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Immunomodulatory effects of Diclofenac in leukocytes through the targeting of Kv1.3 voltage-dependent potassium channels

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ABSTRACT

Kv1.3 plays a crucial role in the activation and proliferation of T-lymphocytes and macrophages. While Kv1.3 is responsible for the voltage-dependent potassium current in T-cells, in macrophages this K^+ current is generated by the association of Kv1.3 and Kv1.5. Patients with autoimmune diseases show a high number of effector memory T cells that are characterized by a high expression of Kv1.3 and Kv1.3 antagonists ameliorate autoimmune disorders in vivo. Diclofenac is a non-steroidal anti-inflammatory drug (NSAID) used in patients who suffer from painful autoimmune diseases such as rheumatoid arthritis. In this study, we show that diclofenac impairs immune response via a mechanism that involves Kv1.3. While diclofenac inhibited Kv1.3 expression in activated macrophages and T-lymphocytes, Kv1.5 remained unaffected. Diclofenac also decreased iNOS levels in Raw 264.7 cells, impairing their activation in response to lipopolysaccharide (LPS). LPS-induced macrophage migration and IL-2 production in stimulated Jurkat T-cells were also blocked by pharmacological doses of diclofenac. These effects were mimicked by Margatoxin, a specific Kv1.3 inhibitor, and Charybdotoxin, which blocks both Kv1.3 and Ca^{2+}-activated K^+ channels ($K_{Ca}3.1$). Because Kv1.3 is a very good target for autoimmune therapies, the effects of diclofenac on Kv1.3 are of high pharmacological relevance.

Key words: Voltage-dependent K^+ channels, inflammation, leukocytes, immunomodulation, non-steroidal anti-inflammatory drugs

Abbreviations: BMDM, bone marrow-derived macrophages; COX, cyclooxygenase; CTX, Charybdotoxin; Kv, voltage-dependent K^+ channels; LPS, lipopolysaccharide; MgTx, Margatoxin; NSAID, non-steroidal anti-inflammatory drug; PHA, phytohematogglutinin; PMA, phorbol ester; $T_{EM,}$ Effector memory T-cells.

1. Introduction

Voltage-dependent potassium channels (Kv) play a crucial role in the repolarization of membrane potential in nerve and muscle, controlling their excitability. In addition, they are involved in the proliferation and activation of leukocytes [1, 2]. Particularly, Kv1.3 performs a key function in the immune system, controlling and leading to proliferation and IL-2 synthesis [3]. Effector memory T-cells (T_{EM}), which express high levels of Kv1.3, are present in autoimmune disorders [4]. In this context, antagonizing the activity of Kv1.3 with highly specific peptides reverses and prevents experimental autoimmune encephalomyelitis in rats [5]. Rheumatoid arthritis is a T-cell mediated autoimmune disease that causes severe inflammation and pain in the articulations and surrounding tissues. Kv1.3-high T_{EM} are abundant in these patients, and selective blockage of Kv1.3 ameliorates arthritis in rats [6]. In addition, Kv1.3, together with Kv1.5, is also present in macrophages, where it controls proliferation and activation processes [2, 7-9]. Macrophages turn the immune response toward inflammation or tolerance. These cells, which also act as antigen-presenting cells, modify the cytokine milieu and the intensity of T-cell signaling. Therefore, the inhibitory effects of Kv1.3 blockers on macrophages and microglia may account, in part, for the benefits of Kv1.3-based therapies.

Diclofenac (2-(2,6-dichloranilino) phenylacetic acid) is a non-steroidal anti-inflammatory drug (NSAID) that inhibits COX-2 (cyclooxygenase) activity and prevents the synthesis of inflammatory mediators. This compound is therapeutically used for pain-suffering patients, including those with rheumatoid arthritis or osteoarthritis. Diclofenac shows anti-nociceptive effects that are reversed by potassium channel blockers [10]. This effect seems to be dependent on the mechanism of each NSAID, as other compounds of this family do not involve potassium channel activity

[11]. ATP-dependent potassium channels (K_{ATP}) play a key role in the anti-nociceptive effect of diclofenac on rat paw [12]. Moreover, diclofenac, and not other NSAIDs, inhibits sodium currents in sensory neurons, which contributes to its analgesic activity [13]. Similarly, diclofenac's anticonvulsant properties involve the activation of heterotetrameric KCNQ2/KCNQ3 channels, which are responsible for the M current that modulates neuronal excitability and firing [14]. Finally, diclofenac also has many effects in the immune system, including impairing migration and accumulation of leukocytes [15-18] and diminishing NO production by macrophages [19, 20]. We here demonstrate that Kv1.3 is involved in the anti-inflammatory mechanism of diclofenac. Our results have clinical relevance because Kv1.3 is considered a very important target in therapies for anti-immune disorders. Our data provide new information about additional benefits of the use of diclofenac in the pharmacological treatment of the progression of rheumatoid arthritis.

2. Materials and methods

2.1. Animals and cell culture

Murine bone marrow-derived macrophages (BMDM) were isolated as described elsewhere [2, 21]. Cells were cultured in DMEM containing 20% FBS and 30% supernatant of L-929 fibroblast (L-cell) conditioned media as a source of Macrophage-Colony Stimulating Factor (MCSF). Raw 264.7 macrophages and Jurkat T-lymphocytes were cultured in RPMI culture media, containing 10% FBS and supplemented with 10 U/mL penicillin and streptomycin and 2 mM L-glutamine. All animal handling was approved by the Ethics Committee of the University of Barcelona and was in accordance with EU regulations.

2.2. Chemicals

Treatments were done in the culture media for 18-24 h. BMDM, Raw macrophages, and Jurkat T-lymphocytes were treated with 100 ng/ml, 1 μg/ml and 10 μg/ml of lipopolysaccharide (LPS, Sigma-Aldrich, Madrid, Spain), respectively. Jurkat T-cells were also stimulated with 5 μg/ml phytohematogglutinin (PHA, Sigma-Aldrich), with 80 nM phorbol ester (PMA, Sigma-Aldrich) or with combinations of both. Controls with DMSO, the vehicle for PMA, were performed to discard possible non-specific side effects. Diclofenac sodium salt (Sigma-Aldrich) was used at concentrations of 1.5 and 15 μM. Margatoxin (MgTx) (10 nM) and Charybdotoxin (CTX) (100 nM) were used to inhibit Kv1.3 alone or Kv1.3 and K_{Ca}3.1, respectively (Alomone, Israel).

