INFECTION AND IMMUNITY, July 1996, p. 2782–2786 Vol. 64, No. 7
0019-9567/96/$04.00+0

Tumor Necrosis Factor Alpha and Interleukin-12 Contribute to Resistance to the Intracellular Bacterium *Brucella abortus* by Different Mechanisms

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Received 9 February 1996/Returned for modification 22 March 1996/Accepted 29 April 1996

Both interleukin-12 (IL-12) and tumor necrosis factor alpha (TNF-α) are produced early in intracellular bacterial infection. Depletion of either IL-12 or TNF-α by a single injection of specific antibody 4 h before the injection of *Brucella abortus* 19 led to the exacerbation of infection 2 weeks later. Whereas the effect of IL-12 depletion on resistance was persistent and exacerbation was still significant 6 weeks later, the bacterial numbers in mice depleted of TNF-α were similar to the bacterial numbers in control infected mice by 6 weeks postinfection. Massive splenomegaly, which is often seen in 2-week *Brucella*-infected mice, was not observed in IL-12- or TNF-α-depleted mice. Both IL-12- and TNF-α-depleted mice showed reduced cell accumulation in the spleen compared with the massive cell accumulation in control infected mice. Granuloma formation in livers was much reduced in IL-12-depleted mice but not in TNF-α-depleted mice. Gamma interferon (IFN-γ) production by cells from TNF-α-depleted mice was not significantly different from that of cells from control infected mice. In contrast, the production of IFN-γ by both $CD4^+$ and $CD8^+$ T cells from IL-12-depleted mice was greatly reduced, compared with that from control infected mice. This effect was still observed when the antibody injection was delayed for up to 7 days postinfection, but injections of anti-IL-12 antibody into mice with established *Brucella* infection had no significant effect on IFN-γ production by T cells. Taken together, these results suggested that IL-12 contributed to resistance mainly via an IFN-γ-dependent pathway and had a profound effect on the induction of acquired cellular resistance. In contrast, TNF-α was involved in resistance possibly via direct action on effector cells and may not be essential for the induction of acquired cellular resistance.

Resistance to facultative bacterial pathogens such as *Brucella abortus* depends on acquired cell-mediated resistance, characterized by the activation of T lymphocytes and subsequent activation of macrophages for increased killing of such organisms. Soluble factors, namely cytokines, are key mediators that lead to the induction of cell-mediated resistance. Interleukin-12 (IL-12) and tumor necrosis factor alpha (TNF-α) are two cytokines, produced mainly by macrophages, which play an important role in resistance to infection with intracellular bacteria. Depletion of IL-12 prior to infection resulted in major exacerbation of infection with *Listeria monocytogenes* (21), *B. abortus* (25), and *Mycobacterium* spp. (3, 18). Like IL-12 depletion, depletion of TNF-α also results in an exacerbation of infection with intracellular bacteria such as *Listeria* spp. (7, 14) and mycobacteria (11).

The effects of IL-12 on resistance to infection with intracellular pathogens could be linked to gamma interferon (IFN-γ) production. The exacerbation of infection by *B. abortus* (25), *Leishmania major* (19), and mycobacteria (18) in IL-12-depleted mice correlated with decreased IFN-γ production. An infusion of recombinant IFN-γ (rIFN-γ) reversed the exacerbation of listerial infection in IL-12-depleted mice (21). Conversely, neutralization of IFN-γ abrogated the protective effect of rIL-12 against infection with *Leishmania major* (19) and *Toxoplasma gondii* (4). On the other hand, although TNF-α has been shown to be a costimulator of IFN-γ production by NK cells from SCID splenocytes in vitro (22), it is not clear whether TNF-α is required for the induction of an IFN-γ-producing Th1 response. In vitro experiments with ovalbumin-specific T-cell receptor transgenic mice showed that TNF-α had no direct effect on Th1 development (8). Furthermore, IFN-γ infusion did not reverse the exacerbation of listerial infection in TNF-α-depleted mice (21), suggesting that the role of TNF-α is most likely different from that exhibited by IL-12 and is, at least partly, independent of IFN-γ.

The infection of mice with attenuated vaccine strain 19 of *B. abortus* leads to a chronic infection lasting up to 9 to 12 weeks, with the activation of T cells to produce IFN-γ apparent at about 2 weeks postinfection (25). This prolonged course of infection allows time to explore the sequential factors involved in induction of cell-mediated immunity required to control these and other intracellular bacteria. In this report, by using neutralizing antibodies to TNF-α and IL-12, we explore the role of endogenous TNF-α and IL-12 in resistance to brucella infection, particularly the influence of TNF-α or IL-12 depletion at the beginning of infection on subsequent activation of an IFN-γ-producing Th1 response. The possible mechanisms by which TNF-α and IL-12 are involved in resistance are discussed.

