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Original article

Analgesic adjuvants modulate morphine-induced immune effects in mice

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ABSTRACT

Background: Macrophages, involved in the pathogenesis of pain, express a variety of receptors enabling responsiveness to certain medications, including adjuvant analgesics (AAs), that are effective in neuropathic pain and include drugs not primarily indicated for pain treatment, such as anticonvulsants or antidepressants. Their analgesic effects are likely associated with immunomodulatory activity, that remain undefined. Thus, current research aimed at examining the impact of AAs on morphine-induced effects exerted on mouse immunity.
Methods: Macrophages from mice treated with morphine with or without gabapentin, amitriptyline or venlafaxine, were either subjected to phagocytosis assay, cultured to evaluate the generation of cytokines, or were pulsed with either corpuscular antigen or hapten and transferred to naive recipients to induce humoral or cellular response, respectively. Active contact hypersensitivity was also elicited in drug-treated mice.
Results: We observed that repeatedly administered morphine and AAs reduced antigen phagocytosis by macrophages. Further, amitriptyline with morphine enhanced basal secretion of cytokines by macrophages, and all drugs tended to decrease LPS-stimulated release of pro-inflammatory cytokines. Morphine and AAs impacted the expression of phagocytosis and antigen-presentation markers on macrophages, which led to the reduced ability of morphine-affected macrophages to induce B-cell secretion of specific antibodies, and the addition of AAs strengthened this effect. Finally, gabapentin and venlafaxine suppressed the contact hypersensitivity reaction, while amitriptyline seemed to have the opposite effect.
Conclusions: Our study demonstrated a significant anti-inflammatory activity of AAs across a broad spectrum of macrophage immune functions, which is likely critical to their analgesic activity supporting the beneficial effect of morphine.

Introduction

Macrophages are an adaptable group of phagocytic cells integral for performing a variety of immune functions. Additionally, several studies showed that macrophages, as well as other phagocytes such as microglia, are particularly important during the generation of pain [1–4] through their production of pro-inflammatory cytokines, growth factors and lipids, which can act upon nociceptors to cause pain [5]. The role of macrophages as phagocytic cells responsible for the initial anti-microbial response and removal of cellular debris and apoptotic bodies is well known, as is their secretory activity and functioning as antigen presenting cells (APC), inducing antibody formation [6] or cell-mediated immunity [7]. Furthermore, cytotoxically-activated macrophages act as effector cells of the delayed-type hypersensitivity reaction against intracellular pathogens and of contact hypersensitivity (CHS) to haptens [7]. Macrophages possess a variety of cell-membrane associated and intracellular receptors, including opioid, serotonin, and norepinephrine receptors [8], and thus can react to a variety of signaling molecules, and even certain medications, including those commonly used in the treatment of pain [9]. Opioids, such as morphine, are commonly used analgesics in treatment of severe pain, and their effects could be inhibited with the use of opioid receptor antagonists, naloxone especially.

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Adjuvant analgesics (AAs) are medications that are not primarily indicated for the treatment of pain, but are effective as analgesics in specific clinical situations, including neuropathic pain [10]. Some of the clinically effective AAs include anticonvulsants, such as gabapentin (GABA), and antidepressants, such as amitriptyline (AMI) and venlafaxine (VENLA) [10]. AMI is a tricyclic antidepressant [11] and VENLA is a serotonin-norepinephrine reuptake inhibitor, both of which act upon serotonin and norepinephrine transporters leading to increased levels of these neurotransmitters at neural synapses [9,12]. GABA is an anticonvulsant drug, which is believed to inhibit voltage-gated calcium channels present at nerve endings [13]. While the clinical efficacy of these AAs is widely accepted, much remains to be elucidated as to how exactly they affect the immune system. Their analgesic effects are likely associated with their immunomodulatory activities. Along these lines, GABA is a well-known calcium-channel inhibitor [13] and calcium signaling is essential for immune cell activation, AMI diminishes production of TNF-α by macrophages [11], and VENLA impairs the innate macrophage response [12,14]. Their effects on the functioning of immune cells, including macrophages, could help to better explain how they lead to analgesia. On the other hand, opioids and AAs possess immunomodulatory properties, but their effects on immunity under homeostatic conditions are weakly understood. Thus, investigation of opioids' and AAs' impact on humoral and cellular immune responses in naive animals will greatly support the understanding of the interaction of analgesics with the immune system, which may also benefit the efficiency of the clinical treatment of pain, as annually reviewed by Bodnar [15].

We previously studied the effects of several opioids and AAs on the innate immune response of macrophages [16]. Thus, the aim of this research was to examine the influence of the AAs (GABA, AMI, and VENLA) on the functions of macrophages in the induction of the humoral and cell-mediated immune responses, when administered independently or together with morphine. We sought to determine the phagocytic activity of macrophages, the release of cytokines and cell-surface markers' expression, and subsequently macrophage antigen presentation resulting in activation of B lymphocytes for humoral response or effector T cells for CHS reaction.

Materials and methods

Animals

We obtained inbred male CBA mice, ranging from eight to ten weeks old, from the Animal Facility of the Faculty of Medicine, Jagiellonian University Medical College, Krakow, Poland. Animal subjects were given free access to food and water. All experiments were conducted according to the guidelines of the Animal Care and Use Committee of the Jagiellonian University Medical College and under ethical approval of the 1 st Local Ethics Committee (approval no. 215/2015).

Drug administration

The following drugs were used: morphine sulfate (02DR0910), naloxone hydrochloride (02BZ0514) (WZF Polfa S.A., Warsaw, Poland), amitriptyline hydrochloride (A8404), gabapentin (G154) and venlafaxine hydrochloride (V7264) (Sigma, St. Louis, MO, USA). All drugs were used as sterile solutions in DPBS for intraperitoneal (*ip*) injections. Mice were treated for eight days with morphine; morphine and naloxone; AMI; morphine and AMI; GABA; morphine and GABA; VENLA; or morphine and VENLA. The following doses were used: morphine 20 mg/kg twice per 24 h, naloxone 1 mg/kg, AMI 10 mg/kg, GABA 5 mg/kg, VENLA 5 mg/kg, all once per 24 h [16–18]. When co-administered, morphine and a particular AA were injected one by one at the same time of day. The naloxone to morphine dosage regimen was based on previous studies and referred to clinical conditions [19]. A control group was established with mice who were not treated with any medications.

Reagents

Several reagents were used, including Dulbecco's phosphate-buffered saline (DPBS), fetal calf serum (FCS), PBS, RPMI1640, thioglycollate medium (Gibco Life Technologies, Grand Island, NY, USA), heparin sodium salt, 2-mercaptoethanol (2-ME), mineral oil (heavy fraction) (Sigma, St. Louis, MO, USA), sheep red blood cells (SRBC) (Graso Biotech, Starogard Gdanski, Poland), and guinea pig complement (Biomed, Lublin, Poland).

