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Full-length Article

Deleterious synergistic effects of distress and surgery on cancer metastasis: Abolishment through an integrated perioperative immune-stimulating stress-inflammatory-reducing intervention

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A B S T R A C T

The perioperative period holds disproportionate impact on long-term cancer outcomes. Nevertheless, perioperative interventions to improve long-term cancer outcomes are not clinical routines, including perioperative stress-reducing or immune-stimulating approaches. Here, mimicking the clinical setting of pre-operative distress, followed by surgery, we examined the separate and combined effects of these events on the efficacy of pre-operative immune stimulation in rats and mice, and on post-operative resistance to tumor metastasis of the syngeneic mammary adenocarcinoma MADB106 in F344 rats and the CT26 colon carcinoma in Balb/C mice. The novel immune stimulating agents, GLA-SE or CpG-C (TLR-4 and TLR-9 agonists, respectively), were employed pre-operatively. Sixteen hours of pre-operative behavioral stressors (i) lowered CpG-C induced plasma IL-12 levels, and reduced resistance to MADB106 and CT-26 experimental metastases, and (ii) worsened the deleterious effects of laparotomy on metastasis in both tumor models. In rats, these effects of pre-operative stress were further studied and successfully abolished by the glucocorticoid receptor antagonist RU-486. Additionally, *in vitro* studies indicated the dampening effect of corticosterone on immune stimulation. Last, we tested a perioperative integrated intervention in the context of pre-operative stress and laparotomy, based on (i) antagonizing the impact of glucocorticoids before surgery, (ii) activating anti-metastatic immunity perioperatively, and (iii) blocking excessive operative and post-operative adrenergic and prostanoid responses. This integrated intervention successfully and completely abolished the deleterious effects of stress and of surgery on post-operative resistance to experimental metastasis. Such and similar integrated approaches can be studied clinically in cancer patients.

1. Introduction

The perioperative period in cancer patients, spanning days to few weeks (depending on surgery type), holds disproportionate impact on long-term cancer outcomes, despite its short duration. This relatively short period encompasses vast psychological distress, which alone or together with the surgical trauma were shown to induce numerous neuroendocrine responses, harm immune efficacy, and promote tumor progression through various mechanisms (Shaashua et al., 2017; Neeman and Ben-Eliyahu, 2013). Thus, the perioperative period entails potential for various interventions aimed at improving long-term cancer outcomes. Such interventions may include (i) prevention of psychological stress responses, (ii) activation of anti-metastatic immune functions, and (iii) blockade of inflammatory and sympathetic stress responses induced by the surgical procedure (Horowitz, 2015). Importantly, stress responses were recently found to reduce the efficacy of various immunotherapies (Levi, 2016). As stress hormones, immune functions, immune activation, and surgical stress-inflammatory responses were suggested or shown to interact in complex manners (Ader, 2007), optimal perioperative approaches to improve long-term cancer outcomes should consider these interactions, as detailed below.

First, stress responses, including prior to surgery, impact immune competence, post-operative recovery, and the efficacy of immune stimulation. Beginning at diagnosis, oncological patients exhibit psychological distress (Seok et al., 2010), reaching peak levels during the perioperative period, resulting in elevated levels of various stress hormones (Thornton et al., 2010). Others and us have shown that stress hormones, including glucocorticoids (GCs) and catecholamines (CAs), harm CMI efficacy *in vivo*, as indicated in lower plasma levels of pro-CMI/pro-inflammatory cytokines (e.g. IL-12, TNF-α, and IFN-γ) (Shaashua et al., 2012; Frick et al., 2009), and in reduced NK-dependent resistance to cancer metastasis (Ben-Eliyahu et al., 2000). Psychological stress also influences various aspects of recovery from the surgical trauma, as both GCs and CAs were shown to directly impair wound healing by reducing wound-site infiltration of neutrophils and levels of pro-inflammatory cytokines (Gouin and Kiecolt-Glaser, 2011). Additionally, higher pre-operative anxiety levels were correlated with increased use of intra-operative anesthetic agents (Maranets and Kain, 1999), as well as with longer time to relaxation during induction of anesthesia (Mitchell, 2003). Last, we have previously shown that when

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immune stimulation (e.g., through the TLR-9 agonist CpG-C) is initiated in the context of psychological stress, its efficacy is diminished, rendering it markedly ineffective (Levi, 2016; Goldfarb et al., 2011).

Second, the surgical stress-inflammatory response is known to affect various aspects of immunity, and to promote malignant progression through various mechanisms (Neeman and Ben-Eliyahu, 2013). However, it is unclear whether (i) psychological stress that precede surgery exacerbates or attenuates these impacts, and (ii) whether stress and surgery-induced immune suppression may partially or completely abolish beneficial effects of a successful pre-operative immune stimulation. Oncological surgeries in patients are most commonly conducted following days or weeks of distress, and it is unclear whether this preceding stress period induce habituation or sensitization of the patient to the various effects of the surgical stress-inflammatory response.

In previous studies others and we introduced various successful pharmacological interventions to overcome the harmful effects of either psychological stress or of surgery. Glucocorticoid and/or β-adrenergic receptor antagonists were found effective and often additive in blocking the effects of psychological stress (Rosenne et al., 2014). Additionally, the deleterious effects of surgery on immunity and on metastatic progression were synergistically attenuated in mice and rats by a combination of β-adrenergic receptor antagonists and COX-2 synthesis inhibitors (Glasner et al., 2010; Benish et al., 2008; Benish and Ben-Eliyahu, 2010). Interestingly, the blockade of glucocorticoid response in the context of surgery was markedly less effective, and seems not feasible clinically (Rosenne et al., 2014). Importantly, the combined inhibition of β-adrenergic and COX-2 signaling was recently tested clinically in two phase-II biomarker trials in breast and colorectal cancer patients, yielding promising results (Shaashua et al., 2017; Haldar et al., 2017).

