Genetic Vaccines and Therapy

Research

Novel non-viral method for transfection of primary leukemia cells and cell lines

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Abstract

Background: Tumor cells such as leukemia and lymphoma cells are possible targets for gene therapy. However, previously leukemia and lymphoma cells have been demonstrated to be resistant to most of non-viral gene transfer methods.

Methods: The aim of this study was to analyze various methods for transfection of primary leukemia cells and leukemia cell lines and to improve the efficiency of gene delivery. Here, we evaluated a novel electroporation based technique called nucleofection. This novel technique uses a combination of special electrical parameters and specific solutions to deliver the DNA directly to the cell nucleus under mild conditions.

Results: Using this technique for gene transfer up to 75% of primary cells derived from three acute myeloid leukemia (AML) patients and K562 cells were transfected with the green flourescent protein (GFP) reporter gene with low cytotoxicity. In addition, 49(+/- 9.7%) of HL60 leukemia cells showed expression of GFP.

Conclusion: The non-viral transfection method described here may have an impact on the use of primary leukemia cells and leukemia cell lines in cancer gene therapy.

Background

Leukemia cells are obvious and attractive targets for gene transfer since these cells are potentially susceptible to immunotherapeutic strategies. Recently, cytokine gene transfer and expression of immunomodulatory genes in various kinds of tumor cells have been shown to mediate tumor regression and antimetastatic effects in several animal models [1]. Many leukemic entities respond to a treatment with interferon-alpha [2]. Therefore, gene transfer of various cytokine genes such as interleukin-2 (IL-2), IL-7 and IL-12 has been envisaged [3,4]. Despite of expressing MHC molecules, leukemia cells are ineffective antigen presenting cells (APC) [5]. Often leukemic cells are unable to stimulate T cells because they lack expression of important co-stimulatory molecules [5]. The use of vectors expressing co-stimulatory molecules or cytokines and the

use of genetically modified cells for therapeutic purposes are likely to have a significant role for patients with leukemia in the future [6,7]. To date, only a few strategies applicable to the therapy of these diseases have reached the point of clinical trial [8,9].

The availability of molecular genetic technology has opened up a large range of potential strategies for the treatment of leukemia. Hematological malignances have several features that make them particularly amenable to gene transfer approaches. The neoplastic cells circulate in the blood, so that large numbers of tumor cells can be harvested and sorted for *ex vivo* manipulation. The efficiency of transduction can easily be monitored *in vitro* and simple blood tests can be used to monitor expression of the transgene or changes in bystander effects following gene transfer. Finally, normal host cells that infiltrate the tumor can be found in the blood, making them accessible for isolation and analysis. Therapeutic approaches using *ex vivo* immunological modification of malignant cells have not been widely investigated in hematological malignancies. One of the prerequesites to these applications is an appropriate vector that can achieve high efficiency gene transfer in leukemic cells, without major cytotoxicity. Adenoviral vectors are able to transduce a wide range of cells [10], however there are only little data concerning their ability to transduce hematopoetic cells [11-13]. Recent reports have described successful gene transfer with adenoviral vectors into chronic myeloid leukemia cells (CML) after preactivation of the target cells [14]. With the use of an adenoviral vector, containing a modified fiber protein, an increased gene transfer in acute myeloid leukemic (AML) cells could be shown [15]. Although virus based systems enhance delivery efficiency, recombinant viral based treatments have been associated with complications that result from highly evolved and complex viral biology and / or host parasites interactions [16]. The future of gene therapy requires the development of efficient and nontoxic delivery mechanisms. However, at the present non-viral methods concerning the transfection of hematopoetic cells [17-19] remain poorly efficient.

Oliver Zelphati et al. tested seven commercially available transfection reagents, and he found out that all tested reagents were inefficient for delivery charged molecules into hematopoetic cell lines and primary AML blasts [20]. Very little data on cell viability and transfection efficiency of primary leukemic cells could be provided, most of the researchers using enzymatic bulk assays or a PCR analysis to detect reporter gene expression.

Studies in our laboratory aimed developing an efficient non-viral DNA delivery system for transfection of leukemia cells. We have analyzed several methods of gene delivery into this cell type. To compare non-viral and viral techniques, leukemia cells were transfected with an adenoviral vector expressing the reporter gene green fluorescent protein (GFP). Electroporation as general approach to the introduction of macromolecules into cells was used as a non-viral method. In addition, gene gun, a helium gas pressure-driven device, that delivers gold microparticles coated with plasmid DNA directly into cells, and a novel electroporation based technique called nucleofection [21] were used.

Here, we describe a new transfection protocol accomplishing highly efficient gene transfer to human chronic and acute myeloid leukemia (AML) cell lines and into primary AML cells derived from three patients.

Methods
Primary leukemia cells
Three untreated AML patients were included in the present study. AML cells were isolated from peripheral blood by Ficoll-Paque density centrifugation (Lymphoprep, Nycomed, Oslo, Norway). Cells were cultured in complete RPMI 1640 with Glutamax (GIBCO, Berlin, Germany) supplemented with 10% heat-inactivated fetal calf serum (FCS) (PAA, Cölbe, Germany), 100 U/ml penicillin and 100 μg/ml streptomycin (Seromed, Berlin, Germany), and 25 mM Hepes (hydroxyethylpiperazine ethane sulfonic acid, GIBCO).

