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The total electric charge and time of application of galvanic currents to macrophages can optimize the release of IL-1β with low cell death

Alejandro Peñin-Franch[1,4], José Antonio García-Vidal[1,2,4], Ana Isabel Gómez[1], Pilar Escolar-Reina[1,2], Francesc Medina-Mirapeix[1,2]✉ & Pablo Pelegrín[1,3]✉

Galvanic current has been emerging as a novel therapy to regenerate chronic tissue lesions, including musculoskeletal and dermatological lesions. Recently, the NLRP3 inflammasome and IL-1β release have been identified as a signaling pathway triggered upon galvanic current application. However, the parameters for the clinical application of galvanic current remain subjective to the experience of the facultative in charge. In this study we used an in vitro model of macrophage culture and application of different combinations of the parameters of galvanic current to study IL-1β production and cell death. Increasing electric charge of galvanic current induces the release of IL-1β, but electric charges equal or higher to 144 mC also increase cell death. The release of IL-1β have a substantial variation within different electric charge of galvanic currents, being increased by decreasing the current and increasing the time of current application. Within the range of current intensities studied, the most optimal protocol for maximizing IL-1β release without inducing cell death was identified at electric charges equal to or near 144 mC, applied over a total duration of approximately 25 s. Our findings lay the groundwork for future in vivo studies assessing different electric charge of galvanic current, with the aim of yielding clinically relevant outcomes.

Keywords Percutaneous needle electrolysis, Galvanic current, Macrophage, Inflammation, IL-1β, Cell death

Inflammation is a tightly regulated host response to infection and tissue injury, aiming to eliminate invading pathogens or restoring tissue homeostasis[1]. The coordination of inflammation involves the production of various cytokines that activate different immune and non-immune cells to coordinate an effector response. However, if cytokine production persist over time, inflammation can becomes a chronic process, leading to tissue disfunction and the onset of various diseases[2]. Among the cytokines involved in inflammation, interleukin (IL)-1β plays a crucial role in the host response to sterile injuries and tissue damage. IL-1β signaling blockage increases not only the risk of infection, but also decreases effective wound healing, confirming that IL-1β is important to initiate an inflammatory response that latter promote tissue repair[3,4]. The production of IL-1β is a tightly controlled process by the activation of inflammasomes, which are multiprotein complexes that promote caspase-1 activation and the subsequent maturation of IL-1β[5]. Of all the inflammasomes, the one formed by the nucleotide-binding domain and leucine-rich repeat receptor with a pyrin domain 3 (NLRP3) is the most extensively studied and implicated in tissue healing[6,7]. It can be activated in response to a wide array of danger signals that disrupt intracellular homeostatic conditions[8,9]. Almost all NLRP3 activators can induce a decrease in intracellular K^+, leading to conformational changes in the inactive NLRP3 structure, which in turn leads to inflammasome formation and caspase-1 activation[10–12]. Caspase-1 is a protease that processes IL-1β into its bioactive form and promotes its cellular release by inducing a specific type of necrotic cell death known as pyroptosis[13]. Therefore, disturbances in the permeability of the plasma membrane are potent inducers of the NLRP3 inflammasome and IL-1β release. Recently, it was discovered that applying a galvanic current to macrophages induces cellular K^+ efflux and the subsequent activation of NLRP3 in macrophages, leading to the release of IL-1β[7].

[1]Biomedical Research Institute of Murcia IMIB-Pascual Parrilla, Murcia, Spain. [2]Department of Physical Therapy, University of Murcia, Murcia, Spain. [3]Department of Biochemistry and Molecular Biology B and Immunology, University of Murcia, Murcia, Spain. [4]Alejandro Peñin-Franch and José Antonio García-Vidal contributed equally to this work. ✉email: mirapeix@um.es; pablopel@um.es

In vivo, the activation of NLRP3 in response to galvanic current was found to be crucial in initiating a tissue repair response in the tendon by enhancing collagen deposition[7]. This could shed light on the clinically beneficial effects of the galvanic current therapy, which has been successfully used in the repair of various musculoskeletal and dermatological lesions, such as tendinopathies and mammary fistula[14–18]. This therapy is applied through a needle, using protocols with a electric charge defined by parameters of current, duration, and the number of pulses or repetitions[17]. However, two recent systematic reviews reported that the choice of dosage for musculoskeletal lesions was inadequately supported by scientific evidence from studies comparing two or more protocols. These studies revealed significant variability and a lack of consensus on the parameters and the total electric charge, which often is less than 80 mC[17,19]. Protocols aimed at dermatological lesions, which often use higher electric charges were not compared either. In this study, we used different protocols for the application of galvanic current, starting from 3 mA and 36 mC of total electric charge, in in vitro cultures of macrophages to study the conditions that maximize IL-1β release while maintaining low toxicity. We found that the production of IL-1β was not always higher when a higher current (3, 6 or 12 mA) was used. Interestingly, with the same total galvanic electric charge, the duration of current application was found to be a significant factor inducing IL-1β release.

Material and methods

Cell culture and treatments

Bone marrow-derived macrophages (BMDMs) were obtained from wild-type C57/BL6 mice. No live mice were utilized in the study, as we used bone marrow from mice humanely euthanized by CO_2 inhalation. All methods were performed according with the Spanish national (RD 1201/2005 and Law 32/2007) and EU (86/609/EEC and 2010/63/EU) legislation, as well as in accordance with ARRIVE guidelines. In this study, no in vivo procedure is performed and according to above mentioned legislation no ethics committee approval is required.

