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Bupivacaine inhibits the TLR4 - and TLR2 - Myd88/NF- κ B pathways in human leukocytes

Running Head: Bupivacaine inhibits TLR4/TLR2

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ABSTRACT

Background. Local anesthetics have anti-inflammatory effects. Because most previous experiments were performed with supra-therapeutic concentrations, we measured the effects of clinically relevant concentrations of bupivacaine on the TLR4- and TLR2-MyD88-NF- κ B pathways.

Methods. We measured TNF- α and PGE2 release, p38 MAP-kinase phosphorylation and translocation of NF- κ B in human peripheral blood mononuclear cells (hPBMC) and human monocytes challenged with LPS (lipopolysaccharide) or Pam3CSK4 (tripalmitoylated lipopeptide Pam3CysSerLys4) in the presence or absence of bupivacaine. Similarly, we measured the effect of bupivacaine on HEK293 cells expressing the hTLR4 and the hTLR2 genes and challenged with LPS or Pam3CSK4. Finally, molecular docking simulations of R(+)- and S(-)-bupivacaine binding to the TLR4-MD-2 complex and to the TLR2/TLR1 heterodimer were performed.

Results. In PBMCs, bupivacaine from 0.1 to 100 μ M inhibited LPS-induced TNF- α and PGE2 secretion, phosphorylation of p38 and nuclear translocation of NF- κ B in monocytes. Bupivacaine similarly inhibited the effects of Pam3CSK4 on TNF- α secretion. Bupivacaine inhibited the effect of LPS on HEK293 cells expressing the human TLR4 receptor and the effect of Pam3CSK4 on HEK293 cells expressing the human TLR2 receptor. Molecular docking showed that bupivacaine binds to the MD-2 co-receptor of TLR4 and to the TLR2 receptor.

Conclusions. Contrary to numerous experiments performed with supratherapeutic doses, our results were obtained with concentrations of bupivacaine as low as 0.1 μ M. We conclude that

bupivacaine modulates the inflammatory reactions such as those observed after surgery or trauma, at least partly by inhibiting the TLR4- and TLR2-NF- κ B pathways.

KEYWORDS: Toll-like receptors, local anesthetics, bupivacaine, inflammation.

LIST OF ABBREVIATIONS

CD14 Cluster of differentiation protein 14

COX-2 Cyclooxygenase 2

ELISA enzyme-linked immunosorbent assay

IL1- β Interleukin 1 beta

MAP-kinase Mitogen-activated protein kinase

MD-2 Myeloid differentiation protein 2

MyD88 Myeloid differentiation primary response 88

NF- κ B Nuclear factor kappa-light-chain-enhancer of activated B cells

Pam3CSK4 Tripalmitoylated lipopeptide Pam3CysSerLys4

PBMC Peripheral blood mononuclear cell

PGE2 prostaglandin E2

qPCR Real-time polymerase chain reaction

RNA Ribonucleic acid

TLR Toll like receptor

TNF- α Tumor Necrosis Factor alpha

XlogP3 predicted logarithm of the n-octanol/water partition coefficient

1. INTRODUCTION

Local anesthetics are small molecules with anti-inflammatory properties [1,2]. These properties are increasingly considered in the perioperative therapeutic strategy [3]. During and after major abdominal or vascular surgery, an inflammatory process occurs and local anesthetics modulate the liberation of inflammatory mediators [4,5]. Local anesthetics

decrease LPS -induced cytokine production in leukocytes, Jurkat cells, macrophages and microglial cultures [6-9]. Bupivacaine decreases *ex-vivo* production of TNF- α and IL1- β by cultured leukocytes from mice subjected to *in-vivo* carrageenan injury [10]. At clinically relevant concentrations, bupivacaine decreases microglial stimulation in rat spinal cord and nucleus of the solitary tract and inhibits axonal transport [11,12]. These intrinsic anti-inflammatory properties may involve pathways different from the voltage gated ion channels blockade. Activation of the TLR4-MD-2 and TLR2/1 complexes by many endogenous and exogenous ligands is involved in numerous diseases, including postoperative pain [13-15]. The two major pathways driven by TLR4 activation are the TRIF and the MyD88 pathways, the latter being considered the predominant rapid proinflammatory system. After activation by LPS, this cascade rapidly induces activation of the canonical NF- κ B components. This pathway induces secretion of proinflammatory mediators such as COX-2, TNF- α and IL1- β and activates MAP-kinases [13-16]. After stimulation by LPS, the TLR4 protein level rapidly increases and NF- κ B rapidly translocates to the nucleus. A previous study has shown that lidocaine at high doses interacts with the TLR4 pathway in murine macrophages [17]. Similarly, articaine inhibits the NF- κ B pathway in kidney from mice receiving LPS [18]. Because TLR4 and TLR2 possess the same adaptor protein MYD88, we explored both pathways to verify our hypothesis that bupivacaine acts on these routes. We measured the effect of bupivacaine at low, clinically relevant concentrations on human leukocytes challenged with LPS and the lipopeptide Pam3CSK4 (tripalmitoylated lipopeptide Pam3CysSerLys4) and performed molecular docking to better understand the relationship between this local anesthetic and the TLR receptors.

2. MATERIALS AND METHODS

2.1. Reagents

RPMI (Roswell Park memorial Institute) 1640 with 1% ultraglutamine was purchased from PAA Laboratories (Velizy-Villacoublay France); racemic bupivacaine and carrageenan from Sigma-Aldrich, Saint Quentin Fallavier, France; ultra-pure LPS from E. Coli 111:B4 and

Pam3CSK4 from InvivoGen (Toulouse, France); Lympholyte-poly and Lympholyte-H from Cederlane (Tebu-bio Le Perrey-en-Yvelines, France); the gradient magnetic cell sorting kit used to isolate monocytes from Miltenyi Biotec (Miltenyi Biotec®, Paris, France); trizol and the Superscript II® reverse transcriptase from Invitrogen (Cergy Pontoise, France); all other reagents from Sigma-Aldrich unless specified otherwise.

2.2. Human leukocytes

After approval by the local ethical committee (CCPPRB of Bicêtre Hospital), informed consent was obtained from ASA1 volunteers. Human peripheral blood mononuclear cells (hPBMCs) were isolated by density gradient using lympholyte, resuspended in RPMI 1640 and cultured or used for isolation of monocytes. Experiments were performed on 1) hPBMCs, 2) hPBMCs enriched in monocytes (adherent cells), or 3) monocytes isolated from hPBMCs using a high-gradient magnetic cell sorting kit (Miltenyi Biotec®, Paris). Cell purity with this technique is at least 94% of CD14 cells. hPBMCs were plated in 24-well plates and monocytes were plated in 96-well plates (final concentration 2×10^6 cells.ml⁻¹) in RPMI 1640 supplemented with 1% L-glutamine. All cells were tested for viability using trypan blue and counted at the time of harvesting.

