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ВЛИЯНИЕ ЭЛЕКТРОПОРАЦИИ ДНК КОНСТРУКЦИЯМИ НА ДЕНДРИТНЫЕ КЛЕТКИ

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Резюме. На сегодняшний день, трансфекция клеток млекопитающих ДНК или РНК конструкциями является единственным методом доставки запрограммированной информации в ядро клетки. Одним из часто используемых методов трансфекции в работе с дендритными клетками, является электропорация. Суть метода состоит в повышении проницаемости мембраны путем проведения электрического импульса через клетку. В связи с повышенной проницаемостью мембраны повышается шанс попадания ДНК или РНК конструкций внутрь клетки, но при этом снижается выживаемость клетки после воздействия тока на плазматическую мембрану клетки.

В исследовании использовали мышей самцов линии C57Bl/6 возраста 2-4 месяца. Из бедренной кости мышей выделяли 20×10^6 клеток костного мозга, после клетки культивировали в полной RPMI-1640 среды в течение 7 суток. Для генерации дендритных клеток из клеток костного мозга, в культуральную среду добавляли 100 нг/мл rmFlt3-L на 0 день. После 7 дней культивирования, клеточную культуру электропорировали контрольными некодирующими плазмидами p5 (EP P5) или пламзмидами pmaxCCR9 (EP CCR9), кодирующими мышиный рецептор хемотаксиса CCR9. В качестве контролей выступали, клеточные культуры электропорированные без плазмид (mock EP) и культуры клеток без электропорации (none EP). Электропорировали 5×10^5 клеток и оставляли на 10 минут. После 10 минут клетки собирались и рассаживались в 24-луночном планшете в 1 мл культуральной среды и кондиционной среды (1:1). Затем добавляли по 50 нг/мл Flt3-L в каждую лунку. На следующий день трансфицированные клетки оценивались с помощью метода проточной цитофлуориметрии и количественной ПЦР.

Установлено, что после электропорации в группах mock EP, EP P5, EP CCR9 относительное количество живых $CD11c^+$ дендритных клеток было достоверно меньше, чем в non EP группе. Более того, в группах EP P5 и EP CCR9 было достоверно меньше живых $CD11c^+$ дендритных клеток, чем в группе mock EP. Экспрессия маркера CD86 было достоверно выше в группах EP P5 и EP CCR9, чем в группах non EP и mock EP. Экспрессия I-Ab среди cDC2s было достоверно выше в группе EP CCR9 по сравнению с группой non EP. У плазмацитоидных дендритных клеток и конвенциональных дендритных клеток 2-го типа, в группах EP CCR9, экспрессия CCR9 была достоверно выше, чем в группе non EP.

Таким образом, в данном исследовании продемонстрирована эффективность электропорации, сопровождающаяся снижением выживаемости и созреванием дендритных клеток.

Ключевые слова: электропорация, плазмацитоидные дендритные клетки, конвенциональные дендритные клетки 2-го типа, ДНК конструкции, жизнеспособность, зрелость

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EFFECT OF DNA CONSTRUCTIONS ELECTROPORATION ON DENDRITIC CELLS

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Abstract. Today transfection of mammalian cell with DNA or RNA construction is the only method for delivering programmed information into the cell nucleus. Electroporation is most commonly used method of transfection in experiments with dendritic cell. The aim of electroporation is to permeabilize the membrane by passing electric impulse through the cell. Due to the increase permeability of the membrane chance DNA or RNA construction getting inside into the cell is increased, wherein survival of the cells is decreased.

