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High mobility group box 1 protein released in the course of aseptic necrosis of tissues sensitizes rats to pyrogenic effects of lipopolysaccharide

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ABSTRACT

It is still an open question as to whether or not aseptic injuries affect the generation of fever due to exogenous pyrogens including bacterial products. Therefore, in the present paper we have investigated the course of endotoxin fever in rats induced with lipopolysaccharide (LPS; given intraperitoneally in a dose of 50 μg/kg) 48 h after subcutaneous administration of turpentine oil (TRP; 0.1 mL per rat) that causes aseptic necrosis of tissues. We found that febrile response was significantly augmented in the animals pre-treated with turpentine compared to control rats (pre-treated with saline), and that observed excessive elevation of body temperature (Tb) was accompanied by enhanced release of fever mediators: interleukin-6 (IL-6) and prostaglandin E_2 (PGE_2) into plasma. Moreover, we found that sensitization to pyrogenic effects of lipopolysaccharide was associated with the increase in plasma level of high mobility group box 1 protein (HMGB1), one of the best-known damage-associated molecular patterns (DAMP), which was recently discovered as inflammatory mediator. Since the injection of anti-HMGB1 antibodies weakened observed hyperpyrexia in the animals pre-treated with turpentine, we conclude that HMGB1 is a plasma-derived factor released in the course of aseptic injury that enhances pyrogenic effects of LPS.

1. Introduction

Lipopolysaccharide (LPS) intrinsic to the cell walls of gram-negative bacteria is the most studied and potent exogenous pyrogen (defined as a substance derived from outside of the host which, when entering the inner environment of an organism, causes fever; see: Glossary of terms for thermal physiology, 2001). Following a specific receptor-mediated recognition process of the bacterial product, LPS triggers fever via mediators called endogenous pyrogens, which are released primarily by the activated immune cells (Roth et al., 2014). These endogenous factors are mostly referred to the cytokines, and tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), and IL-6 (IL-6) have been proven as the mediators and modulators of fever by using several animal models (Kluger et al., 1995; Kozak et al., 1998; Leon, 2002). Pyrogenic cytokines, in turn, initiate liberation of the arachidonic acid from membrane phospholipids and induce cyclooxygenase-2 (COX-2), leading to the enhanced production of prostaglandin E_2 (PGE_2). PGE_2 is thought to play a critical role in affecting the thermoregulatory centers presumably located in the hypothalamus, to start the fever response (Blatteis et al., 2005). Thus, an infection-associated fever is regarded as a physiological response. This statement is supported by the facts that it is an endogenously induced reaction and its size (i.e., ceiling and duration) appears to be highly regulated. The latter is a result of the actions of mechanisms collectively called endogenous antipyresis, which prevents febrile temperature from reaching a dangerously high level (Kluger, 1991; Kozak et al., 2000). It is reflected in clinical observations from a pre-antibiotic era showing that episodes of high fevers (with body temperature exceeding 41 °C) are rare (DuBois, 1949).

A particular type of fever which is aseptic and non-infectious by its nature is observed in patients after: surgical procedures (Fanning et al., 1998), stroke (Reith et al., 1996), myocardial infarction, pulmonary emboli, acute pancreatitis, gastrointestinal hemorrhage, drug administration, sunburn and other disorders (Cunha, 1999). Clinical studies indicate that such patients are particularly susceptible to the subsequent infectious fever (ibid.). In laboratory settings, an aseptic fever is most frequently induced by intramuscular (i.m.) or subcutaneous (s.c.) injection of a minute volume (in order of microliters) of turpentine oil, a tissue necrosis-producing irritant. Local inflammation caused by turpentine induces a robust acute phase response (APR) consisting of fever, hypophagia, cachexia, lethargy, and acute phase protein

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production (Leon, 2002). The mechanism of fever induced by turpentine differs from that of LPS since it does not involve TNF-α (Leon et al., 1997; Kozak et al., 1998). On the other hand, a key role for IL-1β and IL-6 in the turpentine-provoked fever has been demonstrated in studies using a cytokine gene knockout mice (Zheng et al., 1995; Kozak et al., 1998). It is still unclear, however, whether or not the irritant-triggered alterations (e.g., increase level of cytokines resulting in body temperature elevation) are mediated by a specific factor (or factors) released by necrotic cells into the blood, which are then recognized by a proper receptors of the immunocompetent cells. A presumption that such a factor (or factors) may exist was derived, among others, from studies showing an exacerbated fever in response to LPS in rats previously treated with turpentine (Soszynski and Krajewska, 2002).

High mobility group box protein 1 (HMGB1) discovered in the early 70s as one of a group of chromatin-associated proteins (Martinotti et al., 2015) was previously thought to function only as a nuclear factor that enhances transcription. However, recent findings revealed that HMGB1 mediates the immune response to infection and inflammation, functioning as the damage-associated molecular pattern (DAMP) molecule (Yang et al., 2015). HMGB1 exhibits its pro-inflammatory properties when released from the cell. This secretion to the extracellular matrix occurs in two principal ways: actively by inflammatory cells (i.e. endotoxin-stimulated monocytes and macrophages) and passively by necrotic and apoptotic cells (Lee et al., 2014). Main receptors for released HMGB1 protein are RAGE (receptor for advanced glycation end-products) and TLR (toll-like receptor) 4 found on the surface of immune as well as endothelial cells. Finally, several of studies showed the ability of this protein to induce pro-inflammatory cytokine release (for review see Erlandsson et al., 2004).

In the present study we tested a hypothesis, that HMGB1 protein is the blood factor responsible for turpentine-provoked exacerbation to the pyrogenic effects of LPS.

2. Materials and methods

2.1. Animals and body temperature measurements

Male rats [Strain: Wistar Crl: WI(Han)] aged 8–12 weeks and weighing from 250 g to 300 g were purchased from the Mossakowski Medical Research Centre Polish Academy of Sciences.

