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Technical note

A procedure for efficient non-viral siRNA transfection of primary human monocytes using nucleofection

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ABSTRACT

Monocytes are an important constituent of the innate immune system. Therefore, manipulating gene expression of primary human monocytes is a crucial mean to study and characterize the functions of targeted proteins in monocytes. Gene silencing by transfection of cells with small interfering RNA (siRNA) leading to the degradation of the corresponding mRNA and thus to reduced target protein levels is an important tool to investigate gene and protein function of interest. However, non-viral transfection of primary monocytes is challenging because siRNA uptake by these suspended cells is tricky, and the individual cells vary among different donors and do not proliferate. Here, we describe a procedure for efficient non-viral transfection of primary human monocytes isolated from peripheral blood, which maintains cell viability and cell functions, such as responsiveness to stimuli like LPS and IL-10. Nucleofection was used as an electroporation technique that enables efficient introduction of siRNA and silencing of target genes. Using a modification of our previously published protocol for the fast-proliferating THP-1 monocytic cell line, we transfected primary human monocytes with siRNA targeting 5-lipoxygenase (5-LO). In fact, we successfully downregulated 5-LO mRNA resulting in reduced protein levels and enzymatic activity.

1. Introduction

As a crucial component of innate immunity, monocytes are recruited from the blood stream into inflamed tissues, where they differentiate into macrophages. Monocytes/macrophages possess versatile functions in immune defense. They phagocyte pathogens and activate the adaptive immunity, thereby protecting the host. Monocytes/macrophages also promote wound healing or angiogenesis and are involved in the pathogenesis of several diseases by releasing proinflammatory mediators that can promote inflammation and even cancer development (De Palma et al., 2007; Shi and Pamer, 2011). Therefore, monocytes are relevant cells for investigation of their cell biology and regulation of cellular responses within immune defense and inflammation. 5-Lipoxygenase (5-LO) plays a pivotal role in inflammatory and allergic conditions and thus in diseases, such as asthma and cardiovascular disease, but also in several cancers (Peters-Golden and Henderson, 2007). 5-Lipoxygenase initiates the synthesis of leukotrienes (LTs) and other bioactive lipid mediators from arachidonic acid (AA) (Samuelsson et al., 1987). Leukotrienes are proinflammatory mediators. For example, LTB_4 promotes leukocyte chemotaxis, and cysteinyl LTs, such as LTC_4, LTD_4, and LTE_4, cause increased vascular permeability and bronchoconstriction. Among immune cells, including polymorphonuclear leukocytes (PMNL) and mast cells, LTs are formed mainly by monocytes and macrophages. 5-Lipoxygenase is a cytosolic protein, which, upon cell stimulation and intracellular mobilization of Ca^{2+}, translocates to the nuclear membrane. Arachidonic acid is released by the cytosolic phospholipase (cPL)A_2 from phospholipids at this locale where it is subjected to 5-LO for metabolism (Radmark et al., 2007).

Abbreviations: AA, arachidonic acid; 7-AAD, 7-amino actinomycin D; ALOX5, arachidonate 5-lipoxygenase gene; CCL3, chemokine (C-C motif) ligand 3; CCR7, chemokine (C-C motif) receptor 7; CD163, cluster of differentiation 163; FCS, fetal calf serum; 5-H(p)ETE, 5-hydroperoxyeicosatetraenoic acid; IL, interleukin; 5-LO, 5-lipoxygenase; LPS, lipopolysaccharide; LTB_4, leukotriene B_4; PBMC, peripheral blood mononuclear cells; PBS, phosphate-buffered saline; PE, phycoerythrin; PG, prostaglandin; RP-HPLC, reversed-phase high-performance liquid chromatography; RPL37A, ribosomal protein L37A; RT-qPCR, reverse transcriptase quantitative polymerase chain reaction; siRNA, small interfering RNA; SOCS3, suppressor of cytokine signaling 3; TNF, tumor necrosis factor.

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Gene silencing approaches require effective uptake of nucleic acids into the cell. Several methods have been published for the introduction of, e.g., plasmid DNA or siRNA into eukaryotic cells (Burke et al., 2002; Morille et al., 2008). However, monocytes are hard to transfect with commonly used methods (Schnoor et al., 2009). With our previously published protocol, we successfully established a method, based on electroporation, for the efficient transfection of THP-1 macrophages (Schnoor et al., 2009). The THP-1 monocytic leukemia cell line is widely used instead of primary human monocytes for gene knockdown experiments and for investigation of the impairment of the target protein as consequence for the cellular functionality (Preiss et al., 2007; Maess et al., 2014a,b). One advantage of such immortalized monocytic cell line is their fast-proliferating characteristics that allow convenient culturing and marked *de novo* protein synthesis in contrast to primary monocytes isolated from peripheral blood. However, regulation of genes in transformed monocytic cell lines in comparison to non-transformed primary human monocytes may differ (Kohro et al., 2004). Therefore, analysis of gene knockdown in primary monocytes is indispensable to study protein functions in the context of primary, non-transformed cells.

Monocytes/macrophages respond to stimulation with interleukin (IL)-10 or lipopolysaccharide (LPS) with upregulation of so-called alternative activation marker genes (suppressor of cytokine signaling 3 (SOCS3), cluster of differentiation 163 (CD163), IL-10) or classical activation marker genes (IL-1β, tumor necrosis factor (TNF)-α, chemokine (C-C motif) ligand 3 (CCL3), chemokine (C-C motif) receptor 7 (CCR7)), according to the M2 (alternative) and M1 (classical) macrophage polarization types (Ito et al., 1999; Mantovani et al., 2002). Therefore, responsiveness of monocytes after transfection to either LPS (classical activation stimulus) or IL-10 (alternative activation stimulus) is an important control to verify preservation of characteristics of cellular regulation. Here we present a procedure for the efficient non-viral transfection of primary human monocytes using a modified nucleofection procedure, which maintains cell viability and cell functions, such as responsiveness to LPS and IL-10.

