Multifaceted role of the complement system in health and disease: a focus on properdin

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Culture medium used during small interfering RNA (siRNA) transfection determines the maturation status of dendritic cells

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Abstract

Gene silencing using small interfering ribonucleic acids (siRNA) is a powerful method to interfere with gene expression, allowing for the functional exploration of specific genes. siRNA interference can be applied in both cell lines, as well as in primary, non-dividing cell types like dendritic cells. However, the efficacy in different cell types is variable and requires optimization. Here, we showed that the type of culture medium used during lipid-based siRNA-mediated transfection acts as a critical factor, affecting dendritic cell activation. Transfection of immature monocyte-derived dendritic cells in RPMI medium, but not in IMDM, showed increased transcript levels of pro-inflammatory cytokines. Moreover, the expression of co-stimulatory molecules was enhanced, thereby increasing the T cell stimulatory capacity. Our data demonstrates that the choice of medium should be critically examined as one of the variables while optimizing cell transfection.

Introduction

Dendritic cells (DCs) are professional antigen-presenting cells playing an important role in orchestrating adaptive immune responses (1,2). Immature DCs are responsible for the capture, processing and subsequent presentation of antigens in major histocompatibility complexes (MHC) (2). DC maturation can be induced by pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs), via pattern recognition molecules (PRRs) expressed by DCs (3). As part of the PRRs, toll-like receptors (TLRs) are for example able to recognize lipopolysaccharide (LPS, via TLR4), double-stranded RNA (via TLR3) and single-stranded viral RNA (TLR7) (3). TLR ligation on DCs results in upregulation of co-stimulatory molecules CD80, CD86 and CD40 and an increase in cytokine production, including IL-12, converting DCs into the most potent activators of T cells (1–4).

DC biology and function are commonly studied by making use of monocytes as progenitor cell. Monocyte-derived DCs (MoDCs) are generated in vitro by the culture of monocytes, isolated from peripheral blood mononuclear cells (PBMCs), in the presence of granulocyte-macrophage colony-stimulating factor (GM-CSF) and interleukin 4 (IL-4) (5). For the culture and functional analysis of MoDCs, the use of several standard media has been described, including Roswell Park Memorial Institute (RPMI) and Iscove's Modified Dulbecco's Media (IMDM). It has previously been described that the choice of medium used during differentiation affects phenotypical and functional characteristics, including the expression of CD1a and the production of IL-12 (6), underlining the importance of medium during MoDCs culture (6,7).

Downregulation of genes is a powerful way to investigate functional involvement in various processes, including MoDC differentiation and T cell priming. Molecular tools like small-interfering ribonucleic acids (siRNAs) are often used to reduce gene-, and subsequent protein-expression (8). Since immature MoDCs (iDCs) are sensitive for activation by DAMPs and PAMPs, it is plausible that cells can also be sensitive for manipulation by siRNAs (9,10). For this reason, efforts have been put in optimizing the methodology for increasing transfection efficiency of MoDCs without inducing cell maturation (11,12). However, the role of the culture medium used during transfection was not investigated. Therefore, we investigated the effect of RPMI and IMDM during siRNA transfection on the activation status of iDCs. Following transfection, we analyzed the mRNA levels of *IL6* and *IL12B* and the surface expression of maturation markers CD80, CD83, CD86 and HLA-DR on MoDCs. Finally, we assessed whether these changes led to alterations in T cell priming.

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Materials and methods

Monocyte isolation and monocyte-derived dendritic cells (MoDC) generation
Peripheral blood mononuclear cells (PBMCs) were isolated from healthy blood donors (Sanquin, Amsterdam, The Netherlands) using Ficoll density gradient centrifugation (Pharmacy, LUMC, Leiden, The Netherlands). Monocytes were isolated by positive magnetic cell sorting using anti-CD14 MACS microbeads (130-050-201, Miltenyi Biotec, Leiden, The Netherlands), cells were seeded (1.5×10^6 per well, 6 wells plate (Costar, corning Incorporated, New York, NY,USA)) and supplemented by 20 ng/mL IL-4 (PHC0041, Invitrogen, Carlsbad, CA, USA) and 10 ng/mL GM-CSF (Premium grade, 130-093-868, Miltenyi Biotec) in medium (RPMI 1640 (524000-025, Gibco/Life technologies, Breda, The Netherlands) +10% fetal calf serum (FCS; Bondico, Alkmaar, The Netherlands), 90 U/mL penicillin and 90 µg/mL streptomycin (Gibco/Life technologies)). At day 3 of the culture, cells were supplemented with fresh medium containing cytokines. Cells were harvested at day 5 and differentiation was determined by flow cytometry (LSR-II, BD Biosciences, Vianen, The Netherlands) using mouse anti-human CD14 (APC, 1:200, Clone MφP9, cat no. 345787, BD), mouse anti-human DC-SIGN (CD209, PE, 1:50, Clone 9E9A8, cat no. 330106, Biolegend, San Diego, CA, USA) and mouse anti-human CD1a (FITC, 1:50, Clone HI149 (RUO), cat no. 555806, BD Pharmingen) antibodies. MoDCs which were CD1a+DC-SIGN+ CD14- were considered differentiated correctly (5,13). Results were analyzed using FlowJo Software version 10 (Tree Star, Ashland, OR, USA).

