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IL-9 Protects Mice from Gram-Negative Bacterial Shock: Suppression of TNF-α, IL-12, and IFN-γ, and Induction of IL-10

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IL-9 is a T cell-derived cytokine that, similar to the Th2 cytokines IL-4 and IL-10, has been implicated in the response to parasitic infections, allergy, and inflammatory processes. Because both IL-4 and IL-10 can confer protection to mice from septic shock, we investigated whether IL-9 may also be capable of conferring resistance on recipients of an otherwise lethal challenge with *Pseudomonas aeruginosa*. Prophylactic injections of rIL-9 appeared to be most effective in preventing the onset of a lethal shock, according to a pattern that was both dose dependent and time dependent. The protective effect of IL-9 was correlated with marked decreases in the production of the inflammatory mediators TNF-α, IL-12, and IFN-γ, as well as the induction of the anti-inflammatory cytokine IL-10. Sustained levels of IL-9-specific transcripts could be detected in the spleens of mice recovering from sublethal *P. aeruginosa* infection. Therefore, IL-9 may be protective in septic shock via a rather unique mechanism involving a complex modulation of inflammatory and anti-inflammatory mediators. *The Journal of Immunology*, 2000, 164: 4197–4203.

Septic shock results from the uncontrolled sequential release of mediators with proinflammatory activity in response to infection with Gram-negative bacteria and in response to endotoxin (1–4). Endotoxin exerts its effect by inducing potent macrophage activation and release of cytokines such as TNF-α, IL-1, IL-6, IL-12, and IFN-γ (5). IL-12, in concert with TNF-α or B7 costimulation, can further act as a potent inducer of IFN-γ production by T and NK cells (6–8). The central role of proinflammatory cytokines in the pathogenesis of endotoxic shock is underlined by the occurrence of high levels of circulating cytokines in both humans and experimental animals during endotoxemia (9).

During the course of septic shock, triggering of regulatory mechanisms may oppose macrophage activation (10), thus alleviating an overwhelming, dysregulated inflammatory response that contributes to pathology and potentially death in the host. This concept is supported by a large body of evidence showing the ability of anti-cytokine maneuvers to improve outcome in experimental challenge with LPS or Gram-negative bacteria. Administration of neutralizing anti-cytokine Abs (11, 12) or of IL-1R antagonist (13) or gene targeting of proinflammatory cytokines (14) or cytokine receptors (15, 16) can diminish lethality in experimental endotoxemia.

Therapeutic effects in experimental septic shock syndromes are also achieved by the administration of recombinant cytokines with macrophage-deactivating properties. IL-10 can act as a potent modulator of cytokine production and lethality in LPS-induced pathology (17–19). Gene transfer with IL-4 improves survival in lethal endotoxemia (20), and selective, compartmentalized blockade of TNF-α overproduction by IL-4 enhances pulmonary clearance of *Pseudomonas aeruginosa* in mice (21). We have recently shown that rIL-4 may protect animals from death in a toxic shock model with the bacterium, correlating with decreased levels of circulating TNF-α soon after challenge (22). IL-13, a cytokine that shares anti-inflammatory properties with IL-4 and IL-10, protects mice from LPS-induced lethal endotoxemia (20, 23).

IL-9 is a T cell-derived cytokine that acts on various cell types, including T cells, mast cells, erythroid and myeloid progenitors, and B lymphocytes (reviewed in Refs. 24 and 25). In vitro, IL-9 production by Th clones is restricted to the Th2 subset. Evidence indicates that IL-9 is also expressed in vivo during the course of Th2 responses, through the activation of IL-4-dependent (26, 27) and IL-4-independent (28) mechanisms. While IL-9 has been shown to enhance resistance to helminths (29–31), its potential role in acute Gram-negative infection has not been explored yet. Because of the mutual regulation of IL-9 and IL-4 expressions, we have examined the effect of IL-9 administration in a shock model with *P. aeruginosa*, in which prophylactic IL-4 provides protection (22). We found that IL-9 was endowed with marked therapeutic activity in otherwise lethally infected mice. This was concomitant with early down-modulation of proinflammatory cytokines and induction of the anti-inflammatory cytokine IL-10.

Materials and Methods

Mice

Hybrid (BALB/cCr × DBA/2Cr)F_1 ($CD2F_1$) mice were purchased from Charles River Breeding Laboratories (Calco, Milan, Italy). Mice of both sexes, ranging in age from 2 to 4 mo, were used.

Organism and infection

The origin and characteristics of the *P. aeruginosa* strain used in this study have been described elsewhere (32–34). The organism was routinely cultured in tryptic soy broth (Difco Laboratories, Detroit, MI) and incubated

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at 37°C for 18–24 h with constant aeration. For infection, overnight cultures were centrifuged, the soft pellet was resuspended in PBS, and 10^{10} cells (representing the LD_{90}[2] in our model system) were injected i.v. Portions of suitable dilutions were also inoculated onto agar plates for precise enumeration of CFU. All deaths resulting from infection, as proven by clinical signs and histopathological examination, occurred within 3 days of microbial challenge and mostly within the first 24–48 h (mortality was routinely recorded for up to 7 days from challenge). Enumeration of CFU in the blood of infected mice was performed by standard procedures, by inoculating 0.1-ml blood samples onto nutrient agar plates (32).

Cytokines and Abs

Murine rIL-4 and rIL-9 (5×10^7 U/mg) were produced in the baculovirus system, as previously described (35). Murine rIL-12 was a generous gift from Dr. B. Hubbard (Genetics Institute, Cambridge, MA). Murine rTNF-α and rIFN-γ were from Genzyme (Cambridge, MA). Murine rIL-10 was from PharMingen (San Diego, CA).

