Nucleoporation of dendritic cells: efficient gene transfer by electroporation into human monocyte-derived dendritic cells[1]

Petra Lenz[a], Silvia M. Bacot[b], Michelle R. Frazier-Jessen[b], Gerald M. Feldman[b,*]

[a] *Laboratory of Cellular Oncology, National Cancer Institute, NIH, Bethesda, MD 20892, USA*
[b] *Division of Monoclonal Antibodies, Center for Biologics Evaluation and Research, Food and Drug Administration, HFM-564, Bldg. 29A, Rm 3C24, 29 Lincoln Drive, Bethesda, MD 20892, USA*

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Abstract Dendritic cells (DCs) are ideal accessory cells in the developing field of gene therapy. Although viral transfection of DCs has become widespread, non-viral transfection of DCs has shown disappointing results. Recently, a new technique for transfecting primary cells has become available – the Amaxa Nucleofector™. Here, we describe the use of this device in the successful non-viral transfection of human monocyte-derived DCs. Using enhanced green fluorescent protein as a reporter gene DCs were transfectable with efficiencies approaching 60%, remaining responsive to lipopolysaccharide-stimulated cytokine production in short-term experiments (though long-term functional assays were hampered by loss of viability). Although these data demonstrate the ease and efficiency with which human monocyte-derived DCs can now be non-virally transfected, they also suggest the limitations of this technology due to the gradual loss of cell viability. The potential use of this system in the development of DC-based cell and gene therapies will be hampered until cell viability can be maintained.

Key words: Electroporation; Dendritic cell; Gene therapy; Monocyte; Nucleofection; Green fluorescent protein

1. Introduction

Dendritic cells (DCs) are bone marrow-derived leukocytes whose primary function is that of antigen capture and presentation for the initiation of a primary immune response [1–3]. Given their pivotal role in antigen processing as well as their important role in tumor immunology [4–12], the use of ex vivo-manipulated human DCs in augmenting the immune response is an attractive approach in the growing fields of cell-based and gene therapies.

The use of genetically modified primary DCs for research or therapeutic uses has been greatly hampered by the difficulties involved in their successful transfection. Although several authors have reported the use and feasibility of retroviral transduction of human DCs in vitro [4,7,13,14], the safety concerns inherent in the use of engineered viruses has limited their use in the clinical setting. Moreover, low transfection efficiencies with non-viral vectors [15–19] have severely limited this area of therapeutic research. Recently, a new technology was developed that allows the directed electroporation of DNA into the nucleus. This new technology – nucleofection – is thought to be capable of generating transfectants of non-proliferating and otherwise difficult-to-transfect primary cells. We report here on the optimization of a non-viral nucleofection protocol as a successful method of DC gene transfer that is capable of achieving high efficiencies with limited short-term toxicity to the cell.

2. Materials and methods

2.1. Cell culture

Human peripheral blood monocytes were obtained from healthy volunteers by leukapheresis. Monocytes were purified from mononuclear cells by Ficoll-hypaque sedimentation followed by countercurrent centrifugal elutriation [20,21]. Monocytes ($>95\%$ $CD14^+$) were resuspended at a density of $1.5–2.0\times10^6$ cells per ml in complete medium – Dulbecco's modified Eagle's medium (DMEM; Life Technologies, Rockville, MD, USA) containing 10% fetal bovine serum (BioWhittaker, Walkersville, MD, USA), 10 μg/ml gentamicin, 100 ng/ml recombinant human granulocyte/macrophage colony-stimulating factor (generously provided by Immunex Corporation, Seattle, WA, USA) and 100 ng/ml recombinant human interleukin (IL) 4 (generously provided by Schering Plough Corporation, Kenilworth, NJ, USA), and cultured at 37°C for a minimum of 6 days to allow for differentiation into DCs.

