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Interaction of interleukin-6, tumour necrosis factor and interleukin-1 during *Listeria* infection

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SUMMARY

Injected recombinant interleukin-6 (IL-6), tumour necrosis factor (TNF) and IL-1 all protect mice against experimental infection with *Listeria monocytogenes*. We have therefore investigated the interaction of these cytokines during infection. Treatment with recombinant (r)IL-6 enhanced TNF production by spleen cells during the first 2 days of infection. Anti-TNF antibody could totally abolish the protective effect of rIL-6, while the optimal protective function of TNF could not be achieved when IL-6 was neutralized by anti-IL-6 antibody. IL-1 induced a high level of IL-6 in the serum a short time after its administration, and neutralization of IL-6 totally abolished the protective function of rIL-1. The results thus provide further evidence for the complexity of cytokine interaction.

INTRODUCTION

Interleukin-6 (IL-6), tumour necrosis factor (TNF) and IL-1 are among the early cytokines appearing during bacterial infection,[1,2] mainly produced by monocytes. These cytokines mediate a wide array of biological activities, many of them overlapping. Their functions include stimulation of lymphocyte proliferation, enhancement of natural killer (NK) cell activities, stimulation of haemopoisis, alterations in endothelial cell interaction with leucocytes, and augmentation of synthesis of acute-phase proteins by hepatocytes.[3] All three cytokines have been shown to enhance resistance to experimental infection of mice with *Listeria monocytogenes* when recombinant materials are administered before injection of the bacteria.[4–6] Conversely, *in vivo* administration of antibodies against IL-6, TNF and IL-1 results in an increase in bacterial growth in the organs and ultimately in death from listeriosis in mice,[7–9] indicating that endogenously produced IL-6, TNF and IL-1 are crucial in anti-listerial resistance.

Enhanced production of IL-6, TNF and IL-1 occurs very early in infection. TNF and IL-1 are reported to be potent inducers of IL-6, injection of TNF or IL-1 inducing high levels of IL-6 *in vivo*,[10,11] suggesting that these cytokines may regulate each other, so affecting the early immune response to infection. Synergistic effects of TNF and IL-1 in eliminating certain tumours from mice, in inducing neutrophilic infiltration and aggregation and in stimulating the synthesis of thromboxanes have all been reported.[12] It has also been reported that combined administration of TNF and IL-1 resulted in a significant enhancement of resistance to *Listeria* beyond that obtained with either monokine alone.[13] *In vitro*, IL-6 synergizes with IL-1 to control the initial steps in T-cell activation.[11] Indeed, it has been suggested that the effect of IL-1 on thymocyte proliferation is mediated by IL-6 production.[14] In the light of these known interactions, and with an interest primarily in the role of IL-6 in cell-mediated immunity to infection,[9] we undertook to examine the interaction of IL-6 with TNF and IL-1 during experimental infection of mice with *L. monocytogenes*.

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MATERIALS AND METHODS

Infection of mice

Female C57BL/10 mice, 6–8 weeks of age, were infected intravenously with 1×10^4 *L. monocytogenes* strain EGD organisms from 24-hr cultures on horse blood agar plates. The dose was standardized turbidimetrically and checked retrospectively by viable counts. At specified times after infection mice were killed by an overdose of fluothane, and the spleens and livers were homogenized individually for viable bacterial counts, as described previously.[6] The results are presented as the mean $\log_{10}$ viable *L. monocytogenes* cells per spleen or liver ± the standard deviation (SD) for the group of mice. Statistical analysis was based on the Student's *t*-test.

Sample collection

Serum. Under fluothane anaesthetic, mice were bled from the heart with a syringe and 25-gauge needle to collect blood aseptically. The blood was allowed to clot for 1 hr at room

temperature, and the clots were retracted overnight at 4° to collect the serum, which was stored at −20° for assay.

Supernatants. After the preparation of spleen cells as described previously,[9] the cell concentration was adjusted to 2×10^6/ml and cultured with or without 10^6 heat-killed listeriae (HKL) in Dulbecco's modified Eagle's minimal essential medium (DMEM) at 37° in 5% CO_2. Supernatants were collected at 24 hr and stored at −20° until assay.