Mouse bone marrow cells were obtained from leg bones of mice (8–10 weeks of age). Femurs and tibia were removed, and the bone marrow flushed out. The bone marrow cells were resuspended in L-929 conditioned medium composed of DMEM (Lonza) supplemented with 25% L-929 cultured medium, 15% fetal bovine serum (FCS, Life Technologies), 100 U/ml penicillin/streptomycin (Lonza), and 1% L-glutamine (Lonza). L-929 cultured medium was used as a source of macrophage-colony stimulating factor (M-CSF) and was obtained by culturing L-929 mouse fibroblast cell line (ATCC# CCL-1) in DMEM high-glucose (Biowest), with 5% FCS and 100 U/ml penicillin/streptomycin until confluence for 7 days, media was then collected from different cell culture flasks, pulled, filtered through 0.22 µm filters (Millipore) and stored at – 80 °C until use. Bone marrow cells were plated in 150-mm dishes and cultured at 37 °C in the presence of 5% CO_2 with L-929 conditioned medium. After 7 days, the resulting BMDMs were detached by gentle scraping with cold PBS, replated into 6-well plates at a density of 0.42×10^6 cells/cm^2, and used the following day. The macrophage purity of these preparations was ~ 90%, measured routinely by flow cytometry using murine macrophage antigen F4/80. BMDM culture medium was replaced the day after seeding with fresh medium, and cells were primed for 2 h using 1 µg/ml *E. coli* lipopolysaccharide (LPS) serotype O55:B5 (Sigma-Aldrich). The cells were then washed twice with an isotonic buffer composed of 147 mM NaCl, 10 mM HEPES, 13 mM glucose, 2 mM $CaCl_2$, 1 mM $MgCl_2$, and 2 mM KCl, pH 7.4. Subsequently, the cells were treated in OptiMEM (Lonza) with varying intensities and durations of galvanic current (as detailed in the text, figures and figure legends). The cells were then cultured for an additional 6 h. In certain experiments, the specific NLRP3 inhibitor MCC950 (aka CRID3 or CP-456,773, Sigma-Aldrich) was used at a concentration of 10 µM during the galvanic current application and for the subsequent culture period.

Application of electrical current

As previously described[7], the application of galvanic current was current-driven using Physio Invasiva device (Prim) with a custom adaptor for six-well cell culture plates (Fig. 1A) with two electrodes of the same area (8.3 cm^2) are on the same well and were made of surgical steel coated with platinum (30 nm). For this coating and in order to avoid oxidation/reduction of the electrodes, sputtering (Physical Vapour Deposition) was used using the Leica EM ACE 600 High Vacuum sputter Coater device (*Leyca Microsystems GmbH). Using an angled sputter head with stage rotation, this device is capable of generating a most homogeneous profile, minor thickness variations across sample dish and best control of layer thickness. A series of sequential experiments were conducted to analyze the impact of changes in the parameters of galvanic current on IL-1β release. The parameters used in these experiments were electric charge (Q), current (I) and total time (t). The total time depends on the duration and number of pulses, and the total electric charge is determined by the equation $Q = I \; x \; t$. All tested protocols fell within four different electric charges: 36, 72, 144 and 288 mC. The first three electric charges align with the clinical practice of galvanic therapy in humans[17–19], while the last one was selected to explore the effects of higher electric charges. Within each electric charge, variations of the parameters were established as specified in the figure legends. In general, following standard clinical practice[17–19], the current varied between 3 and 6 mA, the number of pulses ranged from 2 to 4, and the duration of pulses was between 3 and 6 s. In the experiments, one of these parameters (i.e., the current, number and/or duration of pulses) was fixed, while the remaining parameters were varied (i.e., doubled or halved). In each experiment, the potential difference (ΔV) and Joule heating effects (Joules) were recorded by Physio Invasiva equipment, and currents densities (J) and charge densities (σ) were calculated.

LDH release measurements

The Cytotoxicity Detection kit (Roche) was used to measure the activity of lactate dehydrogenase (LDH) following the manufacturer's instructions and read in a Synergy Mx plate reader (BioTek). LDH activity was determined in cell supernatants of cells exposed to the different galvanic current protocols as specified in the

