INFECTION AND IMMUNITY, Mar. 1995, p. 1107–1112 Vol. 63, No. 3
0019-9567/95/$04.00+0


# $T_H1$ Cells Trigger Tumor Necrosis Factor Alpha-Mediated Hypersensitivity to *Pseudomonas aeruginosa* after Adoptive Transfer into SCID Mice

REINHARD FRÜH,[1] BARBARA BLUM,[2] HORST MOSSMANN,[3] HORST DOMDEY,[2] AND BERND-ULRICH VON SPECHT[1]*

*Chirurgische Universitätsklinik, Chirurgische Forschung, 79106 Freiburg,[1] Laboratorium für Molekulare Biologie, Genzentrum der Ludwig-Maximilians-Universität, 81375 München[2] and Max-Planck-Institut für Immunbiologie, 79108 Freiburg,[3] Germany*

Received 28 June 1994/Returned for modification 10 October 1994/Accepted 19 December 1994

**Recent experiments have shown that gamma interferon (IFN-γ), either administered or induced in vivo, e.g., by certain bacteria, is a key mediator in inducing hypersensitivity to bacterial lipopolysaccharides. The source of endogenous IFN-γ in this context (natural killer versus $T_H1$ cells) has not been investigated yet. In order to investigate the role of antigen-specific, IFN-γ-producing $T_H1$ cells in murine *Pseudomonas aeruginosa* infection, a murine $T_H1$ cell line was propagated in vitro by using recombinant *P. aeruginosa* outer membrane protein I. Adoptive transfer experiments were performed by intravenous injection of various amounts of $T_H1$ cells into *P. aeruginosa*-challenged SCID mice. Adoptive transfer of $5 \times 10^6$ T cells into SCID mice followed by an intraperitoneal challenge with $1.4 \times 10^6$ CFU of live *P. aeruginosa* resulted in the rapid death of the animals within 12 h postchallenge, whereas transfer of lower T-cell doses and saline as a control did not cause any detrimental effects. After challenge with $2.8 \times 10^6$ CFU of *P. aeruginosa*, similar results were obtained 18 h postchallenge; however, at the end of the 72-h observation period, no significant differences in survival rates were obtained between the groups treated with different amounts of T cells. The rapid death of mice treated with $5 \times 10^6$ T cells was reflected by 860-fold-elevated levels of tumor necrosis factor alpha (TNF-α) present in serum 2 h postchallenge, whereas no significant differences in TNF-α serum levels were detectable in mice treated with lower doses of T cells or with saline. Pretreatment of T-cell-reconstituted SCID mice with neutralizing anti-IFN-γ monoclonal antibodies completely protected mice from bacterial challenge and reduced TNF-α levels in serum. We conclude that under the experimental conditions described here, IFN-γ- and interleukin-2-producing $T_H1$ cells represent an important trigger mechanism inducing TNF-α-mediated hypersensitivity to bacterial endotoxin.**

Tumor necrosis factor alpha (TNF-α) is presently recognized to be the critical mediator of endotoxin-induced shock reactions (6, 30). Infusion of endotoxin or bacteria results in a brief, early serum TNF-α peak sufficient to induce a cascade of secondary cytokines, including interleukin-1 (IL-1), IL-6, and IL-8 (29, 34, 36). The source of TNF-α is the endotoxin-activated macrophage (6). Normal sensitivity to the lethal effects of lipopolysaccharide (LPS) is enhanced under different experimental conditions (treatment with hepatotoxic agents, growing malignant tumors, or infection with bacteria) (16, 17). Recently, several investigators have found that gamma interferon (IFN-γ) plays an essential role in the development of septic shock. Treatment of mice with IFN-γ increased susceptibility to endotoxin associated with increased levels of TNF-α. Treatment with anti-IFN-γ antibodies, however, blocked induction of circulating TNF-α (21, 22). Thus, IFN-γ seems to be a key mediator of endotoxin-related hypersensitivity. Katschinski et al. reported that certain bacteria induce sensitization to LPS by stimulation of endogenous IFN-γ production (26), although in this study, the primary source of IFN-γ ($T_H1$ versus natural killer [NK] cells) was not investigated. Previous studies by Heremans et al. (23) demonstrated that depletion of NK cells but not $CD4^+$ and $CD8^+$ T cells protected NMRI mice against LPS-induced Shwartzman-like reactions, although purified LPS was used, which is unlikely to activate T cells.

Our present study aimed to demonstrate the association of antigen-activated $T_H1$ cells in sensitization to LPS. An experimental adoptive transfer model in the mouse was developed by transfer of syngeneic IFN-γ- and IL-2-producing $T_H1$ cells specific for the inoculated gram-negative pathogen *Pseudomonas aeruginosa*, which represents a major cause of lethal gram-negative sepsis and shock (19, 27). As an antigen for the generation of *P. aeruginosa*-specific T cells, we chose the *P. aeruginosa* outer membrane protein I (OprI), which is highly conserved through all 17 serotypes of the IATS scheme and which has recently been cloned by our group (14). In vitro propagation of antigen-specific T cells requires soluble, nontoxic antigens. Metal chelate affinity chromatography based on the interaction of a stretch of histidine residues linked to the recombinant protein with $Ni^{2+}$-nitrilotriacetic acid (NTA) resin has been shown to provide these highly purified recombinant protein antigens in large amounts (32). The propagation of a syngeneic murine $T_H1$ cell line was performed by repeated in vitro stimulation of in vivo-primed lymph node cells with the purified recombinant *P. aeruginosa* OprI. As an adoptive transfer and challenge model, we used the severe combined immunodeficiency (SCID) mouse, since this mutation is characterized by a defective antigen receptor gene rearrangement and therefore lacks functional T and B cells (7, 8), whereas antigen-presenting cells (APC) and NK cells are not affected (12, 13). Thus, the pathways of infection in the SCID mouse are not

* Corresponding author. Mailing address: Chirurgische Forschung, Chirurgische Univ. Klinik, Hugstetterstrasse 55, D-79106 Freiburg, Germany. Phone: 0761 270-2575. Fax: 0761 270-2579.

modulated by host lymphocytes or specific antibodies to LPS or other bacterial structures.

