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Original research

Effect of shock waves on macrophages: A possible role in tissue regeneration and remodeling

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HIGHLIGHTS

- We report the effects of Shock Waves (SW) on macrophages activity in vitro.
- SW did not induce activation of resting macrophages.
- Low energy SW dampens the induction of the pro-inflammatory profile in M1 macrophages.
- Low energy SW promotes the acquisition of an anti-inflammatory profile with M2 macrophages.

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ABSTRACT

Introduction: Extracorporeal Shock Wave Therapy (ESWT) is broadly used as a non-surgical therapy in various diseases for its pro-angiogenic and anti-inflammatory effects. However, the molecular mechanisms translating tissue exposure to shock waves (SW) in a biological response with potential therapeutic activity are largely unknown. As macrophages take part in both the onset and amplification of the inflammatory response, and well in its resolution, we investigated the effect of SW on their biology.
Methods: Human monocyte-derived macrophages were polarized to classic (M1) pro-inflammatory macrophages or alternative (M2) anti-inflammatory macrophages and exposed to SW ad different intensities. Expression levels of marker genes of macrophage activation were measured by qPCR at different time points.
Results: SW did not induce activation of resting macrophages at any energy level used. Conversely, when used at low energy SW caused a significant inhibition of some M1 marker genes (CD80, COX2, CCL5) in M1 macrophages and a significant synergistic effect for some M2 marker genes (ALOX15, MRC1, CCL18) in M2 macrophages. SW also affected cytokine and chemokine production, inducing in particular a significant increase in IL-10 and reduction in IL-1β production.
Conclusions: Macrophage exposure to low energy SW dampens the induction of the pro-inflammatory profile characterizing M1 macrophages and promotes the acquisition of an anti-inflammatory profile synergizing with macrophage alternative activation.

1. Introduction

Since its original introduction in medicine for kidney stones treatment (lithotripsy) in the early nineties, extracorporeal shock wave therapy (ESWT) has significantly expanded its fields of clinical applications, first to musculoskeletal diseases and later on to regenerative medicine [1–3]. ESWT are presently applied to a wide range of pathologies of different origins and localization, in

Abbreviations: CCL, CC chemokine ligand; CCR, CC chemokine receptor; CXCL, CXC chemokine ligand; ESWT, extracorporeal shock wave therapy; IL, interleukin; IFN, interferon; LPS, lipopolysaccharide; SW, shock waves.

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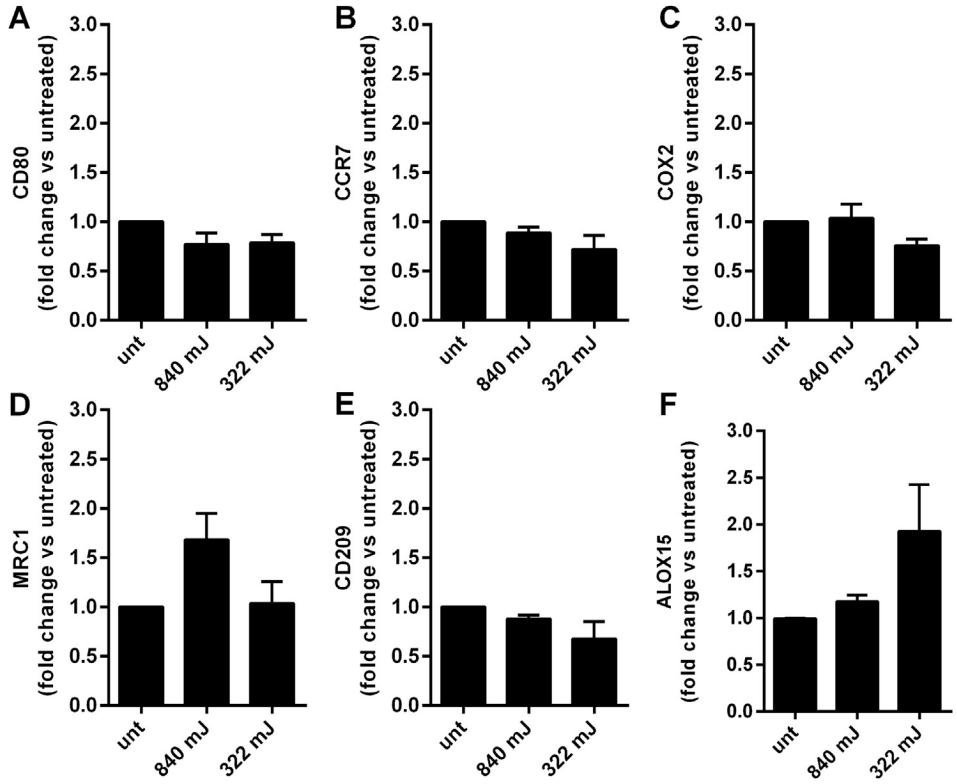