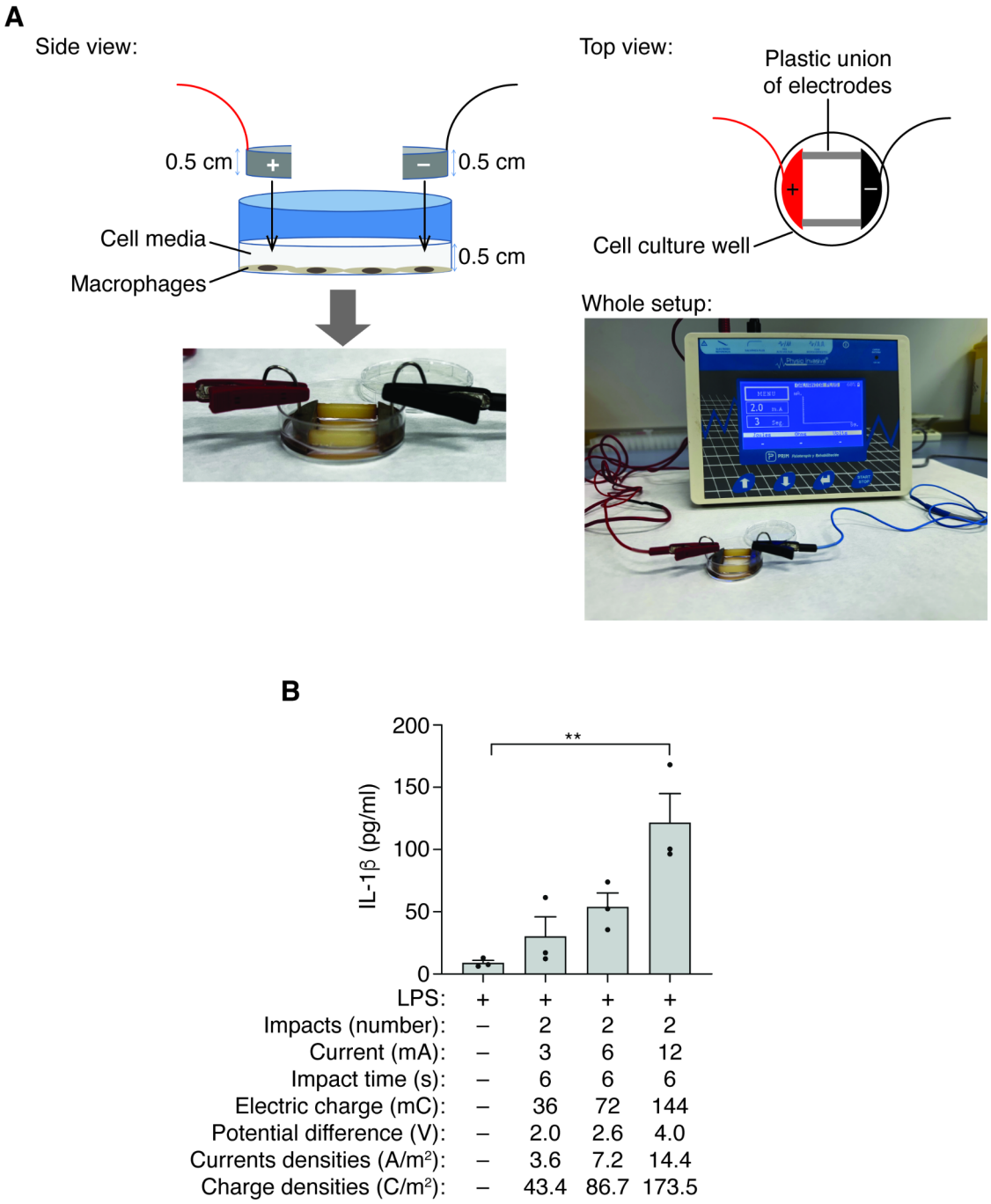

Fig. 1. IL-1β release is induced by increased current. (**A**) Custom adaptor for six-well cell culture plates used in this study to apply galvanic current to macrophage cultures. The two electrodes present the same area (8.3 cm^2) and were made of surgical steel and coated with platinum. (**B**) IL-1β release from mouse bone marrow–derived macrophages (BMDMs) initially treated with LPS (1 μg/ml) for 2 h, then subjected to varying intensities of galvanic current (3, 6, 12 mA, as indicated in the figure) and incubated for an additional 6 h. The central values in the figure represent the mean, while the error bars denote the standard error of the mean (s.e.m.). The data is based on 3 independent experiments. Statistical analysis was performed using a one-way ANOVA, with significance levels indicated as follows: $^{**}p < 0.005$.

figure legends. In the same experiments, LDH activity was also measured in cell lysates obtained by incubating the cells in ice-cold lysis buffer (50 mM Tris-HCl pH 8.0, 150 mM NaCl, 2% Triton X-100) supplemented with 100 µl/ml of protease inhibitor mixture (Sigma-Aldrich) for 30 min on ice and centrifugated at 13,000×*g* for 10 min at 4 °C. The results of the extracellular amount of LDH in galvanic current treated cells was expressed as a percentage of the total amount of LDH present in the lysates of non-treated cells considered as a 100% of the total LDH.

ELISA

After cell stimulation, the concentration of IL-1β in cell supernatants was determined using an ELISA kit purchased from R&D Systems, following the manufacturer's instructions. The results were read using a Synergy Mx plate reader (BioTek).

Yo-Pro-1 uptake assay

Following the stimulation of macrophages with various galvanic current conditions, 2.5 µM of Yo-Pro-1 iodide (Life Technologies) was added. The number of Yo-Pro-1 fluorescent cells was measured after 5 min incubation period using a Nikon Eclipse Ti microscope, equipped with a 20×S Plan Fluor objective (numerical aperture 0.45), a digital Sight DS-QiMc camera (Nikon), a 482 nm/536 nm filter set (Semrock) and NIS Elements software (Nikon). Cell counting was performed using ImageJ software (National Institutes of Health). The total count was derived from 14,092 cells (ranging from 2400 to 4200 cells per condition) across 2 to 3 independent experiments for each condition.

Statistics

Statistical analyses were conducted using GraphPad Prism 9 (Graph-Pad Software, Inc). Initially, a Shapiro–Wilk normality test was performed on all groups to determine the appropriate analysis type. For two-group comparisons, either a nonparametric Mann–Whitney *U* test (without assuming normal distribution of the values) or a parametric unpaired *t*-test (for normally distributed data) was used to ascertain statistical significance. For comparisons involving more than two groups, either a nonparametric Kruskal–Wallis test (without assuming normal distribution of the values) or a parametric one-way ANOVA test (for normally distributed data) was used to determine statistical significance. Data are presented as mean values, with error bars representing the standard error derived from the number of independent assays indicated in the figure legend. These are also overlaid on the histograms as dot plots. *P*-values are indicated are indicated in figure legends, with $p > 0.05$ considered not significant (*ns*).

Results

We have previously found that the application of galvanic current to cultured macrophages triggers the activation of the NLRP3 inflammasome and the subsequent release of the pro-inflammatory cytokine IL-1β[7]. In this study, we sought to determine whether alterations in the various parameters of galvanic current could induce changes in IL-1β release. Initially, we replicated the findings of our previous study[7] and observed that the release of IL-1β increased in a manner dependent on the current and density of the current (Fig. 1B). Therefore, by solely modifying the current (and consequently the electric charge), we were able to regulate the release of pro-inflammatory cytokines. However, this increase was not statistically significant when comparing macrophages treated with 3 mA and 36 mC ($J = 3.6$ A/m^2 and $\sigma = 43.4$ C/m^2). to untreated cells. Joule heating effects showed a range that varied from 0.03 to 0.41 Joules.