2.3. RNA isolation and RT-PCR analysis

Total RNA was isolated by using Nucleospin RNA II (Invitrogen, Carlsbad, CA), which contains DNaseI. Transcriptor reverse transcriptase (Roche, Barcelona, Spain) was used for cDNA synthesis, according to manufacturer's instructions. Primers for PCR analysis were: Kv1.5, accession number NM012972 (F: 5'-GGATCACTCCATCACCAG-3'; R: 5'-GGCTTCCTCCTCCTTCCTTG-3'); and Kv1.3, accession number NM019270 (F: 5'- CTCATCTCCATTGTCATCTTCTG C-3'; R: 5'-TTGAAGTTGGAAACAATCAC-3'). In all cases, negative controls were performed in the absence of the reverse transcriptase reaction.

Real-time PCR primers were: Kv1.5 (F: 5'-TCCGACGGCTGGACTCAATAA-3'; R: 5'-CAGATGGCCTTCTAGGCTGTG-3'); and Kv1.3 (F: 5'-AGTATATGGTGA TCGAAGAGG-3'; R: 5'-AGTGAATATCTTCTTGATGTT-3'). LightCycler FastStart DNA MasterPLUS SYBR Green I (Roche) was used, and the reactions were performed under the following conditions: 95ºC for 5 s, 55ºC for 8 s, and 72ºC for 9 s, preceded by

10 min at 95ºC and followed by 10 min at 95ºC. Melting curves were performed to verify the specificity of the product, and primers for 18S were included as an internal reference [8, 9].

2.4. Protein extracts and western blot

Cells were washed twice in cold phosphate-buffered saline (PBS) and lysed with lysis solution (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1% Triton X-100, and 1 mM EDTA) supplemented with 0.1% pepstatin, 0.1% leupeptin, 0.1% PMSF, and 0.1% aprotinin as protease inhibitors. Cells were disrupted by passing the lysate 10 times through a 25 G syringe. Lysates were centrifuged at 14000 rpm for 10 min to discard cellular debris.

Total proteins (50 μg) were boiled in Laemmli SDS loading buffer and separated on a 10% SDS–PAGE gel. They were transferred to PVDF membranes (Immobilon-P, Millipore, Billerica, MA) and blocked in 5% dry milk supplemented with 0.05% Tween-20 PBS before immunoreaction. Polyclonal antibodies against Kv1.3 (1/200, Alomone), Kv1.5 (1/500, Alomone), and inducible Nitric Oxide Synthase (1/500, Santa Cruz Biotechnology, Santa Cruz, CA) were used. A monoclonal anti-β-actin antibody (Sigma) was used as a control. The specificity of the antibodies was tested with control antigen peptides provided by the manufacturer. Secondary antibodies were purchased from BioRad (Hercules, CA) (anti-rabbit, 1/3000; anti-mouse, 1/10000).

2.5. IL-2 production measurements

IL-2 production in Jurkat T-lymphocytes was measured with an ELISA kit (eBioscience, San Diego, CA), following the manufacturer's instructions. Cells were centrifuged at 1200 rpm for 4 min, and the supernatant was used to quantify the IL-2

concentration. Reactions were performed in 96-well plates coated with the capture antibody. After blocking, standards and samples were incubated with Detection Antibody followed by HRP-Streptavidin. H_3PO_4 (1M) was used as stop solution. Plates were read at 450 nm.

2.6. Migration assays

Confluent BMDM plated in 6-well dishes were scratched with a single-edged razor blade cut. The scratch began at the middle of the dish and extended over an area 7-10 mm wide. The migrating cells were counted at 20x magnification at three distinct sites along the wound (1 mm^2 each), beginning at the scratch line and extending as far out as the cells had migrated. The mean distance between the wound edge and the nuclei of migrating cells was also measured. The results are given as the percentage of migrating cells and migrated distance (μM).

2.7. Electrophysiological Recordings

Whole-cell currents were recorded at room temperature (21-23 °C) using the whole cell patch-clamp technique with an Axopatch 1C amplifier (Axon Instruments, Foster City, CA). Micropipettes of 2-4 MΩ were pulled from borosilicate glass capillary tubes (GD-1; Narishige, Tokyo, Japan) using a P-87 puller (Sutter Instruments, Novato, CA). Electrodes were filled with the following solution (in mM): 80 K-aspartate, 42 KCl, 3 phosphocreatine, 10 KH_2PO_4, 3 MgATP, 5 HEPES-K, and 5 EGTA-K (adjusted to pH 7.25 with KOH). The extracellular solution contained (in mM) 136 NaCl, 4 KCl, 1.8 $CaCl_2$, 1 $MgCl_2$, 10 HEPES-Na, and 10 D-glucose, and it was adjusted to pH 7.40 with NaOH. Macrophages and Jurkat T-cells were clamped to a holding potential of -80 mV. The current-voltage (IV) protocol used consisted of 15 pulses ranging from -80 to

+60 mV, in 10 mV steps. Each pulse was 250 ms long. Peak amplitude (pA) was normalized by capacitance values (pF). Data analysis was performed using the CLAMPFIT utility of PCLAMP 9.2 and Origin 7.0.3 (Microcal Software, Beijing, China).

2.8. Statistical analysis

Real-time PCR, protein expression, migration and IL-2 concentration experiments were repeated at least three times in duplicate. Electrophysiological recordings were performed in 6-10 cells from at least 3 independent experiments. Values are expressed as mean ± S.E.M. The significance of any observed differences was established by Student's *t* test (Graph Pad, PRISM 3.0, La Jolla, CA). A value of $p<0.05$ was considered significant.

3. Results

First we characterized Kv channels expressed in Raw 264.7 macrophages and Jurkat T- lymphocytes. Delayed rectifier K^+ currents were evoked in Raw (Fig. 1A) and Jurkat cells (Fig. 1B) by depolarizing pulses (250 ms) from a holding potential of -80 mV to + 60 mV in 10 mV steps. More depolarizing potentials were needed to open channels in Raw cells (Fig. 1C). While the threshold potential for activation was -30 mV in T-cells, it was approximately -10 mV in macrophages. Moreover, current density was 10-fold higher in T-lymphocytes than in macrophages. RT-PCR experiments showed that while Jurkat T-cells only expressed Kv1.3, Raw macrophages also presented Kv1.5 (Fig. 1D). These results were further confirmed by western blot analysis (Fig. 1E). Furthermore, as expected, Kv currents in macrophages and T-lymphocytes were fully inhibited by 10 nM MgTX and 100 nM CTX (not shown).

Kv1.3 plays an important role in macrophage proliferation and activation, and LPS-induced activation increases Kv1.3 expression. Macrophages, with or without 1 μg/ml LPS, were treated with diclofenac for 24 h and voltage-dependent K^+ currents were analyzed. The presence of 15 μM diclofenac led to a 50% decrease in the current amplitude in macrophages without LPS treatment (Fig 2A). Similar results were obtained in LPS-treated macrophages. However, lower doses of diclofenac (1.5 μM) did not produce apparent changes in activated cells. Surprisingly, not only LPS activation, but also the presence of diclofenac, resulted in a reduction in cell size (Fig 2B). Therefore, we calculated the peak amplitude normalized to cell size (pA/pF) (Fig. 2C). We observed that LPS increased peak current density in the absence of diclofenac ($p<0.05$ vs. control). In addition, while diclofenac (15 μM) had no effects on control Raw cells, it markedly reduced the current density in LPS-stimulated macrophages ($p<0.05$ vs. LPS). Therefore, 15 μM diclofenac led to a significant reduction of K^+ currents in LPS-activated macrophages.