Fig. 1. SW effects on resting macrophages. Resting macrophages were exposed to 322 or 840 mJ SW and after 4 h expression levels of M1 (A–C) and M2 (D–F) markers were analyzed by qPCR. Results were normalized on the housekeeping gene GAPDH and expressed as fold enrichment compared to untreated macrophages (unt). Results are shown as mean ± SEM of 3 independent experiments.

orthopedics (tendinopathies, bone healing disturbances, vascular bone diseases), dermatology/vulnology (wound healing disturbances, ulcers, painful scars) [1], and neurology (spastic hypertonia and related syndromes) [4]. More recently, the positive effects of ESWT on soft tissues and the vascular bed have made it possible its application in clinical practice also for some andrologic disturbances (induratio penis plastic, erectyle disfunctions) [5]. Regenerative and trophic effects have also been demonstrated in ischemic and related heart diseases, although at present ESWT application in this field is still experimental [6,7].

A key point in the ESWT history has been represented by the shift from the mechanical model of lithotripsy to its applications in not-urological fields, where mechanical stimulation is converted in biological reactions in a living tissue. This phenomena is supported by mechanotransduction pathways, which imply the activation of a number of largely unknown cellular events, responsible for the positive effects of ESWT on cell metabolism and cell cycle, which ultimately account for the ductility of the therapy [8]. We can summarize the final effect of ESWT as a general improvement of tissue homeostasis and metabolism, accompanied by improving of the tissue self-healing abilities. Evidence from basic science and clinical studies indicates that this effect involves the ability of shock waves (SW) to support proliferation and differentiation of stem cells, which significantly

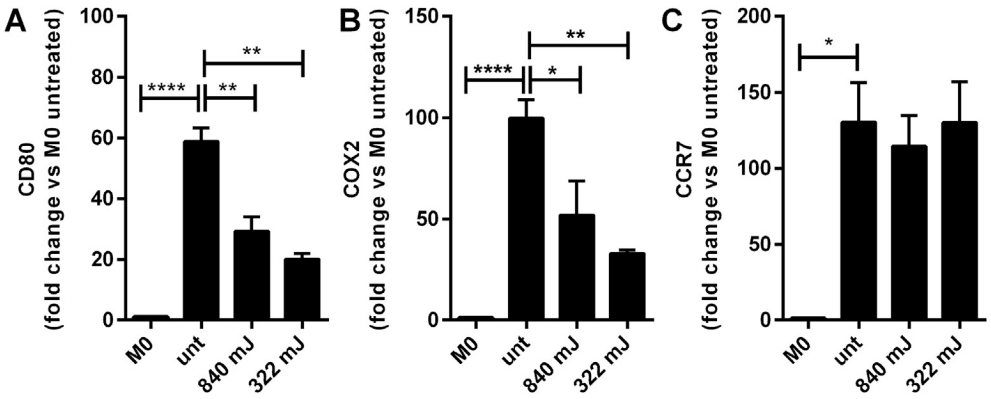

Fig. 2. SW effects on classical activated M1 macrophages. Macrophages were stimulated with LPS plus IFN-γ for 24 h to induce M1 polarization and then exposed to 322 or 840 mJ SW. After 4 h, expression levels of the M1 markers CD80 (A), CCR7 (B), and COX2 (C) were measured by qPCR. Results were normalized on the housekeeping gene GAPDH and expressed as fold enrichment compared to untreated macrophages (unt). Results are shown as mean ± SEM of 3 independent experiments. ****$p < 0.0001$, **$p < 0.01$, and *$p < 0.05$ by Bonferroni's multiple test.

