Contents

Editorial
News and comment
Review
223 Strategies to achieve targeted gene delivery via the receptor-mediated endocytosis pathway
SI Michael & DT Curiel

Papers
233 Tumor cell bystander killing in colonic carcinoma utilizing the Escherichia coli DeoD gene to generate toxic purines
EJ Sorscher, S Peng, Z Bebok, PW Allan, LL Bennett Jr & WB Parker

239 Lipopolysaccharide is a frequent contaminant of plasmid DNA preparations and can be toxic to primary human cells in the presence of adenovirus
M Cohen, A Baker, M Saltik, E Wagner & M Buschle

247 Gene therapy for phenylketonuria: phenotypic correction in a genetically deficient mouse model by adenovirus-mediated hepatic gene transfer
B Fang, RC Eisensmith, XHC Li, MJ Finegold, A Shedlovsky, W Dove & SLC Woo

255 Receptor-mediated gene delivery employing lectin-binding specificity
RK Batra, F Wang-Johanning, E Wagner, RI Garver, Jr & DT Curiel

261 Expression of the human glucocerebrosidase and arylsulfatase A genes in murine and patient primary fibroblasts transduced by an adenovirus-associated virus vector
J-F Wei, F-S Wei, RJ Samulski & JA Barranger

269 Gene therapy for Lewis lung carcinoma with tumor necrosis factor and interleukin 2 cDNAs co-transfected subline
T Ohira, Y Ohe, Y Heike, ER Podack, KJ Olsen, K Nishio, M Nishio, Y Miyahara, Y Funayama, H Ogasawara, H Arioka, H Kato & N Saijo

Calendar
Lipopolysaccharide is a frequent contaminant of plasmid DNA preparations and can be toxic to primary human cells in the presence of adenovirus

Matt Cotten¹, Adam Baker¹, Mediyha Saltik¹, Ernst Wagner¹,² and Michael Buschle²
¹IMP, Research Institute of Molecular Pathology, Dr Bohrgasse 7, 1030 Vienna and ²Bender & Co., Ernst Boehringergasse 5-11, 1121 Vienna, Austria

Introuction

Endotoxin (lipopolysaccharide, LPS) is a major component of the Gram-negative bacterial cell wall. The LPS monomer is a diglucosamine diphosphate bearing six lipid moieties, linked to either two or three 2-keto-3-deoxyoctonate (KDO) residues linked to an inner core of carbohydrate residues linked to the outer (O-antigen) carbohydrate residues (see [1, 2] for details). The LPS molecule is an extremely potent stimulator of the mammalian immune system and a number of mechanisms exist to detect LPS and to respond to the presence of either this molecule or Gram-negative bacteria [3-12]. LPS is a common contaminant of plasmid DNA preparations grown in Escherichia coli. Up to 40% of the surface LPS of E. coli can be released by treating intact bacteria with Tris/EDTA solutions [1, 13], conditions that are normally used in the initial steps of preparing plasmid DNA. The negative charges associated with the lipid A and inner core of LPS cause the LPS molecule to behave like DNA on anion exchange chromatographic resins. The large size of the micellar form of LPS causes the molecule to behave like a large DNA molecule on size exclusion resins. The density of LPS in CsCl is similar to that of plasmid–EtBr complexes (1.37 g/ml versus 1.5 g/ml), so that CsCl-banded DNA can be easily contaminated. We document here a toxicity in primary human skin fibroblasts as well as primary human melanoma cells exposed to LPS-containing DNA or pure LPS in the presence of adenovirus. Toxicity appears at levels of 100 ng/ml free LPS or 100 pg/ml when the LPS is assembled into polylysine/adenovirus complexes. Methods to remove the contaminating LPS and eliminate the toxicity are described.