Enzyme-linked immunosorbent assay kits

The following enzyme-linked immunosorbent assay (ELISA) kits were used: Mouse tumor necrosis factor (TNF) alpha ELISA Ready-SET-Go!® (sensitivity 8 pg/ml, cat. no. 88-7324-22), Mouse IL-6 ELISA Ready-SET-Go!® (sensitivity 4 pg/ml, cat. no. 88-7064-22), Mouse IL-10 Platinum ELISA Test (sensitivity 5 pg/ml, cat. no. BMS614/2), Mouse transforming growth factor (TGF)-beta1 Platinum ELISA Test (sensitivity 7.8 pg/ml, cat. no. BMS608/4), (eBioscience Inc., San Diego, CA, USA); Mouse IL-12p40 BD OptEIA™ Set (sensitivity 15.6 pg/ml, cat. no. 555165; BD Biosciences, San Diego, CA, USA).

Antibodies

Fluorescein isothiocyanate (FITC)-conjugated rat anti-mouse Mac-3 monoclonal antibody (mAb), phycoerythrin (PE)-conjugated rat anti-mouse I-A^k, CD80, CD86, CD40, CD11b, CD14, CD16/32 mAb (BD Pharmingen, San Diego, CA, USA) were used in cytometric analysis.

Harvesting of oil- and thioglycollate-induced peritoneal macrophages

Peritoneal macrophages induced by *ip* injection of 1 ml of mineral oil or 2 ml of thioglycollate medium on day 3 were harvested from mouse donors, who had been treated *ip* with opioids (OPs) and/or AAs. Macrophages were washed out of peritoneal cavities with 5 ml of ice-cold DPBS containing heparin (5 U/ml) and, after control of their viability, used for *in vitro* studies.

Phagocytosis assay

Macrophage phagocytosis was assessed by using a novel method previously validated by us [20]. Briefly, macrophages were pulsed with FITC-coupled SRBC (in a ratio of 1:10) for 20 min at 37 °C. This was followed by osmotic shock-mediated lysis and removal of non-phagocytosed FITC-SRBC. Finally, cells were analyzed by flow cytometry on FACS Calibur (BD Bioscience, San Jose, CA, USA), and acquired data were analyzed firstly with BD CellQuest Pro software, and then with GraphPad Prism and Excel software [20].

Cytokine immunoassays

Macrophages from untreated or drug-treated mouse donors were cultured in 24-well flat bottom plates at a concentration of 2×10^6 cells per well in 2 ml of RPMI1640 with 5% FCS, partially after stimulation with lipopolysaccharide (LPS, 200 ng). Standard conditions of 37 °C and 5% CO_2 were maintained during culturing. Supernatants were collected after 24 h for measurement of IL-6, IL-12p40, and TNF-α and after 48 h for measurement of IL-10 and

TGF-β. Supernatants were stored at −80 °C for subsequent cytokine measurement using ELISA kits according to manufacturers' instructions [12,21,22].

Flow cytometry

Apart from testing CD16/32 expression, peritoneal macrophages from naive or drug-treated mice were incubated with mAb (clone 2.4G2) to block Fc receptors. Cells were then stained with appropriately diluted FITC-conjugated anti-mouse Mac-3 mAb and PE-conjugated mAb against either I-A^k (MHC class II), CD80, CD86, CD40, CD11b, CD14, or CD16/32 surface markers. After washing, macrophages were analyzed by flow cytometry (FACSCalibur, BD Bioscience, San Jose, CA, USA) for expression of selected markers. In each case, 2×10^4 cells were analyzed for data acquisition [12,21,22]. The strategy of cell gating was shown in Suppl. Fig. 1.

Hemolytic plaque forming and direct hemagglutination assays

Thioglycollate-induced peritoneal macrophages were harvested from drug-treated and untreated mice and incubated for 30 min at 37 °C with SRBC, in a ratio of 1:10. Lysis of non-phagocytosed SRBC was then performed *via* osmotic shock. SRBC-pulsed macrophages were subsequently injected *ip* into naive recipients at a concentration of 4×10^6 per mouse. Blood and spleens were collected from these mice seven days after macrophage injection, with spleens being individually weighed at the time of collection. Total anti-SRBC antibody serum titers, as well as IgG antibody titers (from sera pre-incubated with 0.15 M 2-ME) were measured *via* direct hemagglutination and results were expressed as a $\log_2$ of titers. Briefly, two-fold dilutions of tested sera in DPBS were incubated for 90 min at 37 °C with 1% SRBC suspension. The anti-SRBC antibody titer was considered to be the inversed value of the highest dilution, at which hemagglutination still occurred. IgM antibody titers were estimated by subtracting IgG titers from total serum anti-SRBC antibody titers. Further, single cell suspensions of each spleen were prepared in RPMI1640 medium and incubated for 90 min at 37 °C with 1% SRBC suspension in the presence of guinea pig complement. The number of cells producing anti-SRBC antibody was measured *via* hemolytic plaque assay, following the microscope slide technique [12,21,22].

CHS assay

Mice were treated for eight days with one the assayed drug treatments and on the 3rd day were contact sensitized by topical application of 0.15 ml of 5% picryl chloride (PCL) solution in acetone with ethanol mixture on shaved abdomen. Five days later mice were ear challenged by topical application of 0.01 ml of 0.4% PCL in acetone with olive mixture on both sides of both ears to elicit CHS ear swelling response, measured two and twenty-four hours later with an engineer's micrometer (Mitutoyo, Japan) [12,21–23] by observer unaware of the experimental groups [23]. Oil-induced peritoneal macrophages were harvested from mice treated for eight days with one of the above treatments and then labelled with trinitrophenyl hapten (TNP) by 10 min incubation with trinitrobenzene sulfonic acid (TNBSA) in DPBS at a ratio of 2 mg of TNBSA per 10^8 macrophages. After washing, cells were injected intravenously into naive recipients at a dose of 2.5×10^6 cells per mouse. A week later mouse recipients of hapten-labelled macrophages were ear challenged as above to elicit CHS ear swelling response. Simultaneously, naive mice were ear challenged to induce non-specific, background increase in ear thickness due to chemical irritation of skin by hapten and its vehicle. This increase was then subtracted from the results obtained in experimental group to evaluate the net ear swelling response, expressed as delta ± standard error (SEM).

Statistical analysis

All experiments were performed two to four times (N = 2–4). Statistical analysis was performed in GraphPad Prism 8 (GraphPad, San Diego, CA, USA) using a one-way or two-way analysis of variance (ANOVA) with *post hoc* Tukey test to estimate the statistical significance of differences between groups and all ANOVA assumptions were met. In two-way ANOVA the morphine treatment was assigned as column factor, and treatment with AAs was a row factor, while in one-way ANOVA drug-treatment was a main factor.