We next investigated whether variations in the parameters of galvanic current related to total time (number and duration of pulses) could induce changes in IL-1β release without altering the electric charge, current, or total time. We tested this by combining these two parameters in three different electric charges (72, 144 and 288 mC), while keeping the current and the total time constant to 24 s for each one. For a $Q = 72$ mC with a constant current of 3 mA ($J = 3.6$ A/m^2 and $\sigma = 86.7$ C/m^2), we observed an increase in IL-1β release when the pulse duration was increased, rather than when the number of pulses were increased (Fig. 2A). However, when both parameters (pulse duration and number of pulses) were altered, there were no significant differences in IL-1β release for electric charges of 144 mC at 6 mA ($J = 7.2$ A/m^2 and $\sigma = 173.5$ C/m^2), and 288 mC at 12 mA ($J = 14.4$ A/m^2 and $\sigma = 347$ C/m^2) (Fig. 2B,C).

We then asked whether changes in the current and duration could induce a variation in IL-1β release without modifying Q. We tested this by combining these two parameters in two different electric charges (72 and 144 mC), while fixing one of the parameters related to total time (either number or duration of pulses). As the release of IL-1β was clearly dependent on the duration of the pulses at 72 mC, we decide to fix the number of pulses for this electric charge. We found that the increase of IL-1β release following two impacts at $Q = 72$ mC was dependent on the current and duration (i.e., applying 6 mA for a duration of 12 s increased the release of IL-1β more than applying 12 mA for 6 s) (Fig. 3A). In this line, the protocol applying 3 mA for 24 s at an electric charge of 72 mC resulted in a higher increase in IL-1β release ($p = 0.0049$) than the protocol of applying 6 mA for 6 s (i.e., decreasing current and doubling pulse duration) (Fig. 2A). In contrast, for $Q = 144$ mC, a protocol with a current of 3 mA applied over a total time of 48 s induced a significantly higher release of IL-1β than a current of 12 mA applied over 12 s (Fig. 3B). However, that protocol (3 mA over 48 s) induced a similar IL-1β release ($p = 0.1556$) to the protocol of 144 mC obtained with 6 mA over 24 s (i.e., halving the number of pulses and doubling the current) (Fig. 2B). All these protocols activated the NLRP3 inflammasome, as evidenced by the decrease in IL-1β release following treatment with MCC950 (Fig. 3C,D). An increase in the total electric charge led to an increase in the percentage of permeabilized macrophages (Fig. 3E). However, no differences were observed in the number of permeabilized cells when different parameters were used for the 72 and 144

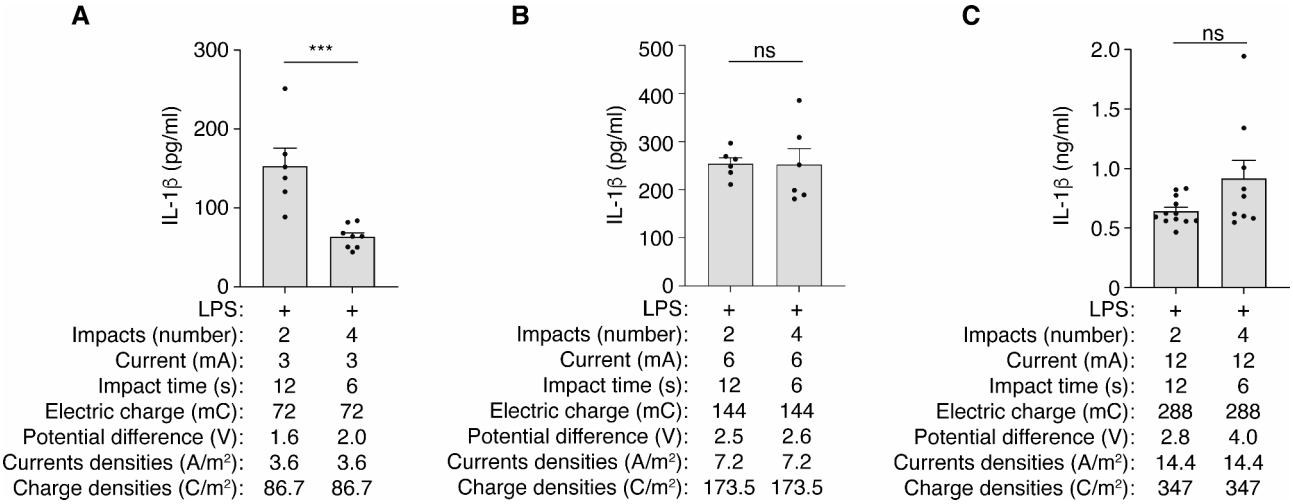

Fig. 2. Enhanced IL-1β release with increased impact duration at a galvanic current electric charge of 72 mC. (**A**) IL-1β release from mouse bone marrow–derived macrophages (BMDMs) treated with LPS (1 µg/ml) for 2 h, followed by the application of different protocols involving an increasing duration or number of impacts of 3 mA of galvanic current at an electric charge of 72 mC (as indicated in the figure) and then incubated for an additional 6 h. (**B**) IL-1β release from BMDMs treated as explained in panel A but with an increased current of 6 mA of galvanic current at electric charge of 144 mC (as indicated in the figure). (**C**) IL-1β release from BMDMs treated as explained in panel A f but with an increased current of 12 mA of galvanic current at electric charges of 288 mC (as indicated in the figure). The central values in the figure represent the mean, while the error bars denote the standard error of the mean (s.e.m.). The data is based on 6–12 independent experiments. A *t*-test was used for statistical analysis in (A) and (B), while a Mann–Whitney test was used in (C). Significance levels are indicated as follows: $***p < 0.0005$, *ns* $p > 0.05$.

mC conditions (Fig. 3E), supporting the idea that cell permeabilization was not dependent on inflammasome activation, as we previously described[7].