Because the association between Kv1.3 and Kv1.5 generates the major voltage-dependent K^+ current in macrophages [7, 9], we wanted to check whether this diclofenac-dependent current decrease was due to changes in the expression of either of these Kv channels. Real-time PCR analysis and, as shown in Fig. 3A, Kv1.3 mRNA expression was increased 2.5-fold with LPS treatment. However, the presence of 15 μM diclofenac counteracted this increase. A lower concentration of diclofenac (1.5 μM) had no effect on either control or LPS-treated macrophages. Unlike Kv1.3, Kv1.5 expression was not affected by any of these treatments. We also undertook western blot experiments to check Kv1.3 and Kv1.5 protein expression (Fig. 3B-D). While 15 μM diclofenac reversed the LPS-induced increase in Kv1.3, no changes were observed for Kv1.5 (Fig. 3 B-D). iNOS expression was checked as a marker of cell activation (Fig. 3

B and E). As expected, LPS-induced activation led to an important increase in iNOS expression. As with Kv1.3, 15 μM diclofenac - but not 1.5 μM - decreased iNOS levels in LPS-stimulated Raw cells.

Macrophage activation was impaired by diclofenac. Therefore, we next wanted to examine its effects on cell migration. To that end, we used primary cultures of bone marrow-derived macrophages (BMDM) because their morphological changes during activation allow for better measurements in this kind of studies. Representative pictures of cell-migration assays are shown in Fig. 4 (A-F). The percentage of migrating macrophages was calculated (Fig. 4G and H). Almost no migration was observed in resting macrophages (Fig 4A and G), regardless of the presence of diclofenac (data not shown). In contrast, LPS stimulated cell migration (Fig 4D and G), and this effect was reversed by the presence of 15 μM diclofenac (Fig 4F and G). No significant changes were observed with 1.5 μM diclofenac on LPS-stimulated BMDM (Fig 4E and G). We also measured the migrated distance (Fig. 4H). In the presence of LPS, both 1.5 μM and 15 μM diclofenac reduced the travelled distance. Margatoxin (MgTx) potently blocks Kv1.3, and Charybdotoxin (CTX) inhibits both Kv1.3 and intermediate-conductance Ca^{2+}-activated K^+ channels ($K_{Ca}3.1$), which are present in leukocytes [22, 23]. Both toxins markedly reduced the number of LPS-activated migrating cells (Fig 4B and C).

According to these results, diclofenac impairs macrophage activation and migration, and these effects are mediated by Kv1.3. Macrophages are involved in the inflammatory response by modifying the extracellular milieu and acting as antigen-presenting cells. Therefore, the level of activation of macrophages strongly influences the immune response carried out by T-lymphocytes. In this context, we also evaluated the effects of diclofenac on T-lymphocytes. We measured IL-2 production, as a marker of T-cell activation, in response to different stimuli (Fig. 5). Fig. 5A shows IL-2 production

induced by the treatment of Jurkat T cells with LPS or PMA. Neither of these stimuli significantly enhanced IL-2 synthesis; on the contrary, PHA triggered a 10-fold increase of IL-2 production (Fig. 5B). Diclofenac, as well as MgTx and CTX, inhibited T-cell IL-2 production of PHA-stimulated lymphocytes. The effect of double stimulation with PHA and PMA is shown in Fig. 5C. Although PMA by itself did not increase IL-2 production (Fig. 5A), it enhanced PHA-induced stimulation, as the double stimulation produced a 450-fold induction of IL-2 synthesis. In this case, only 15 μM diclofenac significantly inhibited T-cell activation. Similar results were obtained with the Kv1.3-blockers MgTx and CTX.

Our results with the Kv1.3-blockers suggested that this protein could be involved in the T-cell immunosuppressive response generated by pharmacological relevant doses of diclofenac. Therefore, we evaluated the effects of diclofenac on Kv1.3 expression in Jurkat cells. Real-time PCR experiments indicated that a 15 μM dose of diclofenac, but not a 1.5 μM dose, decreased gene expression of Kv1.3 in both PHA- and (PHA + PMA)-stimulated cells (Fig. 6A). Similar to the effect observed in macrophages, 15 μM diclofenac showed no significant effect on non-stimulated T-cells. Protein levels were also analyzed (Fig 6B-E). Representative western blots are shown in Fig. 6B and D, together with densitometric quantifications of different experiments (Fig. 6C and E). As can be seen, no changes were observed in PHA (Fig. 6B and C) or (PHA + PMA)-treated cells (Fig. 6D and E). However, in all cases, 15 μM diclofenac significantly decreased Kv1.3 levels in stimulated Jurkat T-lymphocytes.

4. Discussion

This study demonstrates that Kv1.3 contributes to the anti-inflammatory effects of diclofenac, which could provide additional benefits in therapies against autoimmune

diseases associated with inflammation and pain. Moreover, our data also show that the modulation of Kv1.3 in macrophages and T-lymphocytes could lead to important alterations in the immune response in these disorders.

A relationship between potassium channels and diclofenac has been previously described [10, 14]. However, this is the first time that this connection has been established in the immune system. Diclofenac functions as a channel opener, activating ATP-dependent K^+ channels and triggering anti-nociceptive effects by reducing pain sensation in response to different stimuli [12]. This is in agreement with the diclofenac-dependent abrogation of leukocyte activation that we found. In fact, unlike inward-rectifier ATP-dependent K^+ channels, diclofenac inhbits the outward-rectifier K^+ channel Kv1.3. Similar opposite mechanisms occur between Kir2.1 and Kv1.3 during pro-inflammatory insults [2, 24]. Diclofenac also activates KCNQ2/KCNQ3 heteromeric channels in rat cortical neurons, but again, the final effect is an attenuation of their excitability, which is the basis for its anticonvulsant properties [14].

Besides K^+ channels, Na^+ currents are also affected by diclofenac. Diclofenac inhibits Na^+ currents in rat myoblasts [25], which are necessary for muscle development and myoblast differentiation [26, 27]. Furthermore, diclofenac also blocks Na^+ currents in sensory neurons producing analgesia [13]. All of these observations further support the immunosuppressive role of diclofenac via Kv1.3.

