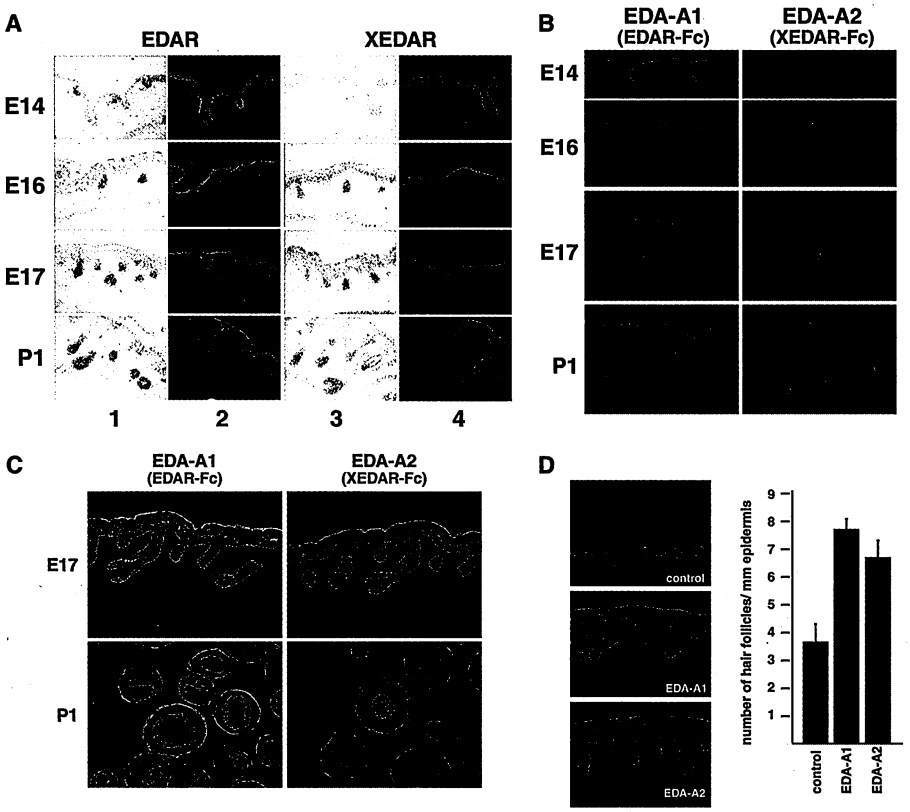

Fig. 4. Expression of EDAR, XEDAR, EDA-A1, and EDA-A2 in mouse skin. (**A**) Expressions of EDAR and XEDAR in mouse skin were examined by in situ hybridization (*23*). The digoxigenin-labeled riboprobes for mouse EDAR and XEDAR were synthesized corresponding to nucleotides 12 to 493 of the EDAR ORF and nucleotides 37 to 767 of the XEDAR ORF, respectively (1 and 3). The same sections were also immunostained with anti-keratin 5 (green) (2 and 4). (**B** and **C**) In situ staining of mouse skin with EDAR-hFc and XEDAR-hFc fusion proteins (red) (*24*). In (C), the mouse skin section was stained twice with anti-keratin 5 (green). A transverse view of P1 hair follicles shows that XEDAR-hFc and EDAR-hFc bind distinct regions. (**D**) Stimulation of epidermis downgrowths by EDA-A1 and EDA-A (*25*). Skin was dissected from CD-1 mouse embryos between E13.5 and E14.5 and cultured for 3 days in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. Purified recombinant Flag-EDA-A1, Flag-EDA-A2, or Flag-Blys (control) was used at a final concentration of 0.2 μg/ml. Skin sections were then immunostained with anti-keratin 5 to assess the number of epidermal downgrowths. The average number of downgrowths per unit length of epidermis was calculated on the basis of a total of 18 organ cultures in three different experiments.