Membrane permeabilization precedes cell death and our previous findings indicated an increase in LDH leakage from the cell under certain high charges of galvanic current[7]. Consequently, we aimed to investigate whether alterations in the parameters (current and duration), without changing the electric charge, could also induce variations in LDH leakage. We compared to protocols at 72 and 144 mC, which demonstrated the highest increase of IL-1β release. At 72 mC, both tested protocols (3 mA and 6 mA) showed no significant differences in LDH leakage (Fig. 4A). In contrast, at 144 mC, the protocol with 3 mA exhibited significantly higher LDH leakage than the protocol with 6 mA (Fig. 4B).

Despite that the release of IL-1β was partially dependent on the different parameters of galvanic current application within certain electric charges, we decided to investigate if the total charge could accurately correlate with IL-1β release. We established a linear regression line with the data set from all galvanic application protocols and then determined its coefficient of determination, R^2. Figure 5A shows that 67.05% of the variance in the release of IL-1β can be explained by the electric charges applied to the cells. In line with this, we aimed to investigate if the data set from all galvanic application protocols could also accurately predict LDH leakage. Figure 5B shows that 60.08% of the variance in the LDH leakage can be explained by the charges of galvanic current applied to the cells.

These data suggest that, in vitro, the parameters of galvanic current can be finely tuned to control different concentrations of IL-1β release, thereby achieving optimized conditions with IL-1β release and low cell death to minimize the toxic effect of the treatment. However, a higher electric charge results in high IL-1β release and a high cell death.

Discussion

In this study, we demonstrate that an increase in the electric charge of galvanic current applied to in vitro cultured and primed macrophages results in an elevated release of IL-1β. Our findings indicate that, within a given electric charge, the release of IL-1β can be modified by adjusting the parameters of current (consistently above 3 mA), number, and/or duration of pulses during the galvanic current application. Furthermore, we discovered that an increase in electric charge is linearly related with cell death, with significant and relevant variations in cell death at a Q = 144 mC. This charge can also be adjusted by varying the parameters of the galvanic current.

In our study faradaic reactions of the current and corrosion were minimized by using surgical steel and platinum-coated electrodes[20] and this coating has been shown to produce only trace amounts of H_2O_2[21]. Nevertheless, faradaic reactions could cause electrolysis of water and oxygen reduction reactions generating reactive oxygen species, which are important triggers of the NLRP3 inflammasome[5]. We could not discard that ROS production due to the faradaic reaction will contribute to NLRP3 activation, as the peroxidation of the plasma membrane lipids, which could compromise the integrity of the plasma membrane inducing its damage