(Warsaw, Poland), and after shipping the rats were allowed to acclimatize for 10 days before starting the experiments started. Animals were kept individually in a room at constant relative humidity (60 ± 5%), temperature (24 ± 1 °C), and with a 12:12 h light - dark photoperiod, with lights on at 7:00 h. Rodent laboratory food and drinking water were provided *ad libitum*. All procedures were approved by the Local Bioethical Committee for Animal Care (permission no. 20/2012 and 20/2016). To monitor core body temperature (Tb), all animals were implanted intra-abdominally with temperature-sensitive miniature biotelemeters (PhysioTel® model TA10TA-F40, Data Sciences International, St. Paul, MN, U.S.A) under sterile conditions (for details see Wrotek et al., 2011a). All surgical procedures were performed at least ten days before the start of experiments.

2.2. Reagents

Local aseptic necrosis of tissues was induced with undiluted turpentine oil (Elissa, Warsaw, Poland). Turpentine was injected subcutaneously (s.c.) into the right hindlimb at a volume of 0.1 mL/rat.

LPS derived from *Escherichia coli* (Sigma-Aldrich, St. Louis, MO, U.S.A) was dissolved in pyrogen-free 0.9% sodium chloride (saline) and injected intraperitoneally (i.p.) at a dose of 50 μg/kg to provokes fever. It has been well established in our laboratory and elsewhere that this dose of LPS provoke a reproducible characteristic febrile rise in Tb in male Wistar rats (see, e.g., Piotrowski et al., 2014; Soszynski et al., 2013; Wrotek et al., 2011a).

Rabbit anti-HMGB1 antibodies (Sigma-Aldrich, St. Louis, MO, USA) were injected i.p. in a dose of 25 μg/rat 1 h prior to the injection of LPS into the animals pre-treated with turpentine. Antibodies were suspended in a volume of 500 μL of phosphate buffered saline. Rabbit IgG (Rockland Immunochemicals, Limerick, PA, USA) was used as a control.

2.3. Preparation of plasma for administration to naive recipient rats

Blood was collected from anesthetized rats (mixture of ketamine/xylazine) 24 h after the turpentine or saline injections, by cardiac puncture into the solution of ethylenediamine tetraacetic acid (EDTA, Sigma-Aldrich, St. Louis, MO, USA). Plasma was separated by centrifugation (20 min, $1000 \times g$) within 30 min of collection and immediately injected into the recipient rats (i.p. injection at a volume of 4 mL/rat).

2.4. ELISA assays

Blood for assay was collected as described above. Plasma was separated by centrifugation (20 min, $1000 \times g$) within 30 min of collection. All samples were kept frozen at −20 °C until assay. To inhibit prostaglandin synthesis by COX-2, indomethacin (Sigma-Aldrich, St. Louis, MO, USA) in a final concentration of approximately 10 μg/mL was added to the blood samples before centrifugation and separation of plasma used for PGE_2 content measurements.

IL-6 and PGE_2 levels in plasma were determined by ELISA kits purchased from R&D Systems (Minneapolis, MN, USA). Plasma HMGB1 concentrations were determined using an EIAab ELISA kit (Wuhan, China). Wells were read with a multi-detection microplate reader (model Synergy HT; BioTek, Winooski, VT, USA).

2.5. Experimental protocols

Experimental procedures are depicted in Fig. 1.

Experiment 1 was performed to investigate changes in plasma HMGB1 concentration in the course of aseptic fever, therefore blood for ELISA was collected from non-implanted rats 7, 12, 24 and 48 h after the administration of turpentine (s.c. injection in a volume of 0.1 mL/rat).

Experiment 2 was set to evaluate the course of LPS fever in rats after aseptic necrosis of tissues produced by a turpentine injection. Since the rise in Tb after s.c. injection of turpentine (0.1 mL/rat) has about a 7 h latency and ends next day, LPS was injected 48 h after the turpentine, when post-turpentine body temperature of rats returned to normal values. Plasma concentration of fever mediators (IL-6 and PGE_2) and HMGB1 was determined by ELISA. Blood for assays was collected from non-implanted animals 4 h after LPS administration, at the time of the greatest increase in body temperature observed with biotelemetry.

Experiment 3 was performed to investigate the possibility of transplantation into naïve animals of the factor released due to aseptic injury that is responsible for a sensitization to the pyrogenic effects of LPS. Therefore, separate groups of rats were given either the plasma isolated from the blood collected 24 h post-turpentine (referred to as 'experimental plasma') or the one isolated from the saline-treated rats (designated to as 'sham plasma'). Forty-eight hours later, all the plasma-recipient rats were treated with a pyrogenic dose of LPS (50 μg/kg i.p.). Time intervals between injections were maintained in the same way as in *experiment 1*. Blood for ELISA assays was collected 4 h after LPS administration.

Experiment 4 was set to investigate the role of HMGB1 in sensitization to pyrogenic effects of lipopolysaccharide observed in rats pretreated with turpentine. Therefore, animals injected with turpentine received HMGB1 antibodies or IgG as a control an hour before LPS. The interval of 48 h between turpentine and LPS injections was maintained as in *experiment 1* as well as the time point of blood collection for ELISAs.