Results

Influence of morphine, AAs and naloxone on macrophage phagocytosis

When we analyzed phagocytosis by macrophages using FITC-coupled SRBC, we observed that the administration of all AAs led to a statistically significant decrease in the number of phagocytosing cells as compared to macrophages from untreated mice, as seen in Fig. 1A. Interestingly, the addition of AA to morphine seems to have a synergistic effect, especially in the case of AMI (Fig. 1A), without influencing the efficiency of the process (Fig. 1B), as compared to macrophages from control mice or mice treated with morphine or AMI alone. Efficiency of phagocytosis by GABA macrophages was slightly enhanced (Fig. 1B), even though the overall number of phagocytosing cells was decreased as was also observed in VENLA macrophages (Fig. 1A). In contrast, affecting morphine effects with naloxone impaired macrophage phagocytic activity (Suppl. Fig. 2A).

Analysis of the efficacy of FITC-SRBC phagocytosis allowed us to differentiate $CD11b^+FITC^+$ macrophages into two subpopulations of low and high efficacy (Fig. 1), that are clearly seen in the case of morphine-treated macrophages (Fig. 1C), and this effect was affected by naloxone (Suppl. Fig. 2A), while addition of AMI to morphine strongly decreased the number of macrophages expressing high phagocytic activity (Fig. 1D). Treatment with GABA alone seems to have the opposite effect to morphine (Fig. 1E), while venlafaxine exhibited the inverse effect when administered with morphine (Fig. 1F).

Influence of AAs on macrophage cytokine secretion

The secretion of cytokines is a key process in the orchestration of both the innate and humoral immune responses. For our study, we measured the secretion of several pro- (TNF-α, IL-6, IL-12p40) and anti-inflammatory (IL-10, TGF-β) cytokines by macrophages harvested from treated and untreated murine donors as shown in Fig. 2. Upon statistical analysis, we discovered some interesting results. Macrophages of mice treated with morphine, with or without AMI, express enhanced basal secretory activity of pro-inflammatory cytokines and IL-10 (Fig. 2). Further, each of the AAs led to a statistically significant reduction in the LPS-stimulated secretion of at least one pro-inflammatory cytokine by macrophages as compared to macrophages from untreated controls (Fig. 2A). The combination of morphine and AMI lead to a significant reduction in the LPS-induced secretion of pro-inflammatory cytokines with a synergistic effect for TNF-α and IL-12p40 secretion (Fig. 2A). All drugs tend to increase LPS-stimulated TGF-β secretion (Fig. 2B). Furthermore, morphine with or without AMI led to a statistically significant increase in the LPS-stimulated secretion of the anti-inflammatory cytokine IL-10 (Fig. 2B). In general, macrophages from mice treated with morphine combined

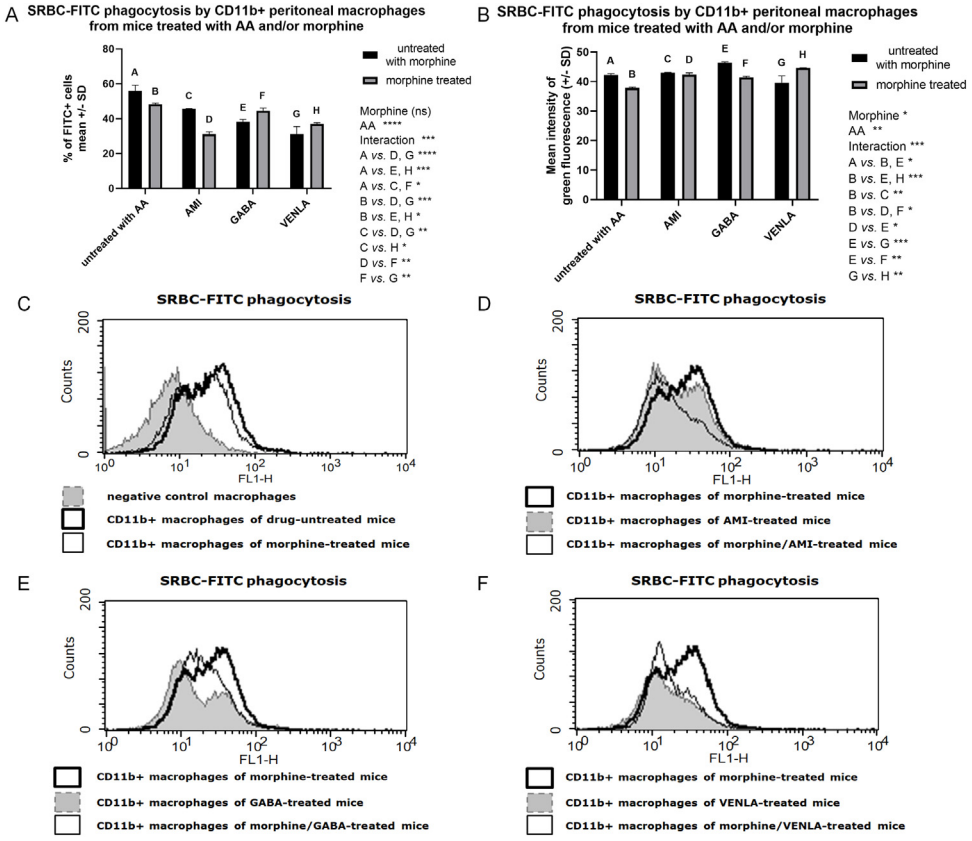

Fig. 1. Analgesic adjuvants (AA) modulate phagocytosis of fluorescein isothiocyanate (FITC)-coupled sheep red blood cells (SRBC) by mouse CD11b$^+$ macrophages. Oil-induced peritoneal macrophages harvested from mice, which received one of the drug treatments were incubated for 20 min in 37 °C water-bath with SRBC-FITC, stained with phycoerythrin (PE)-conjugated rat anti-mouse CD11b monoclonal antibody, and then red and green fluorescence emission by these cells was measured cytometrically. (**A**) The percentage of macrophages of mice treated with respective drug that emit green fluorescence after incubation with SRBC-FITC. (**B**) The geometric mean of emitted green fluorescence by control and drug-treated CD11b$^+$FITC$^+$ macrophages incubated with SRBC-FITC. (**C**) The histogram showing the count of CD11b$^+$ macrophages emitting green fluorescence after pulsing with SRBC-FITC, that were harvested from drug-untreated mice (bold black line) or morphine-treated mice (thin black line) in comparison to negative control macrophages (gray). (**D**) The histogram showing the count of CD11b$^+$ macrophages emitting green fluorescence after pulsing with SRBC-FITC, that were harvested from morphine-treated mice (bold black line), amitriptyline-treated mice (gray) or morphine and amitriptyline-treated mice (thin black line). (**E**) The histogram showing the count of CD11b$^+$ macrophages emitting green fluorescence after pulsing with SRBC-FITC, that were harvested from morphine-treated mice (bold black line), gabapentin-treated mice (gray) or morphine and gabapentin-treated mice (thin black line). (**F**) The histogram showing the count of CD11b$^+$ macrophages emitting green fluorescence after pulsing with SRBC-FITC, that were harvested from morphine-treated mice (bold black line), venlafaxine-treated mice (gray) or morphine and venlafaxine-treated mice (thin black line). AMI: macrophages from mice treated with amitriptyline; GABA: macrophages from mice treated with gabapentin; VENLA: macrophages from mice treated with venlafaxine. (ns) – statistically non-significant, * $p <$ 0.05, ** $p < 0.01$, *** $p < .005$, **** $p < 0.001$; n = 4; N = 2.

with naloxone showed similar cytokine release pattern as macrophages from control, drug-untreated mice (Suppl. Fig. 2B).