In the study male mice C57Bl/6 line 2-4 months old were used. From femur bones was isolated 20×10^6 bone marrow cells, which were cultured in 20 mL of complete RPMI-1640 for 7 days. To generate dendritic cells from BM cells, 100 ng/mL of rmFlt3-L was added to culture media at day 0. After 7 days of cultivation, the cell cultures were electroporated with control noncoding plasmids p5 (EP P5) or pmaxCCR9 encoding mouse chemokine receptor CCR9 (EP CCR9). The controls were cell cultures electroporated without any plasmids (mock EP) and cell cultures without electroporation (none EP). 5×10^5 cells were electroporated and resting for 10 minutes. After 10 minutes, cells were harvested and seeded into 24-well plates in 1 mL of culture medium and conditioning medium (1:1). Then, 50 ng/mL of Flt3-L was added to each well. The next day, transfected cells were collected and used for flow cytometry, qRT-PCR analysis.

It was found that after electroporation in the groups mock EP, EP P5, EP CCR9 relative amount of live $CD11c^+$ dendritic cells was significantly less than in the non EP group. Moreover, in the EP P5 and EP CCR9 groups the frequency of live $CD11c^+$ dendritic cells was significantly less than in the mock EP group. Expression of the CD86 marker in the EP P5 and EP CCR9 groups was significantly higher than in the non EP and mock EP groups. Expression of the I-AB(MHCII) in the EP CCR9 group on cDC2s was significantly higher than in the non EP group. On plasmacytoid DCs (pDCs) and conventional type 2 DCs (cDC2s) in the EP CCR9 group expression of CCR9 was significantly higher than in the non EP group. Therefore, in this study, we demonstrated the effectiveness of electroporation, accompanied by the decrease in the survival rate and maturation of DCs.

Keywords: electroporation, plasmacytoid dendritic cells, conventional dendritic cell type 2, DNA constructions, viability, maturity

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Introduction

Electrotransfection of cells is one of the methods of transfection induced by electric field impulses used recently as an effective technique of foreign DNA introduction into cells of any origin [9, 14]. The aim of electroporation is to permeabilize cell membrane while preserving the cell viability. While applying impulses, irreversible damage occurred to the plasma membrane, from which some cells are not able to recover. The latter depends on cell ability to achieve biochemical balance after inducing intense molecules influx and efflux. If that balance is not attained, cell viability may be lost [4, 9, 13].

Comprehension of electroporation mechanism is important for improving efficiency in experiments with dendritic cells (DCs) both *in vitro* or *in vivo* [5]. Recently, methods of electroporation turned out to be more frequently applied for antigen loading of DCs compared to other methods of transfection. For example, transfection of immature DCs by using DNA or RNA constructs leads to special ability to capture and process antigens followed by their maturity [8]. Within an hour after electroporation, DCs start to express X-box binding protein 1 (XBP1), which is a transcription factor controling biogenesis, thereby reducing stress and promoting cell survival [12]. Moreover, glycolysis in DCs depends on PI3K/Akt, mTORC1, which is one of the most common signaling pathways for proliferation in the stress response. The process is supported by the fact that glucose restriction strongly inhibits activation and lifespan of DCs after stress, which electroporation can induce [11].

In this study, we electroporated cell cultures containing murine $CD11c^+B220^+$pDCs and $CD11c^+SIRP\alpha^+$cDC2s for assessing viability and maturation.

Materials and methods

In the study, male C57Bl/6 mice aged 2-4 months were used, obtained from the SPF vivarium of the Institute of Cytology and Genetics (Novosibirsk, Russia) and maintained at the animal facility of the Research Institute of Fundamental and Clinical Immunology on standard diet, under natural light conditions with unrestricted access to food and water.

An artificial gene encoding mouse CCR9 (UniProtKB-Q9WUT7) was designed and prepared with the optimization of codon composition for its efficient expression in mammalian cells. The artificial gene was subcloned into the pMax-plasmid vector under the control of a CMV promotor added with poly(A)tail. After transformation in Escherichia coli plasmid DNA was purified and sequenced.