As immunotherapy holds great promise in cancer treatment, it could be instrumental to induce immune activation and maintain its efficacy in the perioperative context. Translational animal studies employing various immunotherapies have indeed shown great success when examined in different cancer types and animal models, leading to lower recurrence rates and improved survival (Levi, 2016; Glasner et al., 2010; Goldfarb et al., 2011). However, these treatments have rarely yielded similar clinical success (Colombo and Trinchieri, 2002). According to a recent examination, less than 10% of successful cancer-related translational studies reach clinical significance (Mak et al., 2014), with various speculation for their failure, including interactions with stress responses (Reiche et al., 2004). Also discouraging are known stress responses induced by immunotherapeutic treatments (e.g. elevation in GCs level) (Denicoff et al., 1989; Baker et al., 1989).

Here, we simulated the clinical perioperative context, specifically its two distinct aspects of (i) pre-operative psychological stress, and (ii) the physiological trauma of surgery. We aimed at studying the following questions: (1) Do psychological stress dampen the efficacy of immune stimulation, (2) Do pre-surgical psychological stress exacerbate the deleterious effect of the surgical trauma on immunity and resistance to metastasis, and (3) Can an integrative perioperative intervention that incorporate immune stimulation and pharmacological interventions overcome the deleterious effects of both psychological stress and surgery? The integrated therapeutic intervention we devised and studied combines (i) blockade of pre-operative psychological stress responses (based on GC antagonist), (ii) pre-operative immune stimulation (based on the novel TLR-4 agonist GLA-SE (Matzner et al., 2016) or the TLR-9 agonist CpG-C (Goldfarb et al., 2011), and (iii) blockade of the surgery-induced stress-inflammatory response (based on inhibition of β-adrenergic and COX-2 signaling), together aiming to achieve optimal prevention of post-operative metastatic disease.

2. Materials and methods

2.1. Animals

Male and female Fischer 344 (F344) rats, 3–6 months old, and 8 weeks old male BALB/c mice, were housed 3–4 per cage in our vivarium with *ad-libitum* access to food and water on a 12:12 light–dark cycle at 22–24 °C. Rats were handled 4 times prior to experimentation to reduce potential procedural stress. Age, weight, sex and drug administration were counterbalanced across all experimental procedures. Housing conditions were monitored by the Institutional Animal Care and Use Committee of Tel Aviv University, which also approved all studies described herein.

2.2. Drugs

Glucopyranosyl lipid-A SE (GLA-SE) and its administration: GLA (Immune Design, Seattle, WA), a synthetic TLR-4 agonist (Coler et al., 2010), dissolved in a stable emulsion (SE, see below, 2000 μg GLA in 1 ml SE) was further diluted in PBS for a final concentration of 100 μg/ml.

Stable emulsion (SE) delivery system for GLA: SE (Immune Design, Seattle, WA) is an oil-in-water emulsion manufactured by high shear homogenization. The oil droplets are stabilized by emulsifiers in a bulk aqueous phase, and serve as an adjuvant delivery system (Fox et al., 2008).

CpG-C: CpG-C (Sigma, Israel), a TLR-9 agonist (ODN 2395: 50-TCGTCGTTTTCGGCGCGCGCCG-30) with a phosphorothioate backbone. Purity examination by the limulus amebocyte lysate assay resulted with undetectable levels of endotoxin. CpG-C was dissolved in PBS (phosphatebuffered saline) and was administered intraperitoneally (i.p.) at a dose of 100 μg per animal to mice (Goldfarb et al., 2009).

RU-486: (Sigma, Israel), progesterone and glucocorticoid receptor antagonist. The drug was dissolved in corn oil and was administered s.c. at a dose of 25 mg/kg.

Propranolol: (Sigma, Israel) a non-selective β-adrenergic blocker. The drug was dissolved in a slow release vehicle (see below) and administered s.c. at a dose of 1.5 mg/kg.

Etodolac: a semi-selective COX-2 inhibitor, kindly donated by Taro, Israel. The drug was dissolved in corn oil and was administered s.c. at a dose of 12.5 mg/kg.

Slow release vehicle: The slow release vehicle is an emulsion used to extend absorption time of drugs, and is based on 4 parts PBS, 3 parts mineral oil (Sigma, Israel), and 1 part mannide-monooleate (a non-specific surface active emulsifier, Sigma, Israel). Unpublished data from our laboratory indicated that a β-adrenergic antagonist administered in the slow release vehicle exerted its effects 9 and 12 h following injection, whereas the same dose of drug administered in saline had no effects at these time points.

2.3. Tumor cell lines and their maintenance

MADB106: MADB106 is a selected variant cell line obtained from a pulmonary metastasis of a chemically induced mammary adenocarcinoma (MADB100) in the F344 rat (Barlozzari, 1985). MADB106 tumor cells metastasize only to the lungs, a process that is dependent upon NK cells (Barlozzari, 1985). The lung tumor retention (LTR) of MADB106 cells is highly indicative of the number of metastases that would have developed weeks later (Barlozzari, 1985; Ben-Eliyahu et al., 1996; Shakhar and Ben-Eliyahu, 1998). Additionally, because the metastatic process of MADB106 is sensitive to NK activity predominantly in the first 24 h following inoculation (Barlozzari, 1985; Ben-Eliyahu and Page, 1992), LTR is more reflective of *in vivo* NK activity levels than the number of actual metastases (Shakhar and Ben-Eliyahu, 1998). The MADB106 cell line was maintained in monolayer cultures in complete media (CM) (RPMI-1640 media supplemented with 10% heat-

inactivated fetal calf serum (FCS), 50 μg/mL of gentamicin, 2 mM of L-glutamine, 0.1 mM of non-essential amino-acids, and 1 mM of sodium pyruvate, Biological Industries, Kibbutz Biet Haemek, Israel) in 100% humidity, 5% CO_2 at 37 °C. Cells were removed from the culture flask with 0.25% trypsin solution in PBS, and were washed with CM. This cell line was used for *in vivo* assessment of lung tumor retention (LTR).