signal sequence derived from protrypsin and the hFc region downstream of the XEDAR sequence, resulting in XEDAR-hFc. EDAR-hFc was constructed by fusing the human EDAR (amino acids 1 to 189) to hFc.
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15. Human EDA-A1 (amino acids 241 to 391) or EDA-A2 (amino acids 241 to 389) and human placental AP were amplified by polymerase chain reaction, fused, and cloned into the expression vector pFlag-CMV1 (Sigma) with AP at the NH_2-terminus of EDA-A1 or EDA-A2, respectively. Flag-EDA-A1 or Flag-EDA-A2 was constructed by cloning EDA-A1 (amino acids 179 to 391) or EDA-A2 (amino acids 179 to 389), respectively, into pFlag-CMV1.
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21. Single-letter abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.
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26. We thank L. Closkey, P. Hass, and M. Nagel for help with recombinant protein expression and purification; R. Pitti for help with measuring the affinity of ligand-receptor interaction; K. O'Rourke for proofreading the manuscript; members in V.D.'s laboratory for discussions; and Genentech's antibody technology and DNA synthesis labs.

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CD95/CD95 Ligand Interactions on Epithelial Cells in Host Defense to *Pseudomonas aeruginosa*

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Pseudomonas aeruginosa causes severe infections, particularly of the lung, that are life threatening. Here, we show that *P. aeruginosa* infection induces apoptosis of lung epithelial cells by activation of the endogenous CD95/CD95 ligand system. Deficiency of CD95 or CD95 ligand on epithelial cells prevented apoptosis of lung epithelial cells in vivo as well as in vitro. The importance of CD95/CD95 ligand–mediated lung epithelial cell apoptosis was demonstrated by the rapid development of sepsis in CD95- or CD95 ligand–deficient mice, but not in normal mice, after *P. aeruginosa* infection.

Several pathogens, including some bacteria, viruses, and parasites, are able to trigger apoptosis of mammalian host cells (*1*, *2*). A paradigm for bacteria-induced apoptosis is *Shigella flexneri*, which, upon invasion, induces apoptosis by the activation of caspases (in particular, caspase 1) (*3*). Some other bacteria also induce apoptosis, including *Salmonella typhimurium*, some enteropathogenic *Escherichia coli*, *Listeria monocytogenes*, and *Staphylococcus aureus* (*4*–*7*). However, the molecular mechanisms involved and their relevance to host pathogen interactions are still largely unknown.

P. aeruginosa is clinically one of the most important classes of bacteria, because it induces pneumonia and sepsis in cystic fibrosis or immunocompromised patients. To gain insight into the molecular mecha-

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nisms of *P. aeruginosa* interaction with mammalian cells, we investigated whether the induction of apoptosis contributes to the previously described cytotoxicity of *P. aeruginosa* (*8*). The results (Fig. 1) reveal marked apoptosis of human Chang conjunctiva cells or murine ex vivo lung fibroblasts within 30 min of infection with *P. aeruginosa* strain 762 (*9*).

To look for possible mechanisms by which apoptosis might be initiated during *P. aeruginosa* infection, we examined the involvement of the CD95/CD95 ligand system, one of the most important systems by which apoptosis is triggered in a variety of mammalian cells (*10*). Neutralization of the CD95/CD95 ligand system by the addition of CD95-Fc protein (*11*) to human epithelial cells or by the genetic deficiency of either CD95 or CD95 ligand in ex vivo fibroblasts from *lpr* (lymphoproliferation) or *gld* (generalized lymphoproliferative disease) C3H mice (*12*) almost completely blocked *P. aeruginosa*–mediated apoptosis (Fig. 1). In contrast, fibroblasts from (isogenic) normal C3H mice were highly sensitive to the induction of apoptosis, indicating a crucial role for the CD95/CD95 ligand system in the initiation of cellular apoptosis by *P. aeruginosa* (*13*).