2.2. Transfection of DCs

A red-shifted humanized variant of the green fluorescent protein (GFP) gene driven by the CMV promoter (pEGFP-N1) obtained from Clontech (Palo Alto, CA, USA) was used to gauge transfection efficiency. pEGFP-N1 was propagated in *Escherichia coli* strain DH5α (Gibco BRL, Grand Island, NY, USA) and purified by double banding on cesium chloride gradients. The non-adherent DCs were harvested at days 6 or 7 of culture, washed once in cold phosphate-buffered saline, and resuspended in the specified electroporation buffer to a final concentration of 2×10^7 cells/ml. Five μg of plasmid DNA was mixed with 0.1 ml of cell suspension, transferred to a 2.0 mm electroporation cuvette, and nucleofected with an Amaxa Nucleofector™ apparatus (Amaxa, Cologne, Germany) as described. DNA quantity, cell concentration, and buffer volume were kept constant throughout all experiments. After electroporation, cells were immediately transferred to 2.0 ml of complete medium, and cultured in six-well plates at 37°C until analysis. Viability of cells immediately after transfection was determined by trypan blue exclusion; at all other time points viability was determined by propidium iodide staining. Validation of the GFP data was performed using a dominant-negative MyD88 construct tagged with GFP that was kindly provided by Dr. Cynthia Leifer, NCI, NIH.

*Corresponding author. Fax: (1)-301-827 0852.
E-mail address: feldman@cber.fda.gov (G.M. Feldman).

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2.3. EGFP expression analysis

EGFP expression in transfected cells was determined at specific times post nucleofection by FACS analysis. DCs were harvested and resuspended in ice-cold Hanks' balanced salt solution (Life Technologies) containing 0.1% bovine serum albumin (Life Technologies) and 0.1% sodium azide (Aldrich, Milwaukee, WI, USA). EGFP expression was determined by excitation at 488 nm and analysis at 511 nm. Cellular fluorescence and viability (propidium iodide exclusion) were monitored in a FACSCalibur® (Becton Dickinson, Mansfield, MA, USA) and analyzed using the CellQuest software provided by the manufacturer.

2.4. Immunophenotyping of DCs

DCs were also analyzed for expression of differentiation and activation markers. Cells were stained with phycoerythrin-conjugated monoclonal antibodies raised against the following human surface antigens (all antibodies obtained from Pharmingen, San Diego, CA, USA): CD14 (M5E2, mouse IgG2a), CD11c (B-ly6, mouse IgG1), CD80 (L307.4, mouse IgG1), HLA-DR (G46-6, mouse IgG2a), CD40 (5C3, mouse IgG1), CD83 (HB15e, mouse IgG1), CD86 (IT2.2, mouse IgG2b), and appropriate isotype controls. Cells (10^5 per assay) were incubated with the respective antibody (2.5 μg per sample) for 30–45 min at 4°C prior to washing. Cellular fluorescence was monitored as described previously. DCs, gated by forward and side scatter, expressed high levels of CD11c but were negative for CD14 (data not shown).

2.5. Functional analysis of transfected DCs

To detect intracellular cytokine production, untreated or transfected cells were exposed to lipopolysaccharide (LPS, 1 μg/ml), and 1 h later, the proteosome inhibitor brefeldin A (GolgiPlug, Pharmingen) was added for an additional 5 h. Cells were then fixed and permeabilized using a Cytofix/Cytoperm Kit (Pharmingen) according to the manufacturer's instructions, and stained with FITC-labeled mouse anti-human monoclonal antibodies against IL-1β (IgG1), tumor necrosis factor α (TNFα) (IgG1), IL-6 (IgG2b), IL-12 (IgG1), and their respective isotype controls (all R&D Systems, Minneapolis, MN, USA). Cellular fluorescence was monitored and analyzed as described.

