Small interfering RNA (siRNA) delivery into murine bone marrow-derived macrophages by electroporation

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Abstract

Selective gene silencing by RNA interference (RNAi) is a valuable tool for the targeted manipulation of the development and/or function of cells. Using a fluorescein-labeled non-silencing siRNA duplex, we established a protocol for the electroporation of primary mouse macrophages which routinely yielded >95% transfected cells. Electroporation of siRNAs directed against MAPK1 and CD86 led to an efficient knock-down of cellular protein in bone marrow-derived mouse macrophages (BM-Mϕ). Importantly, the electroporation procedure did not impair the viability of BM-Mϕ, their ability to ingest or degrade E. coli or their capacity to express iNOS mRNA, to produce NO or to upregulate TNF and IL-6 mRNA in response to inflammatory stimuli such as LPS. Therefore, we propose that electroporation of silencing siRNAs into murine BM-Mϕ is a highly efficient method to manipulate gene expression of BM-Mϕ that does not cause toxicity or a non-specific alteration of macrophage biology.

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1. Introduction

The term RNA interference (RNAi) describes a molecular mechanism where small RNA molecules inhibit the expression of a particular gene sharing a homologous sequence. Small interfering RNA (siRNA) molecules usually consist of 20–25 base pairs of double strand RNA and mimic intermediates of the naturally occurring microRNA pathway which was recently discovered to play an important role in mammalian gene regulation. The alteration of the mRNA amount via siRNA transfer is not mediated by inhibition of gene transcription, but by a post-transcriptional sequence-specific degradation of the transcribed and fully processed homologous mRNA. Virtually any gene with a known sequence can be silenced in a very specific and long lasting manner by the design of gene-specific siRNA. Therefore RNAi has been proven to be an elegant tool to study the cellular function of many genes (Tuschl and Borkhardt, 2002; Pei and Tuschl, 2006).

Macrophages play a pivotal role in host defence against various pathogens and are regulating a variety of immunological processes (Bogdan, 2006; Gordon, 2007). To study the immunobiology of macrophages researchers need to analyse primary macrophages rather than immortalized macrophage-like myeloid cell lines, because the phenotype of the latter is frequently very different from primary tissue-derived cells such as bone marrow-derived macrophages (BM-Mϕ). The use of RNAi may be of tremendous help to investigate the function of various genes in BM-Mϕ. To this end the gene-
specific artificial siRNA has to be introduced into the macrophages by some experimental maneuver, since RNA is anionic and hydrophilic and therefore unable to enter cells by passive diffusion. However, transfection of primary macrophages has proven to be difficult (reviewed in: (Zhang et al., 2009)). The transfection of monocytes and macrophages with DEAE-dextran and calcium phosphate was shown to be of limited efficiency (Mack et al., 1998). Macrophage-cell lines such as U937 and RAW264.7 have been successfully transformed by lipofection (Zhang et al., 2001) and related methods operate via crosslinking of macrophage surface receptors (Billiet et al., 2005). However, lipofection was shown to interfere with the biology of macrophages and therefore is not the best method to transfer siRNA into macrophages (Leon-Ponte et al., 2005). Efficient transfection of macrophages has been reported by methods that rely on physical disruption of the cell membranes. These include the transfer of nucleic acids by electroporation as established by Neumann and colleagues 1982 (Neumann et al., 1982) and the nucleoporation technique developed by AMAXA Biosystems, Germany. While electroporation resulted in good transfection efficiencies in the RAW264.7 macrophage-like cell line, electroporation was much more difficult in BM-MΦ (Stacey et al., 1993). In contrast, nucleofection allowed successful transfection of DNA or RNA into BM-MΦ (Weischenfeldt and Porse, 2008; Zhang et al., 2009).

Unfortunately, researchers using the AMAXA nucleofection technique are neither informed in detail about the preprogrammed specific electrical parameters of the AMAXA nucleofector® nor about the composition of the specific solutions designed for optimal transfection of the cells they use. Furthermore, to the best of our knowledge the effects of electroporation and of the transfer of siRNA on the biology of BM-MΦ have not been studied carefully. Therefore, we established a protocol for the efficient transfer of siRNA into BM-MΦ using a conventional electroporator and defined media and investigated in detail whether electroporation or the transfer of non-silencing-siRNA (ns-siRNA) interferes with the biology or viability of macrophages.