Transfection of MoDCs
The protocol for transfection of suspension cells (Dharmacon, Lafayette, CO, USA) was optimized. Transfection complexes were generated in 96 wells plates (flat bottom) by the separate preparation of well 1 and well 2; Well 1 contained 20 µL 5 µM control siRNA (siGENOME Non-Targeting siRNA Pool #2, D-001206-14-20, Dharmacon) in the presence of 80 µL plain medium, either RPMI or IMDM (12440-053, Gibco/Life technologies). Well 2 contained 1.25 µL Dharmafect 1 (T2001-01, Dharmacon) and 98.75 µL plain medium (either RPMI or IMDM). Well 1 and well 2 were incubated for 5 min at room temperature. Next, well 1 was pipetted to well 2 and incubated for 20 min at room temperature. Meanwhile, harvested MoDCs were plated in either RPMI or IMDM medium containing 10% FCS in the presence of IL-4 and GM-CSF (250.000 cells/well, 500 µL, 12 wells plate). Furthermore, 300 µL RPMI or IMDM containing 10% FCS and control cells (L-orient, 1:10 ratio (14)) was added to immature cell (iDCs) cultures. For the generation of mature MoDCs, 300µL RPMI or IMDM +10% FCS containing mouse fibroblast L-cells stably transfected with CD40L (1:10 ratio, (14)), was added. Both L-orient and L-CD40L cells were irradiated before usage (7500 Rad, Gammacell 1000 Elite, Best Theratronics, Kanata, ON, Canada). The generated transfection complexes were pipetted to the iDCs

and L-CD40L MoDCs (mDCs) cultures (final siRNA concentration: 100 nM). Untransfected iDCs (no introduction of siRNA and transfection reagent) and mDCs were taken along as a control. Supernatants of MoDCs were harvested after 48h and kept frozen until further usage (–20˚C). Cells were harvested and partially used for the assessment of cell viability and maturation using flow cytometry. The remaining cells were used for RNA extraction and gene levels analysis using qPCR.

To assess the transfection efficiency, both iDCs and mature MoDCs were transfected with a siGLO (Cyclophilin B Control siRNA D-001610-01-05, Dharmacon) labelled with DY-547, following the same protocol as described above. Transfection efficiency was determined 48 h after transfection using flow cytometry.

Analysis of cell viability and maturation using flow cytometry
To determine the effects of transfection on cell viability and maturation, cells were harvested and washed using FACS buffer (1% BSA, 0.02% NaN3 diluted in PBS). Cell viability was assessed by flow cytometry using Annexin-V (FITC, 1:1000; A700, VPS Diagnostics, Hoeven, The Netherlands) and 7-AAD (PerCP, 1:50; cat no. 559925 BD Biosciences). Annexin-V-FITC was diluted in 1× binding buffer (10× binding buffer, included in the VPS Diagnostics A700 kit, diluted in distilled water). Cells were considered viable when Annexin-V- negative and 7-AAD-negative.

To determine the maturation status, cells were incubated for 30 min with mouse anti human HLA-DR (APC, 1:75, Clone L243, cat no. 347403, BD), mouse anti human CD80 (FITC, 1:50, Clone L307(4), cat no. 557226, BD Pharmingen), mouse anti human CD83 (PeCy7, 1:50, Clone HB15e (RUO), cat no. 561132, BD Pharmingen) and mouse anti human CD86 (V450, Clone 2331 (FUN-1; RUO), cat no. 560357 BD Pharmingen). Cells were washed and resuspended in FACS buffer and acquired (LSR-II, BD Biosciences).

Gene transcription levels in (im)mature MoDCs
mRNA was isolated from ~0.5×10^6 MoDCs using the RNeasy Micro kit (Qiagen) following the manufacturer's instructions. Genomic DNA digestion was performed on the columns using RNase-free DNase. RNA levels were quantified using NanoDrop (ND-1000, Thermo Fisher Scientific, Waltham, MA, USA). Next, reverse transcription for the generation of complementary DNA (cDNA) was performed using the M-MLV First-Strand Synthesis system (Invitrogen). For each PCR reaction, 2 ng cDNA was pipetted into a 384 hard-shell PCR plate (Thin wall, Bio-Rad, Hercules, CA, USA) in combination with primers diluted in IQ SYBR green mix (Bio-Rad). Specific primers were used for human GAPDH (15), IL6, IL12 (16) and Cyclophilin B (17) (Table 1, final primer concentration of 1.25 µM, Biolegio, Nijmegen, The Netherlands). Samples were run (60 degrees, C1000

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Thermal Cycler, (Bio-Rad)) and gene expression levels were determined by calculating the mean values of triplicates followed by the ΔCT (Gene of interest – GAPDH). Copies per GAPDH were calculated using $2^{-\Delta CT}$. Fold changes were calculated by the ratio of the difference between control siRNA and untransfected cells, or iDCs and L-CD40L matured cells.

Table 1: Oligonucleotide sequences used for real-time PCR

Target gene	GenBank accession no.	Product size (bp)	Forward primer (5'- 3')	Reverse primer (5'-3')
Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)	**NM_001357943.2**	175	TTCCAGGAGCGAGATCCCT	CACCCATGACGAACATGGG
Cyclophilin B (CypB;PPIB)	**NM_000942.4**	110	GTCCGTCTTCTTCCTGCTG	CATCTTCATCTCCAATTCGTAGG
Interleukin-6 (IL6)	**NM_001371096.1**	247	AAGCCAGAGCTGTGCAGAT GAGTA	AACAACAATCTGAGGTGCCCA TGC
Interleukin-12B (IL12B)	**NM_002187.2**	122	CAGCAGCTTCTTCATCAGGG	GAGTACTCCAGGTGTCAGG

