Published in final edited form as:

J Immunol Methods. 2010 December 15; 363(1): 21–28. doi:10.1016/j.jim.2010.09.028.

Validation of efficient high-throughput plasmid and siRNA transfection of human monocyte-derived dendritic cells without cell maturation

Robert Bowles^{*}, Sonali Patil^{*}, Hanna Pincas, and Stuart C. Sealfon

Center for Translational Systems Biology and Department of Neurology, Mount Sinai School of Medicine, One Gustave L. Levy Place, New York, NY 10029, USA

Robert Bowles: robert.bowles@mssm.edu; Sonali Patil: sonali.patil@mssm.edu; Hanna Pincas: hanna.pincas@mssm.edu; Stuart C. Sealfon: stuart.sealfon@mssm.edu

Abstract

Transfection of primary immune cells is difficult to achieve at high efficiency and without cell activation and maturation. Dendritic cells (DCs) represent a key link between the innate and adaptive immune systems. Delineating the signaling pathways involved in the activation of human primary DCs and reverse engineering cellular inflammatory pathways have been challenging tasks. We optimized and validated an effective high-throughput transfection protocol, allowing us to transiently express DNA in naïve primary DCs, as well as investigate the effect of gene silencing by RNA interference. Using a high-throughput nucleofection system, monocyte-derived DCs were nucleoporated with a plasmid expressing green fluorescent protein (GFP), and transfection efficiency was determined by flow cytometry, based on GFP expression. To evaluate the effect of nucleoporation on DC maturation, the expression of cell surface markers CD86 and MHCII in GFP-positive cells was analyzed by flow cytometry. We established optimal assay conditions with a cell viability reaching 70%, a transfection efficiency of over 50%, and unchanged CD86 and MHCII expression. We examined the impact of small interfering RNA (siRNA)-mediated knockdown of RIG-I, a key viral recognition receptor, on the induction of the interferon (IFN) response in DCs infected with Newcastle disease virus. RIG-I protein was undetectable by Western blot in siRNA-treated cells. RIG-I knockdown caused a 75% reduction in the induction of IFN- β mRNA compared with the negative control siRNA. This protocol should be a valuable tool for probing the immune response pathways activated in human DCs.

Keywords

Dendritic cells; Nucleofection; High-throughput; Green fluorescent protein; siRNA; Interferon signaling

© 2010 Elsevier B.V. All rights reserved.

Address all correspondence and requests for reprints to: Stuart C. Sealfon, Mount Sinai School of Medicine, Annenberg 14-94; Box 1137, One Gustave L. Levy Place, New York, NY 10029, Tel.: +1 212-241-7075, Fax: +1 212-289-4107.

^{*}These authors contributed equally to this work.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

1. Introduction

Dendritic cells (DCs) are cells of haematopoietic origin that are specialized in the capture, processing, and presentation of antigen in association with molecules of the major histocompatibility complex (MHC) on the cell surface. T cells are stimulated by the MHC-peptide complexes, thereby leading to a primary immune response (Reis e Sousa, 2004). Hence, DCs link the innate immune system with the adaptive immune system. DCs express pattern recognition receptors (PRRs), which detect the presence of pathogens by recognizing conserved structures that are referred to as pathogen-associated molecular patterns (PAMPs). The recognition of a microbial structure by PRRs, which sets off the process of DC maturation, activates an intracellular signaling cascade that results notably in the induction of genes encoding proinflammatory cytokines such as type I interferons (IFNs). Specifically, DC maturation is characterized by an inflammatory response, upregulation of co-stimulatory molecules like CD86 and of the MHC molecules, and migration of DCs to the lymphoid organs, where they stimulate naïve T and B cells (for review, see (Banchereau and Steinman, 1998; Clark et al., 2000; Reis e Sousa, 2004)).

PRRs can be divided into four different families: Toll-like receptors (TLRs) and C-type lectin receptors (CLRs), which are membrane-associated, and NOD-like receptors (NLRs) and RIG-I-like receptors (RLRs), which are cytosolic. TLR ligands comprise bacterial lipopolysaccharide (LPS) and flagellin, while CLRs bind carbohydrate structures that are present on viruses, bacteria, and fungi. NLRs bind bacterial peptidoglycan components, whereas members of the RLR family, which is composed of RIG-I, MDA-5, and LGP2 recognize RNA segments from RNA viruses (Takeuchi and Akira). In particular, RIG-I responds to the Paramyxoviridae, such as Newcastle disease virus (NDV) and Sendai virus (SeV) (Kato et al., 2005; Kato et al., 2006). RIG-I identifies short double-stranded RNA, and the presence of a 5' triphosphate terminus enhances the induced IFN response (Hornung et al., 2006).