Patient 1 was a 71-year-old man with FAB M1 classification (acute myelocytic leukemia). Immunophenotyping for this patient was not done. Patient 2 was a 43-year-old woman with TdT-positive FAB M5b classification (acute monoblastic leukemia) with the following immunophenotype: CD13 (78%), CD14 (66%), CD15 (72%), CD33 (73%), and CD64 (77%). Patient 3 was a 63-year-old woman with FAB M4 EO classification (acute myelomonocytic leukemia) with the following immunophenotype: CD13 (89%), CD14 (15%), CD15 (13%), CD33 (24%) and CD64 (12%).

Cell lines
The following cell lines were analyzed: K562 (human chronic myeloid leukemia cell line) and HL60 (human acute myeloid leukemia cell line), both obtained from Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ, Braunschweig, Germany). The cell lines were grown in complete RPMI 1640 with Glutamax supplemented with 10% heat-inactivated fetal calf serum, 100 U/ml penicillin and 100 μg/ml streptomycin, and were kept in a humid incubator with 5% CO_2 at 37°C. Virus propagation was performed in the Ad5 E1-transformed human embryonic retina cell line 911 [22]. This cell line was grown in Dulbecco's modified eagle medium (DMEM, GIBCO) supplemented with 10% FCS, 100 U/ml penicillin and 100 μg/ml streptomycin.

Adenovirus preparation and infection of leukemic cells

Transfection efficacy was determined with the GFP expressing adenovirus pQB-AdBM5GFP (E1 and E3 deleted replication defective Adenovirus type 5, Quantum Biotechnologies INC., Montreal, Canada). This adenovirus contains a cytomegalovirus (CMV) promotor. Viral stocks were generated as described before [11] and purified by $CsCl_2$ centrifugation [22]. Plaque assays were essentially performed as described by Graham and Prevec [23]. The titer of the Ad-GFP was 5×10^9 plaque forming units (pfu)/ml. For the adenoviral transfection we used our protocol for transfection of lymphoma cells published recently [11]. In brief, adenoviral transfections with double $CsCl_2$ purified Ad-GFP of K562 cells were carried out in 24-well plates with 5×10^5cells in 50 µl phosphate-buffered saline (PBS) with 1 mM $MgCl_2$/1% horse serum (HS), at an MOI of 200. After 2 hours of incubation at 37°C, 5% CO_2, 1 ml of complete culture medium was added to the cells. Because no visible toxic effect in comparison to the controls (only PBS +1 mM $MgCl_2$/1% HS) were observed, it was not necessary to remove the virus.

Expression plasmid for eGFP

The plasmid pMGV was described before [24] and was obtained from Mologen (Berlin, Germany). The vector contains a CMV enhancer promotor sequence from the immediate early gene of the human cytomegalovirus, the GFP open reading frame from *A. victoria*, a simian virus (SV)-40 polyadenylation signal, a self-replication-origin (ori p) and a gene for ampicillin resistance.

Plasmid preparation

The plasmid used for transfection was prepared with the Qiagen EndoFree plasmid kit following the manufactures instructions (Qiagen, Hilden, Germany). This kit removes more than 99% of contaminating endotoxin.

Electroporation of leukemia cells

K562 and HL60 cells were transfected by electroporation at various conditions using the electroporation system easyject plus (Eurogentec, Seraing, Belgium). In brief, 5×10^6 cells were suspended in 500 µl complete RPMI medium, mixed with 30 µg of pMGV-plasmid in a 4 mm electroporation cuvette, and incubated on ice for 10 min. After electroporation with a single pulse (electrical parameters for K562 between 270 volt, 1050 µF and 300 volt, 1800 µF, 99 Ω, and for HL60 electroporation condition differs between 1050 and 1800 µF, 200 – 450 V (in 50 V steps), 99 Ω.) the cells were transferred into complete RPMI medium at a density of 1×10^6cells per ml.

Nucleofection of cells

Primary AML cells, K562 and HL60 cells were transfected by nucleofection with the optimised conditions by using the Nucleofector system from amaxa GmbH (Cologne,

Germany). The Nucleofector technology is a highly efficient non-viral gene transfer method for most primary cells and for hard-to-transfect cell lines [25-27]. This technology is based on the long-known method of electroporation, which has now been significantly improved. Cell-type specific combinations of electrical current and solutions make the technology unique in its ability to transfer polyanionic macromolecules directly into the nucleus. Thus, cells with limited potential to divide, like many medically highly relevant primary cells, are made accessible for efficient gene transfer. The condition for each cell type have been optimised by using the manufactures' guidelines.

After centrifugation 5×10^5 (cell lines) or 1×10^6 (primary cells) cells were suspended in 100 µl prewarmed Nucleofector Solution Kit R (K562, HL60) or Nucleofector Solution Kit T (primary AML cells), containing 10 µg of pMGV-plasmid in a 2 mm electroporation cuvette (amaxa GmbH, Cologne; Germany). The Nucleofector Kits are cell type specific solutions and commercial available for different cell types (amaxa GmbH). The samples were kept in the cuvette only for the time of the pulse. The leukemic cell line K562 was transfected with the electrical setting P-13 and T-02, the HL60 cell line with electrical setting T-01 and S-11. For primary cells we used the electrical setting U-15 and S-04. After nucleofection with optimized programs the cells were transferred immediately into prewarmed complete RPMI medium.

