Journal of Immunological Methods 344 (2009) 109–115

Contents lists available at ScienceDirect

Journal of Immunological Methods

journal homepage: www.elsevier.com/locate/jim

Research paper

Efficient non-viral transfection of THP-1 cells

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ARTICLE INFO

Article history:
Received 5 February 2009
Accepted 26 March 2009
Available online 5 April 2009

Keywords:
Transfection
THP-1
Macrophages
Monocytes
Foam cell formation
TNF-α secretion

ABSTRACT

Macrophages are an important part of the cellular immune system and play a key role during immune responses. Thus, macrophages are interesting targets in basic and clinical research. Primary monocytes or monocyte-derived macrophages do not proliferate on a suitable scale so that their use for functional studies *in vitro* is limited. Immortal proliferating cell lines, such as the human THP-1 monocytic leukemia cell line, are therefore often used instead of primary cells. Transfection is a useful tool to study the function of gene products, but transfection of THP-1 monocytes and pre-differentiated THP-1 macrophages with subsequent differentiation into mature THP-1 macrophages using phorbol esters is usually accompanied by a progressive loss of cell viability. In this study, we describe a simple and rapid approach for efficient transfection of THP-1 monocytes and pre-differentiated THP-1 macrophages using a modified Nucleofection-based approach. The protocol maintains cell viability and functionality, thus allowing efficient transfection of THP-1 cells combined with subsequent differentiation of transfected THP-1 cells into mature macrophages.

1. Introduction

Macrophages, the phagocytic cells of the cellular immune system, play a key role in many disease processes (Lewis and McGee, 2003). Following a local stimulus, circulating blood monocytes emigrate from the blood stream into tissues where the cells differentiate into mature macrophages. Such macrophages interact with other cells of the immune system, neighboring tissue cells as well as epithelial cells that form a first barrier for invading pathogens (Schnoor and Parkos, 2008) in order to process and present antigens and to orchestrate the immune response (Ricardo et al., 2008). Additionally, they are responsible for removing necrotic tissue or invaded microorganisms, and contribute pivotally to wound healing (Lewis and McGee, 2003). Their central role in cell-mediated immunity and inflammation make macrophages an interesting target in basic and clinical research.

Pure human primary monocytes can be obtained from peripheral blood by dextran sedimentation followed by Ficoll density gradient centrifugation (Lee et al., 2006) or leukapharesis combined with counter-current elutriation (Schnoor et al., 2008). However, the amount of primary monocytes or monocyte-derived macrophages for functional studies is limited because the cells do not proliferate on a suitable scale (Lorkowski et al., 2001). Many *in vitro* studies therefore use immortalized proliferating cell lines, such as human THP-1 as well as murine J774A.1 and RAW 264.7, instead. The human THP-1 monocytic leukemia cell line is a well-established and

Abbreviations: ADRP, adipophilin, adipose differentiation-related protein; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GFP, green fluorescent protein; HKG, housekeeping gene(s); LPS, lipopolysaccharide; PAT, protein family comprised of perilipin, adipophilin and TIP47; PI, propidium iodide; PMA, phorbol 12-myristate 13-acetate; SRP14, 14 kDa signal recognition particle; TIP47, 47 kDa MPR tail-interacting protein, mannose 6-phosphate receptor-binding protein 1 (M6PRBP1); TUBA1, α1 tubulin; UBE2D2, ubiquitin-conjugating enzyme E2D 2.