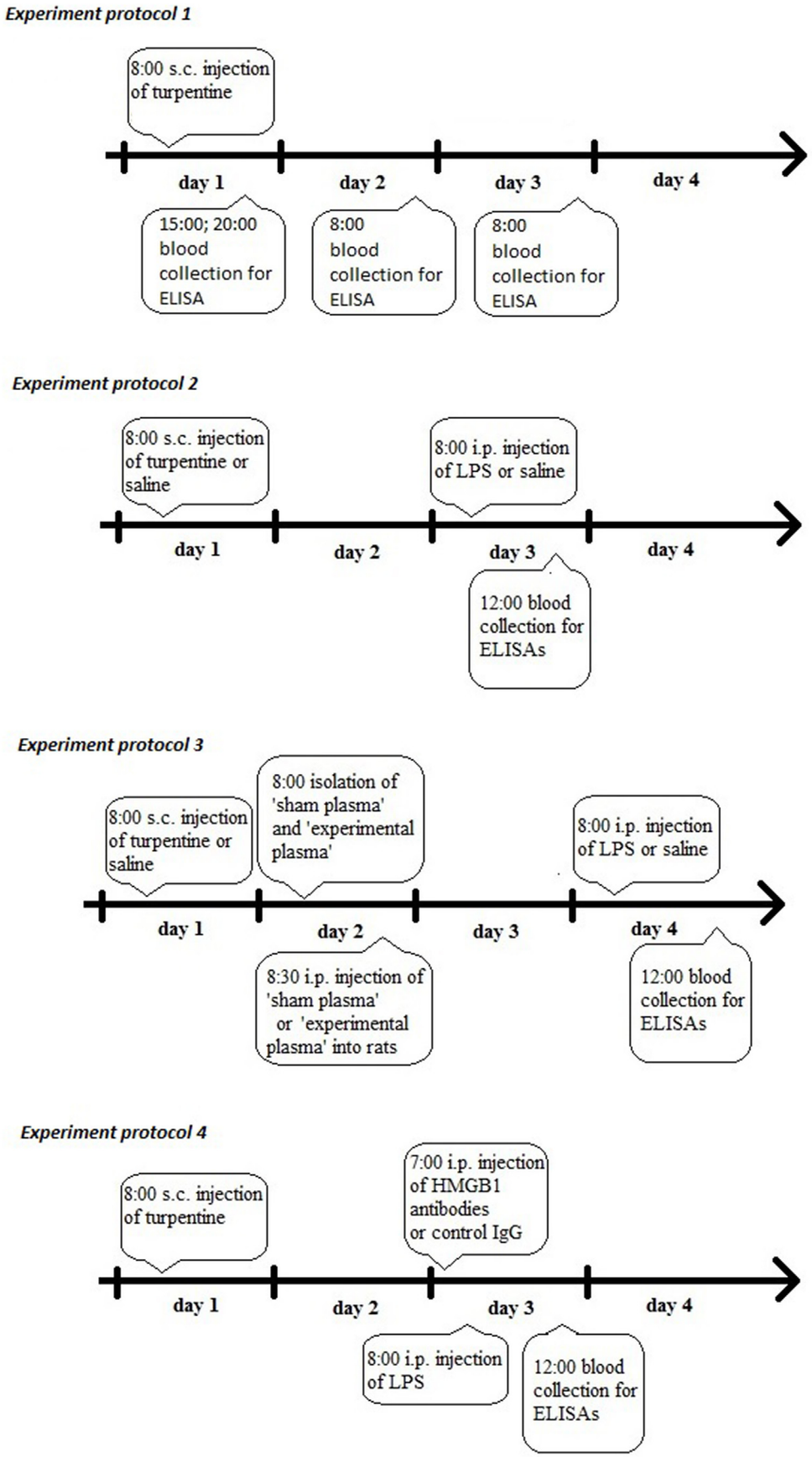

Fig. 1. Schematic diagram showing the experimental procedures. Experimental protocol 1: fever induced by aseptic necrosis of tissues. Experimental protocol 2: induction of endotoxin fever 48 h after injection of turpentine or saline. Experimental protocol 3: induction of endotoxin fever in rats injected with 'experimental plasma' or 'sham plasma'. Experimental protocol 4: induction of endotoxin fever an hour after administration of HMGB1 or control IgG antibodies to rats pre-injected with turpentine.

2.6. Data analysis

Values are reported as means ± standard error mean (S.E.M.). Five-minute temperature recordings were pooled into 20 min averages for presentation. Mean values ± S.E.M. of IL-6, PGE_2, and HMGB1 concentrations in plasma were calculated for four plasma samples, each from different animal in the experimental group, that were assayed in duplicate. ANOVA with repeated measures followed by a Tukey multiple comparison post hoc test was used to determine differences in time-dependent patterns of temperature among groups. ANOVA followed by a Tukey pairwise comparison was used to test for statistical differences among groups at individual time points as well as IL-6, PGE_2, and HMGB1 contents. Differences were considered significant at $P < 0.05$.

3. Results

3.1. Plasma HMGB1 concentration rises in a course of aseptic necrosis of tissues

Injection of turpentine provoked a rise in Tb that started with a latency period lasting for c.a. 8 h. as can be seen in Fig. 2A. Temperature had gradually grown, reaching the highest values (39.11 ± 0.11 °C) 14 h post-injection. Afterwards, a 10 h-lasting gradual decrease of Tb towards normal was observed and we have not found any significant changes in body temperature of rats treated with turpentine the following day in comparison to saline treated animals. While saline injection did not cause HMGB1 release into blood (Fig. 2B), turpentine administration provoked an increase in plasma HMGB1 concentration observed already 7 h post-injection (410 ± 145 pg/mL) that gradually grew reaching 1942 ± 328 pg/mL 48 h post-injection.