Dendritic cells were generated from the bone marrow (BM) of UBC-GFP mice. First, 20×10^6 BM cells isolated from femur bones were cultured for 7 days in 75-cm2 flasks (TPP, Switzerland) together with 20 mL of complete RPMI-1640 supplemented with 10% FCS (Biowest, France), 2 mM L-glutamine (Biolot, Russia), 10 mM HEPES (Biolot, Russia), 5×10^{-4} M 2-mercaptoethanol (Sigma-Aldrich, USA), 80 μg/mL gentamycin (KRKA, Slovenia), and 100 μg/mL benzylpenicillin (Biolot, Russia). To generate dendritic cells from BM cells, 100 ng/mL of rmFlt3-L (R&D Systems, USA) was added at day 0. Half of the growth factor-containing medium was changed every 2-3 days.

After 7 days of experiment, cell cultures were harvested and electroporated with pmaxCCR9 (EP CCR9), without plasmid (MockEP) or left untreated (non EP group). Cell cultures electroporated with non-coding plasmid p5 (EP P5) were used as control for assessing effective electroporation. For electroporation, 5×10^6 of cells were resuspended in 50 μL of OptiMEM (Thermo Fisher Scientific, USA) with or without 50 μg of pmaxCCR9 and transferred to 1-mm cuvettes (BTX, USA). Electroporation was carried out using a BTX 830 electroporator (BTX, USA) with a single pulse (strength 200 V, pulse duration 40 μs). Electroporation was followed by 10 minutes of resting, after which cells were seeded into six-well plates (TPP, Switzerland) in 5 mL of culture medium and conditioning medium (1:1). Next, 50 ng/mL of Flt3-L was added to each well. One day after transfection, cells were collected and used for flow cytometry, qRT-PCR analysis.

For flow cytometry, 5×10^5 cells were collected and incubated in the dark at room temperature with an appropriate combination of fluorescently labeled monoclonal antibodies, in staining buffer, for 20 minutes. For DC staining, anti-CD11c-PE/Cy7 (N418), anti-B220-Bv510 (RA3-6B2), anti-SIRPα-PerCP/Cy5.5(P84), anti-CCR9-PE (9B1), anti-I-Ab-APC (AF6-120.1), anti-CD83-FITC (Mitchel-19) and anti-CD86-APC/Cy7 (GL-1) (BioLegend, USA) antibodies were used. DAPI was used to stain dead cells.

To analyze the gene expression profiles of nonEP, MockEP, and EP CCR9 DCs, total RNA was isolated using TRIZOL reagent in accordance with the manufacturer's instructions (Invitrogen, Carlsbad, CA, USA).

RNA concentrations were determined by measuring the absorbance at 260 nm using a Nanodrop 2000c spectrophotometer (Wilmington, DE, USA). Reverse transcription of RNA into cDNA was performed using an MMLV cDNA Synthesis Kit (Evrogen, Russia).

Subsequently, SYBR® Green technology was used for relative mRNA quantification by qPCR in a CFX96 C1000 thermal cycler (Bio-Rad). qPCR reactions were conducted at 95°C for 4 minutes, followed by 40 cycles at 95 °C for 10 s, 65 °C for 15 s, and 72 °C for 30 s. All primer sets were obtained from Biosan (Russia).

qPCR was performed in triplicate and resulting mRNA levels were normalized to levels of the reference gene phosphoglycerate kinase 1. Melt curve analysis was performed to confirm the specificity of the amplified product. The ΔΔCt method was used for data processing and analysis [7].

Statistical analysis was performed using Prism 8.0 (GraphPad Software, USA). According to the Shapiro–Wilk normality test, all data were distributed normally. Data were analyzed by parametric one-way ANOVA or two-way ANOVA with Tukey's corrected multiple comparisons. P-values ≤ 0.05 were considered statistically significant.

Results and discussion

The effect of electroporation on the viability of dendritic cells.

After electroporation, a relative amount of live $CD11c^+$DCs in the mock EP, EP P5, EP CCR9 groups was significantly lowered compared to the non EP group (Figure 1A). Moreover, in the groups with plasmid-performed electroporation (EP P5 and EP CCR9), relative amount of live $CD11c^+$DCs was significantly decreased compared to mock EP group (Figure 1A).