3. Results

3.1. Optimization of cDNA transfection in DCs

Immature DCs derived from human monocytes were used to optimize the nucleofection conditions. Over 80 separate conditions were tested, and transfection efficiencies were determined by FACS analysis of EGFP expression. As shown in Fig. 1 (which represents a subset of the overall data), analysis of cells 24 h post transfection demonstrated a high percentage of EGFP expression under optimized experimental conditions. Of the various electrical settings and buffer conditions used, the best nucleofection efficiencies were observed with protocol T-01 and both buffers T and V (48.0 ± 8.2%, range of 30–60%, and 44.2 ± 7.0%, range of 25–59%, respectively). These two sets of conditions were used in subsequent experiments.

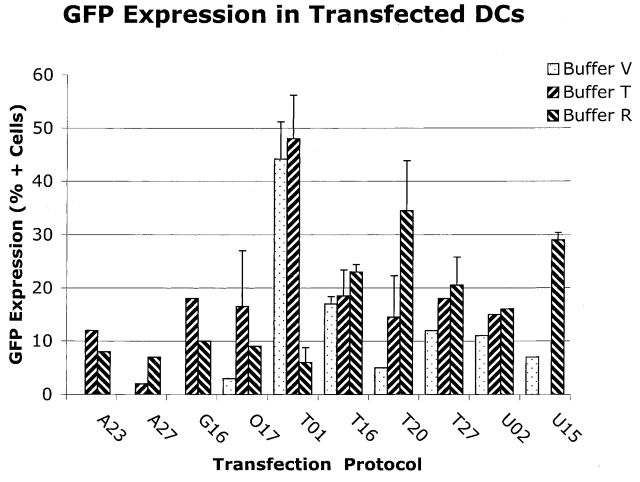

Fig. 1. Expression of EGFP in nucleofected DCs. Day 7 monocyte-derived DCs were resuspended (2×10^7 cells/ml) in buffer R (right cross hatch), buffer T (left cross hatch), or buffer V (stippled), and nucleofected using the settings described. Cells were then transferred to complete DMEM, and incubated at 5% CO_2 and 37°C for 24 h. Cellular expression of EGFP was determined by FACS analysis as described. Data represent the mean and its standard error of four to seven individual experiments.