3.2. Endotoxin fever is accelerated and augmented in turpentine pre-treated rats

The thermal response of rats injected i.p. with LPS (50 μg/kg) 48 h post-turpentine and/or saline administration are summarized in Fig. 3A. As can be seen, the second dose of saline (1 mL/kg i.p.) injected two days after the first subcutaneous saline injection (0.1 mL/rat; "saline/saline" group in Fig. 3A) did not significantly affect body temperature in the rats. Injection of saline into rats pre-treated with turpentine did not significantly affect Tb of the rats, in comparison to the "saline/saline" group, therefore this data was not shown on the graph. Injection of LPS into animals pretreated subcutaneously with saline ("saline/LPS" group in Fig. 3A) induced a biphasic fever that started at about 120 min post-injection. The first peak of the Tb rise (38.20 ± 0.09 °C) was reached 160 min post-injection. The second peak of the endotoxin fever and maximum Tb elevation (38.59 ± 0.11 °C) was reached 220 min post-injection. After which, a 6 h lasting gradual decrease of Tb towards normal was observed. Pre-treatment with turpentine significantly accelerated and augmented the febrile response to LPS given 48 h afterwards. Rise in body temperature started almost immediately after the LPS administration and the first peak of Tb (38.93 ± 0.12 °C) was observed 120 min post-injection. The second peak, with a maximum value of 39.11 ± 0.23 °C, was achieved between 240 and 300 min post-injection. Mean body temperature of "turpentine/LPS" animals was significantly ($p < 0.001$) higher between 60 and 180 min post-injection (calculated mean Tb 38.46 ± 0.15 °C) compared to that of "saline/LPS" group of rats (mean Tb 37.70 ± 0.09 °C). It was also significantly higher ($p < 0.01$) during the next 4 h, measured between 200 and 440 min post-injection (38.81 ± 0.28 for "turpentine/LPS" and 38.35 ± 0.11 for "saline/LPS", respectively).

3.3. Pre-injection with turpentine enhances stimulation of IL-6, PGE_2, and HMGB1 release during endotoxin fever in rats

To determine the changes in concentration of fever mediators (IL-6 and PGE_2) and HMGB1 protein in plasma, blood was collected from non-implanted rats (that obtained the same injections as the implanted groups described above) 4 h after LPS injection. This particular time-point was adjusted to the most advanced changes in Tb after LPS administration in the control animals. As shown in Fig. 3B and C, a single injection of LPS caused significant increase ($p < 0.001$) in plasma IL-6 (1789 ± 98 pg/mL) and PGE_2 (1527 ± 31 pg/mL) concentrations compared to animals treated with saline (56 ± 11 pg/mL for IL-6 and 624 ± 32 pg/mL for PGE_2, respectively). More than twice as high ($p < 0.001$) of a concentration of IL-6 and PGE_2 was measured in the plasma of rats pre-injected with turpentine (4228 ± 250 pg/mL for IL-6 and 2729 ± 316 pg/mL for PGE_2). In contrast, in rats injected with saline 48 h after turpentine administration, plasma concentration of IL-6 (281 ± 29 pg/mL) was low, yet still significantly higher than in "saline/saline" group ($p < 0.001$). There was however no difference in plasma PGE_2 concentration between "turpentine/saline" (598 ± 81 pg/mL) and saline-only treated rats ($p > 0.05$). We also found a significant increase in plasma concentration of HMGB1 protein in animals treated with LPS 48 h after saline administration (2105 ± 305 pg/mL) that did not differ ($p > 0.05$) from animals pre-treated with turpentine and injected with saline after 48 h (1943 ± 329 pg/mL) as showed in Fig. 3D. Furthermore, plasma levels of HMGB1 measured in animals that received LPS 48 h after turpentine was more than twice as high (5806 ± 916 pg/mL) in comparison to the previously mentioned "saline/LPS" and "turpentine/saline" animals ($p < 0.001$). Plasma HMGB1 in animals treated twice with saline was below the detection limit of the ELISA kit used in the experiments (EIAab, cat. no. E0399r; sensitivity of assay 156 pg/mL).

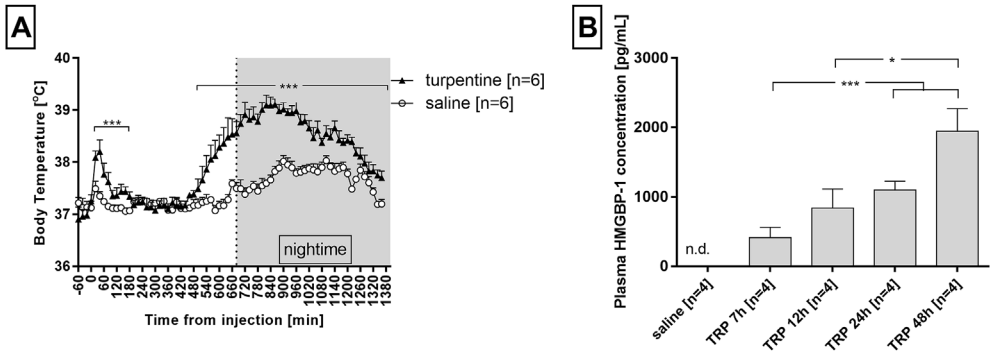

Fig. 2. Panel A demonstrates changes of body temperature (°C) over time (min) of rats treated at time "0" with turpentine (closed triangles) or saline (open circles) in a s.c. dose of 0.1 mL/rat. Sample size is indicated in parentheses. Values are means ± S.E.M. at 20-min averages. Asterisk indicates significant difference (***$p < 0.001$) between "saline" and "turpentine" groups. Panel B illustrates changes in plasma levels of HMGB-1 in animals treated with turpentine. Values are means ± S.E.M. Letter n indicates sample size in a respective group. Asterisk indicates significant difference (*$p < 0.05$; ***$p < 0.001$).