In the EP P5 and EP CCR9 groups there was a decreased level of pDCs compared to the non EP and mock EP groups. Percentage of cDC2s in the EP P5 and EP CCR9 groups was lowered compared to the non EP group (Figure 1B).

The phenotype of DCs after electroporation.

We determined the phenotype of DCs after electroporation by examining specific markers of maturity CD83, CD86 and I-Ab (MHCII).

The CD83 DC maturity marker did not significantly changed in proportion of cell expression and the mean value of fluorescence (Figure 2A, E).

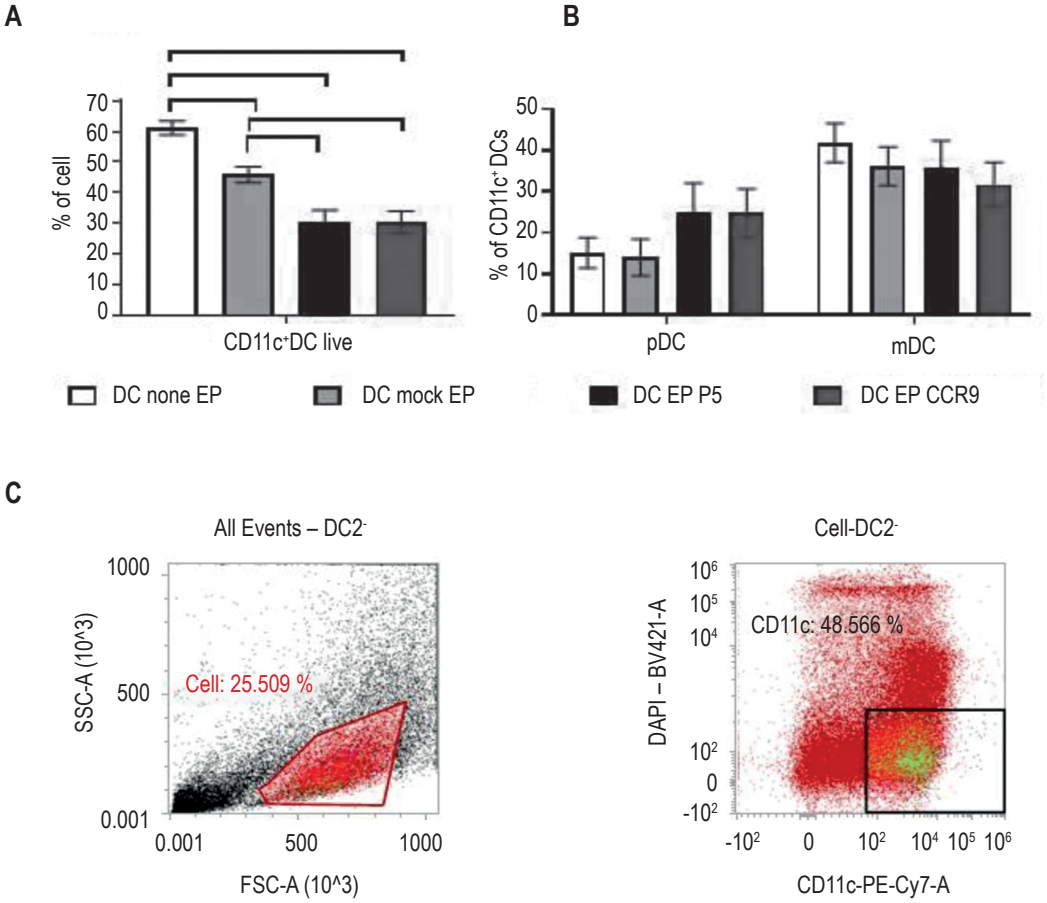

Figure 1. A, relative amount of CD11c⁺DCs live. B, relative amount of pDCs and cDC2s. C, flow cytometry gating strategy of dendric cells, CD11c⁺live DCs

Note. Data are presented as mean ± SEM. Brackets indicate significant differences (One-way ANOVA (A), two-way ANOVA (B) with Tukey's multiple comparisons test, $p < 0.05$).