2. Materials and methods

2.1. Preparation of macrophages

BM-MΦ were generated from 6 to 9 weeks old female C57Bl/6 mice as described (Schleicher et al., 2005; Schleicher and Bogdan, 2009). Briefly, total BM cells were cultured in hydrophobic Teflon bags (DuPont, purchased via Cadillac Plastic, Karlsruhe, Germany) with 10% CO2/90% humidified air in Dulbecco’s modified Eagle’s medium (Invitrogen, Carlsbad, CA) supplemented with 0.05 mmol/L 2-ME. 1% nonessential amino acids, 10% fetal calf serum (Sigma-Aldrich, Deisenhofen, Germany), 5% horse serum (Cell Concepts, Umkirch, Germany), and 15% IS29 cell-conditioned medium. After 8 days of culture the cell population regularly contained more than 90% macrophages (CD11bhigh F4/80high).

2.2. siRNA duplexes

A non-silencing siRNA duplex (ns-siRNA), a fluorescein (FITC)-labeled ns-siRNA duplex (ns-FL-siRNA), and a silencing siRNA duplex targeted against the MAPK1 were obtained from Qiagen (Hilden, Germany). The ns-FL-siRNA duplex contained a modification of the sense oligonucleotide (3’-Fluorescein, 6-FAM[3Fl]). The ns-FL-siRNA duplex and the ns-siRNA duplex were directed against the following non-coding gene sequence AATTTCCGAACGTTCACGT (sense: UUCUCGGACGUCCGUCGdTdT, antisense: ACUGAC ACGUUCCGAGAAdTdT). In case of MAPK1 duplexes, the target sequence was AACTGACCTCCAGAACAAAG (sense: CUGGACUUCAGGAAAGAAdTdT, antisense: UGUUUCUGGAAUCCAGAdTdT). siRNA oligonucleotides directed against CD86 (NM_019388) were purchased from Pharmacia (Thermo Fisher Scientific, Epsom, United Kingdom) using Pharmacia’s pre-validated siRNA database: CD86 pre-validated siRNA (catalog L-058966). The duplexes were dissolved in siRNA suspension Buffer (Qiagen) to a final concentration of 0.3 µg/µL, heated for 1 min to 90 °C and incubated at 37 °C for 60 min. Dissolved duplexes were stored in aliquots at −70 °C.

2.3. siRNA transfer via electroporation

siRNA duplexes were transferred to a 4-mm cuvette (Molecular Bioproducts, purchased via VWR International, Germany) and filled up to a final volume of 50 µL with OptimEM (Invitrogen). 50 µL of the cell suspension (containing 2 × 106 BM-MΦ) were added and pulsed in a GenePulser Xcell (Bio-Rad, München, Germany). Pulse conditions were 400 V, 200 µF, and 100 Ω. After electroporation, cells were transferred to RPMI 1640 medium supplemented with 300 mg/L L-Glutamine and 2000 mg/L NaHCO3. 30 min later an equal amount of RPMI 1640 medium (with 300 mg/L L-Glutamine and 2000 mg/L NaHCO3) plus 20% FCS, 0.01 mol/L HEPES and 0.1 mmol/L 2-ME was added resulting in a final concentration of RPMI 1640 medium (with 300 mg/L L-Glutamine and 2000 mg/L NaHCO3) of 10% FCS, 0.005 mol/L HEPES and 0.05 mmol/L 2-ME.

2.4. Viability of BM-MΦ — MTT-assay

A modification of the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT, Sigma-Aldrich) reduction assay (Mosmann, 1983) was used. 0.5 × 106 macrophages were seeded in 150 µL in 96 well-flat bottom plates and were treated as indicated. 24 h after stimulation the cells were pulsed with 10 µL of 10 mg/mL MTT reagent for 4 h at 37 °C, followed by the addition of 100 µL 10% (w/w) SDS/0.01 N HCl over night. After this period, microtiter plates were read at 550 nm in an ELISA plate reader to measure formazan production. The results are expressed as absolute optical density (O.D.) readings.