The ability to dissect the molecular networks underlying DC response to various pathogens is crucial to a better understanding of the regulation of these signaling pathways and their induced genes. This should also help to develop antibacterial and antiviral therapies, as well as cancer treatments. Nevertheless, examining those molecular and biochemical events has been impeded by the difficulty in transfecting primary DCs effectively. Although retroviral transduction of DCs *in vitro* has been previously reported and may represent a valuable tool in tumor immunotherapy, it is limited by the fact that engineered viruses are a labor-intensive process and can represent a biohazard risk (Henderson et al., 1996; Reeves et al., 1996; Aicher et al., 1997; Thomas et al., 2003). On the other hand, efficient electroporation of human monocyte-derived DCs (MoDCs) with mRNA was previously demonstrated without apparent induction of DC maturation (Van Tendeloo et al., 2001); in contrast, three years earlier, neither electroporation nor lipofection of MoDCs with plasmid DNA were reported successful (Van Tendeloo et al., 1998). Laderach and his coworkers employed an electroporation procedure to knock down NF κ B p50 with small interfering RNA (siRNA), and showed the absence of significant toxicity to the cells, of DC maturation or of a type-I IFN response (Laderach et al., 2003). Feldman's group reported efficient nucleoporation of human DCs with a plasmid expressing green fluorescent protein (GFP), using the Amaxa Nucleofector (Lonza Walkersville Inc.) in electroporation cuvettes, yet their technique resulted in gradual loss of cell viability (Lenz et al., 2003). Nucleofection is a relatively recent transfection method optimized to transfect primary cell types (Hamm et al., 2002), which are generally difficult to transfect, by enabling directed electroporation of nucleic acids to the nucleus. In summary, few data describing high-efficiency transfection of MoDCs with both DNA and siRNA have been reported thus far.

Herein, we report the optimization of a high-throughput nucleoporation method as an efficient way of transfecting primary human DCs with either plasmid DNA or siRNA, with a limited toxicity to the cell and no induction of DC maturation. Using this electroporation protocol, we provide evidence for successful transfection of MoDCs with siRNA and effective silencing of targeted gene RIG-I at both the mRNA and protein levels. To our knowledge, this work allows for the first time the design of high-throughput loss-of-function studies in primary human DCs, providing new opportunities for the characterization of signaling pathways in these cells.

2. Materials and Methods

2.1. Generation of DCs

Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll density gradient centrifugation (Histopaque, Sigma-Aldrich, St. Louis, MO) from buffy coats of healthy anonymous donors (New York Blood Center, New York, NY). CD14⁺ monocytes were immunomagnetically purified by using a MACS CD14 isolation kit (Miltenyi Biotec, Bergisch Gladbach, Germany). After elution from the Midi MACS LS columns, CD14⁺ monocytes were plated (1×10^6 cells/ml) in DC growth medium, which contains RPMI 1640 medium (Invitrogen, Carlsbad, CA) supplemented with 10% fetal calf serum (HyClone, Logan, UT), 2mM 1-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin (Invitrogen), along with 500 U/ml GM-CSF and 1000 U/ml human IL-4 (PeproTech, Rocky Hill, NJ). Monocytes were differentiated into naïve DCs by a 5- to 6-day incubation at 37°C and 5% CO₂ in DC growth medium.

2.2. Transfection of DCs with plasmid DNA

Naïve DCs were transfected with pmaxGFP in the Nucleofector 96-well Shuttle, using the Cell Line Optimization 96-well Nucleofector Kit (Lonza Walkersville Inc., Walkersville, MD). Cells were initially transfected using one of the kit's nucleoporation buffers, also referred to as Nucleofector Solutions SE, SF, and SG, in combination with one of 31 standard nucleoporation programs provided by the manufacturer. As indicated in the Results, the Human Monocyte 96-well Nucleofector Solution was employed next, along with the standard nucleoporation programs as well as additional exploration of transfection parameter space. Briefly, prior to transfection, cells were pelleted and resuspended in the specified nucleoporation buffer. The cell suspension (5×10^5 cells) was combined with plasmid DNA (0.4 µg), and the mixture (20 µl) was transferred to each well of a 96-well Nucleocuvette plate. Immediately after transfection, 80 µl of DC growth medium were added to each well using a multichannel pipette, and cells were incubated for 10 min at 37°C. Transfected cells were then transferred to ScreenMates 0.75 ml Matrix tubes (Thermo Fisher Scientific, Hudson, NH) containing 100 µl of pre-warmed DC growth medium, and incubated for another 48 h at 37°C and 5% CO₂, to allow expression of pmaxGFP. Viability of cells was determined by trypan blue exclusion 48 h after transfection.

2.3. Transfection of DCs with siRNA

Cells (5×10^5 per well) were transfected with 0.25µg of either RIG-I targeting or non-silencing control siRNA in the Nucleofector 96-well Shuttle system using the monocyte-specific buffer in combination with nucleoporation program FF168, according to the manufacturer's recommendations (Lonza Walkersville Inc.). RIG-I (DDX58) siRNA ON-TARGETplus Set of 4 oligonucleotides and fluorescent negative control siRNA (siGLO RISC-Free Control siRNA) were obtained from Dharmacon (Lafayette, CO). DDX58 target sequences were as follows: 5'-CGUAAGAGUGAUAGAGGAA-3', 5'-GAUUGUAGAGAAAGGUAUA-3', 5'-CAGCAGGAUUCGAUGAGAU-3', 5'-GGUAUAGAGUUACAGGCAU-3' (GenBank accession no.: NM_014314).