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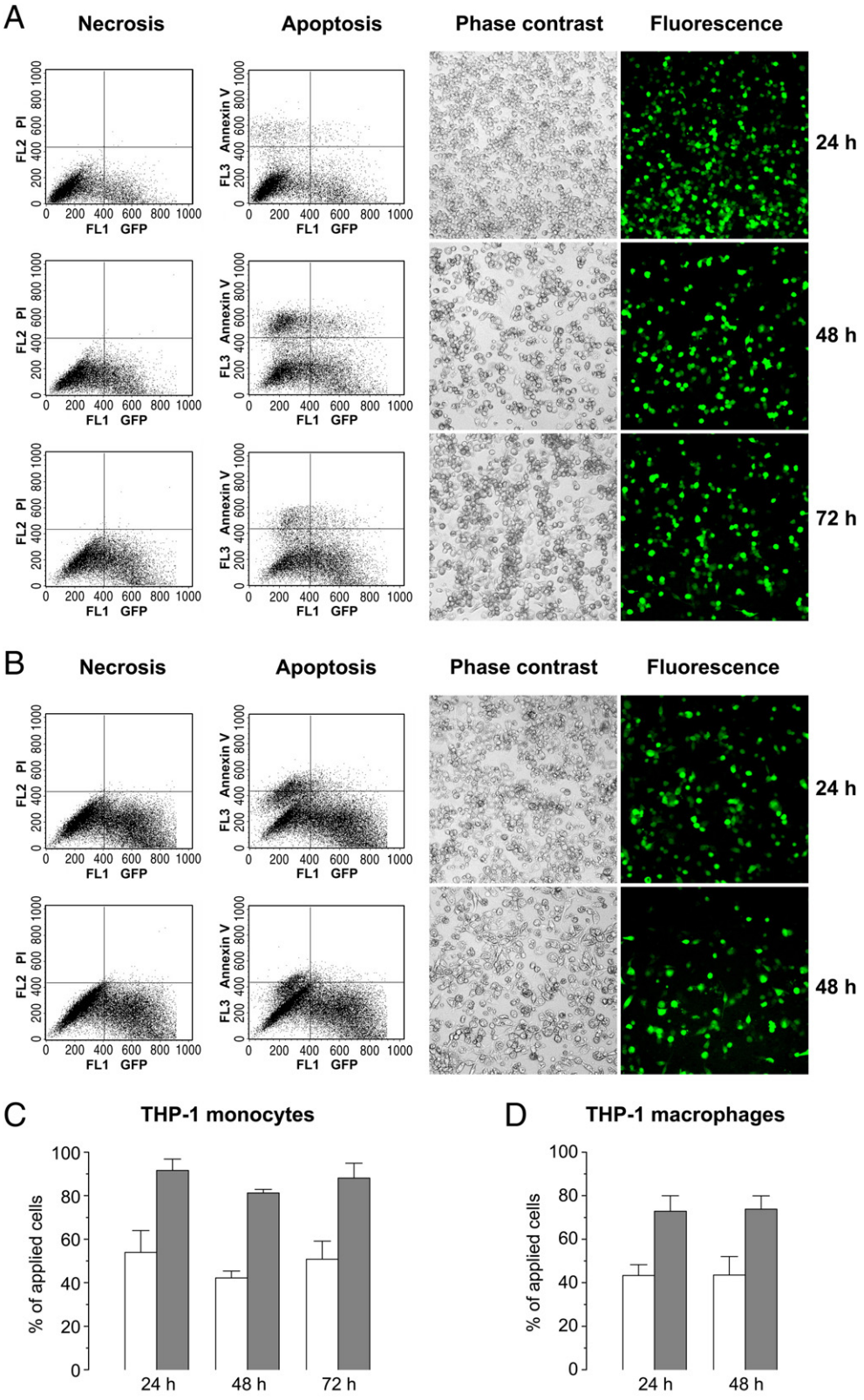
doi:10.1016/j.jim.2009.03.014


A
Necrosis
Apoptosis
Phase contrast
Fluorescence
FL2 PI
FL3 Annexin V
FL1 GFP
24 h
48 h
72 h
B
Necrosis
Apoptosis
Phase contrast
Fluorescence
24 h
48 h
C
THP-1 monocytes
% of applied cells
24 h
48 h
72 h
D
THP-1 macrophages
% of applied cells
24 h
48 h

widely used model for investigating the biological function of human monocytes and macrophages (Auwerx, 1991; Cassol et al., 2006; Preiss et al., 2007). Phorbol esters such as phorbol 12-myristate 13-acetate (PMA) allow differentiation of THP-1 monocytes into macrophage-like cells which mimic many of the characteristic features of human primary macrophages (Tsuchiya et al., 1982; Auwerx, 1991).