Correspondence: Matt Cotten, IMP, Research Institute of Molecular Pathology, Dr Bohrgasse 7, 1030 Vienna, Austria

Results

Fractionation of CsCl gradient to show position of LPS relative to DNA

The plasmid pCLuc was grown in E. coli DH5α in the presence of 100 μg/ml ampicillin and a cleared lysate of the bacteria was prepared and fractionated by CsCl density gradient centrifugation (see Materials and methods). The gradient was subsequently fractionated (2 ml fractions) and fractions from the gradient (2 ml) were assayed for plasmid DNA content (A260 nm and gel electrophoresis) and LPS content (Limulus assay). The results are shown in Figure 1. We find that the original cleared lysate contains LPS at more than 500 000 endotoxin units (EU)/ml (>500 μg/ml; results not

Figure 1 Location of LPS in CsCl density gradient purity of plasmid DNA. A cleared lysate of E. coli bearing an ampicillin resistance plasmid was prepared and subjected to CsCl density gradient centrifugation in the presence of ethidium bromide as described in the Materials and methods. After 18 h at 200 000 × g (Beckman VTi50 rotor) the gradient was partitioned into 22 fractions. Fractions were assayed for plasmid DNA content by agarose gel electrophoresis and for LPS content by limulus assay.
LPS content of DNA prepared by different methods

Column chromatographic methods of purifying plasmid DNA fractionate the DNA based largely on its charge density. The LPS molecule possesses a high negative charge density due to the presence of phosphate groups on the lipid A moiety as well as phosphate and carboxyl groups on the octolonic acid sugar groups (reviewed in [1, 2]). Because of the negative charge density of LPS as well as the molecule’s ability to assemble into high molecular weight micelles, the LPS has a structure that shares features with the chemistry and size of plasmid DNA. Ion-exchange resins used for purifying plasmid DNA (e.g., Qiagen, Nucleobond) generate plasmid preparations that contain significant quantities of LPS (see Table 1). Like DNA, LPS precipitates from ethanol and isopropanol solutions. Furthermore, because the LPS is not detected by either ethidium bromide staining in agarose electrophoresis or by absorbance at 260 nm, the presence of LPS can be largely undetected in DNA preparations. Table 1 lists the LPS contents of plasmid DNA prepared by a variety of methods.

We have subjected three types of DNA preparations to two methods of LPS removal. A simple and effective method of LPS removal employs the detergent Triton X-114. At temperatures below its cloud point of 20°C, Triton X-114 is miscible with aqueous solutions; at temperatures above 20°C, the detergent partitions into a separate phase [14]. This phase partitioning can be used to extract the lipophilic LPS molecule from aqueous protein solutions [15] as well as DNA preparations [16]. An alternative LPS removal method employs polymyxin B, a cyclic fungal peptide that binds the lipid A/KDO component of LPS with high affinity [9, 17, 18]. Chromatographic resins bearing this peptide can be used to remove LPS from protein or DNA solutions. We obtain similar LPS removal success with either method (Table 1). Both the Triton extraction method and the polymyxin resin generate DNA preparations with comparable absence of LPS (<0.1 EU/6 μg DNA). We have occasionally encountered difficulty using either method to remove the LPS that contaminates CsCl purified DNA. It is possible that the CsCl purification enriches for a form of LPS that does not avidly bind either the detergent phase or the polymyxin. Sodium dodecylsulfate/polyacrylamide gel electrophoresis (SDS/PAGE) resolution of DNA samples stained with an LPS-specific silver stain [19, 20] demonstrates that the form of LPS most frequently found contaminating DNA preparations is a higher molecular weight, heterogeneous form of LPS consistent with the smooth LPS phenotype of the E. coli strains used to propagate the plasmid DNA (results not shown).

Toxicity of LPS to primary cells in the presence of adenovirus

In an initial study to determine if LPS could generate a toxicity to mammalian cells, cultures of primary human melanoma cells were exposed to purified LPS samples (from either E. coli 0111:B4 or Salmonella minnesota) in the absence or presence of psoralen-inactivated adenovirus d11014. After 48 h, the cultures were processed to determine surviving cells by washing the cell monolayers, fixing with formaldehyde and staining with crystal violet (Figure 2) or incubating with the viability stain MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium) (Figure 3).