2.5. Viability of BM-MΦ — trypan blue staining

The viability of the untreated and electroporated cells was determined by a trypan blue exclusion assay. 1 × 106 macrophages were seeded into a 96 well-flat-bottomed plate. 24 h later the culture supernatant was removed and 0.04% (w/v) trypan blue in PBS was added to the wells and the percentage of trypan blue-positive cells was determined immediately.

2.6. Viability of BM-Mϕ — Diff-Quick stain

Either mock or ns-siRNA electroporated macrophages or untreated macrophages were seeded into eight-well LabTek® Permanox Chambers (Nunc, Naperville, IL) at 2 × 10⁵ macrophages/well. 24 h later the cells were stimulated or left untreated. 24 h after the indicated stimulus the cells were stained with Diff-Quick® (Dade Behring, Marburg, Germany).

2.7. Analysis of the cell surface phenotype

For surface phenotyping the following fluorochrome (FITC-, PE-, or APC-) -labeled antibodies were used (all from BD Biosciences, Heidelberg, Germany unless otherwise stated): anti-CD16/18 (2.4G2); anti-CD11b (M1/70), anti-F4/80 (CI:A3-1; Serotec; Düsseldorf, Germany), anti-CD86 (GL1). The specificity of the stainings was verified by the use of isotype control mAbs. All analyses were performed on a FACSCalibur (BD Biosciences).

2.8. Bacterial infection of BM-Mϕ

BM-Mϕ were infected with E. coli bacteria (HB101 strain) grown to stationary phase. The multiplicity of infection (MOI) was adjusted by reading the culture density at the optical density of 600 nm. The actual MOI of each experiment was determined by plating dilutions of the inoculum onto agar plates for the determination of the number of colony-forming units (CFU). For synchronization of infection, the culture plates were centrifuged at 500 × g for 5 min. BM-Mϕ were infected (MOI 10) for 60 min at 37 °C and 5% CO₂. After infection, cells were washed twice with PBS to remove non-internalized bacteria. To kill residual extracellular bacteria gentamicin was added at 100 µg × ml⁻¹ for 1 h, followed by 25 µg × ml⁻¹ gentamicin for the rest of the experiment. After cellular lysis using 0.1% Triton X-100 in PBS the number of viable intracellular bacteria was determined by serial dilution in 0.05% Tween 80 in PBS and subsequent plating on Mueller-Hinton agar plate to enumerate CFU. Relative survival is given as the ratio of CFU at 24 h and 2 h after infection.

2.9. RNA isolation, reverse transcription, real-time PCR and relative quantification

Total RNA from cell culture experiments was extracted with Trizol (Invitrogen) according to the manufacturer's instruction and analyzed by real-time RT-PCR. 1–2 µg of total RNA was reverse transcribed using the High Capacity cDNA Archive kit (Applied Biosystems, Foster City, CA). Real-time PCR was performed on an ABI Prism 7900 sequence detector (Applied Biosystems) using Taqman Universal Mastermix and Assays-on-Demand (Applied Biosystems), which include pre-validated primers were purchased from Qiagen. Specificity and length of PCR products were verified by melting point analyses.

2.10. Immunoblotting

At indicated time-points macrophage monolayers were lysed using a PE-lysis buffer (6.65 M Urea, 1% SDS, Tris [tris(hydroxymethyl)aminomethane] HCl, pH 6.8, 5 mM DTT) in the presence of a protease inhibitor cocktail (Roche Diagnostics, Mannheim). Lysates were diluted with SDS-PAGE sample buffer. 60 µg of protein was separated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis and transferred onto polyvinylidene difluoride membrane (Millipore, Schwabach, Germany). MAPK1/ MAPK3 was detected using an ERK1/2 (MAPK3/MAPK1) specific antibody (Cell Signaling Technology, via New England Biolabs, Frankfurt a.M., Germany), Hsp90 by using an HSP90-specific antibody from Santa Cruz Biotechnology (Santa Cruz, CA). In both cases bound antibodies were visualized by ECL technology.

3. Results

3.1. BM-Mϕ are efficiently electroporated with siRNA

We previously established that BM-DC can be highly efficiently transfected with siRNA using an electroporation technique (Jantsch et al., 2008). Applying this protocol to BM-Mϕ, we were able to achieve a transfection efficiency of routinely >95%, when 2 × 10⁶ BM-Mϕ were electroporated in 100 µL Opti-MEM with 6 µg ns-FL-siRNA using pulse conditions of 400 V, 150 µF, and 100 Ω (Fig. 1A and B).