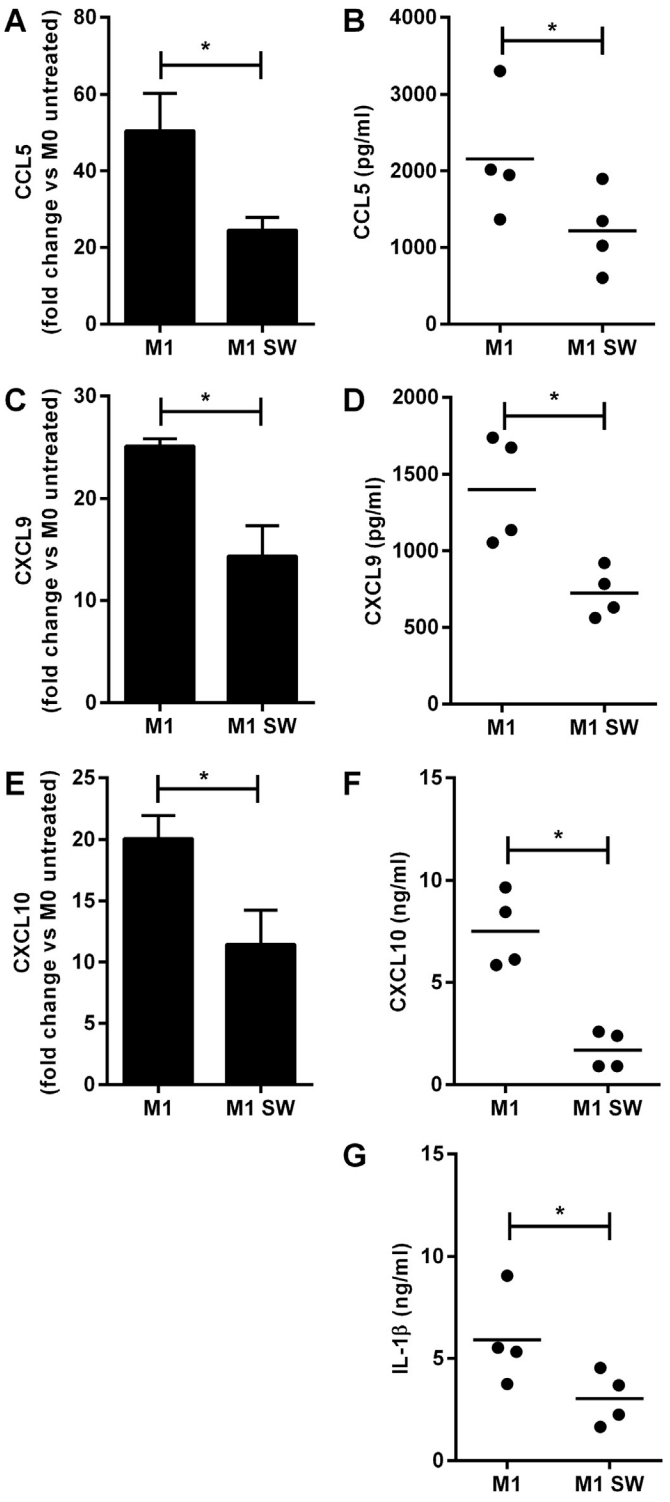

Fig. 3. SW inhibits expression and secretion of pro-inflammatory cytokines by M1 macrophages. Macrophages were stimulated with LPS plus IFN-γ for 24 h (M1) and subsequently exposed or not to 322 mJ SW (M1 SW). After 4 h, gene transcript levels and cytokine concentrations in cell-free supernatants were measured for CCL5 (A and B), CXCL9 (C and D), CXCL10 (E and F), and IL-1β (G). Results are expressed as mean ± SEM of 4 independent experiments. *$p < 0.05$ by t-test.

contribute to tissue healing, but besides stem cells, many other cell targets, including endothelial cells, bone cells, and small unmyelinated nerve fibers, have been involved in ESWT therapeutic potential [1–3,7,9–15].