Influence of morphine, AAs and naloxone on macrophages and their cell surface marker expression

Analysis of the percentage of cells expressing low (SSC^{low} population) or high (SSC^{high} population) granularity revealed a decreased percentage of low granularity macrophages in the case of cells from mice treated with morphine and naloxone, or with AMI, while the populations of macrophages from mice treated with AMI and morphine reflected those of control and morphine-treated mice. Conversely, an increased percentage of low granularity macrophages was observed, when cells had been obtained from donors treated with GABA or VENLA with or without morphine (Table 1). The gating strategy was depicted in Suppl. Fig. 1.

The expression of several cell surface markers involved in antigen presentation (DR, CD80, CD86, CD40) and phagocytosis (CD11b, CD14, CD32/16) by macrophages is shown in Table 2. Generally, we observed that the administration of morphine led to a decrease in the expression of all analyzed cell surface markers on both populations of macrophages. Interestingly, this decrease was generally reversed by the addition of AMI, while naloxone, mu opioid receptor antagonist, increased the expression of analyzed markers over the values of macrophages from control and morphine-treated mice. GABA decreased the macrophage expression of antigen presentation markers even when administered with morphine, especially in the case of the SSC^{low} population of macrophages. VENLA alone slightly upregulated the expression of antigen presentation markers on SSC^{high} macrophages, with the opposite effect in the case of the SSC^{low} population.

Influence of morphine, AAs and naloxone on the antigen presenting macrophage-induced humoral response measured in plaque forming assay

The results of plaque forming assay enumerating B cells producing SRBC-specific antibodies are shown in Fig. 3A. One can clearly see that spleens from mice transferred with SRBC-pulsed macrophages of mice treated with morphine had statistically significantly less effector B cells than spleens from recipients of drug-unaffected, SRBC-pulsed macrophages. What is intriguing, this effect was also seen in mice who received macrophages of

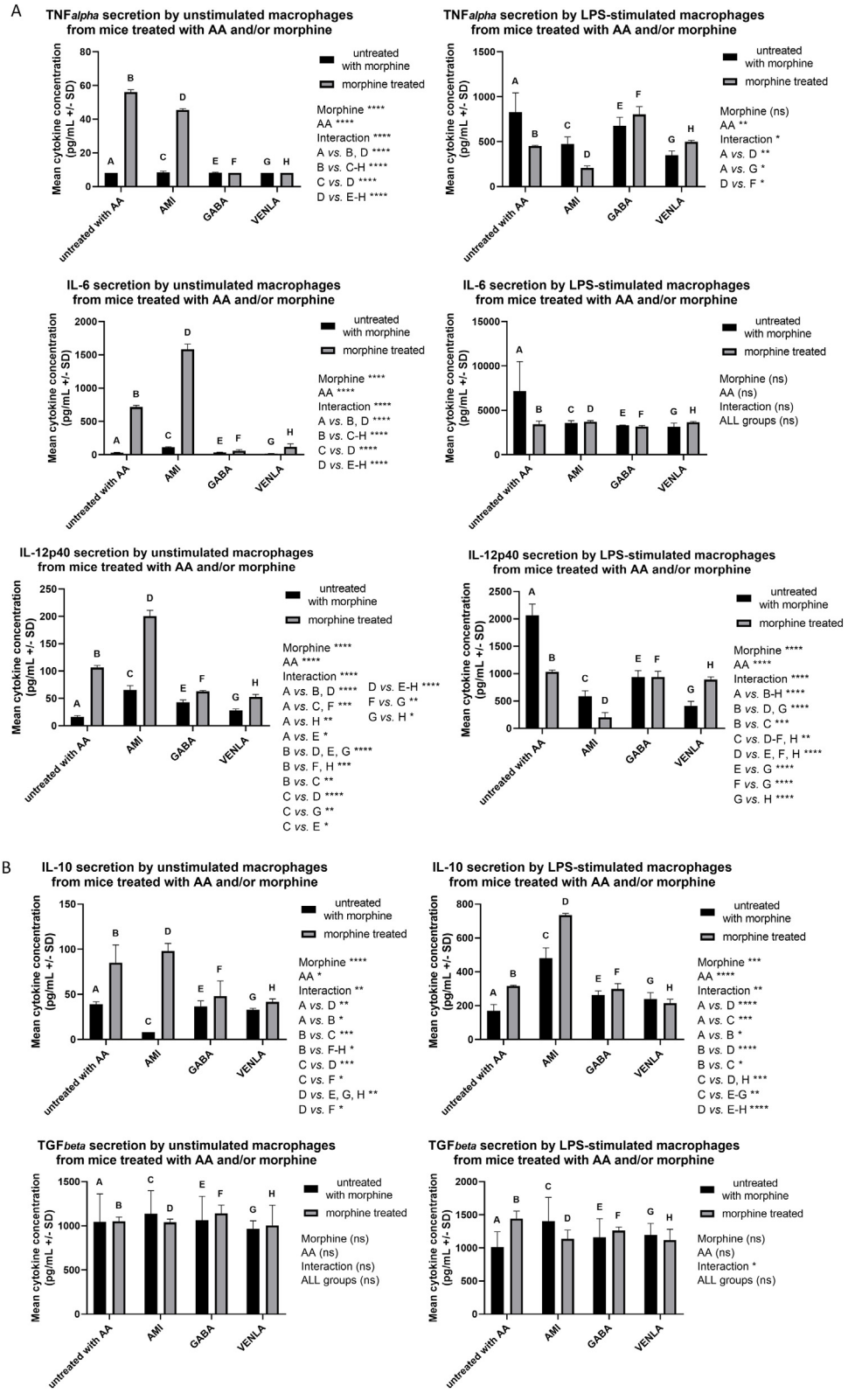

Fig. 2. Cytokine secretion by macrophages is affected by the administration of analgesic adjuvants (AA). Oil-induced peritoneal macrophages harvested from mice, which received one of the above treatments for eight days, were cultured in standard conditions at a concentration of 2×10^6 cells per well in 2 ml of culture medium. Half of the cultures were stimulated with LPS (200 ng). Enzyme-linked immunosorbent assay (ELISA) was performed in order to measure (**A**) the concentration of TNF-α, IL-6 and IL-12p40 in supernatants collected after 24 h of culture and (**B**) the concentration of IL-10 and TGF-β in supernatants collected after 48 h of culture. (ns) – statistically non-significant, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$, **** $p < 0.001$; n = 3–5; N = 2. AMI: macrophages from mice treated with amitriptyline; GABA: macrophages from mice treated with gabapentin; VENLA: macrophages from mice treated with venlafaxine.