MoDCs : T cell co-culture

IDCs were harvested as described previously. Pan T cells were purified from peripheral blood leukocytes (PBL, remaining fraction after monocyte-isolation) using Pan T cell isolation kit (130-096-535, Miltenyi Biotec). The isolated Pan T cells were frozen at −80˚C (IMDM containing 10% FCS, 10% DMSO and 90 U/mL penicillin and 90 µg/mL streptomycin) and thawed on the day iDCs were used for the co-culture. Pan T cells were labelled with 1 µM carboxyfluorescein succinimidyl ester (CFSE; cat no. C34554, Thermo Fisher Scientific) diluted in PBS. IDCs, either untransfected or transfected with control siRNA in IMDM or RPMI medium, were co-cultured with allogeneic Pan T cells (1:10 ratio, 10^5 Pan T cells vs 10^4 MoDCs) in IMDM containing 10% FCS and 90 U/mL penicillin and 90 µg/mL streptomycin. After 6 days, supernatants and cells were harvested. Cells were stained using mouse anti-human CD3 (PE; 1:50, cat no. 555333, BD Biosciences), mouse anti-human CD4 (V450; 1:50, Clone RPA-T4 (RUO), cat no. 560345, BD Biosciences) and mouse anti-human CD8 (APC; 1:50, SK1 clone, cat no. 345775 BD Biosciences) and analyzed using flow cytometry (LSR-II; BD Biosciences). Gating strategy: viable cells (FSC-A vs SSC-A) and single cells (FSC-A vs FSC-H) were selected. CD4$^+$ and CD8$^+$ T cells were selected by gating CD3$^+$CD4$^+$ and CD3$^+$CD8$^+$, respectively. T cell proliferation was determined by analysing the CFSE dilution of CD3$^+$CD4$^+$ and CD3$^+$CD8$^+$ T cells on histograms.

Statistics

Statistical analysis between multiple groups were performed using a two-way ANOVA and Bonferroni post hoc tests were used to test for statistical significance. Significance was defined as $P \leq 0.05$. For statistical analysis and graphical representations Prism v.8 was used (Graphpad Software, San Diego, CA, USA).

Results

Transfection in IMDM or RPMI medium gives a similar efficiency and cell viability

To assess the effect of the culture medium used during the transfection of MoDCs, monocytes were differentiation into immature dendritic cells (iDCs). As expected, iDCs expressed CD1a and DC-SIGN and were CD14 negative (Figure 1A). Both iDCs and L-CD40L matured MoDCs (mDCs) were transfected, after which the transfection efficiency was determined using a fluorescently labelled siRNA (siGLO, Figure 1B). The use of RPMI or IMDM medium did not significantly affect the transfection efficiency of both iDCs and mDCs, although, transfection of cells in RPMI medium was more heterogeneous (Figure 1C). Transfection of iDCs and mDCs with siGLO, next to a fluorescently labelled siRNA also a specific siRNA targeting the Cyclophilin B gene, resulted in reduced Cyclophilin B expression, preferentially in cells transfected in IMDM (Figure 1D). Transfection of iDCs and mDCs in RPMI resulted in a decreased cell viability, as was assessed by flow cytometry using 7-AAD and Annexin-V staining (Figure 1E-F). Transfection of iDCs and mDCs in IMDM medium did not affect the cell viability.