2.4. Infection of DCs

Twenty-four hours after transfection, DCs were spun down and the medium was completely removed. Cells were resuspended in DC growth medium containing RPMI 1640, 100 U/ml penicillin, and 100 µg/ml streptomycin (Invitrogen) at 1×10^6 cells/ml, in the presence of NDV (titer = 4×10^8) at a multiplicity of infection of 1. After a 45-min incubation at 37°C and 5% CO₂, fresh DC growth medium containing RPMI 1640, 10% fetal calf serum (HyClone), 100 U/ml penicillin, and 100 µg/ml streptomycin, was added back to the infected cells (1×10^6 cells/ml) for the remainder of the infection. Cells were harvested after 10 h. Following a similar protocol, untransfected DCs were infected with a recombinant NDV that expresses GFP, NDV-GFP at a multiplicity of infection of 1. NDV-GFP was kindly provided by Adolfo Garcia-Sastre (Park et al., 2003).

2.5. Flow cytometry analysis

Forty-eight hours after transfection with pmaxGFP (Lonza Walkersville Inc.), cells were fixed with 1.5% paraformaldehyde (Sigma-Aldrich, St. Louis, MO) and incubated at room temperature for 15 min. Cells were gently pelleted (centrifuged at 40,000 g for 5 min), washed twice in PBS (Invitrogen), and resuspended in PharmingenStain Buffer (BSA; BD Biosciences, San Jose, CA) at 5×10^5 cells/ml. Cells were stained with allophycocyanin (APC)-conjugated monoclonal antibodies against MHCII, and phycoerythrin (PE)-conjugated monoclonal antibodies against CD86 (BD Biosciences, San Jose, CA), and then incubated at room temperature for 30 min. Gates were initially set to exclude dead cells and cellular debris. The green fluorescent cell population was then gated and scored as GFP-positive, and expression analysis of maturation markers MHCII and CD86 was performed within the GFP-positive subpopulation. Determination of the percentage of transfected cells was based on the inclusion of cells exhibiting high levels of green fluorescence and the exclusion of autofluorescent, untransfected cells. Cells were assayed on a FACScan flow cytometer (BD Biosciences), and data were analyzed using the FlowJo software (Tree Star, Ashland, OR).

2.6. Quantitative real-time PCR

RNA was isolated from cells using the ChargeSwitch Total RNA Cell Kit (Invitrogen) according to the manufacturer's protocol, except for the use of 50 µl of magnetic beads and 5 µl of elution buffer. RNA was quantified using a Nanodrop spectrophotometer (Nanodrop Technologies, Wilmington, DE). cDNA was synthesized from total RNA using AffinityScript Multiple Temperature Reverse Transcriptase (Agilent Technologies, Wilmington, DE) and oligo(dT)₁₈ as a primer. Quantitative real-time PCR was carried out with PlatinumTaq DNA polymerase (Invitrogen) and a SYBR Green-containing buffer (Molecular Probes, Invitrogen) using an ABI Prism 7900 HT thermocycler (Applied Biosystems, Foster City, CA), as previously described (Yuen et al., 2002). Each transcript in each sample was assayed in triplicate, and the mean cycle threshold (Ct) was used to calculate copy number. Three housekeeping genes were used for global normalization in each experiment: ribosomal protein S11 (Rps11), tubulin (Tuba), and β-actin (Actb). The following primers were used: NDV-HN (sense: 5'-GACAATGCTTGATGGTGAAC-3', antisense: 5'-CAATGCTGAGACAATAGGTC-3'), NDV-NP (sense: 5'-GGATCTGATGAGAGCGGTAG-3', antisense: 5'-TTGTCAATCAATACCCCCAG-3'), RIG-I (sense: 5'-AAAGCCTTGGCATGTTACAC-3', antisense: 5'-GGCTTGGGATGTGGTCTACT-3'), IFNβ (sense: 5'-GTCAGAGTGGAATCCTAAG-3', antisense: 5'-ACAGCATCTGCTGGTTGAAG-3'), MxA (sense: 5'-CGTGGTGATTTAGCAGGAAG-3', antisense: 5'-TGCAAGGTGGAGCGATTCTG-3'), Rps11 (sense: 5'-CGAGGGCACCTACATAGACA-3', antisense: 5'-GAGATAGTCCCGGCGGATGA-3'), Actb (sense: 5'-GCCTCAACACCTCAAACCAC-3',

antisense, 5'-CCACAGCTGAGAGGGAAATC-3'), Tuba (sense: 5'-AGCGCCCAACCTACACTAAC-3', antisense: 5'-GGGAAGTGGATGCGAGGGTA-3').