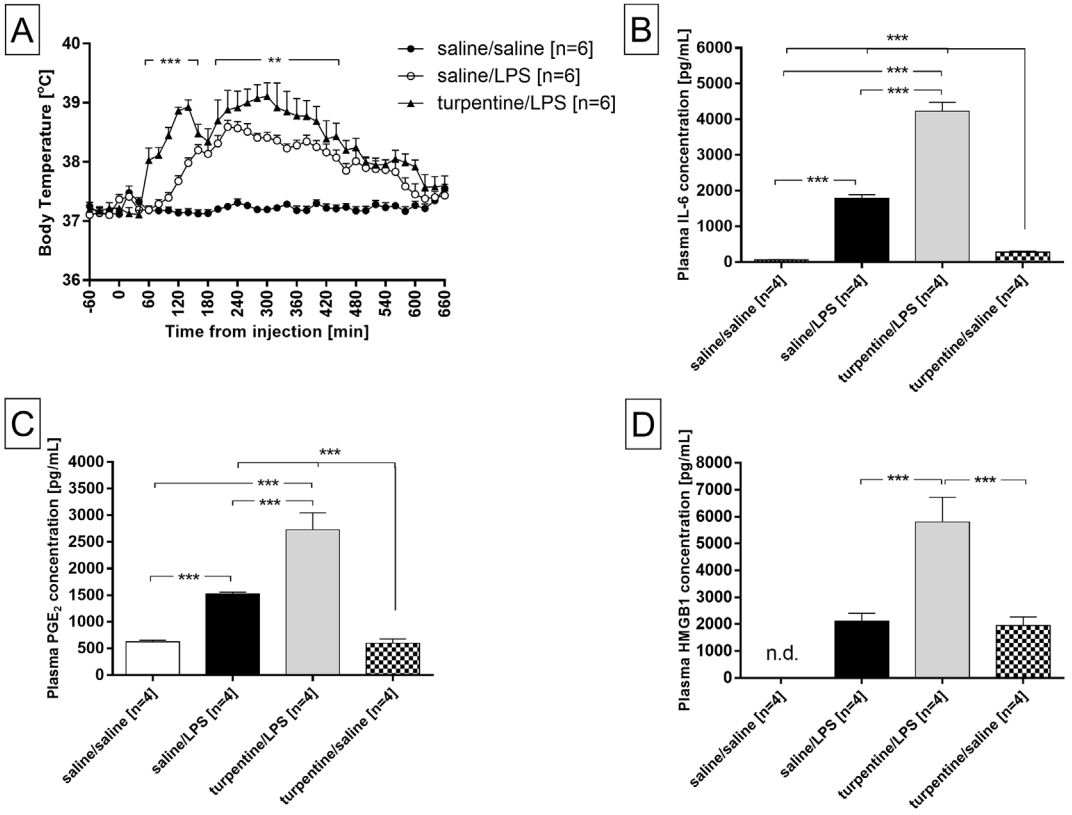

Fig. 3. Panel A depicts the effect of LPS (50 μg/kg injected i.p. at time "0") on body temperature of rats pre-treated 48 h earlier with turpentine (closed triangles) or saline (open circles). Closed circles represent Tb of animals pre-treated s.c. with saline and injected i.p. with saline after 48 h. Sample size is indicated in parentheses. Values are means ± S.E.M. at 20-min averages. Asterisk indicates significant difference (**$p < 0.01$; ***$p < 0.001$) between "saline/LPS" and "turpentine/LPS" groups. Panels below illustrate changes in plasma levels of IL-6 (panel B), PGE2 (panel C) and HMGB1 (panel D) in animals pre-treated with saline or turpentine during LPS fever induced after 48 h. Blood for ELISAs were collected 4 h after LPS injection. Values are means ± S.E.M. Letter n indicates sample size in a respective group. Asterisk indicates significant difference (***$p < 0.001$).

3.4. Plasma obtained from turpentine-treated rats augments the LPS fever in recipients

Injection of the 'experimental plasma' as well as 'sham plasma' differentially affected normal day-time Tb of the recipient rats. Animals treated with 'experimental plasma' responded with a short-lasting rise in Tb, whereas rats injected with 'sham plasma' showed a temporary drop of Tb (data not shown). However, animals in both experimental groups revealed a normal circadian night-time rise in Tb. There were also no significant changes in the course of body temperature during next 24 h compared to saline-treated rats. As can be seen in Fig. 4A recipients of 'experimental plasma' responded with significantly higher LPS-induced fever than that of rats injected with 'sham plasma'. The maximum value of Tb reached at 320 min post-injection (39.17 ± 0.12 °C) was significantly ($p < 0.001$) higher than that of the "sham plasma/LPS" group (38.55 ± 0.07 °C). In the course of a fever, a mean body temperature in "experimental plasma/LPS" rats calculated between 160 and 460 min was also significantly higher than that of "sham plasma/LPS" group (respectively 38.93 ± 0.08 °C and 38.09 ± 0.17 °C; $p < 0.001$). Injection of saline into rats pre-treated with 'experimental plasma' or 'sham plasma' did not provoke any significant changes in Tb in comparison to rats pre-treated with saline (data not shown).

3.5. Pre-injection of 'experimental plasma' obtained from turpentine-treated rats increases plasma IL-6, PGE_2 and HMGB1 concentration in LPS-treated animals

As shown in Fig. 4B and C, injection of LPS into rats that were treated with 'experimental plasma' 48 h before LPS caused significant increase ($p < 0.001$) in plasma levels of IL-6 (4114 ± 709 pg/mL) and PGE_2 (2775 ± 132 pg/mL) in comparison to animals pre-treated with 'sham plasma' (2035 ± 105 pg/mL for IL-6 and 1474 ± 21 pg/mL for PGE_2, respectively). There were no significant changes ($p > 0.05$) between both IL-6 and PGE_2 concentration in animals pre-treated with 'experimental plasma' or 'sham plasma' and those injected after 48 h with saline.

Although the plasma HMGB1 protein concentration in "experimental plasma/LPS" (1641 ± 358 pg/mL), "sham plasma/LPS" (1575 ± 357 pg/mL), and "experimental plasma/saline" (900 ± 270 pg/mL) experimental groups did not significantly differ ($p > 0.05$) as can be seen in Fig. 4D, it was significantly ($p < 0.001$) higher in all these groups than in rats treated with 'sham plasma' and saline (225 ± 58 pg/mL).