2. Materials and methods

2.1. Materials

RPMI 1640 medium with L-glutamine, penicillin, streptomycin, and human serum were from PAA Laboratories (Pasching, Austria). Cell culture flasks and well plates were from Greiner bio-one (Frickenhausen, Germany). Mouse anti-5-LO monoclonal antibody was a kind gift by Dr. Dieter Steinhilber (Goethe University Frankfurt, Germany). Lipopolysaccharide (LPS) and all other chemicals were from Sigma-Aldrich (Steinheim, Germany), unless indicated otherwise.

2.2. Isolation of monocytes

Leukocyte concentrates were prepared from blood of healthy adult human donors who had not taken any anti-inflammatory medication for the last 10 days (Institute of Transfusion Medicine at the University Hospital Jena, Germany). Peripheral blood mononuclear cells (PBMC) were isolated by dextran sedimentation and centrifugation on lymphocyte separation medium (PAA Laboratories, Pasching, Austria). PBMC were washed with ice-cold phosphate-buffered saline (PBS) and plated (2×10^7 cells/ml) in culture flasks containing RPMI 1640 medium supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine, and 10% (v/v) fetal calf serum (FCS). After 1.5 h at 37 °C and 5% CO_2, non-adherent cells were removed by washing with PBS. For transfection, adherent monocytes were detached using Accutase I (PAA Laboratories, Pasching, Austria) for 15–30 min at 37 °C, 5% CO_2, and resuspended in 0.1 % (w/v) glucose in PBS.

2.3. Nucleofection

Nucleofection was performed using the Human Monocyte Nucleofector Kit (Lonza, Cologne, Germany) with an adoption of our previously described method (Schnoor et al., 2009). For transfection, detached monocytes (2.5×10^6 cells) were resuspended in 100 μl of Human Monocyte Nucleofector Solution (Lonza, Cologne, Germany) and transferred into a nucleofection cuvette. One microgram of siRNA was added and electroporation was performed in the Nucleofector 2b device (Lonza, Cologne, Germany) using the Y-001 program. Cells were then transferred into a fresh tube containing 500 μl of transfection medium (Lymphocyte Growth Medium (LGM)-3 (Lonza, Cologne, Germany) supplemented with 20% (v/v) human serum, 1% (v/v) non-essential amino acids, 2 mM L-glutamine, 1% (v/v) sodium pyruvate). Transfected cells in suspension were then transferred dropwise into two wells of a 12 well-plate containing 1.75 ml pre-warmed transfection medium. Within our previously published original protocol, the Amaxa Human Monocyte Nucleofector Medium (Lonza, Cologne, Germany) was used. Because of the unavailability of that culture medium, we accordingly confirmed suitability and used the LGM-3 medium. After 4 h, medium was removed, replaced by fresh transfection medium (see above, additionally containing 100 U/ml penicillin and 100 μg/ml streptomycin), and cells were incubated for the indicated times. 24 h after transfection, fresh transfection medium (containing 10% (v/v) human serum) was added and cells were cultured as indicated in the figures. The following siRNAs were used: on-target plus human ALOX5 siRNA SMARTpool, on-target plus non-targeting siRNA (Thermo Scientific Dharmacon, Lafayette, CO), Alexa 488 siRNA (Qiagen, Hilden, Germany).

2.4. Flow cytometry analysis

For flow cytometry analysis, cells were detached using Accutase I solution (PAA Laboratories, Pasching, Austria) for 15–30 min at 37 °C and 5% CO_2. After detachment, cells were centrifuged, resuspended and analyzed with an Attune Acoustic Focusing Cytometer (Life Technologies, Carlsbad, CA). For determination of cell viability and analysis of apoptosis or necrosis, cells were stained with phycoerythrin (PE)-labeled annexin V and 7-amino actinomycin D (7-AAD), respectively, using the Apoptosis Detection Kit I (BD Biosciences, Heidelberg, Germany) according to the manufacturer's instructions. For determination of transfection efficiency, Alexa Fluor 488 labeled non-targeting control siRNA (Qiagen, Hilden, Germany) was used.

2.5. Life cell imaging

For imaging of fluorescently labeled Alexa Fluor 488 siRNA uptake, monocytes (1.25×10^6 per dish) were plated after nucleofection with fluorescent siRNA into glass bottom dishes (MatTek Corporation, MA) containing pre-warmed transfection medium and incubated at 37 °C and 5% CO_2 atmosphere for 24 h. For microscopic imaging, monocytes were washed with pre-warmed PBS and imaging buffer (PBS containing 0.1% (w/v) glucose, 1 mM $CaCl_2$ and 1 mM $MgCl_2$) was added. Images were taken with an AxioCam MR3 camera (Carl Zeiss, Jena, Germany) and were acquired, cut, linearly adjusted in the overall brightness and contrast, and exported to TIF format using AxioVision 4.8 software. The microscope incubator (Axio Observer Z1 inverted microscope, LCl Plan-Neofluar 63x/1.3 Imm Corr DIC M27 objective, Carl Zeiss, Jena, Germany) was used at 37 °C and 5% CO_2.

2.6. Quantitative real-time RT-PCR (RT-qPCR)

After transfection and incubation of monocytes, culture medium was removed and total RNA from monocytes was isolated using the Nucleospin RNA XS Kit (Macherey-Nagel, Düren, Germany) according to the manufacturer's instructions. For cDNA synthesis, the Superscript