The adoptive transfer study presented here will provide evidence that antigen-specific $T_H1$ cells trigger LPS hypersensitivity by release of IFN-γ upon bacterial infection.

## MATERIALS AND METHODS

**Animals.** Female BALB/c mice were purchased from the Zentralinstitut für Versuchstierzucht, Hannover, Germany. Female C.B-17 *scid*/*scid* mice (SCID) were purchased from Bomholtgard Breeding Centre, Ry, Denmark, and kept under sterile conditions in a laminar flow unit. All mice were used at an age of 6 to 8 weeks.

**Bacterial strains and growth conditions.** Strains of *P. aeruginosa* serogroup 1 (ATCC 33348) and serogroup 12 (ATCC 33359) were obtained as a gift from A. Bauernfeind, Max-von-Pettenkofer-Institut, Munich, Germany. Bacteria were grown and adjusted to the required concentrations as described previously (38).

**Preparation of native OprI.** For immunization of lymph node donor mice, OPRs of *P. aeruginosa* serogroup 12 were isolated by detergent extraction as described by Inouye et al. (25). Crude bacterial lysate was obtained by sonication of live *P. aeruginosa* serogroup 1 in a Branson sonifier. Protein content was determined by the Bio-Rad protein assay (Bio-Rad, Hercules, Calif.).

**Preparation and purification of recombinant *P. aeruginosa* OprI from *Escherichia coli*.** The *P. aeruginosa* OprI gene sequence contained in plasmid pITaq1 (14) was amplified by PCR with the two primers 5′-GCGGATCCATCGAGGG TAGAATGTGCAGGCCACTCCAAGAAACCG-3′ and 5′-CCAAGCTTATT ACTTGCGGCTGGCTTTTTC-3′. Amplified DNA was purified, digested with *Hin*dIII and *Bam*HI, and ligated into the corresponding sites of the expression vector pQE8 (Diagen, Düsseldorf, Germany) downstream of a sequence encoding an oligo-histidine peptide of six residues. The resulting construct, pQE8-I, was transformed into competent M15/pREP4 cells (Diagen). Expression of the recombinant OprI-$(His)_6$ fusion protein was induced by 4 h of incubation in 1 liter of TB medium containing 1 mM IPTG (isopropylthiogalactopyranoside) (31). Cells (6 g, wet weight) were suspended for 1 h at room temperature in 30 ml of lysis buffer (6 M guanidinium HCl, 0.1 M $NaH_2PO_4$, 0.1 M Tris [pH 8.0]). After 20 min of centrifugation at 10,000 × *g*, the supernatant was loaded onto an equilibrated Ni-NTA column (9-ml bed volume; Diagen). The column was washed first with 50 ml of lysis buffer and subsequently with 50 ml of 8 M urea–0.1 M $NaH_2PO_4$–0.1 M Tris (pH 8.0)–50 ml of 50 mM sodium phosphate (pH 8.0)–100 mM KCl–0.1% Tween 20–10 mM β-mercaptoethanol–10 mM EDTA–10 mM EGTA (ethylene glycol tetraacetic acid). The recombinant protein was eluted with 250 mM imidazole in the same buffer.

**SDS-PAGE and immunoblotting of OprI.** Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on 12% slab gels and Western immunoblotting were performed as described previously (33). After blotting, nitrocellulose sheets were incubated with 1:90-diluted 2A1 (28) hybridoma supernatant. Binding was visualized with alkaline phosphatase-linked goat anti-mouse immunoglobulin G (IgG) diluted 1:3,000 and 5-bromo-4-chloro-3-indolyl-phosphate as the substrate (Bio-Rad).

**Generation of OprI-specific T cells.** T lymphocytes were obtained and cultured as described by Corradin et al. (11). Briefly, BALB/c mice were injected subcutaneously at the base of the tail with 100 μl (100 μg) of OPRs of *P. aeruginosa* serogroup 12 emulsified in complete Freund's adjuvant. Nine days later, the mice were killed, and the inguinal and periaortic lymph nodes were removed aseptically. Lymph nodes were teased into a single-cell suspension in ice-cold RPMI 1640 with a tissue homogenizer (Braun Melsungen). The lymph node cell suspension was washed twice. The cell pellet was suspended in complete T-cell medium (CTCM; RPMI 1640 supplemented with 2.0 g of $NaHCO_3$ per liter, 10% fetal bovine serum, $5 \times 10^{-5}$ M 2-mercaptoethanol, 2 mM L-glutamine, 100 U of penicillin, and 100 μg of streptomycin per ml). Viable cells were counted by trypan blue exclusion and adjusted to a concentration of $2 \times 10^6$/ml. Then 12 ml of the cell suspension was incubated in 50-ml upright tissue culture flasks (Costar, Cambridge, Mass.) in the presence of 25 μg of recombinant OprI and 20 U of recombinant murine IL-2 (rIL-2; Boehringer, Mannheim, Germany) per ml at 37°C in a 5% $CO_2$ incubator.

**Preparation of irradiated APC.** To obtain syngeneic APC, spleens of female BALB/c mice were removed aseptically. A single-cell suspension was prepared in ice-cold RPMI 1640. Erythrocytes were lysed with 0.17 M $NH_4Cl$. The remaining cells were irradiated with 3,000 rads in a $^{137}Cs$ source. APC were then suspended in CTCM at $4 \times 10^6$ cells per ml.