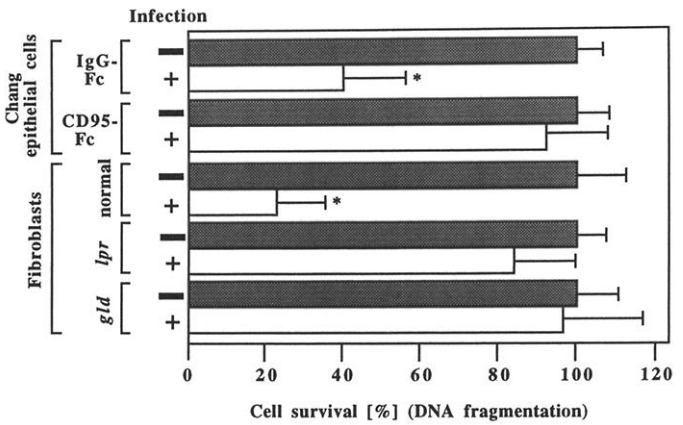

Fig. 1. *P. aeruginosa* induces apoptosis of epithelial cells by the CD95/CD95 ligand system. Human Chang conjunctiva cells or normal murine ex vivo lung fibroblasts undergo apoptosis after infection with *P. aeruginosa* strain 762 for 30 min (9). Genetic deficiency of CD95 or CD95 ligand in *lpr* or *gld* fibroblasts, as well as neutralization of CD95 ligand on Chang epithelial cells by treatment with soluble CD95-Fc protein (5 μg/ml), prevented *P. aeruginosa*–triggered apoptosis. Treatment with control immunoglobulin G (IgG)–Fc did not affect *P. aeruginosa*–induced apoptosis. Shown are the mean ± SD of three independent experiments (*$P < 0.05$, *t* test).

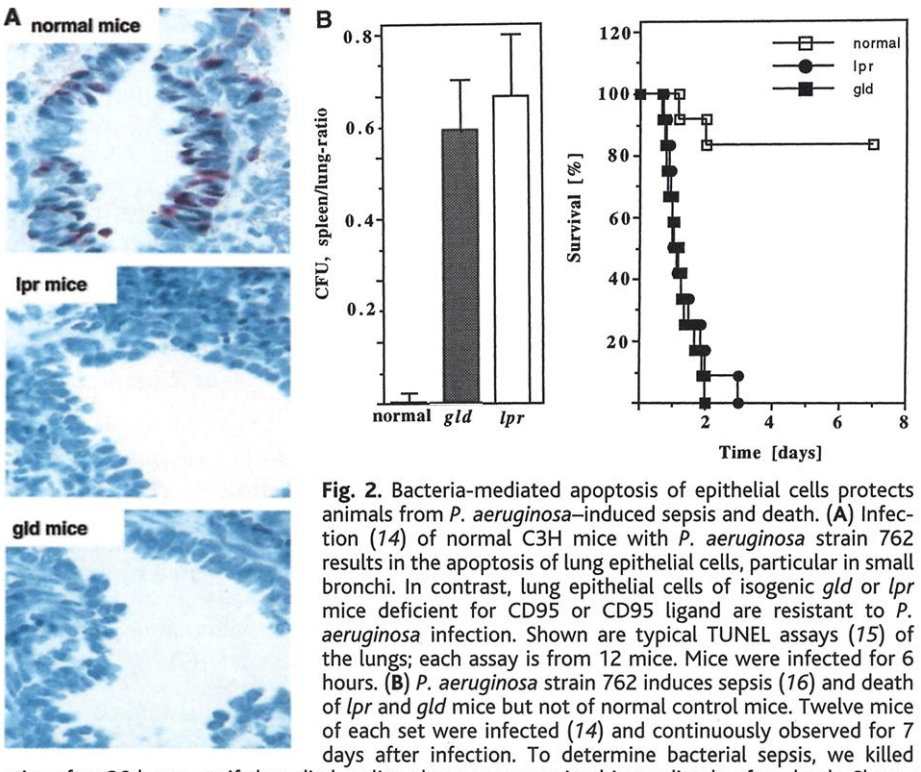

Fig. 2. Bacteria-mediated apoptosis of epithelial cells protects animals from *P. aeruginosa*–induced sepsis and death. (**A**) Infection (*14*) of normal C3H mice with *P. aeruginosa* strain 762 results in the apoptosis of lung epithelial cells, particular in small bronchi. In contrast, lung epithelial cells of isogenic *gld* or *lpr* mice deficient for CD95 or CD95 ligand are resistant to *P. aeruginosa* infection. Shown are typical TUNEL assays (*15*) of the lungs; each assay is from 12 mice. Mice were infected for 6 hours. (**B**) *P. aeruginosa* strain 762 induces sepsis (*16*) and death of *lpr* and *gld* mice but not of normal control mice. Twelve mice of each set were infected (*14*) and continuously observed for 7 days after infection. To determine bacterial sepsis, we killed mice after 36 hours, or if they died earlier, they were examined immediately after death. Shown is the ratio of *P. aeruginosa* CFUs grown from the spleen to the number of CFUs grown from the lung. This minimizes variations due to the infection procedure. Error bars indicate SD.