2.3. HEK293 cells and cell culture

HEK293 hTLR4 and hTLR2 cells were purchased from InvivoGen, Toulouse, France. These cells are stably transfected with the human hTLR4, MD-2 and CD14 co-receptors genes or with the human hTLR2 and CD14 co-receptor and express an inducible SEAP (secreted embryonic alkaline phosphatase) reporter gene responding to NF-κB and AP-1 stimulation. The HEK Null is the parental cell line. Cells were cultured in Dulbecco's Modified Eagle Medium supplemented with 4.5 g.l⁻¹ glucose, 10% (v/v) heat inactivated fetal bovine serum, 50 U.ml⁻¹ penicillin, 50 mg.ml⁻¹ streptomycin, 100 mg.ml⁻¹ Normocin™ and 2 mM L-glutamine

in 96 well plates. Hygromycin B (200 $\mu\text{g.ml}^{-1}$) and Zeocin™ (100 $\mu\text{g.ml}^{-1}$) were used as selection agents.

2.4. Reverse transcription and qPCR

Reverse transcription followed by TaqMan real time PCR was performed in hPBMCs. Total RNA was extracted using Trizol and reverse transcription was carried out with the Superscript II® reverse transcriptase. TaqMan real-time PCR assays were performed on an ABI Prism 7300 sequence detector (Applied Biosystems, Courtaboeuf France). Primers and probes were from Applied Biosystems (Hs00152939_m1 encoding for the human toll-like receptor 4 isoform C and isoform A precursors and Hs00765730_m1 encoding for the human nuclear factor NF-kappa-B p105 subunit isoforms 1 and 2). The 18S ribosomal ribonucleic acid (rRNA) was used as control.

2.5. Western blots

Cells were lysed using RIPA (Radio-Immunoprecipitation Assay) lysis buffer and the total protein extracts were separated by SDS Page (Sodium Dodecyl Sulphate–polyacrylamide gel electrophoresis). Primary antibodies were from Novus Biologicals Cambridge UK: mouse monoclonal TLR4 Antibody [IMGEX: IMG-5031A] and rabbit polyclonal MyD88 Antibody [IMGEX: IMG-178]. Loading normalization used β -actin sc-47778 (Santa-Cruz, Cliniscience, Nanterre, France). Proteins were visualized using the quantitative Odyssey Fc imaging system (Li-Cor Biosciences, Lincoln, NE, USA) and analyzed using Image Studio software.

2.6. PGE₂, TNF- α , p38 MAP-kinase and NF- κ B assays

PGE2 was measured with a commercial ELISA kit (Cayman, Interchim Montluçon, France). TNF- α was measured with a commercial ELISA kit (Duoset™, R&D systems, Abingdon, United Kingdom). p38 MAPK and phospho-p38 MAPK were measured with a highly specific cell-based ELISA kit (FACE, Active-Motif, La Hulpe, Belgium). The P55 and P60 forms of NF- κ B were measured in nuclear extracts from monocytes using the highly sensitive TransAM® NF- κ B kit (Active-Motif, La Hulpe, Belgium).

2.7. Experiments (Table 1)

2.7.1. Experiments on human leukocytes

In the following experiments, each volunteer's batch received all treatments within the same experiment. TNF- α and PGE2 concentrations were measured in the dishes supernatant.

Inhibition of LPS- and PAM3CSK4-induced leucocytes priming by bupivacaine.

Human PBMCs were pretreated with bupivacaine 0, 1.5, 7.5 and 75 μ M (for TNF- α effect) or 0, 0.1, 1, 10 and 100 μ M (for PGE2 effect). Similarly, monocytes were pretreated with bupivacaine 0, 0.1, 1, 10 μ M. Six hours later, the cells were stimulated with LPS 100 ng.ml⁻¹ (hPBMCs) or 10 ng.ml⁻¹ (monocytes) or Pam3CSK4 200 ng.ml⁻¹ (hPBMCs) or 20 ng.ml⁻¹ (monocytes) during 18h. The bupivacaine PGE2 or TNF- α concentration-effect relation was modeled with an Emax model: $E = E_0 - \frac{E_{\max} C}{ED50 + C}$, where E is the measured concentration of TNF- α or PGE2, E₀ is the maximum effect of LPS or Pam3CSK4, E_{max} is the maximum effect of bupivacaine and C is bupivacaine concentration.

Effect of bupivacaine on TLR4 and MYD88 protein concentration

Adherent hPBMCs from six volunteers were pretreated with 0, 0.1, 1, 10, 100 μM bupivacaine six hours before LPS challenge (100 ng.ml^{-1}) during 45 minutes. After lysis, cell extracts were separated, and protein levels measured by immunoblotting.

Effect of bupivacaine on p38 MAP-kinase phosphorylation

Adherent hPBMCs from eight volunteers were pretreated with 0, 0.1, 1, 10, 100 μM bupivacaine six hours before LPS challenge (100 ng.ml^{-1}) during 45 minutes. Thereafter, cells were harvested and phospho- and total p38 MAP-kinase assay was performed on cell lysate.

Effect of bupivacaine on mRNA expression of hTLR4 and hNF- κB p105 subunit

Reverse transcription followed by TacMan real time PCR was performed in total cell lysate from adherent hPBMCs ($n=6$ volunteers). Bupivacaine (0, 1, 10, 100 μM) was added to the cells six hours before addition of LPS (100 ng.ml^{-1}) or medium during 45 minutes before harvesting.

Effect of bupivacaine on NF- κB translocation after LPS challenge

Monocytes from six volunteers were pretreated with bupivacaine 1 μM or saline (each subject was his/her own control) during 45 minutes before addition of LPS 10 ng.ml^{-1} during 45 minutes before harvesting. The P55 and P60 forms of NF- κB were assayed in the nuclear extract.

2.7.2. Experiment on HEK293 cells

Bupivacaine (0, 0.1, 1, 10, 100 μM) and either LPS, 10 ng.ml^{-1} (HEK293 hTLR4 cells), Pam3CSK4, 50 ng.ml^{-1} (HEK293 hTLR2 cells) or culture medium alone were added on cells cultured in 96 wells for 6 hours. Null cells were also stimulated with 100 ng.ml^{-1} TNF- α (positive

control). Eight wells/condition were performed in duplicate. SEAP secretion was measured using QUANTI-Blue® at 620 nM.