Particle bombardement of leukemic cells (Gene gun)

Here we used a Biolistic PDS-1000/He unit (BioRad; Munich, Germany). Gold particles (1.0 µm, ABCR) were washed twice in 70% ethanol followed by washing twice in aqua dest. and were concentrated at 60 mg/ml. 6.3 mg gold particles (0.9 mg/macrocarrier) and 42 µg plasmid-DNA were mixed by pipetting (total volume: 56 µl). Afterwards, 504 µl isopropanol was added drop by drop during vortexing the gold/DNA suspension. 7 macrocarriers (Bio-Rad) were overlayed with 80 µl gold/DNA/isopropanol suspension and air-dried. Transfection was performed by 20 Hg below atmospheric pressure, 2200 or 1550 psi (rupture disks) at different positions.

1×10^8 cells were transferred to transwell dishes (Corning costar, 3.0 µm pore size). Short before transfection supernatant was removed by pipetting. After transfection cells was harvest, resuspended in medium and cultured in cell culture flask. 24 hours after transfection expression of transgene was measured by flow cytometry.

Growth curves after transfection

Cell viability was determinated by trypan blue exclusion. PBS was used for control transfection. Non-transfected and GFP transfected cells were counted after adenoviral

infection, nucleofection, and particle bombardment 24 – 72 hours after transfection.

Immunofluorescence and flow cytometric studies
Leukemia cells (5×10^5 cells) were washed with PBS and stained with 10 μl monoclonal CD80-FITC and CD86-PE antibody (Pharmingen, Heidelberg, Germany) in a total volume of 50 μl for 15 minutes. Isotype-matched antibodies were used as controls. Stained cells were washed with PBS/1% BSA and subsequently analyzed using an Epics XL flow cytometry system (Coulter-Immunotech, Hamburg, Germany). Background staining using irrelevant antibodies was less than 2%. 10^5 cells were analyzed for each sample.

To analyze the percentage of GFP positive cells we measured 5×10^5 cells. Cells were washed with PBS, resuspended in 1 ml PBS and 10 μg/ml propidiumiodid (PI) was added immediately before flow cytometric analysis. Lymphocytes were gated based on their scatter profile and cells were evaluated for GFP expression. The transfection efficiency was determined 24 hours up to 72 hours posttransfection. Nucleofected primary cells were also measured after four hours.

Results
Expression of co-stimulatory molecules
In order to elucidate the possibility of co-stimulatory molecules-mediated gene therapy for leukemia cells we analyzed the expression of CD80 and CD86. We determined the expression of these receptors on the cell surface of primary AML cells and leukemic cell lines. The primary cells derived from three AML patients did not express CD80 receptors and only a low level of CD86 (4.5 +/- 1.6 %). The leukemic cell lines K562 and HL60 did not express CD80 receptors as determined by immunophenotyping and FACS analysis. In contrast expression of CD86 was found on the cell surface of K562 (72.4+/-5.1%) and HL60 (76.5+/- 0.5%) cells.

Adenoviral gene transfer
In preliminary experiments using the leukemic cell line K562, we observed that these cells could be successfully transfected with an adenoviral vector expressing the reporter gene GFP [28]. At an MOI of 200, 49+/-4% of the cells showed a positive GFP signal after 72 hours in flow cytometric analysis (Figure 1a). These results were in accordance with previous reports [14,15,29], which showed that leukemic cells could be efficiently transfected with adenoviral vectors. Roddie and coworkers showed high adenoviral transduction efficiency in three of four leukemia cell lines but not in HL60 [30].

### Transfection of leukemia cells using electroporation
To compare the new nucleofection technology (nucleofector, amaxa) with the standard electroporation techniques (easyject plus, BioRad), we transfected K562 cells with pMGV with various electrical parameters as described in the literature [17,18,31]. Transfection efficiency was determined 24, 48, and 72 hours after electroporation by flow cytometric and fluorescence microscopical analysis. For K562 cells the transfection efficiency was 15.5+/-3.5% (Table 1). Cell viability determined by trypan blue exclusion, was markedly impaired by electroporation (data not shown). These results are in accordance with previous reports [17,18]. For HL60 cells maximum transfection efficiency was 30.2+/-5.6% (1050 μF, 450 V, 99Ω) with high toxicity 66.2+/-6.8% (Table 1).

Transfection of leukemia cells using gene gun
We transfected HL60 and K562 cells by gene gun technique. Various parameters were examined. Here we used 2200 or 1550 psi (rupture disks) at different positions (second, third, and fourth position). The transfection efficiency was determined and quantified by the expression of the encoding plasmid pMGV after 24 and 48 hours posttransfection. In summary, the transfection rates were 3.0+/-1% for HL60 and 1.5+/-0.5% for K562 cells (Table 1).