Cytokines

Murine recombinant (r)IL-6 was synthesized as a fusion protein using a *lac* operon-inducible plasmid in *Escherichia coli* and purified as described previously.[15] The specific activity of the recombinant protein, as measured in the hybridoma growth assay,[11] was 2×10^8 U/mg. One U/ml was defined as zero stimulation, with half maximal stimulation requiring 16 U/ml. Endotoxin contamination of these solutions was less than 0·25 EU/mg of protein using the *Limulus* amoebocyte lysate assay (Associates of Cape Cod, Cape Cod, MA). Purified rTNF, also produced in *E. coli*, displayed specific activity of $1{\cdot}2 \times 10^7$ U/mg. Human recombinant (hr)IL-1α was provided by P. J. Lomedico (Hoffman-La Roche, Nutley, NJ). The lyophilized material was reconstituted to 2×10^8 U/ml (D10 assay; protein concentration, 0·68 mg/ml; endotoxin, < 0·5 EU/ml) with pyrogen-free saline, and stored at 4°.

Cytokine bioassay

IL-6 was measured by survival and proliferation of the IL-6-dependent cell line 7TD1, compared with purified rIL-6.[11] TNF activity was assayed by the cytotoxic activity against WEHI-164 clone 13,[16] again compared with a rTNF standard. All assays were performed in triplicate and standard preparations of the appropriate cytokine were included in each assay. Specificity was checked with neutralising antibodies.

Antibodies

Polyclonal antisera to IL-6 and TNF were raised by injecting rabbits intramuscularly with 50 μg recombinant cytokine in Freund's complete adjuvant, followed by two weekly injections of 50 μg cytokine in incomplete Freund's adjuvant. Thereafter rabbits were boosted monthly without adjuvant. Serum was collected 2 weeks after each boost. It was semi-purified by 50% $(NH_4)_2SO_4$ precipitation, followed by exhaustive dialysis against phosphate-buffered saline (PBS). The protein content was determined by absorption at 280 nm. Using *in vitro* bioassays, 1 ml of anti-IL-6 serum was found to neutralize at least 10^5 U IL-6, while 1 ml of anti-TNF serum neutralized at least 10^6 U TNF activity. Serum from non-immunized rabbits was collected and $(NH_4)_2SO_4$ precipitated for use as control globulin.

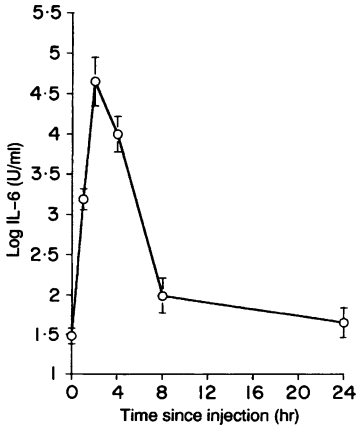

Figure 1. IL-6 production induced by rIL-1α *in vivo*. Mice were injected with 10^5 U rIL-1α i.p. and serum was collected at different time points. IL-6 in the serum was determined by bioassay. Results show IL-6 log mean ± SD of five mice.

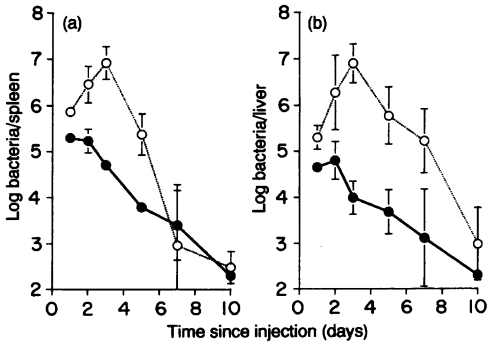

Figure 2. Effect of rIL-1 on bacterial numbers during *Listeria* infection. Mice were infected with 10^4 *Listeria* i.v. 4 hr after injection of 10^5 U rIL-1α/mouse (●) or 0·2 ml of saline (○) i.p. Each point represents the mean and SD of five mice. Differences between rIL-6-treated mice and control mice were significant on days 2–5 in spleens (a) and days 2–7 in livers (b) ($P < 0{\cdot}01$) by Student's *t*-test.

In vivo *depletion of cytokines*

To deplete endogenous IL-6 or TNF, each mouse was given a single intraperitoneal (i.p.) injection of 0·5 ml (5 mg anti-IL-6 antibody or 0·5 mg anti-TNF) of specific polyclonal antibody 4 or 24 hr before *Listeria* infection. Control mice treated with 0·5 ml normal immunoglobulin (5 mg for anti-IL-6 antibody treatment control, or 0·5 mg for anti-TNF treatment control) were also included.