We suggest that diclofenac modulates Kv1.3 via a still unidentified mechanism in leukocytes. Unlike diclofenac, most NSAIDs involve no K^+ channel activity in their anti-nociceptive effects, indicating distinct mechanisms of action [11]. Several NSAIDs have been tested, and only flufenamic acid partially mimics diclofenac's effects on Na^+ currents in neurons [13]. Authors argue that the structure of NSAIDs, and not the inhibition of COX, must be involved [13]. In this context, diclofenac specifically

decreases Kv1.3, but not Kv1.5, expression. Therefore, by changing the heterotetrameric composition of the channelosome, leukocytes may be able to fine-tune the immune response [9].

There is growing experimental support for the existence of a diclofenac-dependent attenuation of the immune response. Diclofenac inhibits the accumulation of leukocytes and the formation of lesions [16, 17]. In addition, it also blocks the migration of leukocytes [15, 18]. Our results also indicated a reduction in macrophage migration. With 15 μM diclofenac, the number of migrating LPS-activated BMDM decreased, as did the distance they travelled. Paradoxically, some studies have shown that diclofenac enhances B-16 murine melanoma cell migration [28]. However, these discrepancies could be explained by the NSAID concentrations used. Liu et al. (2004) used 100 μM diclofenac, whereas clinically available doses of diclofenac reach a maximum concentration of 10 μg/ml in blood, which is equivalent to 30 μM [20]. Similarly high concentrations of diclofenac (100 μM) also affect rat cerebellar granule cells in ways contrary to what our study shows, activating transient outward K^+ currents [29]. In our study, we tested two concentrations of diclofenac, both of clinical relevance, which in all cases trigger dose-dependent effects. Higher doses (150 μM) cause cell death, thus making it impossible to perform functional studies (data not shown).

MgTx and CTX, which block Kv1.3, mimicked the effects of diclofenac on LPS-stimulated migrating cells. Kv1.3, which interacts with β1-integrin, is important in leukocyte migration because its opening activates T-cell integrin function [30]. Our results further support a Kv1.3-mediated cell migration mechanism and indicate that diclofenac partially impairs macrophage migration via Kv1.3.

Similar to our results, diclofenac partially blocks NO production in Raw cells [19, 20]. Although both Kv1.3 and Kv1.5 are present in macrophages, Kv1.3 is the main

isoform responsible for cell activation [8, 9]. The incubation of BMDM with Margatoxin partially blocks iNOS induction in response to LPS or TNFα, which involve Kv1.3 in the activating response [2, 8]. We demonstrate that the decrease in iNOS is concomitant with a reduction in Kv1.3. Kv currents are generated mainly by Kv1.3 homomeric complexes and by heterotetrameric combinations of Kv1.3 and Kv1.5 in macrophages [7, 9, 31]. Low Kv1.3/Kv1.5 ratios, as found in Raw cells, impair pharmacological treatments based on Kv1.3 and dull the control of proliferation and activation [8, 9]. Therefore, our results show a potent effect of diclofenac on Kv1.3-driven macrophage activation. This is the first time that a relationship has been shown between a NSAID mechanism and Kv1.3 in the immune system.

Kv1.3 plays a key role in the initiation of the immune response in T-lymphocytes. Kv1.3 is the responsible for triggering the calcium influx necessary for IL-2 synthesis. The Ca^{2+}-activated K^+ channel $K_{Ca}3.1$, the main K_{Ca} channel in T-cells, sustains this calcium influx [1, 3]. Charybdotoxin, which inhibits Kv1.3 and $K_{Ca}3.1$, blocks IL-2 production and mitogenesis in T-cells [32]. However, $K_{Ca}2.2$, which is charybdotoxin-insensitive [33-35], is the main K_{Ca} channel involved in the Ca^{2+} signaling in Jurkat cells [36]. Therefore, CTX solely targets Kv1.3 in our model. Diclofenac inhibited IL-2 production in stimulated T-cells, leading to the attenuation of the immune response. Furthermore, both MgTx and CTX mimicked these effects, suggesting an important role of Kv1.3 in diclofenac-induced immunosuppression. We also demonstrated the inhibitory effect of diclofenac on Kv1.3 expression. However, PHA and PHA + PMA stimulation did not increase Kv1.3, possibly due to the cell's origin. Only effector memory T cells (T_{EM}) markedly increase their number of Kv1.3 channels when they receive activation stimuli, acquiring the $Kv1.3^{high}K_{Ca}3.1^{low}$ phenotype. Naïve and central memory T-lymphocytes only show a very slight increase in Kv1.3 [35, 37].

Jurkat T cells come from a human leukemia, and they do not differentiate into T_{EM}. In addition, unlike macrophages, Kv1.3 function in T-lymphocytes is not always coincident with an increase in expression [38, 39]. However, our data show that Kv1.3 expression is reduced by diclofenac, impairing T-cell function. This result may be important for rheumatoid arthritis patients who are treated with diclofenac because of its anti-inflammatory and pain killing effects. Parallel actions of this NSAID on Kv1.3 could be attenuating the lymphocyte-enhanced immune response because Kv1.3 is a very good target for T-cell-mediated autoimmune disorders [6]. Selective blockade of Kv1.3 reverses these types of disorders *in vivo* [6].

In summary, we provide strong evidence of the inhibitory effects of diclofenac on Kv1.3 expression in macrophages and T-lymphocytes. These changes are selective, as Kv1.5 is not altered by this treatment. Kv1.3 inhibition is accompanied by impaired inflammatory response in these cells. In macrophages, we observed a decrease in iNOS expression and a blockage in their migration in response to LPS. In T-cells, IL-2 production is reduced. These changes were mimicked by treatment with Margatoxin and Charybdotoxin, which block Kv1.3. Our results suggest that diclofenac ameliorates rheumatoid arthritis, and probably other inflammatory autoimmune diseases, by targeting Kv1.3 in leukocytes. Therefore, in addition to the well-known anti-inflammatory and anti-nociceptive effects of diclofenac, this drug could also aid in the prognosis of the disease by ameliorating its progression.

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FOOTNOTES

NV and MD contributed equally. CV and AF contributed equally.

FIGURE LEGENDS

Figure 1. Macrophages and T lymphocytes express voltage-dependent K^+ channels. Representative traces of outward K^+ currents evoked in Raw 264.7 macrophages (A) and Jurkat T cells (B). Cells were held at -80 mV, and currents were elicited by depolarizing pulses that increased in 10-mV steps (250 ms in duration) from -80 to +60 mV. (C) Current density *versus* voltage relationship of K^+ currents in Jurkat T cells (○) and Raw 264.7 macrophages (●). Current densities are much higher in Jurkat lymphocytes. The inset highlights K^+ currents in Raw. (D) mRNA expression of Kv1.3 and Kv1.5 in Raw 264.7 macrophages and Jurkat T lymphocytes. E, Kv1.3 and Kv1.5 protein expression in both cell types.