In most recent years a key role in tissue repair and healing has also been attributed to the innate immunity system, and macrophages in particular play an important role in this setting, both for their key role to control shift from acute inflammation to either its chronicization or resolution phases and for their ability to recruit and stimulate stem cells [16]. Resting macrophages populate virtually any tissue and are specifically devoted to sensing of endogenous as well as exogenous danger signals. Upon exposure to pro-inflammatory stimuli, both tissue resident macrophages as well as de novo generated macrophages derived from local differentiation at injured sites of circulating monocytes become "classically activated" (or M1) macrophages [17], which exert a range of proinflammatory activities to provide a protective response to pathogens. Macrophages are highly plastic cells and during the course of the inflammatory process they integrate signals from the microenvironment and coordinate the evolution of the local inflammatory response, sustaining its chronicization if tissue damage persists and on the opposite providing clues for its resolution if the tissue insult is ceased and anti-inflammatory mediators dominates. In this last setting macrophages become "alterative activated" (M2) or M2-like macrophages and acquire a distinct functional profile [16,18]. Several events characterize the resolution phase of inflammation, including engulfment of apoptotic neutrophils by efferocytosis and subsequent polarization of macrophages associated to their ability to generate anti-inflammatory and pro-resolving mediators [16]. This event is also characterized by lipid mediator class switching, in which pro-inflammatory lipid mediators generated by the activity of cyclooxygenase-2 (COX2) in M1 macrophages are substituted by pro-resolving lipid mediators generated by 12/15-lipoxygenases (ALOX12/15) in M2 macrophages [19].

Mechanotransduction is a process active in different cell types allowing sensing and processing of mechanical information provided by the extracellular environment and the conversion of biomechanical forces in biochemical responses influencing cellular functions such as migration, proliferation, differentiation, and apoptosis [8]. Originally developed in adhesion biology, where integrins have been shown to facilitate force transmission between the extracellular matrix and the intracellular actin cytoskeleton, mechanosensing has then be shown to rearrange proteins laterally within the membrane, thus inducing conformational changes and regulating their activity. Mechanotransduction has been extensively investigated in endothelial cells exposed to shear stress, but more recent evidence indicates its role in a number of other cell types, including fibroblasts, bone cells, and mesenchymal stem cells [20]. Though implications for their biology are largely undefined, macrophages also have recently been demonstrated to be endowed with mechanosensing properties [21]. Considering the clinical evidence that ESWT promotes resolution of inflammatory processes and tissue regeneration and the key role of macrophages in these events, the aim of this work was to investigate the effects of SW stimulation on macrophage biology in a well-controlled in vitro setting.

2. Materials and methods

2.1. Reagents

LPS from *Escherichia coli* serotype 055:B5 was purchased from Sigma, human recombinant IFNγ and IL-4 were purchased from R&D Systems.

2.2. Macrophage generation and polarization

Human monocyte were isolated from healthy donor buffy coats in compliance with indications by the ethical committee of Istituto Clinico Humanitas, Milan, Italy, and macrophages were generated and polarized to M1 and M2 mcrophages as described in detail in SI Materials and methods.

2.3. SW treatment

Macrophage culture T25 flasks were completely filled up with RPMI 1640 medium to prevent air interference with impulse transmission and then exposed to SW, in a patented thermostated plexiglass waterbath, filled up with degassed water to avoid cavitation, an heater plate to avoid thermic shock to cells during treatment, and a wedge-shaped absorber on the back wall, to prevent interference by waves reflection [22].

The cell culture flasks were hold at 5 cm from the SW source represented by an electrohydraulic defocused OP155-Orthogold100 device (MTS Medical UG, Konstanz, Germany) and were exposed to 400 SW shots at frequency 3.5 Hz/s and Energy Flux Dentisities of 0.1 or 0.03 mJ/mm^2, corresponding to 840 and 322 mJ total energy, respectively. After exposure to SW, cell cultures were placed in fresh medium and incubated at 37 °C for indicated time. Untreated cell cultures were manipulated as SW-treated cultures.

2.4. RNA isolation and gene expression analysis

Total RNA was purified using TRIzol (Ambion) according to the manufacturer's instructions and one microgram was reverse transcribed using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems) according to manufacturer's guidelines. Quantitative RT-PCR was performed as described in SI Materials and methods.

2.5. ELISA assay

Antibodies and detection reagents for ELISA assays were purchased from R&D Systems and used according to the manufacturer's instructions. Samples were diluted so that the optical density fell within the optimal portion of a log standard curve.

2.6. Statistical analysis

Results are expressed as mean ± SEM and statistical significance was based on non-parametric t-test or non-parametric ANOVA followed by post hoc Bonferroni's test performed using the GraphPad Prism 5 software (GraphPad Software).