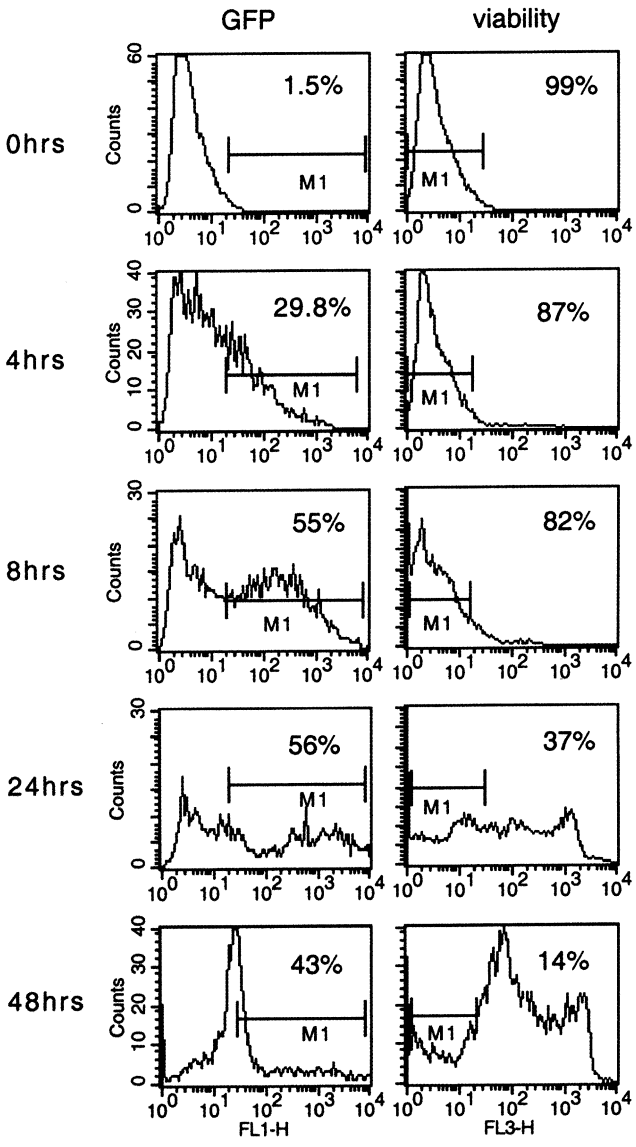

Fig. 2. Time course of EGFP expression in nucleofected DCs. Day 7 monocyte-derived DCs were resuspended in buffer V, and nucleofected using protocol T-01. Cells were then transferred to complete medium and incubated as described. Cells were removed at the specified times post treatment, and expression of EGFP as well as viability determined by FACS analysis. Numbers in the upper right indicate the percentage of EGFP-expressing or viable cells, respectively, and are representative of a minimum of four experiments.

3.2. Kinetics of EGFP expression

Since the specified conditions demonstrated a significant level of EGFP expression at 24 h post transfection, the kinetics of that response was determined as a function of time.

Using the electrical program T-01 and buffer V, cells were transfected with EGFP and the efficiency of transfection determined up to 48 h post treatment. As seen in Fig. 2, the efficiency of DC transfection was as high as 30% as early as 4 h after nucleofection. By 8 h post nucleofection transfection efficiency under these conditions was up to 55%, where it remained over the next 24 h (Fig. 2). By 48 h post nucleofection the transfection efficiency was still high, reaching 40%. The cell viability over this time period reflected the damage that generally occurs upon transfection – during the initial 8 h post treatment cell viability remained high, staying in the 80–90% range. By 24 h however, cell viability had dropped to under 40%, continuing to drop to 14% by 48 h post treatment.

3.3. Effect of nucleofection on phenotypic characteristics of immature DCs

Given the cellular damage that these transfection methods incur over time, an important consideration when working with any cell type but especially DCs is the impact of any treatment on cell phenotype and function. To assess the effect of nucleofection on these parameters, we performed FACS analysis on untransfected or transfected DCs 24 h after treatment in either buffer T or buffer V (Fig. 3). Under these conditions nucleofection did not induce DC maturation or activation. With the exception of a slight but consistent down-regulation of CD80, the phenotype remained stable over 24 h after treatment.

3.4. LPS-induced activation and differentiation of transfected DCs

To assess the ability of transfected cells to respond in a functional manner to external stimuli untreated and EGFP-transfected DCs were stimulated with LPS, and cytokine production was determined by intracellular cytokine staining. As shown in Fig. 4, the nucleofection procedure alone did not induce any of the inflammatory cytokines tested (TNFα, IL-1β, IL-6, IL-12), whereas exposure to LPS induced high levels of TNFα and IL-1β, moderate levels of IL-6, and no IL-12 (Fig. 4). Transfected cells that were subsequently treated with LPS exhibited a different pattern of cytokine production, indicating that nucleofection affects gene expression in DCs (Fig. 4). Whereas IL-1β, TNFα and IL-6 responses to LPS in nucleofected DCs were substantially decreased, IL-12 was induced. This latter finding is of particular interest considering the important role of IL-12 in the induction of Th1 T cell responses. A more dramatic decrease in LPS responsiveness was observed in regard to LPS-induced phenotypic characteristics. Transfected immature DCs subsequently treated with LPS failed to undergo phenotypic activation or maturation as evidenced by the unchanged surface expression of the costimulatory molecules CD40 and CD80, and the activation marker CD83 (data not shown).