To analyze the importance of *P. aeruginosa*–triggered, CD95-mediated apoptosis in vivo, isogenic *lpr*, *gld*, or wild-type mice were intranasally infected (*14*) with *P. aeruginosa* strain 762, and the induction of apoptosis in lung epithelial cells was determined by TUNEL (terminal deoxynucleotidyl transferase–mediated deoxyuridine triphosphate nick-end labeling) 6 hours after infection (*15*). Infection of normal mice resulted in the rapid (within 3 to 4 hours) induction of apoptosis in epithelial cells of small bronchi (Fig. 2A). In contrast, lungs from infected *lpr* or *gld* mice did not display apoptotic cells.

To address the question of whether CD95-mediated apoptosis of lung epithelial cells induced by *P. aeruginosa* functions as part of the immune defense or as part of the bacterial attack against the host, we infected normal, *lpr*, or *gld* mice with *P. aeruginosa* (*14*) and monitored the development of *P. aeruginosa* colonization of the spleen (*16*), indicative of sepsis and, finally, death. None of the wild-type mice showed substantial numbers of *P. aeruginosa* in the spleen upon infection, and only 10% of these animals died in a 60-day observation period after infection (Fig. 2B). In contrast, all *lpr* and *gld* mice developed sepsis, and 100% of the animals died within 96 hours of infection.

Thus, the high susceptibility of *lpr* or *gld* mice to *P. aeruginosa* infections was critically associated with the failure of lung epithelial cells to be activated by CD95 and to undergo apoptosis upon infection. However, the high susceptibility of these mice might also have been caused by a malfunction of the immune system, thereby permitting the bacteria to induce generalized infection. To differentiate between these possibilities, we ablated the immune system of normal, *gld*, or *lpr* mice by irradiation and reconstituted these animals with bone marrow cells (BMCs) from syngenic *lpr* or *gld* mice, in the case of irradiated wild-type mice, or with BMCs from syngenic normal mice, in the case of irradiated *lpr* and *gld* mice (*17*); the success of the transplant was confirmed by the induction or absence, respectively, of activation-induced cell death (AICD) (*18*) in ex vivo splenic lymphoblasts (*19*) (Fig. 3A). Mice were intranasally infected (*14*) with *P. aeruginosa* and monitored for the development of sepsis (Fig. 3B). Normal mice transplanted with BMCs from *lpr* or *gld* mice were as resistant to infection as untransplanted normal

mice were (Fig. 3B). Likewise, *lpr* or *gld* mice transplanted with normal BMCs were as susceptible to infection with *P. aeruginosa* as untransplanted *lpr* or *gld* mice were and died of sepsis. Consistent with this, apoptosis of lung epithelial cells was only observed in normal mice, regardless of whether they had been transplanted with BMCs from *lpr* or *gld* mice or had been left untransplanted (Fig. 3C) (*14*). *lpr* or *gld* mice remained resistant to the induction of apoptosis in lung epithelial cells, despite transplantation with normal BMCs. This excludes a predominant role for the immune system and suggests a pivotal function of lung epithelial cells in the primary response to *P. aeruginosa* in the lung.

Several studies have demonstrated the cytotoxicity of *P. aeruginosa* toxins (*20*); however, the mechanisms of cell death triggered by these factors are unknown. Our work suggests that the up-regulation of the CD95/CD95 ligand system constitutes one of the major mechanisms mediating this cytotoxicity. It could be speculated that bacterial polysaccharides (*21*) induce a translocation of secretory vesicles, which have been previously shown to store CD95 and CD95 ligand (*22, 23*), resulting in increased CD95/CD95 ligand surface expression and the induction of apoptosis.