3. Results

3.1. SW do not activate resting macrophages

To understand if SW could activate macrophages, we stimulated resting in vitro generated monocyte-derived macrophages with SW at different energy and after 4 h from SW exposure expression levels of marker genes of macrophage activation were measured by qPCR. As shown in Fig. 1, SW had no modulatory effects on the expression of the M1 markers (CD80, CCR7, COX2). We observed a trend to increased expression for the M2 genes MRC1 and ALOX15, even though results did not reach statistical significance, while the M2 marker CD209 was unaffected. Overall, we conclude that SW are *per se* unable to induce activation of resting macrophages.

3.2. SW inhibit M1 activation

Normal adult wound healing is a sequence of events involving haemostasis, inflammation, matrix deposition, and remodeling [23]. Classical activated (M1) macrophages are engaged at the initial inflammatory phase, causing tissue damage through the secretion of pro-inflammatory cytokines (TNFα, IL-1β, IL-6, IL-12), proteinases, and ROS [16]. They are also known as a relevant cell source of arachidonic acid metabolites generated by cyclooxygenase-2 (COX2), which have been involved in wound healing [24]. As SW have been previously reported to suppress the early pro-inflammatory immune response [25], we examined their effects on the expression of marker genes associated with M1 polarization. When macrophages polarized to M1 by LPS and IFN-γ treatment were treated with SW at different intensities, we observed a significant inhibition of the M1 marker genes CD80 and COX2, particularly evident when low-doses SW were used (Fig. 2A and B). Conversely, no effect was evident for CCR7 (Fig. 2C). To better clarify the relevance of this effect, we analyzed the effect on gene expression and secretion of pro-inflammatory cytokines and chemokines of low energy SW. As shown in Fig. 3, SW had a significant inhibitory effect on CCL5 gene expression levels, which corresponded to a significant reduction of CCL5 levels detected in the supernatant by ELISA (Fig. 3A and B, respectively). Similar effects were observed for the inflammatory chemokines CXCL9

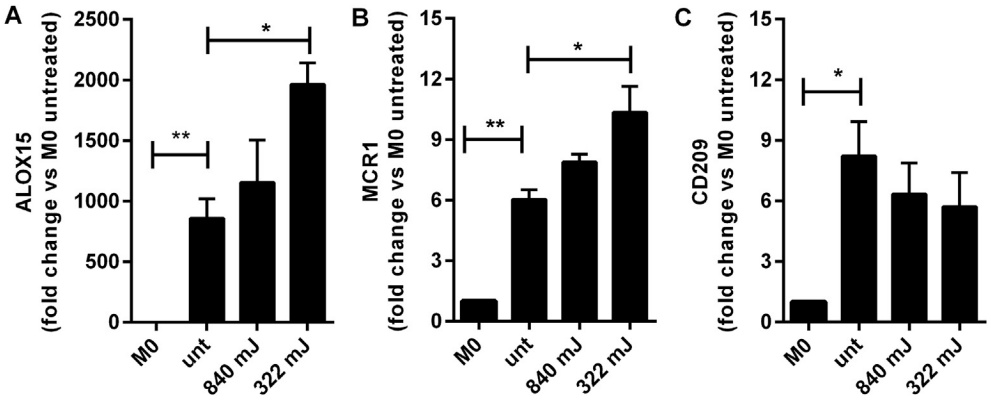

Fig. 4. SW effects on alternative activated M2 macrophages. Expression levels of M2 marker genes MRC1 (A), CD209 (B), and ALOX5 (C) in M2 macrophages exposed or not (unt) to SW at indicated energy. Resting macrophages (M0) are shown for comparison. Results from 3 independent experiments were normalized on the housekeeping gene GAPDH and expressed as mean ± SEM fold enrichment relative to M0. $^{**}p < 0.01$ and $^{*}p < 0.05$ by Bonferroni's multiple test.

(Fig. 3C and D) and CXCL10 (Fig. 3E and F) and the primary pro-inflammatory cytokine IL-1β (Fig. 3G). Overall, these results suggest that SW have an inhibitory effect on inflammatory macrophages, particularly when used at low doses.