Rat anti-mouse IL-12 p40 mAb C17.8 was from Genzyme. Rabbit anti-mouse IL-12 Ab was a generous gift from Dr. F. Fallarino (University of Perugia, Perugia, Italy) (36). Anti-mouse IFN-γ mAbs AN-18.17.24 and XMG1.2 and anti-mouse TNF-α MP6-XT3 were purified from hybridoma culture supernatants by means of affinity chromatography. Anti-mouse IL-10 mAbs JES5-2A5 and biotinylated SXC-1 were from PharMingen. For in vivo neutralization experiments, the SXC-1 mAb was purified from hybridoma culture supernatants using chromatography on Thiophilic gel that retains IgM (Affiland, Ans-Liege, Belgium).

In vivo treatments

LPS from *P. aeruginosa* (serotype 10) was obtained from Sigma (St. Louis, MO). For in vivo challenge, mice were injected i.p. with a predetermined LD_{90} (850 μg) of LPS, and survival was monitored over the next 7 days. rIL-4 was administered i.p. 24 h before bacterial challenge, at the dose of 3 μg/mouse given as a complex with 30 μg of anti-IL-4 mAb 11B11 (22). Various doses of rIL-9 were administered i.p. 1 and/or 24 h before bacterial challenge, unless otherwise stated. Heat inactivation of IL-9 to be used as a control treatment was achieved by autoclaving. Pentoxifylline (PTX; Sigma) was administered i.p. in PBS at 30 mg/kg 1 h before infection (34). For neutralization of IL-10, mice received 500 μg of SXC-1 mAb i.p. 2 h before bacterial challenge.

Cytokine assays

TNF bioactivity in sera was measured as cytotoxic activity to WEHI 164 clone 13 murine fibrosarcoma cells, obtained through the courtesy of P. van der Bruggen (Ludwig Institute for Cancer Research, Brussels, Belgium). The assay was performed as described (34), in the presence of LiCl, to optimize sensitivity to TNF-mediated cytotoxicity and using a tetrazolium-based colorimetric assay to estimate mortality of WEHI cells. The specificity of the assay was determined by incubating samples with the neutralizing rat anti-mouse TNF-α mAb MP6-XT3. TNF titers were expressed as ng/ml, calculated by reference to a standard curve constructed with known amounts of rTNF-α.

Plasma levels of IL-12 p40 were assayed by sandwich ELISA using C17.8 mAb and biotinylated polyclonal rabbit anti-mouse IL-12 Ab. The sandwich ELISA for IFN-γ measurements involved the use of AN-18.17.24 mAb and biotinylated XMG1.2 mAb (37). IL-10 measurements involved the use of mAb JES5-2A5 and biotinylated SXC-1 mAb (38). The sensitivity limit was ~20 pg/ml for all cytokines assayed by ELISA.

RNA preparation and PCR

These procedures, involving 30 cycles of amplification with an annealing temperature of 60°C, were previously described in detail (34, 38). β-actin primers were purchased from Clontech Laboratories (Palo Alto, CA). The sequences of 5′ sense and 3′ antisense primers of IL-9 and IL-10 were as follows: 5′ IL-9, GAT GAT TGT ACC ACA CCG TG; 3′ IL-9, CCT TTG CAT CTC TGT CTT CTG G; 5′ IL-10, TCC TTA ATG CAG GAC TTT AAG GGT TAC TTG; and 3′ IL-10, GAC ACC TTG GTC TTG GAG CTT ATT AAA ATC.

The amplified DNA size was 540 bp for β-actin, 234 bp for IL-9, and 256 bp for IL-10. The PCR products were analyzed by 1.5% agarose gel electrophoresis and visualized by ethidium bromide staining.

[2] Abbreviations used in this paper: LD_{90}, lethal dose 90, inoculum size or dose at which 90% mortality occurs; PTX, pentoxifylline.

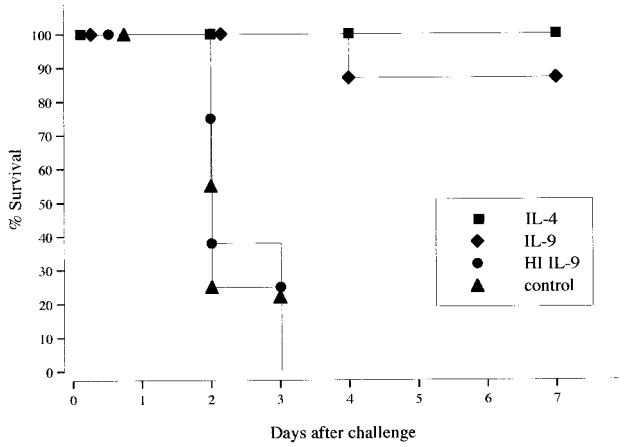

FIGURE 1. IL-9 protects mice from *P. aeruginosa* challenge. Before lethal bacterial infection, the animals received IL-4 (3 μg/mouse), IL-9 (4 μg/mouse twice), heat-inactivated (HI) IL-9, or control PBS, the IL-4-treated mice representing the positive control for 100% protection against development of lethal shock. Survival was monitored over the next 7 days. Shown is one of six experiments with similar results.

Statistical analysis

In the in vivo infection experiments, each experimental group consisted of at least eight animals and survival curves were analyzed by the Kaplan-Meier method. In the in vitro assays of cytokine determinations, Student's *t* test was employed to analyze differences between groups.

Results

Effect of IL-9 administration on P. aeruginosa*-induced shock*

We have used a well-established model of toxic shock with *P. aeruginosa* to determine whether IL-9, a Th2-associated cytokine, is capable of exerting immunomodulatory effects similar to those induced by IL-4 administration (22). In concurrence with IL-4 or IL-9 treatment, mice were challenged i.p. with 10^{10} *P. aeruginosa* cells. IL-4 was administered 24 h before challenge at the optimal dose of 3 μg/mouse, given as a complex with 30 μg of anti-IL-4 mAb 11B11, which is known to improve bioavailability and increase the effectiveness of IL-4 therapy (22). IL-9 was administered twice at 4 μg/mouse, 24 and 1 h before challenge. Control mice received either PBS or heat-inactivated IL-9. In several independent experiments, IL-9 reproducibly and significantly ($p < 0.01$) protected mice against the onset of a lethal shock, with the adopted treatment schedule curing ~80% of the infected animals (representative experiment shown in Fig. 1).

