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Different permeabilization patterns of splenocytes and thymocytes to combination of pulsed electric and magnetic field treatments

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ABSTRACT

Genetic manipulation of T cells is frequently inefficient, however, when combined with physical methods (i.e. electroporation) a promising alliance with immunotherapy can be formed. This study presents new data on permeabilization of murine thymocytes and splenocytes as a T cell model using pulsed electric (PEF) and electromagnetic field (EMF). The 300 ns, 500 ns, 2 μs and 100 μs pulse bursts in a broad range of PEF 0–8 kV/cm were applied separately and in combination with 3.3 T, 0.2 kV/cm EMF pulses. The permeabilization efficiency was evaluated using fluorescent dye (YO-PRO-1) and flow cytometry. It was shown that a >14% increase in thymocytes permeabilization is achieved when electroporation is applied in combination with EMF, however splenocytes responded in a different manner – a statistically significant ($P < 0.05$) reduction in permeabilization was observed. The cytokine secretion patterns were mainly unaltered independently on the applied treatment parameters determined by secretion of IFNγ, IL-4 and IL-17 – the main cytokines of Th1, Th2 and Th17 cells. The results of this study are useful for development of pulsed power protocols for effective genetic modification of T cells.

1. Introduction

T lymphocytes are fundamental for the control of the immunological responses and thus, their deficiency or defects in development increase the susceptibility of patients to infections, immune dysregulation and autoimmunities and, in case of cancer, accelerate tumor growth [1–3]. At the same time, the gene modified T cells provide a versatile tool for immunotherapy and have been successfully used for treatment of cancer in humans [4–6]. Gene modified T cells might provide a safer way to overcome tumor immunosuppression, however challenges still remain in targeting certain liquid and solid tumors [7,8]. Also, genetic manipulation of T cells is frequently inefficient, however, when combined with physical methods (i.e. electroporation) the promising alliance with immunotherapy is formed, including but not limited to the emerging CRISPR/Cas9-based gene therapy [8,9].

Electroporation is a pulsed electric field (PEF) induced phenomenon, which triggers increased permeability of cells to molecules by means of pore formation in plasma membrane of the cell [10–12]. The process can be reversible or irreversible depending on the applied PEF parameters [13–16]. However, the selectivity of the electroporation may be a concern if excessive electroporation is used, which requires a balance between electrotransfection efficiency and cell viability [17,18]. Therefore, usually a parametric analysis of cell response is performed in a broad range of PEF in order to determine the optimal protocol [19,20]. In case of primary T cells, the area is poorly covered – the ms range protocols are established [21], however the permeabilization dynamics in microsecond or sub-microsecond range are not known.

Also as an alternative for electroporation, the contactless pulsed electromagnetic field (EMF) method has been proposed recently [22–24]. It is a newly discovered phenomenon of reversible and non-reversible cell permeabilization, which is triggered by high time-varying magnetic field (high dB/dt, where B – magnetic flux density), strong enough to induce high PEF in the sample. The EMF induced electroporation is currently the dominant idea of the mechanism of effect, however the permeabilization occurs already in 5–20 V/cm, which is lower than the established thresholds (0.2–1 kV/cm range) for conventional electroporation [22,25,26]. As a result, the phenomenon is not fully understood, however it has already proved to be effective for

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tumor treatment in vivo and has high applicability due to contactless nature of the method [22,26]. The additive EMF effect when combined with conventional electroporation was also confirmed [27].

Therefore, in this work, we focus the response of murine lymphocytes to nanosecond and microsecond electroporation since there is limited information regarding permeabilization efficacy of these cells, existence of immune response or equivalent pulse parameters that are required for successful permeabilization. Additionally, we provide an insight on the interaction of PEF and EMF methods with splenocytes and thymocytes as a model, which was never done before. We investigated if electroporation could change T cell properties and the pattern of cytokine secretion after the stimulation determined by analysis of secretion of IFNγ, IL-4 and IL-17: the main cytokines of Th1, Th2 and Th17 cells, respectively.

2. Materials & methods

2.1. Pulsed power setups

Up to 3 kV, 100 ns–1 ms square wave high voltage pulse generator was used for electroporation [28]. The setup generated pulsed electric field (0–8 kV/cm) using two protocols: 1) single pulse protocol (2 μs or 100 μs) and 2) high frequency (1 kHz) burst of five nanosecond range pulses (100–950 ns). The pulses were generated in a commercially available 1 mm gap electroporation cuvette (Biorad, Hercules, USA).

Up to 3.3 T high *dB/dt* pulsed magnetic field setup was used for contactless treatment [29]. It was based on Marx circuit topology. The total discharge voltage was 23 kV, resulting in up to 5 kA current in the coil (2 layers, 6 windings), which was compatible with 0.2 ml polymerase chain reaction (PCR) sterile tubes (Quali, SC, USA). A sequence of 50 pulses was used at repetition frequency of 0.25 Hz (total treatment time of 3 min 20 s). The peak value of induced electric field in the sample was 0.2 kV/cm.