Transfection of leukemic cells by nucleofection
With the aim of developing a gene therapy protocol for leukemia, we were interested in identifying the most effective non-viral method of DNA delivery into primary leukemia cells and leukemia cell lines. Several approaches have been developed to enhance the efficiency of non-viral gene transfer via naked DNA including gene gun and electroporation. Here, we tested a novel electroporation based technique called nucleofection. This electroporation based technique combines cell type specific solutions with mild conditions which guarantee high efficiency and low cell death rates. Physical approach allow DNA to penetrate directly the cell membrane and bypass endosomes / lysosomes, thus avoiding enzymatic degradation. The DNA may also be directly delivered to the nucleus by nucleofection. Transfection efficiency was determined 24 hours after nucleofection by flow cytometric assays. Figure 2 shows the flow cytometry analysis of the three primary AML cells 24 hours after nucleofection. Cells were pulsed with two different programs (U15, S04) and show transfection relative efficiencies up to 71,5% (+/- 1.8) (program S04) with low toxicity (5,4% +/-0.2). Flow cytometric analysis four hours after nucleofection showed same transfection efficiencies like the 24 hours measurement (data not shown) The transfection data of the primary AML cells of three patients and flow cytometric analysis are shown in figure 2a and 2b. Figures 1b (K562 cells) and 1c (HL60 cells) show the primary data of one
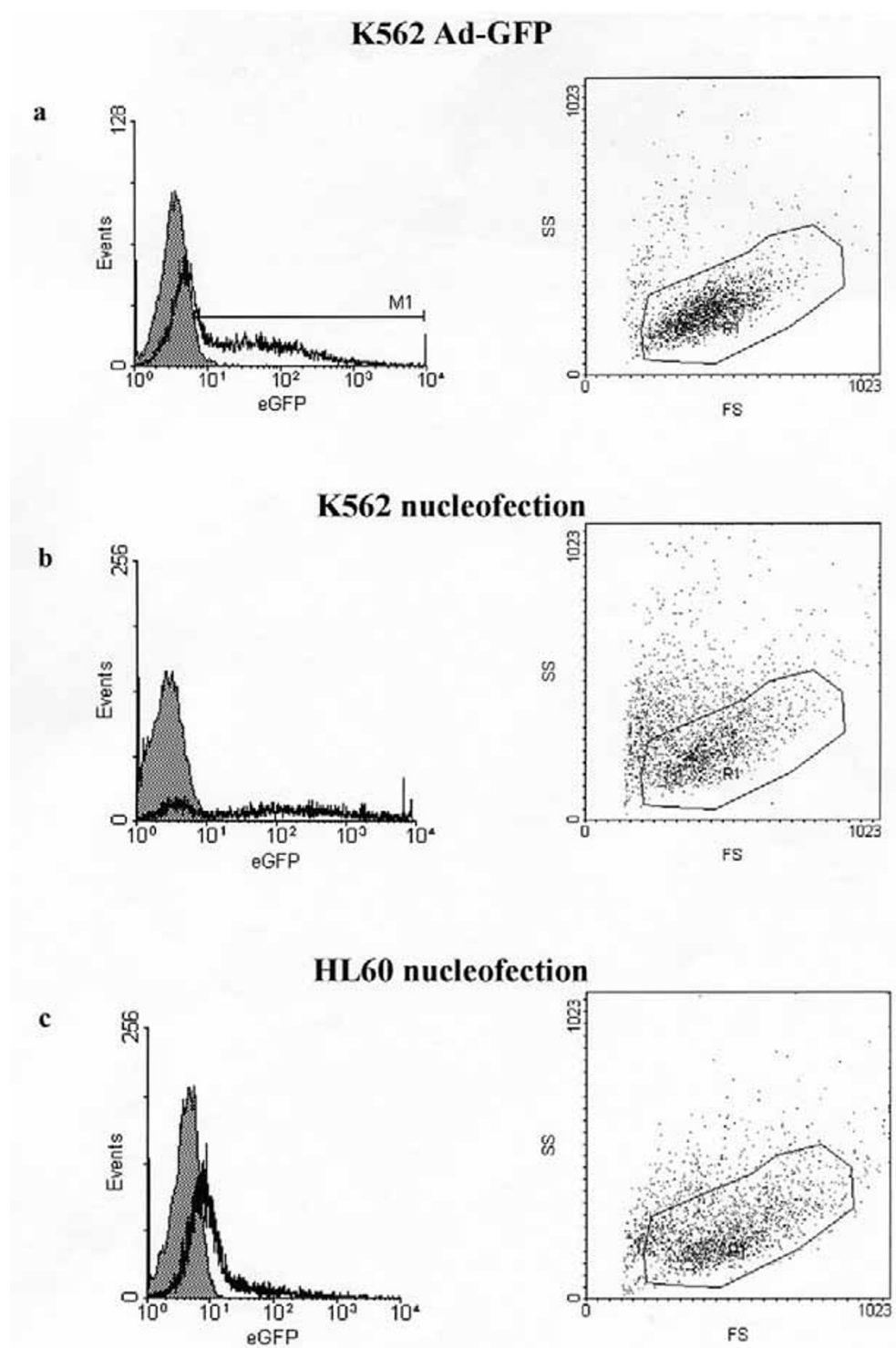
Figure 1
Primary data of GFP and eGFP transfected leukemic cell lines. Lymphocytes were gated based on their scatter profile (figures on the right) and cells were evaluated for transgene expression. a) GFP-expression of K562 cells. Cells were transfected with Ad-GFP at an MOI of 200 and assayed by flow cytometry 72 hours posttransfection. The overlay of the shaded histogram represents the background fluorescence of untreated cells. Positive transfected cells expressed intracellular green fluorescent protein. b) eGFP-expression of K562 cells 24 hours after nucleofection. c) eGFP-expression of HL60 cells 24 hours after nucleofection. Data are shown from one representative experiment.

Table 1: Comparison of various methods in the transfection efficiency of leukemic cells. Transfections were performed with an expression plasmid for eGFP or adenoviral GFP expressing vector as described in materials and methods. 24 hours after transfections cells were harvested and assayed by flow cytometry. Ad-GFP transfected cells were analyzed 72 hours after transduction. Results of three separate experiments are presented (ND, not done).