Figure 2. Diclofenac decreases current density in activated Raw 264.7 macrophages. Cells were incubated for 18-24 h with LPS and/or diclofenac (DIC). (A) Representative traces of outward K^+ currents evoked in control and 15 μM diclofenac-treated Raw 264.7 cells (left). Right, representative traces of 1 μg/μl LPS-activated Raw cells with and without diclofenac. Two concentrations of diclofenac were tested: 1.5 μM, grey current record; 15 μM, black current record. Cells were held at -80 mV, and currents were elicited by a depolarizing pulse of +60 mV lasting 250 ms. (B) Cell size calculated as cell capacitance in pF for the analyzed populations of cells. *, $p < 0.05$ vs. control without diclofenac. (C) Current density analysis of the different groups. Current intensity (pA) at -60 mV was normalized by cell size (pF). Significant changes were found for LPS (**, $p<0.01$ *vs*. control) and for 15 μM diclofenac treatment (*, $p<0.05$ *vs*. LPS) by Student's *t* test.

Figure 3. Diclofenac selectively inhibits Kv1.3 expression in LPS-activated Raw 264.7 macrophages. Cells were incubated for 18-24 h in the presence or absence of 1 μg/μl LPS with/without 1.5 and 15 μM diclofenac. (A) Kv1.3 (white) and Kv1.5 (black) mRNA expression by real-time PCR for the treatments indicated. Significant differences were found for Kv1.3 in LPS-activated macrophages (*, $p<0.05$ *vs.* control) and for LPS-activated macrophages treated with 15 μM diclofenac (***, $p<0.001$ *vs.* LPS). (B) Representative western Blot analysis of Kv1.3 and Kv1.5 protein expression for the treatments indicated. (C-E) Quantification of data in panel B: Kv1.3 (C), Kv1.5 (D) and iNOS (E). Significant differences were found for Kv1.3 in LPS-activated macrophages (*, $p<0.05$ *vs.* control) and in LPS-activated macrophages treated with 15 μM diclofenac (***, $p<0.001$ *vs.* LPS). Also iNOS showed significant changes after treatment with LPS (***, $p<0.05$ *vs*. control) and 15 μM diclofenac (***, $p<0.05$ *vs*. LPS). All values were normalized by β-actin expression and compared to the control in the absence of diclofenac (0). AU: arbitrary units. White bars, no addition; Grey bars, 1.5 μM diclofenac; black bars, 15 μM diclofenac.

Figure 4. Diclofenac impairs migration of bone marrow derived macrophages (BMDM). Experimental procedures are described in the Materials and Methods section. Cells were incubated for18-24 h with 100 ng/μl of LPS in the presence or absence of 10 nM MgTx, 100 nM CTX or diclofenac (DIC) at the concentrations indicated. (A-F) Representative images of the different groups. Migrating cells are found at the right of the scratch. Bars represent 500 μm. (A) control cells without addition. (B) LPS-activated cells in the presence of MgTx. (C) LPS-activated cells in the presence of CTX. (D-F) LPS-activated cells in the absence (D) or the presence of 1.5 μM (E) and 15 μM (F) diclofenac. (G) Percentage of migrating cells. Significant differences were found in

LPS-activated BMDM treated with 15 μM diclofenac (**, p<0.01 *vs.* LPS). (H) Distance travelled by the migrating macrophages (μm). Distance was measured between the scratch and the nucleus of the cell. Significant differences were found in LPS-activated BMDM treated with diclofenac at both concentrations (**, p<0.01 and ***, p<0.001 for LPS *vs.* 1.5 and 15 μM diclofenac, respectively). White bars, no diclofenac; Grey bars, 1.5 μM diclofenac; Black bars, 15 μM diclofenac.

Figure 5. Inhibitory effects of diclofenac and K^+ channel blockers on IL-2 production by stimulated Jurkat T lymphocytes. Cells were stimulated with 10 μg/μl LPS, 5 μg/ml PHA, 80 nM PMA and PHA (5 μg/ml) + PMA (80 nM) for 18-24 h. Simultaneously, 10 nM Margatoxin (MgTx), 100 nM Charybdotoxin (CTX), or diclofenac (DIC) were added at the concentrations indicated. Supernatant was collected and processed for an ELISA analysis. (A) IL-2 production (pg/ml) of control (Ctrl), LPS and PMA-stimulated Jurkat cells. (B) IL-2 production (pg/ml) of control (Crtl) and PHA-stimulated Jurkat T cells. Significant differences were found in PHA-stimulated cells (***, p<0.01 *vs.* Crtl), MgTx, CTX, 1.5 μM diclofenac and 15 μM diclofenac (***, p<0.001; ***, p<0.001; **, p<0.01; *, p<0.05, respectively *vs.* PHA without diclofenac). (C) IL-2 production (ng/ml) of control (Ctrl) and (PHA + PMA)-stimulated Jurkat T lymphocytes. Significant differences were found in PHA + PMA-stimulated cells (***, p<0.001 *vs.* Ctrl) and cells treated with MgTx, CTX and 15 μM diclofenac (*, p<0.05; ***, p<0.001; *, p<0.05, respectively *vs.* PHA + PMA without diclofenac).

Figure 6. Diclofenac decreases Kv1.3 expression in activated T-lymphocytes. Cells were stimulated with either 5 μg/ml PHA or combination of PHA (5 μg/ml) + 80 nM PMA for 18-24 h. (A) Kv1.3 mRNA expression in control, PHA-stimulated and

PHA+PMA-stimulated Jurkat T cells in the presence or absence of diclofenac (DIC) at the concentrations indicated. Significant differences were found with 15 μM DIC in PHA and PHA+PMA- stimulated lymphocytes (*, $p<0.05$; **$p<0.01$ *vs.* PHA and PHA+PMA in the absence of DIC (0), respectively). (B-E) Kv1.3 protein expression in control, PHA-stimulated and PHA+PMA-stimulated Jurkat T cells in the presence or absence of DIC. B, Representative western blot analysis in the presence or absence of PHA. (C) Quantification of the relative Kv1.3 protein expression from experiments presented in panel B. (D) Representative western blot analysis in the presence or absence of PHA+PMA. (E) Quantification of the relative Kv1.3 protein expression from experiments presented in panel D. All values were normalized by β-actin expression and compared to the control in the absence of DIC (0). *, $p<0.05$; **, $p<0.01$ *vs.* PHA+PMA and PHA in the absence (0) of DIC, respectively. White bars, no diclofenac; Grey bars, 1.5 μM diclofenac; Black bars, 15 μM diclofenac. AU, arbitrary units.