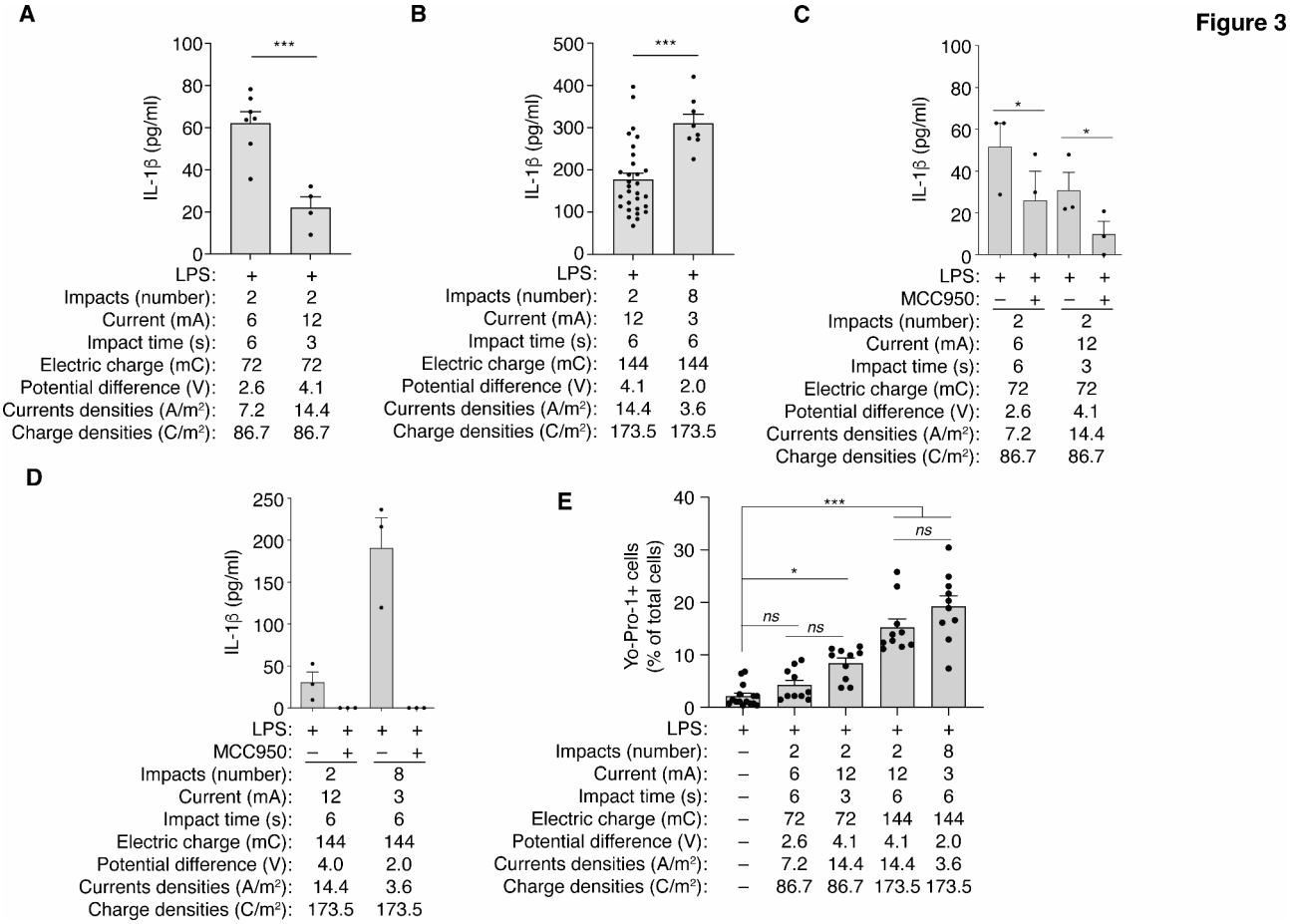

Fig. 3. Higher IL-1β release with decreased current and increased duration or number of impacts. (**A**) IL-1β release from mouse bone marrow–derived macrophages (BMDMs) treated with LPS (1 µg/ml) for 2 h, followed by the application of different protocols involving an equal number of impacts and increasing current or duration of the impacts of galvanic current at an electric charge of 72 mC (as indicated in the figure) and then incubated for an additional 6 h. (**B**) IL-1β release from BMDMs treated as explained in panel A but with equal duration of impacts and increasing current or number of the impacts at an electric charge of 144 mC (as indicated in the figure). (**C**, **D**) IL-1β release from BMDMs treated as explained in panel A (**C**) or in panel B (**D**) but treating the macrophages with the NLRP3 inhibitor MCC950 (10 µM) during and after galvanic current application (as indicated in the figure). (**E**) Percentage of Yo-Pro-1 positive BMDMs after treatment with galvanic current as explained in panel A and B (as indicated in the figure). The central values in the figure represent the mean, while the error bars denote the standard error of the mean (s.e.m.). The data is based on 3–30 independent experiments. A *t*-test was used for statistical analysis in panels A,B,C, and a Kruskal–Wallis test for panel E. Significance levels are indicated as follows: $^{***}p < 0.0005$, $^{*}p < 0.05$, *ns* $p > 0.05$.

and permeabilization upon electric pulses application[22–25]. These processes will impact on the cellular function of the macrophages, as K^+ ion leakage, NLRP3 activation and IL-1β release[5,22], as observed in our study. On the other hand, we recorded the Joule heating effect of the passage of current, showing that even with the highest electrical charges of 144 mC, it generated a minimal amount of Joules (0.41 Joules). This follows the line of other studies that, using electrodes with a smaller area and similar dosages, hardly generated any thermal variations[26].

The initial step of our study successfully replicated and validated the findings of a previous study[7], which demonstrated that increasing the electric charge of the galvanic current applied to LPS-primed macrophages (by increasing the current from 3 to 12 mA, while maintaining a constant duration) resulted in an elevated release of IL-1β. This release was attributed to the activation of the NLRP3 inflammasome and was found to contribute to tendon regeneration in an animal model[7]. Furthermore, our current study also explored the relationship between electric charge and the release of IL-1β from macrophages. We experimented with different combinations of parameters, such as increasing current while reducing duration. As a result of these comprehensive experiments, we discovered two additional significant findings.

First, we discovered that an increase in the electric charge within the range of 36 to 288 mC has a linear relationship with the release of IL-1β from macrophages. Comparisons with previous similar in vitro studies for IL-1β release are not feasible, as there are no current reports of the effect of galvanic current on inflammasome activation and IL-1β release. However, a study reported that nanoseconds pulsed electric field also induced