3.2. Electroporation with siRNA does not affect the viability of BM-Mϕ

To test whether electroporation and transfer of siRNA affect the viability of BM-Mϕ, we performed MTT assays as well as trypan blue exclusion assays 24 h after electroporation. We did not observe any significant differences in the cell survival, when untreated BM-Mϕ were compared with either mock or ns-siRNA electroporated BM-Mϕ. We measured an increase in the formazan production after stimulation with LPS compared to unstimulated controls (Fig. 2A), irrespective whether the cells were electroporated or not. In accordance with these results we also observed no toxicity of mock or ns-siRNA electroporation on BM-Mϕ in trypan blue exclusion assays. Again, we noted slightly improved cell viability in cultures treated with LPS compared to untreated BM-Mϕ.

Fig. 1. Efficiency of transfection of BM-Mø by electroporation using fluorescein-labeled ns-siRNA duplex. (A) 6 µg ns-FL siRNA were electroporated into 2 × 10^6 BM-Mø with 400 V, 150 µF and 100 Ω in a total volume of 100 µL Opti-MEM. (B) 2 × 10^6 BM-Mø were left untreated, mock electroporated or electroporated with 6 µg ns-FL-siRNA with 400 V, 150 µF and 100 Ω in a total volume of 100 µL Opti-MEM. The mean percentages + SD of fluorescein-positive cells after the indicated treatment for four experiments are shown.

Fig. 2. Electroporation and transfer of ns-siRNA do not affect the viability of BM-Mø. (A) Electroporation of BM-Mø does not affect viability. BM-Mø were left untreated, electroporated with 400 V, 150 µF and 100 Ω with no siRNA (mock) or 6 µg ns-siRNA. 24 h later BM-Mø were stimulated +/− 10 ng/mL LPS for 24 h and MTT-assay was performed. Formazan production was measured as described in Materials and Methods using a ELISA reader at 550 nm. Mean + SD of three independent experiments are given. (B) Electroporation does not cause toxicity of BM-Mø. BM-Mø were treated as described in A. To assess viability trypan blue exclusion assays were performed. The percentage of dead cells was calculated by dividing trypan blue cells with all cells counted. Means + SD of three independent experiments are given. (C) Electroporation of BM-Mø does not affect the morphology of BM-Mø. BM-Mø were treated as described in A. 24 h thereafter BM-Mø were stimulated in the presence or absence of 10 ng/mL LPS for additional 24 h and Diff-Quick stain was performed. Representative images out of three independent experiments are shown.

independent of the electroporation procedure (Fig. 2B). In order to see, whether electroporation might alter the BM-Mφ morphology, we performed Diff-Quick-staining and analyzed the morphology of LPS-stimulated or unstimulated BM-Mφ, which were either mock or ns-siRNA electroporated or left untreated prior to stimulation. In these experiments the morphology of BM-Mφ after electroporation was indistinguishable from that of untreated controls (Fig. 2C). We conclude that neither electroporation nor the presence of siRNA molecules affect the viability of BM-Mφ.

3.3. Electroporation neither activates BM-Mφ nor does it impede LPS-induced activation

To investigate whether electroporation and treatment with siRNA activate BM-Mφ or modulate the capacity of BM-Mφ to express inducible nitric oxide synthase (iNOS), to generate NO or to upregulate proinflammatory cytokine mRNA, we stimulated untreated, mock electroporated, or ns-siRNA electroporated BM-Mφ with 10 ng/mL LPS or left the cells unstimulated. We did not detect any effect of mock electroporation or ns-siRNA electroporation on the ability of BM-Mφ to produce NO (Fig. 3A) or express iNOS mRNA (Fig. 3B) in response to LPS. Furthermore, in the absence of LPS neither mock electroporation nor electroporation with ns-siRNA induced the expression of iNOS or the production of NO by BM-Mφ (Fig. 3A and B). Analysis of the mRNA expression levels of two proinflammatory cytokines (IL-6 and TNF) revealed no influence of either mock electroporation or ns-siRNA electroporation on the ability of BM-Mφ to express TNF (Fig. 3C) or IL-6 (Fig. 3D) in response to LPS at three different time-points of stimulation. Electroporation alone failed to induce TNF or IL-6 mRNA (Fig. 3C and D). From these data we conclude that electroporation and transfer of siRNA neither activate BM-Mφ nor impair the LPS responsiveness of these cells.