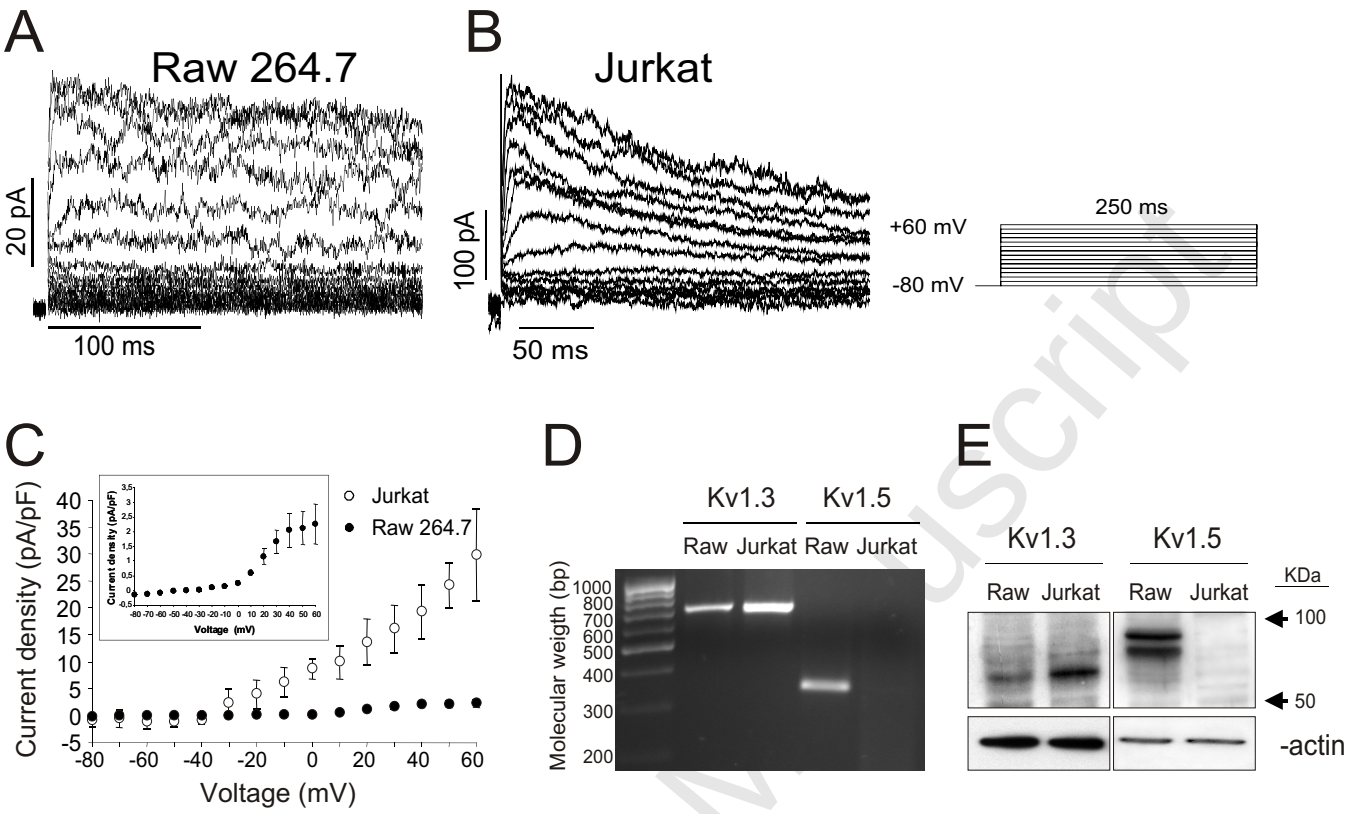


Figure 1

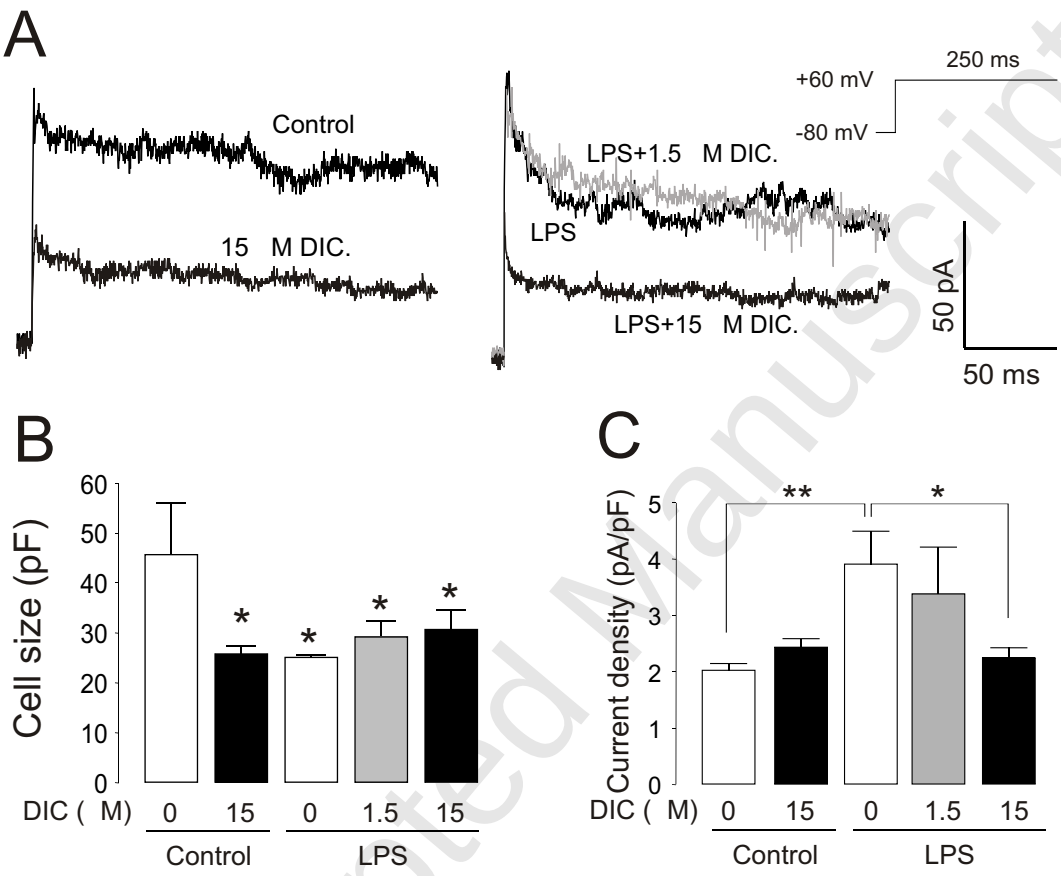
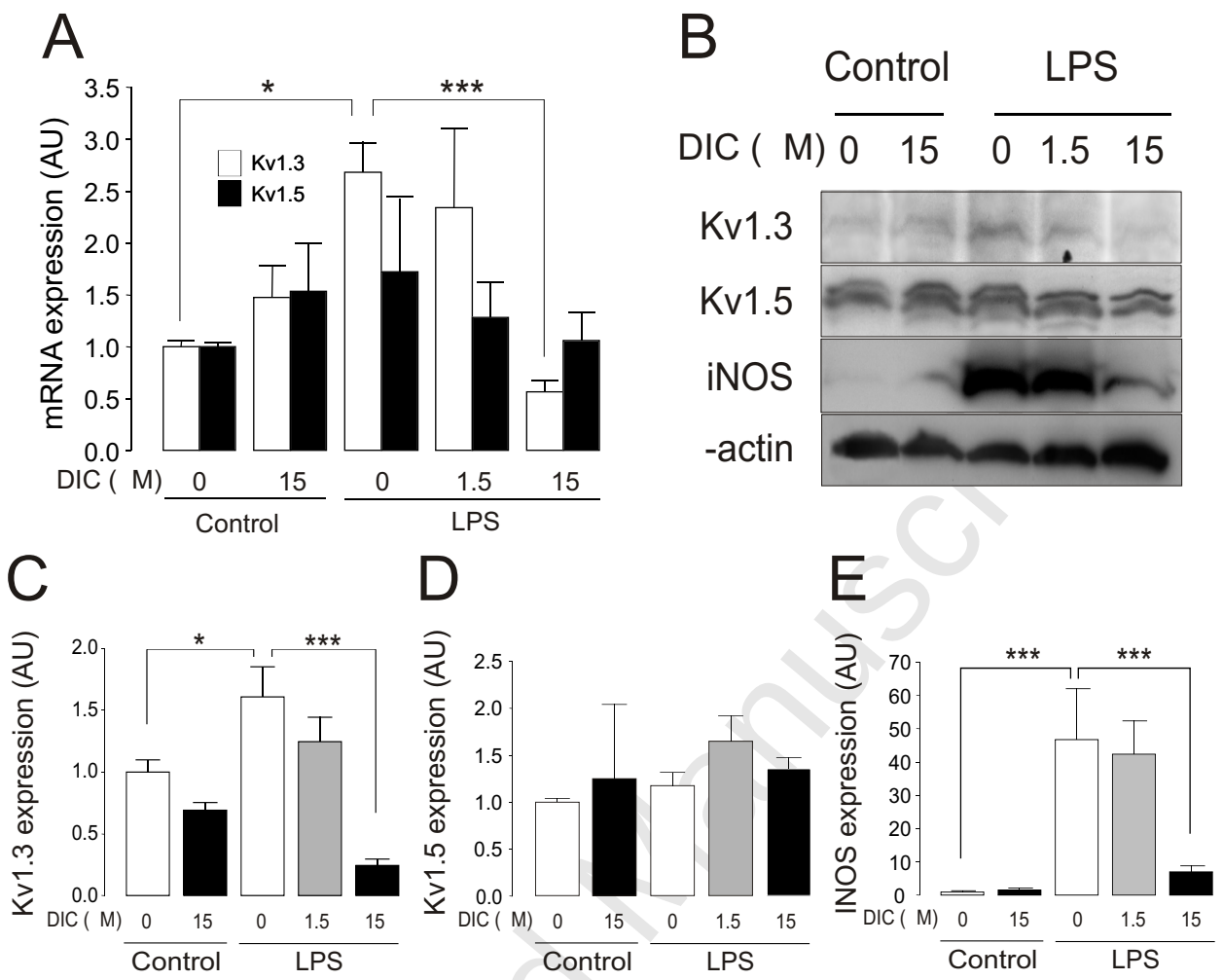