2.7. Western blotting

Cells (2.5×10^6 cells pooled from 5 wells) were lysed in RIPA Lysis Buffer supplemented with protease inhibitor PMSF, sodium orthovanadate, and a protease inhibitor cocktail, according to the manufacturer's instructions (Santa Cruz Biotechnology, Santa Cruz, CA). Protein concentrations were determined using the Bio-Rad DC Protein Assay (Bio-Rad, Hercules, CA). For each condition, 20 µg of protein were separated on a Ready Gel Tris-HCl Gel with a 4–15% precast linear gradient polyacrylamide gel (Bio-Rad), and transferred to Hybond-P PVDF membranes (GE Healthcare, Piscataway, NJ). Kaleidoscope Prestained Standards (Bio-Rad) were also run on the gel as molecular weight markers. The membranes were blocked in 5% milk in Tris-buffered saline/0.1% Tween 20 (TBST) for 1 h at room temperature, and subsequently incubated with the primary antibodies overnight at 4°C. After a few washes with TBST, the membranes were incubated with the secondary horseradish peroxidase (HRP)-conjugated goat anti-mouse or goat anti-rabbit antibodies (1/5000 dilution) at room temperature for 1 h (Santa Cruz Biotechnology). Membranes were washed extensively with TBST and rinsed in Tris-buffered saline. The HRP immunocomplexes were detected using the Pierce ECL Western blotting Substrate (ThermoFisher Scientific, Waltham, MA). The autoradiographs were scanned on a ScanMaker 6800 and analyzed using Microtek ScanWizard 5 software (Microtek, Cerritos, CA). Primary antibodies were: mouse monoclonal anti-RIG-I antibody, obtained from the Mount Sinai Hybridoma Center (1.5 µg/ml) and rabbit polyclonal anti-GAPDH antibody (1/1000 dilution) (Bethyl Laboratories, Montgomery, TX).

3. Results

3.1 Optimization of transfection conditions

DCs were transfected with a GFP-expressing plasmid, and transfection efficiency was determined by flow cytometry analysis of GFP expression 48 hours after transfection. Untransfected cells were used as controls for autofluorescence. GFP-transfected cells exhibited an increase in fluorescence. The percentage of transfected cells was determined by establishing a gate well above the level of autofluorescence. Cells within the gate were scored GFP-positive. DC maturation, which is initiated by the recognition of a pathogen, is notably characterized by an up-regulation of cell surface markers CD86 and MHCII and production of cytokines such as IFNs (Ueno et al., 2007). Therefore, the percentage of mature cells within the GFP-positive sub-population was calculated based upon concomitant expression of cell surface markers CD86 and MHCII, as determined by FACS; i.e. cells positive for both markers were scored as mature. Untransfected cells were used as a negative control for background maturation levels. Because DC maturation occurs following infection with a pathogen, DCs infected with NDV-GFP, a recombinant NDV that expresses GFP, were used as a positive control. For clarity purposes, we chose to graphically represent the percentage of immature cells, which was inferred from the percentage of mature cells within the transfected sub-population. The negative control was adjusted to 100 % of immature cells, and all data were normalized against it.

To optimize transfection conditions, we tested various buffer conditions and nucleoporation programs using the Amaxa Nucleofector technology. The use of SF buffer in combination with some specific programs (e.g. FF150, FF137, and FF138) resulted in transfection efficiencies above 50%. However, the percentage of immature cells was markedly lower than that of the untransfected control, namely below 60% (data not shown). Because our cells were monocyte-derived dendritic cells, we then decided to test the manufacturer's

Human Monocyte-specific buffer in conjunction with various electroporation programs. Among the four programs (FF150, FF130, FF137 and FF138) which elicited transfection rates above 50% in the absence or near absence of DC maturation, FF137 and FF138 gave the highest transfection rates (Figure 1A). Under the latter transfection conditions, cells showed a viability of 70% at 48-h post transfection, as determined by trypan blue staining (data not shown), which reflected a limited cellular damage resulting from our transfection protocol. Figure 1B illustrates the FACS analysis of CD86 and MHCII expression when using transfection programs FF137 and FF138. Transfection conditions were further optimized by testing seven additional programs recommended by Amaxa technical support (Figure 2). All tested programs, except for FP137 and FP158, yielded similar rates of transfection and maturation as FF137 and FF138.

To confirm that our transfection method did not induce DC maturation, we analyzed the expression of the IFN β gene by quantitative real-time PCR (qRT-PCR). No significant upregulation of IFN β was observed when using either of the seven most effective transfection programs (data not shown). We further analyzed the expression level of MxA, which is known to be an IFN-inducible protein in DCs, and thus represents a sensitive marker for IFN expression (Ronni et al., 1993; Osterlund et al., 2005). MxA gene expression was stimulated 20-fold under all seven aforementioned transfection conditions, suggesting the occurrence of a minimal level of induction of IFN. Nonetheless, the absence of any significant induction of IFN β gene expression in transfected DCs prompted us to perform siRNA knockdown experiments and demonstrate the usefulness of our method for functional assays. Program FF168 was selected in all subsequent experiments due to its lowest maturation rate.