Transfection of expression vectors or small interfering RNAs is widely used to study the function of gene products and has been successfully applied to many cell models (Luo and Saltzman, 2000; Gresch et al., 2004). However, THP-1 cells are notoriously difficult to transfect. Viral transfection of THP-1 cells has been described (Lee et al., 2004), but this approach is time-consuming, requires specially equipped laboratories with high security levels and may cause various unknown side-effects (Thomas et al., 2003). Non-viral transfection of THP-1 monocytes has also been described (Martinet et al., 2003; Lee et al., 2005), but without maintaining viability and functionality during differentiation of transfected THP-1 cells into macrophages. Therefore, most studies using transfected THP-1 cells avoid maturation. Since characteristic macrophage functions can only be investigated in fully matured THP-1 macrophages, a non-viral transfection method maintaining viability is required. To solve the problems associated with the transfection of THP-1 cells, we chose the Nucleofector technology which has been successfully applied to many different cell types (Lakshmipathy et al., 2007; Han et al., 2008). This technology combines cell-specific electric pulses and reagents which allow transfection of eukaryotic cells at high efficiency and reproducibility due to a direct transfer of the vector into the nucleus (Martinet et al., 2003). However, the protocol for transfecting THP-1 cells recommended by the manufacturer works efficiently only with THP-1 monocytes without subsequent differentiation into macrophages. Phorbol ester-mediated differentiation of transfected THP-1 monocytes results in an almost complete loss of cell viability after 24 h. We therefore describe here a modified Nucleofection procedure which allows simple and efficient transfection of THP-1 monocytes as well as pre-differentiated THP-1 macrophages which can be then efficiently transformed into fully-mature macrophages.

2. Materials and methods

2.1. Cells

The THP-1 monocytic cell line was purchased from the American Tissue Culture Collection (ATCC; Rockville, MD). Cells were maintained in L-glutamine-containing RPMI 1640 (Sigma, Deisenhofen, Germany) supplemented with 10% fetal bovine serum (PAA, Cölbe, Germany), 1% non-essential amino acids, 1% sodium pyruvate and 0.1 mg/ml penicillin/streptomycin/L-glutamine (Sigma) as previously described (Stolle et al., 2007). Transfection was performed using THP-1 monocytes that were split 24 h prior to transfection or with THP-1 macrophages pre-differentiated for 48 h in supplemented RPMI 1640 containing 100 ng/ml PMA (Sigma) and 50 μM 2-mercaptoethanol (Sigma).