However, the frequency of CD86⁺pDCs and cDC2s in the EP P5 and EP CCR9 groups was significantly higher than in the non EP and mock EP groups (Figure 2B). Mean fluorescence intensity (MFI) of CD86 on cDC2s in the EP P5 and EP CCR9 groups was significantly higher than in the non EP and mock EP groups (2F). The frequency of I-Ab⁺ cDC2s in the EP CCR9 group was significantly higher than in the non EP group (Figure 2C). MFI of I-Ab on pDC and cDC2s in the EP CCR9 group was higher than in the non EP and mock EP groups (Figure 2G).

DCs electroporated with pmaxCCR9 DNA-plasmids express higher levels of CCR9.

Flow cytometry analysis showed that in the EP CCR9 group percentage of CCR9⁺pDCs and cDC2s was significantly higher than in the non EP group (Figure 2D). In addition, relative amount of CCR9⁺cDC2s in the EP CCR9 group was significantly higher than in the mock EP group (Figure 2D).

According to the expression levels of native CCR9 RNA and plasmid CCR9 RNA measured by qRT-PCR, the DCs electroporated with pmaxCCR9 (EP CCR9) expressed 94.4-fold higher levels of bulk CCR9 RNA compared with non-electroporated DCs (nonEP) (median 2^-ΔΔCt 119.46 and 1.27, respectively, N = 4). Mock electroporated DCs (MockEP) expressed bulk CCR9 RNA that was 1.4-fold lower than that of nonEP DCs (median 2^-ΔΔCt 0.85 and 1.27, respectively, N = 4).

The electrostatic charge of the membrane is an element responsible for maintaining the physiological identity and function of cell organelles [2]. The electrostatic charge and the balance of electrons in the environment depend on the shape of the cells, e.g., cells with oval shape have uniformly distributed electrostatic charge, whereas those with processes (dendrites) or irregularly shaped cells display different electric charge on the cell membrane, and therefore

