Video Article

Optimized Protocol for Efficient Transfection of Dendritic Cells without Cell Maturation

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Abstract

Dendritic cells (DCs) can be considered sentinels of the immune system which play a critical role in its initiation and response to infection[1]. Detection of pathogenic antigen by naïve DCs is through pattern recognition receptors (PRRs) which are able to recognize specific conserved structures referred to as pathogen-associated molecular patterns (PAMPS). Detection of PAMPs by DCs triggers an intracellular signaling cascade resulting in their activation and transformation to mature DCs. This process is typically characterized by production of type 1 interferon along with other proinflammatory cytokines, upregulation of cell surface markers such as MHCII and CD86 and migration of the mature DC to draining lymph nodes, where interaction with T cells initiates the adaptive immune response[2,3]. Thus, DCs link the innate and adaptive immune systems.

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. It should also help facilitate the development of DC-based vaccines against infectious diseases and tumors. However, this line of research has been severely impeded by the difficulty of transfecting primary DCs[4].

Virus transduction methods, such as the lentiviral system, are typically used, but carry many limitations such as complexity and bio-hazardous risk (with the associated costs)[5,6,7,8]. Additionally, the delivery of viral gene products increases the immunogenicity of those transduced DCs [9,10,11,12]. Electroporation has been used with mixed results[13,14,15], but we are the first to report the use of a high-throughput transfection protocol and conclusively demonstrate its utility.

In this report we summarize an optimized commercial protocol for high-throughput transfection of human primary DCs, with limited cell toxicity and an absence of DC maturation[16]. Transfection efficiency (of GFP plasmid) and cell viability were more than 50% and 70% respectively. FACS analysis established the absence of increase in expression of the maturation markers CD86 and MHCII in transfected cells, while qRT-PCR demonstrated no upregulation of *IFNβ*. Using this electroporation protocol, we provide evidence for successful transfection of DCs with siRNA and effective knock down of targeted gene RIG-I, a key viral recognition receptor[16,17], at both the mRNA and protein levels.

Protocol

1. Program the Amaxa 96 well shuttle Nucleofector

1. Open a new parameter file.
2. Select the number of wells you will be using for standard transfection by dragging the cursor over the 96 well plate diagram. Use a minimum of 3 wells to pool for each experimental sample.
3. Input the program code: in part1 select 'FF' and in part2 select '168' from the pull down menus
4. From Solution box select 'Monocyte, human'
5. Under Control Option select 'standard'.
6. Click on Apply.
7. To include a no-transfection control, select further wells from the diagram as required and then choose 'No Program Control' from Control Option and click on Apply.
8. Select any remaining unused wells on the plate diagram and click on Undefine.

2. Prepare DCs for transfection

1. All work should be done under sterile conditions in a cell culture hood where possible. Prepare the nucleofection solution by adding 96-well supplement to Human Monocyte 96-well Nucleofector Solution in the ratio of 450 to 2025. Mix and allow to warm to room temperature. You will need 20µl of nucleofection solution per well. Make an excess of 10% to allow for pipetting error.
2. Place the number of nucleocuvette modules you need into the nucleocuvette plate in the correct orientation ie inserting the first one in to rows 1 and 2.
3. Determine the number of DCs needed for your experiment calculating on 500,000 cells per well and pellet by centrifuging at 400g for 10min. Carefully remove the supernatant.
4. Resuspend the cells in the nucleofection solution by gently pipetting up and down a few times.
5. Divide the correct volume of resuspended cells into eppendorfs labeled for the particular treatment eg GLO and RIG-I.
6. Add 0.25µg siRNA per 500,000 cells and mix by pipetting. Use non-targeting siRNA in your no-transfection control sample.
7. Pipette 20µl of the above mixtures into the nucleocuvette modules, according to your experimental layout, ensuring the liquid is delivered to the bottom of the well.

8. Cover the nucleocuvette plate with the lid and tap the plate on a hard surface a couple of times to help ensure removal of air bubbles.