**Long-term culture of T lymphocytes.** Four days after in vitro cultivation of lymph node cells, viable, blastlike cells were counted, washed twice, and resuspended in CTCM together with APC ($4 \times 10^5$ T cells and $2 \times 10^6$ APC) in 50-ml tissue flasks. Recombinant OprI (25 μg/ml) and 20 U of rIL-2 were added. Fresh medium and rIL-2 were substituted after 5 days. Cycles of restimulation with antigen and APC were repeated every 10 days for 10 restimulation cycles.

**Lymphocyte proliferation assay.** To determine the antigenic specificity of established T lymphocyte lines, cells were tested for their ability to proliferate in the presence of either recombinant or native OprI. At 10 to 12 days after antigenic restimulation, viable, blastlike cells were counted and seeded at $2 \times 10^4$ cells per well (50 μl) into 96-well flat-bottomed microculture plates (Costar). Then $2 \times 10^5$ APC (50 μl) were added per well, followed by addition of 100 μl of recombinant OprI or *P. aeruginosa* serogroup 1 lysate in triplicate at serial dilutions. After 54 h in culture, cells were pulsed with 1 μCi of [*methyl*-$^3$H]thymidine. Incubation was continued, and cells were harvested after another 18 h. [*methyl*-$^3$H]thymidine incorporation was counted in a liquid scintillation counter and expressed in counts per minute (cpm) as mean values of triplicate cultures.

**Phenotype and cytokine secretion of OprI-specific T cells.** Ten days following stimulation with antigen, T lymphocyte cultures were assessed by flow cytometry for the percentage of cells expressing the surface markers L3T4 and Lyt2.2. Phycoerythrin-conjugated anti-L3T4 (rat IgG2a) and fluorescein-conjugated anti-Lyt2.2 (rat IgG2a) antibodies were purchased from Dianova (Hamburg, Germany). Samples were analyzed on a Becton Dickinson FACScan. Rat IgG2a isotype antibodies were used to assess nonspecific binding of direct conjugated antibodies. Lymphocytes were gated forward scatter versus side scatter.

For detection of cytokines released by activated T cells, culture supernatants were prepared by restimulating $2 \times 10^5$ T cells per ml with 25 μg of recombinant OprI per ml in the presence of $2 \times 10^6$ APC per ml in total volumes of 1.5 ml in 24-well tissue culture plates. T-cell cultures were used at least 10 days following antigenic restimulation and cultured in the absence of exogenous IL-2. After 24 h, cell-free culture supernatants were collected and frozen at −20°C until assayed. For detection of TNF-α, concanavalin A (ConA)-stimulated T-cell supernatants were prepared in order to avoid the presence of TNF-α produced by APC. T cells isolated by density centrifugation were incubated at $2 \times 10^6$/ml with 2.5 μg of ConA per ml in 24-well tissue culture plates in total volumes of 1.5 ml. Supernatants were collected after 1, 2, 4, 8, 24, and 48 h.

IL-2 was assayed by the ability of T-cell culture supernatants to support the proliferation of the IL-2-dependent CTLL-2 cell line (18). Results were expressed as international units (IU) per milliliter by comparison with a standard curve of rIL-2.

IL-4 was assayed by the ability of T-cell culture supernatants to support the proliferation of the IL-2- and IL-4-dependent CT.4S cell line (24) (kindly provided by W. E. Paul, National Institutes of Health) in the presence of the IL-2 receptor antibody 5A2 (1:90-diluted hybridoma supernatant). Results were expressed as IU per milliliter by comparison with a standard curve of rIL-4 (Boehringer).

IFN-γ present in T-cell culture supernatants was assayed by an enzyme-linked immunosorbent assay (ELISA) (Holland Biotechnology BV, Leiden, The Netherlands). Levels of IFN-γ were expressed by comparison with the murine recombinant IFN-γ standard.

**Adoptive transfer and challenge experiments.** Ten days after the 10th antigenic restimulation, viable cells were isolated by density centrifugation (Histopaque 1.083; Sigma), washed twice, counted, and resuspended in sterile saline. Groups of six mice were injected in the lateral tail vein with different amounts of T cells ($5 \times 10^3$, $5 \times 10^4$, $5 \times 10^5$, and $5 \times 10^6$). Control groups were injected with sterile saline. Four hours later, all mice were challenged intraperitoneally with either $1.4 \times 10^6$ or $2.8 \times 10^6$ live *P. aeruginosa* serogroup 1 organisms suspended in sterile saline. Survival rates were monitored for the following 72 h. For determination of TNF-α, blood samples were drawn from the tail veins of five additional T-cell-reconstituted groups before or 2 and 4 h after challenge with $2.8 \times 10^6$ *P. aeruginosa.*

**In vivo neutralization of IFN-γ.** Groups of five mice were injected intraperitoneally with either 500 μg of neutralizing rat anti-mouse IFN-γ monoclonal antibodies (MAbs) (clone DB-1; Biosource) or 500 μg of human albumin (Curasan, Kleinostheim, Germany) 2 h prior to intravenous reconstitution with $5 \times 10^6$ T cells. Challenge with $1.4 \times 10^6$ CFU of *P. aeruginosa* serogroup 1 was performed 4 h following reconstitution with T cells. Blood samples for the detection of TNF-α were drawn 180 min postchallenge, and survival rates were monitored for the following 72 h.

**TNF-α bioassay.** The content of TNF-α in individual serum samples and in T-cell culture supernatants was determined in a cytotoxicity assay with the TNF-sensitive L929 cell line (kindly provided by C. Galanos, Max-Planck-Institut für Immunbiologie, Freiburg, Germany) in the presence of 1 μg of dactinomycin per ml as described previously (2). TNF-α activity was expressed as units per milliliter, 1 U being the amount of TNF-α causing 50% lysis of L929 cells. Recombinant TNF-α (Boehringer) was used as a standard control. The neutralizing anti-TNF-α MAb MP6-XT3 (1 μg/ml) (Pharmingen, San Diego, Calif.) was used as a control to exclude the presence of TNF-β.