2.8. Molecular docking

Lidocaine, bupivacaine, amitriptyline, LPS-lipidA from E.coli and Pam3CSK4 structures were obtained from PubChem as SDF files and translated on-line into pdb files using the NCI translator Cactus. The structure of LPS from E.coli was used after retrieving from the crystal structures of the human TLR4-human MD-2-E.coli LPS complex (obtained from the RCSB protein data bank as pdb file (PDB entries: 3FXI) [19]. Similarly, the crystal structure of the human TLR2/1-Tri-Acylated Lipopeptide human heterodimer complex was obtained from the RCSB protein data bank as pdb file (PDB entries: 2Z7X) [20]. The ligands and HOH residues were removed using Molegro Molecular Viewer (Molegro, Aarhus Denmark). Polar hydrogens and Gasteiger charges were added and a 20x20x20 or 40x40x40 Å grid was initially constructed using AutoDock 4.0 (The Scripps Research Institute, USA). Flexibility of ligands was considered by maximizing torsion number. MD2 pocket was kept rigid, whereas residues surrounding TLR2 pocket were made flexibles [20,21]. Docking was performed using AutoDock Vina using a 0.375 Å spacing grid [22]. Visualization and residue-ligand analysis were performed using Biovia Discovery Studio (Dassault, San Diego, USA). Binding of LPS and PAM3CSK4 were also calculated in order to validate our procedures. Because dimerization of TLR receptors is induced by ligand binding, we also computed ligand binding to each monomer (TLR4 or TLR2).

2.9. Statistics

Pharmacodynamic fitting was performed using NONMEM VI, level 1 (GloboMax, LLC, Hanover, MD). ED50, the concentration of bupivacaine leading to half-maximum effect is

reported as the population value and 95 % confidence interval (95% CI) calculated using likelihood profiling.

Other statistical procedures were performed using R. The number of subjects needed in each experiment was calculated using a 0.05 alpha error (bilateral), a power of 80 % and a Cohen d estimated from our experience or from the literature. All data were checked for departure from normality using the Shapiro-Wilk test and qq plots. ANOVAs comparing the effects of bupivacaine and mitogens have been performed using the procedure lme because of the unbalanced nature of the data sets and correlations. The effects on HEK293 cells were compared using two-way ANOVA (Null cells were not included in the ANOVAs). ANOVAs were followed by post-hoc comparisons with the Bonferroni-Holms correction. To limit the errors inflation, we deliberately limited the comparisons to control and bupivacaine groups, control and LPS groups and LPS and LPS+bupivacaine groups.

Data are reported as the mean and 95% confidence interval or the standard error of the mean in the figures for clarity. $P < 0.05$ was considered significant.

3. RESULTS

See table 2 and figures 1-4 for statistical significance.

3.1. Bupivacaine inhibits PGE2 and TNF- α secretion by stimulated leukocytes *in-vitro* (Figure 1 and figure 4).

LPS induced PGE2 and TNF- α secretion by cultured leukocytes. In hPBMCs, E_{max} was 10000 and 6500 $\text{pg}\cdot\text{ml}^{-1}$ for PGE2 and TNF- α respectively. In human monocytes, E_{max} was 299 $\text{pg}\cdot\text{ml}^{-1}$ TNF- α . Similarly, Pam3CSK4 induced TNF- α production by the cultured leukocytes. E_{max} was 479 $\text{pg}\cdot\text{ml}^{-1}$ and 519 $\text{pg}\cdot\text{ml}^{-1}$ TNF- α in hPBMCs and monocytes respectively.

3.2. Bupivacaine inhibits the LPS-induced increase in TLR4 and MyD88 protein content, p38 phosphorylation and TLR4-mediated activation of NF- κ B (Figure 2)

Bupivacaine inhibited 1) the LPS-induced increase in TLR4 mRNA expression and TLR4 and MyD88 protein content in hPBMCs, 2) the LPS-induced phosphorylation of p38 MAP-kinase in adherent cells from hPBMCs and 3) the LPS-induced increase in NF- κ B p105 mRNA in adherent PBMCs and the translocation of p50 and p65 subunits of NF- κ B in monocytes. Bupivacaine alone did not modify any of these parameters.

3.3. Bupivacaine inhibits LPS- and Pam3CSK4-induced NF- κ B secreted embryonic alkaline phosphatase (SEAP) in HEK293 cells stably expressing hTLR4, MD-2 and CD14, and hTLR2 and CD14 respectively (Figure 2f and figure 4c).

Neither LPS, Pam3CSK4 nor bupivacaine had any effect on SEAP secretion in Null HEK193 cells. Bupivacaine inhibited the LPS- and PAM3CSK4-induced SEAP secretion in HEK293 cells expressing TLR4 and TLR2. Bupivacaine alone did not induce SEAP secretion at all concentrations.

3.4. In-silico docking simulations of bupivacaine binding to the TLR4-MD-2 complex (Figure 3) and to the TLR2/1 heterodimer (Figure 4d)

Amitriptyline and lidocaine bound in the MD-2 pocket with affinities of $\Delta G^\circ = -8.6$ and -6.1 kCal.mol⁻¹ respectively. Bupivacaine binding was stereospecific with affinities of -7.6 and -6.9 kCal.mol⁻¹ for the R(+) and S(-) enantiomers respectively. Bupivacaine bound to the TLR2/TLR1 heterodimer with a similar affinity of -6.9 kCal.mol⁻¹ for both enantiomers. Lipid A and LPS bound to the MD-2 pocket with respective affinities of -26.3 and -40.7 kCal.mol⁻¹ and Pam3CSK4 bound to the TLR2/TLR1 heterodimer with an affinity of -15.3 kCal.mol⁻¹.

Bupivacaine binding to the monomers (TLR4-MD2 or TLR2) was identical to binding to the dimers.

4. DISCUSSION

Our study shows that the anti-inflammatory effect of bupivacaine on human leukocytes is at least partly mediated by inhibition of the TLR4-MyD88-NF- κ B pathway. Bupivacaine also inhibited the Pam3CSK4-induced TLR2-NF- κ B pathway. Interestingly, this effect occurred at low unbound, clinically observed concentrations - the threshold for CNS toxicity in volunteers is $\approx 1 \mu\text{M}$ free bupivacaine concentration [23]. In human PBMCs and monocytes, bupivacaine pretreatment inhibited the secretion of inflammatory mediators induced by LPS or Pam3CSK4. The dose-effect relationship showed that the ED50 of bupivacaine was very low and eight to ten times lower in the monocytes than in the PBMCs experiment. This is consistent with the fact that monocytes/macrophages are primary cells expressing TLR4 and TLR2. Similar results have been observed in macrophage challenged with LPS and treated with bupivacaine [6].