2.2. Nucleofection

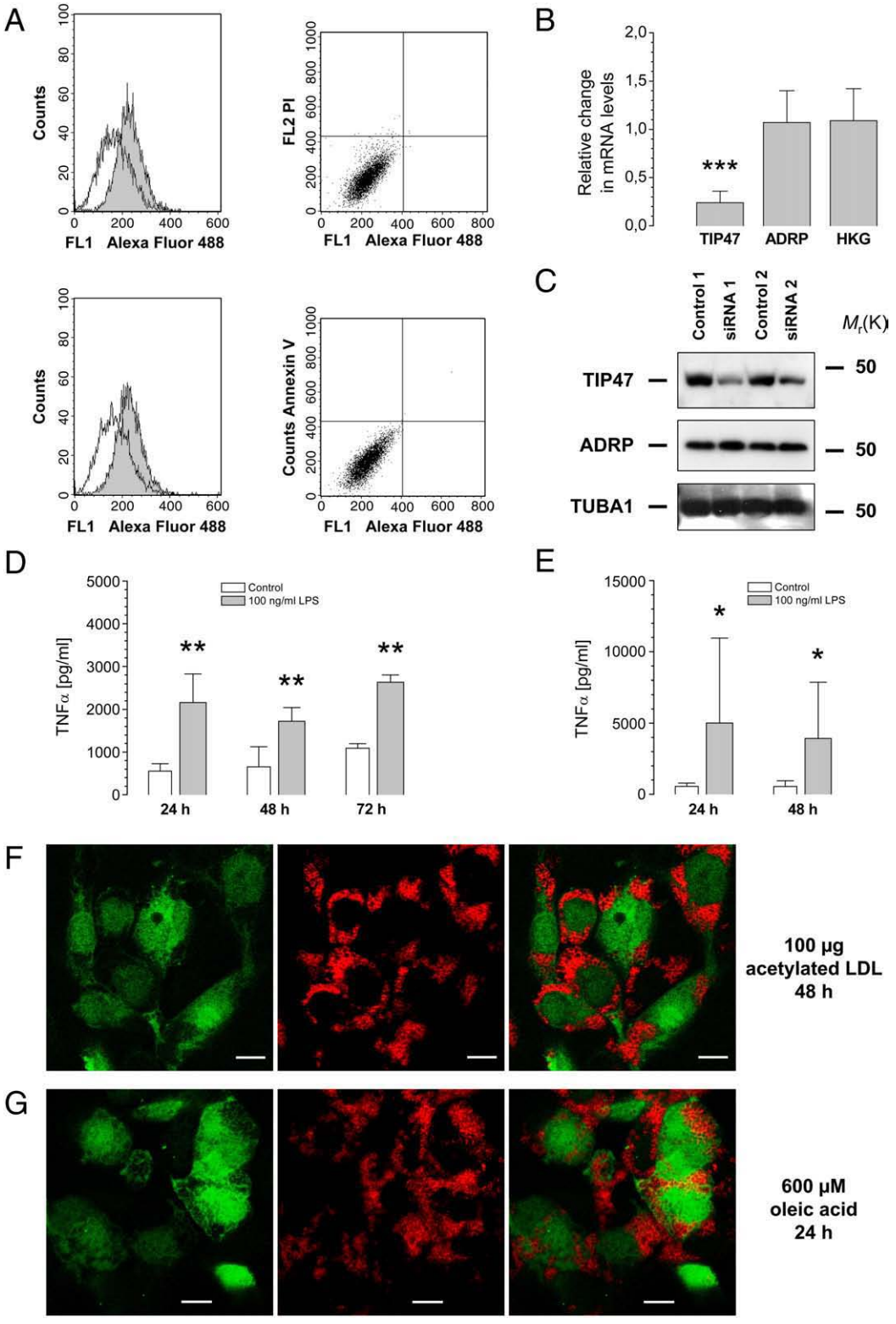
Transfection of THP-1 cells was performed using the Nucleofector II (Amaxa, Cologne, Germany). Compared to the protocol recommended by the manufacturer (Amaxa), we made the following changes: We used the Human Monocyte Nucleofector Kit, which was actually developed for the transfection of primary human monocytes, instead of the THP-1 Cell Line Nucleofector Kit V (Amaxa). Additionally, 20% autologous human serum (PAA) instead of 10% fetal bovine serum was used for supplementation of culture medium. Furthermore, the number of cells was adjusted to $2.5 \cdot 10^6$ cells per transfection cuvette, and the amount of pmaxGFP plasmid (Amaxa) was reduced to 0.5 μg per cuvette. For gene silencing, 1 μg of siRNA directed against TIP47 or scrambled siRNA (Qiagen, Hilden, Germany) were used. A further improvement was achieved by introducing a recovery phase of 4 h after transfection in Human Monocyte Nucleofector Medium (Amaxa) supplemented with 20% human serum, 1% non-essential amino acids, 1% sodium pyruvate and 0.1 mg/ml penicillin/streptomycin/L-glutamine at 37 °C and 5% carbon dioxide. Monocytes of a single transfection cuvette were transferred into two wells of a 24-well cell culture plate or into a single well of a 6-well plate (Biochrom, Berlin, Germany) containing 1.5 ml fresh Human Monocyte Nucleofector Medium (Amaxa) supplemented as described above and containing 100 ng/ml PMA and 50 μM 2-mercaptoethanol. For transfection of pre-matured macrophages, $1.5 \cdot 10^7$ THP-1 cells were seeded in a 75 cm^2 cell culture flask and treated with 100 ng/ml PMA and 50 μM 2-mercaptoethanol for 48 h. Cells were detached prior to transfection using Accutase I (PAA) for 10 min at 37 °C (Lee et al., 2007). Detachment efficiency was up to 100% and Accutase I treatment did not cause necrosis or apoptosis as determined by flow cytometry using propidium iodide and annexin V staining (data not shown). Use of Accutase is essential since THP-1 macrophages are resistant to trypsin treatment. Transfected cells of a single cuvette were split directly into two wells of a 24-well cell culture plate or into one well of a 6-well plate for subsequent adherence. Transfection medium was replaced after a recovery phase of 4 h by Human Monocyte Nucleofector Medium supplemented with 1% non-essential amino acids, 1% sodium pyruvate and 0.1 mg/ml penicillin/streptomycin/L-glutamine as well as 100 ng/ml PMA and 50 μM 2-mercaptoethanol to continue differentiation. Detailed step-by-step protocols for the transfection of THP-1 monocytes and pre-matured THP-1 macrophages are provided as online supplemental material.