IL6 and IL12B levels increased in MoDCs transfected in RPMI medium

Next, we assessed the *IL6* and *IL12B* mRNA levels in iDCs. Although *IL6* and *IL12B* could be detected in iDCs, the transcript levels were not affected by transfection with control siRNA in IMDM medium. In contrast, both *IL6* (Figure 2A) and *IL12B* (Figure 2B) expression levels were increased when iDCs were transfected with control siRNA in RPMI medium. When iDCs were activated with CD40L in either IMDM or RPMI, a similar level of activation was observed (Figure 2C-D). This indicates that the increase in *IL6* and *IL12B* expression is a specific effect of transfection of iDCs in the presence of RPMI medium.
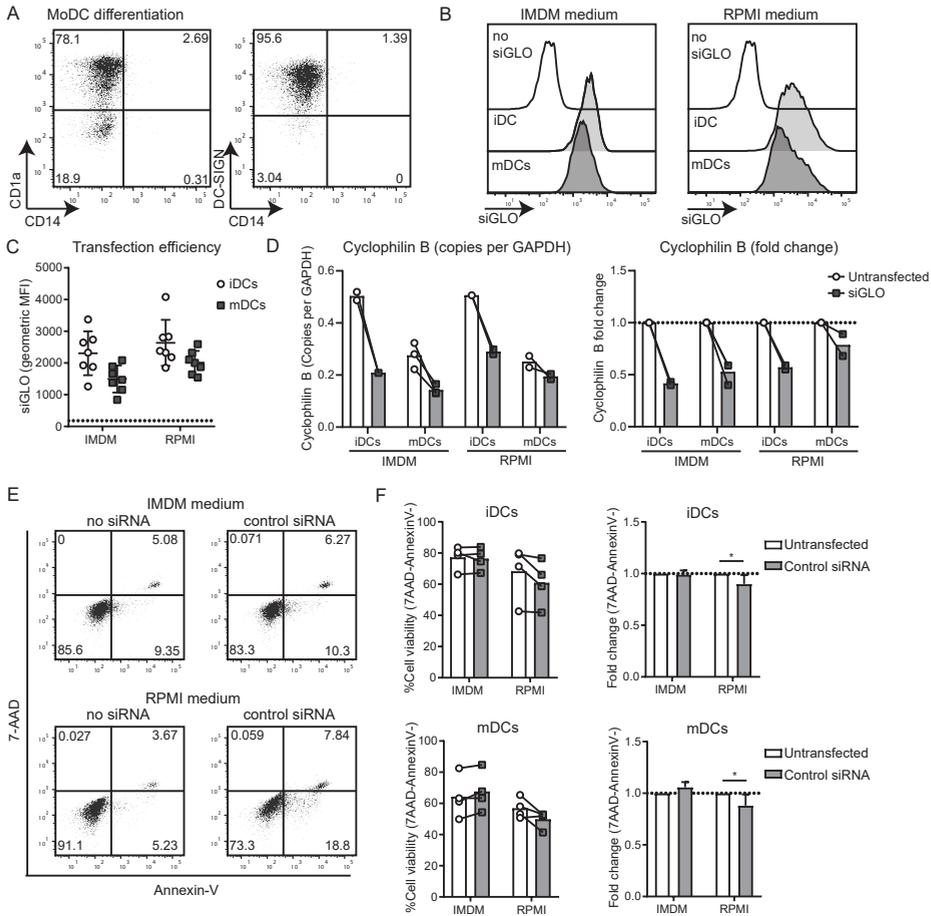
Figure 1. The type of medium used during transfection of MoDCs affects the cell viability.
Monocytes were isolated from PBMCs and iDCs were generated by culturing monocytes for 5 days in
the presence of IL-4 and GM-CSF. **(A)** FACS analysis of MoDC differentiation using cell surface markers
CD14, DC-SIGN and CD1a. Numbers represent the percentage of cells expressing the indicated markers.
(B, C) IDCs were harvested at day 5 and transfected for 48 h using 100 nM DY-547 labelled siGLO
in either RPMI or IMDM medium. In addition, for the generation of mature MoDCs (mDCs), mouse
fibroblast L-cells expressing L-CD40L (2.5×10^5 MoDCs vs 2.5×10^4 L-CD40L expressing cells) were
added simultaneously. IDCs were cultured with control cells (2.5×10^5 MoDCs vs 2.5×10^4 L-orient cells).
Transfection efficiency of iDCs (light grey histogram) and mDCs (dark grey histogram) was analyzed by
FACS and compared to untransfected MoDCs (white histogram). siGLO positivity was quantified using
geometric MFI values. Data are shown as mean ±SD of seven individual experiments. **(D)** Cyclophilin B
copies per GAPDH are shown and were used to calculate the fold change of Cyclophilin B expression
in siGLO transfected iDCs and mDCs (grey bars) compared to untransfected iDCs (white histogram).
(E) FACS analysis of the viability of iDCs after transfection with 100 nM control siRNA compared to
untransfected cells using 7-AAD and Annexin-V (representative of $n = 4$). **(F)** Representation of the
percentage of viable cells and quantification of the viability of iDCs and mDCs after transfection with 100
nM control siRNA (grey bars) compared to untransfected cells (white bars). Data points are presented
as the mean ± SD of four different donors. Bars represent the means. Two-way ANOVA, Bonferonni,
* P ≤ .05.

Transfection of iDcs in RPMI induced cell maturation

Next, we questioned whether the type of medium also affected the expression of co-stimulatory molecules. Similar to the expression of *IL6* and *IL12B*, transfection with control siRNA in IMDM medium did not induce alterations in the cell surface expression of DC activation markers CD80 (Figure 3A), CD83 (Figure 3B), CD86 (Figure 3C) or HLA-DR (Figure 3D, left histograms). However, iDCs transfected with control siRNA (Figure 3, grey histograms and bars) in RPMI medium significantly increased the expression of CD80 (Figure 3A), CD86 (Figure 3C) and HLA-DR (Figure 3D) when compared to untransfected iDCs (Figure 3, white histograms and bars). Furthermore, iDCs transfected with siGLO in RPMI had a significant increase in CD86 surface expression (Figure 3B).
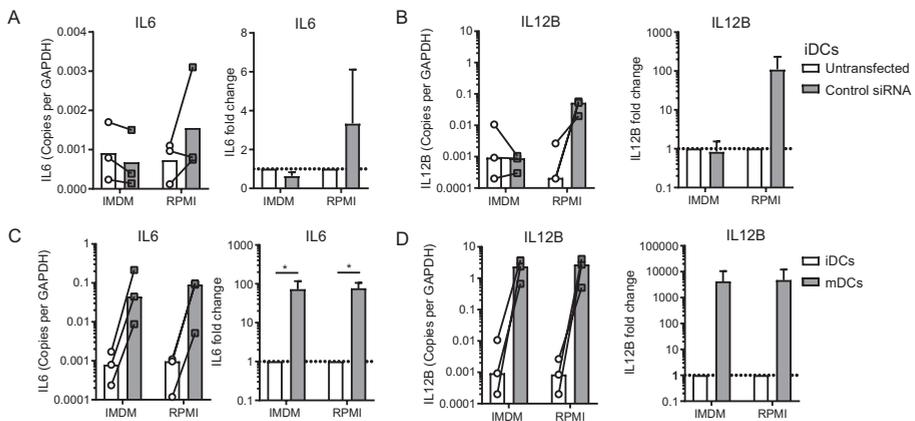
Figure 2. Upregulation of *IL6* and *IL12B* mRNA expression in iDCs transfected in RPMI medium.
IDCs or mDCs were transfected for 48 h in the absence (white bars) or presence of control siRNA (grey bars) in both RPMI and IMDM, followed by RNA isolation and cDNA synthesis. Transcript levels of *GAPDH*, *IL6* and *IL12B* were determined by RT-PCR. **(A, B)** *IL6* and *IL12B* copies per *GAPDH* are shown and were used to calculate the fold change of *IL6* and *IL12B* expression in control siRNA transfected iDCs (grey bars) compared to untransfected iDCs (white bars) of cells transfected in RPMI and IMDM. **(C, D)** *IL6* and *IL12B* expression by both iDCs (white bars) and mDCs (grey bars), cultured in RPMI or IMDM medium. *IL6* and *IL12B* copies per *GAPDH* are shown and were used to calculate the fold change of *IL6* and *IL12B* expression in mDCs (grey bars) compared to iDCs (white bars). Data are presented as the mean ± SD of three different donors. Bars represent the means. Two-way ANOVA, Bonferonni, * P ≤ .05.