## RESULTS

**Purification of recombinant *P. aeruginosa* OprI.** To obtain large amounts of purified OprI, metal chelate affinity chromatography was performed. Figure 1A shows the purification steps for recombinant OprI containing an amino-terminal peptide tag of six histidine residues. This procedure allowed us to prepare about 30 mg of recombinant OprI from a 1-liter culture of IPTG-induced recombinant *E. coli* with a purity of at least 95% as estimated by SDS-PAGE (Fig. 1B). In the recom-

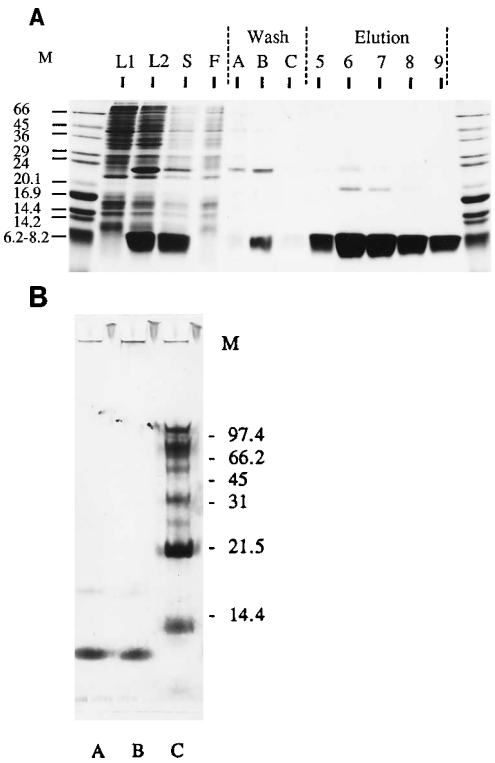


FIG. 1. (A) SDS-PAGE of the purification steps for recombinant OprI. Before loading the samples onto the 17% polyacrylamide gel, the proteins were diluted (dilutions are indicated in parentheses) so that signals of similar intensity were obtained. L1, lysate of cells before IPTG induction ($10^{-4}$); L2, lysate of cells 5 h after IPTG induction ($3 \times 10^{-5}$); S, supernatant of guanidinium-lysed cells ($3 \times 10^{-5}$); F, flowthrough of the supernatant of guanidinium-lysed cells through the Ni-NTA column ($3 \times 10^{-5}$); Wash A ($2 \times 10^{-5}$); Wash B, buffer B ($2 \times 10^{-4}$); Wash C, buffer C ($2 \times 10^{-4}$); Elutions 5 to 9, fractions 5 to 9, respectively ($5 \times 10^{-4}$); M, molecular size markers (in kilodaltons). (B) Silver staining of purified recombinant OprI. OprI was subjected to SDS-PAGE as in panel A. Gels were stained with a commercial staining kit (Pharmacia, Freiburg, Germany). Lanes A and B, pools of recombinant OprI after the last purification step; lane C, molecular size markers.

binant OprI preparation, the 8-kDa band corresponding to native OprI could be demonstrated by Western blotting after reaction with the OprI-specific MAb 2A1 (data not shown).

**Antigenic specificity of established T cells.** The specific incorporation of [$^3$H]thymidine by $2 \times 10^4$ T cells and $2 \times 10^5$ APC in the presence of both recombinant and native OprI contained in *P. aeruginosa* serogroup 12 lysate is shown in Table 1. Significant proliferative responses were obtained for both antigen preparations, with maximum stimulation indices of 32.5 in the case of recombinant OprI and 26.3 in the case of native OprI. After incubation of T cells with *E. coli* M15/pREP4 lysate (the host strain for recombinant OprI) as well as after incubation with OprI in the absence of APC (data not shown), background activities were obtained.

**Phenotype and cytokine secretion of OprI-specific T cells.** The percentage of in vitro-propagated T cells expressing the L3T4 and Lyt2.2 surface markers was determined by flow cytometric analysis of double-stained samples. Nearly 99% of gated cells were positive for the murine T helper cell marker L3T4, and 1% stained double positive for L3T4 and Lyt2.2. In 24-h supernatants of $2 \times 10^5$ OprI-specific T cells, 88 U of IL-2 per ml was detected by a CTLL-2 assay, 8,000 ng of IFN-γ per ml was detected by ELISA, and 2.11 U of TNF-α per ml was detected by a TNF bioassay. No IL-4 was measurable in culture supernatants with the CT.4S assay.

TABLE 1. [$^3$H]thymidine incorporation by T lymphocytes[a]

| Antigen | Concn (μg/ml) | [$^3$H]thymidine incorporation (mean cpm ± SEM) |
|---|---|---|
| None (medium only) | | 4,327 ± 574 |
| Recombinant OprI | 100 | 140,670 ± 3,262 |
| *P. aeruginosa* lysate | 50 | 113,233 ± 2,939 |
| *E. coli* M15 lysate | 50 | 7,027 ± 193 |
| ConA | 2.5 | 96,741 ± 2,382 |

[a] [$^3$H]thymidine incorporation by the established T-lymphocyte line in response to recombinant OprI or sonicated *P. aeruginosa* was determined during the 10th restimulation period. T cells ($2 \times 10^4$) were incubated with $2 \times 10^5$ APC in the presence of serial dilutions of recombinant OprI or sonicated *P. aeruginosa*. Controls were incubated with either sonicated *E. coli* M15/pREP4, ConA, or medium alone. Values are means of triplicate cultures ± standard error of the mean.