Radiolabeling of MADB106 tumor cells for the assessment of lung tumor retention: Tumor cell DNA radiolabeling for assessment of LTR was accomplished by adding 0.5 μCi/ml of ^{125}Iododeoxyuridine (^{125}IDUR, Eisenberg bros., Israel) to the cell culture for 24 h.

CT26: The CT26 murine colon carcinoma cell line is a chemically-induced undifferentiated carcinoma, syngeneic to the Balb/C strain (Corbett, 1975). Cells were grown in monolayer cultures in CM, at 37 °C, 100% humidity, and 5% CO_2. Cells were removed from the culture flask with a 0.25% trypsin solution in PBS, washed once in PBS containing 0.1 mg/ml BSA (335 × g for 10 min), and adjusted to a final concentration of 2×10^5/ml in PBS-BSA for spleen injection at a volume of 100 μl per animal. Cells were kept on ice during the entire injection procedure in each of the experiments. No effects of duration of this pre-inoculation, *in vitro* tumor cell maintenance, on the number of developing liver metastases, were evident.

2.4. Stress paradigms

Tilt-light: Animals remained in their home cages, with free access to food and water, for a total period of 16 h. The cages were placed in a 45° tilted position, resulting in a reduced available floor space, in a fully lighted room for the entire session. This stress protocol was initiated 3–5 h before the onset of the dark period.

The paradigm started at the beginning of the animals activity hours (dark hours), and during this entire time rats remained in their original cages, but were placed in a room with constant lightning and in a 45° tilted position.

Wet-cage: Rats were placed in cages filled with room-temperature water to the height of 2 cm, with free access to food and water, for a total period of 16 h. The cages were placed in a fully lighted room for the entire session. This stress protocol was initiated 3–5 h before the onset of the dark period.

2.5. Experimental laparotomy

The procedure has been described in details elsewhere (Melamed et al., 2005). Briefly, rats were anesthetized and maintained with 2.5% isoflurane and a 4 cm midline abdominal incision was performed. The small intestine was externalized, rubbed with a PBS-soaked gauze pad and left hydrated with a PBS-soaked gauze pad for 30 mins. Finally, the intestine was internalized and the abdomen sutured.

2.6. Surgical procedure for CT26 tumor cell injection through the spleen

Mice were anesthetized and maintained with 2.5% isoflurane and a 0.5 cm abdominal incision was performed adjacent to the spleen (a left flank incision approximately 2 cm left of the abdominal midline). 2×10^4 CT26 tumor cells in 100 μl PBS were injected into the spleen (and naturally drained into the splenic and portal veins, and deposited in the liver), using a 31G needle, which was maintained in the spleen tissue for two minutes following injection. A 4/0 blue polypropylene monofilament non-absorbable suture was placed across the hilum of the spleen to prevent bleeding, and a splenectomy was then performed. After the excision of the contaminated spleen, the peritoneum and skin were sutured. The animals were allowed to recover in their home cages.

Assessment of metastatic development: Animals were monitored daily for general well-being after tumor injection, and euthanized with an overdose of isoflurane on the 20th day. Livers were then harvested and weighed, and surface-hepatic metastases were counted by an experimenter blind to the experimental group of each animal. Metastases were identified as being greater than 1 mm in diameter, forming a spherical solid and distinct formation.

2.7. Inoculation with MADB106 tumor cells and assessment of lung tumor retention (LTR)

Rats were lightly anesthetized with isoflurane, and 4×10^5/kg MADB106 tumor cells in 2 ml/kg PBS containing 0.1% bovine serum albumin (BSA) were injected into their tail vein. For assessment of LTR, animals were sacrificed with CO_2 24 h after inoculation with ^{125}IDUR-labeled tumor cells, their lungs were removed and placed in a γ-counter to assess percent radioactivity retained in this organ. LTR was calculated using the following formula: (radioactivity count of lung – background radioactivity) × 100/(radioactivity count of the total injected cell suspension – background radioactivity).

2.8. Assessment of plasma corticosterone levels

Blood for assessment of plasma corticosterone (CORT) levels was drawn from the heart into heparinized test tubes. Plasma CORT levels were measured employing ELISA (AssayPro, St. Charles, MO), per manufacturer's instructions.

2.9. In-vitro studies of IL-12 induced production

Pooled blood from F344 animals was washed once with PBS and twice with CM, each in a 4-fold dilution, 10 min at 456 g, followed by supernatant removal to restore the original volume.

A 960 μl blood aliquot was placed in a well, and added with 20 μl of corticosterone, in a final concentration of 10^{-7} M, 10^{-8} M, 10^{-9} M, or 0 (saline control). Samples were incubated at 100% humidity, 5% CO_2, and 37 °C for 2 h, and then added with 20 μl of GLA in a final concentration of 100 ng/ml, or saline, and returned to incubation for another 22 h. At the end of incubation supernatant was collected for IL-12 levels analysis employing ELISA (eBioscience, San-Diego, CA), per manufacturer's instructions.