The TLR4 pathway is a major player in inflammation [13]. LPS rapidly induced an increase in TLR4 mRNA expression and an increase in TLR4 and MyD88 protein levels (Figure 2). Similarly, an increase in the NF- κ B p105 subunit mRNA was observed 45 minutes after LPS challenge. This may correspond to a feed-back related to the rapid degradation of this NF- κ B precursor, which has been observed after LPS challenge in murine RAW264.7 macrophages [17]. This was accompanied by nuclear translocation of the p50 and p65 NF- κ B subunits, whose concentration increased by three-fold in the monocytic nuclear extract. This activation of the canonical NF- κ B pathway induced the secretion of proinflammatory mediators and the phosphorylation of the p38 MAP-kinase. Bupivacaine produced a similar effect on the TLR2/TLR1 pathway.

To strengthen these results, we used two HEK293 cells lines stably transfected to express 1) the human TLR4, MD-2 and CD14 co-receptors and 2) the human TLR2 and CD14 co-receptor. After stimulation, reporter activity increased by three-fold in the two cell lines and bupivacaine totally inhibited this effect from the lower dose (0.1 μ M).

Bupivacaine alone had no effect on any experiment, but bupivacaine pretreatment inhibited partly, but never totally, the effects of LPS and Pam3CSK4 in leukocytes as demonstrated by the significant E_{max} in the dose-effect experiments.

In an experiment using murine macrophages, Lee et al. already showed that 50 μ M lidocaine was able to attenuate the stimulation of the TLR4-NF- κ B pathway induced by LPS [21]. However, these authors added the NOS inhibitor L-NAME to all their cell cultures. They concluded that the observed effects were related to voltage-gated sodium channel blockade, in line with their previous conclusion [6].

To further characterize this effect and to quantify the affinity of bupivacaine to receptors, we modeled bupivacaine binding to the TLR4-MD-2 complex and to the TLR2/TLR1 heterodimer by in-silico docking. Amitriptyline possesses anti-inflammatory properties and is known to bind to the TLR4-MD-2 dimer [24,25]. We then calculated the Gibbs free energy of binding of amitriptyline and lidocaine in addition to bupivacaine because these molecules cover a wide range of hydrophobicity ($XlogP3 = 5.0, 3.4$ and 2.3 for amitriptyline, bupivacaine and lidocaine respectively). The affinity to the MD-2 pocket was -8.6 and -6.1 $kCal.mol^{-1}$ for amitriptyline and lidocaine respectively. Bupivacaine binding was stereospecific with affinity of -7.6 and -6.9 $kCal.mol^{-1}$ for the R(+) and S(-) enantiomers respectively. As expected, interactions between ligands and macromolecules were hydrophobic and the affinities were inversely proportional to $XlogP3$. We calculated an affinity of -40.7 $kCal.mol^{-1}$ for LPS, similar to the value of -38.5 $kCal.mol^{-1}$ reported by Qiu et al [26]. The difference in affinity between LPS from E. Coli and R(+)-bupivacaine was then $\Delta\Delta G^\circ = -33.2$ $kCal.mol^{-1}$, which is important and likely explains that bupivacaine only moderately suppresses TLR4 functions. Bupivacaine bound to TLR2 with a $\Delta G^\circ = -6.9$ $kCal.mol^{-1}$, similar for the R(+) and S(-) enantiomers and close to the affinity

to MD2. In our simulations, Pam3CSK4 had an affinity of $-15.3 \text{ kCal.mol}^{-1}$ very similar to the value of $-15.25 \text{ kCal.mol}^{-1}$ reported by Su et al. [21].

Although, conversion between free energy (ΔG°) and dissociation constant (K_D), or inhibition constant (IC_{50}), must be done with great care, our results show that bupivacaine binding to the MD-2 and TLR2 pockets is strong enough to explain the effects observed at very low bupivacaine concentration (Table 3). Bupivacaine affinity to TLR4-MD-2 and to TLR2 was in the range of the affinity to alpha-1 glycoprotein in serum and to most voltage-gated channels (Table 3) [27-32]. However, the full correspondence between affinity of binding to a specific site and inhibition of effect has been questioned [33]. Since dimerization and activation of the TLR receptors is induced by their specific ligands, we also tested the hypothesis that ligand binding to protein may differ between the monomeric receptor and the active, homo- or heterodimer in a similar way to binding to ionic channels [34]. Binding was similar for both states.

Local anesthetics are small hydrophobic molecules, and it is not surprising to observe pleiotropic effects not related to their nerve blocking effect. For example, local anesthetics at very low concentrations interfere with the G protein-coupled receptors [35-36]. Other small molecules interfere with the TLR pathways. Volatile anesthetics have been shown to attenuate the TLR4 and TLR2 systems activation [37,38]. On the contrary, opioids and particularly morphine may activate the TLR4 pathway [39,40].

5. CONCLUSION

These experiments show that bupivacaine inhibits the TLR4 and TLR2 pathways at very low concentrations, (as low as $0.1 \mu\text{M}$) contrary to numerous experiments performed with supratherapeutic doses. Interestingly, in the absence of an inflammatory insult, we were unable to show any effect of bupivacaine. This effect appears noticeable only when pathogen-associated ligands are in excess in the environment surrounding the receptor. We may

conclude that bupivacaine, and likely other local anesthetics, modulate the inflammatory reactions such as those observed after surgery or trauma, at least partly by inhibiting the TLR4- and TLR2-NF- κ B pathways.

Conflicts of interest

None.

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Author contribution

Marie Binczak M.D. This author helped to design the study, made major contribution to experiments, initial analysis and first draft.

Emilien Purenne M.D. This author helped to design the study, made major contribution to experiments, initial analysis and first draft.

Hélène Beloeil M.D, Ph.D. This author helped to design the study, made minor contribution to experiments, made significant contribution to initial analysis and first draft.

Dan Benhamou M.D. This author made significant contribution to initial analysis, first draft and revision.

Jean Xavier Mazoit M.D., Ph.D. This principal investigator was responsible for the conception and design of the study, made minor contributions to experiments, performed molecular docking and statistical analysis, made major contribution to initial analysis, first draft and revision.

All authors discussed and approved the final manuscript as submitted.