3.5. Nucleofection of mature DCs

Given that the lack of phenotypic maturation and activa-

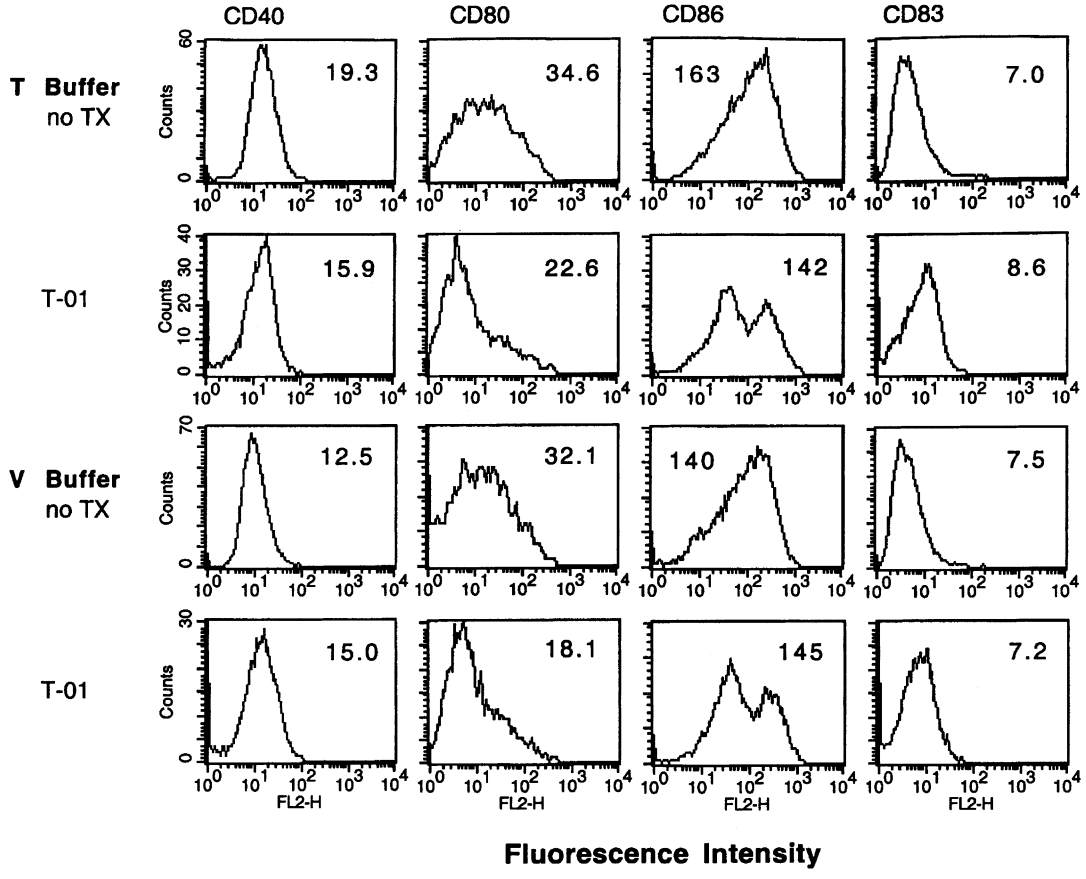

Fig. 3. Surface expression of differentiation markers on monocyte-derived DCs. Day 7 monocyte-derived DCs were resuspended in buffer T or buffer V and either nucleofected using protocol T-01 or left untouched ('no TX'). Cells were then transferred to complete medium and incubated as described. After 24 h cells were stained for surface expression of CD40, CD80, CD86, and CD83 as described in Section 2. Numbers represent the mean fluorescence intensity of the histograms. Shown are the results of one representative experiment out of a minimum of four experiments.