We find that incubation of primary cells with LPS alone (up to 250 μg/ml) or psoralen-inactivated adenovirus alone (up to 10⁷ virus particles/cell) has no toxic effect (Figure 2a). However, combined treatment of these cells with mature adenovirus (10⁵ virus particles/cell) plus LPS (down to 50 ng/ml) kills these cultures, as measured by crystal violet staining (Figure 2a).

Purified preparations of immature adenovirus particles (with a density of 1.31 g/cm³) possess the full complement of outer capsid proteins yet are defective at cellular entry and cytoplasmic release of co-endocytosed material [21, 22] (M.C. and M.S., unpublished results). These immature adenovirus particles are an assembly intermediate, are deficient for viral DNA (hence their lighter density) and are inactive in the pH-dependent membrane disruption reaction that is required for adenovirus entry into host cells (M.C. and M.S., unpublished results). Exposure to LPS in the presence of immature adenovirus particles does not result in cellular toxicity (Figure 2b), demonstrating that cytoplasmic entry of the adenovirus (and possibly co-entry of LPS) is required for toxicity.

When the viability stain MTT is used to measure toxicity in the presence of mature virus particles and LPS, similar results are obtained (Figure 3). No toxicity is apparent with LPS alone, a slight decline in viability is observed with adenovirus alone (10 000 or 100 000 virus particles per cell) but a severe decline in viability is observed with the higher virus input in the presence of LPS (Figure 3).

Table 1 LPS content of DNA preparations

<table>
<thead>
<tr>
<th>Method</th>
<th>No. of samples</th>
<th>Mean LPS content (EU/6 μg DNA)</th>
<th>Range (EU/6 μg DNA)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA preparation</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CsCl</td>
<td>13</td>
<td>4.3</td>
<td>0.6 – &gt;25</td>
</tr>
<tr>
<td>Qiagen</td>
<td>5</td>
<td>10.3</td>
<td>3.8 – &gt;25</td>
</tr>
<tr>
<td>Nucleobond</td>
<td>7</td>
<td>20.2</td>
<td>0.5 – &gt;25</td>
</tr>
<tr>
<td>Treatment to remove LPS</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CsCl/Triton X-114</td>
<td>7</td>
<td>0.09</td>
<td>0.05 – 0.195</td>
</tr>
<tr>
<td>Qiagen/Triton X-114</td>
<td>4</td>
<td>0.08</td>
<td>0.016 – 0.2</td>
</tr>
<tr>
<td>Nucleobond/polymyxin</td>
<td>7</td>
<td>0.02</td>
<td>0.001 – 0.075</td>
</tr>
</tbody>
</table>

Note: 1 EU (endotoxin unit) = 1 ng LPS
Figure 2 LPS toxicity in the presence of adenovirus. Primary human melanoma cells (isolate H 225) were plated at 40,000 cells per well of a 24-well dish. The cells were exposed to the indicated quantities of LPS (S. minnesota wild type) and adenovirus particles to cell (a) d1014, psoralen-inactivated; (b) d1014, light. 1.31 g/cm^3 particles) in 1 ml of DMEM/2% horse serum for 2 h at 37°C, after which 1 ml of RPMI 1640/10% FCS was added. After 48 h, the cell samples were washed once with HBS, fixed in formaldehyde and stained with crystal violet (see Materials and methods).

Similar toxicities are observed with E. coli 0111:B4 LPS (smooth), S. minnesota wild type LPS (smooth) and S. minnesota Re 595 LPS (rough E; results not shown). The virus and LPS must be exposed to cells simultaneously to generate the toxicity. Exposure to virus or LPS alone for 2 h followed by washing and then exposure to the second component does not result in toxicity (results not shown). The toxicity does not appear to involve adenovirus gene expression. No viral gene expression can be detected with psoralen-inactivated adenovirus d1014 (results not shown) and similar levels of LPS toxicity are observed with either psoralen-inactivated adenovirus d1014 or non-inactivated d1014. Although we have not yet performed an exhaustive survey, the toxic response appears to be a function of primary cell cultures and the toxic response does not occur with established cell lines (e.g. HeLa, Vero, 3T3 or K562). In addition, there appear to be species differences in the response to LPS/adenovirus toxicity. We have found that primary mouse muscle fibroblasts are sensitive to the toxicity but primary chicken embryo fibroblasts and primary dog muscle fibroblasts are not sensitive.