RESULTS

Interaction of IL-6 and IL-1 during infection

To assess induction of IL-6 by doses of rIL-1α known to protect against listeriosis,[4] mice were injected with 10^5 U rIL-1α i.p. and bled at intervals after injection for assay of serum IL-6. One hour after injection of rIL-1α, a high level of IL-6 appeared in the serum with a peak at 2 hr after injection (Fig. 1). By 4 hr, the IL-6 level began to decline but was still high. Between 8 and 24 hr it returned to normal.

Mice were therefore infected with 10^4 listeriae 4 hr after i.p. injection of 10^5 U rIL-1α, the standard time interval used in earlier experiments to protect mice.[4] Bacterial numbers in spleen and liver of rIL-1 treated mice were much lower than those of control mice (Fig. 2). Significant differences could be observed between 2 and 5 days in spleens and 2 and 7 days in livers after infection. However, infection with listeriae following IL-1 treatment induced only a low level of IL-6 in the serum (Fig. 3). This low level of IL-6 was maintained until 3 days

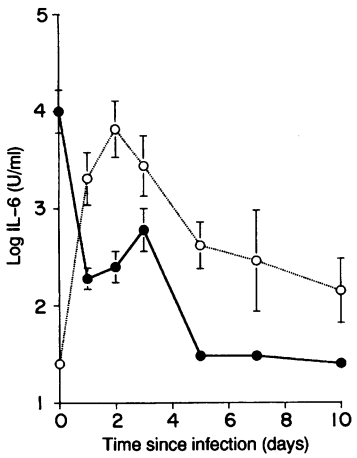

Figure 3. Effect of rIL-6 administration *in vivo* on the production of TNF by cultured spleen cells. Spleen cell suspensions were prepared from mice infected with 10^4 listeriae 4 hr after injection of 50 μg rIL-6 (●) or 0·2 ml of saline (○). Each point represents the mean and SD of triplicate cultures. No TNF was produced by cells from uninfected mice, whether injected with rIL-6 or not, by any of the cells cultured without listerial antigen. The specific cytotoxicity of TNF was proved by anti-TNF antibody.

post-infection, after which IL-6 was undetectable in serum of the mice. In contrast, control mice without the treatment of IL-1 preinfection expressed a high level of IL-6 in serum 1 day after infection, with a peak at 2 days post-infection. Elevated IL-6 could be detected up to 10 days post-infection in mice without rIL-1 treatment.

In order to study the involvement of IL-6 in protection by IL-1, mice were first treated with anti-IL-6 antibody and injected with rIL-1α 24 hr later. Four hours after injection, mice were challenged with 10^4 listeriae. Bacterial numbers in spleen and liver were determined at 5 days post-infection, where strong protection by rIL-1α had been observed. Several controls were also included (Table 1). Deletion of endogenous IL-6 by administration of anti-IL-6 antibody totally abolished the protective effect of rIL-1α. Bacterial numbers in the mice treated with anti-IL-6 antibody and rIL-1α reached a similar level to that in the mice treated with anti-IL-6 antibody alone. Administration of control immunoglobulin to the mice had no effect on the protective function of rIL-1α.

Interaction of IL-6 and TNF during infection

Mice were injected with 50 μg rIL-6 4 hr before infection with 1×10^4 *L. monocytogenes*, a procedure which we have previously shown to produce maximum protection 5 days post-infection.[6] TNF activity in serum from both rIL-6-treated and untreated mice was undetectable. However, when spleen cells from infected mice were cultured with HKL for 24 hr, TNF activity in the supernatants from rIL-6-treated mice was higher than that in supernatants from infected but untreated control mice at 1–2 days post-infection (Fig. 4). After that time, the TNF level in the supernatants of cultured spleen cells from control mice increased rapidly and no significant difference was observed between the two groups after 5 days of infection.