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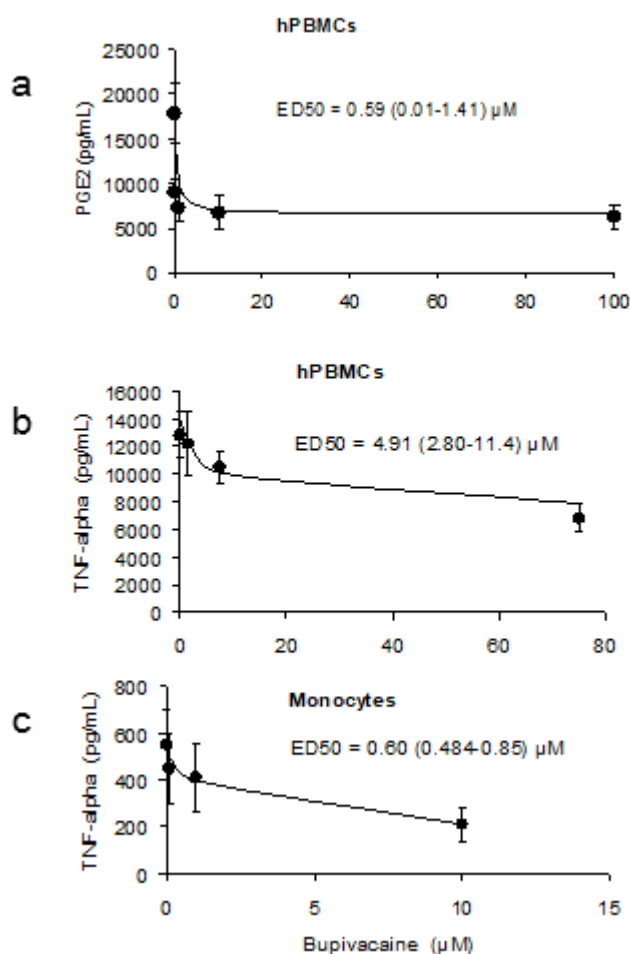


Figure 1. Low doses of bupivacaine inhibit PGE2 and TNF- α secretion by stimulated leukocytes in-vitro.

Inhibition of (a) PGE2 and (b) TNF- α secretion by bupivacaine in hPBMCs. Cells were pretreated by bupivacaine before LPS challenge ($100 \text{ ng}\cdot\text{ml}^{-1}$) during 18 hours. c) Inhibition of TNF- α secretion by bupivacaine in human monocytes. Cells were pretreated by bupivacaine before LPS challenge (10 ng/ml) during 18 hours. ED50 of TNF- α was eight times lower in the monocyte experiment, consistent with the relative concentration of monocytes in PBMCs (the concentration of cells was $2 \cdot 10^6 \cdot \text{ml}^{-1}$, similar in the two experiments).

Data are estimated typical values (95% confidence interval).

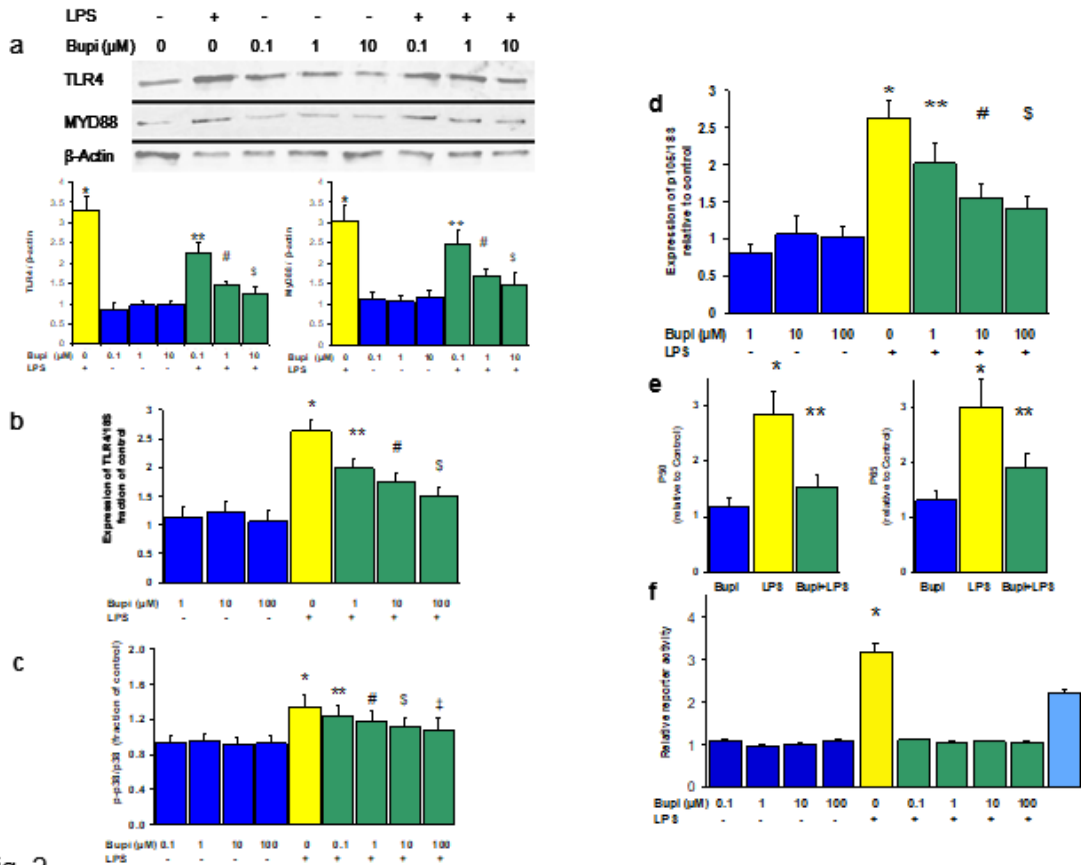


Fig. 2

Figure 2. Bupivacaine (Bupi) inhibits the LPS-induced increase in TLR4 and MyD88 protein content, p38 phosphorylation and TLR4-mediated activation of NF-κB. Data are reported as the response ratio relative to negative control.

a) Effect of LPS and bupivacaine on TLR4 and MyD88 protein content in adherent PBMCs. Cells were cultured in the presence of bupivacaine or culture medium alone and then subjected to LPS (10 ng.ml⁻¹) for 45 minutes. Bupivacaine alone did not modify the TLR4 and MyD88 content (p=0.432 and p=0.326 respectively). LPS increased by three to four-fold the protein content (* p=0.0008 and p=0.0027 vs. control for TLR4 and MyD88 respectively). Pretreatment by bupivacaine inhibited the increase in TLR4 protein content (** p=0.02, # p=0.007, \$ p=0.0003 LPS vs. LPS + Bupi 0.1, 1 and 10 μM respectively). Inhibition of the MyD88 protein content increase was only significant for the higher dose (** p=0.08, # p=0.08, \$ p=0.04 LPS vs. LPS + Bupi 0.1, 1 and 10 μM respectively).