Purification of DNA eliminates toxicity

Several preparations of pGS-hIL-2, a plasmid bearing the human interleukin 2 (IL-2) gene driven by the CMV promoter, were purified with a modified Nucleobond protocol (see Materials and methods) and the LPS content was found to range from 0.5 to >25 EU/6 μg DNA (Figure 4). Each plasmid preparation was subjected to polymyxin B chromatography or Triton X-114 extraction to remove LPS. After treatment, all samples contained less than 0.1 EU/6 μg DNA. The original and the purified DNA preparations were then assembled into adenovirus streptavidin–polylysine–(StrpL)–transferrin–polylysine (TfpL) complexes and transfected into primary human melanoma cells. The culture supernatants at 24–48 h post-transfection were harvested and the IL-2 content was measured by enzyme-linked immunosorbent assay (ELISA). In all cases we find that LPS removal leads to increased gene expression (from 3–26-fold; Figure 4). From the morphology of the transfected cultures at 48 h post-transfection, cellular toxicity could easily account for the poor IL-2 expression from the LPS-bearing DNA samples.

LPS assembled into polylysine complexes is more toxic than free LPS
The observation of toxicity that initiated this investigation was due to LPS contamination of DNA
which was seldom more than 5–10 EU/6 μg DNA (comparable to 5–10 ng of LPS, used in 2–5 ml of culture medium = 1–5 ng/ml) However, the toxicity demonstrated with the addition of pure LPS to cell cultures requires the presence of >50 ng/ml of pure LPS for toxicity measured by crystal violet staining (Figure 2) or >5 μg/ml LPS for toxicity by MTT reduction (Figure 3). Simple charge considerations suggest that the negatively charged LPS molecule could interact with the positively charged polylysine of our transfection complexes. We considered the possibility that polylysine-LPS complexes, bound to adenovirus might be present in transfection complexes prepared with LPS-contaminated DNA preparations and the loading of LPS onto polylysine-adenovirus complexes might enhance the cellular interactions of the LPS molecule and increase the toxicity of the LPS molecule. We tested this idea directly in the following experiment.

Standard biotinylated-adenovirus–StrpL–TfpL–DNA complexes were prepared. However, the DNA used (Triton X-114 extracted to remove LPS) was premixed with known quantities of pure LPS prior to the assembly of the polylysine complexes. The complexes were then supplied to primary fibroblasts and primary melanoma cells and the resulting toxicity was quantitated by MTT assay (Figure 5). We begin to observe toxicity at concentrations of LPS of 5 ng/ml, obtained with adenovirus/polylysine/DNA complexes containing as little as 10 ng of LPS/6 μg DNA (Figure 5). These are concentrations comparable to those that produce toxicity with contaminated DNA and are toxicities at 10–1000 times lower concentrations than the conditions that generate toxicity with free LPS and adenovirus mixtures.
not observed below LPS concentrations of 5–50 ng/ml. This difference in concentration probably reflects the mode of assay. With the luciferase assay we are measuring the fate of the cells that actually endocytose the DNA–virus–LPS complex whereas with the MTT assay we are measuring the fate of the entire culture.

The toxicity does not require the high virus/cell ratios used in these experiments. When the delivery of a luciferase marker gene is used to follow the fate of the cells that ingest the DNA–LPS complex, the toxicity at 10^6 virus per cell is comparable to that at 10^5 virus per cell. Therefore, the toxicity due to LPS contamination appears under the same conditions employed for efficient DNA delivery.