For research of the interaction between EMF and PEF procedures, the concomitant delivery of magnetic field pulses after electric field pulses was used. The delivery of electric pulses after magnetic pulses was not studied due to the limitations of the experimental setups.

The waveforms of applied pulses are shown in Fig. 1.

2.2. Thymocyte and splenocyte preparation for cell permeabilization assay

The 6–9 weeks old BALB/c mice were killed by cervical dislocation. All applicable national and institutional guidelines were followed (license Nr. LT61-903). Thymus and spleens were removed and mashed through the cell strainer into the 3.5 cm Petri dish with RPMI medium (ThermoFisher Scientific Inc., USA). Cells were centrifuged at 300 ×*g* for 5 min at room temperature. Thymocytes were resuspended in RPMI medium at concentration of 3×10^7 cells/ml. Splenocytes were resuspended in 15 ml of 0,16 M NH_4Cl to lyse erythrocytes and incubated for 5 min before centrifugation. The centrifuged cells were resuspended in RPMI medium at concentration of 3×10^7 cells/ml.

2.3. Permeabilization assay

For analysis of cell permeabilization the YO-PRO-1 (YP) (ThermoFisher Scientific Inc., USA) fluorescence dye was used. Just before electroporation 63 μl of cell suspension were mixed with 7 μl of 10 μM YP to obtain final dye concentration of 1 μM. For PEF permeabilization, the 60 μl of the resultant suspension was transferred to electroporation cuvette. After electroporation, 30 μl (half of initial 60 μl) was instantly transferred to PCR tube, following by exposure to EMF (50 pulses). Further, the PEF only and PEF + EMF treated cells were transferred to separate 1.5 ml tubes (Eppendorf, Hamburg, Germany), incubated for additional 10 min at room temperature and analyzed using flow cytometry (Amnis, Seattle, USA). The visualization of the applied treatment methodology is presented in Schematic 1.

As it can be seen in Schematic 1 the total treatment time was >3 min. Application of high current pulses results in Joule heating, however in this work the coil has been designed taking into account the thermal effects. Firstly, the high cross-section copper wire was used (1.5 mm × 2 mm), which ensured lower active resistance and higher thermal energy dissipation. Secondly, the pulses were submicrosecond with a total oscillating waveform energy of 5–6 J. After the 50 pulses (3.3 T, 0.25 Hz) sequence the measured

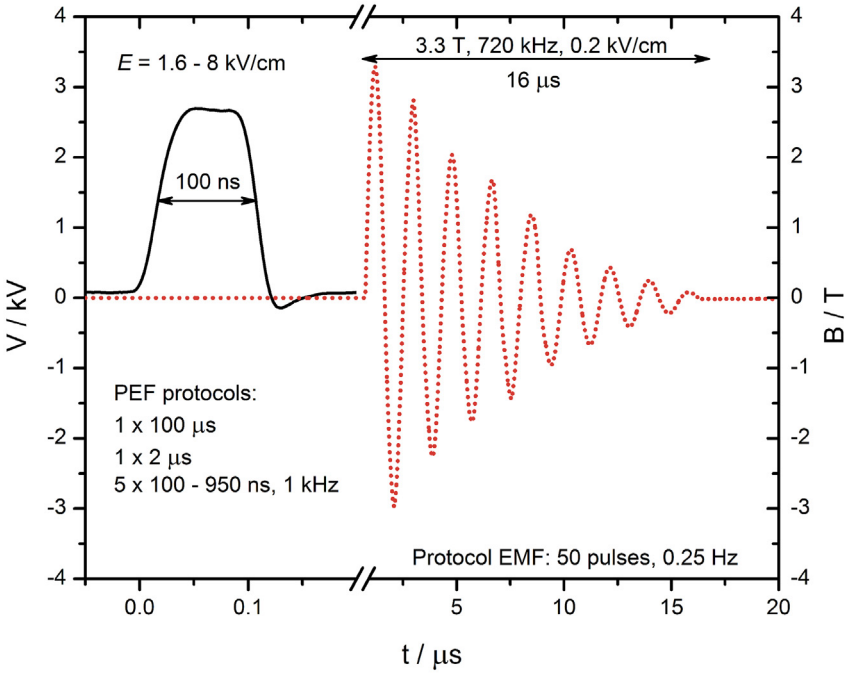

Fig. 1. The waveforms of the applied nanosecond electric and magnetic field pulses. The pulses have been measured using a calibrated loop sensor (VGTU, Vilnius, Lithuania), a DPO4034 oscilloscope (Tektronix, Beaverton, OR, United States), post-processed in OriginPro Software (OriginLab, Northampton, MA, United States).