3. Transfect DCs

1. Insert plate into the Nucleofector 96-well shuttle tray, click on Upload and then start.
2. Follow progress of transfection process on the lap top display; a black cross on a green background signifies a successful transfection in that well, whereas a black bar on a red background means it was unsuccessful.
3. On completion of the transfection process, remove the plate and add 80µl of DC growth medium to each well using a multichannel pipette.
4. Incubate plate for 10 min at 37°C and 5% CO_2.
5. Transfer the 100µl volume from the nucleocuvettes into matrix tubes containing 100µl of pre-warmed DC growth medium, maintaining the correct orientation.
6. Remove and discard those tubes where transfection did not occur.
7. Incubate at 37°C and 5% CO_2 for 24h or other desired time interval.

4. Infect cells with NDV

1. Remove matrix tubes from the incubator to the cell culture hood and pool tubes for each experimental sample into eppendorfs
2. Pellet the cells gently by spinning in a desk top centrifuge for 5 min and remove supernatant.
3. Resuspend the cells in serum-free growth medium containing NDV at an MOI of 1 and incubate at 37°C and 5% CO_2 for 45 min, with eppendorfs loosely covered in a sterile fashion.
4. Add 900µl of DC growth medium, and re-incubate for 8-10 h.

5. Harvest cells

1. Pellet cells by spinning eppendorfs in a desk top centrifuge and remove supernatant.
2. Harvest cells for RNA or protein extraction according to your protocol.

6. Representative results:

Using our optimized protocol we transfected DCs with *RIGI*-targeting siRNA for 24 h and then infected the cells with NDV (a paramyxovirus detected by RIG-I) to stimulate the interferon response pathway. By qRT-PCR analysis we demonstrated knock down of the gene by 75% at the transcription level. We also observed a similar reduction in the expression of *IFNβ*, which is a downstream effector of RIG-I in the IFN signaling cascade. Furthermore, we observed that the expression of *IFNβ* in non-infected, control-transfected cells was not detectable while that of *MxA*, an *IFNβ* downstream response gene, was minimal (Figure 1A).

A second transfection of DCs with *RIGI*-targeting siRNA was performed using enough cells to include Western blot analysis. Where the RT-PCR results were similar to what we saw previously (62% and 66% knock down of *RIG-I* and *IFNβ* expression respectively) (Figure 1B), the Western blot probed for RIG-I revealed that the expression of this gene had been completely blocked (Figure 1C).