3.6. Injection with anti-HMGB1 antibodies weakened hyperpyrexia to LPS observed in animals pre-treated with turpentine

As can be seen in Fig. 5A, animals injected with anti-HMGB1 antibodies responded with weakened fever (38.19 ± 0.18 °C measured between 220 and 420 min post-injection) induced by LPS 48 h after turpentine administration, as compared to control rats treated with rabbit IgG (38.85 ± 0.25 °C respectively; $p < 0.01$). Also, the first peak of Tb rise after LPS injection was significantly ($p < 0.01$) higher in rats from the experimental group "TRP/IgG/LPS" (39.36 ± 0.11 °C reached 140 min post-injection) than in the "TRP/HMGB1ab/LPS" group (39.04 ± 0.15 °C). This phenomenon was accompanied with decrease in plasma IL-6 (7870 ± 178 pg/mL) and PGE_2

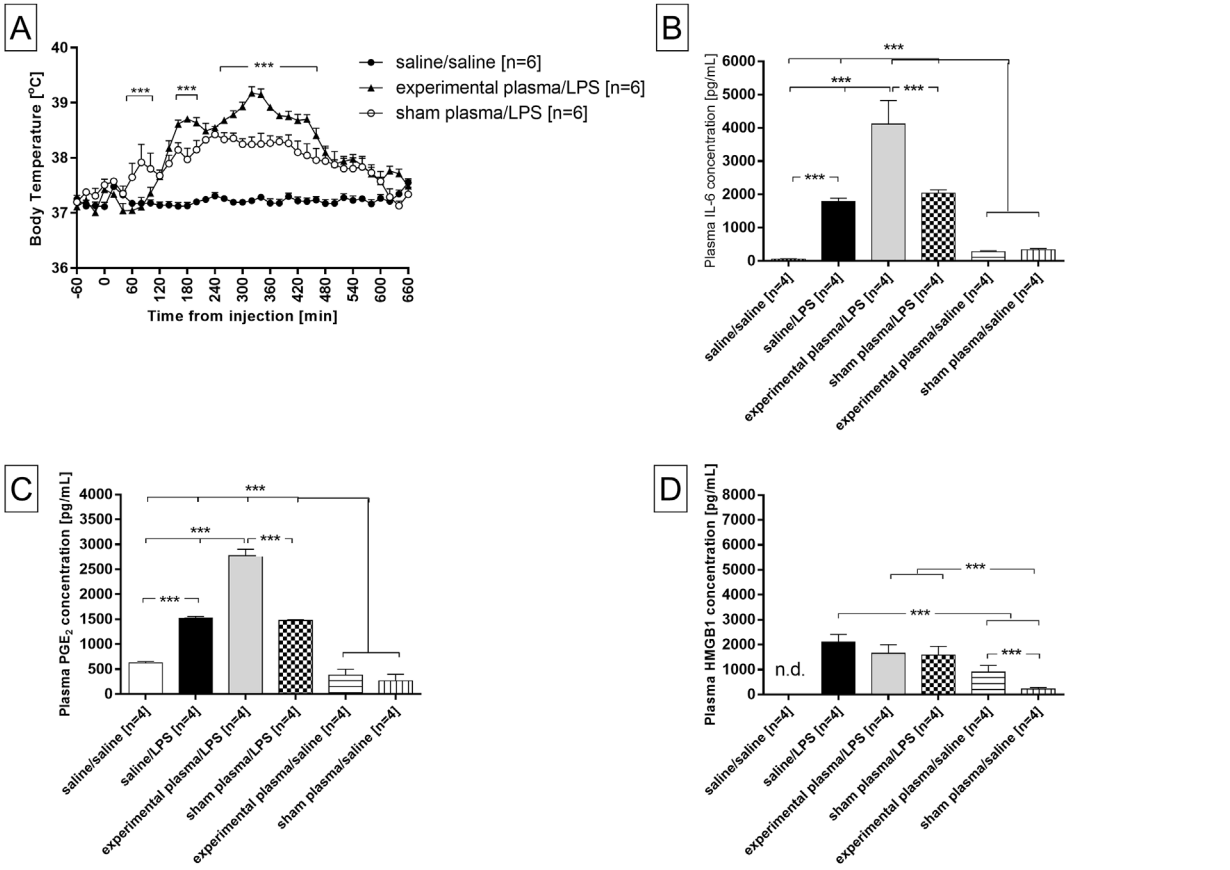

Fig. 4. Panel A demonstrates changes of body temperature (°C) over time (min) of rats pre-injected with 'experimental plasma' (closed triangles) or 'sham plasma' (open circles) and after 48 h (at time "0") treated with LPS (50 μg/kg i.p.). Closed circles represent Tb of animals pre-treated s.c. with saline and injected i.p. with saline after 48 h. Sample size is shown in parentheses. Values are means ± S.E.M. at 20-min averages. Asterisk indicates significant difference (***$p < 0.001$) between "sham plasma/LPS" and "experimental plasma/LPS" groups. Panels below represent changes in plasma levels of IL-6 (panel B), PGE2 (panel C) and HMGB1 (panel D) in animals pre-treated with saline, 'experimental plasma' (gray bar) or 'sham plasma' (checker bar) during LPS fever induced after 48 h. Blood for ELISAs were collected 4 h after LPS injection. Injection of saline was used as a control. Values are means ± S.E.M. Letter n indicates sample size in a respective group. Asterisk indicates significant difference (***$p < 0.001$).

(469 ± 240 pg/mL) concentration measured 4 h after LPS administration in "TRP/HMGB1ab/LPS" rats in comparison to "TRP/IgG/LPS" animals (8898 ± 263 pg/mL for IL-6, $p < 0{,}001$ and 1172 ± 132 pg/mL for PGE2, $p < 0{,}05$) as shown in Fig. 5B and C.

4. Discussion

Fever is regarded as an important defense mechanism. There are number of data supporting the hypothesis that an increase of body temperature in response to infections is beneficial, serving to protect the infected host by facilitating the activation of the immune defense (Kluger et al., 1996). Nevertheless, there are clinical situations in which fever can be detrimental and for certain patients, even a relatively modest increases in core temperature encountered during fever are deleterious and should therefore be suppressed (Mackowiak, 2000). That includes cardiovascular or pulmonary disorders, sepsis, neurologic injury or stroke (Wrotek et al., 2011b; Cunha, 2002; Albrecht et al., 1998; Styrt and Sugarman, 1990).