In a series of experiments not reported in this work, we also examined a variety of IL-9 doses and regimens (including prophylactic vs postchallenge administration) for optimal antibacterial protection. As a rule, the prophylactic administration of two IL-9 doses (4 μg/mouse, 24 and 1 h before challenge) proved to be the most effective therapy. As an example, Fig. 2 compares this dosage with 1 or 5 μg of IL-9 administered once (−24 h) or twice (−24 and −1 h) before bacterial challenge. It is interesting to note that the administration of 4 μg of IL-9 24 h before challenge was associated with a 50% cure rate even in the absence of a second (at −1 h) exposure to the cytokine. Although a single IL-9 treatment near the time of infection (4 μg/mouse, up to 3 h postchallenge) would appear to marginally benefit the hosts, no therapeutic activity was observed when IL-9 treatment was delayed until 6 h after *Pseudomonas* challenge (data not shown).

Viable bacteria were enumerated in peripheral blood samples from *Pseudomonas*-infected mice treated with the optimally protective regimen of IL-9 (4 μg/mouse, 24 and 1 h before challenge).

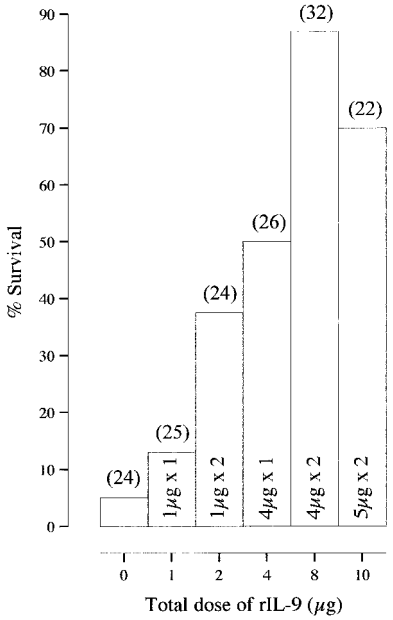

FIGURE 2. Effect of different IL-9 dosages on survival of mice infected with *P. aeruginosa*. Lethally infected mice were treated with one or two IL-9 doses at 1 and/or 24 h before challenge (indicated). Survival was monitored over the next 7 days. Compiled data are from three experiments, each consisting of at least eight mice per group per experiment. Numbers in parentheses indicate total animals tested per group.

No significant differences were found between control and IL-9-treated mice soon after challenge. Both types of recipients experienced a >50-fold reduction in bacterial burden during the first 12 h of infection (from $>5 \times 10^8$ CFU/ml at 10 min to $<0.1 \times 10^8$ CFU/ml of blood at 12 h, a time when control mice would begin to succumb). However, in mice cured by IL-9 therapy, bacterial number had decreased to an undetectable level by 48 h of infection.

Effect of IL-9 administration on production of proinflammatory cytokines

Using the same experimental model of lethal or sublethal *P. aeruginosa* infection, we have previously demonstrated a strong association between the development of a fatal septic shock and production of TNF-α (32–34). In addition, IL-12 and IFN-γ are also proinflammatory cytokines with a putative pathogenetic role in septic shock and endotoxemia (6–8). We therefore measured serum levels of TNF-α, IL-12 p40, and IFN-γ in mice challenged with *P. aeruginosa* or LPS and treated with IL-9. Mice received IL-9 at 24 and 1 h before challenge with *P. aeruginosa* cells or an LD_{90} of LPS. Circulating levels of TNF-α, IL-12 p40, and IFN-γ were measured at 2, 4, 6, 8, and 24 h postchallenge (Fig. 3). With both live bacteria and LPS, IL-9 exerted a dramatic effect on the early expression of the cytokines being assayed. At 2, 4, and 6 h from challenge, the respective levels of TNF-α, IL-12 p40, and IFN-γ were greatly reduced. The most dramatic effect occurred with TNF-α, whose expression was reduced by 10-fold in *P. aeruginosa*-infected mice and >2-fold in animals challenged with LPS. Interestingly, despite the reduction induced by IL-9, the high baseline expression of circulating TNF-α in the latter mice still resulted in death of the majority of the animals, an effect that could not be opposed by adding a third administration of IL-9 at 1 or 4 h postchallenge (data not shown).

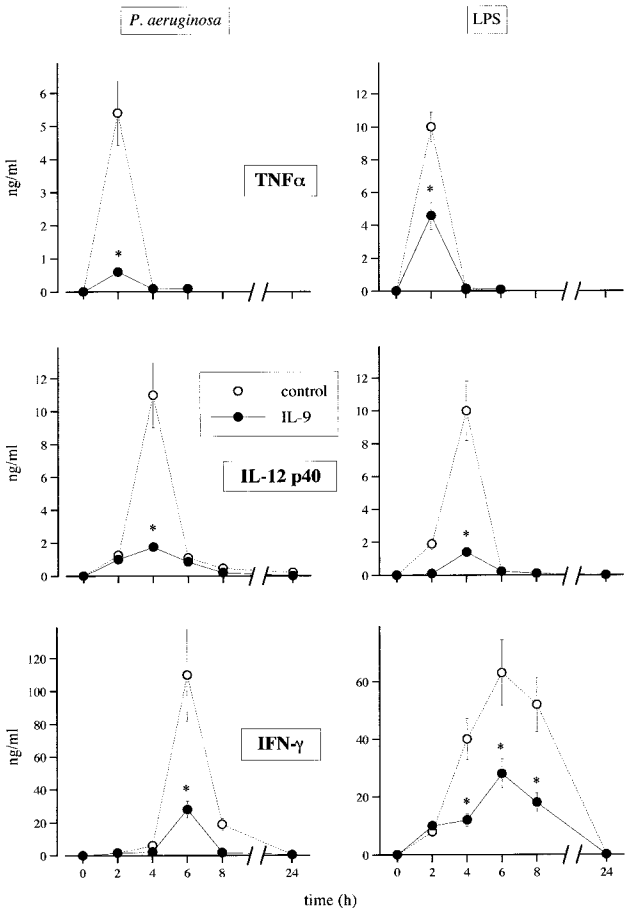

FIGURE 3. Serum levels of proinflammatory cytokines in IL-9-treated mice challenged with *P. aeruginosa* or LPS. Following administration of 4 μg IL-9 or vehicle alone (control) at −24 and −1 h, mice were challenged with live bacteria or LPS. Serum was obtained from six mice per time point, cytokine levels being measured at different times postchallenge. The values represent the means ± SE for bioassay (TNF-α) or ELISA (IL-12 p40 and IFN-γ) data from individual mice. *, $p < 0.01–0.001$ (IL-9-treated vs control mice).