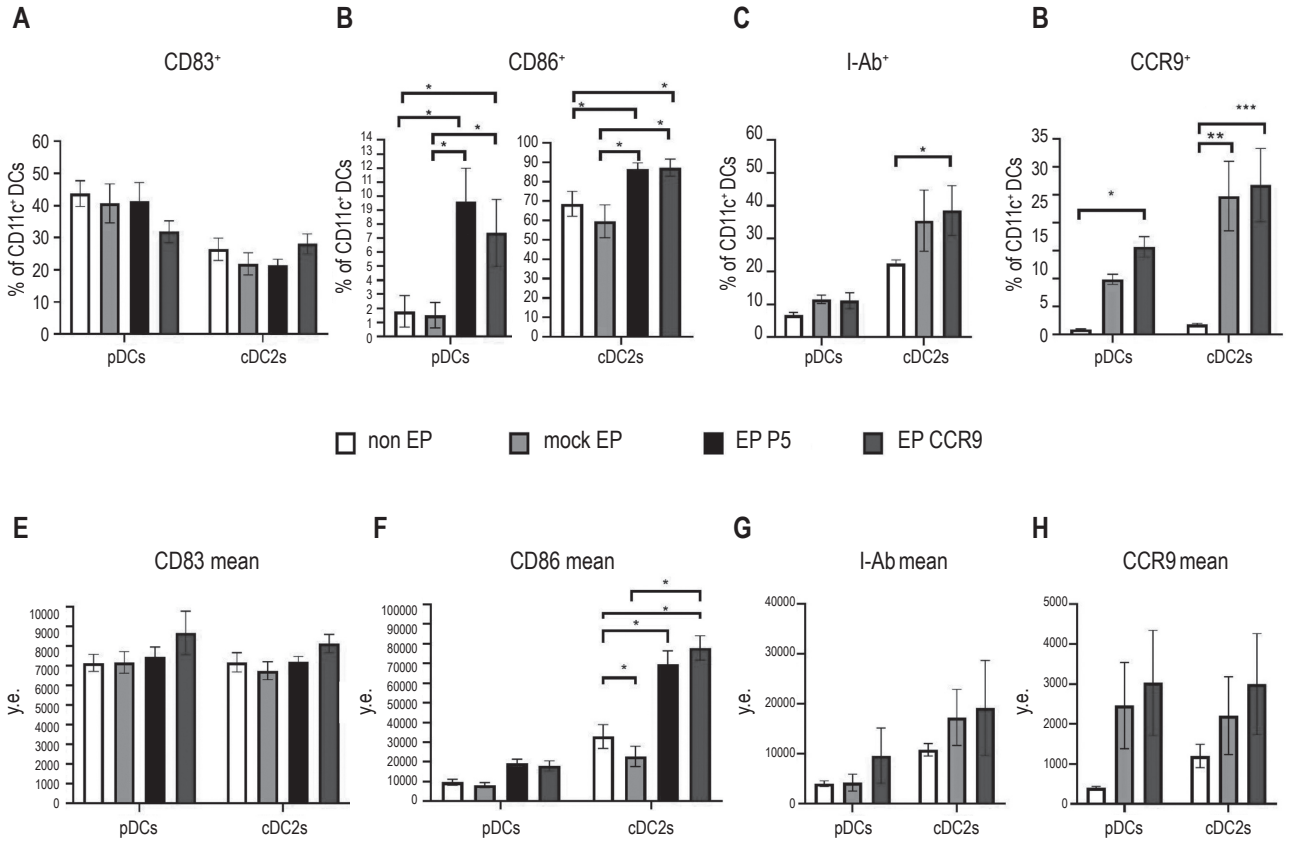

Figure 2. Relative amount of CD83 (A), CD86(B), I-Ab (C), CCR9 (D) positive DCs and mean of fluorescence intensity of CD83 (E), CD86 (F), I-Ab (G), CCR9 (H) on DCs (n = 6)

Note. Data are presented as mean ± SEM. Brackets indicate significant differences (Two-way ANOVA with Tukey's multiple comparisons test. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$).

are less resistant to electric impulse [10]. Dendritic cells are generally of oval shape with characteristic dendrites suggesting that DCs in the groups of mock EP, EP P5 and EP CCR9 cells were less viable compared to non EP dendritic cells (Figure 1A).

Cluster of differentiation 86 (CD86) is an important marker of dendritic cell maturity. Upregulated CD86 expression leads to increased I-Ab(MHCII) surface level and potential for T-cells activation [1, 6, 8]. In this study, we observed that pDCs and cDC2s had a higher level of CD86 expression in the EP P5 and EP CCR9 groups. We confirmed an increase of I-Ab expression on cDC2s after electroporation by DNA construction encoding-CCR9 (Figure 2C).

After electroporation, qPCR data showed increased levels of bulk CCR9 RNA in the EP CCR9 group compared to the non EP group. Moreover, flow cytometry analysis showed an increase of CCR9 expression on pDCs and cDC2s in the EP CCR9 group, which confirms the effectiveness of electroporation as a method for DNA construct transfection (Figure 2D). CCR9 is a chemokine that regulates the migration of dendritic cells from the bone marrow to the thymus for participation in negative deletion of the na ve T-cell [3].

Conclusion

The current study demonstrated a potential for dendritic cell electroporation with DNA constructions encoding plasmids CCR9. Out data confirmed the low viability and maturation of dendritic cell after electroporation, but the effectiveness of the method for delivery of DNA construction into the cell as well.

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