3.3. SW enhance M2 activation

In the late inflammatory phase macrophages acquire a phenotype distinct from the pro-inflammatory M1 polarization and related to the alternative M2 polarization [16], contributing to the wound healing process by producing anti-inflammatory cytokines and growth factors promoting tissue regeneration and matrix deposition [16]. Having observed an inhibitory effect of SW on the pro-inflammatory functions of M1 macrophages, we next investigated their impact on M2 macrophages polarized with IL-4 for 24 h. When exposed to SW, M2 macrophages showed a significant over-induction for the M2 markers ALOX15, a 12/15-lipoxygenase that produce anti-inflammatory metabolites in contracts with the role of COX2 [16], and the macrophage mannose receptor MRC1 (Fig. 4A and B, respectively), while no effect was evident for CD209 (Fig. 4C). As the effect of SW on M2 markers was most evident when low energy SW were used, as observed for M1 genes, we then investigated the effect of low energy SW on gene expression and secretion of cytokines and chemokines associated to M2 polarization. SW-treated M2 macrophage released a significantly higher amount of IL-10 and CCL18, which correlated with a corresponding increase in transcripts (Fig. 5A–D). Conversely, no effect was evident for CCL17 (Fig. 5E and F). Overall, these results suggest that SW have an inhibitory effect on inflammatory macrophages, particularly when used at low doses. We conclude that low energy SW synergize for M2 activation to significantly increase expression of some M2 genes and anti-inflammatory cytokines.

3.4. SW outcome on lipoxygenase family

Lipoxygenase enzymes catalyze the unsaturated fatty acids oxidation, producing pro- or anti-inflammatory metabolites. The human genome includes six functional lipoxygenase genes (ALOX15, ALOX15B, ALOX12, ALOX12B, ALOXE3, ALOX5), which encode for six different LOX isoforms [26]. M1 macrophages express ALOX5, which plays a major role in the biosynthesis of pro-inflammatory leukotrienes, while ALOX15, ALOX15B, and ALOX12 are expressed in M2 macrophages associated to resolution of inflammation [27] and in carotid plaque macrophages [28]. We investigated the action of SW on macrophage metabolism evaluating their effect on ALOX15 genes in M2 macrophages. As shown in Fig. 6, though IL-4 was able to induce all three ALOX isoforms in macrophages, low energy SW synergized with IL-4 exclusively for ALOX15 (Fig. 6A), while no additive effects were observed for ALOX15B and ALOX12 (Fig. 6B and C, respectively).