In the light of the above early activation of TNF production by spleen cells, the possibility that TNF was responsible for protection by IL-6 was investigated. Mice were injected i.p. with 0·5 mg anti-TNF antibody and 50 μg rIL-6, and 4 hr later these animals were infected with 10^4 listeriae. Bacterial numbers in organs were assayed 4 days post-infection. Control groups treated with normal immunoglobulin or PBS were included. Table 2 shows that depletion of endogenous TNF by administration of anti-TNF antibody resulted in the complete abrogation of the protective effect of rIL-6. Nor was rIL-6 able to mitigate the exacerbating effect of anti-TNF antibody. Bacterial numbers in the mice treated with anti-TNF antibody and rIL-6 reached the same level as those in the mice treated with anti-TNF antibody alone. Administration of control immunoglobulin to the mice had no effect on the protective

Table 1. The effect of anti-IL-6 antibody on protective function of rIL-1α during *Listeria* infection†

Treatment	Mean log bacteria no./organ ± SD	
	Spleen	Liver
PBS	5·45 ± 0·20	5·09 ± 0·53
rIL-1α	4·08 ± 0·50	4·07 ± 0·46
Anti-IL-6 antibody + rIL-1α	5·59 ± 0·45	6·54 ± 0·88
Anti-IL-6 antibody	6·20 ± 0·64	7·05 ± 0·67
Control immunoglobulin + rIL-1α	4·68 ± 0·36	3·85 ± 0·51

Spleen: PBS vs rIL-1α: * ; rIL-1α vs Anti-IL-6 antibody + rIL-1α: * ; PBS vs Anti-IL-6 antibody + rIL-1α: ** ; Anti-IL-6 antibody + rIL-1α vs Anti-IL-6 antibody: NS ; rIL-1α vs Control immunoglobulin + rIL-1α: *** ; Anti-IL-6 antibody vs Control immunoglobulin + rIL-1α: NS

Liver: PBS vs rIL-1α: *** ; rIL-1α vs Anti-IL-6 antibody + rIL-1α: * ; PBS vs Anti-IL-6 antibody + rIL-1α: * ; Anti-IL-6 antibody + rIL-1α vs Anti-IL-6 antibody: NS ; rIL-1α vs Control immunoglobulin + rIL-1α: NS ; Anti-IL-6 antibody vs Control immunoglobulin + rIL-1α: *

† C57BL10 mice were treated i.p. with 5 mg of anti-IL-6 antibody, control immunoglobulin or PBS, and injected with 10^5 U rIL-1α/mouse 24 hr later. Mice were challenged with *Listeria* 4 hr after rIL-1a. Groups of mice without rIL-1a treatment were included. Bacterial numbers in spleen and liver were determined at 5 days post-infection.

* $P < 0·001$; ** $P < 0·01$; *** $P < 0·05$; by Student's *t*-test.

NS, not significant.

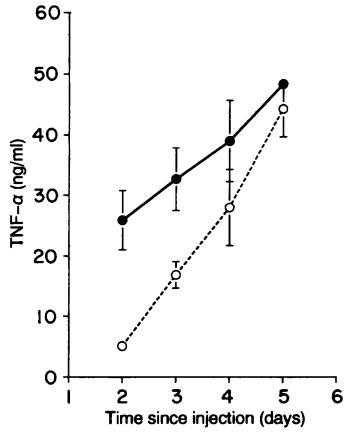

Figure 4. IL-6 in serum of mice treated with rIL-1α (●) or pyrogen-free saline (○) before *Listeria* infection. Mice were injected with 10^5 U rIL-1α i.p. 4 hr before *Listeria* infection and serum was collected at different time points. IL-6 in the serum was determined by bioassay. Results show IL-6 log mean ± SD of five mice.

function of rIL-6. Similar results were obtained in each of two experiments.

In order to study the converse involvement of IL-6 in protection by TNF, mice were first treated with 5 mg anti-IL-6 antibody and 24 hr later were injected with 10^5 U rTNF. Four hours after injection with rTNF, mice were challenged with 10^4 listeriae. Bacterial numbers in spleen and liver were determined at 5 days post-infection. Several controls were also included. Depletion of endogenous IL-6 production by administration of anti-IL-6 antibody only partially inhibited the protective effects of rTNF (Table 3). Bacterial numbers in mice treated with rTNF and anti-IL-6 antibody were significantly lower than those in mice treated with anti-IL-6 antibody alone, and higher than those in mice treated with rTNF and control immunoglobulin. In other words, rTNF may have the ability to reduce exacerbation by anti-IL-6 antibody.