MATERIALS AND METHODS

Mice and bacteria. CBA mice were bred by pedigreed brother-sister mating in the Department of Microbiology, University of Melbourne. They were housed under conditions of isolation and fed sterile pellets and water to maintain their infection-free status. The *B. abortus* attenuated vaccine strain, strain 19, was originally obtained from the Commonwealth Serum Laboratories (Melbourne, Australia). *Brucella* organisms were maintained by weekly subculture on horse blood agar. For infection, mice were injected intravenously with 5×10^5 *B.*

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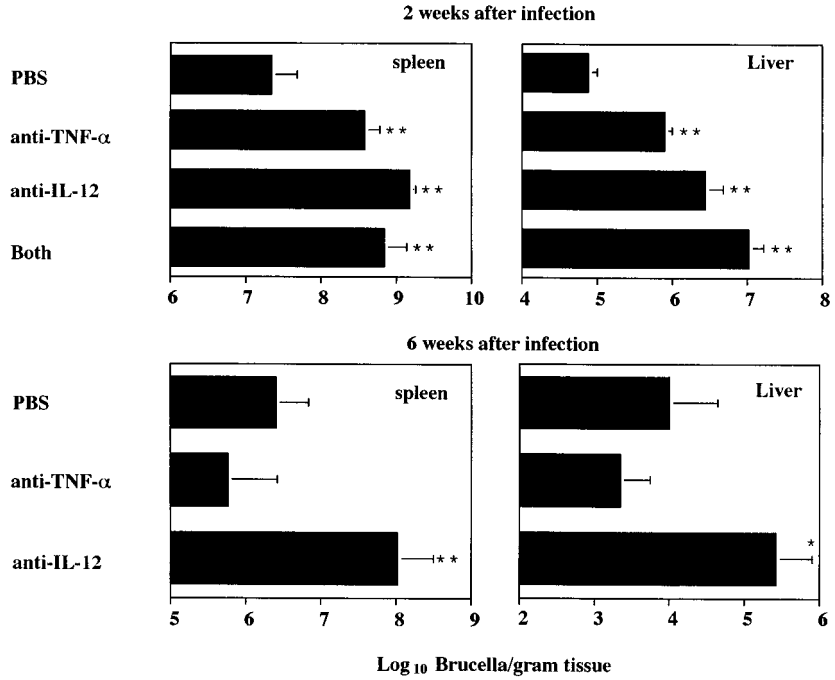

FIG. 1. Effects of neutralization of TNF-α and IL-12 on brucella infection. Four hours before intravenous injection with 5×10^5 *B. abortus* 19 cells, mice were injected intraperitoneally with 0.2 ml of sterile phosphate-buffered saline (PBS), 2 mg of polyclonal rabbit anti-mouse TNF-α antibody, 2 mg of anti-IL-12 MAb, or 1 mg each of anti-IL-12 antibody and anti-TNF-α antibody. Mice were killed 2 or 6 weeks later. The spleen and liver from each mouse were weighed, and the bacterial numbers in weighed portions were counted. Data are the means ± standard deviations of log bacterial numbers in organs from five mice per group. The significance of the difference in bacterial numbers between depleted mice and control mice was assessed by Student's *t* test. *, $P < 0.01$; **, $P < 0.001$.

abortus 19 cells from a 24-h culture on horse blood agar. Soluble *Brucella* protein (SBP) was used as the recall antigen in spleen lymphocyte cultures; its preparation has been described elsewhere (26).

MAbs. Anti-IL-12 monoclonal antibody (MAb) was prepared from the hybridoma C15.6 (rat immunoglobulin G1; kindly provided by G. Trinchieri, Wistar Institute, Philadelphia, Pa.). Isotype control MAbs were prepared from an anti-influenza virus hemagglutinin hybridoma (gift of M. Anders, Department of Microbiology, University of Melbourne, Melbourne, Australia). Polyclonal rabbit anti-mouse TNF-α was prepared from the sera of rabbits which had been injected intramuscularly with 50 μg of rTNF-α in Freund's complete adjuvant and given monthly booster injections without adjuvant. Gamma globulin was purified by 50% saturated ammonia sulfate precipitation. One milligram of anti-TNF-α protein neutralized more than 10^5 U of TNF activity. Control gamma globulin was prepared from preimmune rabbit serum.