3.5. Electroporation of BM-Mφ does not alter the rate of bacterial uptake or the antibacterial capacity

To investigate whether siRNA electroporation influences the ability of BM-Mφ to kill pathogens, we infected mock electroporated, ns-siRNA electroporated and untreated BM-Mφ with E.coli (MOI of 10) and performed gentamicin-protection assays. We did not observe a significant difference in the rate of bacterial uptake when comparing mock or ns-siRNA electroporated BM-Mφ with non-electroporated cells (Fig. 4A). Likewise, when analyzing the antibacterial capacity of untreated, mock electroporated or ns-siRNA electroporated BM-Mφ, we observed no inhibitory effect of electroporation on the capacity of BM-Mφ to kill E. coli (Fig. 4B). Thus, both the phagocytosis and the killing of E. coli remain unaffected by the electroporation procedure.

3.6. Delivery of siRNA duplexes into BM-Mφ results in effective gene silencing

To test whether the electroporation of siRNA into BM-Mφ is indeed able to silence genes in BM-Mφ, we performed experiments with a pre-designed duplex against the MAPK1.

![Fig. 3](image-url)
BM-Mφ were electroporated with 6 µg of siRNA and incubated for 24 h. After additional 24 h total RNA was isolated, reverse transcribed, and a real-time RT-PCR was performed for MAPK1. The expression of the gene encoding the HPRT served as an internal control. The normalized MAPK1 mRNA/HPRT ratio of untreated cells was set to 1 and compared to cells that were electroporated without siRNA (mock), with ns-siRNA or a siRNA duplex directed against MAPK1. Mock electroporation of BM-Mφ or electroporation of ns-siRNA did not alter MAPK1 mRNA levels as compared to untreated controls. In contrast, electroporation of MAPK1 siRNA duplexes reduced the expression of MAPK1 mRNA level by approximately 70% (Fig. 5A). To investigate the effects of MAPK1 silencing on protein level, cellular lysates were prepared 4 days after siRNA electroporation and subjected to immunoblotting using an ERK-specific antibody recognizing MAPK3 (ERK1) and MAPK1 (ERK2) in their un-phosphorylated state. BM-Mφ treated with MAPK1-specific siRNA inhibited only the expression of MAPK1, whereas MAPK3 remained unaffected (Fig. 5B). In addition, we transferred siRNA directed against the costimulatory molecule CD86 via electroporation. 24 h later the cells were stimulated with LPS for one day or left untreated. The expression of CD86 on the surface of BM-Mφ was analyzed by flow-cytometry. When the CD86 specific siRNA was used, BM-Mφ did not upregulate CD86, whereas the LPS-induced upregulation of CD86 remained intact after mock electroporation or electroporation with ns-siRNA (Fig. 5C and D). Thus, transfer of specific siRNA, but not the electroporation procedure alone interferes with the biology of macrophages.

4. Discussion

Transfection of primary immune cells with siRNA has become an important tool to define the role of single genes for the development, differentiation and function of these cells, but is frequently hampered by a lack of efficiency. Here, we describe for the first time a highly efficient electroporation protocol for the delivery of siRNA into BM-Mφ. We carefully tested whether this method alters the immunobiology of BM-Mφ in order to provide a solid basis for its specificity. Our data clearly show that neither the electroporation procedure nor the electroporation of ns-siRNA affected the function and phenotype of BM-Mφ. In contrast, the highly efficient transfer of specific siRNA duplexes into BM-Mφ led to a robust knock down of cellular proteins such as MAPK1 and CD86 protein. Therefore, we propose electroporation as a suitable method to transfer siRNA into BM-Mφ in order to manipulate gene expression in these cells.

Previous studies suggested that myeloid cells which are known to be difficult to transfect can be successfully targeted by electroporation. siRNA was effectively transferred into human blood monocyte-derived DC via electroporation and no detrimental effects on the biology of these cells were observed (Prechtel et al., 2006). Likewise, we could demonstrate that electroporation can also be used for efficient transfection of mouse BM-DC with siRNA, whereas lipofection appeared to be toxic (Jantsch et al., 2008). In line with these results van Tendeloo et al. showed that lipofection alters the function of hematopoietic cells and is inferior to electroporation (Van Tendeloo et al., 2001). Further, lipofection interfered with various biological responses of macrophages (Leon-Ponte et al., 2005) essentially questioning the use of this method for the transfection of macrophages.