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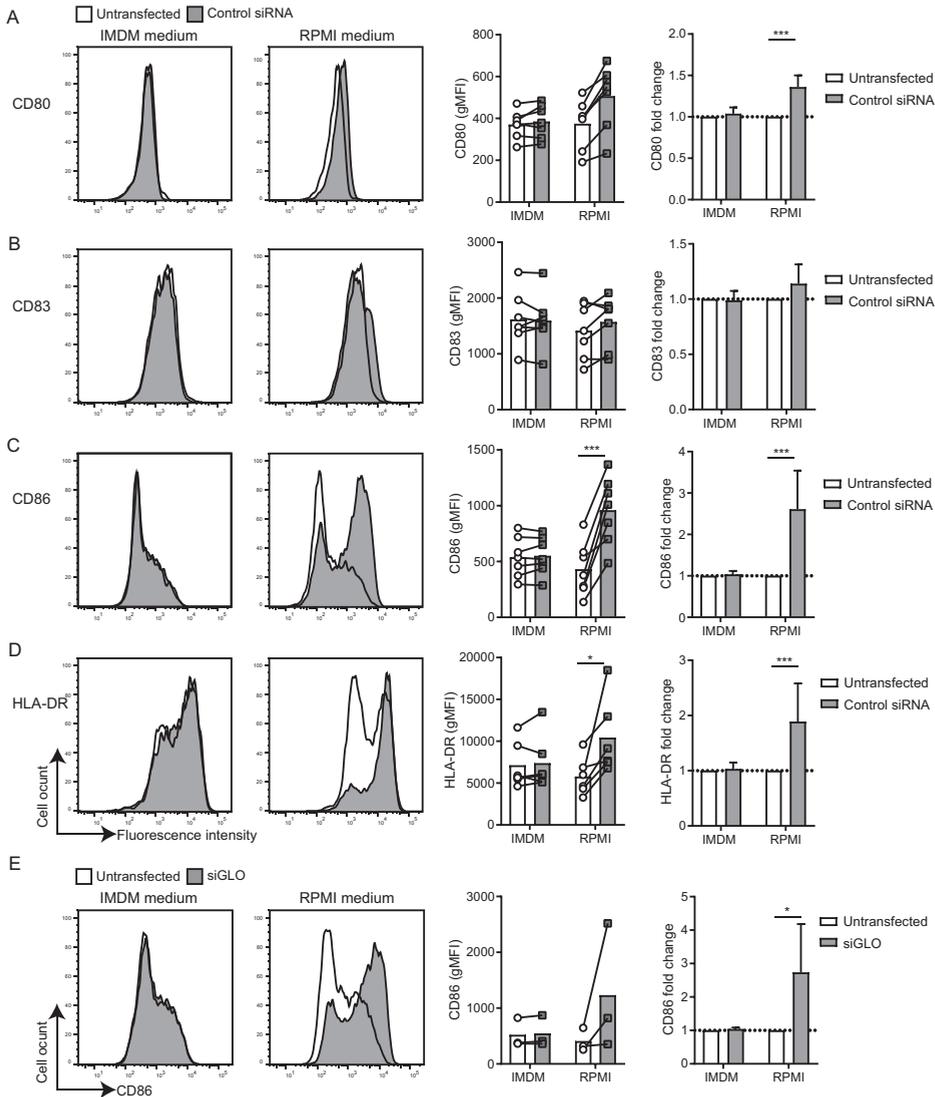
Figure 3. Transfection of iDCs in RPMI medium increases expression of CD80, CD86 and HLA-DR maturation markers. (A-D) IDCs were harvested 48 h after transfection. Surface expression of **(A)** CD80, **(B)** CD83, **(C)** CD86 and **(D)** HLA-DR was determined by flow cytometry. Histograms represent the maturation marker expression on untransfected (white histogram) and control siRNA transfected (grey histogram) iDCs. Geometric MFI values are shown and were used to calculate the fold change of maturation marker expression on control siRNA transfected iDCs (grey bars) compared to untransfected iDCs (white bars). Data are presented as the mean ± SD of six (HLA-DR) or seven (CD80, CD83, CD86) different donors. **(E)** Representation of the CD86 surface expression on iDCs transfected in either RPMI or IMDM with siGLO (grey histogram), compared to untransfected iDCs (white histogram, $n = 3$). Geometric MFI values are shown for the CD86 expression and were used to calculate the fold change of CD86 expression on control siRNA transfected iDCs (grey bars) compared to untransfected iDCs (white bars). Bars represent the means. Two-way ANOVA, Bonferonni, * $P \le .05$, ** $P \le .01$, *** $P \le .001$.