2.3. Flow cytometry and microscopy

Transfection efficiency and cell viability were monitored by means of flow cytometry using a FACScalibur (Becton Dickinson, Heidelberg, Germany) as well as fluorescence and

Fig. 1. Transfection efficiency and cell viability of THP-1 cells after transfection with the GFP encoding expression vector pmaxGFP. Flow cytometric analyses and microscopic analyses of A) THP-1 monocytes and B) pre-differentiated THP-1 macrophages. Propidium iodide (PI) and annexin V staining show necrosis and apoptosis at indicated times after transfection. Phase contrast and fluorescence microscopy reveal normal cell morphology and GFP expression in THP-1 cells at indicated time points after transfection. Transfection efficiencies (white bars) and cell viability (grey bars) are displayed for THP-1 monocytes (C) and THP-1 macrophages (D). All values are means ± standard deviation of at least three independent experiments.

phase contrast microscopy using a Leica DMIRE2 microscope (Wetzlar, Germany). Transfection efficiency was quantified by flow-cytometric analysis of green fluorescent protein (GFP)-positive cells. Cell viability was determined by analyzing the number of necrotic and apoptotic cells by flow cytometry using propidium iodide and annexin V staining.



2.4. Real-time RT–PCR

Isolation of RNA, synthesis of cDNA and real-time RT–PCR using the ABI PRISM 7900HT Sequence Detection System (Applied Biosystems, Weiterstadt, Germany) and the Quanti-Tect SYBR Green PCR kit (Qiagen) were performed as previously described (Stolle et al., 2005). Primers were from Invitrogen (Karlsruhe, Germany) and were as follows: TIP47 forward, 5′-GGCCCTAAGCCTGATGGAAA-3′; TIP47 reverse, 5′-CTGGCC TTCCACCAGCTTCT-3′; adipophilin (ADRP) forward, 5′-CTG ATG AGT CCC ACT GTG CTG A-3′; ADRP reverse, 5′-TGT GGC ACG TGG TCT GGA G-3′; SRP14 forward, 5′-AGC ACTG TGG TGA GCT CCA AG-3′; SRP14 reverse, 5′-TCA GCC CAT CCA TGT TAG CTC TA-3′, GAPDH forward, 5′-CAA CAG CGA CAC CCA CTC CT-3′; GAPDH reverse, 5′-CAC CCT GTT GCT GTA GCC AAA-3′; UBE2D2 forward, 5′-CCA GAT GAT CCT TTA GTG CCT GAG-3′; UBE2D2 reverse, 5′-ACA TCG CAT ACT TCT GAG TCC ATT C-3′. Transcript levels of the lipid droplet-associated PAT protein family members TIP47 and ADRP were normalized to the expression of housekeeping genes glyceraldehyde-3-phosphate dehydrogenase (GAPDH), 14 kDa signal recognition particle (SRP14), or ubiquitin-conjugating enzyme E2D 2 (UBE2D2).

2.5. Foam cell formation and immunofluorescence staining

To induce foam cell formation, GFP-transfected THP-1 macrophages were loaded either with 100 μg/ml acetylated low-density lipoproteins (acLDL) for 48 h or with 600 μmol oleic acid complexed to 1 μmol bovine serum albumin for 24 h. Cells were then fixed in 4% paraformaldehyde, blocked and permeabilized in 1% BSA containing 0.05% Tween 20. After incubation with anti-adipophilin for 1 h, cells were washed three times, incubated with Alexa 568-conjugated species-specific secondary antibody for 1 h and washed again three times. After embedding in fluorescence mounting medium (DAKO Cytomation, Hamburg, Germany) foam cell formation of THP-1 macrophages was analyzed by using an Axiovert 200 M LSM510 confocal laser scanning microscope (Carl Zeiss, Jena, Germany).