DISCUSSION

Numerous studies on the interactions between cytokines have been published. Overlaps between the immunological functions IL-6, TNF and IL-1 have been recognized.[3] The three cytokines derive from a similar cellular source and express their role at a similar period of time during infection. As endogenous IL-6[9] as well as TNF[5] and IL-1[8] are now shown to be protective factors during infection, the question arises as to how IL-6, with its multitude of biological activities, fits into the network of interactive cytokines regulating the immune system.

The protective role of injected rIL-1 against *Listeria* infection has been previously reported.[4,17] IL-1 given before *Listeria* infection has been shown to enhance T-cell activation,[4] promote haemopoiesis[4] and to be involved in the chemotaxis of neutrophils.[17] Conversely, anti-IL-1 antibody[8] or anti-IL-1-receptor antibody[18] exacerbates *Listeria* infection. However, IL-1 is not present in the peripheral circulation of mice during sublethal listeriosis.[18]

IL-1 has been shown to be a potential inducer of IL-6 in mice.[19] It has been shown here that injection of rIL-1 i.p. into mice could induce a high level of IL-6 in serum after 2–4 hr. The IL-6 production induced by rIL-1 was transient and returned to normal 24 hr later. Because several effects of IL-1 are thought to be mediated through induction of IL-6,[12] the possible role of IL-6 in rIL-1-enhanced host defence was investigated. Neutralization of IL-6 by specific anti-IL-6 antibody totally abolished the protective effect of rIL-1 when administered to mice before *Listeria* infection. This result suggested that the protective role of IL-1 against *Listeria* infection required the presence of IL-6. Interestingly, mice infected with *Listeria* after IL-1 treatment ultimately produced less IL-6 in their serum than did untreated mice. This presumably reflected the lower numbers of bacteria, as it has been shown that serum IL-6 levels correlate with bacterial dose.[6]

Both IL-6 and IL-1 are stimulators of T-cell activation. It was reported that *in vivo* administration of rIL-1 before *Listeria* infection could increase T-cell activation, as determined by an early increase in the production of interferon-γ (IFN-γ) *in*

Table 2. The effect of anti-TNF antibody on protective function of rIL-6 during *Listeria* infection†

	Mean log bacteria no./organ ± SD	
Treatment	Spleen	Liver
PBS	6·50 ± 0·55	6·81 ± 0·09
rIL-6	4·94 ± 0·55	4·63 ± 0·63
Anti-TNF antibody + rIL-6	8·77 ± 0·13	8·78 ± 0·14
Anti-TNF antibody	8·85 ± 0·05	8·88 ± 0·07
Control immunoglobulin + rIL-6	4·88 ± 0·23	4·20 ± 0·25

† C57BL10 mice were treated i.p. with 1 mg of anti-TNF antibody (in 0·5 ml PBS), control immunoglobulin (in 0·5 ml PBS) or PBS (0·5 ml) in combination with 50 μg rIL-6/mouse. Groups of mice without the treatment of rIL-6 were included. All mice were challenged with 10^4 listeriae 4 hr later. Bacterial numbers in spleen and liver were determined at 4 days post-infection.

* $P < 0{\cdot}001$; $P < 0{\cdot}01$; by Student's *t*-test.

NS, not significant.

Table 3. The effect of anti-IL-6 antibody on protective function of rTNF during *Listeria* infection†

Treatment	Mean log bacteria no./organ ± SD	
	Spleen	Liver
PBS	5·69 ± 0·42	6·26 ± 0·78
rTNF	4·24 ± 0·24	4·11 ± 0·65
Anti-IL-6 antibody + rTNF	5·20 ± 0·38	6·94 ± 0·49
Anti-IL-6 antibody	6·44 ± 0·25	7·65 ± 0·67
Control immunoglobulin + rTNF	4·67 ± 0·34	5·30 ± 1·10

† C57BL10 mice were treated i.p. with 5 mg of anti-IL-6 antibody, control immunoglobulin or PBS, and injected with 2 μg of rTNF/mouse 24 hr later. Mice were challenged with *Listeria* 4 hr after rTNF. Groups of mice without rTNF treatment were included. Bacterial numbers in spleen and liver were determined at 5 days post-infection.