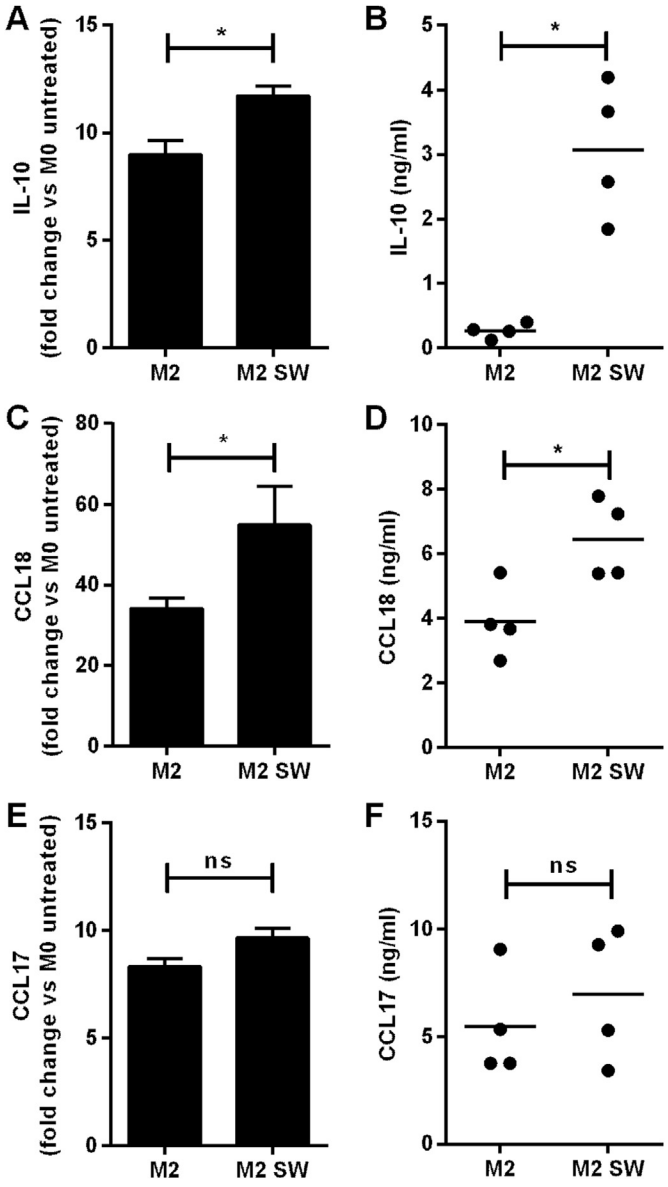

Fig. 5. SW effects on M2-related cytokines in alternative activated macrophages. Macrophages were stimulated with IL-4 for 24 h (M2) and subsequently exposed or not to 322 mJ SW (M2 SW). After 4 h, gene transcript levels and cytokine concentrations in cell-free supernatants were measured for IL-10 (A and B), CCL18 (C and D), and CCL17 (E and F). Results are expressed as mean ± SEM of 4 independent experiments. *$p < 0.05$ by t-test.

4. Discussion

SW are biphasic high-energy acoustic waves characterized by an initial positive very rapid phase of high amplitude, followed at a distance of microseconds by a sudden phase of mild depression responsible of the biological activity on living tissues [1]. In agreement with the definition proposed by Huang C and collaborators as "a therapeutic intervention that reduce and reverse injury to damaged tissues or promote the homeostasis of healthy tissues by mechanical means at the molecular, cellular, or tissue level" [8] SW are today considered a form of mechanotherapy. As for most forms of mechanotherapy in clinical use, ESWT mainly focus on improving tissue regeneration and, consistent with this, clinical results indicates that besides reduction and/or inhibition of apoptosis, the clinical efficacy of ESWT is tightly related to its ability to improve neovascularization and matrix remodeling in tissues [1–3,7,11,14,15,20,29,30].

In order to optimize their clinical applications, each type of mechanical intervention aimed to exert a therapeutic effect should be understood at the molecular and cellular level. The mechanisms of action of SW is associated to mechano-transduction, a process by which physical forces are sensed, transduced, and then transformed into intracellular biochemistry and gene expression [8,14]. A rapidly increasing body of evidence demonstrates that mechanotransduction events after exposure to SW are not only dose-dependent but also can be different in different cell types, as osteoblasts, endothelial cells, tenocytes, and stem cells at different levels of differentiation have all been