3.2. Functional studies: siRNA knockdown of RIG-I

We sought to perturb a key viral recognition receptor, RIG-I, by siRNA knockdown, and study its effect on activation of the IFN signaling pathway induced by NDV infection. To this aim, DCs were transfected with siRNA targeting RIG-I, followed by infection with NDV. Expression levels of RIG-I, IFN β and MxA were analyzed by qRT-PCR. Data revealed a 75% inhibition of RIG-I expression by RIG-I siRNA, as compared to the control siRNA (Figure 3A). Similarly, transcript levels of IFN β were reduced by 75% in the presence of RIG-I siRNA, as compared to the control siRNA. In contrast, MxA RNA expression was unchanged by RIG-I siRNA treatment. In uninfected DCs, whether the cells had been treated or not with RIG-I siRNA, the mRNA levels of RIG-I, IFN β and MxA were very low and comparable to one another. These results implied that in the absence of a virus-mediated stimulation of the IFN signaling pathway, genes involved in this pathway are presumably expressed at a basal, barely detectable level; therefore, under these basal conditions, RIG-I siRNA has no gene silencing effect on either RIG-I or downstream genes. Moreover, control siRNA-treated uninfected cells exhibited negligible expression levels of RIG-I, IFN β , and MxA, when compared to control siRNA-treated NDV-infected DCs. Thus, activation of MxA gene expression as a consequence of the transfection procedure appeared insignificant compared to that resulting from virus-mediated stimulation of the IFN signaling cascade. Those results reinforced the notion that our nucleoporation protocol was an effective experimental model for characterizing signaling pathways in primary DCs.

To confirm the effect of RIG-I siRNA knockdown on IFN signaling response to NDV, the same experiment was repeated in primary DCs prepared from another buffy coat, and qRT-PCR analysis of RIG-I, IFN β and MxA gene expression was carried out. Additionally, RIG-I protein levels were determined by Western blot. RIG-I and IFN β RNA levels were reduced by 62% and 66%, respectively, as compared to the control siRNA treatment (Figure 3B). These results were analogous to those found originally. As for the untransfected cells used as an additional control, they showed RIG-I and IFN β expression levels comparable to those

obtained in control siRNA-treated DCs; however, MxA mRNAs were slightly decreased, once again suggestive of a minimal induction of the MxA gene due to the transfection procedure. Western blot analysis showed undetectable RIG-I protein levels (Figure 3C, lanes 5 and 6), as compared to the control siRNA conditions (lanes 3 and 4). Hence, the qRT-PCR data were confirmed at the protein level. As expected, untransfected cells displayed amounts of RIG-I protein comparable to cells treated with control siRNA. Therefore, the marked inhibition of expression of RIG-I and IFN β in knocked down DCs strongly supports the validity and strength of our transfection protocol.

4. Discussion

At present, the molecular bases of DC maturation and activation in response to pathogen infection as well as exposure to inflammatory cytokines/chemokines are not fully understood. Characterizing those signaling cascades and downstream target genes may help develop DC-based vaccines against infectious diseases and against tumors. The ability to transiently transfect expression vectors into primary DCs and perturb components of the DC signaling network through RNA interference (RNAi)-based gene function analyses, should provide new insights into the molecular mechanisms regulating these processes. Even though the utilization of viral transduction has proven fairly effective in generating genetically modified DCs, a number of drawbacks are associated with this gene delivery method. Compared to plasmids, viral vectors are more costly to produce and biosafety precautions are required for handling them (Mincheff et al., 2001). Additionally, the delivery of viral gene products increases the immunogenicity of those transduced DCs (Jooss et al., 1998; Mitchell and Nair, 2000; Mincheff et al., 2001; Roth et al., 2002). Finally, the retroviral genome can transform the host DC by integrating into the host cell DNA (Mulders et al., 1998). Alternatively, many non-viral transfection methods have either been inefficient, or led to a limited cell survival (Van Tendeloo et al., 1998; Lenz et al., 2003). In contrast, a group of researchers electroporated human MoDCs with mRNA (Van Tendeloo et al., 2001) without inducing DC maturation, based on an expression analysis of markers CD80 and CD83. Furthermore, the authors observed stimulation of the cytotoxic T lymphocyte (CTL) immune response when DCs were transfected with RNA and then matured with LPS + TNF α . Laderach and his colleagues effectively knocked down NF κ B p50 with siRNA using electroporation, without causing significant toxicity to the cells or inducing a type-I IFN response. Still, the data obtained from the ELISA analysis of IFN α expression were not illustrated; additionally, their electroporation procedure was not high-throughput (Laderach et al., 2003). Another study described an efficient electroporation method for delivering siRNA into MoDCs. Although the authors investigated the effects of siRNA on cell viability and the expression of DC maturation markers, they did not confirm the effect of gene silencing at the protein level, nor did they examine a possible induction of the IFN signaling pathway (Prechtel et al., 2006). Recently, Flatekval and Sioud (2009) used the nucleofection technology to knockdown IDO gene expression in DCs. However, potential effects of the transfection conditions on cell survival or on activation of the IFN response were not shown (Flatekval and Sioud, 2009).

In this work, we present an optimized protocol for high-throughput nucleoporation of primary human MoDCs, with limited cell toxicity and an absence of DC maturation. Transfection efficiency and cell viability, which were determined 48 hours after transfection, were more than 50% and 70%, respectively. FACS analysis established the absence of expression of the maturation markers CD86 and MHCII in transfected cells. As induction of the IFN signaling is another major attribute of mature DCs, we measured the expression levels of the IFN β gene in transfected cells. We observed no significant upregulation of the IFN β gene, which initially suggested an absence of activation of this pathway by our transfection method. However, when we analyzed the expression level of MxA, we detected