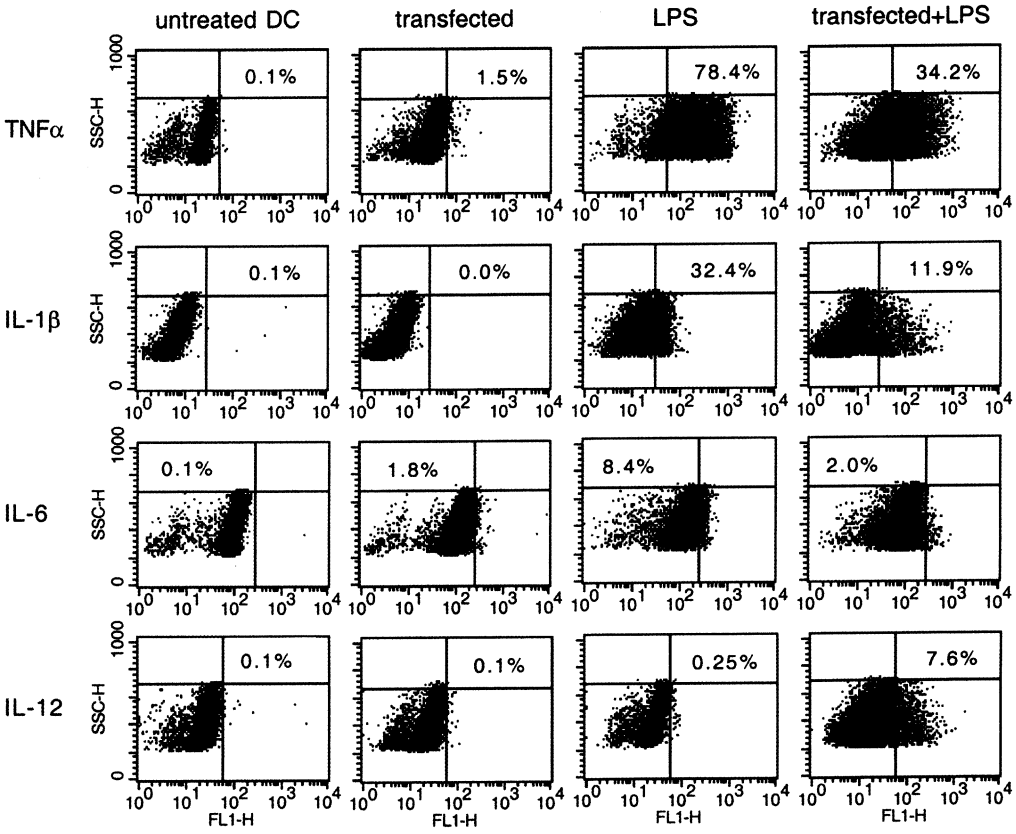

Fig. 4. Induction of inflammatory cytokines in nucleofected DCs. Day 7 monocyte-derived DCs were nucleofected as described, and immediately exposed to LPS (1 μg/ml) for 1 h followed by brefeldin A for 5 h. The intracellular cytokines TNFα, IL-1β, IL-6 and IL-12 were quantified by flow cytometry (FL1) as described in Section 2. Numbers represent the percentage of cytokine-producing DCs, and are representative of three separate experiments.

tion after nucleofection would be a major drawback for many DC applications, we investigated whether matured DCs can be nucleofected efficiently, and whether the procedure conserves their mature phenotype. Immature DCs were pretreated with LPS for 24 h, transfected, and assayed for marker expression to demonstrate terminal maturation (Fig. 5A). Using the optimized transfection procedures identified earlier, transfection efficiencies reached up to 39% at 8 h after nucleofection (Fig. 5B). At 24 h post nucleofection, cells retained their mature phenotype, and exhibited similar viability and EGFP expression as their immature counterparts (data not shown).

3.6. Validation of DC transfection efficiency with GFP

To determine whether the data obtained with GFP as the transfecting construct can be expanded to other plasmids, we attempted to transfect a similar pEGFP-N1 construct into which amino acids 152–296 of the MyD88 sequence (corresponding to the C-terminal binding domain but lacking the N-terminal death domain) was subcloned. As shown in Fig. 6, high transfection efficiency and excellent viability were observed within 6 h post transfection, demonstrating the ability to apply these methodologies to different constructs.