2.10. Flow cytometry

Standard procedures were used to prepare cells for flow cytometric analysis (Melamed et al., 2005). Lymphocytes were identified based on their FSC and SSC location, and monocytes were identified by the APC-conjugated anti-NKR-P1 mAb (Biolegend, San Diego, CA) as being NKR-P1^{dim} (CD161^{dim}) cells. Flow cytometry analysis was conducted using a FACScan (Becton Dickinson). To assess the total number of cells per μl of sample (or a specific cell subtype), 300 polystyrene microbeads (20 μm, Duke Scientific, Palo Alto) per μl sample were added to each sample, and the following formula was used: (# of cells in sample/# of microbeads in sample) × 300.

2.11. Statistical analysis

One, two, or three-way factorial analyses of variance (ANOVA) with a pre-determined significance level of 0.05 were conducted. Provided significant group differences were found, Fisher's protected least significant differences (Fisher's PLSD) contrasts were performed to compare specific pairs of groups, based on a priori hypotheses.

When SD significantly differed between groups of the same experiment, per-wise comparisons were conducted employing student's *t*-test.

3. Procedures and results

3.1. An overlook of the experimental flow and rationale

I. Aiming to establish a mild prolonged stress paradigm, to better

simulate clinical aspect of pre-operative stress, we first studied physiological characteristics of the tilt-light stress paradigm, and compared it to our previously used wet-cage stress paradigm (Levi, 2016). On face value, the tilt-light stress paradigm is based more on psychological perturbations of the animal standard environment, than on physiological perturbations, which are common in many other stress paradigms (e.g. cold-water immersion or wet-cage) (Exp. 1–2).

II. We than tested whether either the tilt-light or the wet-cage stress paradigms dampen the efficacy of immune stimulation by either the TLR-9 agonist CpG-C or the TLR-4 agonist GLA (Exp. 2–3).

III. As the tilt-light stress paradigm reduced the efficacy of immune stimulation, we continued by testing whether blocking the glucocorticoid response by RU-486 can prevent such deleterious effects of psychological stress. Simultaneously, we examined possible direct effects of GLA immune stimulation on levels of stress hormones (Exp. 3–5).

IV. As in the clinical setting psychological stress precedes surgery, we studied whether pre-surgical psychological stress exacerbate the deleterious effect of the surgical trauma on immunity and resistance to metastasis (Exp. 6), and found additive or synergistic effects, which we than attempted to block by attenuating glucocorticoid signaling (Exp. 7).

V. Following a successful pre-surgical immune stimulation and blockade of the deleterious effects of pre-surgical psychological stress (II-IV), we tested an integrative perioperative intervention that incorporate immune stimulation and pharmacological interventions to overcome the deleterious effects of both psychological stress and of surgery (Exp. 8).

3.1.1. Exp. 1: The tilt-light stress paradigm in rats: Elevated corticosterone levels and altered composition of circulating immunocytes

We first examined the stressful nature of the tilt-light stress paradigm in rats, by assessing plasma corticosterone levels and numbers of circulating leukocytes. We collected peripheral blood from the heart of female F344 rats 2 and 16 h following initiation of the stress paradigm, and compared outcomes to matched controls maintained in their home cages in the vivarium. Corticosterone levels showed expected circadian rhythm (higher at the beginning of the dark phase), and the stress paradigm elevated these levels at both time points tested ($F(1,42) = 18.905$, $p < 0.01$) (Fig. 1A). Total lymphocyte and monocyte numbers were reduced 2 h following the induction of stress, but not at the 16 h timepoint ($F(1,43) = 5.948$, and $F(1,43) = 2.903$, respectively. $p < 0.01$ for both) (Fig. 1B–C).

3.1.2. Exp. 2: The deleterious effects of stress on the efficacy of CpG-C immune stimulation in mice are stress-paradigm dependent

We compared the effects of the tilt-light and the wet-cage stress paradigms on the efficacy of immune stimulation in mice, employing the TLR-9 agonist CpG-C. Male and female C57Bl/6J mice were subjected to either wet-cage, or the tilt-light stress paradigms for a period of 16 h, or maintained in their home cages in the vivarium. Two hours following the beginning of stress, 100 μg of CpG-C was injected i.p. Immediately at the end of stress, peripheral blood was withdrawn from the hearts of all animals. IL-12 is a prominent Th1 cytokine induced by CpG-C and GLA-SE, and a potent activator of NK activity and anti-metastatic immunity (Goldfarb et al., 2011; Matzner et al., 2016; Tsimopoulou et al., 2015). It was thus used as an index for the efficacy of immune stimulation.

Both stress paradigms significantly reduced baseline IL-12 levels by approximately 2-fold (from ~10 pg/ml to ~5 pg/ml). CpG-C significantly elevated IL-12 levels, under both control and stress conditions ($p < 0.05$ for both). Importantly, the tilt-light and the wet-cage paradigms reduced the efficacy of CpG-C, resulting in significantly lower levels of IL-12 under these stress conditions, as well as lower IL-12 elevation ratio in the stress conditions compared to the control