Quantitation of infection. Mice were killed by CO_2 narcosis at 14 days postinfection, and their spleens and livers were removed and weighed. Weighed fragments of spleens and livers were homogenized in normal saline with an Ultra Turrax homogenizer (Janke and Kunkel K. G., Breisgau, Germany). The numbers of *B. abortus* cells in these organs were established by plating serial 10-fold dilutions of organ homogenates in saline on a horse blood agar plate.

Culture for cytokine production. Spleen cells (2×10^6/ml) were cultured in Dulbecco's modified Eagle's medium (Gibco Laboratories, Grand Island, N.Y.) with 10% fetal calf serum and 60 μg of SBP per ml for 24 h. For T-cell culture, $CD4^+$-enriched T cells were cultured at 2×10^6 cells with or without antigen (same concentration as stated above) in a 2-ml volume in the presence of 4×10^6 irradiated normal spleen cells as antigen-presenting cells (APC). For $CD8^+$-enriched cells, 100 U of rIL-2 per ml was also present in the culture. Culture supernatants were harvested 72 h later. Negative selection of $CD4^+$ T cells and positive selection of $CD8^+$ T cells have been described previously (26).

Cytokine assay. IFN-γ was assayed by the suppression of proliferation of WEHI-279 cells (17) in comparison with an IFN-γ standard (catalog no. Gg 02-901-533) supplied by the National Institutes of Health, Bethesda, Md. The specificity of the IFN-γ assay was checked with an anti-IFN-γ monoclonal antibody (6).

NO_2^- assay. Nitric oxide (NO) production by spleen cells cultured as described above for cytokine production was determined by the Griess reaction (5). Briefly, culture supernatants (50 μl) were mixed with 100 ml of 1% sulfanilamide (Sigma, St. Louis, Mo.) and 100 μl of 0.1% *N*-(1-naphthyl)ethylenediamine dihydrochloride in 2.5% polyphosphoric acid (Sigma) at room temperature for 5 min. A_{540} was measured. NO_2^- was quantified by comparison to the $Na(NO_2)$ (Sigma) standard.

Histology. Fragments of livers and spleens from mice were fixed in 10% formalin-buffered solution. Tissue sections were prepared by Central Laboratory, Pathology Department, University of Melbourne. Slides were stained with hematoxylin and eosin for microscopic examination and microphotography.

Statistics. The statistical significance of the data of bacterial counts was determined by Student's *t* test.

RESULTS

Effects of the depletion of IL-12 or TNF-α on bacterial numbers. Intraperitoneal administration of polyclonal rabbit anti-mouse TNF-α antibody greatly exacerbated subsequent infection with *B. abortus*. The numbers of brucellae per gram of spleen or liver at 2 weeks postinfection in TNF-α-depleted mice were more than 10-fold higher than those in control infected mice ($P < 0.001$) (Fig. 1). An exacerbation of *Brucella* infection was also observed in mice which had been intraperitoneally injected with anti-mouse IL-12 MAb (Fig. 1), as we had demonstrated previously (25). Simultaneous injection of anti-IL-12 antibody and anti-TNF-α antibody showed no additive or synergistic effect on infection. An injection of preimmune rabbit serum globulin or isotype control MAb had no effect on brucella infection (data not shown). Such experiments were repeated five times with similar findings.

Although TNF-α depletion with polyclonal antibody resulted in the exacerbation of infection at 2 weeks postinfection, these mice recovered from infection as efficiently as did control infected mice at 6 weeks postinfection (Fig. 1). In contrast, mice with their IL-12 depleted by one injection of antibody before infection still showed significant exacerbation of infection at 6 weeks ($P < 0.001$).

Effects of the depletion of IL-12 or TNF-α on inflammatory response to *Brucella* infection. The infection of mice with *B. abortus* 19 normally resulted in marked splenomegaly within 2 weeks of infection. However, mice depleted of TNF-α or IL-12 and subsequently subjected to brucella infection had much

TABLE 1. Effects of IL-12 and TNF-α depletion on spleen size during *Brucella* infection[a]

Treatment	Spleen wt (mg)	
	2 wk	6 wk
PBS	498 ± 25	332 ± 83
Anti-IL-12	111 ± 9	110 ± 25
Anti-TNF-α	161 ± 10	132 ± 13
Normal	64 ± 1	60 ± 7