**Discussion**

Previous work has demonstrated the utility of adenovirus particles for enhancing the delivery of DNA to eukaryotic cells [23–28]. Most evidence suggests that the primary function of the adenovirus particle in these applications is to increase the cytoplasmic entry of endocytosed material, a function that was initially characterized in detail by the Pastan and Carrasco groups [29, 30]. This enhanced intracellular delivery of material applies to toxins as well as to the DNA molecules that we seek to deliver. Endotoxin (LPS) is a common contaminant of E. coli-grown plasmid DNA preparations. These LPS contaminations are simple to overlook because the molecule is not visualized either by ethidium bromide staining or by absorbance at 260 nm, the two methods commonly used to analyze and quantify DNA preparations. We have demonstrated here that the levels of endotoxin contaminating DNA preparations can be toxic to primary cells in the presence of entry-active adenovirus particles. We have demonstrated that DNA purified with techniques for removing contaminating LPS molecules enhances gene expression in primary cells and eliminates the toxicity.

It is not yet clear if the toxicity is due to physical damage to the cell by the LPS, if the cytoplasmic delivery of LPS is a signal or if the combined interaction of adenovirus particles and LPS molecules activates a toxic signalling pathway. Experiments to clarify the mechanism generating this toxicity are in progress.

The use of adenovirus particles to enhance receptor-mediated gene delivery is a powerful technique, generating high levels of transient gene expression in a variety of cell types. However, the use of this system in vivo for long-term expression has been hampered by the rapid decay in gene expression [31]. Our observation that the LPS commonly contaminating DNA preparations is responsible for a toxicity, and the demonstration here of a simple remedy for this problem, should increase the utility of this transfection method for primary cells.

**Materials and methods**

**DNA preparation**

All DNA plasmids carried either the β-lactamase ampicillin resistance gene or the tetracycline resistance gene and were propagated in the bacterial strains HB101 or DH5α in the presence of 100 μg/ml ampicillin or 5 μg/ml tetracycline in either LB or Terrific medium [32]. Saturated overnight cultures of the plasmid-transformed bacterial strains were prepared, collected by centrifugation and processed for plasmid DNA purification in the following manner.

CsCl. This procedure is described in detail [33]. Briefly, the bacterial pellet from a 1-liter culture was suspended in 10 ml of 20% (w/v) sucrose, 10 mM EDTA, 50 mM Tris pH 7.5 (solution 1) incubated on ice for 10 min, 2.2 ml lysozyme (10 mg/ml in solution 1) was added for an additional 10 min on ice, 5 ml 0.2 M EDTA pH 7 was added and the sample was incubated for 10 min on ice and finally, 10 ml 2% (v/v) Triton X-114, 60 mM EDTA and 40 mM Tris pH 7.5 were added followed by incubation for 15 min on ice. This lysate was then centrifuged for 30 min (Sorvall SS34, 17K) and 28.5 g CsCl and 400 μl ethidium bromide (10 mg/ml) were added to the supernatant (26 ml initial volume). The material was centrifuged for >18 h in a Beckman VTi50 rotor at 200 000 × g at 20°C. The lower of the two ethidium-rich bands was collected and centrifuged again, in a Beckman VTi65 rotor for >4 h at 350 000 × g at 20°C. The ethidium-rich band was again harvested, extracted with CsCl-saturated isopropanol until the pink ethidium color was gone, dialyzed extensively against TE (10 mM Tris, 0.1 mM EDTA pH 7.4), mixed with 1/10 vol. 3 M sodium acetate pH 5.0 and precipitated with 3 vol. of ethanol at −20°C. The collected DNA precipitate was further processed with RNase A, proteinase K, phenol/chloroform and chloroform, reprecipitated and the final DNA pellet was suspended in TE and quantified by optical absorbance, assuming that 0.05 mg/ml DNA has an absorbance at 260 nm of 1.

**Qiagen and Nucleobond**. Qiagen (Diagen GmbH, Hilden, Germany) and Nucleobond (Macherey-Nagel, Düren, Germany) plasmid DNA resins were used following the directions supplied by the manufacturers, with the
exception that Nucleobond columns were rinsed four times with high stringency wash buffer (rather than twice) following binding of the DNA to the resin.