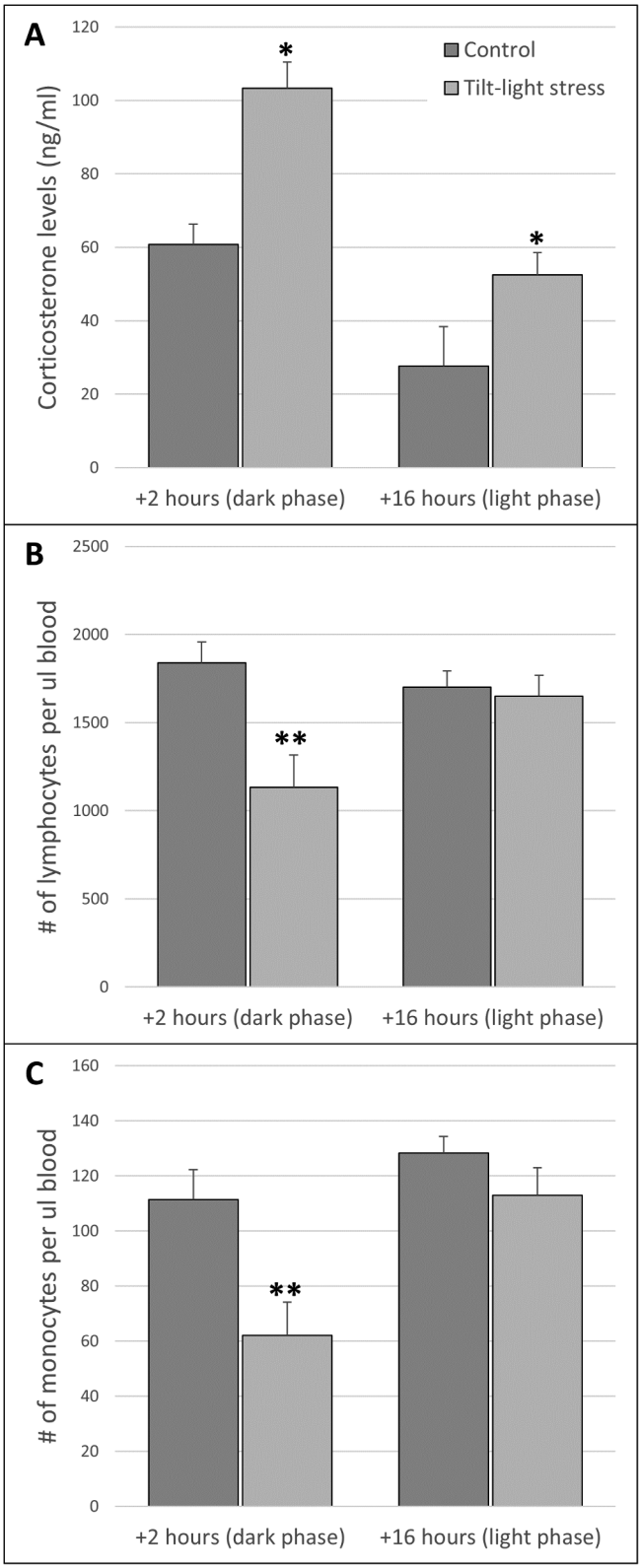


Fig. 1. The effects of the tilt-light stress paradigms were examined in rats at 2 and 16 hours following initiation, which occurred during the beginning of the dark and light phases, respectively (n = 4–6 per group). (A) Stress elevated corticosterone levels across time-points, and at each time-point alone (*). The stress paradigm lowered the total number of circulating lymphocytes (B) and monocytes (C) two hours following its initiation (**), but not at the 16 hours time-point.

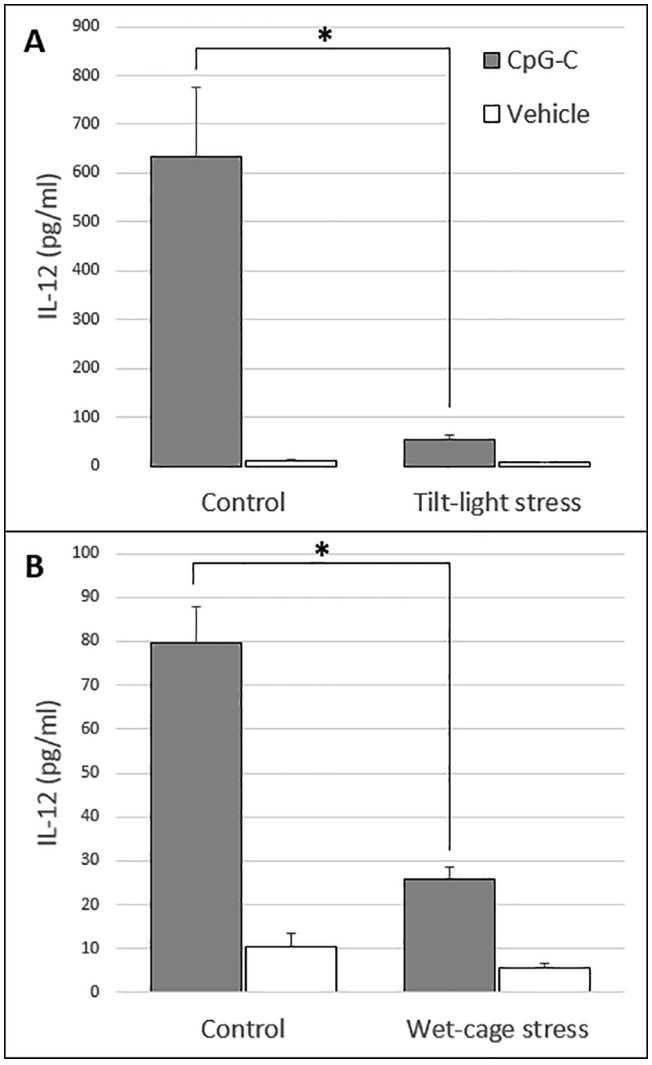


Fig. 2. Mice were subjected or not to a 16 hours stress exposure, and were administered with CpG-C or vehicle 2 hours following stress initiation (n = 7–10 per group in each of the three stress paradigms). IL-12 plasma levels were assessed at the end of the stress. CpG-C elevated IL-12 levels in all experiments, and this elevation was attenuated partly by the tilt-light stress (A) and wet-cage stress (B) paradigms (*).

conditions ($F(1,31) = 36.71$, and $F(1,68) = 11$, respectively. $p < 0.01$ for both) (Fig. 2).