Table 1
Cytometric analysis of cell populations of macrophages obtained from donors treated with morphine and adjuvants. Oil-induced peritoneal macrophages from untreated mice or donors treated for eight days with one of the above treatments were analyzed cytometrically. According to the side scatter (SSC) value, macrophages were classified as cells of low (SSC^{low}) or high (SSC^{high}) granularity. Results are presented as the percentage of macrophages belonging to the particular population. n = 3, N = 2. AMI - amitriptyline; GABA - gabapentin; VENLA - venlafaxine. The strategy of cell gating was shown in Suppl. Fig. 1.

Populations of macrophages from mice treated with morphine ± analgesic adjuvants		
Macrophages from mice treated with	percentage of cells (mean ± SD)	
	SSC^{low} population	SSC^{high} population
untreated (control)	73.8 ± 1.43	25.6 ± 1.43
Morphine	71.2 ± 1.82	27.3 ± 1.92
Morphine and Naloxone	63.7 ± 1.39 **, #	35.7 ± 1.40 **, #
AMI	64.1 ± 0.87 ***, #	35.2 ± 0.92 ***,#
Morphine and AMI	71.6 ± 1.37 §§	27.4 ± 1.42 §§
GABA	84.8 ± 0.75 ****, ###	14.6 ± 0.76 ****, ###
Morphine and GABA	85.3 ± 1.01 ****, ###	13.9 ± 0.98 ****, ###
VENLA	83.9 ± 0.52 ***, ##	15.4 ± 0.56 ***, ##
Morphine and VENLA	80.1 ± 0.52 **, # $	18.6 ± 0.50 **, #, $

Statistical significance of differences (number of symbol repeats express the strength of significance).
* group *vs.* control.
# group *vs.* Morphine.
§ AMI *vs.* Morphine and AMI.
$ VENLA *vs.* Morphine and VENLA.

donors administered with morphine together with AAs. This effect was blocked, however, when the opioid antagonist naloxone was administered to macrophage donors, leading to an increase in the number of effector B lymphocytes as compared to independent morphine administration to macrophage donors (Suppl. Fig. 2C). Interestingly, treatment of macrophage donors with VENLA, even when administered independently, led to a decrease in the number of effector B cells by nearly one-half.

Influence of AAs on anti-SRBC antibody serum titers

While our data from the measurement of anti-SRBC antibody titers did not allow extensive statistical analysis, they do support the general trends shown in our analysis of B cell activation, as shown in Fig. 3B. Again, we can observe that morphine treatment of macrophage donors led to a decrease in the murine humoral immune response, with decreased production of anti-SRBC antibodies being observed. AAs also led to decreased antibody production, with VENLA leading to the greatest decrease. GABA and VENLA when administered separately led to acceleration of maturation of humoral response in macrophage recipients expressed as increasing IgG titers in comparison to IgM.

Influence of AAs on morphine effects in CHS reaction

Antidepressant AAs, when administered with morphine, tended to suppress the early phase of active CHS reaction, while GABA slightly increased the activity of morphine (Fig. 4A). In contrast, mice treated with morphine and GABA developed suppressed antigen-specific CHS late phase ear swelling, similarly to mice treated with VENLA with or without morphine, while AMI seemed to enhance the response, especially when administered with morphine (Fig. 4B). However, when the CHS reaction was induced by hapten-labelled macrophages, we observed increased CHS early phase ear swelling in recipients of macrophages from morphine-treated donors, whereas macrophages from GABA-treated mice appeared to weakly induce the CHS early phase reaction (Fig. 4C). Interestingly, ear swelling in the CHS late phase was significantly suppressed in the case of recipients of macrophages from donors treated with GABA or VENLA and also when GABA was administered with morphine (Fig. 4D). It is worth nothing that naloxone treatment reversed morphine's effects in the late phases of CHS, both in actively sensitized mice and in hapten-labelled macrophage recipients, as reported previously [22].

Discussion

In patients suffering from cancer-related pain, OP titration is frequently performed with the goal of achieving adequate pain relief [10]. It is estimated, however, that treatment resistant pain occurs in 10–20% of patients with advanced cancer [24].Thus, achieving adequate analgesia in patients suffering from advanced neoplastic disease is often a challenge for physicians. When titration with OPs alone is inadequate, the addition of an AA to a patient's medication regimen may help bring about pain relief, especially in the context of hyperalgesia or neuropathic pain [10]. Our results suggest that macrophages influenced by morphine and AAs undergo anti-inflammatory activation, which may help to explain the clinical analgesic effects of AAs. Furthermore, the presumed anti-nociceptive effect of drug-influenced macrophages may differ, depending on the actual inflammatory-related cause of pain. Additionally, one of the leading advantages of co-administration of AAs is related to their opioid sparing effects. Finally, one can speculate that AAs may also affect the subjective perception of pain, inducing an overall beneficial effect on the patient's condition.

While the OPs are well studied regarding their influence on the immune system [8,16,20–22], the effects of AAs remain unclear. Our study focused on three of the most widely used AAs in the clinical treatment of pain and lends support to the idea that the analgesic activity of these medications is also due to their influence on the functioning of the immune system. Current results indicate a significant suppressive effect of AAs on the murine innate immune response closely related to the induction of the adaptive humoral response. As shown here, AAs reduce phagocytic activity in the case of $CD11b^{+}$ macrophages pulsed with SRBC, while the effect of OPs depends on the studied population of phagocytes [20]. In addition, we have previously demonstrated the immunomodulatory effects of AAs on the oxygen burst [16] activated to neutralize the phagocytosed antigens for their further processing in macrophages.

When added to morphine treatment, AMI seems to exert its effect at the stage of phagocytosis and GABA and VENLA also enhance reactive oxygen intermediates' production. However, these effects seem to be compensated by altered expression of cell-surface markers involved in the process of phagocytosis.

Another pathway leading to induction of humoral response involves cytokine signaling. In general AMI with or without morphine enhanced basal secretion of both pro- and anti-inflammatory cytokines by macrophages, but significantly decreased the secretion of pro-inflammatory cytokines when macrophages were stimulated with LPS, which was also true in the case of VENLA. These observations are in line with previously reported effects of VENLA and another tricyclic antidepressant, imipramine [12,14]. AMI has also been reported to alleviate hyperalgesia [11] and septic shock [25] through the blockade of TNF-α signaling. The addition of VENLA to a culture of dendritic cells led to inhibition of the release of pro-inflammatory cytokines, including IL-12p40 [26], which was similarly demonstrated here. Surprisingly, we demonstrated that morphine treatment induced basal TNF-α secretion by unstimulated mouse macrophages, which may be explained by activation of nuclear factor kappa B by morphine itself [27] or TLR-4 by active morphine metabolite [28].