Combined effects of PTX and postchallenge IL-9 treatment

Phosphodiesterase inhibitors, which modulate the production of TNF-α, are known to improve outcome in experimental sepsis (9). We have previously shown that PTX, a phosphodiesterase inhibitor, is beneficial in mice given a lethal inoculum of *P. aeruginosa*. The protection afforded by PTX has been taken to indicate that TNF-α overproduction is an important mechanism of pathogenesis in our septic shock model (34). We therefore wanted to examine whether the therapeutic effect of IL-9 administered postchallenge could be amplified by combined treatment with suboptimal doses of PTX. Mice were administered the phosphodiesterase inhibitor at 30 mg/kg 1 h before challenge with *P. aeruginosa*. At 3 h of infection, the animals were treated with 4 μg/mouse IL-9. Fig. 4 shows that neither PTX alone nor IL-9 alone resulted in significant protection. However, combined treatment with IL-9 and PTX led to survival of most of the infected animals.

Induction of IL-10 by IL-9 treatment of infected mice

The apparently synergic effects of PTX and IL-9 administration in Fig. 4 suggested that TNF-α inhibition might not be the only mechanism involved in the therapeutic activity of IL-9 in our model system. As we have previously shown that the anti-

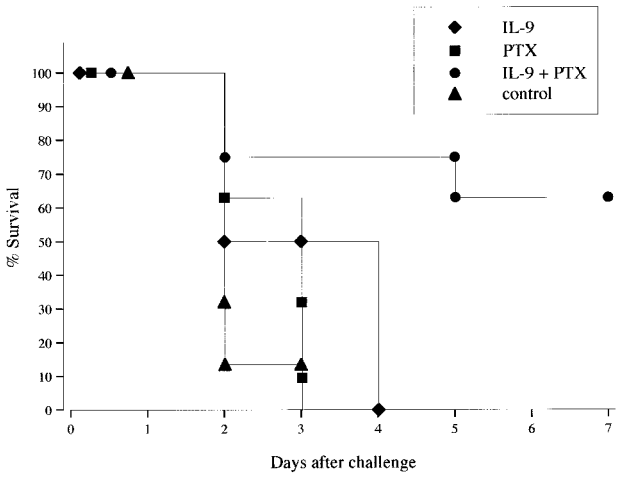

FIGURE 4. Survival of *Pseudomonas*-infected mice treated with a TNF-α inhibitor and IL-9. Mice were given PTX 1 h before challenge with *P. aeruginosa* and treated with IL-9 at 3 h of infection. Data are from one representative experiment of three performed with eight mice per group per experiment.

inflammatory cytokine IL-4 is produced during the course of infection with *P. aeruginosa* (22), we measured serum levels of the anti-inflammatory cytokines, IL-4 and IL-10, in mice challenged with the bacterium or LPS and treated with IL-9 (Fig. 5). The animals were given IL-9 24 and 1 h before either type of challenge, and serum cytokine levels were measured by ELISA at 2, 4, 6, 8, and 24 h. Although the limited production of IL-4 peaking at 4 h of infection was apparently unaffected by IL-9 treatment, a striking increase was observed at 2 h in the IL-10 levels of *Pseudomonas*-infected and IL-9-treated mice. Although to a lesser extent, this effect was also evident after challenge with LPS.

The expression of IL-10 in *Pseudomonas*-infected mice and its induction by IL-9 were also studied by analysis of specific transcripts in the spleen (Fig. 6). At 0.5, 1, 2, 4, and 8 h of infection, we measured mRNA for IL-10 in IL-9-treated and untreated mice. Although transcripts specific for IL-10 were hardly detectable in the spleens of control mice, these transcripts were greatly expressed soon after infection in the animals receiving IL-9.

Effect of IL-10 neutralization on IL-9 therapeutic activity

To directly test the hypothesis that endogenous IL-10 could mediate or contribute to the therapeutic activity of IL-9, we examined

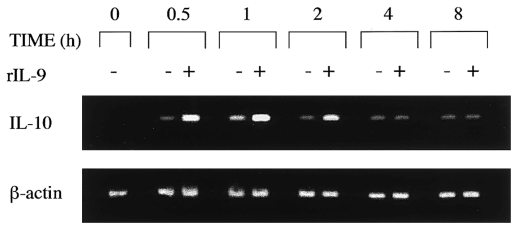

FIGURE 6. IL-9 induces IL-10 mRNA expression in response to host challenge with *P. aeruginosa*. RNA was isolated from control (−) or IL-9-treated (+) mice either uninfected (time 0) or infected for different times, and the resulting cDNA was used in the PCR normalized to β-actin. After amplification, 10 μl of the reaction mix was removed, analyzed by 1.5% agarose gel electrophoresis, and visualized by ethidium bromide staining.

the effect of IL-10 neutralization in *Pseudomonas*-infected mice treated with 4 μg rIL-9 at 24 and 1 h before challenge. A single injection of anti-IL-10 mAb administered 2 h before infection resulted in 100% mortality in both IL-9-treated and untreated mice (data not shown), thus apparently reversing the beneficial effect of IL-9 therapy. However, in experiments not reported in this work, we found that independent of any possible cotreatment, IL-10 neutralization resulted per se in abnormal susceptibility to challenge, as demonstrated by the development of a lethal shock in mice administered otherwise sublethal inocula (e.g., 10^9 *P. aeruginosa* cells). This suggested that a basal production of IL-10 is an absolute requirement for the host to cope with infection in our experimental model. Therefore, the deleterious effect of IL-10 neutralization could not be taken as definite evidence for an obligatory role of increased IL-10 secretion in the therapeutic activity of rIL-9. In line with these observations was the finding that, both in IL-9-treated and untreated mice, neutralization of IL-10 resulted in a 5-fold increase in the levels of circulating TNF-α soon after infection (15.3 $\pm$ 1.8 and 16 $\pm$ 2.1 ng/ml of TNF-α for anti-IL-10-treated mice with or without concurrent IL-9 therapy, respectively).