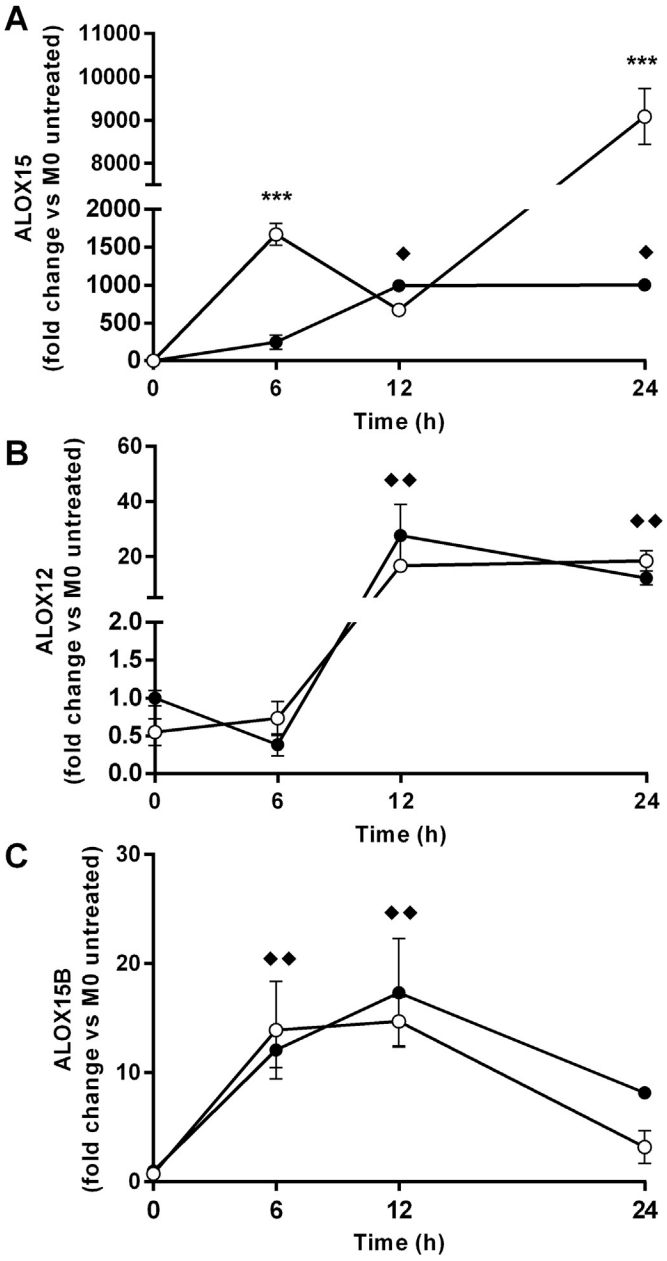

Fig. 6. SW effect on the expression of lipoxygenase family genes in M2 macrophages. Macrophages were stimulated for indicated time with IL-4 and then exposed (white symbols) or not (black symbols) to 332 mJ SW for 4 additional hours. Total RNA was then collected and expression levels of lipoxygenase family genes (ALOX12, panel A; ALOX15B, panel B; ALOX15, panel C) were evaluated by q-PCR. Results are expressed as mean ± SEM of expression fold change compared to resting untreated macrophages (M0) of 3 independent experiments. Statistics has been calculated using Bonferroni's multiple test to compare untreated M2 to M0 (◆$p < 0.05$, ◆◆$p < 0.01$) and SW-treated M2 to untreated M2 (***$p < 0.0001$).

demonstrated to be responsive to SW but have different optimal pattern and range of mechanical stimulation and develop different biological answers, including upregulation of TGF-β1 expression and NO production, suppression of NF-kB activity and pro-inflammatory cytokines production [7,8,14,25]. More recently, SW application in an experimental setting based on intramuscular silicone injection resulted in a lesser dense fibrous capsule and, when applied in multiple sessions, in active degradation of the fibrous envelope accompanied by synergistic alterations in pro- and anti-fibrotic proteins (TGF-β1 and matrix metalloproteinase 2, respectively), leading the authors to conclude that SW decelerate capsule formation and may contribute to fibrotic tissue resorption [31]. The promotion of regenerative events instead of simply healing processes by ESWT may have multiple applications and is presently considered of particular interest in cardiology, where restoring of tissue integrity, instead of fibrous tissue, is crucial for cardiac function. Consistent with this, recent evidence indicates that ESWT improves ventricular function in ischemic heart failure [6,32].

The body of scientific data demonstrating that ESWT can induce tissue healing and regeneration through mechanotransduction has brought to the present view of SW as immunomodulators during the wound healing process [7,25], which is also well in line with recent evidence indicating that SW influence the TLR3 pathway.

Though macrophages have a well-known pivotal role in wound healing and tissue regeneration, their biological responses to SW exposure has never been investigated. We here show that macrophages are sensitive to SW exposure. SW did not induce activation of resting macrophages, but if applied to pre-activated macrophages were able to fine tuning their functional profile. Of interest, SW inhibited the expression of M1 marker genes (CD80, COX2, CCL5) in M1 macrophages and significantly reduced their ability to secrete the pro-inflammatory cytokine IL-1β. Conversely, when applied to M2 macrophages SW showed a synergistic effect for the induction of some M2 marker genes (ALOX15, MRC1, CCL18) and induce a significant increase in the production of the anti-inflammatory IL-10. These results contribute to our understanding of the mechanisms of action of ESWT and add macrophages to the list of target cells potentially contributing to the therapeutic effect of SW. From the clinical point of view, the possibility to early modulate the interplayed chain of biological events of tissue regeneration in which macrophages are involved by SW exposure opens new perspectives and insights for counteract a number of acquired pathological conditions for which fibrosis is a critical element, including post-traumatic sequelae with muscle and skin lesions. Further studies investigating ESWT in the early phase of recovery are required in order to evaluate the potential of SW to reduce the fibrous component in regenerating tissues, but our results suggest that macrophages are likely to play a key role on this scenario.

Ethical approval

Ethical approval was not requested to perform the “in vitro study”.

Sources of funding

None.

Author contribution

Naths Grazia Sukubo, Massimo Locati and M. Cristina D'Agostino designed the study;

Naths Grazia Sukubo performed research and analysed data;

Naths Grazia Sukubo, Elisabetta Tibalt, Massimo Locati and Maria Cristina D'Agostino wrote the paper.

Massimo Locati, M. Cristina d'Agostino and Stefano Respizzi revised the final version of the work.

Conflict of interest

None of the authors have any conflict of interest.

Guarantor

None.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ijsu.2015.07.719.

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