Triton X-114 extraction. Triton X-114 (Sigma) was passed through three 0°C/30°C temperature cycles (as described by Bordier [14]) to isolate a homogeneous preparation of the detergent. Extraction of LPS from DNA samples was performed as follows, a modification of previously published methods [15, 16]. DNA samples at 0.5–1.5 mg/ml in TE were adjusted to 0.3 M sodium acetate pH 7.5. Triton X-114 (3 μl per 100 μl DNA solution) was added and the samples were vortexed thoroughly and incubated on ice for 10 min. The samples were then transferred to 30°C for 5 min to allow the two phases to separate, centrifuged in a prewarmed Eppendorf centrifuge (ca. 30°C) for 2 min at 2000 and the aqueous phase was transferred to a fresh Eppendorf tube. This extraction was repeated two additional times and the DNA in the final aqueous phase was precipitated with 0.6 vol. of isopropanol at room temperature. The precipitate was collected by centrifugation, washed twice with 80% ethanol (−20°C), air-dried, resuspended in TE and quantified.

Polymyxin B chromatography
A volume of polymyxin B resin slurry (Affi-Prep polymyxin, BioRad) equal to the DNA sample volume was briefly exposed to 3 vol. 0.1 N NaOH, followed by three washes of five resin volumes with TE (10 mM Tris, 0.1 mM EDTA pH 7.4). The pelleted resin was resuspended with the DNA samples (in TE at 0.8–1.2 mg/ml) and the mixture was agitated by rotation overnight at 4°C. The sample was then transferred to a disposable column (BioRad, Poly-Prep) that had been pretreated with 0.1 N NaOH and washed with TE. The eluate was collected, the resin was washed with an additional bed volume of TE and the eluate plus wash were pooled. The DNA in this pooled sample was precipitated with 1/10 vol. 3 M sodium acetate pH 5 and 2 vol. of ethanol. The precipitate was collected by centrifugation, washed twice with 80% ethanol, dried, dissolved in TE and quantified as described above.

LPS preparations
As specified in the figure legends, the pure LPS preparations used in these studies were either a smooth LPS from S. minnesota, extracted by the phenol/water method and further purified by gel filtration, a similar preparation of LPS from E. coli 0111:B4 or LPS from the S. minnesota rough mutant Re 595 (all from Sigma). All three LPS types yielded similar results. LPS preparations were suspended in LPS-free water by sonication for 5 min in a Transsonix 570/H (360 W) sonicator bath. Serial dilutions were prepared in LPS-free water with 30 s of vortexing between dilutions. The final dilutions were sonicated for 5 min before use.

Virus procurement, biotinylation and psoralen inactivation
The E4-defective adenovirus 5, dl1014 [34] was grown on the complementing cell line W162 [35]. Pellets of infected cells were suspended in 20 mM hydroxyethylpiperazine sulfonate (HEPES) pH 7.4, 1 mM phenylmethylsulfonyl fluoride (PMSF) at 2 ml per 3 × 10^7 cells and subjected to three freeze/thaw cycles (liquid nitrogen, 37°C). The suspension was then mixed with an equal volume of Freon and vortexed for 3 × 30-s bursts and centrifuged 10 min at 1700 × g (Heraeus Sepatech, 2705 rotor). The aqueous phase (upper) was saved and the Freon phase was vortexed with 1/5 vol. 20 mM Hepes pH 7.4 and centrifuged as before. The aqueous phases were pooled, transferred to a Beckman VTi50 centrifuge tube (15 ml/tube) and underlayered with 15 ml of 1.2 g/cm^3 CsCl, 20 mM Hepes pH 7.4 and then with 7 ml of 1.45 g/cm^3 CsCl, 20 mM Hepes pH 7.4. The samples were centrifuged at 200 000 × g in a Beckman VT150 rotor for 40 min at 20°C. The lower opalescent band of mature virus particles at 1.34–1.35 g/cm^3 (as measured by refractive index) and the upper band (immature particles at 1.31–1.32 g/cm^3) were collected separately and centrifuged to equilibrium (>4 h) at 350 000 × g in a VTi65 rotor. The opalescent virus bands (either 1.31 g/cm^3 for immature or 1.34 g/cm^3 for mature) were harvested and either processed directly for biotinylation and psoralen inactivation or diluted with an equal volume of 86% glycerol (Fluka) and stored at −80°C.