Expression of IL-9-specific transcripts in the spleens of Pseudomonas*-infected mice*

To investigate the possible role of endogenous IL-9 in Gram-negative bacterial shock, we monitored mRNA levels of IL-9 in the spleens of mice undergoing lethal vs sublethal challenge with *P. aeruginosa*. Spleens were harvested at 0.5, 2, 4, 8, 24, or 120 h after inoculation of 10^{10} or 10^9 *P. aeruginosa* cells, and PCR-

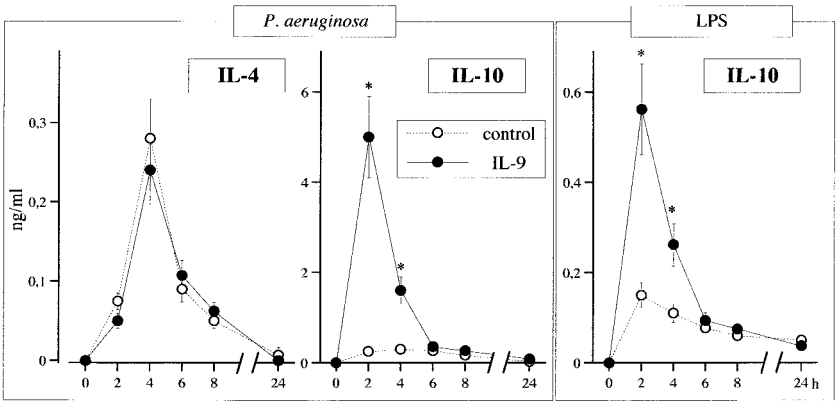

FIGURE 5. Circulating levels of IL-4 and IL-10 in mice challenged with *P. aeruginosa* or LPS and treated twice with 4 μg/mouse IL-9. At different times after challenge, sera were assayed for IL-4 and IL-10 contents. Control mice were treated with IL-9 vehicle alone. No significant IL-10 production was induced by IL-9 in additional control mice (not reported in the figure) receiving LPS vehicle alone. Data are the means $\pm$ SE for six individual mice per time point. *, $p < 0.01$–0.001 (IL-9-treated vs control mice).

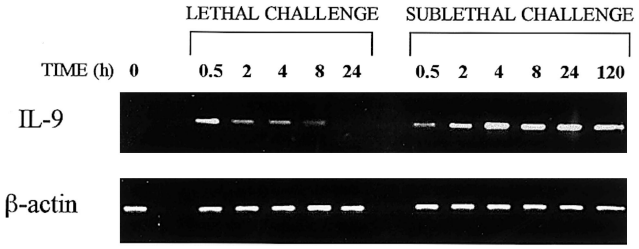

FIGURE 7. IL-9 mRNA expressions revealed by PCR normalized to β-actin in the spleens of mice receiving lethal or sublethal challenge with *P. aeruginosa*. Spleen samples from uninfected (time 0) or infected mice were prepared at different time points after challenge. One experiment representative of three.

assisted mRNA amplification was performed using specific primers (Fig. 7). The timing and pattern of message detection appeared to differ qualitatively in the spleens of lethally vs sublethally infected mice. High levels of specific messages were detected throughout the course of infection (up to day 5) in mice eventually recovering from disease. In contrast, in mice developing lethal shock, a progressive reduction in IL-9 mRNA expression was observed starting 30 min postinfection. Barely detectable transcript levels were found at 24 h, a time when the animals began to die.

Discussion

IL-9 is a pleiotropic cytokine produced primarily by Th cells that was originally described as a growth factor for some T cell lines and mucosal type mast cells. Several additional activities have been ascribed to the cytokine, including erythroid differentiation, Ig production, neuronal differentiation, and expression of granzymes and high affinity IgE receptor in Th clones (reviewed in Refs. 24 and 25). Because of its restricted production by Th2 clones in vitro (27) and its expression in Th2-type responses in vivo (29–31), IL-9 is considered to be a Th2 cytokine that can be induced via IL-4-dependent (26, 27) and IL-4-independent (28) pathways. IL-10 dependence of IL-9 production has also been described in different models (28, 39). Although IL-9 has been implicated in the response to parasitic infections (29–31, 40), allergy (41), and inflammatory processes (42), its potential role in antibacterial host defense is totally unexplored.

Septic shock is an often fatal condition, and excessive production of proinflammatory cytokines is thought to contribute significantly to its lethality. This concept is supported by much evidence derived from studies in animal and in human systems in which the systemic injection of LPS or live bacteria or the presence of bacterial infection is associated with the sustained production of proinflammatory cytokines. Various studies in animal models have demonstrated the protective effect of anti-cytokine maneuvers, including the administration or induced expression of the Th2 cytokines IL-10 (17–19) and IL-4 (20). In addition, the anti-inflammatory cytokine IL-13 has been found to provide protection from LPS-induced endotoxemia in a manner that is similar to but distinct from that of IL-10 (23). We have recently shown that rIL-4 may exert a dual effect on the resistance of mice to *P. aeruginosa*-induced septic shock and production of TNF-α. Improved survival and decreased TNF-α production are observed when the cytokine is administered 24 h before challenge, whereas, paradoxically, increased mortality and overproduction of TNF-α occur when the rIL-4 is given near the time of infection (22).