b) Expression of TLR4 mRNA in adherent hPBMCs. Cells were pretreated with bupivacaine 0, 1, 10, 100 μM before addition of LPS (100 ng.ml⁻¹) or medium during 45 minutes. RT-qPCR

was performed in cell lysates. Bupivacaine alone did not modify the expression of TLR4 mRNA ($p=0.829$), which was markedly increased by LPS (* $p=0.002$). Pretreatment by bupivacaine inhibited this increase in a dose-dependent manner (** $p=0.02$, # $p=0.04$, \$ $p=0.04$ LPS vs. LPS + Bupi 1, 10 and 100 μM respectively).

c) Phosphorylation of p38 MAP-kinase in adherent hPBMCs. Cells were cultured with bupivacaine and then subjected to LPS stimulation (100 ng.ml^{-1}) for 45 minutes. Phospho- and total p38 were measured in adherent cells. LPS significantly increased phosphorylation ($p=0.01$). Bupivacaine alone had no effect on phosphorylation ($p=0.368$), whereas it inhibited p38 phosphorylation. This inhibition was only statistically significant for the higher dose (** $p=0.22$, # $p=0.09$, \$ $p=0.052$, ‡ $p=0.03$ LPS vs. LPS + Bupi 0.1, 1, 10 and 100 μM respectively).

d) Expression of NF- κB p105 mRNA in adherent PBMCs. Cells were pretreated with bupivacaine (0, 1, 10, 100 μM) before addition of LPS (100 ng.ml^{-1}) or medium during 45 minutes. RT-qPCR was performed in cell lysates. Bupivacaine alone did not modify the expression of NF- κB p105 mRNA ($p=0.67$), which was markedly increased by LPS (* $p=0.0005$). Pretreatment by bupivacaine inhibited this increase in a dose-dependent manner (** $p=0.04$, # $p=0.0003$, \$ $p=0.02$ LPS vs. LPS + Bupi 1, 10 and 100 μM respectively).

e) P50 and P65 forms of NF- κB in human monocytes nuclear extracts. Cells were plated in the presence of bupivacaine (1 μM) or culture medium for 60 minutes and subjected to LPS stimulation (10 ng.ml^{-1}) during 45 minutes. Bupivacaine alone did not induce P50 or P65 translocation ($p=0.21$ and $p=0.13$ respectively). LPS induced the translocation of both P50 and P65 (* $p=0.007$ and $p=0.01$ respectively). Bupivacaine partly inhibited the translocation of NF- κB induced by LPS (** $p=0.012$ and $p=0.03$, LPS vs. LPS + Bupi respectively).

f) Effect of bupivacaine on HEK293 cells expressing the human TLR4, MD-2 and CD14 co-receptors genes and expressing an inducible embryonic alkaline phosphatase (SEAP) reporter gene responding to NF- κB . Bupivacaine was added at clinically relevant concentrations (0.1-1 μM) and higher (10-100 μM) prior to stimulation with 10 ng.ml^{-1} LPS or culture medium alone. Bupivacaine alone at all concentrations did not induce SEAP secretion ($p=0.99$). LPS significantly induced SEAP secretion and this secretion was totally abolished

by bupivacaine (* $p < 0.0001$ vs. Bupi and LPS + Bupi). Null cells were used as positive control. Neither LPS nor bupivacaine had any effect, but cells responded to TNF- α (positive control, light blue histogram on the right).

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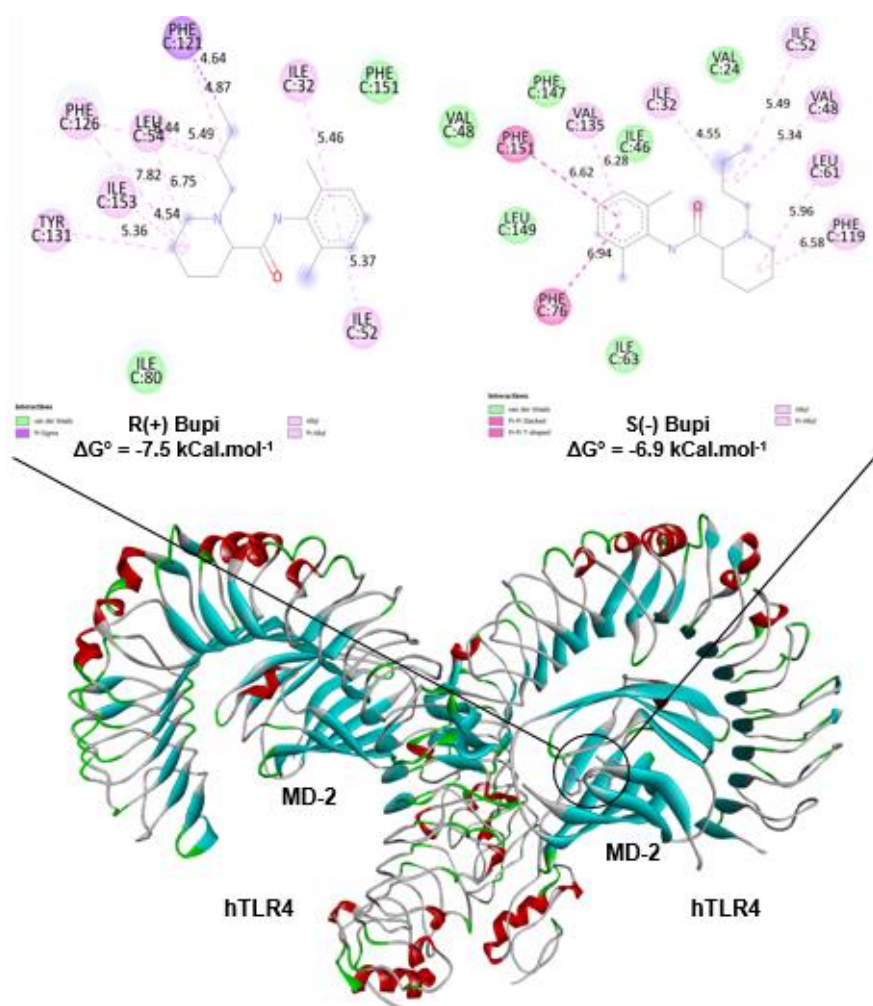


Figure 3. Molecular docking of bupivacaine binding to MD2.

