate virulence and avirulence. TNF and IFN-γ, but not IL-4 and IL-10, were induced endogenously in mice in association with the multiplication and clearance of inoculated bacteria, and cytokine production depended largely on the virulence level of *R. equi*. Results obtained by neutralization of TNF and IFN-γ *in vivo* by mAb clearly indicated the essential role of cytokines in the control and survival of virulent *R. equi* infection, but they did not affect avirulent *R. equi*. These data suggested that TNF and IFN-γ were secreted in response to microbial stimuli, and that TNF was required early in infection to limit replication of bacteria within the organs. This study also showed that the absence of the virulence plasmid prevented ATCC 33701 from increasing its systemic infection in cytokine-depleted mice.

Depletion of TNF *in vivo* by anti-TNF mAb led to a lethal course of virulent *R. equi* infection in mice given a sublethal dose, in contrast with the results of studies by Nordmann et al.[20] They observed only inhibition of the ability of euthymic Swiss mice to clear an intravenous challenge at 7 days postinoculation by anti-TNF and/or anti-IFN-γ antibodies.[20] This discrepancy might be explained by a difference in the virulence of *R. equi* used in the two studies because no effects of TNF neutralization were observed in the mice infected with avirulent *R. equi* in this study.

The number of bacteria in the organs of mice inoculated intravenously with virulent *R. equi* and treated with mAb to IFN-γ did not show significant reduction by day 4 (Table 2), and rose significantly at day 7 and 14. The effect of IFN-γ neutralization by mAb on the course of pulmonary *R. equi* infection was examined by Kanaly et al.[22] who showed that BALB/c mice treated with mAb failed to clear pulmonary *R. equi* infection, resulting in the development of pulmonary granulomas. These and our data suggest that secretion of IFN-γ is essential for immune clearance of *R. equi* from mice.

Macrophage activation is a key component of the protective immune response to *R. equi* infection because the key event in the pathogenesis of *R. equi* infection in mice is considered to be the ability of virulent *R. equi* to survive and multiply in macrophages.[29] A previous study showed that the pathogenesis of *R. equi* infection involves at least two important virulence determinants, both of which play critical roles in the disease: one is the virulence plasmid, which is required for *R. equi* to grow within host cells; and the other is the granulomagenic activity that is related to the lipid and cell wall contents of the species, which induces the characteristic pathological changes.[26] The present study clearly showed a relationship between virulence of *R. equi* strains and their capacity to induce TNF secretion. More recently, Giguere & Prescott[30] evaluated intracellular survival and measured cytokine induction by mouse macrophages infected with a virulent *R. equi* strain, 103[+], its avirulent plasmid-cured derivative, 103[−], and heat-killed 103[+]. The number of bacteria in the macrophages infected with 103[−] and heat-killed 103[+] decreased progressively, whereas the 103[+] numbers remained constant over 48 hr. However, their study found no difference in IL-12 and TNF production between virulent and avirulent *R. equi*.[30] This difference from might reflect the *in vitro* nature of Giguere & Prescott's work.

The release of cytokines is an important part of the immune response against an infection, and several studies have described the importance of TNF and IFN-γ in the immune response against *R. equi*.[20,22,23] Indeed, *in vivo* neutralization of TNF and IFN-γ exacerbates *R. equi* infection.[20,22] The present study clearly showed that the induction of TNF and IFN-γ in serum and organs of mice infected with virulent *R. equi* corresponded with the proliferation of *R. equi* in the organs; i.e. virulent *R. equi* induced higher cytokine secretion than did avirulent *R. equi*. The ability of macrophages to produce TNF appears to be a critical step in the activation of the first line of defence against foreign organisms, and it is not surprising that several pathogens have evolved virulence mechanisms that interfere with the host's cytokine responses. Interference with TNF-α has been reported for several bacteria, such as *Yersinia*, brucellae and *Mycobacterium avium*.[31–33] Mice infected with wild-type *Y. pestis* produce much less TNF-α than mice infected with *Y. pestis* cured of a virulence plasmid (pYV), indicating that a factor suppressing TNF-α synthesis is encoded by the pYV plasmid.[34] Therefore, virulent *R. equi* is quite different from those pathogens that induce suppression of TNF release.

A study by Nordmann et al.[20] showed that *in vivo* TNF and IFN-γ serum levels in *R. equi*-infected mice remained below the level of detectability in mice infected with an AIDS isolate of *R. equi*. The strain used in Nordmann's study was not virulent because the LD$_{50}$ of the strain demonstrated $> 10^8$ bacteria in BALB/c mice by the intravenous route, suggesting that it was avirulent. This contention is supported by the results of our study in which low or no levels of TNF and IFN-γ were detected in the serum of mice infected with avirulent *R. equi*.

T helper 1 (Th1) cells are defined by their ability to produce IFN-γ and IL-2, while Th2 cells produce IL-4, IL-5 and IL-10.[35,36] In mice and humans, many infectious agents produce preferential Th1 (type 1) or Th2 (type 2) immune responses.[37] The relative balance of type 1 versus type 2 responses determines the outcome, including the ability of infected hosts to control a number of intracellular pathogens.[23,28,37] This effect is mediated by the respective lymphokines produced.[35,36] In this study, *in vivo* administration of anti-IFN-γ or anti-TNF mAb accelerated the death of mice inoculated with a sublethal dose of virulent *R. equi*, and no IL-4 or IL-10 could be detected in mice with virulent and avirulent *R. equi* infections. These results suggest that immune clearance of *R. equi* appears to be a Th1-like response and that IFN-γ is a primary mediator.[4,22,23]

In conclusion, TNF and IFN-γ were found to be critical cytokines that offered protection against primary infection with a sublethal dose of *R. equi* strains of virulence and intermediate virulence, but they did not suppress avirulent *R. equi* infection.

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