Table 2
Cytometric analysis of cell surface markers related to phagocytic activity or antigen presentation of macrophages obtained from donors treated with morphine and adjuvants. The level of expression of markers related to phagocytosis (CD14, CD11b, CD16/32) and antigen presentation (DR, CD80, CD86, CD40) on the surface of oil-induced peritoneal macrophages of SSC^{low} (Table 2a) or SSC^{high} (Table 2b) populations, which were collected from mice treated for eight days with one of the above treatments, was analyzed cytometrically. Results are presented as the percentage of macrophages expressing a particular marker within either the total population of analyzed macrophages or the Mac3+ subpopulation of macrophages. n = 3, N = 2. AMI - amitriptyline; GABA - gabapentin; VENLA - venlafaxine.

Macrophages from mice treated with	macrophage population	percentage of SSC-low cells expressing particular marker (mean ± SD)							
		Mac3	CD14	CD80	CD86	DR	CD11b	CD32/16	CD40
untreated (control)	TOTAL		4.6 ± 0.21	51,0 ± 0.99	48.8 ± 1.33	40.9 ± 0.64	50.9 ± 1.48	34.0 ± 1.41	15.8 ± 2.55
	Mac3+	5.1 ± 1.13	2.0 ± 0.92	6.6 ± 0.99	5.3 ± 0.49	4.1 ± 0.49	5.4 ± 0.57	12.2 ± 3.75	4.0 ± 0.57
Morphine	TOTAL		15.4 ± 0.49	46.3 ± 0.14	36.6 ± 0.07	32.2 ± 0.42	50.4 ± 0.99	28.3 ± 1.13	12.5 ± 0.07
	Mac3+	6.8 ± 0.21	4.0 ± 3.6	5.8 ± 0.07	4.7 ± 0.64	4.0 ± 0.00	4.6 ± 0.28	10.9 ± 0.07	2.5 ± 0.07
Morphine and naloxone	TOTAL		7.5 ± 0.21	58.7 ± 1.70	53.4 ± 0.64	44.6 ± 3.18	60.8 ± 5.23	39.1 ± 0.64	20.8 ± 1.06
	Mac3+	10.7 ± 1.63	5.8 ± 0.10	13.8 ± 11.9	11.9 ± 0.14	9.8 ± 1.34	19.2 ± 5.44	32.1 ± 1.27	9.7 ± 0.99
AMI	TOTAL		6.4 ± 0.14	54.1 ± 1.41	47.8 ± 0.64	24.0 ± 1.13	68.6 ± 3.54	38.1 ± 0.14	14.8 ± 0.14
	Mac3+	7.7 ± 0.49	3.3 ± 0.07	6.7 ± 0.64	8.9 ± 0.21	5.3 ± 0.28	9.0 ± 2.47	29.0 ± 0.07	5.1 ± 0.14
Morphine and AMI	TOTAL		7.1 ± 0.14	48.2 ± 0.07	41.8 ± 0.71	24.1 ± 0.07	66.2 ± 0.85	29.6 ± 0.21	14.2 ± 4.60
	Mac3+	8.3 ± 0.92	6.3 ± 0.14	10.4 ± 0.35	10.6 ± 0.35	8.2 ± 0.92	11.2 ± 0.00	19.7 ± 0.14	4.6 ± 0.00
GABA	TOTAL		15.2 ± 11.03	39.6 ± 0.49	36.5 ± 1.77	24.8 ± 0.07	57.6 ± 0.64	20.1 ± 1.63	14.3 ± 1.56
	Mac3+	4.1 ± 0.77	1.6 ± 0.64	3.3 ± 0.14	4.4 ± 1.27	3.4 ± 0.07	4.4 ± 0.78	7.4 ± 0.85	2.7 ± 0.42
Morphine and GABA	TOTAL		0.8 ± 0.21	37.0 ± 0.21	38.0 ± 0.35	23.4 ± 0.85	61.0 ± 0.42	18.5 ± 0.99	12.5 ± 0.57
	Mac3+	2.3 ± 1.77	0.3 ± 0.07	2.9 ± 0.21	3.2 ± 0.14	2.4 ± 0.57	2.8 ± 0.42	6.3 ± 1.13	2.1 ± 0.14
VENLA	TOTAL		0.7 ± 0.21	43.4 ± 2.69	44.8 ± 1.27	33.7 ± 4.81	58.8 ± 0.07	25.7 ± 2.9	17.9 ± 5.09
	Mac3+	6.2 ± 1.06	0.4 ± 0.07	5.3 ± 1.34	4.2 ± 0.28	4.0 ± 0.49	5.3 ± 0.14	11.7 ± 1.20	3.1 ± 0.21
Morphine and VENLA	TOTAL		0.7 ± 0.07	39.1 ± 0.78	41.7 ± 0.49	26.6 ± 0.07	56.7 ± 0.07	22.4 ± 2.26	13.0 ± 1.20
	Mac3+	5.3 ± 0.00	0.3 ± 0.00	4.6 ± 0.71	5.2 ± 0.64	4.1 ± 0.35	5.4 ± 0.28	7.2 ± 0.42	2.7 ± 0.21

Macrophages from mice treated with	macrophage population	percentage of SSC-high cells expressing particular marker (mean ± SD)							
		Mac3	CD14	CD80	CD86	DR	CD11b	CD32/16	CD40
untreated (control)	TOTAL		31.8 ± 0.78	71.8 ± 0.21	49.0 ± 0.0	33.8 ± 0.71	90.8 ± 0.57	56.7 ± 1.20	36.5 ± 0.42
	Mac3+	39.9 ± 3.4	30.2 ± 1.63	38.2 ± 2.19	38.9 ± 0.78	29.2 ± 0.07	41.00 ± 0.99	39.0 ± 0.42	34.0 ± 0.07
Morphine	TOTAL		33.5 ± 0.14	70.4 ± 1.06	37.3 ± 0.14	21.2 ± 0.28	92.9 ± 0.28	46.5 ± 1.63	19.3 ± 0.14
	Mac3+	32.4 ± 1.56	23.1 ± 5.66	31.7 ± 0.71	26.5 ± 1.70	18.2 ± 1.48	31.4 ± 1.20	30.0 ± 1.77	17.5 ± 2.40
Morphine and naloxone	TOTAL		48.6 ± 0.00	85.8 ± 0.78	63.9 ± 0.07	48.5 ± 0.42	94.4 ± 0.78	66.4 ± 0.49	52.1 ± 0.07
	Mac3+	62.0 ± 1.34	48.3 ± 0.42	54.9 ± 1.12	56.1 ± 0.78	46.7 ± 0.07	64.9 ± 0.64	61.7 ± 0.07	50.9 ± 0.14
AMI	TOTAL		27.3 ± 0.42	77.9 ± 1.34	45.4 ± 0.49	26.7 ± 0.00	94.8 ± 2.26	45.5 ± 0.85	28.0 ± 0.14
	Mac3+	32.6 ± 0.28	26.2 ± 0.14	32.1 ± 1.48	32.9 ± 1.06	25.1 ± 0.07	34.7 ± 2.47	34.2 ± 0.07	26.3 ± 0.64
Morphine and AMI	TOTAL		40.1 ± 0.07	83.5 ± 1.20	59.1 ± 0.78	42.1 ± 0.28	96.9 ± 0.78	37.3 ± 5.23	38.8 ± 4.10
	Mac3+	50.2 ± 0.35	39.8 ± 0.14	49.0 ± 1.56	49.7 ± 0.57	40.5 ± 1.06	54.1 ± 1.07	30.6 ± 2.72	33.6 ± 1.70
GABA	TOTAL		38.0 ± 8.56	79.4 ± 1.77	45.2 ± 1.98	35.2 ± 0.77	83.6 ± 3.89	44.1 ± 3.04	34.4 ± 0.42
	Mac3+	32.5 ± 0.07	27.3 ± 3.25	32.4 ± 1.13	31.3 ± 0.49	27.2 ± 0.00	31.4 ± 2.40	35.0 ± 3.68	29.4 ± 2.05
Morphine and GABA	TOTAL		12.9 ± 0.35	78.8 ± 1.13	45.7 ± 0.21	31.0 ± 0.64	86.3 ± 1.20	40.3 ± 0.78	28.5 ± 0.00
	Mac3+	29.3 ± 2.90	12.2 ± 0.21	25.7 ± 0.14	26.7 ± 0.14	24.4 ± 2.90	28.8 ± 0.42	33.4 ± 0.42	24.1 ± 0.00
VENLA	TOTAL		13.4 ± 0.14	83.9 ± 1.06	54.3 ± 0.07	50.0 ± 7.99	95.1 ± 1.13	49.9 ± 2.55	41.0 ± 1.91
	Mac3+	40.5 ± 4.45	13.2 ± 1.2	37.7 ± 1.20	36.5 ± 0.85	33.8 ± 0.28	38.8 ± 0.42	42.9 ± 0.57	34.3 ± 0.99
Morphine and VENLA	TOTAL		18.4 ± 0.00	86.3 ± 0.85	63.5 ± 0.14	50.9 ± 1.27	56.3 ± 0.85	56.9 ± 2.62	42.3 ± 0.28
	Mac3+	46.9 ± 0.71	18.1 ± 1.34	47.5 ± 0.42	46.0 ± 1.34	41.2 ± 0.35	47.6 ± 1.20	47.0 ± 0.92	36.4 ± 2.90