Activation of CD95 in lung epithelial cells may protect the host from *P. aeruginosa* infection by at least two mechanisms: First, apoptosis of infected cells results in the targeting of *P. aeruginosa* into apoptotic bodies, which are rapidly internalized by other cells. Fusion of those phagosomes with lysosomes then results in digestion of the bacteria. In contrast, internalization of *P. aeruginosa* without apoptosis of the host cell (as in *lpr* or *gld* mice) might permit the bacterium to block maturation of the phagosome, promote intracellular survival and even growth of the bacterium before transcytosis, and protect bacteria from the host immune system. Second, *P. aeruginosa*–mediated activation of CD95 might stimulate nuclear factor κB (*24*), c-Jun NH_2-terminal kinase (*25*), GADD153 (*26*), and phospholipase A_2 (*27*) or may interfere with the functions of growth factor receptors (*28*), resulting in the secretion of defensins and/or cytokines from epithelial cells. CD95-triggered secretion of defensins and/or cytokines into bronchi may then kill extracellular bacteria. Those factors may also prevent the penetration of bacteria through the epithelial cell barrier, even in situations where excessive apoptosis of epithelial cells occurs.

The concerted action of CD95-dependent lysosomal degradation of *P. aeruginosa* in

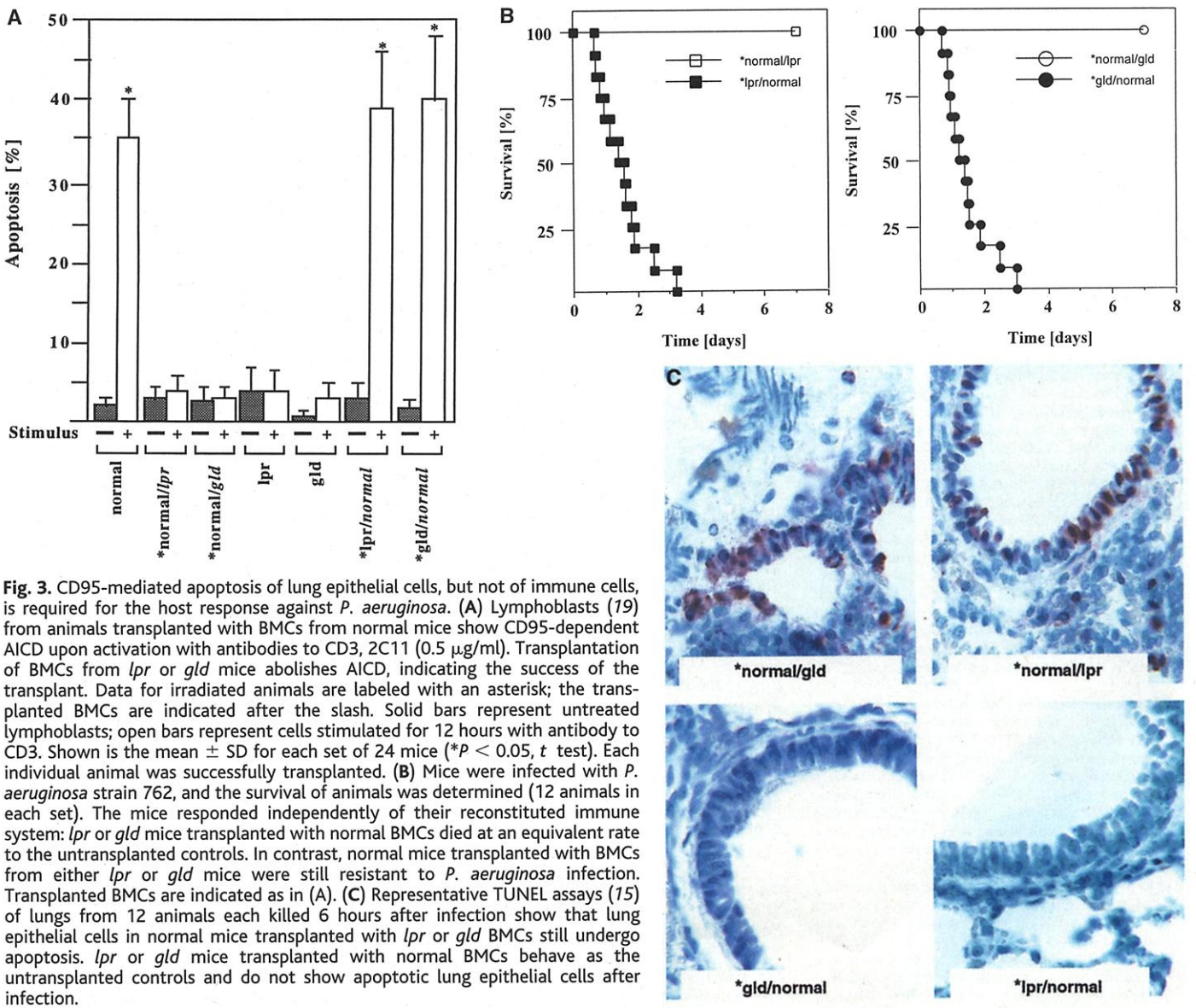