After incubation of $2 \times 10^6$ T cells per ml in the absence of APC, maximum TNF-α concentrations of 80 U/ml were obtained 8 h after addition of 2.5 μg of ConA per ml.

**Adoptive transfer and challenge experiments.** The survival rates of groups of six SCID mice, each reconstituted with various amounts of T cells after intraperitoneal challenge with $1.4 \times 10^6$ live *P. aeruginosa* serogroup 1 cells, are presented in Fig. 2. Reconstitution of the control group with $5 \times 10^6$ T cells without subsequent bacterial challenge did not cause any visible detrimental effects (data not shown). All mice in the saline control group as well as in the groups treated with $5 \times 10^3$, $5 \times 10^4$, and $5 \times 10^5$ T cells survived the 72-h observation period. In contrast, in the group that was treated with $5 \times 10^6$ T cells, five of six animals died rapidly, within the first 12 h. Thus, transfer of $5 \times 10^6$ T cells apparently enhanced the susceptibility of animals to the effects of *P. aeruginosa* infection. Figure 3 shows the same experiment after challenge with $2.8 \times 10^6$ live *P. aeruginosa*. No significant differences in survival rates were obtained after 72 h, although the time to death differed depending on the number of T cells applied. At 18 h postchallenge, five of six mice in the group treated with $5 \times 10^6$ T cells had died, whereas only one mouse in the group treated with $5 \times 10^4$ cells died. Thus, at 18 h postchallenge, significant differences in survival rates ($P \leq 0.0024$) were obtained, as calculated by a two-tailed Fisher exact test.

**TNF-α in serum samples.** TNF-α serum levels in *P. aerugi-*

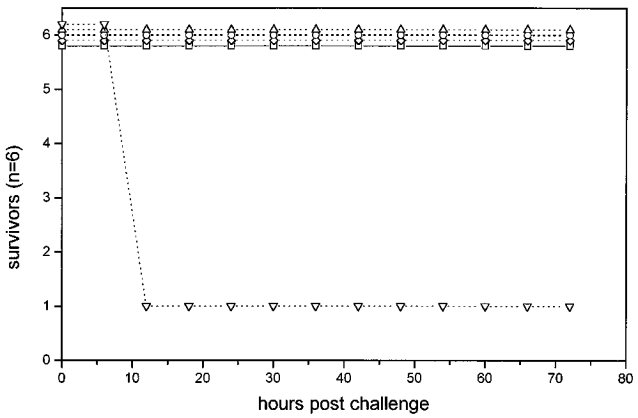


FIG. 2. Survival of SCID mice reconstituted with $5 \times 10^3$ (◇), $5 \times 10^4$ (○), $5 \times 10^5$ (△), or $5 \times 10^6$ (▽) OprI-specific T cells or saline (□) after intraperitoneal challenge with $1.4 \times 10^6$ CFU of *P. aeruginosa* serogroup 1.

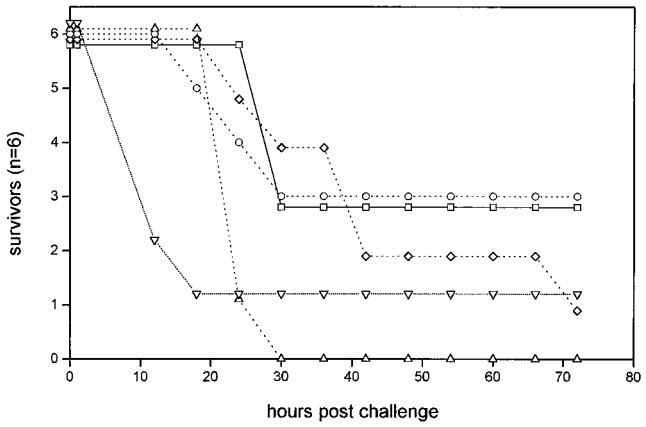


FIG. 3. Survival of SCID mice reconstituted with $5 \times 10^3$ (◇), $5 \times 10^4$ (○), $5 \times 10^5$ (△), or $5 \times 10^6$ (▽) OprI-specific T cells or saline (□) after intraperitoneal challenge with $2.8 \times 10^6$ CFU of *P. aeruginosa* serogroup 1.

*nosa*-challenged SCID mice are shown in Fig. 4. TNF-α was not detectable immediately before challenge. At 2 h postchallenge, however, TNF-α was detectable in all groups, including those animals which were not treated with T cells (saline control). No significant differences were obtained between TNF-α levels in saline-treated SCID mice (11.29 ± 3.74 U/ml) and animals receiving $5 \times 10^3$ (15.08 ± 2.60 U/ml), $5 \times 10^4$ (13.61 ± 2.22 U/ml), or $5 \times 10^5$ (17.94 ± 2.08 U/ml) T cells. However, mice injected with $5 \times 10^6$ T cells demonstrated an 860-fold increase in serum TNF-α levels, measuring 9,465 ± 1,350 U/ml. At 4 h postchallenge, increased levels of TNF-α, exceeding those measured at 2 h postchallenge in all other groups, were still detectable in the group treated with $5 \times 10^6$ T cells (37.05 ± 18.5 U/ml). No significant differences were observed among the groups treated with $5 \times 10^5$, $5 \times 10^4$, or $5 \times 10^3$ T cells or saline.

**In vivo neutralization of IFN-γ in T-cell-reconstituted SCID mice.** The effects of pretreatment of mice with anti-IFN-γ MAbs on survival and serum TNF-α levels following bacterial challenge were determined. A single injection of 500 μg of anti-IFN-γ MAbs completely protected all the mice (five of five), whereas all five albumin-treated control mice died, accompanied by a significant decrease in serum TNF-α levels (7 ± 2.9 U/ml) in antibody-treated animals compared with the controls (523 ± 114 U/ml) measured 180 min postchallenge.

