Activation of proinflammatory cytokine IL-1β upon electroporation

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Abstract *- Electroporation is a frequently used method for increasing permeability of the cell membrane, but can also cause damage to the cell: cytosol leakage, ROS formation, osmotic swelling, necrosis and induction of apoptosis. Cell stress and cell damage can also trigger inflammasome activation. In this paper we analysed weather electroporation of macrophages* in vitro *can trigger NLRP3 inflammasome activation and subsequent secretion of IL-1β. Cells were exposed to electric pulses and quantity of secreted IL-1β was determined. Our results show that the observed IL-1β secretion was not NLRP3 inflammasome dependent but indicate that nevertheless, electroporation triggers a proinflammatory immune response through IL-1β secretion.*

1 Introduction

Electroporation is a physical method that uses externally applied electric fields to transiently increase the permeability of the cell membrane by formation of hydrophilic pores, through which substances like nucleic acids or membrane impermeable drugs can enter the cells [1]. The number, surface area and stability of the pores, which determine electroporation efficiency, depend on parameters of electric pulses like electric field strength, duration, number of pulses and repetition frequency [2] and need to be optimized for the chosen application and the used cell type. Electroporation is used for gene transfection, cell fusion, insertion of proteins into the cell membrane, electrochemotherapy and others [3].

However, electroporation also causes cell damage and cell stress. Although longer permeated state of the membrane enables the passage of more hydrophilic molecules of interest into the cell, at the same time cytosolic metabolites escape the cell interior, affecting cell homeostasis [4]. Also, reactive oxygen species can be generated at the permeabilized areas [5] and osmotic swelling may occur [6], which can also lead to rapture of the membrane. Increased membrane permeability may change the balance of ions inside the cell, activate cellular nucleases, damage cellular DNA directly and can lead to apoptosis [7].

Cell stress and cell damage can also trigger inflammasome activation. Inflammasomes are protein complexes expressed in myeloid cells and participate in the innate immune system. The most investigated NLRP3 inflammasome is composed of a sensor NLRP3 (NACHT, LRR and PYD domains-containing protein 3), an adaptor ASC (apoptosis-associated speck-like protein) and a pro-caspase-1. Upon activation inflammasomes self-assemble, which leads to the autoactivation of pro-caspase-1. The active caspase-1 in turn cleaves and activates pro-IL-1β and pro-IL-18 [8], [9]. These cytokines trigger the proinflammatory response to pathogen-associated molecular patterns as well as endogenous 'danger signals'[8].

Several different signals have been shown to activate NLRP3-inflammasome, from ATP [10], microbial pore-forming toxins [11], serum amyloid A [12], prion proteins [13], uric acid crystals [14], cholesterol crystals [15] and others. In this paper we analysed if electroporation of macrophages *in vitro* also triggers NLRP3 inflammasome activation and subsequent secretion of IL-1β.

2 Material and Methods

Cell Culturing

Immortalized macrophages from NLRP3-deficient mice (NLRP3-KO) and corresponding wild-type control (WT)(C57BL/6) were a kind gift of K. A. Fitzgerald and prepared as described previously [16]. Cells were cultured in DMEM supplemented with 10% FBS. For electroporation experiments, cells were seeded in 8 well LabTek Chambered Slides (Nunc).

Permeabilization

The permeabilization threshold and the extent of permeabilization were obtained by electroporation of cells in RPMI (Sigma Aldrich) electroporation buffer containing 0.15 mM propidium iodide (PI) (Sigma-Aldrich). Cells were electroporated with 8×2 ms pulses with repetition frequency of 1 Hz and increasing electric field strength (from E = 0.3 kV/cm to E = 1.2 kV/cm with 0.1 kV/cm step increase). Cell

in negative control (NC) were not exposed to electric pulses while cells in positive control (PC) were electroporated with E = 1.4 kV/cm to obtain maximal permeabilization. After 3 min incubation at room temperature to allow PI to enter the permeabilized cells, electroporation buffer was removed and the PI fluorescence intensity was measured using spectrofluorimeter Tecan Infinite M200 (Tecan, Grödig, Austria).