2.6. Western blotting

Equal amounts of protein were separated by SDS–PAGE and transferred electrophoretically to polyvinylidene difluoride membranes (Millipore, Bedford, MA). Tubulin blots of cell lysates were performed to confirm cell viability and the authenticity of siRNA-mediated down-regulation of TIP47. After incubation in PBS containing 5% skim-milk for 1 h to block unspecific binding, the membranes were probed with anti-human TIP47 antibody (GP30; Progen Biotechnik, Heidelberg, Germany), anti-human adipophilin (AP125; Progen Biotechnik) or mouse anti-α-tubulin (Sigma) for 1 h at 37 °C, washed three times in PBS containing 0.5% skim-milk and 0.05% Tween® 20 for 10 min each, incubated with peroxidase-conjugated species-specific secondary antibody (1:10000; DAKO Cytomation) for 1 h at 37 °C, and treated with ECL (Amersham Pharmacia). Chemiluminescence signals were recorded on Hyperfilm X-ray films (Amersham Pharmacia).

2.7. ELISA

TNF-α release by transfected and differentiated THP-1 macrophages in response to 1 μg/ml lipopolysaccharide (LPS, Sigma) was analyzed using the TNF-α ELISA Kit (Biosource, Hamburg, Germany) according to the manufacturer's instructions.

3. Results and discussion

The Nucleofection protocol for the transfection of THP-1 cells, which is recommended by the manufacturer, works efficiently only with THP-1 monocytes if subsequent differentiation is avoided. All experiments performed in our lab according to the manufacturer's instructions resulted in an almost complete loss of cell viability after 24 h exposure to PMA (data not shown). We therefore modified the Nucleofection procedure as described in the Materials and methods section and below in order to maintain viability and functionality of transfected cells after PMA-mediated differentiation into macrophages.

Although use of the Human Monocyte Nucleofector Kit instead of the THP-1 Cell Line Nucleofector Kit V already increased the number of viable cells, the number of living cells was still insufficient for functional studies (data not shown). In search of conditions that increase cell viability, supplementation of the culture medium with 20% autologous human serum (PAA) instead of 10% fetal bovine serum proved best (data not shown). Reducing the number of cells to $2.5 \cdot 10^6$ cells per transfection and the amount of pmaxGFP plasmid (Amaxa) to 0.5 μg per transfection also increased the number of viable cells. Implementing the modified Nucleofection procedure as described in the Materials and Methods section improved significantly transfection efficiency and the number of viable cells.

Phase contrast pictures taken at the indicated time points after transfection revealed normal cell morphology (Fig. 1A and B). Particularly, no shrinkage or blebbing, indicating cell

Fig. 2. RNA interference in THP-1 cells and functional analysis of transfected THP-1 cells. A) Flow cytometric analyses revealed 100% transfection efficiency of THP-1 monocytes transfected with Alexa Fluor 488 labeled negative control siRNA for 24 h (grey) compared to control cells (white). No necrotic (top) or apoptotic cells (bottom) were observed. B) Real-time RT–PCR analysis showed specific down-regulation of TIP47 mRNA expression, whereas expression of adipophilin (ADRP) mRNA was not affected. Expression of TIP47 and adipophilin mRNA was normalized to housekeeping genes (HKG) SRP14, GAPDH or UBE2D2. C) Western blot analyses demonstrated specific down-regulation of TIP47 protein without changes in adipophilin or α1 tubulin (TUBA1) protein levels. Samples of two independent experiments are shown. Secreted TNF-α was measured at indicated time points by means of ELISA in transfected and differentiated THP-1 monocytes (D), or pre-differentiated THP-1 macrophages (E). The cells were either non-stimulated or stimulated with 100 ng/ml LPS in serum-free, PMA-containing medium for 24 h following the indicated periods of differentiation. Confocal microscopy of THP-1 foam cells incubated 24 h after transfection with 100 μg/ml acetylated low-density lipoproteins (LDL) (F) or 600 μmol oleic acid complexed to 1 μmol bovine serum albumin (G) for 24 h. Lipid droplet accumulation during foam cell formation was visualized using a monoclonal anti-adipophilin antibody (red). Expression of GFP is shown in green. Bars: 10 μm. Values are means ± standard deviation of at least three independent experiments. *$p<0.05$; **$p<0.01$; ***$p<0.001$.

death, were observed. Green fluorescent protein was expressed at various levels in different cells. In case of THP-1 monocytes cell viability was usually above 80% after transfection and transfection efficiency was more than 50%, whereas THP-1 macrophages showed a viability of around 75% and a transfection efficiency of more than 40% (Fig. 1C and D). Interestingly, levels of apoptosis and necrosis were low in GFP-positive cells whereas only GFP-negative cells showed noteworthy levels of apoptosis (scatter plots shown in Fig. 1A and B).