MoDCs transfected in the presence of RPMI have increased T cell stimulatory capacities

The upregulation of co-stimulatory markers upon transfection in RPMI medium suggests an effect on the ability of transfected DCs to stimulate T cells. To investigate this, either untransfected or control siRNA treated iDCs, transfected in the presence of RPMI or IMDM medium, where co-cultured with allogeneic T cells. T cell proliferation was assessed by flow cytometry determining CFSE dilution. Co-culture of T cells with untransfected iDCs in RPMI showed some CD8+ (8.97%) and CD4+ (12.9%) T cell proliferation (Figure 4A). Proliferation was increased when T cells were co-cultured with iDCs transfected with control siRNA in RPMI medium (Figure 4A, CD8+:15.4%, CD4+:25.8%). Quantification of the T cell proliferation showed that DCs transfected with control siRNA in IMDM medium did not affect T cell proliferation, whereas transfection in RPMI significantly increased CD4 T cell proliferation (Figure 4B). In direct comparison, untransfected iDCs showed the same dose-dependent induction of allogeneic CD4+ T cell proliferation, and increased proliferation was observed when iDCs were transfected in RPMI, but not in IMDM (Figure 4C). These results showed that the choice of medium during the siRNA transfection of iDCs affects their T cell stimulatory capacities.

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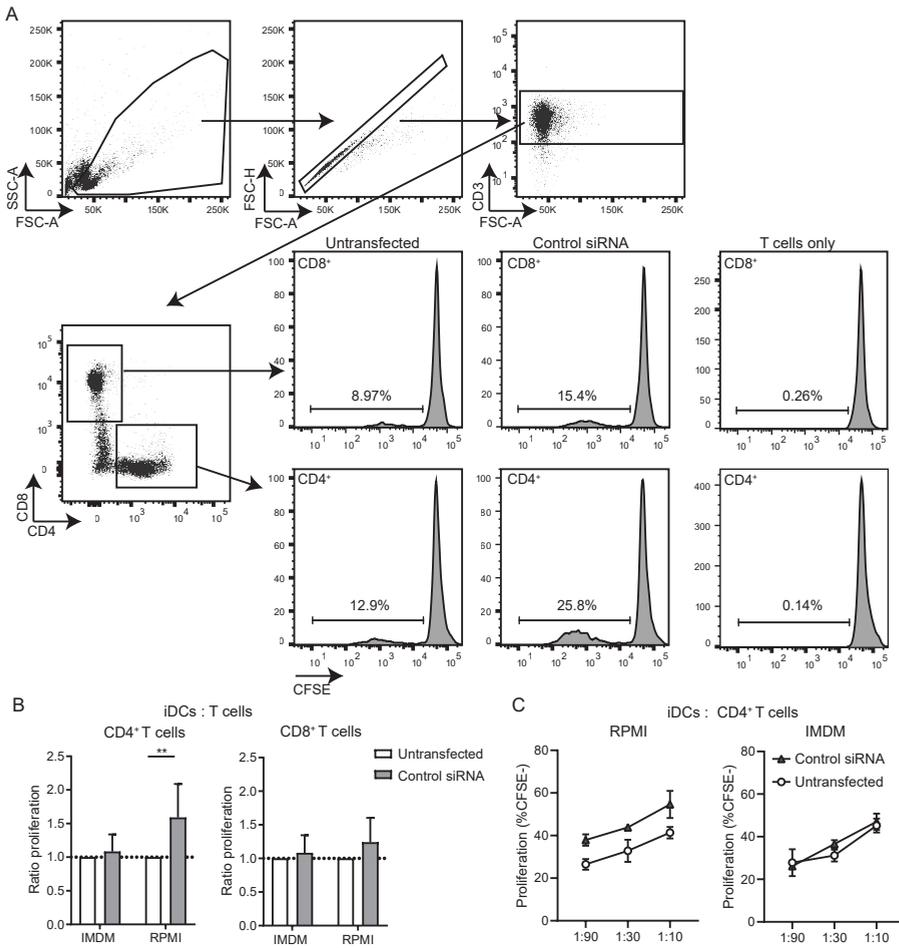
Figure 4. IDCs transfected with control siRNA in RPMI medium induces allogeneic T cells proliferation upon co-culture. IDCs were harvested 48 h after transfection and co-cultured with carboxyfluorescein diacetate succinimidyl ester (CFSE)-labelled allogeneic Pan T cells, isolated from peripheral blood (10^4 iDCs vs 10^5 Pan T cells). **(A)** After 6 days, cells were harvested, stained for T cell surface markers and analyzed using flow cytometry. CFSE dilution of $CD3^+CD4^+$ and $CD3^+CD8^+$ T cells was used to determine T cell proliferation. Data shown represents the FACS analysis of at least three individual experiments. **(B)** Quantification of $CD4^+$ and $CD8^+$ T cell proliferation, determined by the percentage of CFSE-negative cells. Effects of iDCs transfected with control siRNA (grey bars) in either RPMI ($CD4^+$ cells $n = 4$; $CD8^+$ cells $n = 3$) or IMDM ($CD4^+$ cells $n = 6$; $CD8^+$ cells $n = 5$) on $CD4^+$ and $CD8^+$ T cell proliferation is shown as a ratio compared to untransfected cells (white bars). Data points are presented as the mean ± SD. **(C)** Quantification of $CD4^+$ T cell proliferation when a fixed amount of T cells (10^5) were co-cultured with allogeneic iDCs (1.1×10^3, 3.3×10^3 and 10^4) ($n = 1$). iDCs were transfected in RPMI or IMDM with control siRNA (grey triangle) or were left untransfected (white circle). Error bars represent the technical replicates ($n = 3$). Two-way ANOVA, Bonferonni * P ≤ .05.