Virus biotinylation with N-hydroxysuccinimide-biotin (Pierce), inactivation with 8-methoxypsoralen/360 nm UV light and purification by gel filtration using a Pharmacia PD10 column equilibrated with HEPES-buffered saline (HBS, 150 mM NaCl, 20 mM HEPES pH 7.4/40% glycerol were performed as described previously [21, 22]. Virus samples were quantified by protein concentration (BioRad Bradford assay with bovine serum albumin, BSA, as a standard) using the relationship of 1 mg/ml protein = 3.4 × 10^12 adenovirus particles/ml [36].

Cell culture
Primary human melanoma cultures were isolated and grown in RPMI-1640 medium (GIBCO/BRL) supplemented with 100 IU/ml penicillin, 100 μg/ml streptomycin, 2 mM l-glutamine, 1% sodium pyruvate and 10% heat-inactivated (30 min, 56°C) fetal calf serum (RPMI/10% FCS). Primary human skin fibroblasts were isolated from skin biopsies and propagated in Dulbecco’s modified Eagle’s medium (DMEM) plus 2 mM glutamine, 50 μg/ml gentamicin and 10% heat-inactivated fetal calf serum (DMEM/10% FCS). The primary fibroblast cultures were used at passage 5–10; primary melanoma cultures were used at passage 16–25 for these experiments.

Preparation of adenovirus–DNA complexes
StrpL and TtpL were prepared as previously described [25, 37]. Samples of biotinylated, psoralen-inactivated adenovirus dl1014 (8 μl, 1 × 10^12 particles/ml) were diluted into 150 μl HBS and mixed with 1 μg StrpL in 150 μl HBS for 30 min at room temperature. Aliquots of 6 μg plasmid DNA were diluted in 100 μl containing the indicated quantities of LPS (see figure legends). The DNA
(LPS) solutions were then mixed with the adenovirus–StrepL solution for 30 min at room temperature. Finally, a 100-µl aliquot of HBS containing 5 µg TfpL was added to each sample, followed by incubation for 30 min at room temperature. Aliquots of these transfection complexes were then supplied to cells as described in the figure legends (generally 5–50 µl per 20,000–50,000 cells).

Cell viability assays
Crystal violet staining was performed 48–72 h after exposure of cell samples to test agents. The culture medium was removed, the cell layer was washed once with HBS, fixed for 5 min with 4% formaldehyde/150 mM NaCl and then stained for 10 min with 0.1% crystal violet in 2% ethanol. The staining solution was then removed and the well washed once with phosphate-buffered saline (PBS) and once with distilled water. An alternative determination of cell viability was performed using the MTT reduction assay [38] as modified by Promega.

Endotoxin (LPS) assays
LPS was measured with the BioWhittaker QCL-1000 chromogenic Limulus assay, based on the Limulus amoebocyte clotting reaction (reviewed in [39]). All solutions used for DNA and virus preparations as well as TfpL, StrepL and adenovirus preparations were demonstrated to be LPS free (<0.1 endotoxin units/50 µl solution) before use.

Miscellaneous
Luciferase gene expression was measured as described previously [33]. Human IL-2 in cell culture supernatants was determined between 24 and 48 h after transfection by ELISA (T Cell Diagnostics Inc., Cambridge, MA, USA). Supernatants were collected, centrifuged at about 3000 × g in an Eppendorf Microfuge to remove debris and stored at −20°C until assayed. Values reported are in BRMP (Biological Response Modifier Program) units per 10^5 cells per 24 h.

Acknowledgments
We are grateful for the support and encouragement of Max Bünzstiel. We acknowledge the excellent technical assistance of Claudia Denk, Ursula Scheer, Helen Kirlappos and Alexandra Sinski. We thank Gary Ketner for the adenovirus dl1014 strain and the W162 cell line. We also thank Susanne Schreiber for her help in melanoma cell procurement, Gotthold Schaffner for the plasmid pGShIL2-tet and Lisa Ballou and Susanna Chiocca for their critical reading of the manuscript.

References