Figure 2



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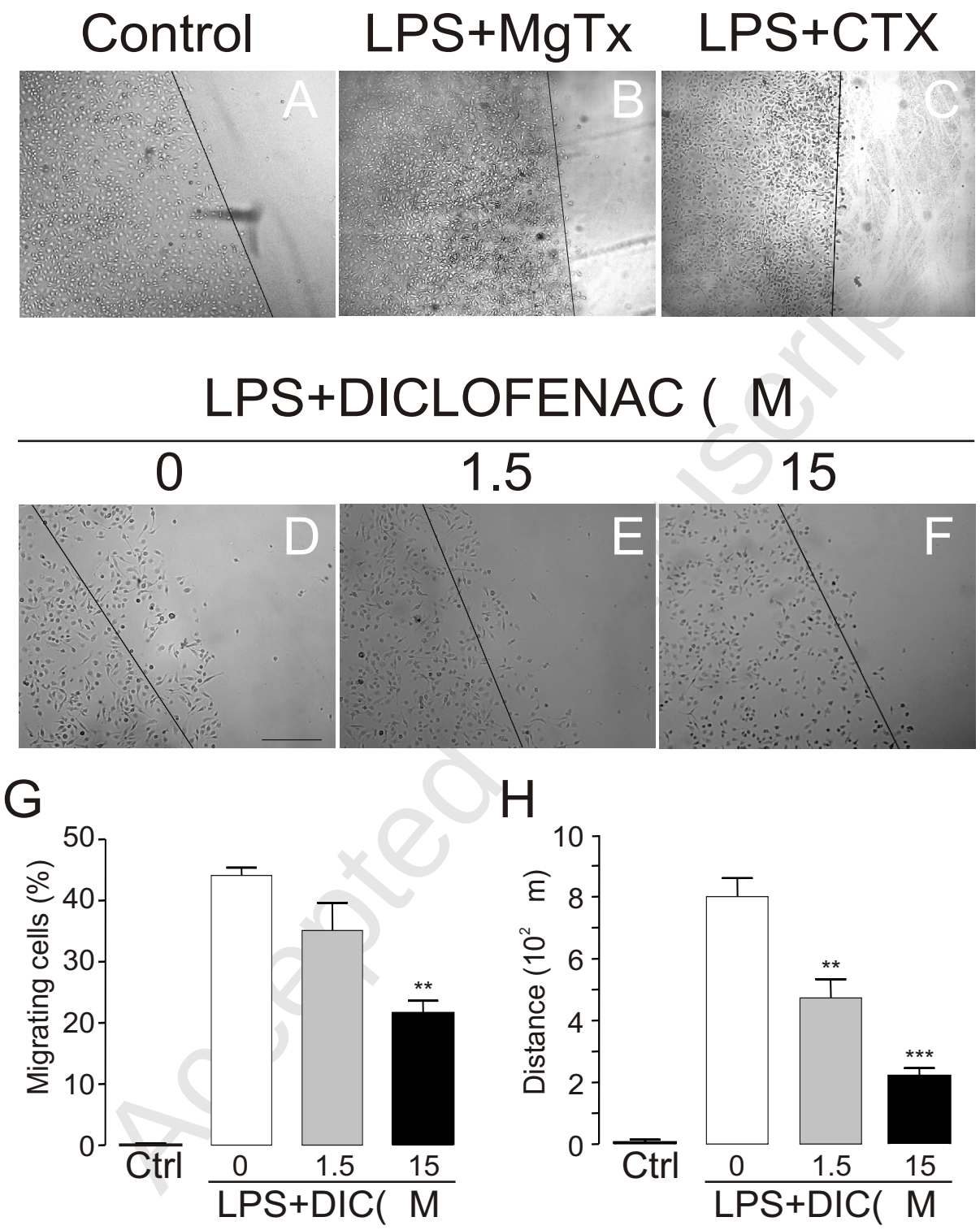