Electroporation

RPMI cell culture medium and low conductivity iso-osmolar electroporation buffer NaPB (10 mM Na_2PO_4/NaH_2PO_4, 1mM $MgCl_2$, 250 mM sucrose; pH 7.4; Sigma-Aldrich) were used as electroporation buffers. Before pulsation, growth medium was removed and 200 µl of electroporation buffer was added to each well. Cells were then incubated for 10 min at 37° to allow the cell membrane to reseal, after which 90 µl medium was removed for IL-1β measurement. Cells in control sample were treated with the same protocol, but without exposure to electric pulses (E = 0 kV/cm).

Electric pulses were generated by Jouan GHT 1287B generator (Jouan, st. Herblain, France). A pair of parallel wire electrodes with 7 mm distance between them (d) was used. All pulsing protocols consisted of 8 consecutive square pulses of frequency 1 Hz and 2 ms duration. Three amplitudes of electric fields (E) were used: 0.9 kV/cm (U = 630 V), E = 1.1 kV/cm (U = 770 V) and E = 1.3 kV/cm (U = 910 V). Electric field strength (E) can be calculated by the formula E = U / d, where U denotes applied voltage and d the electrode distance.

IL-1β ELISA

Cells were primed with ultra-pure LPS (100 ng/ml, Invivogen) in serum-free DMEM for 5–6 h after which medium was removed and replaced with electroporation buffer. For positive control, inflammasome activator ATP in DMEM was added for 1 h. The concentration of secreted IL-1β was measured by ELISA (e-Bioscience) according to manufacturer's instructions.

3 Results and Discussion

Macrophages are important cells of the immune system. Their primary role is to recognize, internalize and mediate the interactions with microbial and altered-self components through a range of plasma membrane receptors. Consequently they can trigger an appropriate immune response through secretion of various cytokines and other signalling molecules [17]. An important response pathway is also activation of the NLRP3 inflammasome, which makes macrophages suitable cells for the study of inflammasome activation.

Permeabilization

To verify the role of NLRP3 inflammasome in macrophage response to electroporation, wild type (WT) macrophages and NLRP3 inflammasome knock-out (NLRP3-KO) macrophages were used. Optimal pulsing protocols for both cell lines were determined with permeabilization experiment. Cells were exposed to pulse protocols with increasing field strength in the presence of membrane impermeable fluorescent dye propidium iodide (PI). PI fluorescence intensity is thus a measure of permeabilization efficiency. Effect of increasing electric field strength on permeabilization of WT and NLRP3-KO cells is shown in Figure 1. The permeabilization threshold was between 0.3 and 0.4 kV/cm for WT (Figure 1A) and between 0.4 and 0.5 kV/cm for NLRP3-KO macrophages cells (Figure 1B). Consistently with increasing electric field strength, PI fluorescence intensity increased, indicating that more cells were electroporated. Based on these results, pulses with E = 0.9 kV/cm, E = 1.1 kV/cm and E = 1.3 kV/cm were used for further experiments.

NLRP3 inflammasome activation

Activation of inflammasome was observed through secretion of IL-1β, the effector molecule of the

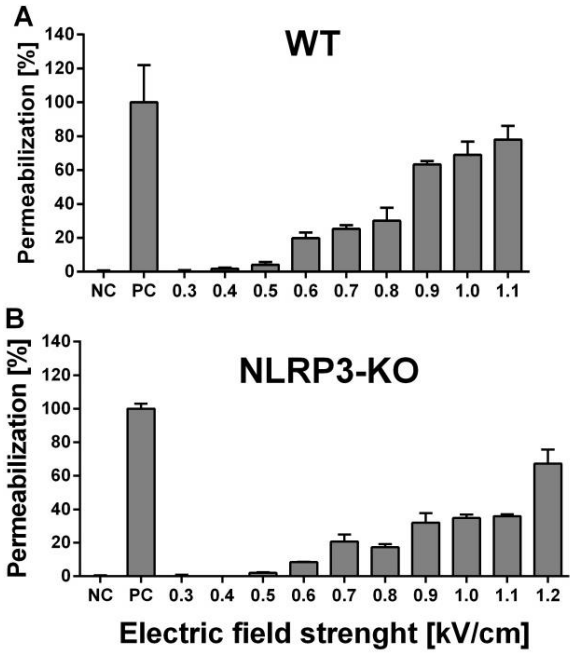

Figure 1: Effect of increasing electric field strength on permeabilization of macrophages for (A) wild type (WT) cells and (B) NLRP3-deficient (NLRP3-KO) cells. Cells were electroporated in the presence of 0.15 mM PI using a train of 8 pulses of 2 ms and increasing field strength E. Cells in the negative control sample (NC) were not electroporated (E = 0 kV/cm) and cells in the positive control (PC) were electroporated with pulses with E = 1.4 kV/cm. The PI fluorescence intensity was measured using spectrofluorimeter. Mean and standard error for two experiments are shown.

activated pathway. IL-1β activation, however, is a two-step process. NF-κB activation is required to initiate transcription of pro-IL-1β and the inflammasome activation to produce the active form of IL-1β by caspase-1 mediated proteolysis of its precursor [9].