In the light of these prior results we were particularly interested to establish a protocol which relied on electroporation as a mean to transfer siRNA into BM-Mφ. Our data illustrate that the very same protocol used for a highly efficient transfer of siRNA into BM-Mφ is also suitable for transferring siRNA in BM-Mφ. This is likely due to the fact that both cell types are derived from the same myeloid precursors and thereby may display a similar composition of biophysical properties thus allowing a successful transfection with identical electroporation parameters. Since identical parameters for electroporation of BM-DC and BM-Mφ can be applied, a direct comparison of the function of various genes in BM-DC and BM-Mφ differentiated from the same bone marrow precursors is now possible. This may encourage future comparative functional genomic analyses of BM-DC and BM-Mφ.

Another strategy to transfer siRNA into mammalian cells is to use viral vector-mediated expression of short hairpin RNA (shRNA). In such a system the shRNA hairpin structure is cleaved by the cellular machinery into siRNA molecules (Paddison et al., 2002). This approach is particularly advantageous when cells are transduced that are hard to transfect (Arts et al., 2003; Rubinson et al., 2003). A protocol for the successful
transfer of shRNA constructs using a lentiviral vector has been published (Lee and Reiner, 2009). Nevertheless, it has been reported that viral-mediated shRNA expression and in consequence silencing of targeted genes may be quite variable (Sachse and Echeverri, 2004). Furthermore, electroporation of specific siRNA duplexes may be less time-consuming, since retroviral transduction of shRNA requires cloning of the viral constructs and the production of the virus.

Since several reports describe nucleic acids from pathogens as potential immunostimulatory agents that interact with Toll-like receptors (TLR) or cytosolic pattern recognition receptors (e.g. RIG-I or MDA-5 helicases), we wanted to carefully investigate potential immunostimulatory side effects of siRNA transfection into macrophages. Cella et al. described that long double-stranded RNA representing an intermediate during replication of some viruses can induce chemokine secretion in DC (Cella et al., 1999). TLR3 (Alexopoulou et al., 2001), the serine-threonine protein kinase R (PKR) (Meurs et al., 1992) and cytoplasmatic helicase proteins like RIG-I (Yoneyama et al., 2004) and MDA-5 (Andrejeva et al., 2004) (Kato et al., 2006) have been shown to detect long double-stranded RNA (>30 nucleotides) in the cytoplasm and to mount proinflammatory responses. The capacity of RNA to induce inflammatory responses depends on its sequence as demonstrated by several groups. In plasmacytoid DC a sequence-specific TLR 7-dependent...
recognition of single-stranded RNA of nine bases (5′-GUCCUUCUCAA-3′) can elicit inflammatory effects (Hornung et al., 2005). These RNA molecules were termed siRNA (immunostimulatory siRNA). A sequence-dependent activation by siRNA was also observed in human DC and other immune cells (Sioud, 2005; Judge et al., 2005). In contrast to these results it has been reported that a 21-nucleotide long siRNA molecule activated TLR3 and suppressed angiogenesis in a sequence and target-non-specific manner (Kleiman et al., 2008). In the present study, however, neither electroporation alone nor the delivery of siRNA molecules induced any activation of BM-Mϕ supporting the notion that siRNA being shorter than 30 bp and lacking a pathogen-derived sequence or structure (e.g. a 5′-triphosphate end generated by viral polymerases allowing recognition via RIG-I (Hornung et al., 2006)) is not inducing an innate immune response.

siRNA exists in macrophages as a mechanism of post-transcriptional regulation of gene expression encoded by so-called micro RNAs (Androulidaki et al., 2009). Our data clearly indicate that RNAi can be used to silence genes of interest by introducing specific artificial siRNA molecules representing intermediates of the natural occurring miRNA pathway without mounting an innate immune response. Thus, siRNA knock down by electroporation is feasible in BM-Mϕ to investigate the function of various genes and may help to unravel the immunobiology of macrophages.

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