a 20-fold increase in gene expression under each of the transfection conditions. This seemed to indicate a minimum level of induction of the IFN signaling pathway. The expression level of the IFN β gene was unchanged in the presence of neutralizing anti-IFN β antibodies added immediately after transfection (a saturating amount of anti-IFN β antibody was added based on a personal communication from James G. Wetmur; data not shown), which was consistent with no detectable increase of IFN β gene expression in transfected cells. Similarly, the expression level of the MxA gene did not vary significantly in the presence of neutralizing anti-IFN β antibodies, indicating a possible IFN-independent activation of the gene (data not shown). IFN-independent activation of MxA was previously proposed by Ronni and his collaborators, who observed an induction of the MxA protein by influenza virus in PBMCs in the absence of detectable IFN (Ronni et al., 1995). On the other hand, because MxA expression seems sensitive to very low concentrations of IFN β , the observed upregulation of MxA mRNA could reflect a minimal - below the detection limit of the qRT-PCR assay - level of activation of IFN β expression due to the transfection procedure. Nonetheless, the absence of a detectable DC activation supports the idea that a subtle increase in IFN β expression should not interfere with the analysis of the immune response signaling. Indeed, the results of our siRNA knockdown experiments confirmed that RIG-I gene silencing was effective in NDV-activated DCs. Specifically, RIG-I siRNA knockdown not only inhibited RIG-I expression at both the RNA and protein levels, but it also indirectly impeded the mRNA expression of IFN β , which is a downstream effector of RIG-I in the IFN signaling cascade. Despite an indirect inhibitory effect of RIG-I siRNA on IFN β , MxA expression remained unaffected. Again, one cannot exclude the possibility of an IFN-independent activation of MxA as a result of the nucleoporation process itself. Nonetheless, it is noteworthy that activation of MxA gene expression by our transfection method is minor compared to that caused by virus-mediated stimulation of the IFN signaling cascade. Altogether, our data support the utilization of this high-throughput method for high-efficiency transfection of human primary DCs and gene knockdown studies.

In summary, we report the optimization of a commercial protocol for efficient nucleofection of DCs with minimal cell activation. This technique, which solves a difficult impediment to research in this field, should benefit researchers who study DC signaling and may contribute to advancement in DC-based immunotherapy.

Acknowledgments

This project was supported by NIH NIAID Contract No. HHSN2662000500021C. We thank Ming Chen for his technical assistance.

Abbreviations

DC	Dendritic cell
CLR	C-Type lectin receptor
GFP	green fluorescent protein
IFN	interferon
LPS	lipopolysaccharide
MDA-5	melanoma differentiation-associated gene 5
MHC	major histocompatibility complex
MoDC	monocyte-derived DC
NDV	Newcastle disease virus

NLR	nucleotide-binding oligomerization domain (NOD)-like receptor
PAMP	pathogen-associated molecular pattern
PBMCs	peripheral blood mononuclear cells
PRR	pattern recognition receptor
qRT-PCR	quantitative real-time PCR
RIG-I	retinoic acid-inducible gene-I
RLR	RIG-I-like receptor
RNAi	RNA interference
SeV	Sendai virus
siRNA	small interfering RNA
TLR	Toll-like receptor

References

- Aicher A, Westermann J, Cayeux S, Willmsky G, Daemen K, Blankenstein T, Uckert W, Dorken B, Pezzutto A. Successful retroviral mediated transduction of a reporter gene in human dendritic cells: feasibility of therapy with gene-modified antigen presenting cells. *Exp Hematol*. 1997; 25:39–44. [PubMed: 8989905]
- Banchereau J, Steinman RM. Dendritic cells and the control of immunity. *Nature*. 1998; 392:245–52. [PubMed: 9521319]
- Clark GJ, Angel N, Kato M, Lopez JA, MacDonald K, Vuckovic S, Hart DN. The role of dendritic cells in the innate immune system. *Microbes Infect*. 2000; 2:257–72. [PubMed: 10758402]
- Flatekval GF, Sioud M. Modulation of dendritic cell maturation and function with mono- and bifunctional small interfering RNAs targeting indoleamine 2,3-dioxygenase. *Immunology*. 2009; 128:e837–48. [PubMed: 19740345]
- Hamm A, Krott N, Breibach I, Blindt R, Bosserhoff AK. Efficient transfection method for primary cells. *Tissue Eng*. 2002; 8:235–45. [PubMed: 12031113]
- Henderson RA, Nimgaonkar MT, Watkins SC, Robbins PD, Ball ED, Finn OJ. Human dendritic cells genetically engineered to express high levels of the human epithelial tumor antigen mucin (MUC-1). *Cancer Res*. 1996; 56:3763–70. [PubMed: 8706022]
- Hornung V, Ellegast J, Kim S, Brzozka K, Jung A, Kato H, Poeck H, Akira S, Conzelmann KK, Schlee M, Endres S, Hartmann G. 5'-Triphosphate RNA is the ligand for RIG-I. *Science*. 2006; 314:994–7. [PubMed: 17038590]
- Jooss K, Yang Y, Fisher KJ, Wilson JM. Transduction of dendritic cells by DNA viral vectors directs the immune response to transgene products in muscle fibers. *J Virol*. 1998; 72:4212–23. [PubMed: 9557710]
- Kato H, Sato S, Yoneyama M, Yamamoto M, Uematsu S, Matsui K, Tsujimura T, Takeda K, Fujita T, Takeuchi O, Akira S. Cell type-specific involvement of RIG-I in antiviral response. *Immunity*. 2005; 23:19–28. [PubMed: 16039576]
- Kato H, Takeuchi O, Sato S, Yoneyama M, Yamamoto M, Matsui K, Uematsu S, Jung A, Kawai T, Ishii KJ, Yamaguchi O, Otsu K, Tsujimura T, Koh CS, Reis e Sousa C, Matsuura Y, Fujita T, Akira S. Differential roles of MDA5 and RIG-I helicases in the recognition of RNA viruses. *Nature*. 2006; 441:101–5. [PubMed: 16625202]
- Laderach D, Compagno D, Danos O, Vainchenker W, Galy A. RNA interference shows critical requirement for NF-kappa B p50 in the production of IL-12 by human dendritic cells. *J Immunol*. 2003; 171:1750–7. [PubMed: 12902474]