[a] Mice were injected with either 0.2 ml of sterile PBS, 1 mg of anti-IL-12 MAb, or 2 mg of polyclonal rabbit anti-mouse TNF-α antibody and then injected later intravenously with 5×10^5 *B. abortus* 19 cells. Mice were killed 2 or 6 weeks later. Data are the means ± standard deviations for five mice per group and are typical of three repeated experiments.

smaller spleens than did control infected mice and only twice the normal spleen weight, compared with more than seven times the normal weight in control infected mice (Table 1). Histological examination revealed numerous granulomas in the livers of control mice and TNF-α-depleted mice 2 weeks after infection. However, granulomas were rarely seen in IL-12-depleted mice. The spleens in control infected mice had massive cell accumulation in the marginal zone and red pulp, while the spleens of IL-12- or TNF-α-depleted, *Brucella*-infected mice had a structure similar to that of a normal spleen (data not shown).

By 6 weeks postinfection, splenomegaly in control infected mice was reduced, although they were still five times the normal weight. Cell infiltration in the spleen was also reduced. Spleens from IL-12- and TNF-α-depleted mice were similar to those from normal mice. Liver lesions in control infected mice and TNF-α-depleted mice had decreased. Small granulomas were observed in 6-week infected mice with IL-12 depletion.

Effects of IL-12 or TNF-α depletion on IFN-γ production by T cells. As shown previously, IL-12 depletion resulted in diminished IFN-γ production by splenic T cells (25). Here, IFN-γ production by both $CD4^+$ T cells and $CD8^+$ T cells was inhibited in IL-12-depleted mice (Table 2). IFN-γ production by $CD8^+$ T cells from *Brucella*-infected mice was IL-2 dependent in our in vitro bulk culture system, but $CD8^+$ T cells from uninfected mice did not produce significant amounts of IFN-γ in the presence of exogenous IL-2. In contrast to the profound effect of IL-12 on IFN-γ production by spleen cells, both $CD4^+$ T cells and $CD8^+$ T cells from TNF-α-depleted, *Brucella*-infected mice were capable of producing IFN-γ. The results shown in Table 2 are from 2-week infected mice. Similar results were obtained from three repeated experiments.

Effects of the time of anti-IL-12 MAb injection on brucella infection. To test when IL-12 is required to influence resistance to brucella infection and IFN-γ production by spleen cells, mice were given anti-IL-12 antibody before or after infection. The results showed that delaying the injection of anti-IL-12 antibody until 7 days after infection still significantly exacerbated infection and inhibited IFN-γ production by spleen cells (Table 3). Because of the absence of macrophage-activating IFN-γ, there was no enhanced NO production in IL-12-depleted mice. When the effects of TNF-α were tested in the same way, the results were more complicated. Although mice that had been given anti-TNF-α antibody 2 or 7 days after infection still had higher numbers of bacteria in their spleens and livers than did control infected mice, delayed injection of anti-TNF-α antibody had only a moderate effect on splenomegaly and had no effect on IFN-γ and NO production by spleen cells (Table 3).

Effects of in vivo and in vitro IL-12 depletion on IFN-γ production by spleen cells from mice with established *Brucella* infections. Mice were infected with 5×10^5 brucellae for 10 weeks and then given 2 mg of anti-IL-12 antibody. IFN-γ production by spleen cells from these mice was not significantly different from that by spleen cells from untreated infected mice (Table 4). Similarly, the addition of anti-IL-12 antibody to in vitro cultures of spleen cells from *Brucella*-infected mice had no dramatic effect on IFN-γ production by spleen cells (Tables 4 and 5). Thus, it appears that once IFN-γ production is established, IL-12 is not required.

DISCUSSION

Macrophage-derived cytokines play an important role in the induction and expression of immunity to infection by intracellular bacteria. Two such cytokines, IL-12 and TNF-α, have been demonstrated to be essential for resistance to these infections. This report shows that by TNF-α or IL-12 depletion in vivo both cytokines are involved in the resistance to *Brucella* infection. While TNF-α contributes to resistance mainly by an IFN-γ-independent pathway, IL-12 mediates antibacterial immunity by regulating IFN-γ production.