Because of the interdependence and similarity of IL-9 activities with those of Th2 cytokines, we have investigated any possible effect of IL-9 administration on the development of a fatal shock according to conditions under which prophylactic IL-4 confers protection. One or two injections of rIL-9 increased survival to an extent similar to IL-4 (Fig. 1) and in a dose-dependent manner (Fig. 2) when a lethal inoculum of *P. aeruginosa* was administered to mice. The effect appeared to be IL-9 specific because survival was not affected in mice receiving heat-inactivated IL-9 (Fig. 1). The recombinant cytokine provided optimal protection when administered 1 day and 1 h before challenge (Fig. 2); however, this protection decreased in a time-dependent manner as the administration of IL-9 was delayed until a maximum of 3 h after challenge (data not shown). Nevertheless, under the latter conditions of administration, the therapeutic activity of IL-9 could be greatly amplified by cotreatment with an inhibitor of TNF-α production (Fig. 4). As a matter of fact, the protective effect of IL-9 correlated with remarkable decreases in the production of circulating TNF-α, IL-12, and IFN-γ (Fig. 3).

Several considerations can be made from these experiments. First, it is likely that TNF-α, IL-12, and IFN-γ are major proinflammatory cytokines whose production in the host in response to live bacteria or LPS is down-modulated by IL-9. Because anti-inflammatory cytokines are known to be released as a regulatory mechanism in septic shock (10), it is possible that endogenous IL-9 may contribute, either directly or indirectly, to a compensatory response in septic shock. Of interest, in the early hours after bacterial challenge, we did not detect increased clearance of the bacterium from the blood of mice cured by IL-9 therapy relative to controls succumbing to challenge. This suggested that the beneficial effect of the exogenous cytokine occurred primarily through interference with the cascade of events ultimately leading to the onset of severe shock. Furthermore, we obtained evidence for the early expression of IL-9-specific transcripts in the spleens of mice challenged with *Pseudomonas*. This expression lasted for at least 5 days postchallenge in mice recovering from sublethal infection, but was reduced in the hours preceding death in the lethally infected mice (Fig. 7). Finally, preliminary experiments have shown that neutralization of endogenous IL-9 by specific Ab may exacerbate the course of sublethal challenge with the bacterium (data not shown).

Second, rIL-9 does not share the paradoxical effect of rIL-4 on resistance of mice to *P. aeruginosa*-induced septic shock as a function of time of administration relative to microbial challenge (22). In fact, rIL-4 appears to exert inhibitory or stimulatory effects on TNF-α production depending on timing of exposure, and such disparate effects correlate with the respective cure or exacerbation of *P. aeruginosa* infection.

Third, the therapeutic activity of rIL-9 in our model may involve multiple mechanisms. Because the peak in TNF-α production after challenge with live bacteria or LPS is an early event during the course of lethal endotoxemia, it is likely that TNF-α acts as an initiator in the cascade of endogenous mediators that will direct the inflammatory and metabolic responses eventually leading to severe shock and organ failure (34). The ineffectiveness of postchallenge therapy with IL-9 could be due to failure to interfere with the initial TNF-α release. However, the synergic effects of early TNF-α inhibition by PTX (which is per se ineffective under the adopted treatment conditions; Fig. 4) and deferred IL-9 therapy suggest that the recombinant cytokine may activate additional mechanisms that are fully protective once the initial TNF-α response has been reduced. That the combined activity of PTX and IL-9 cannot be simply explained by additive effects on TNF-α production (each treatment partially inhibiting TNF-α) is suggested by the fact that IL-9 administration occurred at a time (i.e., 3 h postchallenge) when circulating levels of TNF-α had already begun to decline (Fig. 3 and our unpublished data). On the other

hand, it is interesting to note that TNF-α production was inhibited by PTX treatment alone, while onset of endotoxic shock was not inhibited. This further substantiates the concept that a reduction in circulating TNF-α levels may be necessary but not sufficient for blocking development of Gram-negative bacterial shock.

In vitro studies have shown that IL-10 is a potent macrophage-deactivating cytokine capable of suppressing the induction of proinflammatory cytokines and up-regulating the production of IL-1R antagonist (43, 44). By virtue of its ability to suppress IL-12 release by macrophages, IL-10 also inhibits the production of IFN-γ by T and NK cells (6–8). In vivo studies have shown that selective IL-10 ablation by serotherapy or gene disruption will result in high levels of circulating TNF-α and IFN-γ and in enhanced endotoxin sensitivity (17–19). We found that the administration of an optimally protective regimen of IL-9 was associated with the early (at 2 h) appearance of high levels of circulating IL-10 in mice challenged with live bacteria. In contrast, the limited IL-4 response induced by *Pseudomonas* challenge at 4 h was not significantly affected by IL-9 administration (Fig. 5). IL-10-specific transcripts could be detected in the spleens of IL-9-treated and infected mice (Fig. 6). Of interest, the IL-10 response induced by IL-9 in LPS-challenged mice was much lower than in *Pseudomonas*-infected mice, and no IL-10 induction was observed in the absence of bacterial or LPS challenge (Fig. 5 and data not shown). As mentioned above, the LPS-treated mice would experience sustained TNF-α production and would eventually die.

An IL-10 dependence of IL-9 effects could not be firmly established by the use of anti-IL-10 mAb in our model, as the ablation of endogenous IL-10 would abnormally increase susceptibility to challenge and levels of circulating TNF-α in both IL-9-treated and untreated mice. Yet, our present data suggest a complex, mutual regulation of IL-9 and IL-10 productions within the context of the innate/inflammatory response to infection. Both the cellular source and the mechanism of IL-10 induction by IL-9 are unclear, yet it is possible that one major target of IL-9 activity is represented by the monocyte/macrophage, which could be both deactivated and primed to release IL-10 upon IL-9 treatment. IL-9 could also be acting on different cell types, including B and T lymphocytes, resulting in the early induction of IL-10 that would in turn act on macrophages. This would explain the generalized suppression of the proinflammatory cytokines, TNF-α, IL-12, and IFN-γ, all of which are known to be down-regulated by IL-10 (6, 17, 19).