To demonstrate broad applicability of our procedure, RNA interference using siRNA duplexes was performed. As shown in Fig. 2A, transfection of THP-1 monocytes with Alexa Fluor 488 labeled negative control siRNA (Qiagen, Hilden, Germany) yielded transfection efficiencies of 100% with almost no necrosis or apoptosis (Fig. 2A). Comparable results were obtained using pre-differentiated THP-1 macrophages (data not shown). Additionally, we transfected THP-1 monocytes with siRNA directed against TIP47 (Qiagen), a member of the PAT protein family comprised of perilipin, adipophilin and TIP47, which are expressed in THP-1 cells and locate to cytosolic compartments as wells as to cytosolic lipid droplets (Robenek et al., 2005a).

Real-time RT–PCR analyses revealed that TIP47 mRNA levels were reduced by more than 75% compared to negative control siRNA treated samples (Fig. 2C). Transcript levels of adipophilin (ADRP), another PAT protein family member also locating to cytosolic lipid droplets (Robenek et al., 2005a), were not changed, demonstrating specificity of siRNA-mediated silencing of TIP47 expression. Western blots confirmed specific down-regulation of TIP47 protein levels, whereas expression of adipophilin protein was not affected (Fig. 2C and D). Expression of housekeeping genes and α1-tubulin confirmed cell viability and authenticity of TIP47 down-regulation (Fig. 2C and D).

Next, we turned to the question whether THP-1 cells transfected by our modified Nucleofection procedure still show macrophage-characteristic functions after PMA treatment. Mature macrophages secrete TNF-α upon classical activation by lipopolysaccharides (LPS) (Duffield, 2003). Therefore, TNF-α release by transfected and matured THP-1 macrophages in response to LPS (Sigma) was analyzed. LPS significantly induced TNF-α release in both types of transfected THP-1 macrophages (Fig. 2E and F) confirming that THP-1 macrophages can be classically activated after transfection. Of note, Nucleofection in the presence of DNA slightly increased basal secretion of TNF-α even in the absence of LPS. Recent findings by Muruve et al. that inflammasome activation occurs as a result of transfected cytosolic DNA may explain this observation (Muruve et al., 2008).

Foam cell formation is another functional characteristic of mature macrophages. Foam cell formation occurs in the arterial wall and contributes to the formation of atherosclerotic plaques which may rupture and cause myocardial infarction (Cullen et al., 2005). Macrophages take up atherogenic modified lipoproteins via scavenger receptors in a process that is not subjected to negative feedback regulation (Ross, 1993). The cells store the cholesterol contained within these lipoproteins as cholesteryl esters, and the fatty acids as triglycerides in cytosolic lipid droplets (Robenek et al., 2005b). To induce foam cell formation, transfected THP-1 macrophages were loaded either with acetylated LDL or oleic acid. Both, acetylated LDL and oleic acid, induced foam cell formation of transfected THP-1 macrophages expressing GFP as demonstrated by abundant staining of lipid droplet-associated adipophilin (Fig. 2F and G). This further proves functionality of the transfected mature THP-1 macrophages.

In summary, we present a Nucleofection-based approach for simple and efficient transfection of both, human THP-1 monocytes and THP-1 macrophages, yielding viable and functional monocytes or mature macrophages as required. Our protocol can be easily used to study various monocyte and macrophage-specific processes and functions by means of transgenic expression or gene silencing in a standardized cell culture model.

4. Competing interest statement

The authors declare no competing interests.

Acknowledgements

This work was supported by grants from the Deutsche Infarktforschungshilfe, the Ernest-Solvay-Stiftung and the Deutsche Forschungsgemeinschaft (SCHN 1155-1/1 to MS). We thank Renate Kwiotek and Thomas Böking for their excellent technical assistance.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jim.2009.03.014.

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