The induction of a Th1 response is required not only for recovery from primary infection by intracellular pathogens (1, 4) but also, perhaps more importantly, to counter efficiently secondary infection. Therefore, production of the characteristic Th1 cytokine, IFN-γ, is regarded as an important marker for the induction of cellular immunity (10). It has been demonstrated that TNF-α is required for IFN-γ production by NK cells in vitro (24), although TNF-α depletion did not affect early in vivo IFN-γ production, presumably by NK cells, during *Listeria* infection (15). Furthermore, in vitro experiments using

TABLE 2. Effects of IL-12 and TNF-α depletion on IFN-γ production by splenocytes[a]

Treatment	IFN-γ (U/ml)[b]									
	Unseparated[c]		$CD4^{+d}$				$CD8^{+d}$			
	Medium	SBP	Medium	SBP	APC	SBP + APC	Medium	SBP	APC	SBP + APC
PBS	3 ± 0.3	16 ± 1	<1	2 ± 1	<1	11 ± 1	<1	2 ± 1	<1	9 ± 1
Anti-IL-12	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1
Anti-TNF-α	4 ± 1	21 ± 1	<1	<1	<1	16 ± 2	<1	8 ± 2	<1	21 ± 7
Normal	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1

[a] See Table 1, footnote *a*.
[b] Data are the means ± standard deviations of triplicate cultures from one of three repeated experiments.
[c] Whole spleen cells cultured for 24 h with or without SBP.
[d] T-cell subpopulations cultured for 72 h with APC and SBP antigen, controls of T cells alone, T cells plus SBP, or T cells plus APC.

TABLE 3. Effects of IL-12 or TNF-α depletion and time of antibody injection[a] on *Brucella* infection

Antibody treatment	Time of antibody administration	Spleen wt (mg)[b]	Log brucellae/g of tissue[b]		IFN-γ (U/ml)[c]		NO (μM)[c]
			Spleen	Liver	Without SBP	With SBP	
IL-12	−4 h	110 ± 7	9.16 ± 0.25**	6.64 ± 0.02**	<1	<1	12 ± 4
	2 days	113 ± 15	9.14 ± 0.06**	6.78 ± 0.17**	<1	<1	6 ± 3
	7 days	183 ± 40	8.72 ± 0.39**	5.79 ± 0.16**	<1	5 ± 1	5 ± 3
TNF-α	−4 h	160 ± 17	8.64 ± 0.17**	6.33 ± 0.21**	24 ± 11	48 ± 6	14 ± 1
	2 days	350 ± 66	8.30 ± 0.34*	5.89 ± 0.27**	65 ± 14	62 ± 3	31 ± 2
	7 days	283 ± 31	9.06 ± 0.08**	6.34 ± 0.06**	63 ± 13	128 ± 20	38 ± 3
None		507 ± 35	7.52 ± 0.11	5.14 ± 0.15	9 ± 1	29 ± 1	28 ± 2
Normal		70 ± 10			<1	<1	10 ± 1

[a] Mice were given one intraperitoneal injection of 2 mg of anti-IL-12 antibody or anti-TNF-α antibody 4 h before (−4 h) infection or 2 or 7 days after infection. Mice were infected intravenously with 5×10^5 *B. abortus* 19 cells and killed 2 weeks after infection. The results from one of two repeated experiments are shown.

[b] Log numbers of brucellae in organs and spleen weights are the means ± standard deviations for five mice per group. The bacterial numbers in organs of depleted mice were significantly different from those of control infected mice. *, $P < 0.01$; **, $P < 0.001$ by Student's *t* test.

[c] Unseparated spleen cells were cultured for 24 h with or without SBP antigen. Data for IFN-γ and NO (only without SBP) production are the means ± standard deviations of triplicates from each sample.

ovalbumin-specific T-cell receptor transgenic T cells demonstrated that TNF-α was not required for differentiation of Th0 cells to IFN-γ-producing Th1 cells (8). This study showed that depletion of TNF-α before *Brucella* infection did not abrogate IFN-γ production by T cells in response to *Brucella* antigens. It suggests that TNF-α is not involved in inducing the activation of IFN-γ-producing T cells. In contrast to TNF-α-depleted mice, IL-12-depleted mice showed significant inhibition of IFN-γ production by spleen cells to *Brucella* antigens. This is consistent with the well-known function of IL-12, that is, stimulating NK cells and T cells to produce IFN-γ and favoring a Th1 cell response (20). As shown in this study, IFN-γ production by both $CD4^+$ and $CD8^+$ T cells was decreased in IL-12-depleted, *Brucella*-infected mice.