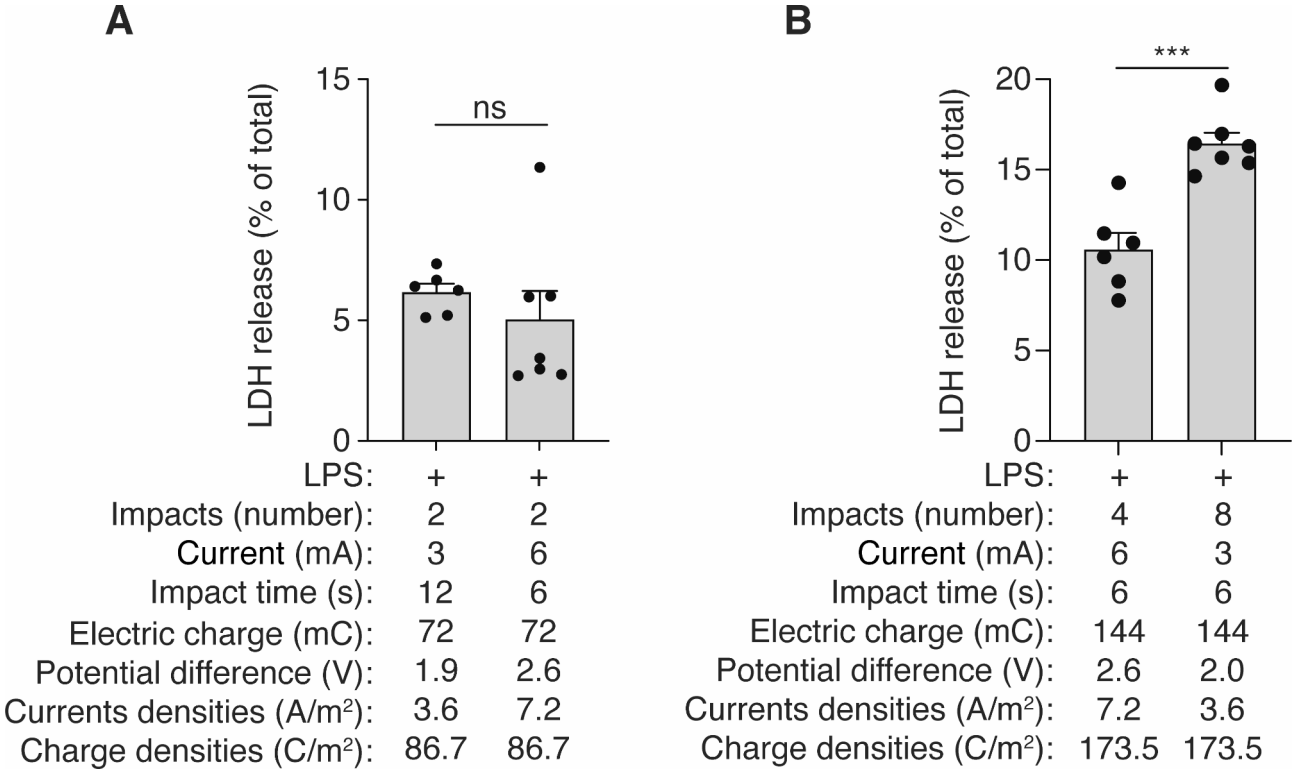

Fig. 4. Electric charges of 144 mC results in higher cell death when increase the total time. (**A**) LHD leakage from mouse bone marrow derived macrophages (BMDMs) treated for 2 h with LPS (1 µg/ml), then different protocols with equal number of impacts and increasing current or duration of impacts of galvanic current at electric charge of 72 mC were applicated (as indicated in the figure) and incubated for an additional 6 h. (**B**) LHD leakage from BMDMs treated for 2 h with LPS (1 µg/ml), then different protocols with equal duration of impacts and increasing current or number of impacts of galvanic current at electric charge of 144 mC were applicated (as indicated in the figure) and incubated for 6 h. Percentage (%) to total in the graphs means that extracellular LDH determinations are represented as a percentage of the total intracellular LDH from viable macrophages. Centre values represent the mean and error bars represent s.e.m.; $n = 5–12$ independent experiments. Mann–Whitney test for A; *t*-test for B; $^{***}p < 0.0005$, ns $p > 0.05$.

an NLRP3 dependent IL-1β release increasing with the electric charge applied to the macrophages[27], this is consistent with our results applying galvanic current. Different protocols of galvanic current have been tested in an animal model, aiming to determine the optimal physiological response of hyperirritable nodules within taut bands of skeletal muscle, also found higher effectiveness using larger charges[28]. Various clinical studies have reported clinical improvements using galvanic currents for the treatment of musculoskeletal[14–17] or dermatological lesions[18]. These improvements were observed using treatment dosages within the first half of that range (i.e., below 144 mC). To our knowledge, no study compared the clinical efficacy between different charges of galvanic current for treatment. The findings of our study could underscore research hypotheses about the appropriate dosage of galvanic current and stimulate subsequent clinical studies. This could help reduce the current uncertainty between electric charges[17,19].

Second, we discovered that the release of IL-1β exhibits significant variation across different electric charges of galvanic currents, such as 72 and 144 mC. This variation could be partially attribute to changes in the parameters of the galvanic current, which could lead to increased activation of the NLRP3 inflammasome[7]. In the case of the 144 mC, our findings indicated that the combination of the two key parameters of total time (number and duration of pulses) had similar impact on IL-1β release, and thus, they did not account the substantial variation. In contrast, our study demonstrated that the release of IL-1β generally increased with decreasing current and increasing total time of galvanic current application. This might be due to the fact that extended periods of low current intensities could cause more significant alterations to the plasma membrane, potentially leading to leakage of intracellular K^+ ion, a critical trigger for the NLRP3 inflammasome. This aligns with the understanding that intracellular K^+ efflux induces NLRP3 inflammasome activation[7,10], and also that current application can activate various ion channels in the cell, such as the voltage-dependent $K_v1.1$ K^+ channel[29]. Interestingly, we found that two protocols (3 mA applied for 48 s and 6 mA for 24 s) exhibited a maximum and similar effect in controlling IL-1β release. Therefore, our study suggests that this range of galvanic current intensities could likely be the most optimal protocol to achieve the highest IL-1β release at electric charge equal to or near 144 mC. This discovery could elucidate why typical dermatological applications of galvanic current