It is assumed that even minor aseptic damage may affect the immune response to further contact with pathogens. Clinicians believe that some patients in intense care are so ill not because the pathogen is overwhelming their body defense mechanisms, but due to their excessive immune response to it. The evidence that aseptic injury may affect the febrile response to LPS was provided by Soszynski and Krajewska (2002) during their research on tolerance to different pyrogens. They found that there is a lack of tolerance to pyrogenic effects of turpentine as well as that there is no cross-tolerance between LPS and turpentine. In their study, turpentine administrated twice intramuscular in 48 h intervals enhanced LPS-induced fever in rats (Soszynski and Krajewska, 2002). In our experiments, aseptic fever was induced by turpentine oil injected once at an s.c. dose of 0.1 mL that provoked rise in Tb in rats. Injection of turpentine significantly potentiated fever to LPS injected after 48 h confirming the observations of Soszynski and Krajewska. In our experiment the endotoxin fever induced 48 h post-turpentine started almost immediately after LPS injection and the changes in Tb were significantly augmented during both characteristic phases of fever. Furthermore, we found that the blood concentration of the fever mediators (PGE_2 and IL-6) measured 4 h after LPS administration, were two-fold higher in rats pre-treated with turpentine as compared to reference group "saline/LPS".

We hypothesized, therefore, that there is a factor released into the blood in the course of the aseptic injury that affects the immune response to further contact with bacterial products. To test this assumption, naïve rats were injected with plasma isolated one day after the subcutaneous administration of turpentine, and treated with LPS 48 h afterwards. Injection of this 'experimental plasma' alone didn't provoke significant changes in Tb (data not shown), however, recipient rats developed significantly higher fever in response to LPS (Fig. 4A) comparable to the one observed in the "turpentine/LPS" group of animals (Fig. 2A). Augmented elevation in Tb was accompanied by enhanced concentration of plasma IL-6 and PGE_2 (Fig. 4B and C). Obtained results proved that a humoral factor involved in the genesis of pyrogenic