We have shown here that the depletion of endogenous IL-12 production by a single injection of anti-IL-12 MAb at the beginning of infection resulted in the abrogation of IFN-γ production at 2 to 4 weeks postinfection and significantly inhibited IFN-γ production at even 12 weeks after infection. These results demonstrate that IL-12 is very potent in the induction of a Th1 response. On the other hand, in vivo depletion of IL-12 in mice with established brucella infections or neutralization of IL-12 in cultures of spleen cells from mice with established infections did not dramatically affect IFN-γ production by spleen cells. Similar results were revealed for murine *T. gondii* infection; treatment with anti-IL-12 exacerbated acute infection but had no effect on chronic infection (4). This also agrees with in vitro work showing that IL-12 was needed only during the first few days of clonal expansion of T cells and that the ability to produce high titers of IFN-γ was then stably maintained by clones even in the absence of IL-12 (13).

It has been shown that TNF-α is required for the influx of phagocytes to the site of infection (23), for granuloma formation (11), and for macrophage activation (2, 12). Granuloma formation localizes pathogens and exposes them to phagocytosis. The injection of anti-TNF-α immunoglobulin G had a dramatic suppressive effect on the development of granulomas in patients with tuberculosis (11). However, TNF-α depletion in this study did not result in a reduced number of granulomas in the liver, although at the same time spleen size and cell accumulation in the spleen were reduced compared with those of control infected mice. It is possible that one injection of anti-TNF-α antibody before infection is not sufficient to neutralize totally the TNF-α produced during 2 weeks of infection or that its role is replaced by TNF-β produced by T cells. TNF receptor gene-disrupted mice could help clarify the role of TNF-α in granuloma formation during *Brucella* infection. On the other hand, IL-12-depleted mice showed no granuloma formation in the first 2 weeks of infection. Cell infiltration in the spleen was also not dramatic. This could be due to a lack of IFN-γ stimulated by IL-12 since IFN-γ has also been implicated in granuloma formation (9).

Another interesting point from this study is that although TNF-α depletion resulted in exacerbated infection at 2 weeks, those mice recovered from infection as efficiently as did control infected mice by 6 weeks postinfection, without the excessive

TABLE 4. Effects of IL-12 depletion on IFN-γ production in mice with established *Brucella* infections[a]

Treatment	IFN-γ (U/ml)	
	Without SBP	With SBP
PBS	<1	570 ± 2
Anti-IL-12	<1	593 ± 8
Normal	<1	<1

[a] Mice were injected intravenously with 5×10^5 *B. abortus* cells. Ten weeks later, mice were injected intraperitoneally with 0.2 ml of sterile PBS or 2 mg of anti-IL-12 MAb. Mice were killed 7 days later. Spleen cells were prepared and cultured at 2×10^6 cells per ml with 60 μg of SBP per ml in 2-ml volumes for 24 h. Data are the means ± standard deviations of triplicates from each sample. These experiments were repeated twice.

TABLE 5. Effects of in vitro IL-12 neutralization on IFN-γ production by spleen cells from mice with established *Brucella* infections[a]

Treatment	Antibody	IFN-γ (U/ml)	
		Without SBP	With SBP
Infected	−	<1	76 ± 2
	+	<1	67 ± 6
Normal	−	<1	<1
	+	<1	<1

[a] Mice were injected intravenously with 5×10^5 *B. abortus* cells. Four weeks later, spleen cells from mice were prepared and cultured at 2×10^6 cells per ml with 60 μg of SBP per ml in the absence (−) or presence (+) of 100 μg of anti-IL-12 antibody (C17.8) per ml in 2-ml volumes for 24 h. Data are the means ± standard deviations of triplicates from each sample. These experiments were repeated twice.

splenomegaly, massive cell accumulation in the spleen, and vigorous production of NO by spleen cells observed in control infected mice. This indicates that TNF-α acts as a two-edged sword, contributing to both immunity and immunopathology. This is also true for IL-12, as IL-12 can mediate toxicity (16). A delicate balance is needed to maximize their potential for enhancement of immunity and to minimize their potential for involvement in pathology.

Thus, TNF-α and IL-12 are both required for resistance to *Brucella* infection, although their roles are quite different. While TNF-α did not affect IFN-γ production, IL-12 depletion dramatically reduced IFN-γ production by T cells. This study suggests that the depletion of cytokines by a neutralizing antibody is a useful tool to understand what effects cytokines produced at a very early stage of infection have on subsequent activation of antigen-specific T cells. Together with other means such as cytokine- and cytokine receptor gene-disrupted mice, it enhances our understanding of the role of cytokines in immune responses to intracellular bacteria and parasites.

ACKNOWLEDGMENT

This work was supported by a project grant from the Australian National Health and Medical Research Council.

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Editor: R. E. McCallum