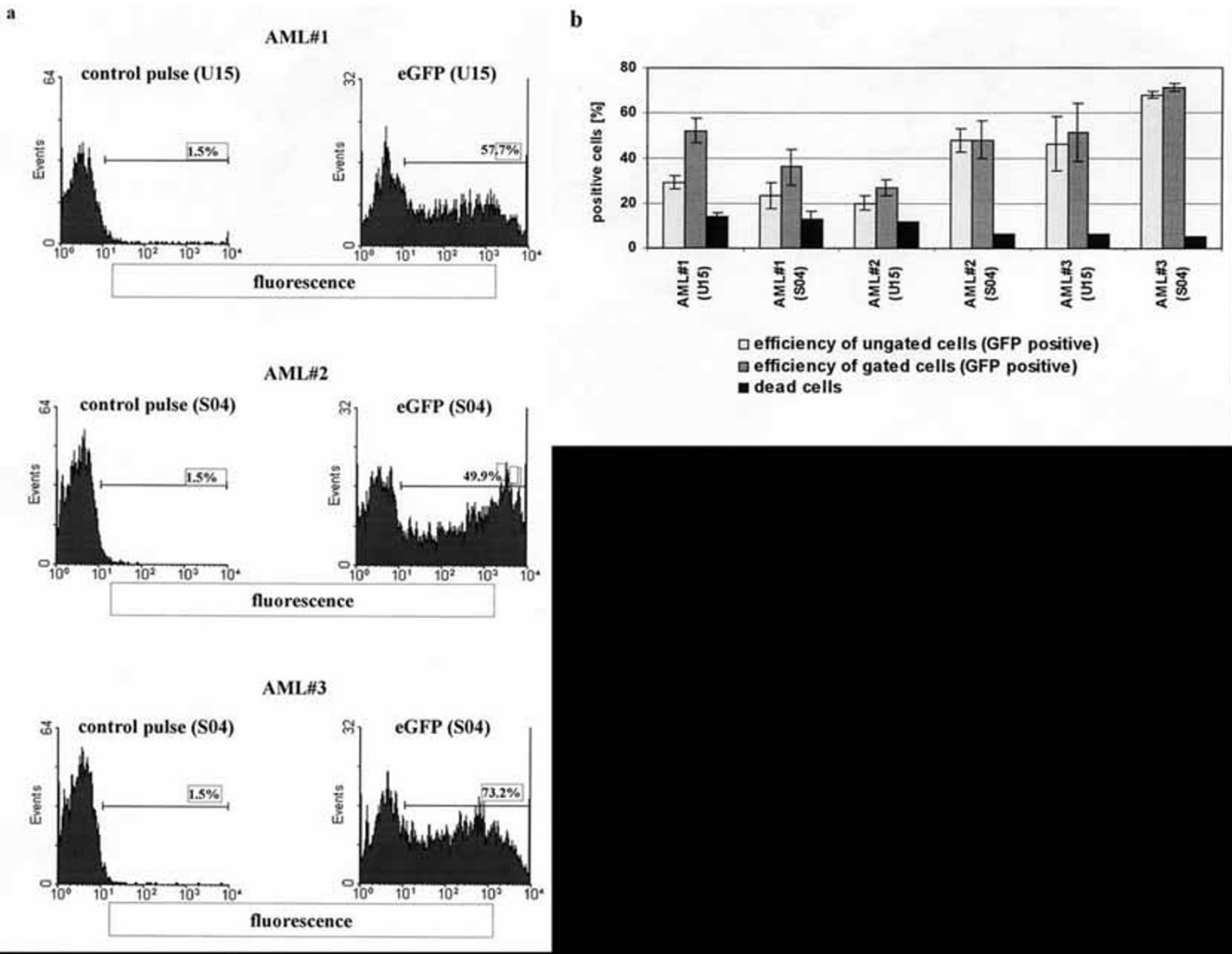| Cell line	Adenoviral gene transfer (MOI 200) GFP-pos. cells [%]	Electroporation GFP-pos. cells [%]	Gene gun GFP-pos. cells [%]	Nucleofection GFP-pos. cells [%]
primary AML cells	ND	ND	ND	60.3 +/- 9.7
K562	49 +/- 4	15.5 +/- 3.5	1.5 +/- 0.5	74.7 +/- 8.0
HL60	ND	30.2 +/- 5.6	3.0 +/- 1	49.0 +/- 9.7