Discussion

The choice of medium used during the culture of MoDCs is important, since it has been shown that the usage of either IMDM or RPMI during the MoDC differentiation results in functionally different cells (6,7). In functional experiments, for example during the co-culture of MoDCs with allogeneic T cells, IMDM medium is often used. Here, we studied the effect of culture medium used during the transfection of MoDC. We compared RPMI and IMDM and observed that, although there were just small differences in transfection efficiency and cell viability, only transfection in RPMI resulted in activation of iDC, as shown by increased expression of cytokines and co-stimulatory molecules.

MoDCs are widely used as a model to investigate DC biology and effort has been put in the investigation of the genes using RNA interference (RNAi). For the introduction of RNAi into cells, various approaches like electroporation and lipid based systems can be applied (18). However, the effective transfection of primary dendritic cells and macrophages is rather difficult (19,20) and optimization is therefore important. Using a fluorescently labelled siRNA (siGLO), which targets the Cyclophilin B gene, we showed that both iDCs and mDCs are transfected comparably in both RPMI and IMDM. However, suppression of Cyclophilin B mRNA levels was less pronounced upon transfection in RPMI. Moreover, iDCs transfected with a siRNA targeting Cyclophilin B in RPMI, resulted in an increased CD86 surface expression.

Since dendritic cells are well equipped for the detection of PAMPs and DAMPs, the exposure to siRNA could potentially induce dendritic cell activation (9,10). This might be especially important in the transfection of iDCs, since the induced activation of these cells would complicate the functional outcome of the factors studied. We showed that iDCs transfected in RPMI with a control siRNA upregulated the mRNA levels of *IL6* and *IL12B*, which was not seen in iDCs transfected in IMDM. Nevertheless, induction of *IL6* and *IL12B* expression upon L-CD40L-induced maturation was comparable for cells cultured in RPMI and IMDM, indicating that this difference is exclusive for medium used during transfection. In line with this, also the surface expression of co-stimulatory markers CD80, CD83, CD86 and HLA-DR was increased on iDCs when transfected with control siRNA in RPMI medium. This indicates that the transfection of iDCs, both by a control siRNA or by a targeting siRNA, alters the iDC activation state upon transfection in RPMI medium, which was not observed for iDCs transfected in IMDM medium.

During liposomal transfection, lipids, for example cationic lipids, are mixed with the negatively charged nucleic acids. This complex facilitates cellular uptake and protects the nucleic acids against enzymatic degradation during endocytosis (21,22). There are

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different types of cationic lipids and the choice depends on the cell type or cell line which needs to be transfected, as was shown for the transfection of plasmacytoid dendritic cells (23). In addition, the size of cationic lipids, siRNA concentration, cell density and factors like the presence of serum should be taken into account when optimizing the transfection protocols (18,24). Also the source of serum is an important factor for culture medium. Therefore, we investigated the substitution of ΔFCS for ΔNHS in the media used during the transfection of iDCs. We observed an increased CD86 expression when iDCs were transfected in RPMI-ΔNHS as well as in IMDM-ΔNHS (data not shown). The composition and concentrations of ingredients vary between RPMI and IMDM medium, and typically, IMDM is considered to be a more nutrient rich medium. Further research is needed to identify the factor (or factors) responsible for the "iDCs activation" during the transfection of these cells in RPMI medium.

To confirm that iDCs activated by transfection and showing an increased expression of cytokines and co-stimulatory molecules were functionally different, co-culture experiments were performed. Co-culture of "activated" iDCs with allogeneic T cells resulted in an increased CD4$^+$ T cell proliferation. Importantly, iDC transfected in IMDM, and which were not activated, showed a T cell stimulatory capacity similar to non-treated cells. Overall, these data show that the choice of medium is important, not only during the differentiation, but also during the transfection of iDCs. So far, in the various protocols available for the transfection of cells, the choice of medium is not taken into account as one of the variables which should be optimized. It is rather stated that the cells should be cultured in their regular medium. We therefore propose that the choice of medium used during the transfection of MoDCs should be included as one of the variables for further optimization.

Acknowledgments

References

1. Banchereau J, Briere F, Caux C, Davoust J, Lebecque S, Liu YJ, et al. Immunobiology of dendritic cells. Annu Rev Immunol. 2000;18:767-811. doi: 10.1146/annurev.immunol.18.1.767.

2. Banchereau J, Steinman RM. Dendritic cells and the control of immunity. Nature. 1998 Mar 19;392(6673):245-52. doi: 10.1038/32588.

3. Iwasaki A, Medzhitov R. Toll-like receptor control of the adaptive immune responses. Nat Immunol. 2004 Oct;5(10):987-95. doi: 10.1038/ni1112.

4. Liu YJ. Dendritic cell subsets and lineages, and their functions in innate and adaptive immunity. Cell. 2001 Aug 10;106(3):259-62. doi: 10.1016/s0092-8674(01)00456-1.

5. Sallusto F, Lanzavecchia A. Efficient presentation of soluble antigen by cultured human dendritic cells is maintained by granulocyte/macrophage colony-stimulating factor plus interleukin 4 and downregulated by tumor necrosis factor alpha. J Exp Med. 1994 Apr 1;179(4):1109-18. doi: 10.1084/jem.179.4.1109.