On the other hand, LPS-stimulated secretion of TGF-β was slightly increased under the influence of studied AAs. Furthermore, GABA administered with morphine led to a significant increase in the LPS-stimulated secretion of IL-10. This synergistic effect was observed as well in the case of rats subjected to the tail-flick test [29]. The anti-inflammatory cytokine upregulation by GABA was also described in other conditions [30]. It was even suggested that GABA attenuates morphine tolerance through IL-10-dependent mechanisms [31]. On the other hand, GABA diminishes IL-6 expression in the pathogenesis of experimental neuropathic pain that likely is involved in its analgesic activity [32]. Thus, the negative effect on pro-inflammatory cytokines and activation of anti-inflammatory IL-10 secretion mediated by AAs is thought to be crucial for pain relief, especially when of a neuropathic origin [5,13]. Antidepressants may also exert their anti-inflammatory effect through the inhibition of nuclear factor kappa B [14] that controls the expression of pro-inflammatory genes [13]. According to the latest discoveries, the development of basal and neuropathic pain is under the influence of several miRNAs of regulatory function, including the miR-183 cluster [33].

All of the above effects are likely responsible for AA induced suppression of humoral response that was expressed as a decreased number of activated B-cells in recipients of SRBC-pulsed macrophages. However, VENLA and GABA seemed to slightly accelerate maturation of B cells producing SRBC antigen-specific antibodies.

One can postulate that such impairment of humoral immunity may affect patients' response to vaccines as well as immunity against extracellular pathogens. It was already suggested for some OPs [21]. Several clinical studies, however, suggest that these immunosuppressive effects are not clinically relevant regarding infection risk and vaccination. While some studies have suggested that treatment of depressed patients with antidepressant drugs, including VENLA and AMI, can increase the risk of clostridium difficile infection [34,35], serious doubts about their clinical validity have been raised [36]. The results of a meta-analysis showed that GABA did not lead to an increased risk of infection [37]. One study even demonstrated that treatment with antidepressants led to an increased cellular immune response to herpes zoster infection after vaccination [38]. Thus, while the immunomodulatory effects of AAs seem to be critical to their analgesic activity, they seem to have little clinical effect on patients' immunity against pathogens.

Interestingly, our research findings indicated the tendency of GABA and VENLA to suppress CHS reaction, especially when co-administered with morphine to donors of macrophages or mice

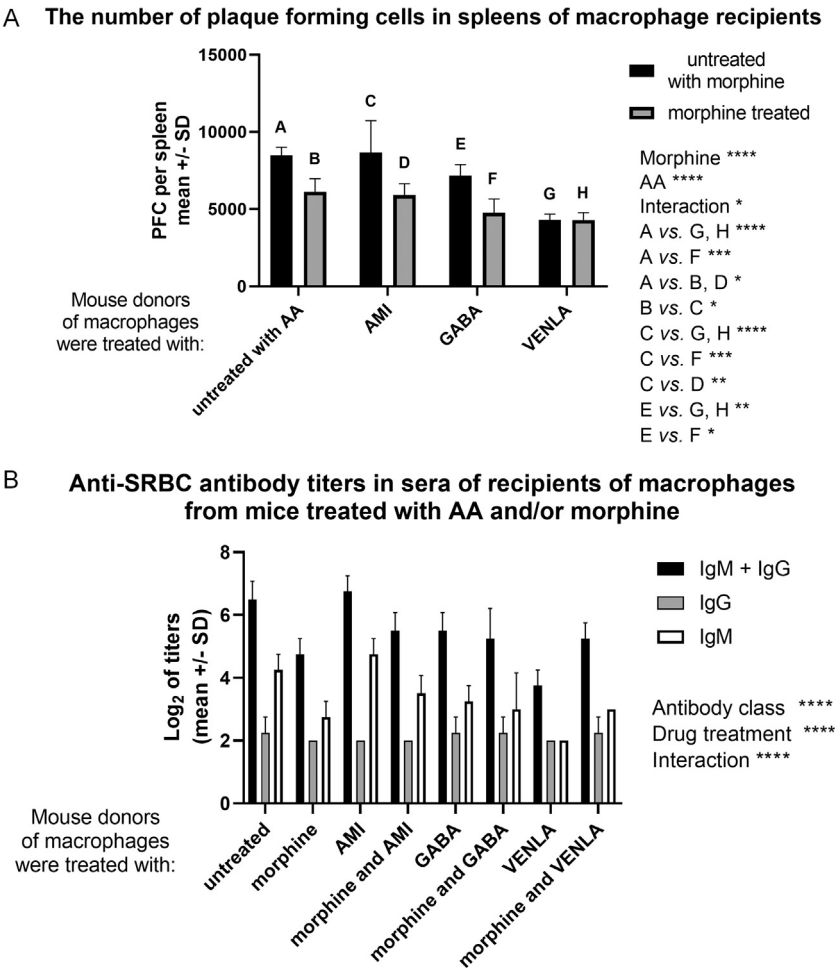

Fig. 3. Analgesic adjuvants (AA) impact humoral immune response. Mouse donors of macrophages were treated for eight days with one of the above treatments. Then, isolated thioglycollate-induced peritoneal macrophages, after pulsing with sheep red blood cells (SRBC), had been injected intraperitoneally into recipients. Sera and spleens were collected individually seven days later. (**A**) The number of plaque-forming cells (PFC), representing anti-SRBC antibody-producing B cells, in spleens of macrophage recipients was estimated through a plaque-forming assay and is presented as the mean number of PFCs per spleen. (**B**) SRBC-specific antibody titers in sera of donor mice were assessed *via* direct hemagglutination assay and are presented as the mean value of log2 of antibody titer. (ns) – statistically non-significant, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.005$, $^{****}p < 0.001$; n = 5; N = 3. AMI: amitriptyline; GABA: gabapentin; VENLA: venlafaxine.