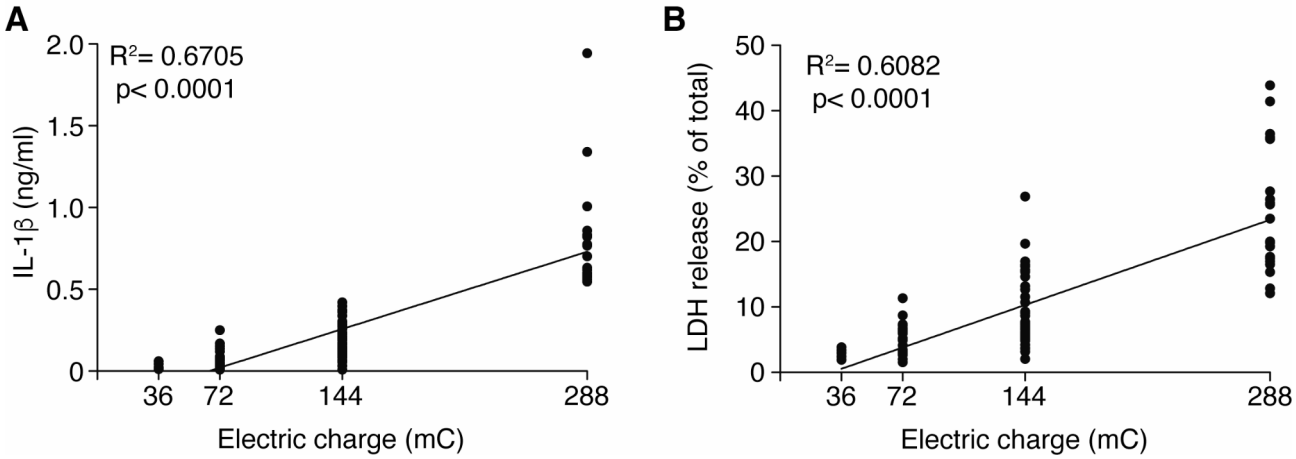

Fig. 5. Correlation of increased electric charges of galvanic current with IL-1β and LDH release. (**A**, **B**) The correlation of IL-1β release (**A**) and LDH leakage (**B**) with total electric charges of galvanic current is shown. Mouse bone marrow–derived macrophages were treated with LPS (1 μg/ml) for 2 h, followed by different protocols of galvanic current with total electric chargess of 36, 72, 144, and 288 mC (as indicated in the figure), and incubated for an additional 6 h. Percentage (%) to total in the graphs means that extracellular LDH determinations are represented as a percentage of the total intracellular LDH from viable macrophages. The data is based on 6–51 samples from at least 3 independent experiments. Statistical analysis was performed using least squares regression and an F-test for both (A) and (B).

use protocols within this range for the treatment of mammary fistulas (e.g., 5 mA for a total time of 25 s), and yield relevant clinical outcomes[18].

Within the electric charge of 72 mC, we identify a single protocol (3 mA applied for 24 s) that resulted in the highest increase of IL-1β release. However, we were unable to suggest an interval of intensities with a maximum effect. Nevertheless, our study also revealed that at this electric charges, the variation in IL-1β release could be explained by the parameters of the current. This suggests an increased plasma membrane permeability to K^+ with the current, and a corresponding increase in the activation of NLRP3. Indeed, the activation of the NLRP3 inflammasome is proportional to the amount of cellular K^+ efflux[10,11]. Consistent with the results at the electric charge of 144 mC, the IL-1β release also increased by decreasing current and increasing the total time of the galvanic current application. Moreover, unlike the results at the electric charge of 144 mC, the release of IL-1β increased even when the total time was adjusted by increasing the duration of the pulses and decreasing the number of pulses. This differential response between the two electric charges when the number and duration of pulses were combined suggests that the response to the parameters of the current may be complex and not systematic across all electric charges. In this context, it could be hypothesized that short applications of galvanic current might lead to a rapid closing of K^+ channels, resulting in a minor decrease of cellular K^+ and low NLRP3 activation. Further studies are needed to investigate how the application time of galvanic current affects intracellular K^+.

Based on the results obtained concerning cell death (i.e., LDH leakage exceeding approximately 15%), it appears that clinical applications of electric charges above 144 mC, and even some at 144 mC, would like result in significant tissue necrosis. This is confirmed by the measurement of plasma membrane permeabilization by Yo-Pro-1 uptake, which increased at 144 mC, but no significance was found among the different protocols used to achieve this electric charge. These findings are also in line with a previous study, which revealed that the bactericidal effect of galvanic current on *Staphylococcus aureus* also depends on the electric charge. This study found that electric charges equal to or higher than 144 mC substantially increased the bactericidal effect compared to electric charges equal to or lower than 125 mC[30]. Furthermore, as previously suggested by that study, we observed that the protocol of 3 mA for 48 s, rather than the protocol of 6 mA for 24 s (both with a total electric charge of 144 mC), resulted in higher cell death. This is probably due to severe plasma membrane disruption that cannot by recovered, making its clinical application not advisable.

Our study does have several limitations. Firstly, we observed IL-1β release from a 3 mA and 36 mC, but did not conduct experiments with galvanic current intensities below 3 mA or electric charges lower than 36 mC. The release of IL-1β from macrophages treated with 3 mA and 36 mC was higher compared to untreated cells, but this increase was not statistically significant. Although such a concentration of IL-1β could have a biological effect in vivo, intensities below 3 mA are unlikely to promote significant IL-1β release. Secondly, our experimental approach was using mouse–derived macrophages cultured in vitro, which were primed with bacterial LPS. This is a well–established model for studying NLRP3 inflammasome activation and IL-1β release. However, the in vitro response of these cultured macrophages to the different electric charges of galvanic current will undoubtedly differ from a clinical scenario. In a clinical sterile setting, there are no potential infections to prime immune cells, and a mixture of cells exposed to the current can respond with different mechanisms, not just the activation of NLRP3. Third, pH variations were not controlled, and therefore it is possible that local pH changes occur around the electrodes at the time of application due to water electrolysis. However, there is evidence that this variation will be transient and normalizes after few seconds of current application due to the Phosphate-Buffered Saline (PBS) medium used[31]. Fourth, the potential galvanostatic effect was not taken into account in the results. Our study presents the impact of electric charges ranging from 36 to 288 mC on the release of the proinflammatory cytokine IL-1β from macrophages. However, it was limited in its examination of the molecular mechanisms that control the effect of varying electric charges on IL-1β release and cell death. In conclusion, we discovered that modifying the parameters of the galvanic current can differentially regulate the release of IL-1β, helping to optimize conditions to minimize the toxic effects of the treatment.

Data availability

The datasets analysed during the current study are available from the corresponding author on reasonable request.

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Author contributions

A.P-F. and A.I.G. performed the experimental work; A.P-F., J.A.G-V., P.E-R. and P.P. analyzed the data, interpreted results, conceived the experiments, prepared the figures and paper writing; F.M-M. and P.P. conceived the project, provided funding and overall supervision of this study.

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Declarations

Competing interests

A.P-F's contract was supported by MVClinic Institute and Prim. P.P. declares that he is an inventor in a patent filed on March 2020 by the Fundación para la Formación e Investigación Sanitaria de la Región de Murcia (PCT/EP2020/056729) for a method to identify NLRP3-immunocompromised sepsis patients. P.P. is consultant and co-founder of Viva in vitro diagnostics SL. The remaining authors declare no competing interests.

Additional information

Correspondence and requests for materials should be addressed to F.M.-M. or P.P.

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