6. Chang CC, Wright A, Punnonen J. Monocyte-derived CD1a+ and CD1a- dendritic cell subsets differ in their cytokine production profiles, susceptibilities to transfection, and capacities to direct Th cell differentiation. J Immunol. 2000 Oct 1;165(7):3584-91. doi: 10.4049/jimmunol.165.7.3584.

7. Ilchmann A, Krause M, Heilmann M, Burgdorf S, Vieths S, Toda M. Impact of culture medium on maturation of bone marrow-derived murine dendritic cells via the aryl hydrocarbon receptor. Mol Immunol. 2012 May;51(1):42-50. doi: 10.1016/j.molimm.2012.02.005.

8. Chow A, Brown BD, Merad M. Studying the mononuclear phagocyte system in the molecular age. Nat Rev Immunol. 2011 Oct 25;11(11):788-98. doi: 10.1038/nri3087.

9. Sioud M. Induction of inflammatory cytokines and interferon responses by double-stranded and single-stranded siRNAs is sequence-dependent and requires endosomal localization. J Mol Biol. 2005 May 20;348(5):1079-90. doi: 10.1016/j.jmb.2005.03.013.

10. Judge AD, Sood V, Shaw JR, Fang D, McClintock K, MacLachlan I. Sequence-dependent stimulation of the mammalian innate immune response by synthetic siRNA. Nat Biotechnol. 2005 Apr;23(4):457-62. doi: 10.1038/nbt1081.

11. Bowles R, Patil S, Pincas H, Sealfon SC. Optimized protocol for efficient transfection of dendritic cells without cell maturation. J Vis Exp. 2011 Jul 8;(53):e2766. doi: 10.3791/2766.

12. Bowles R, Patil S, Pincas H, Sealfon SC. Validation of efficient high-throughput plasmid and siRNA transfection of human monocyte-derived dendritic cells without cell maturation. J Immunol Methods. 2010 Dec 15;363(1):21-8. doi: 10.1016/j.jim.2010.09.028.

13. Geijtenbeek TB, Torensma R, van Vliet SJ, van Duijnhoven GC, Adema GJ, van Kooyk Y, et al. Identification of DC-SIGN, a novel dendritic cell-specific ICAM-3 receptor that supports primary immune responses. Cell. 2000 Mar 3;100(5):575-85. doi: 10.1016/s0092-8674(00)80693-5.

14. Garrone P, Neidhardt EM, Garcia E, Galibert L, van Kooten C, Banchereau J. Fas ligation induces apoptosis of CD40-activated human B lymphocytes. J Exp Med. 1995 Nov 1;182(5):1265-73. doi: 10.1084/jem.182.5.1265.

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15. Dixon KO, O'Flynn J, Klar-Mohamad N, Daha MR, van Kooten C. Properdin and factor H production by human dendritic cells modulates their T-cell stimulatory capacity and is regulated by IFN-γ. Eur J Immunol. 2017 Mar;47(3):470-480. doi: 10.1002/eji.201646703.

16. Dixon KO, van der Kooij SW, Vignali DA, van Kooten C. Human tolerogenic dendritic cells produce IL-35 in the absence of other IL-12 family members. Eur J Immunol. 2015 Jun;45(6):1736-47. doi: 10.1002/eji.201445217.

17. Cao XM, Luo XG, Liang JH, Zhang C, Meng XP, Guo DW. Critical selection of internal control genes for quantitative real-time RT-PCR studies in lipopolysaccharide-stimulated human THP-1 and K562 cells. Biochem Biophys Res Commun. 2012 Oct 19;427(2):366-72. doi: 10.1016/j.bbrc.2012.09.066.

18. Schiffelers RM, Woodle MC, Scaria P. Pharmaceutical prospects for RNA interference. Pharm Res. 2004 Jan;21(1):1-7. doi: 10.1023/b:pham.0000012145.49054.6c.

19. Van Tendeloo VF, Snoeck HW, Lardon F, Vanham GL, Nijs G, Lenjou M, et al. 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 May;5(5):700-7. doi: 10.1038/sj.gt.3300626.

20. Martinez FO. Analysis of gene expression and gene silencing in human macrophages. Curr Protoc Immunol. 2012 Feb;Chapter 14:Unit 14.28.1-23. doi: 10.1002/0471142735.im1428s96.

21. Troegeler A, Lastrucci C, Duval C, Tanne A, Cougoule C, Maridonneau-Parini I, et al. An efficient siRNA-mediated gene silencing in primary human monocytes, dendritic cells and macrophages. Immunol Cell Biol. 2014 Sep;92(8):699-708. doi: 10.1038/icb.2014.39.

22. Gooding M, Browne LP, Quinteiro FM, Selwood DL. siRNA delivery: from lipids to cell-penetrating peptides and their mimics. Chem Biol Drug Des. 2012 Dec;80(6):787-809. doi: 10.1111/cbdd.12052.

23. Smith N, Vidalain PO, Nisole S, Herbeuval JP. An efficient method for gene silencing in human primary plasmacytoid dendritic cells: silencing of the TLR7/IRF-7 pathway as a proof of concept. Sci Rep. 2016 Jul 14;6:29891. doi: 10.1038/srep29891.

24. Inoh Y, Nagai M, Matsushita K, Nakanishi M, Furuno T. Gene transfection efficiency into dendritic cells is influenced by the size of cationic liposomes/DNA complexes. Eur J Pharm Sci. 2017 May 1;102:230-236. doi: 10.1016/j.ejps.2017.03.023.

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