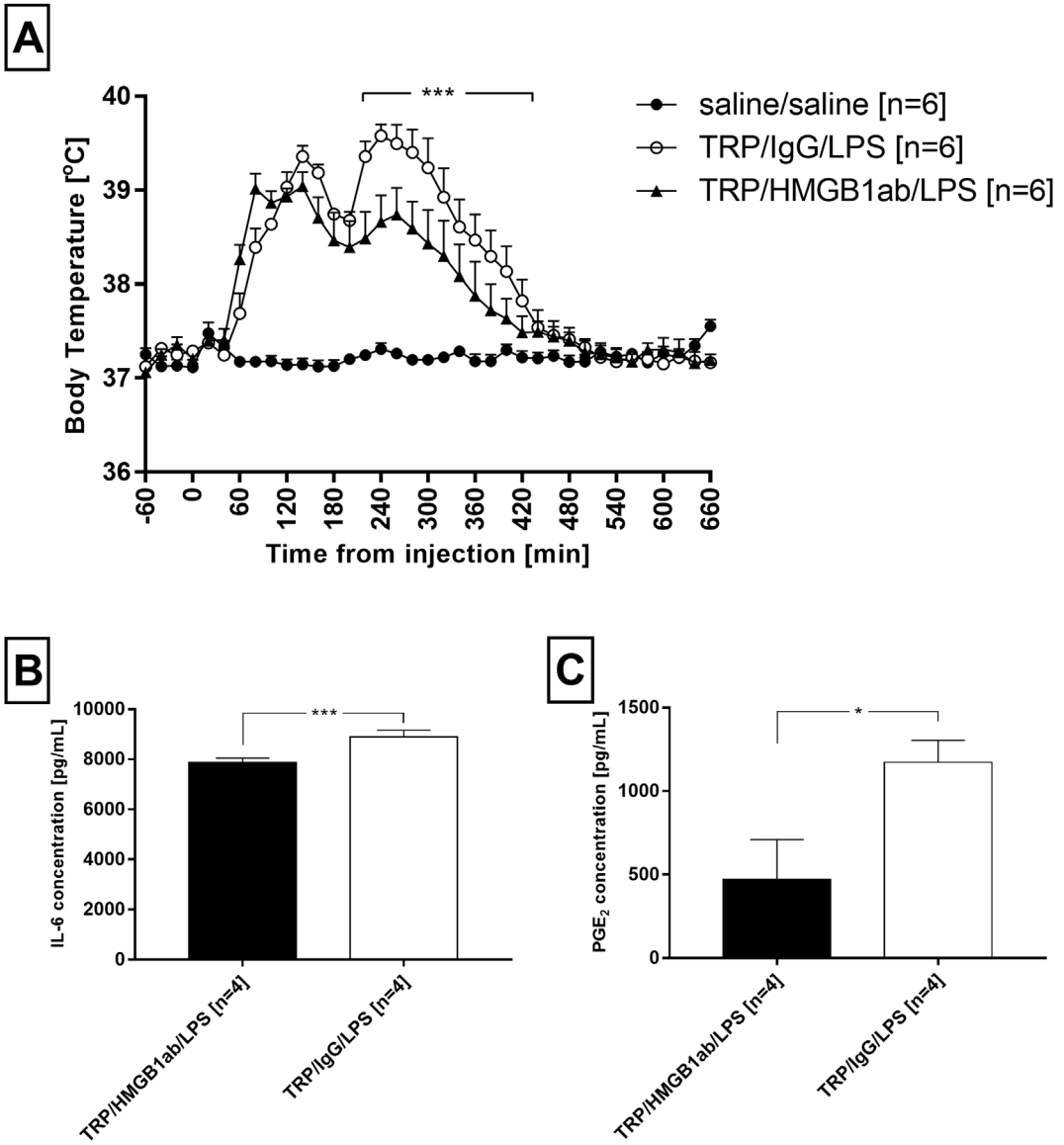

Fig. 5. Changes of body temperature (°C) over time (min) of rats pre-treated with turpentine (0.1 mL s.c.) and after 47 h (at time "-60") with HMGB1 antibodies (25 μg in 500 μL of sterile PBS; closed triangles) or with control IgG (open circles). An hour after antibodies administration (at time "0") rats were injected with LPS (50 μg/kg injected i.p.). Closed circles represent Tb of animals pre-treated s.c. with saline and injected i.p. with saline after 48 h. Values are means ± S.E.M. at 20-min averages. Asterisk indicates significant difference (***$p < 0.001$) between "turpentine/HMGB1/LPS" and "turpentine/IgG/LPS" groups. Panels below illustrate changes in plasma levels of IL-6 (panel B) and PGE2 (panel C) in both experimental groups. Blood for ELISAs was collected 4 h after LPS injection. Values are means ± S.E.M. Letter n indicates sample size in a respective group. Asterisk indicates significant difference (*$p < 0.05$; ***$p < 0.001$).

hypersensitivity to LPS released due to aseptic injury can be transplanted into naïve animals. This factor cannot induce fever by itself, at least just not in this particular concentration, since the injection of 'experimental plasma' had no significant effect on rats Tb. Therefore, most likely, it can lower the threshold of excitability of the physiological components of the mechanisms involved in the generation of infectious fever. In the next experiments, we examined HMGB1 as a potential factor responsible for the results described above.

HMGB1 protein is actively released by stimulation of the immune cells including monocytes, macrophages, and endothelial cells with exogenous pathogen-derived molecules and is passively released by ischemia or cell injury (for rev. see Yang et al., 2010; Andersson et al., 2011). Indeed, we found that HMGB1 is increasingly released into plasma in the course of aseptic necrosis of tissues (Fig. 2B) as well as after LPS injection (Fig. 3D). Not surprisingly, in saline-treated control animals plasma HMGB1 concentration was below the detection limit of the ELISA kit used for experiments. It was, however, more than two-fold higher in animals injected with LPS two days after turpentine in comparison to the mentioned "saline/LPS" and "turpentine/saline" groups. It should be noted that elevated levels of plasma HMGB1 corresponds well with the increase in IL-6 and PGE_2 concentrations. Recent studies strongly suggest, that extracellular HMGB1 acts as a pro-inflammatory cytokine and activates cytokine release from immune cells through TLRs-dependent signaling pathways (Yang et al., 2002; Chen et al., 2004, 2012; Wang et al., 2004) as well as promotes the PGE_2 biosynthesis pathway (Jaulmes et al., 2006; Leclerc et al., 2013).

As can be seen in Fig. 4D, plasma HMGB1 level in the animals pre-injected with 'experimental plasma' and with saline after 48 h, did not significantly differ from "experimental plasma/LPS" and "sham plasma/LPS' groups. Yet, it was still four times higher than in the "sham plasma/saline" experimental group. Presence of HMGB1 protein alone did not translate into elevation in IL-6 or PGE_2 plasma concentrations as can be seen in Fig. 4B and C, however it sensitizes rats to further contact with LPS. The mechanism underlying this observation is still unknown,

but we found that maintaining the 48 h interval between turpentine and LPS administration is crucial for developing hyperpyretic response (data not shown).

In the last experiment, we tested whether or not blocking of HMGB1 activity in animals pre-treated with turpentine will affect endotoxin fever. In fact, injection of HMGB1 antibodies an hour before LPS significantly reduced febrile rise in Tb (as can be seen in Fig. 5A) as a consequence of reduction in IL-6 and PGE_2 release and thus clearly demonstrates, that HMGB1 is one among the crucial factors involved in the mechanism of sensitization to the pyrogenic effects of LPS in rats after aseptic necrosis of tissues.

It is obvious that further studies are needed to fully understand the mechanism underline the actions of HMGB1. It has been proved, that while intraperitoneal injection of HMGB1 into rats (in a dose of 1 μg/kg) did not cause any elevation of Tb, intracerebroventricular injections cause dose-dependent fever (O'Connor et al., 2003). This fact indicates that the concentration of HMBG1in 'experimental plasma' administrated into rats in our experiments could be too low to induce fever. It also suggests the participation of activation of neuronal pathways in observed effects of HMGB1. The protein concentration was, however, sufficient to sensitize rats to further contact with LPS. Since other studies demonstrate that HMGB-1 increase the release of pyrogenic cytokines from Kupfer cells through the activation of TLR2 and TLR4 (Chen et al., 2012) and activates the synthesis/release of PGE_2 (Jaulmes et al., 2006; Leclerc et al., 2013) we presume that observed phenomenon is, at least in part, the effect of the activation and priming of macrophages. Our preliminary data seems to support this hypothesis. Peripheral blood mononuclear cells (PBMCs) isolated from rats 48 h after turpentine or 'experimental plasma' injections were more sensitive to LPS when stimulated *in vitro* and produced significantly higher levels of fever mediators compared to cells isolated from saline and 'sham plasma' treated rats (data not shown). The hypothesis of priming should also explain why the febrile response in rats pre-injected with turpentine was not only higher, but also starts almost immediately after LPS administration.

5. Conclusion

Aseptic injuries associated with sterile physical damage, such as a burn or bruise as well as inner organ damages and fractures, may lead to serious consequences for health and immunity. We showed, that even minor necrosis of tissues strongly affects febrile response to further contact with infectious stimuli. This fact should be considered especially in departments of critical medical care. Our data constitutes also the first experimental evidence that turpentine-induced aseptic fever is a useful model for studying the biological role of HMGB1 in inflammation. In the light of presented results, a promising role of HMGB1 protein as a marker of inflammation and novel therapeutic target should be noticed.

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Declarations of interest

None.

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