Figure 4

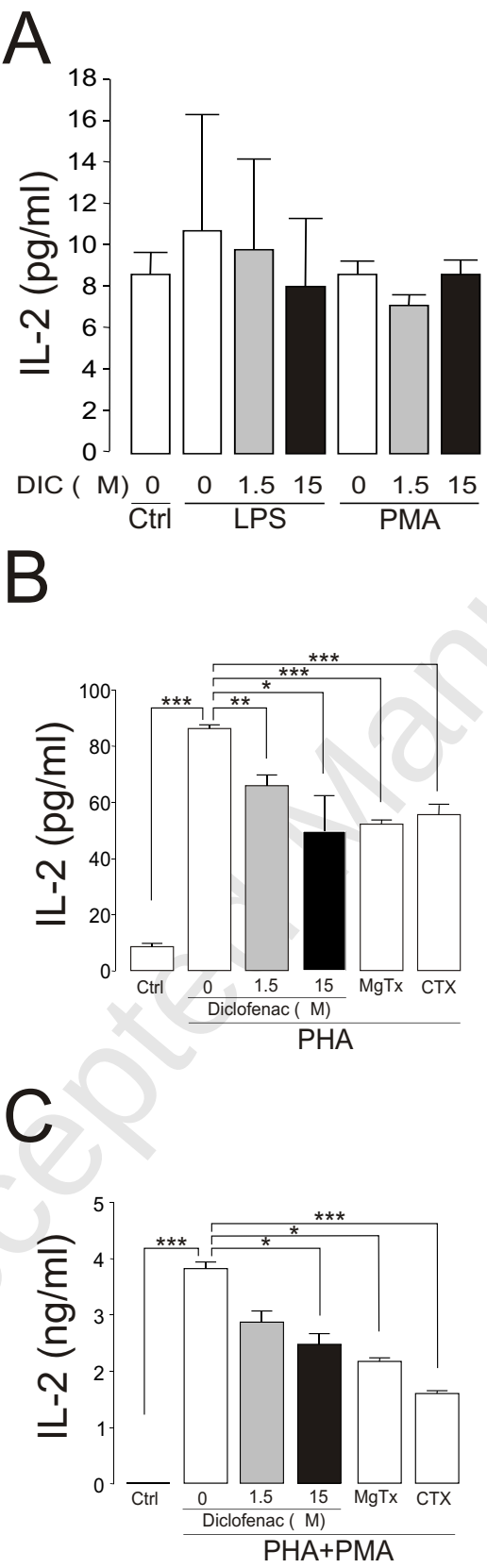

Figure 5

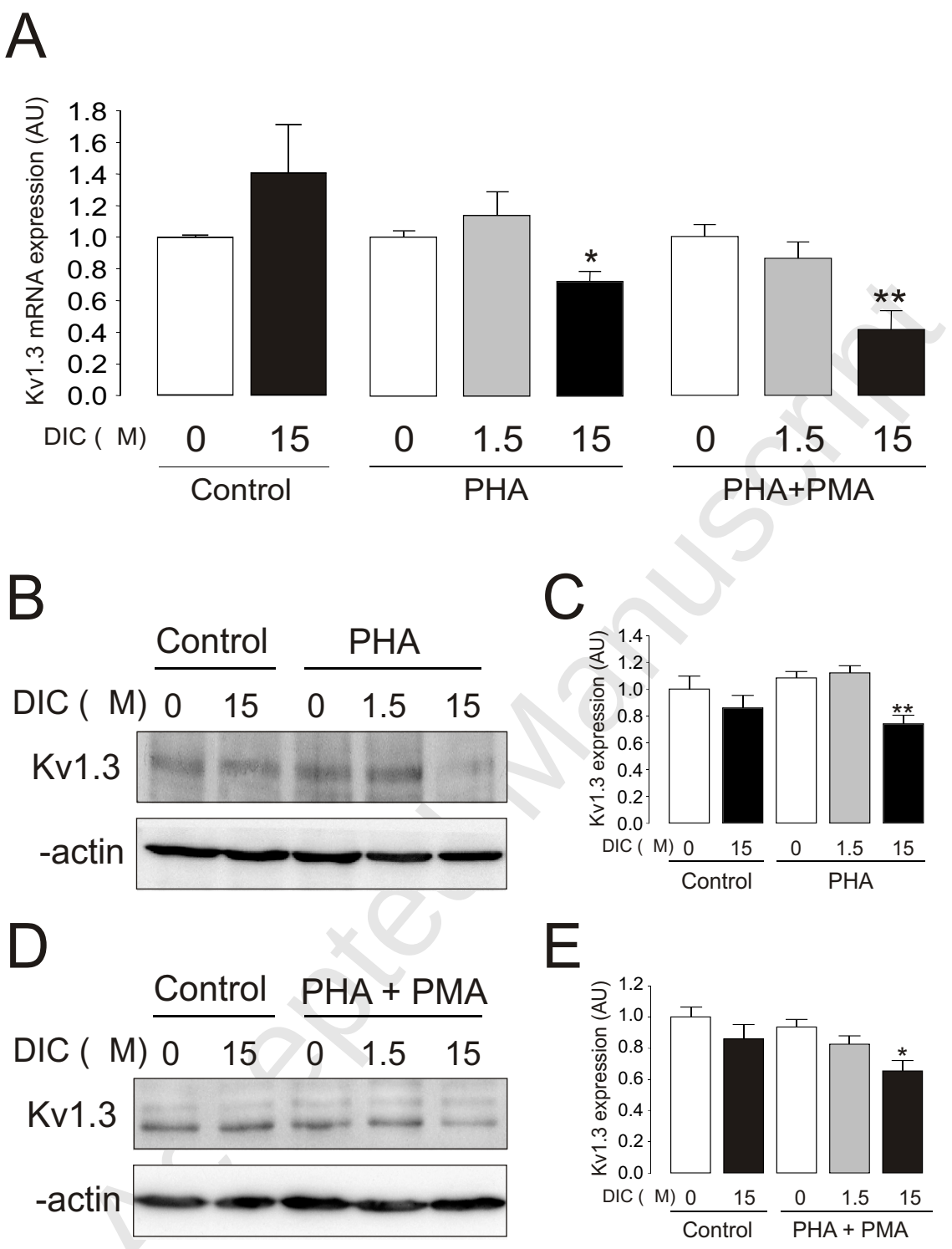

Figure 6

*Graphical Abstract