Figure 2
Nucleofection mediated gene transfer in primary leukemic cells. eGFP expression in AML cells after exposure to optimized pulses. After 24 hours cells were harvested and analyzed by flow cytometric analysis. a) Representative flow cytomeric analysis for each of the three patients. Control cells were pulsed without DNA and showed no eGFP expression (left side); percentage of positive transfected cells is shown on the right. b) Gated and ungated transfection efficiencies of the primary AML cells. Percentage of dead cells was determined by PI staining. The figure represents data from two experiments, respectively. Data are presented as mean +/- standard error of the mean.
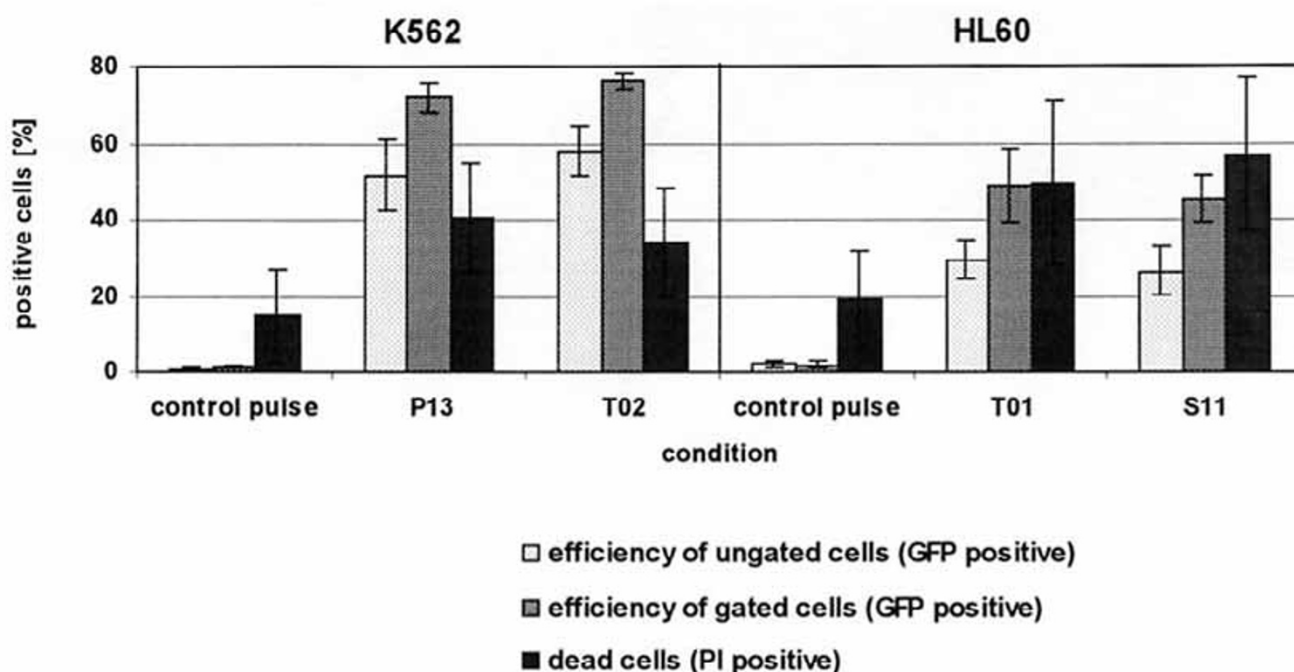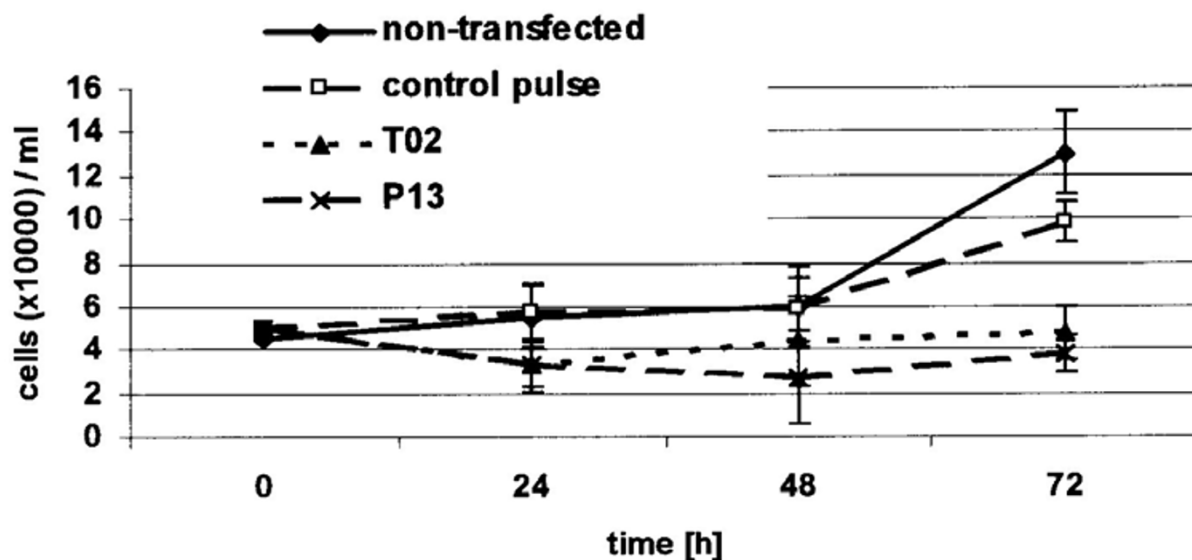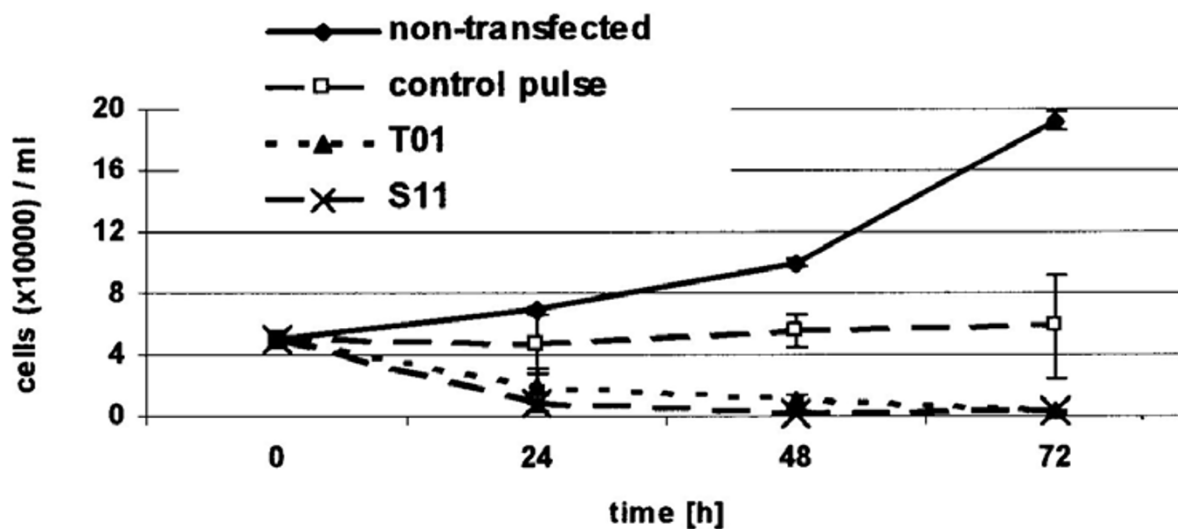
Figure 3
Nucleofection mediated gene transfer in leukemia cell lines. eGFP expression in K562 and HL60 cells after exposure to the optimized pulses. After 24 hours cells were harvested and analyzed by flow cytometric analysis. GFP positivity was assayed in gated as well as in ungated cell populations. Control cells were pulsed without DNA and showed no eGFP expression. Percentage of dead cells was determined by PI staining. The figure represents data from five separate experiments, respectively. Data are presented as mean +/- standard error of the mean.