3.1.3. Exp. 3: The efficacy of immune stimulation is reduced or completely abolished by ongoing stress in rats, partially through glucocorticoids-dependent mechanisms

In this study we aimed at assessing the effects of the tilt-light and the wet-cage stress paradigms on the efficacy of GLA-SE immune stimulation in rats. Male F344 rats were subjected to either the tilt-light or the wet-cage stress paradigms for a period of 16 h, or remained in their home cages at the vivarium (controls). To counteract the potential effects of stress, 30 min prior to stress initiation half of the animals in each group received the glucocorticoid receptor antagonist RU-486. Two hours following stress initiation, half of the animals in each group was injected subcutaneously with 10 μg of the TLR-4 agonist GLA-SE. At the end of the 16 h stress session, all animals were inoculated with radiolabeled MADB106 cancer cells for LTR assessment.

In both experiments, GLA-SE immune stimulation reduced LTR levels in non-stressed animals ($p < 0.05$ for both). This beneficial effect was partially reduced when GLA-SE was administered during the tilt-light stress paradigm (Fig. 3A), and completely abolished under the wet-cage stress condition (Fig. 3B). RU-486 administration prior to stress significantly improved LTR in the immune-stimulated animals in both stress paradigms ($p < 0.05$ for both), allowing a more effective GLA-SE immune stimulation under the tilt-light stress condition (see Fig. 3A).

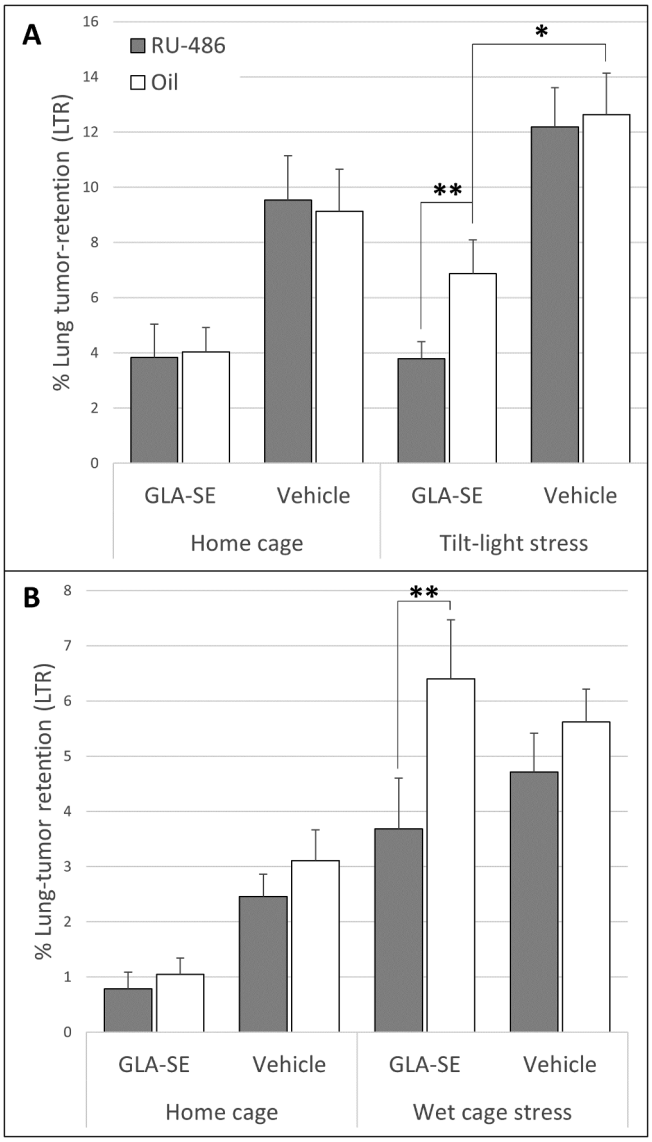


Fig. 3. The effects of ongoing stress on GLA-SE immune stimulation. Rats were subjected or not to 16 hours of either the tilt-light (A) or the wet-cage (B) stress paradigm, and GLA-SE or vehicle were administered 2 hours following stress initiation (n = 13–20 per group). MADB106 tumor cells were administered immediately following stress, and lungs were harvested 24 hours later for the assessment of lung tumor retention (LTR).In both stress paradigms, GLA-SE immune stimulation reduced LTR levels under no-stress conditions, and the stress paradigms elevated LTR levels. (A) The beneficial effect of GLA-SE was partially reduced when administered during the tilt-light stress paradigm (*), and (B) completely and significantly abolished when administered during the wet-cage stress paradigm. RU-486 administration prior to stress significantly improved the effects of GLA-SE immune stimulation in both stress paradigms (**), improving immune stimulation efficacy under the tilt-light stress condition (A).

3.1.4. Exp. 4: Corticosterone reduces the in-vitro immune stimulating effects of GLA

Given the *in vivo* capability of the glucocorticoid receptor antagonist

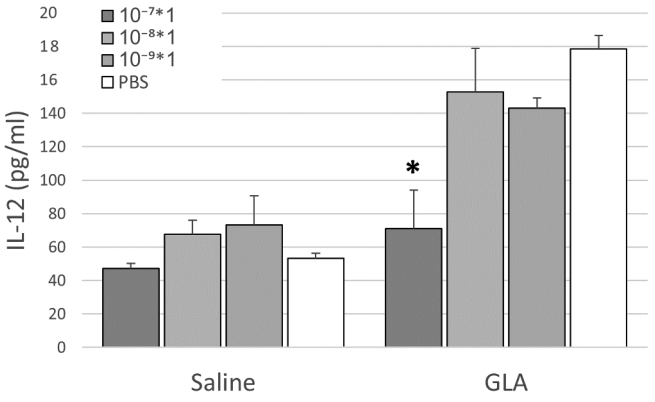


Fig. 4. Corticosterone was added to blood aliquots from naïve rats. GLA or saline was added 2 hours later, and supernatants were collected following an overnight incubation for assessment of IL-12 levels. GLA significantly elevated IL-12 levels, and corticosterone in the concentration of 10^{-7}M (stress physiological levels) completely abolished this elevation (*), without affecting the release of IL-12 in the absence of GLA.