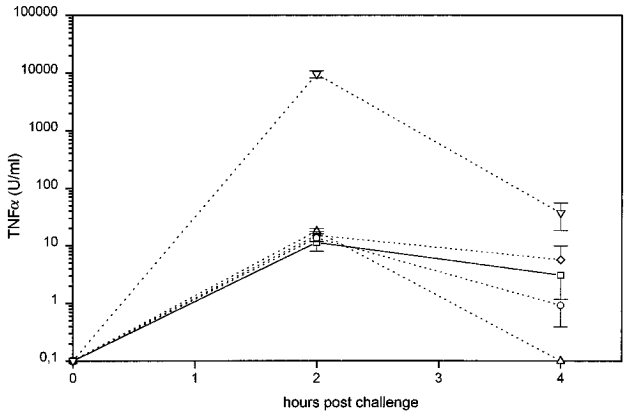


FIG. 4. Time course of TNF-α production by SCID mice reconstituted with $5 \times 10^3$ (◇), $5 \times 10^4$ (○), $5 \times 10^5$ (△), or $5 \times 10^6$ (▽) OprI-specific T cells or saline (□) after intraperitoneal challenge with $2.8 \times 10^6$ CFU of *P. aeruginosa* serogroup 1. Serum for TNF-α estimation was collected 1 h before or 2 and 4 h after challenge. Each point of the curve represents the results (mean ± standard error of the mean) for six individual animals.

## DISCUSSION

Despite the lack of functional T and B cells, naive SCID mice show a relatively high resistance against challenge with *P. aeruginosa*. In former studies, a 50% lethal dose ($LD_{50}$) of 2.49 $\times 10^6$ CFU after challenge with *P. aeruginosa* serogroup 1 was calculated by the probit regression model (data not shown), whereas after challenge of immunocompetent BALB/c mice, an $LD_{50}$ of $2.17 \times 10^7$ CFU was calculated (38). This observation indicates that effective, non-antigen-specific and antibody-independent defense mechanisms are present in the SCID mouse. The cellular effectors responsible for the clearance of *P. aeruginosa* in the SCID mouse are likely to be APC and NK cells, which are not affected by the SCID mutation (12, 13). The T-cell-independent mechanism of macrophage activation defined in the SCID mouse, which has been confirmed for a number of bacterial pathogens and lipopolysaccharides (3, 4), also seems to be responsible for the clearance of *P. aeruginosa*. Stimulation of macrophages by bacterial endotoxin results in release of TNF-α, leading to the activation of NK cells (39). Activated NK cells react by subsequent release of IFN-γ and thus augment macrophage activation, followed by increased antigen presentation and phagocytosis. This is supported by our finding that in vitro incubation of SCID mouse splenocytes with a lysate of *P. aeruginosa* promotes the secretion of significant levels of IFN-γ into the culture supernatants (data not shown). Consequently, IFN-γ secreted by adoptive transferred antigen-specific $T_H1$ cells should increase resistance against the particular pathogen. In fact, our present study demonstrates that opposite findings were obtained after transfer of *P. aeruginosa* OprI-specific $T_H1$ cells into *P. aeruginosa*-challenged SCID mice. Whereas transfer of $5 \times 10^3$, $5 \times 10^4$, and $5 \times 10^5$ T cells did not exhibit any significant effects on survival rates and serum TNF-α levels of mice compared with the saline controls, transfer of $5 \times 10^6$ T cells induced rapid death of the mice within 12 h after challenge with $1.4 \times 10^6$ *P. aeruginosa* cells. Obviously, after transfer of $5 \times 10^6$ T cells, a critical threshold was exceeded, inducing hypersensitivity to bacterial challenge. The rapid killing of the animals was reflected by an immense increase in serum TNF-α levels. Whereas no differences in TNF-α levels were obtained between the saline control group and the groups treated with $5 \times 10^3$, $5 \times 10^4$, and $5 \times 10^5$ T cells, transfer of $5 \times 10^6$ T cells resulted in an 860-fold increase in serum TNF-α levels measured 2 h postchallenge. Thus, under the experimental conditions presented here, $T_H1$ cells seem to play a key role in inducing TNF-α-mediated hypersensitivity to bacterial endotoxin, although beneficial effects of antigen-specific $T_H1$ cells cannot be excluded without performing dose titration experiments with smaller bacterial inocula and assessing CFU in various tissues.

Following in vitro stimulation with recombinant OprI, the established T cells produced IFN-γ, IL-2, and TNF-α. Consequently, the established *P. aeruginosa* OprI-specific T cells could be identified as $T_H1$ cells (9, 15). After adoptive transfer into *P. aeruginosa*-challenged SCID mice, cytokine release by activated $T_H1$ cells has several implications. IFN-γ is activating mononuclear phagocytes, resulting in enhanced endotoxin-induced TNF-α release (10), phagocytosis, and presentation of bacterial antigens. IL-2 is known to be a strong activator of NK cells, resulting in further secretion of IFN-γ by NK cells (20).

High levels of IFN-γ produced by both $T_H1$ cells and NK cells finally promote excessive TNF-α release by mononuclear cells. Consequently, a positive-feedback loop is generated, resulting in an overwhelming immune response, leading to the development of septic shock. The role of IFN-γ as an initiating signal is supported by our findings that pretreatment of T-cell-reconstituted SCID mice with anti-IFN-γ MAbs both protected mice from bacterial challenge and significantly reduced serum TNF-α levels. Furthermore, the sensitivity of mice to TNF is enhanced by the ability of IFN-γ to induce increased expression of cellular TNF receptors (1, 35). Thus, IFN-γ not only promotes increased TNF-α levels but also augments the toxic activities of TNF-α.