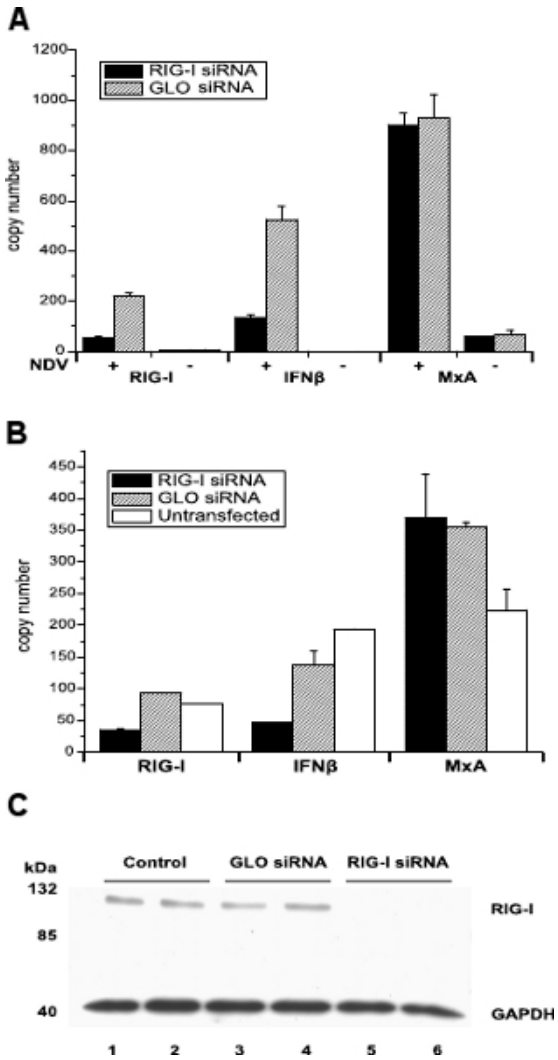

Figure 1. (A) MoDCs were transfected with either siRNA targeting RIG-I or nonspecific GLO siRNA and the effect on the expression of RIG-I, *IFNβ* and *MxA* determined by qRT-PCR. The transfection protocol used a monocyte-specific buffer and nucleoporation program FF168 (Lonza Walkersville Inc.) Following incubation for 24 h, transfected cells were either infected with NDV (+) or left uninfected (-). After a further incubation for 10 h, cells were harvested and RNA extracted Transcript levels represent the results of two replicate experiments. Taken from Bowles *et al* [16]. **(B)** MoDCs obtained from a different buffy coat as used for Figure 1A were again transfected with either RIG-I-targeting siRNA or nonspecific GLO siRNA using the same transfection protocol as above. An additional control using untransfected MoDCs was incorporated into the experiment. Following a 24 h incubation all cells were infected with NDV and incubated for a further 10 h before being harvested for both RNA and protein extraction. Transcript levels of RIG-I, *IFNβ* and *MxA* as determined by qRT-PCR represent the results of two replicate experiments. Taken from Bowles *et al* [16]. **(C)** Lysates from cells described in Figure 1B above were analyzed by Western blot. Lanes 1 and 2, lysates from untransfected cells; lanes 3 and 4, lysates from GLO siRNA transfected cells; lanes 5 and 6, lysates from cells transfected with RIG-I-targeting siRNA. Samples were probed for RIG-I and also GAPDH (as a loading control) and were run in duplicate. Taken from Bowles *et al* [16].

Discussion

Efficient transfection of naïve primary dendritic cells is important for high throughput analysis and reverse engineering of cellular inflammatory pathways in this key cell mediating the innate-adaptive immune transition. However, most investigators find that these cells are difficult to transfect both efficiently and without the transfection procedure inducing cell maturation when using standard transfection techniques. We investigated whether these limitations could be overcome by high throughput protocol optimization using a simultaneous, independent 96-well commercial nuclear transfection system (Lonza).

Using fluorescent activated cell sorting (FACS), we measured a GFP-expressing plasmid as a marker for transfection efficiency and CD86 and MHCII immunoreactivity as a readout of cell maturation. A series of experiments evaluating buffers and electroporation programs ultimately identified conditions showing >50% transfection and an absence of increase in maturation markers.

To further investigate possible activation of DCs by the nucleofection process we analyzed the expression levels of *IFNβ* and its downstream response gene *MxA* at the mRNA level. *MxA* expression is exquisitely sensitive to *IFNβ* production and can be used as a bioassay to detect very low levels of the cytokine18. Through qRT-PCR analysis we saw no detectable *IFNβ* expression but did see some upregulation of *MxA* thus indicating above-baseline *IFNβ* induction. However this minimal upregulation of *MxA* in non-infected, control-transfected cells was negligible compared to that resulting from virus-mediated stimulation of the IFNβ signaling cascade (Figure 1A) and confirmed that we could successfully use our nucleoporation protocol to transfect DCs without activating them to any significant level.

The utility of our optimized protocol was confirmed by Western blot analysis. Transfection of DCs with siRNA targeting RIG-I caused a complete loss of detectable RIG-I protein (Figure 1C). We should note that for successful perturbation of other genes, the amount of siRNA used and length of transfection time may have to be optimized.

To our knowledge, this work allows for the first time the design of high-throughput loss-of-function studies in primary human DCs, thus solving a difficult impediment to research in this field. This should provide new opportunities for the study of DC signaling and may contribute to advancement in developing DC-based immunotherapies.

Disclosures

No conflicts of interest declared.

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