undergoing active sensitization. In contrast, AMI administered with or without morphine, enhanced CHS reaction in actively sensitized mice. This corresponds with our observation that AMI with morphine increased the basal secretion of IL-12p40 by macrophages, while all AAs decreased the production of this cytokine, crucial for induction of CHS, in the case of stimulated macrophages. Along these lines, hapten-labelled macrophages from morphine-treated mice were previously shown to more potently activate CHS reaction after adoptive transfer [22], which was also currently observed. Similarly, administering macrophage donors with imipramine, representing tricyclic antidepressants, increased macrophage inducing activity in the early and late phases of CHS reaction [12], which was true also for AMI, especially at the early phase of CHS reaction. Conversely, in prior [12] and current studies, macrophages from VENLA-treated mice expressed reduced ability to induce the late phase of CHS reaction, as did macrophages from mice treated with GABA with or without morphine. Analogously, treatment of mice with GABA and VENLA, also together with morphine, affected the induction of active CHS reaction at the late phase. However, when administered separately, these AAs also inhibited an early phase of active CHS reaction. Thus, it could be assumed that the suppressive activity of GABA and VENLA may be associated with impairment of the recruitment of antigen-specific effector cells to the site of hapten deposition [12]. Altogether, our findings support the observation that AAs express rather immunosuppressive properties.

In conclusion, the results of our study demonstrate significant anti-inflammatory activity of AAs across a broad spectrum of immune functions, but also suggesting venlafaxine as AA exerting the most consistent effects. Importantly, they support the beneficial, analgesic effects of morphine. Despite the fact that our study was limited to animal subjects, our results may help to explain the exact analgesic mechanism of action of AAs in humans.

Current studies deliberately examined the influence of morphine and AAs on mouse immune response under homeostatic conditions, which allowed us to limit other variables that could affect immunity and the action or metabolism of administered drugs, such as the activation of hypothalamic-pituitary-adrenal axis and secretion of endogenous opiates and glucocorticoids, known to be potently immunosuppressive. However, nociception and the perception of pain is indispensably associated with activation of inflammatory reactions, and thus the possibility that analgesics may act differently on activated than on resting immune cells could not be excluded. Furthermore, chronic pain in clinical conditions occurs as a symptom of diseases that often are linked with dysregulation of immune response, which may possibly be exacerbated by therapy, with opioids and AAs adversely affecting

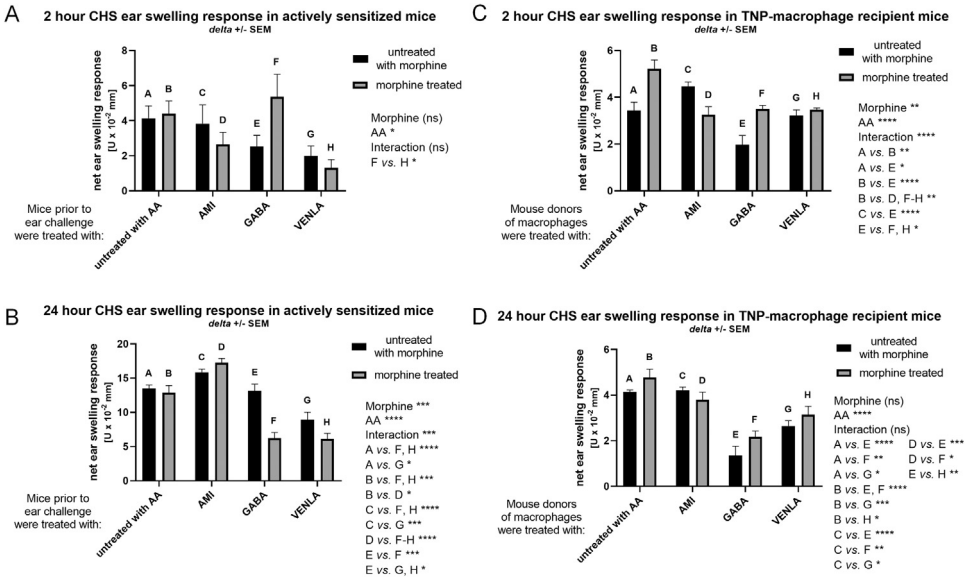

Fig. 4. Analgesic adjuvants (AA) impact morphine effects induced in cellular immune response. Mice were treated for eight days with morphine or one of the analgesic adjuvants, or with drugs in combination. On the 3rd day mice were contact sensitized with picryl chloride (PCL), and 5 days later ear challenged to elicit contact hypersensitivity (CHS) ear swelling response, measured (A) 2 h and (B) 24 h later. Otherwise, mouse donors of macrophages were treated for eight days with one of the above treatments. Then, isolated oil-induced peritoneal macrophages, after labelling with trinitrophenyl hapten (TNP), had been injected intravenously into naive recipients. Seven days later mice were ear challenged with PCL to elicit CHS ear swelling response, measured (C) 2 h and (D) 24 h later. Results, after subtracting background increase in ear thickness of non-sensitized, but ear challenged mice, were expressed as delta ± standard error (SEM). (ns) – statistically non-significant, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$, **** $p < 0.001$; n = 4–5; N = 2-4. AMI - amitriptyline; GABA - gabapentin; VENLA - venlafaxine.

immune system. Finally, it was observed that chronic morphine treatment causes a strong analgesic effect in naive mice on the first days, which is followed by a gradual decrease of morphine's analgesic activity over the next several days. Thus, one can speculate that modulating influence of AAs on morphine-induced immune effects may also change in time. However, all these aspects require further investigation.

Conflict of interest

All authors declare that they have no conflict of interest.

Author contributions

Michael Kozlowski: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Visualization, Writing - original draft. Katarzyna Nazimek: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Visualization, Writing - review and editing. Bernadeta Nowak: Data curation, Formal analysis, Investigation, Methodology. Iwona Filipczak-Bryniarska: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation. Krzysztof Bryniarski: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Project administration, Writing - review and editing.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.pharep.2019.04.016.

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