- Lenz P, Bacot SM, Frazier-Jessen MR, Feldman GM. Nucleoporation of dendritic cells: efficient gene transfer by electroporation into human monocyte-derived dendritic cells. *FEBS Lett.* 2003; 538:149–54. [PubMed: 12633869]
- Mincheff M, Altankova I, Zoubak S, Tchakarov S, Botev C, Petrov S, Krusteva E, Kurteva G, Kurtev P, Dimitrov V, Ilieva M, Georgiev G, Lissitchkov T, Chernozemski I, Meryman HT. In vivo transfection and/or cross-priming of dendritic cells following DNA and adenoviral immunizations for immunotherapy of cancer--changes in peripheral mononuclear subsets and intracellular IL-4 and IFN-gamma lymphokine profile. *Crit Rev Oncol Hematol.* 2001; 39:125–32. [PubMed: 11418309]
- Mitchell DA, Nair SK. RNA-transfected dendritic cells in cancer immunotherapy. *J Clin Invest.* 2000; 106:1065–9. [PubMed: 11067858]
- Mulders P, Pang S, Dannull J, Kaboo R, Hinkel A, Michel K, Tso CL, Roth M, Beldegrun A. Highly efficient and consistent gene transfer into dendritic cells utilizing a combination of ultraviolet-irradiated adenovirus and poly(L-lysine) conjugates. *Cancer Res.* 1998; 58:956–61. [PubMed: 9500456]
- Osterlund P, Veckman V, Siren J, Klucher KM, Hiscott J, Matikainen S, Julkunen I. Gene expression and antiviral activity of alpha/beta interferons and interleukin-29 in virus-infected human myeloid dendritic cells. *J Virol.* 2005; 79:9608–17. [PubMed: 16014923]
- Park MS, Shaw ML, Munoz-Jordan J, Cros JF, Nakaya T, Bouvier N, Palese P, Garcia-Sastre A, Basler CF. Newcastle disease virus (NDV)-based assay demonstrates interferon-antagonist activity for the NDV V protein and the Nipah virus V, W, and C proteins. *J Virol.* 2003; 77:1501–11. [PubMed: 12502864]
- Prechtel AT, Turza NM, Theodoridis AA, Kummer M, Steinkasserer A. Small interfering RNA (siRNA) delivery into monocyte-derived dendritic cells by electroporation. *J Immunol Methods.* 2006; 311:139–52. [PubMed: 16556448]
- Reeves ME, Royal RE, Lam JS, Rosenberg SA, Hwu P. Retroviral transduction of human dendritic cells with a tumor-associated antigen gene. *Cancer Res.* 1996; 56:5672–7. [PubMed: 8971174]
- Reis e Sousa C. Activation of dendritic cells: translating innate into adaptive immunity. *Curr Opin Immunol.* 2004; 16:21–5. [PubMed: 14734106]
- Ronni T, Melen K, Malygin A, Julkunen I. Control of IFN-inducible MxA gene expression in human cells. *J Immunol.* 1993; 150:1715–26. [PubMed: 7679692]
- Ronni T, Sareneva T, Pirhonen J, Julkunen I. Activation of IFN-alpha, IFN-gamma, MxA, and IFN regulatory factor 1 genes in influenza A virus-infected human peripheral blood mononuclear cells. *J Immunol.* 1995; 154:2764–74. [PubMed: 7876547]
- Roth MD, Cheng Q, Harui A, Basak SK, Mitani K, Low TA, Kiertscher SM. Helper-dependent adenoviral vectors efficiently express transgenes in human dendritic cells but still stimulate antiviral immune responses. *J Immunol.* 2002; 169:4651–6. [PubMed: 12370405]
- Takeuchi O, Akira S. Pattern recognition receptors and inflammation. *Cell.* 140:805–20. [PubMed: 20303872]
- Thomas CE, Ehrhardt A, Kay MA. Progress and problems with the use of viral vectors for gene therapy. *Nat Rev Genet.* 2003; 4:346–58. [PubMed: 12728277]
- Ueno H, Klechevsky E, Morita R, Aspori C, Cao T, Matsui T, Di Pucchio T, Connolly J, Fay JW, Pascual V, Palucka AK, Banchereau J. Dendritic cell subsets in health and disease. *Immunol Rev.* 2007; 219:118–42. [PubMed: 17850486]
- Van Tendeloo VF, Ponsaerts P, Lardon F, Nijs G, Lenjou M, Van Broeckhoven C, Van Bockstaele DR, Berneman ZN. Highly efficient gene delivery by mRNA electroporation in human hematopoietic cells: superiority to lipofection and passive pulsing of mRNA and to electroporation of plasmid cDNA for tumor antigen loading of dendritic cells. *Blood.* 2001; 98:49–56. [PubMed: 11418462]
- Van Tendeloo VF, Snoeck HW, Lardon F, Vanham GL, Nijs G, Lenjou M, Hendriks L, Van Broeckhoven C, Moulijn A, Rodrigus I, Verdonk P, Van Bockstaele DR, Berneman ZN. Nonviral transfection of distinct types of human dendritic cells: high-efficiency gene transfer by electroporation into hematopoietic progenitor- but not monocyte-derived dendritic cells. *Gene Ther.* 1998; 5:700–7. [PubMed: 9797876]