In-silico docking simulations of bupivacaine (Bupi) binding to the TLR4-MD2 complex were performed using the crystal structure of the human TLR4-human MD-2-E.coli LPS complex. Bupivacaine bound to MD-2 with an affinity of $\Delta G^\circ = -7.5$ and $-6.9 \text{ kCal.mol}^{-1}$ for the R(+) and S(-) enantiomers respectively. Interactions are hydrophobic (diagram on top showing the interactions and distances between Bupi and MD-2 residues).

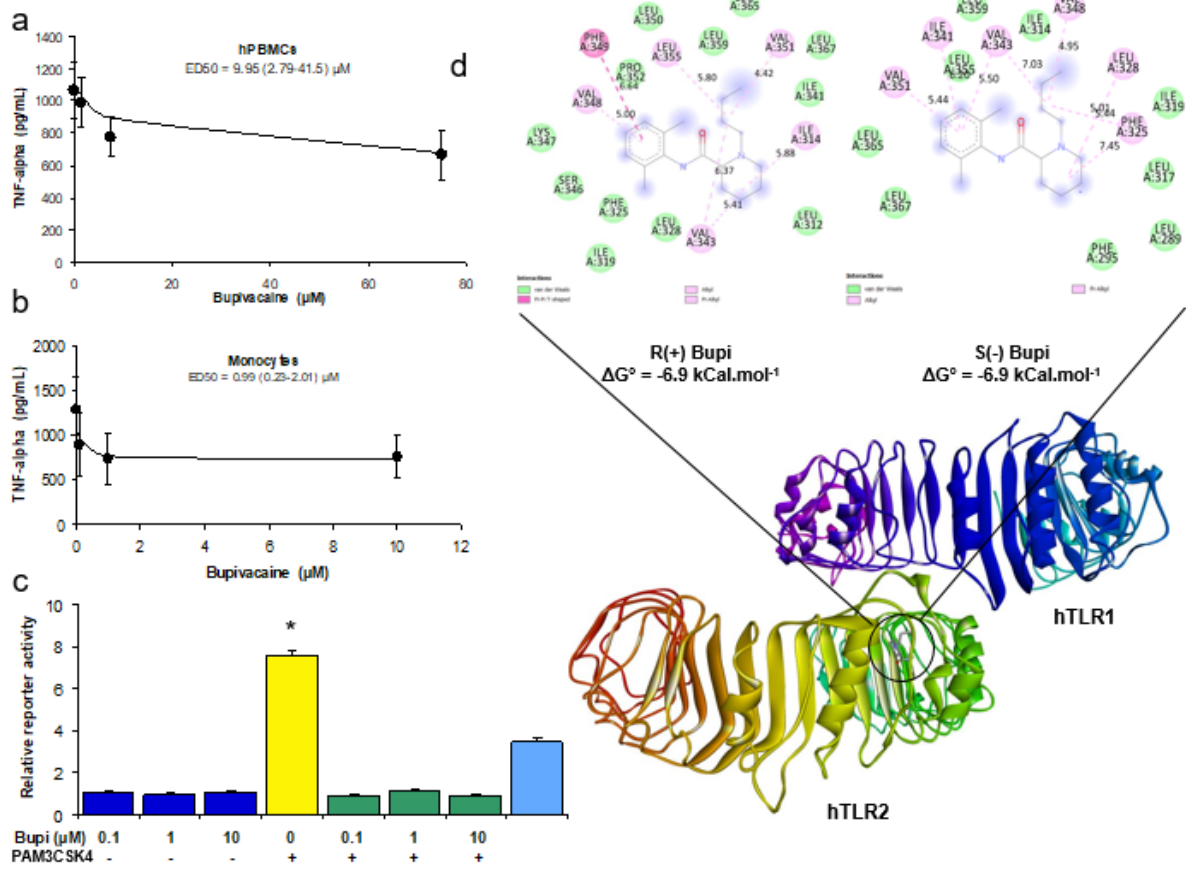


Figure 4. Bupivacaine inhibits TLR2-mediated secretion of TNF- α in human leukocytes, HEK293 cells expressing hTLR2 and CD14 (left). Molecular docking of bupivacaine binding to the TLR2/TLR1 heterodimer (right).

a) Inhibition of TNF- α secretion by bupivacaine in hPBMCs. Cells were pretreated by bupivacaine before PAM3CSK4 challenge (200 ng.ml⁻¹).

b) Inhibition of TNF- α secretion by bupivacaine in human monocytes. Cells were pretreated by bupivacaine before PAM3CSK4 challenge (20 ng.ml⁻¹).

ED50 of TNF- α was ten times lower in the monocyte experiment, consistent with the relative concentration of monocytes in PBMCs (the concentration of cells was $2 \cdot 10^6 \cdot \text{ml}^{-1}$, similar in the two experiments).

c) Effect of bupivacaine (Bupi) on HEK293 cells expressing the hTLR2/hTLR1 receptor and expressing an embryonic alkaline phosphatase (SEAP) reporter gene responding to NF- κB . Bupivacaine was added at clinically relevant concentrations (0.1-10 μM) prior to stimulation with 20 ng.ml⁻¹ PAM3CSK4 or culture medium alone. Bupivacaine alone at all concentrations

did not induce SEAP secretion ($p=0.99$). PAM3CSK4 induced SEAP secretion and this secretion was totally abolished by bupivacaine (* $p<0.0001$ vs. Bupi and PAM3CSK4 + Bupi). Null cells were used as positive control. Neither PAM3CSK4 nor bupivacaine had any effect, but cells responded to TNF- α (positive control, light blue histogram on the right).

d) In-silico docking simulations of bupivacaine binding to the TLR2/TLR1 heterodimer were performed using the crystal structure of the human TLR2/TLR1-PAM3CSK4 complex. Bupivacaine bound to TLR2 with an affinity of $\Delta G^\circ = -6.9$ kCal.mol⁻¹ for both enantiomers. Interactions are hydrophobic (diagram on top showing the interactions and distances between Bupi and TLR2 residues).

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Table 1. Summary of experiments.