Fig. 3. CD95-mediated apoptosis of lung epithelial cells, but not of immune cells, is required for the host response against *P. aeruginosa*. (**A**) Lymphoblasts (*19*) from animals transplanted with BMCs from normal mice show CD95-dependent AICD upon activation with antibodies to CD3, 2C11 (0.5 μg/ml). Transplantation of BMCs from *lpr* or *gld* mice abolishes AICD, indicating the success of the transplant. Data for irradiated animals are labeled with an asterisk; the transplanted BMCs are indicated after the slash. Solid bars represent untreated lymphoblasts; open bars represent cells stimulated for 12 hours with antibody to CD3. Shown is the mean ± SD for each set of 24 mice ($^*P < 0.05$, *t* test). Each individual animal was successfully transplanted. (**B**) Mice were infected with *P. aeruginosa* strain 762, and the survival of animals was determined (12 animals in each set). The mice responded independently of their reconstituted immune system: *lpr* or *gld* mice transplanted with normal BMCs died at an equivalent rate to the untransplanted controls. In contrast, normal mice transplanted with BMCs from either *lpr* or *gld* mice were still resistant to *P. aeruginosa* infection. Transplanted BMCs are indicated as in (A). (**C**) Representative TUNEL assays (*15*) of lungs from 12 animals each killed 6 hours after infection show that lung epithelial cells in normal mice transplanted with *lpr* or *gld* BMCs still undergo apoptosis. *lpr* or *gld* mice transplanted with normal BMCs behave as the untransplanted controls and do not show apoptotic lung epithelial cells after infection.

apoptotic bodies and the secreted bacteriotoxic substances may impede intra- and extracellular bacteria. This would explain the pivotal role observed for the CD95/CD95 ligand system in host defense against infection with *P. aeruginosa*.