Yuen T, Zhang W, Ebersole BJ, Sealfon SC. Monitoring G-protein-coupled receptor signaling with DNA microarrays and real-time polymerase chain reaction. *Methods Enzymol.* 2002; 345:556–69. [PubMed: 11665639]

Figure 1. Optimization of DC transfection conditions using a monocyte-specific buffer

(A) MoDCs were transfected with a GFP-expressing plasmid in the monocyte-specific buffer provided by the manufacturer (Lonza Walkersville Inc.). Various nucleoporation programs were tested. Cells were then transferred to DC growth medium, and incubated at 37°C and 5% CO₂ for 48 h. Transfection efficiency (or percentage of transfected cells) was defined as the percentage of GFP-positive cells, as described in the Results. The percentage of immature cells within the GFP-positive subpopulation was inferred from the percentage of cells expressing both CD86 and MHCII maturation markers concomitantly. Untransfected cells were used as a negative control for DC maturation (100% immature cells), and the data were normalized against it. Data represent the results of three replicate experiments. (B) Flow cytometry dot plots for CD86 and MHCII cell surface maturation markers: MoDCs were transfected with a GFP-expressing plasmid in the monocyte-specific buffer using either nucleoporation program FF137 or FF138 (Panels (iii) and (iv)). We measured the orange fluorescence (PE) for CD86-positive cells and the red fluorescence (APC) for MHCII-positive cells. Untransfected cells (double negative) were used as a negative control for DC maturation (Panel (i)). NDV-GFP-infected cells (double positive) were used as a positive

control for DC maturation (Panel (ii)): those cells were infected with a recombinant NDV expressing GFP for 18 hours.

Figure 2. Refinement of DC transfection conditions using a monocyte-specific buffer

MoDCs were transfected with a GFP-expressing plasmid in the monocyte-specific buffer provided by the manufacturer (Lonza Walkersville Inc.). Another seven nucleoporin programs were tested, in addition to FF137 and FF138. Cells were then transferred to DC growth medium, and incubated at 37°C and 5% CO₂ for 48 h. Transfection efficiency (or percentage of transfected cells) was defined as the percentage of GFP-positive cells, as described in the Results. The percentage of immature cells within the GFP-positive subpopulation was inferred from the percentage of cells expressing both CD86 and MHCII maturation markers concomitantly. Untransfected cells were used as a negative control for DC maturation (100% immature cells), and the data were normalized against it. Cells infected with a recombinant NDV that expresses GFP (NDV-GFP) were used as a positive control for GFP expression and DC maturation.

Figure 3. Effect of siRNA-mediated knockdown of RIG-I on the expression of RIG-I, IFNβ, and MxA in DCs

(A) MoDCs were transfected with either siRNA targeting human RIG-I or nonspecific siRNA (GLO negative control) in the monocyte-specific buffer in combination with nucleoporation program FF168 (Lonza Walkersville Inc.). Following a 24-h incubation at 37°C and 5% CO₂, transfected MoDCs were either infected with NDV (+) at a multiplicity of infection of 1, or left uninfected (-). Infected and uninfected cells were then incubated for another 10 h, and harvested for RNA extraction. Transcript levels of RIG-I, IFNβ, and MxA were determined by qRT-PCR. Data represent the results of two replicate experiments. (B) MoDCs obtained from a different buffy coat were transfected with either RIG-I siRNA or nonspecific GLO siRNA in the monocyte-specific buffer in combination with nucleoporation program FF168. Untransfected MoDCs were used as an additional control. Following a 24-h incubation at 37°C and 5% CO₂, all cells were infected with NDV at a multiplicity of infection of 1. Infected cells were then incubated for another 10 h, and harvested for both RNA extraction and protein extraction. Transcript levels of RIG-I, IFNβ, and MxA were determined by qRT-PCR. Data represent the results of two replicate experiments. (C) Western blot analysis was performed on lysates from the cells described in (B) to quantify the protein levels of RIG-I after siRNA knockdown: lanes 5 and 6, MoDCs transfected with RIG-I siRNA; lanes 3 and 4, MoDCs transfected with nonspecific GLO siRNA; lanes 1 and 2, untransfected MoDCs. Samples were analyzed in duplicates, probed for RIG-I and GAPDH (as a loading control).