In conclusion, our data suggest that IL-9 provides protection from *Pseudomonas*-induced lethal shock via multiple mechanisms that may include suppression of proinflammatory cytokines and induction of IL-10. Although a possible IL-10 dependence of IL-9 production has previously been described in different experimental models, our data provide the first evidence for the occurrence of bidirectional influences between IL-9 and IL-10 productions in innate immunity. Due to its potent and multiple activities involving modulation of proinflammatory and anti-inflammatory cytokines, IL-9 can be added to the list of cytokine immunomodulators that might be beneficial in the treatment of septic shock.

References

1. Tracey, K. J., B. Beutler, S. F. Lowry, J. Merryweather, S. Wolpe, I. W. Milsark, R. J. Hariri, T. J. Fahey, A. Zentella, J. D. Albert, et al. 1986. Shock and tissue injury induced by recombinant human cachectin. *Science 234:470.*
2. Alexander, H. R., G. M. Doherty, C. M. Buresh, D. J. Venzon, and J. A. Norton. 1991. A recombinant human receptor antagonist to interleukin 1 improves survival after lethal endotoxemia in mice. *J. Exp. Med. 173:1029.*
3. Doherty, G. M., J. R. Lange, H. N. Langstein, H. R. Alexander, C. M. Buresh, and J. A. Norton. 1992. Evidence for IFN-γ as a mediator of the lethality of endotoxin and tumor necrosis factor α. *J. Immunol. 149:1666.*
4. Wysocka, M., M. Kubin, L. Q. Vieira, L. Ozmen, G. Garotta, P. Scott, and G. Trinchieri. 1995. Interleukin-12 is required for interferon-γ production and lethality in lipopolysaccharide-induced shock in mice. *Eur. J. Immunol. 25:672.*
5. Van Deuren, M., A. S. M. Dofferhoff, and J. W. M. van der Meer. 1992. Cytokines and the response to infection. *J. Pathol. 168:349.*
6. D'Andrea, A., M. Aste-Amezaga, N. M. Valiante, X. Ma, M. Kubin, and G. Trinchieri. 1993. Interleukin 10 (IL-10) inhibits human lymphocyte interferon-γ production by suppressing natural killer cell stimulatory factor/IL-12 synthesis in accessory cells. *J. Exp. Med. 178:1041.*
7. Murphy, E. E., G. Terres, S. E. Macatonia, C.-S. Hsieh, J. Mattson, L. Lanier, M. Wysocka, G. Trinchieri, K. Murhpy, and A. O'Garra. 1994. B7 and interleukin 12 cooperate for proliferation and interferon γ production by mouse T helper clones that are unresponsive to B7 costimulation. *J. Exp. Med. 180:223.*
8. Kubin, M., M. Kamoun, and G. Trinchieri. 1994. Interleukin 12 synergizes with B7/CD28 interaction in inducing efficient proliferation and cytokine production of human T cells. *J. Exp. Med. 180:211.*
9. Beishuizen, A., I. Vermes, and C. Haanen. 1998. Endogenous mediators in sepsis and septic shock. *Adv. Clin. Chem. 33:55.*
10. Heumann, D., M. P. Glauser, and T. Calandra. 1998. Monocyte deactivation in septic shock. *Curr. Opin. Infect. Dis. 11:279.*
11. Beutler, B., I. W. Milsarek, and A. C. Cerami. 1985. Passive immunization against cachectin/tumor necrosis factor protects mice from lethal effect of endotoxin. *Science 229:689.*
12. Heinzel, F. P. 1990. The role of IFN-γ in the pathology of experimental endotoxemia. *J. Immunol. 145:2920.*
13. Ohlsson, K., P. Bjork, M. Bergenfeld, R. Hageman, and R. C. Thompson. 1990. Interleukin-1 receptor antagonist reduces mortality from endotoxin shock. *Nature 348:550.*
14. Bozza, M., A. R. Satoskar, G. Lin, B. Lu, A. A. Humbles, C. Gerard, and J. R. David. 1999. Targeted disruption of migration inhibitory factor gene reveals its critical role in sepsis. *J. Exp. Med. 189:341.*
15. Pfeffer, K., T. Matsuyama, T. M. Kundig, A. Wakeham, K. Kishihara, A. Shahinian, K. Wiegmann, P. M. Ohashi, M. Kronke, and T. W. Mak. 1993. Mice deficient for the 55 kd tumor necrosis factor receptor are resistant to endotoxic shock, yet succumb to *L. monocytogenes* infection. *Cell 73:457.*
16. Car, B. D., V. M. Eng, B. Schnyder, L. Ozmen, S. Huang, P. Gallay, D. Heumann, M. Aguet, and B. Riffel. 1994. Interferon γ receptor deficient mice are resistant to endotoxic shock. *J. Exp. Med. 179:1437.*
17. Marchant, A., C. Bruyns, P. Vandenabeele, M. Ducarme, C. Gerard, A. Delvaux, D. De Groote, D. Abramowicz, T. Velu, and M. Goldman. 1994. Interleukin-10 controls interferon-γ and tumor necrosis factor production during experimental endotoxemia. *Eur. J. Immunol. 24:1167.*
18. Howard, M., T. Muchamuel, S. Andrade, and S. Menon. 1993. Interleukin 10 protects mice from lethal endotoxemia. *J. Exp. Med. 177:1205.*
19. Gerard, C., C. Bruyns, A. Marchant, D. Abramowicz, P. Vandenabeele, A. Delvaux, W. Fiers, M. Goldman, and T. Velu. 1993. Interleukin 10 reduces the release of tumor necrosis factor and prevents lethality in experimental endotoxemia. *J. Exp. Med. 177:547.*
20. Baumhofer, J. M., B. G. Beinhauer, J. E. Wang, H. Brandmeier, K. Geissler, E. Losert, R. Philip, G. Aversa, and M. G. Rogy. 1998. Gene transfer with IL-4 and IL-13 improves survival in lethal endotoxemia in the mouse and ameliorates peritoneal macrophages immune competence. *Eur. J. Immunol. 28:610.*
21. Jain-Vora, S., A. M. LeVine, Z. Chroneos, G. F. Ross, W. M. Hull, and J. A. Whitsett. 1998. Interleukin-4 enhances pulmonary clearance of *Pseudomonas aeruginosa. Infect. Immun. 66:4229.*
22. Giampietri, A., U. Grohmann, C. Vacca, M. C. Fioretti, P. Puccetti, and F. Campanile. 2000. Dual effect of IL-4 on resistance to systemic Gram-negative infection and production of TNF-α. *Cytokine. In press.*
23. Muchamuel, T., S. Menon, P. Pisacane, M. C. Howerd, and D. A. Cockayne. 1997. IL-13 protects mice from lipopolysaccharide-induced lethal endotoxemia. *J. Immunol. 158:2898.*
24. Renauld, J.-C., F. Houssiau, J. Louahed, A. Vink, J. Van Snick, and C. Uyttenhove. 1993. Interleukin-9. *Adv. Immunol. 54:79.*
25. Demoulin, J. B., and J.-C. Renauld. 1998. Interleukin 9 and its receptor: an overview of structure and function. *Int. Rev. Immunol. 16:345.*
26. Kopf, M., G. Le Gros, M. Bachmann, M. C. Lamers, H. Bluethmann, and G. Köhler. 1993. Disruption of IL-4 gene blocks Th2 cytokine responses. *Nature 362:245.*
27. Gessner, A., H. Blum, and M. Röllinghoff. 1993. Differential regulation of IL-9 expression after infection with *Leishmania major* in susceptible and resistant mice. *Immunobiology 189:419.*
28. Monteyne, P., J.-C. Renauld, J. Van Broeck, D. W. Dunne, F. Brombacher, and J.-P. Coutelier. 1997. IL-4-independent regulation of in vivo IL-9 expression. *J. Immunol. 159:2616.*
29. Grencis, R. K., L. Hultner, and K. J. Else. 1991. Host protective immunity to *Trichinella spiralis* in mice: activation of Th cell subsets and lymphokine secretion in mice expressing different response phenotypes. *Immunology 74:329.*
30. Svetic, A., K. B. Madden, X. Zhou, P. Lu, I. M. Katona, F. D. Finkelman, J. F. Urban, and W. C. Gause. 1993. A primary intestinal helminthic infection rapidly induces a gut-associated elevation of Th2-associated cytokines and IL-3. *J. Immunol. 150:3434.*
31. Faulkner, H., J.-C. Renauld, J. Van Snick, and R. K. Grencis. 1998. Interleukin-9 enhances resistance to the intestinal nematode *Trichuris muris. Infect. Immun. 66:3832.*
32. Campanile, F., L. Binaglia, D. Boraschi, A. Tagliabue, M. C. Fioretti, and P. Puccetti. 1990. Antibacterial resistance induced by recombinant interleukin 1