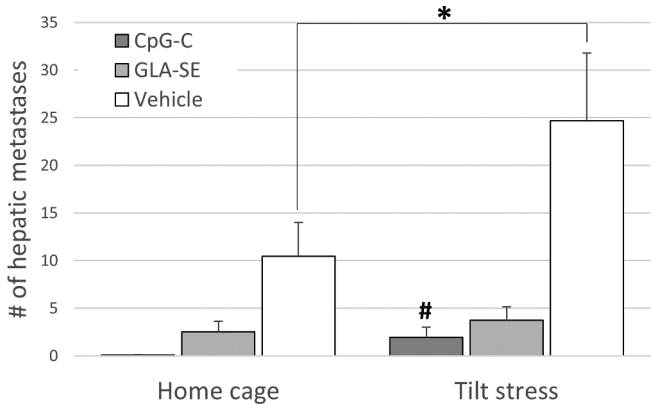


Fig. 5. Male and female Balb/C mice were subjected or not to the tilt-light stress paradigm for a period of 16 hours, and 2 hours following stress initiation, were injected with either GLA-SE, CpG-C, or saline (controls) (n = 13–15 per group). Immediately at the end of stress, all mice underwent abdominal surgery, and were injected with CT26 tumor cells. Hepatic metastases were enumerated 3 weeks later. Stress prior to surgery significantly elevated the number of hepatic metastases (*). GLA-SE and CpG-C immune stimulation reduced the number of metastases under no-stress condition, and this reduction was less effective under stress condition (#).

RU-486 to block the deleterious effects of stress on GLA immune stimulation, we here aimed to assess the direct *in vitro* effects of corticosterone on the efficacy of GLA in stimulating leukocytes to induce cytokine responses. Pooled blood from naïve (non-stressed) F344 male rats was washed to discard endogenous cytokines. Corticosterone was added to blood aliquots, reaching final concentrations of 10^{-7}M, 10^{-8}M, 10^{-9}M, or 0 (saline control). Two hours later (as in the *in vivo* studies), 100 ng GLA/ml (or saline) was added to the blood aliquots, and the samples were further incubated overnight, before supernatants were harvested and assayed for IL-12 levels.

GLA significantly elevated IL-12 levels ($F(1,12) = 42.256$, $p < 0.01$), and corticosterone in the concentration of 10^{-7}M (stress physiological levels) completely abolished this elevation ($p < 0.05$), without affecting the release of IL-12 in the absence of GLA (Fig. 4).

3.1.5. Exp. 5: GLA-SE immune stimulation in rats increase corticosterone levels in a time-dependent manner

To assess possible effects of GLA-SE immune stimulation on levels of stress hormones, we collected peripheral blood from the heart of male F344 rats 2, 4, 6 and 12 h following subcutaneous administration of 10 μg of the TLR-4 agonist GLA-SE (For control levels, samples from animals were collected 4 and 12 h following saline administration). To overcome circadian rhythm in corticosterone levels, all samples were collected simultaneously at the end of the light phase. A significant elevation in corticosterone levels from ~100 ng/ml (SEM = 35) in the control groups to ~250 ng/ml (SEM = 65) was evident only at the 6 and 12 h time points ($(F(4,24) = 3.294$, $p < 0.05$ for both) (see Supplementary Fig. 1).

3.1.6. Exp. 6: Tilt-light stress prior to surgery in mice worsens post-operative resistance to metastases and reduce the efficacy of immune stimulation

Here we aimed at assessing the effects of pre-operative stress and immune stimulation in an additional tumor model and additional immune-stimulating agent, and examine long-term outcomes (metastatic growth). Male and female Balb/C mice were subjected to the tilt-light stress paradigm for a period of 16 h, or remained in their home cages at the vivarium (controls). Two hours following stress initiation, animals in each group were injected with either 10 μg of the TLR-4 agonist GLA-SE, 100 μg of the TLR-9 agonist CpG-C, or saline (controls). Immediately at the end of the stress session all mice underwent abdominal surgery, and were injected with CT26 tumor cells. Hepatic metastases were enumerated 3 weeks later.

Stress prior to surgery significantly elevated the number of hepatic metastases ($F(1,75) = 4.369$, $p < 0.05$). GLA-SE and CpG-C immune stimulation reduced the number of metastases under no-stress condition ($p < 0.01$ for both), and this reduction was less effective under stress condition $p < 0.05$ for CpG-C between no-stress and stress conditions) (Fig. 5).

3.1.7. Exp. 7: The tilt-light stress paradigm in rats prior to surgery worsen post-operative outcomes through glucocorticoids-dependent mechanisms

As psychological stress precede surgery in cancer patients, we here aimed at assessing the effects of pre-operative stress on the effects of surgery. Male F344 Rats were subjected to the tilt-light stress paradigm for a period of 16 h, or remained in their home cages at the vivarium (controls). To counteract the potential effects of this pre-operative stress, 30 min prior to stress initiation half of the animals in each group received the glucocorticoid receptor antagonist RU-486. Laparotomy was conducted in half of the animals in each group immediately following the 16 h stress session, followed by the inoculation of radiolabeled MADB106 for assessment of lung tumor retention (LTR). Surgery alone elevated LTR levels ($F(1,62) = 87.456$, $p < 0.01$), and this elevation was significantly higher when animals were subjected to pre-operative stress ($p < 0.05$). RU-486 administration prior to stress initiation completely abolished this effect, lowering LTR levels to those of the surgery alone group. Neither stress nor RU-486 had any effect when not followed by surgery (Fig. 6).