4. Discussion

The genetic manipulation of DCs has become a major focus of research, due to its potential in the development of vaccines for treatment of cancer, chronic, and infectious diseases. Though the use of viral transduction has been shown to be effective for this purpose [4,10,11,22–28], the application of these methods has certain disadvantages. Besides an increase in the immunogenicity of virus-manipulated DCs due to the transfer of viral proteins [29–32], the risk of oncogenesis or mutagenesis from the insertion of the viral genome into host cell DNA remains a serious safety concern [33–35]. On the other hand, the non-viral transfection of DCs has proven to be difficult, partially due to the fact that these cells are non-dividing. Thus, reports of DC transfection by non-viral methods have been few: electroporation of DCs, when successful, has consistently given transfection efficiencies in the range of 10–15% [15–19,36–38]. Attempts at lipid-mediated transfer of genetic material into DCs have provided similar results [39–44].

Here, we report on a simple method of electroporation of DCs that provides a rapid and efficient way of transferring genetic material into monocyte-derived immature and mature DCs. This transfection procedure retains most of the phenotypic characteristics of DCs and their ability to secrete inflammatory cytokine upon exposure to external stimuli. However, it has certain deleterious effects on DCs – most significantly the limited survival over 48 h and their unresponsiveness to LPS-induced maturation. The latter problem could be circumvented by nucleofection of previously matured DCs.

The low viability at 24 h seen in our experiments is similar to that observed with other electroporation systems and other cell types. It might be partially attributed to the physical damage that occurs when cells are exposed to short-duration but high-intensity electrical discharges. Whether the changes