Target	Intervention	Measure	Number of subjects or conditions
hPBMCs	Culture ± Bupivacaine ± LPS	PGE2 dose-effect	N = 8 volunteers
	Culture ± Bupivacaine ± LPS or Pam3CSK4	TNF- α dose-effect	N = 8 volunteers
Human monocytes	Culture ± Bupivacaine ± LPS or Pam3CSK4	TNF- α dose-effect	N = 6 volunteers
Adherent hPBMCs	Culture ± Bupivacaine ± LPS	phospho p38/p38 MAPkinase	N = 8 volunteers
	Culture ± Bupivacaine ± LPS	mRNA expression of hTLR4 and hNF- κ B p105	N = 6 volunteers
	Culture ± Bupivacaine ± LPS	Western blot TLR4 and MyD88	N = 6 volunteers
Human monocytes	Culture ± Bupivacaine ± LPS	Nuclear translocation of P50 and P65 forms of NF- κ B	N = 6 volunteers
HEK293 blue hTLR4 and hTLR2 cells	Culture ± Bupivacaine ± LPS or Pam3CSK4	NF- κ B reporter assay	Eight wells/condition
hTLR4-MD-2 and hTLR1/2 complexes	Molecular docking	Lidocaine, bupivacaine, amitriptyline, LPS, LPS-Lipid A, PAM3CSK4 binding to MD-2 or TLR2	

hPBMC is human peripheral blood mononuclear cell, hTLR4 is human Toll-Like Receptor 4, hTLR2 is human Toll-Like Receptor 2, MD-2 is Myeloid Differentiation factor 2

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Table 2. Effect of bupivacaine on adherent hPBMCs/monocytes and hTLR4/hTLR2 HEK293 cells. All values are relative to control.

	Bupivacaine				LPS	LPS + Bupivacaine				
	0.1 μ M	1 μ M	10 μ M	100 μ M		0.1 μ M	1 μ M	10 μ M	100 μ M	
TLR4/β-Actin (protein content in cells)	0.87 \pm 0.37	0.96 \pm 0.23	0.98 \pm 0.18		3.31 \pm 0.78	2.25 \pm 0.62	1.45 \pm 0.26	1.23 \pm 0.47		
	p=0.432 vs. control				p=0.0008 vs. control	p=0.02 vs. LPS	p=0.007 vs. LPS	p=0.0003 vs. LPS		
MyD88/β-Actin (protein content in cells)	1.11 \pm 0.46	1.07 \pm 0.30	1.16 \pm 0.40		3.04 \pm 0.91	2.46 \pm 0.85	1.70 \pm 0.39	1.48 \pm 0.68		
	p=0.326 vs. control				p=0.0027 vs. control	p=0.08 vs. LPS	p=0.08 vs. LPS	p=0.04 vs. LPS		
TLR4/18S		1.13 \pm 0.40	1.21 \pm 0.49	1.06 \pm 0.51	2.65 \pm 0.41		1.99 \pm 0.37	1.73 \pm 0.43	1.49 \pm 0.41	
		p=0.829 vs. control				p=0.002 vs. control		p=0.02 vs. LPS	p=0.04 vs. LPS	p=0.04 vs. LPS
phospho-p38/p38 (protein content in cells)	0.94 \pm 0.18	0.95 \pm 0.07	0.91 \pm 0.08	0.93 \pm 0.09	1.34 \pm 0.13	1.24 \pm 0.13	1.18 \pm 0.12	1.12 \pm 0.10	1.08 \pm 0.13	
	p=0.368 vs. control				p=0.01 vs. control	p=0.22 vs. LPS	p=0.09 vs. LPS	p=0.05 vs. LPS	p=0.03 vs. LPS	
p105/18S		0.82 \pm 0.11	1.06 \pm 0.24	1.03 \pm 0.13	2.63 \pm 0.24		2.02 \pm 0.28	1.56 \pm 0.17	1.41 \pm 0.17	
		p=0.67 vs. control				p=0.0005 vs. control		p=0.04 vs. LPS	p=0.0003 vs. LPS	p=0.02 vs. LPS
P50 (protein content in nucleus)		1.19 \pm 0.32			2.85 \pm 1.02		1.54 \pm 0.55			
		p=0.21 vs. control			p=0.007 vs. control		p=0.012 vs. LPS			
		1.31 \pm 0.42			3.0 \pm 1.26		1.92 \pm 0.65			

P65 (protein content in nucleus)		p=0.13 vs. control			p=0.01 vs. control		p=0.03 vs. LPS			
										Null cells
HEK TLR4 (reporter activity)	1.08±0.05	0.97±0.08	1.02±0.04	1.09±0.03	3.17±0.60	1.11±0.06	±0.06	1.06±0.06	1.05±0.06	2.21±0.18
	p=0.99 vs. control				p<0.0001 vs. control	p<0.0001 vs. LPS	p<0.0001 vs. LPS	p<0.0001 vs. LPS	p<0.0001 vs. LPS	p<0.0001 vs. control
HEK TLR2 (reporter activity)	1.04±0.14	0.98±0.09	1.09±0.10		7.55±0.62	0.94±0.09	1.15±0.18	0.96±0.11		3.45±0.50
	p=0.99 vs. control				p<0.0001 vs. control	p<0.0001 vs. LPS	p<0.0001 vs. LPS	p<0.0001 vs. LPS	p<0.0001 vs. LPS	p<0.0001 vs. control

Table 3. Binding affinity of bupivacaine for different channels or receptors. The following relationship was used for conversion :

$\Delta G_{\text{Binding}} = RT \ln K_D$. We assumed equivalence between K_D and IC_{50} , then calculated $\Delta G_{\text{Binding}} \approx RT \ln IC_{50}$. Calculated variables are in red. When available, data are from inactivated state. R is the Boltzmann constant, T is temperature in Kelvin (37 °C), K_D is the dissociation constant, IC_{50} the concentration leading to half-maximum inhibition, HSA is human serum albumin and AAG is alpha-1 acid glycoprotein.

Receptor	K _D /IC ₅₀ (μM)		ΔG° (kCal)		Author
	R(+)	S(-)	R(+)	S(-)	
TLR4 (MD-2)	4.4	13.6	-7.6	-6.9	Present study 2022
TLR2	13.6	13.6	-6.9	-6.9	Present study 2022
Na channels					
Unspecified (IC ₅₀)	8.6	12	-7.2	-7.0	Nau 1999 ref 26
Heart hH1 (IC ₅₀)	3	4.5	-7.8	-7.6	Nau 2000 ref 27
Potassium channels					
Kv1.5 (K _D)	4.1	27	-7.6	-6.5	Valenzuela 1995 ref 28
hERG (IC ₅₀)	12		-7.0		Siebrands 2007 ref 29
Ryanodyne (IC₅₀)					
	31.4		-6.4		Kariya 2012 ref 30
Serum					
HSA (K _D)	114	99.5	-5.6	-5.7	Mazoit 1996 ref 31
AAG (K _D)	1.11	3.34	-8.5	-7.8	