The immense concentrations of TNF-α detected in serum samples of mice treated with $5 \times 10^6$ T cells (9,465 ± 1,350 U/ml) are unlikely to be produced by $T_H1$ cells themselves. Even after in vitro stimulation of $2 \times 10^6$ T cells with the T-cell mitogen ConA, maximum concentrations of 80 U/ml could be observed. Furthermore, if $5 \times 10^6$ transferred T cells are capable of producing 9,465 U of TNF-α per ml, lower doses of T cells should also deliver sufficient concentrations of TNF-α to be detectable in serum. In fact, that was not the case, since no significant differences in TNF-α levels were obtained between the saline control group and the groups treated with $5 \times 10^3$, $5 \times 10^4$, and $5 \times 10^5$ T cells. The death of the animals was not strictly correlated to the elevation of serum TNF-α levels. Although the animals reconstituted with $5 \times 10^6$ T cells died more rapidly than the animals reconstituted with lower T-cell doses or saline, significant differences in survival rates were not obtained after 72 h. Obviously, a distinct threshold in serum TNF-α level is required for triggering lethal shock reactions in mice. However, provided that a sufficient bacterial dose is applied, nonreconstituted SCID mice are also capable of generating corresponding TNF-α serum levels, according to recent observations by Heremans et al., who demonstrated that naive SCID mice can be primed for the LPS-induced Shwartzman reaction (22).

The data presented are in accordance with recent studies showing IFN-γ to be the essential cytokine inducing sensitization to LPS (21, 23, 26). The critical aspect of our findings, however, is that under experimental conditions, $T_H1$ cells deliver sufficient amounts of IFN-γ to trigger LPS hypersensitivity. Future studies on the pathogenesis of endotoxin-induced shock should therefore also consider the role of $T_H1$ cells with respect to their possible association with the development of endotoxin hypersensitivity.

## ACKNOWLEDGMENTS

The excellent secretarial assistance of Gerlinde Moroder is gratefully acknowledged. We thank Anja Schmidt, Marion Dürr, and Sylvia Mallok for expert technical assistance.

This work was supported by grant 01KI 8910/4 from the Bundesminister für Forschung und Technologie to Horst Domdey and Bernd-Ulrich von Specht and by grant E/B41G/L0407/L5921 from the Bundesminister für Verteidigung to Bernd-Ulrich von Specht.