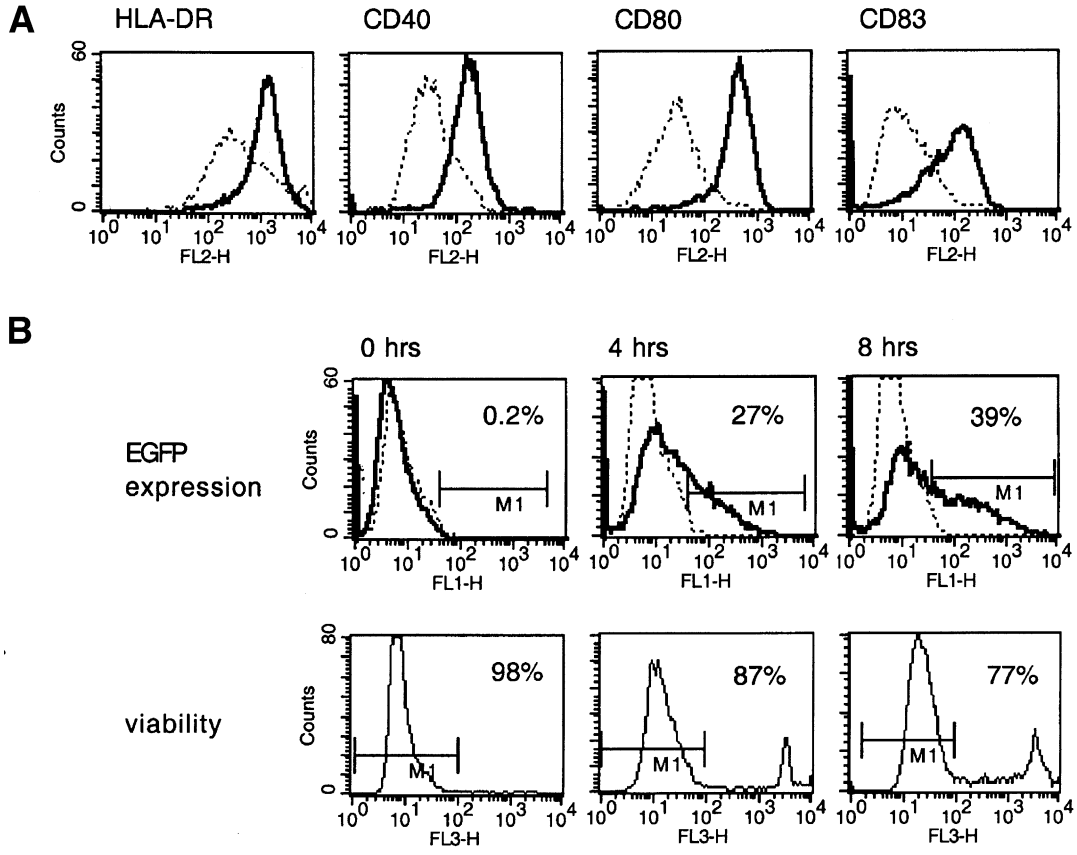

Fig. 5. Nucleofection of LPS-matured DC. Immature DCs (dotted lines) were exposed to LPS (1 μg/ml) for 24 h and maturation (heavy lines) determined by flow cytometry (A). LPS-matured DCs (B) were assayed for EGFP expression and viability before or 4 and 8 h after nucleofection. EGFP expression in nucleofected cells (heavy line) is shown as compared to mock-transfected cells (dotted line). Numbers in the upper right indicate percentage of EGFP-positive or viable cells, respectively. Shown are results of one representative experiment out of three.

in DC responsiveness to LPS stimulation reflect the cellular damage that ultimately results in the observed decreases in viability, or a more direct inhibition of some cellular pathway, is unknown. In any case, the high transfection efficiency obtained within the first 24 h makes nucleofection a powerful procedure for gene transfer into DCs in short-term experiments. Furthermore, despite the low survival at later time points, the continued high transfection efficiency might make this method useful even for some long-term assays and might provide distinct advantages over other methods of transfection that provide higher rates of viability but result in significantly lower transfection efficiencies.

For the time being, the nature of the electrical parameters that the Amaxa Nucleofector™ uses and the composition of the buffers that are required remain a proprietary secret. However, this system remains the only successful method to date of achieving a rapid and efficient short-term transfer of plasmid DNA into monocyte-derived immature or mature DCs, suggestive of the promise it lends to the developing fields of gene therapy and DC research.

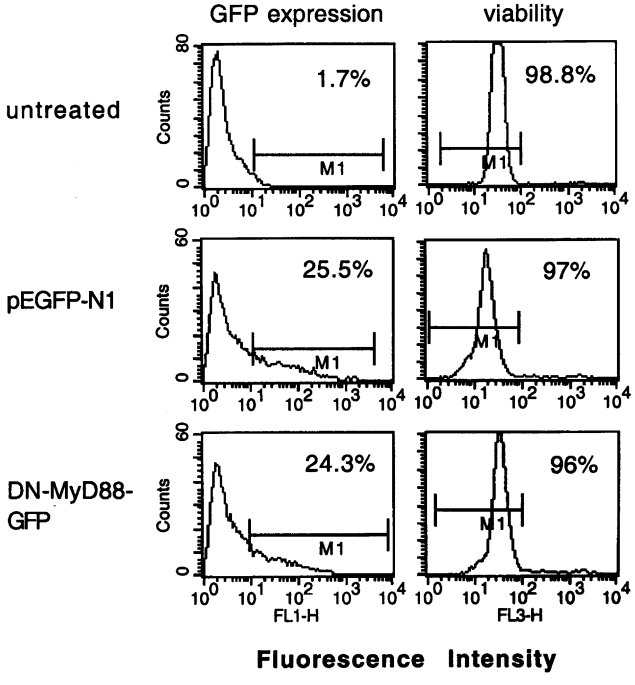

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Fig. 6. Expression of DN-MyD88-GFP in nucleofected DCs. Day 7 monocyte-derived DCs were resuspended in buffer V, and nucleofected using protocol T-01. Cells were then transferred to complete medium and incubated as described. Cells were removed at 6 h post treatment, and expression of pEGFP-N1 and DN-MyD88-GFP (pEGFP-N1 into which a portion of MyD88 was subcloned) as well as viability determined by FACS analysis. Numbers in the upper right indicate the percentage of GFP-expressing or viable cells, respectively.

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