representative experiment with the optimized electrical parameters. The shaded histogram represents the background fluorescence of transfected cells without DNA, positive transfected cells express intracellular green fluorescent protein. Various electrical parameters were assayed to optimize the transfection efficiency of the leukemic cell lines K562 and HL60. Figure 3 demonstrates the two optimized programs for each cell line (K562, HL60) and shows the absolute percentage of GFP positive cells and the relative efficiency of the transfected gated cells. Viability was determined by PI staining. By balancing survival rate and transfection efficiency optimal program for K562 resulted in a population of 76.1 +/- 2.3% positive viable cells in the gate. The program T02 showed low toxicity with 34.0 +/- 14.6% dead cells 24 hours after nucleofection. Gene transfer into leukemic HL60 cell line showed relative efficiencies ranging from 33.4 +/-14.9 to 49.0 +/- 9.7%. Due to the higher percentage of dead cells (49.6 +/- 21.5 up to 63.0 +/- 16%) the relative efficiency was lower in comparison with K562 cells. These results demonstrated a high efficient gene transfer with the nucleofection technique. The time course of GFP transgene

expression after 72 hours showed a constant expression of GFP in the cell line K562 (relative efficiency: 74.7 +/- 8%, program T02). The transgene expression in HL60 cells decreased to 24.3 +/- 9.1% after 72 hours (program T01). Fourteen days after transfection the percentage of transgene expressing cells decreased to 3%. These results indicate that nucleofection mediated gene expression in cells was transient.

Growth curves of transfected leukemia cell lines
Cell counts were determined 24, 48 and 72 hours after transfection. Following transfection with various parameters cell numbers differed from untransfected and control transfected leukemia cells (Fig. 4). In the case of HL60 cells the viability of transfected cells decreased rapidly (Fig. 4b). As shown in figure 4a nucleofection of K562 cells with different electrical parameters revealed a small reduction of cell number over a three day period. Control transfected cells (without DNA) showed only a reduction of cell number in comparison to non-transfected cells (1.3 × 10^6 cells/ml versus 9.8 × 10^5 cells/ml). The cell proliferation of transfected HL60 cells was strongly retarded

Figure 4
Growth curves of K562 (a) and HL60 (b) cells, transfected by nucleofection technique. Cell proliferation was measured by trypan blue staining and cell count. The figure represents data from five separate experiments. Data are presented as mean +/- standard error of the mean.

(untransfected cells after 72 hours 1.9 × 10⁶ cells/ml versus transfected cells 4 × 10⁴ cells/ml), the non-transfected control cells proliferated continuously. Transfection of the HL60 cells lines with nucleofection resulted in a stagnation of cell growth. After 72 hours cell lines continued to proliferate however at a slower rate than the control (data not shown).

Discussion

Chemotherapy and allogeneic bone marrow transplantation (BMT) are the conventional treatment strategies for acute myelogenous leukemia (AML) [32-35]. Complete remissions can be achieved in the majority of patients, but disease recurrence remains a frequent subsequent of treatment failure. For example, in patients whose AML blasts bear complex chromosomal mutations the risk of leukemia relapse is very high, and in AML patients over 60 years of age the five year survival rate after established treatment regimens is below 20% [36]. Unfortunately, therapeutic options for patients with recurrent leukemia are still limited and the prognosis is poor [37]. Second marrow transplants from the same donor may be considered for patients with disease relapse after BMT, but the mortality, treatment-related morbidity and risk of further relapse are high [38,39].

Alternative or additional treatment strategies are provided by immunotherapeutic approaches. Successful employment of donor lymphocyte infusions (DLI) in patients with relapsed chronic myelogenous leukemia (CML) after allogeneic BMT gave reason for the application to acute leukemia patients, but the treatment turned out to be far less effective [40-43]. In other cases of refractory or relapsed AML, infusions of anti-CD33 antibody-conjugated antitumor agents have been successfully used [44]. Furthermore, gene therapy has emerged as a promising approach to provide new treatment options.