To activate NF-κB and initiate transcription of pro-IL-1β, cells were primed for 6h with LPS, after which electroporation was performed to potentially activate the inflammasome. 10 min after electroporation, IL-1β release was determined with ELISA.

Interestingly, electroporation triggered IL-1β secretion in both WT and NLRP3-KO macrophage cells (Figure 2). Secretion increased with increasing field strength as expected, but despite having a non-functional NLRP3 inflammasome, NLPR3-KO macrophages secreted more IL-1β than WT cells. This was observed for electroporation in both electroporation buffers; RPMI medium (Figure 2A) and NaPB electroporation buffer (Figure 2B). These results indicate that electroporation triggers the release of IL-1β, but the activation is not dependent on NLRP3 inflammasome.

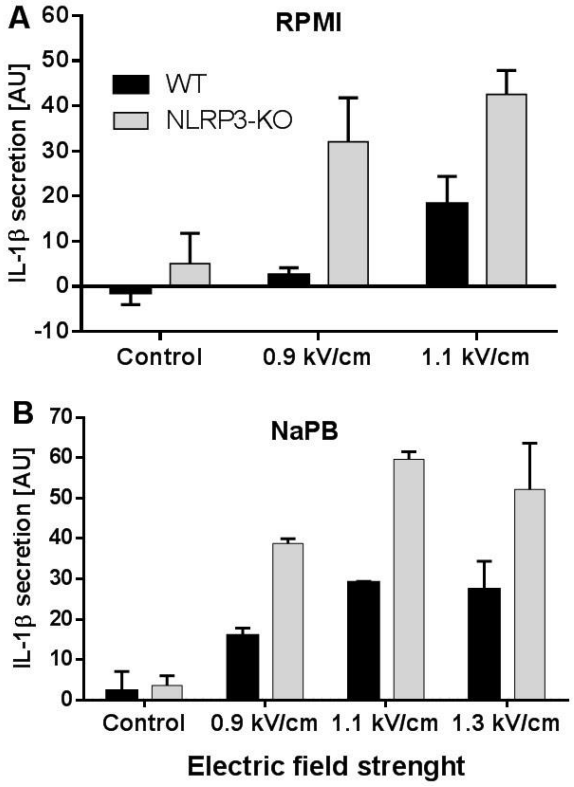

Figure 2: Wild type (WT) and NLRP3 inflammasome knock-out (NLRP3-KO) macrophages release IL-1β upon electroporation in both (A) RPMI electroporation buffer and in (B) low conductivity iso-osmolar electroporation buffer NaPB. Immortalized WT and NLRP3-KO macrophages were primed for 6h with LPS and electroporated. IL-1β release was assessed 10 min after electroporation with IL-1β ELISA. Mean and standard error of two experiments is shown.

Membrane pore formation upon electroporation causes several events that can trigger an immune response and IL-1β release. Cytosolic cell components can leak through the pores and together with remnants of necrotic cells bind to damage-associated molecular pattern (DAMP) receptors, which recognize atypically located self-components [18]. Activation of these receptors induces immune responses that are critical for host defence and tissue repair programs. One of such NLRP3 inflammasome activators is ATP [10], which also leaks out of the electroporated cells. Similarly, ROS formation on electroporated portions of the membrane could activate the NLRP3 inflammasome as well [19]. Another trigger of caspase-1 activation is thought to be the efflux of cytosolic K^+ [8].

Despite that, the IL-1β release that we observed was not NLRP3 inflammasome dependent. The observed IL-1β activation might have been triggered by another member of NLRP protein family or NLRP-related protein called IPAF (ICE protease-activating factor), which have also been shown to activate caspase-1 [20]. Moreover, IL-1β can also be activated independently of caspase-1 activation [21].

In conclusion, our experiments showed that electroporation can trigger release of IL-1β independently of NLRP3 inflammasome, which elicits a proinflammatory response. Further experiments are required to determine the exact mechanism of IL-1β activation.

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