* $P < 0{\cdot}001$; $P < 0{\cdot}01$; *** $P < 0{\cdot}05$; by Student's *t*-test.

NS, not significant.

vitro.[4] A similar effect was observed by *in vivo* administration of rIL-6.[9] These results suggested that both IL-6 and IL-1 could activate T cells and induce IFN-γ production. Results from *in vitro* lymphocyte cultures have shown that both IL-6 and IL-1 are important accessory factors for T-cell proliferation and differentiation.[20] They induced T-cell proliferation and differentiation by increasing the number of IL-2 receptors on T cells and production of IL-2 by T cells. However, they acted on different steps: IL-6 mainly augmented the expression of IL-2 receptor expression on T cells, while IL-1 significantly stimulated IL-2 production by T cells.[11] Nevertheless, it is not clear whether the augmented T-cell activity in IL-6- or IL-1-treated, *Listeria*-infected mice reflects a direct or indirect effect on T-cell activation.

The present results and previous studies[7] show that endogenously produced TNF plays an essential role in antibacterial resistance at an early stage of infection. Recombinant TNF administered to mice resulted in enhanced resistance to lethal challenge with *L. monocytogenes*. Administration of anti-TNF antibody to mice converted a sublethal *Listeria* infection into a lethal infection. This happened, however, only if the anti-TNF antibody was given early in infection, as was also the case with antibody.[9] The role of TNF was also observed in TNF-deficient mice, which readily succumb to infection with *Listeria*.[21]

As a multi-functional cytokine, TNF may mediate the resistance against *Listeria* infection by several pathways. TNF can alter leucocyte recruitment and trafficking *in vivo*,[22] prime neutrophils for oxidative burst activity[23] and activate macrophages.[7] TNF is also involved in the formation of granulomas during infection.[24] As both protection by rTNF and exacerbation by anti-TNF antibody were observed in T-cell deficient (athymic nude) mice,[5,25] TNF produced in *Listeria*-infected organs was presumed to function in a T-cell independent resistance mechanism, via IFN-γ production by NK cells. However, Nakane *et al.*[26] reported that the main role of TNF in resistance to listeriosis in mice may be due to direct enhancement of the listericidal activity of macrophages, not to increasing IFN-γ production. They showed that neutralization of TNF *in vivo* did not affect the titres of IFN-γ in the bloodstream but greatly lowered the listericidal activity of macrophages.

It has been reported that the intravenous injection of TNF could elicit the appearance of IL-6 in the blood.[27] TNF was reported to be responsible for the production of IL-6 *in vivo* following the inoculation of a lethal dose of the Gram-negative bacterium, *E. coli*,[1] as treatment with anti-TNF IgG greatly reduced IL-6 appearing in the circulation. However, Havell & Sehgal[28] reported that IL-6 production in mice undergoing either a sublethal or lethal infection with *Listeria*, occurred primarily through a TNF-independent mechanism. Neutralization of TNF by anti-TNF antibody resulted in the progressive multiplication of bacteria in infected organs and the progressive augmentation of IL-6 production in spleen and serum.

It was not known whether IL-6 could affect TNF production during *Listeria* infection. TNF does not appear in the serum of sublethally infected mice, but the present results show that administration of rIL-6 before infection enhanced the production of TNF by the cultured spleen cells from the treated mice at an early stage of infection. Moreover, when rIL-6 in combination with anti-TNF antibody was administered to mice before infection, the protective effect of rIL-6 was abrogated. On the other hand, the protective effect of rTNF was only partially inhibited when rTNF was administered in combination with anti-IL-6 antibody before infection. These results suggest that, for IL-6 to express its protective function, it requires the presence of TNF, and the co-operation of IL-6 is necessary for an optimal protective effect of TNF.

Hence, there is an intimate interrelationship among these three cytokines. The protective effect of rIL-1 is dependent on IL-6, and that of rIL-6 depends on TNF. It is not possible to state that this is a simple chain reaction of IL-1 inducing IL-6, which induces TNF. Firstly, both IL-1 and TNF are involved in resistance of severe combined immunodeficiency (SCID) mice in the absence of T cells, while IL-6 does not protect SCID mice.[9] Secondly, we have shown that antibody to IL-6 at least

partially abrogates the protective effect of rTNF, suggesting they act side by side rather than in a series. This is further confirmation of the complexity of the cytokine network.

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