in myelosuppressed mice: effect of treatment schedule and correlation with colony-stimulating activity in the bloodstream. *Cell. Immunol. 128:250.*
33. Campanile, F., A. Giampietri, U. Grohmann, L. Binaglia, M. C. Fioretti, and P. Puccetti. 1993. Protective effect of the cyclooxygenase inhibitor indomethacin against *Pseudomonas aeruginosa* infection in neutropenic mice. *Cell. Immunol. 147:341.*
34. Campanile, F., A. Giampietri, U. Grohmann, M. L. Belladonna, M. C. Fioretti, and P. Puccetti. 1996. Evidence for tumor necrosis factor α as a mediator of the toxicity of a cyclooxygenase inhibitor in Gram-negative sepsis. *Eur. J. Pharmacol. 307:191.*
35. Druez, C., P. Coulie, C. Uyttenhove, and J. Van Snick. 1990. Functional and biochemical characterization of mouse P40/IL-9 receptors. *J. Immunol. 145:2494.*
36. Fallarino, F., C. Uyttenhove, T. Boon, and T. F. Gajewski. 1996. Endogenous IL-12 is necessary for rejection of P815 tumor variants in vivo. *J. Immunol. 156:1095.*
37. Grohmann, U., R. Bianchi, E. Ayroldi, M. L. Belladonna, D. Surace, M. C. Fioretti, and P. Puccetti. 1997. A tumor-associated and self antigen peptide presented by dendritic cells may induce T cell anergy in vivo, but IL-12 can prevent or revert the anergic state. *J. Immunol. 158:3593.*
38. Grohmann, U., R. Bianchi, M. L. Belladonna, C. Vacca, S. Silla, E. Ayroldi, M. C. Fioretti, and P. Puccetti. 1999. IL-12 acts selectively on CD8α^- dendritic cells to enhance presentation of a tumor peptide in vivo. *J. Immunol. 163:3100.*
39. Houssiau, F. A., L. Schandené, M. Stevens, C. Cambiaso, M. Goldman, J. Van Snick, and J. C. Renauld. 1995. A cascade of cytokines is responsible for IL-9 expression in human T cells. *J. Immunol. 154:2624.*
40. Else, K. J., L. Hultner, and R. K. Grencis. 1992. Cellular immune responses to the murine nematode parasite *Trichuris muris*. II. Differential induction of Th-cell subsets in resistant versus susceptible mice. *Immunology 75:232.*
41. Petit-Frere, C., B. Dugas, P. Braquet, and J. M. Mencia-Huerta. 1993. Interleukin-9 potentiates the interleukin-4-induced IgE and IgG1 release from murine B lymphocytes. *Immunology 79:146.*
42. Louahed, J., A. Kermouni, J. Van Snick, and J.-C. Renauld. 1995. IL-9 induces expression of granzymes and high-affinity IgE receptor in murine T helper clones. *J. Immunol. 154:5061.*
43. Fiorentino, D. F., A. Zlotnik, T. R. Mosmann, M. Howard, and A. O'Garra. 1991. IL-10 inhibits cytokine production by activated macrophages. *J. Immunol. 147:3815.*
44. Howard, M., A. O'Garra, H. Ishida, R. de Waal Malefyt, and J. De Vries. 1992. Biological properties of interleukin 10. *J. Clin. Immunol. 12:239.*