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9. Human Chang epithelial cells or murine fibroblasts were grown in Dulbecco's minimum essential medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine, penicillin (100 units/ml), and streptomycin (100 μg/ml). Murine fibroblasts were isolated from lungs by dissection of the tissue into ~2 mm^3 particles. Those particles were cultured for 3 days, the cultures were intensively washed, and the fibroblasts were recultured for an additional 5 days. All cells were infected (cell:bacteria ratio, 1:1000) with two well-defined laboratory strains of *P. aeruginosa*, American Type Culture Collection (ATCC) 27853 and PAO-1, as well as three clinical isolates, strains 762, 769, and 696, derived from sputum or urine. Infection time was 30 min for strain 762, 90 min each for 769 and ATCC 27853, and 6 hours for PAO-1 and 696. All strains induced apoptosis comparable to the values shown in Fig. 1. Infection experiments performed with human WI-38 epithelial cells revealed very similar results. Apoptosis was determined by metabolic labeling of the cells for 24 hours with 1 μCi/ml [^{3}H]thymidine (8.3 Ci/mmol) (NEN/DuPont). Cells were disrupted by freezing and thawing, and unfragmented genomic DNA was collected by filtration through glass fiber filters (Pharmacia) and measured by liquid scintillation counting. This method permitted easy separation of cellular from bacterial DNA. Cellular apoptosis was confirmed by staining the cells with fluorescein isothiocyanate–annexin V or ethidium bromide/propidium iodide, followed by flow cytometry or analysis of typical morphological changes of cells using fluorescence microscopy of cells stained with ethidium bromide/propidium iodide.
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14. Mice were infected by nasal application of 7×10^8 or 13×10^9 colony forming units (CFUs) of *P. aeruginosa* strains 762 or ATCC 27853 (total volume, 15 μl), respectively. Shown are the results with *P. aeruginosa* 762, but all in vivo experiments were repeated with ATCC 27853, revealing almost identical results. Equal infection of the mice was controlled by measuring bacterial counts in a small part of the lung obtained from each animal. Lungs were weighed then homogenized in 300 μl of sterile RPMI; 100 μl of the homogenates were spotted onto agar plates then cultured overnight, and *P. aeruginosa* CFUs were counted. Bacterial counts in the lungs 6 hours after infection (Figs. 2A and 3C) were $4 \pm 0.5 \times 10^5$, $5 \pm 1 \times 10^5$, or $4.7 \pm 0.8 \times 10^5$ bacteria per gram of lung in normal, *lpr*, or *gld* mice, respectively. Bacterial counts in the lungs 36 hours after infection (Fig. 2B) revealed $5.2 \pm 0.7 \times 10^5$, $6.3 \pm 0.8 \times 10^5$, or $6.5 \pm 0.9 \times 10^5$ bacteria per gram of lung in normal, *lpr*, or *gld* mice, respectively. After 72 hours, we detected $0.5 \pm 0.3 \times 10^5$, $10.1 \pm 0.8 \times 10^5$, or $11.9 \pm 1 \times 10^5$ bacteria per gram of lung in normal, *lpr*, or *gld* mice, respectively. In all survival experiments (Figs. 2B and 3B), four additional mice were infected and killed after 6 hours, confirming similar amounts of bacteria in the lung (4×10^5 to 6×10^5 bacteria per gram of lung).
15. Lungs were fixed for 2 days and embedded in paraffin; 6-μm sections of lung were cut, removed from paraffin, and digested with proteinase K for 2 min, and endogenous peroxidase was blocked with H_2O_2. Sections were stained with terminal deoxynucleotidyl transferase in the presence of biotinylated deoxyuridine triphosphate and developed with the ABC complex and 3-amino-9-ethylcarbazole. Counterstaining was done in hematoxylin.
16. Bacterial counts in spleens were determined as described for the lungs.
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Specific Mutations Induced by Triplex-Forming Oligonucleotides in Mice

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Triplex-forming oligonucleotides (TFOs) recognize and bind to specific duplex DNA sequences and have been used extensively to modify gene function in cells. Although germ line mutations can be incorporated by means of embryonic stem cell technology, little progress has been made toward introducing mutations in somatic cells of living organisms. Here we demonstrate that TFOs can induce mutations at specific genomic sites in somatic cells of adult mice. Mutation detection was facilitated by the use of transgenic mice bearing chromosomal copies of the *supF* and *cII* reporter genes. Mice treated with a *supF*-targeted TFO displayed about fivefold greater mutation frequencies in the *supF* gene compared with mice treated with a scrambled sequence control oligomer. No mutagenesis was detected in the control gene (*cII*) with either oligonucleotide. These results demonstrate that site-specific, TFO-directed genome modification can be accomplished in intact animals.

By inducing site-specific mutations in the genome, heritable changes can be achieved in gene function and expression. Triplex formation, through recognition of a specific region of duplex DNA by a single-stranded TFO, offers an attractive strategy to modify a mammalian genome. TFOs have so far been used successfully to modify genes and gene function in cells in vitro (*1*). With a view toward a therapeutic application, we have investigated the potential of a triplex approach to target specific mutations in somatic cells of mice in vivo. Transgenic mice (C57BL/6 mice containing multiple copies of a chromosomally integrated λsupFG1 vector, designated 3340) were previously generated (*2*) containing a 30–base pair (bp) triplex-target site within the *supFG1* mutation–reporter gene. We previously demonstrated TFO-targeted mutations in the *supFG1* gene in vitro on intracellular plasmid targets and on a chromosomal locus in a fibroblast cell line established from these mice (*3*–*5*). The results revealed 10- to 100-fold induction of site-specific mutations in cells treated with the specific TFO. Although psoralen conjugation to the TFO, coupled with UVA (ultraviolet, long wave) irradiation, generally increased the frequency of mutations, targeted mutagenesis was seen even without psoralen photoproduct generation, suggesting a substantial triplex-mediated process of mutagenesis. On the basis of these results, the oligonucleotides in this work were not conjugated to psoralen, and no other DNA-damaging agent (or mutagen) was required to induce mutagenesis. This affords an advantage

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