## REFERENCES

1. **Aggarwal, B. B., T. E. Eessalu, and P. Hass.** 1985. Characterization of receptors for human tumor necrosis factor and their regulation by γ-interferon. Nature (London) **318:**665–667.
2. **Aggarwal, B. B., W. J. Kohr, P. E. Hass, B. Moffat, S. A. Spencer, W. J. Henzel, T. S. Bringman, G. E. Nedwin, D. V. Goedel, and R. N. Harkins.** 1985. Human tumor necrosis factor: production, purification, and characterization. J. Biol. Chem. **260:**2345–2354.
3. **Bancroft, G. J., R. D. Schreiber, G. C. Bosma, M. J. Bosma, and E. R. Unanue.** 1987. A T cell-independent mechanism of macrophage activation by interferon-γ. J. Immunol. **139:**1104–1107.
4. **Bancroft, G. J., R. D. Sheehan, R. D. Schreiber, and E. R. Unanue.** 1989. Tumor necrosis factor is involved in the T cell-independent pathway of macrophage activation in scid mice. J. Immunol. **143:**127–130.
5. **Beutler, B., and A. Cerami.** 1988. Cachectin/tumor necrosis factor: an endogenous mediator of shock and inflammation. Annu. Rev. Biochem. **57:**505–516.
6. **Beutler, B., I. W. Milsark, and A. Cerami.** 1985. Passive immunization against cachectin/tumor necrosis factor (TNF) protects from the lethal effects of endotoxin. Science **229:**869–871.
7. **Bosma, G. C., R. P. Custer, and M. Bosma.** 1983. A severe combined immunodeficiency in the mouse. Nature (London) **301:**527–530.
8. **Bosma, G. C., M. T. Davisson, N. R. Reutsch, H. O. Sweet, L. D. Shultz, and M. Bosma.** 1989. The mouse mutation severe combined immunodeficiency (scid) is on chromosome 16. Immunogenetics **29:**54–57.
9. **Bottomly, K.** 1988. A functional dichotomy in CD4+ T lymphocytes. Immunol. Today **9:**268–274.
10. **Collart, M. A., D. Belin, J. D. Vassalli, S. De Kossodo, and P. Vassalli.** 1986. Gamma interferon enhances macrophage transcription of the tumor necrosis factor/cachectin, interleukin 1, and urokinase genes, which are controlled by short-lived repressors. J. Exp. Med. **164:**2113–2118.
11. **Corradin, G., H. M. Ettlinger, and J. M. Chiller.** 1977. Lymphocyte specificity to protein antigens. 1. Characterization of the antigen-induced in vitro T cell-dependent proliferative response with lymph node cells from primed mice. J. Immunol. **119:**1048–1053.
12. **Czitrom, A. A., S. Edward, R. A. Phillips, M. J. Bosma, P. Marrack, and W. Kappler.** 1985. The function of antigen-presenting cells in mice with severe combined immunodeficiency. J. Immunol. **134:**2276–2280.
13. **Dorshkind, K., S. B. Pollack, M. J. Bosma, and R. A. Phillips.** 1984. Natural killer (NK) cells are present in mice with severe combined immunodeficiency (scid). J. Immunol. **134:**3798–3801.
14. **Duchêne, M., C. Barron, A. Schweizer, B.-U. von Specht, and H. Domdey.** 1989. *Pseudomonas aeruginosa* outer membrane lipoprotein I gene: molecular cloning, sequence, and expression in *Escherichia coli*. J. Bacteriol. **171:** 4130–4137.
15. **Fitch, F. W., M. D. McKisic, D. W. Lancki, and T. F. Gajewski.** 1993. Differential regulation of murine T lymphocyte subsets. Annu. Rev. Immunol. **11:**29–48.
16. **Freudenberg, M. A., and C. Galanos.** 1991. Tumor necrosis factor alpha mediates lethal activity of killed gram-negative and gram-positive bacteria in D-galactosamine-treated mice. Infect. Immun. **59:**2110–2115.
17. **Galanos, C., M. A. Freudenberg, and W. Reutter.** 1979. Galactosamine-induced sensitization to the lethal effects of endotoxin. Proc. Natl. Acad. Sci. USA **76:**5939–5943.
18. **Gearing, A. J. H., and C. R. Bird.** 1987. Production and assay of interleukin 2, p. 291–301. *In* M. J. Clemens, A. G. Morris, and A. J. H. Gearing (ed.), Lymphokines and interferons: a practical approach. IRL Press, Oxford.
19. **Griffith, S. J., C. Nathan, R. K. Selander, W. Chamberlin, S. Gordon, S. Kabins, and R. A. Weinstein.** 1989. The epidemiology of *Pseudomonas aeruginosa* in oncology patients in a general hospital. J. Infect. Dis. **160:**1030–1036.
20. **Handa, K., R. Suzuki, H. Matsui, Y. Shimizu, and K. Kumagai.** 1983. Natural killer (NK) cells as a responder to interleukin-2. II. IL-2-induced interferon-γ production. J. Immunol. **130:**988–992.
21. **Heinzel, F. P.** 1990. The role of IFN-γ in the pathology of experimental endotoxemia. J. Immunol. **145:**2920–2924.
22. **Heremans, H., C. Dillen, J. Van Damme, and A. Billiau.** 1994. Essential role for natural killer cells in the lethal lipopolysaccharide-induced Shwartzman-like reaction in mice. Eur. J. Immunol. **24:**1155–1160.
23. **Heremans, H., J. Van Damme, C. Dillen, R. Dijkmans, and A. Billiau.** 1990. Interferon-γ, a mediator of lethal lipopolysaccharide-induced Shwartzman-like shock reactions in mice. J. Exp. Med. **171:**1853–1869.
24. **Hu-Li, J., C. Ohara, C. Watyson, W. Tsang, and W. E. Paul.** 1989. Derivation of a T cell line that is highly responsive to IL-4 and IL-2 (CT.4R) and of an IL-2 hyporesponsive mutant of that line (CT.4S). J. Immunol. **142:**800–807.
25. **Inouye, S., K. Takeishi, N. Lee, M. De Martini, A. Hirashima, and M. Inouye.** 1979. Lipoprotein from the outer membrane of *Escherichia coli*: purification, paracrystallization, and some properties of its free form. J. Bacteriol. **127:**555–563.
26. **Katschinski, T., C. Galanos, A. Coumbos, and M. A. Freudenberg.** 1992. Gamma interferon mediates *Propionibacterium acnes*-induced hypersensitivity to lipopolysaccharide in mice. Infect. Immun. **60:**1994–2001.
27. **Korvick, J. A., J. W. Marsh, T. E. Starzl, and V. L. Yu.** 1991. *Pseudomonas aeruginosa* bacteremia in patients undergoing liver transplantation: an emerging problem. Surgery **109:**62–68.
28. **Marget, M., A. Eckhardt, W. Ehret, B.-U. von Specht, M. Duchêne, and H. Domdey.** 1988. Cloning and characterization of cDNAs coding for the heavy and light chains of a monoclonal antibody specific for *Pseudomonas aeruginosa* outer membrane protein I. Gene **74:**335–345.
29. **Nawroth, P. P., I. Bank, D. Handley, J. Cassimeris, L. Chess, and D. Stern.** 1986. Tumor necrosis factor/cachectin interacts with endothelial cell receptors to induce release of interleukin 1. J. Exp. Med. **163:**1363–1375.

30. **Old, L. J.** 1985. Tumor necrosis factor (TNF). Science **230:**630–632.
31. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
32. **Takacs, B. J., and M. F. Girard.** 1991. Preparation of clinical grade proteins produced by recombinant DNA technologies. J. Immunol. Methods **143:** 231–240.
33. **Towbin, M., T. Staehelin, and J. Gordon.** 1979. Electrophoretic transfer of proteins from polyacrylamide gel to nitrocellulose sheets: procedure and some applications. Proc. Natl. Acad. Sci. USA **76:**4350–4354.
34. **Tracey, K. J., and S. F. Lowry.** 1990. The role of cytokine mediators in septic shock. Adv. Surg. **23:**21–56.
35. **Tsujimoto, M., Y. P. Yip, and J. Vilcek.** 1986. Interferon-γ enhances expression of cellular receptors for tumor necrosis factor. J. Immunol. **136:**2441–2444.
36. **Van Snick, J.** 1990. Interleukin-6: an overview. Annu. Rev. Immunol. **8:**253–278.
37. **Van Zee, K. J., L. E. DeForge, E. Fischer, M. A. Marano, J. S. Kenney, D. G. Remick, S. F. Lowry, and L. L. Moldawer.** 1991. IL-8 in septic shock, endotoxemia, and after IL-1 administration. J. Immunol. **146:**3478–3482.
38. **von Specht, B.-U., G. Strigl, W. Ehret, and W. Brendel.** 1987. Protective effect of an outer membrane vaccine against *Pseudomonas aeruginosa* infection. Infection **15:**408–412.
39. **Wherry, J. C., R. D. Schreiber, and E. R. Unanue.** 1992. Regulation of gamma interferon production by natural killer cells in *scid* mice: roles of tumor necrosis factor and bacterial stimuli. Infect. Immun. **59:**1709–1715.