Leukemia cells are considered as suitable targets for gene therapy. Cytogenetic studies of leukemia cells have identified mutations, chromosomal aberrations the failure of expression of co-stimulatory molecules [1,5,9]. Co-stimulatory molecules such as CD80 (B7.1) and CD86 (B7.2) that are necessary to bind CD28 on T-cells, to maintain production of IL-2 after initial T cells activation, have been shown to be lacking in acute leukemic cells, resulting in T-cell anergy [5]. The lack of expression of CD80 could also be detected in the leukemic cell lines used here. Vectors expressing co-stimulatory molecules or cytokines have been suggested for gene therapy strategies [1,3]. Adenovirus based vectors can be used for targeted gene transfer to AML [15] and after stimulation of the target cells CML and B-CLL could be efficiently transfected by adenoviral vectors. Similar results could be obtained by the use of primary cells [14]. We previously demonstrated efficient gene transfer in Burkitt lymphoma (BL) cell lines and primary lymphoma cells after transfection with adenoviral vectors [11] and could here show similar results in transfection efficiency of the leukemic cell line K562 by use of adenoviral vectors. Recent reports have described the successful gene transfer in the cell line HL60 and primary cells derived from AML patients up to 100% of positive cells after adenoviral gene transfer [15]. Although viral vectors induce long term, high gene expression, phenomena such as the possibility of creating recombination competent adenovirus (RCA) induction of the host immune response are cutting back the use of viral delivery.

Since the efficiency of non-viral gene transfer by naked DNA is lower than that of viral delivery, both chemical and physical techniques have been used to increase the efficiency of DNA uptake and expression. The physical gene transfer approaches allow DNA to penetrate directly the cell membrane and bypass endosomes / lysosomes, thus avoiding enzymatic degradation. The DNA may also be delivered directly to the nucleus by gene gun, electroporation and novel electroporation based technique called nucleofection. Physical gene transfer methods, unlike viral vectors, do not require cell type specific receptors, are safe, highly reproducible and time saving. Due to low transfection efficiency most investigations were made with established cell lines after stable transfection and selection [19]. Even many transfection reagents which show high gene transfer efficiency in common adherent cell lines are not suitable to transfect establish blood cell lines or primary leukemia cells from patients. All samples showed a transfection rate of below 5% positive cells [20]. There is no data concerning efficient non viral gene transfer into primary leukemic cells with gene gun or standard electroporation.

The use of electrotransfer for DNA delivery to eukaryotic cells in vitro has been well known and widely used in basic research. However, it is only recently that electric fields have been used to enhance DNA transfer to animal cells in vivo, and this is known as DNA electrotransfer or in vivo DNA electroporation. This is especially useful to transfect whole tissues or tumors. As well as exciting applications in developmental biology, in vivo DNA electrotransfer is also being used to transfer genes to skeletal muscle and drive expression of therapeutically active proteins and to examine exogenous gene and protein function in normal adult cells situated within the complex environment of a tissue and organ system in vivo [45]. However, the use of in vivo electroporation has just begun and so far nothing has been published of in vivo transfection of cells of the blood system.

Here, we established an optimized non-viral gene delivery into leukemic cells as a first step towards a gene therapy approach. We compared the efficiency of adenoviral mediated gene transfer with the efficiency obtained by electroporation, particle bombardment and nucleofection into leukemic cells *in vitro*. Using established human leukemic cell lines we have analyzed the standard techniques with the novel nucleofection technique. We have examined different electrical programs with the new nucleofector advice to determine the effects on the transfection efficiency and viability of the cells.

In this study we have shown that the novel non-viral transfection technique called nucleofection is an efficient way to transfect not only AML cells lines but also primary AML cells. Here, we achieved transfection efficiency for primary cells from three AML patients up to 75 % with low toxicity after 24 hours (< 20 %). After 72 hours the toxicity inside the lymphocyte gate increased up to 40–45 % (non-nucleofected primary cells 25 %). However, the ability of nucleofection to mediate gene transfer into non-dividing cells and the feasibility to transfect a high range of cells makes it attractive for gene delivery *in vitro*. Nucleofection does only very rarely result in nuclear integration of the transgene. Loss of gene expression during propagation of cells is most likely to be due to loss of the transgene rather than due to loss of transgene expression. In terms of cell numbers, K562 and HL60 cells cease proliferation 72 hrs after nucleofection. It is expected that these cells finally enter cell death. This, however, makes the procedure less suitable for biochemical or pharmacological studies due to severe cell damage during nucleoporation. However, in immunotherapeutic approaches, sustained gene transfer is not essential. Thus, the transient gene expression achieved by use of nucleofection is an available tool for gene therapy.

Conclusions
The ability to efficiently manipulate gene expression in leukemia using non-viral methods should facilitate the functional characterization of pathways affecting lymphocytes physiology. In conclusion, we present a protocol of a new gene transfer method leading to highly efficient gene transfer in primary leukemic cells and established cell lines without major toxicity and low risk of insertional mutagenesis or induction of the host immune response. This protocol should have an important impact on the use of hematopoetic cells in cancer gene therapy protocols.

List of abbreviations
AML, acute myeloid leukemia; APC, antigen presenting cell; BMT, bone marrow transplantation; CML, chronic myelogenous leukemia; DLI, donor lymphocyte infusion; FCS, fetal calf serum; GFP, green fluorescent protein; HS, horse serum; IL-2, interleukin-2; ND, not done.

Competing interests
PB is presently working at Amaxa, he was not when performing the experiments described herein.

Authors contributions
FS, PB, MM and AM carried out the studies, BS was clinically involved and helped in carrying out the studies, ISW participated in the design of the study and its coordination. All authors read and approved the final manuscript.

Acknowledgments

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