3.1.8. Exp. 8: An integrated immunostimulatory, anti-stress, and anti-inflammatory treatment completely abolished the deleterious effects of pre-operative stress and of surgery

Following the establishment of the deleterious effects of pre-operative stress on (i) post-operative outcomes and on (ii) the efficacy of immune stimulation, and the attenuation of these effects by RU-486, we aimed at testing an integrated perioperative treatment in the context of pre-operative stress and surgery. This integrated treatment was conceived to address the entire clinical context of cancer surgery, where we hope such integrated approaches would be studied clinically.

Both the pre-operative stress and the impact of surgery were simulated, alone or together. The treatments included pre-operative immune stimulation with GLA-SE, with or without blockade of the effects of pre-operative stress by RU-486. Based on previous studies in mice and rats, the integrated approach included the blockade of a stress-inflammatory response to surgery, through the combine use of the β-adrenergic blocker, propranolol, and the COX-2 inhibitor, etodolac,

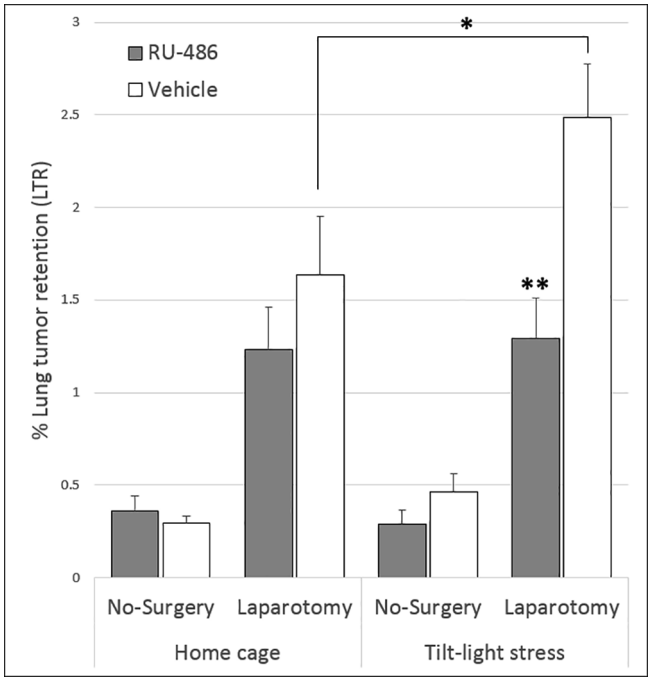


Fig. 6. The effects of pre-operative stress on the outcomes of surgery. Rats were subjected or not to 16 hours of the tilt-light stress, followed by laparotomy or no-surgery (n = 9–11 per group). MADB106 tumor cells were administered immediately following surgery and lungs were harvested 24 hours later for the assessment of lung tumor retention (LTR). Surgery elevated LTR levels, and pre-operative stress worsened this effect (*). Administration of the glucocorticoid receptor antagonist RU-486 prior to stress initiation completely abolished the effects of pre-operative stress on post-surgery outcomes (**). Neither stress nor RU-486 had any effect when not followed by surgery.

30 min prior to laparotomy (Glasner et al., 2010). MADB106 tumor cells were administered immediately following surgery, and lungs were harvested for LTR assessment 24 h later. A schematic presentation of the timing of each drug treatment and stress manipulation is indicated in Fig. 7A. Of 24 potential experimental conditions in a complete design (Fig. 7B), we employed 12 conditions that reflect the most clinically relevant settings: (i) No pre- or peri-operative pharmacological intervention (the current clinical situation); (ii) blockade of stress-inflammatory response to surgery alone (aiming only at the surgical effects); (iii) pre-operative stress blockade and immune-stimulation (aiming at the pre-surgical stress effects); and (iv) complete pre- and peri-operative intervention (aiming at the entire pre-operative and surgical-related effects). Each of these conditions was compared to their respective controls, along with additional required controls for the validation of the surgical effects.

Stress and surgery have each significantly elevated LTR levels ($p < 0.01$ for both). When stress preceded surgery, LTR levels were significantly higher than those of each alone ($p < 0.01$). The combined treatment of RU-486, GLA-SE, and propranolol + etodolac completely abolished each of these three effects ($p < 0.01$), reducing LTR levels to those of naïve animals (Fig. 7C). In the 6 groups that underwent both pre-operative stress and surgery, each of the partial treatments significantly lowered LTR levels ($p < 0.01$), but the full treatment was significantly more effective, and the only one that reduced LTR levels to those of naïve animals.

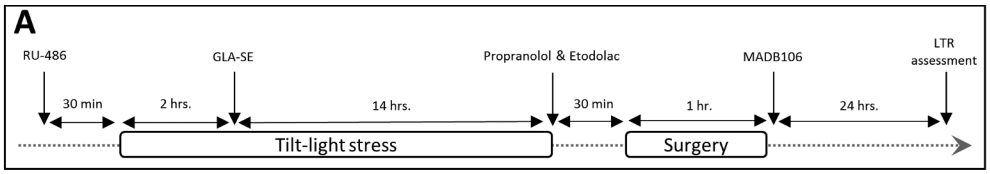
4. Discussion

In the current study, we examined the effects of pre-operative psychological stress and of the surgical trauma (alone or together) on the efficacy of pre-operative immune stimulation and on post-operative outcomes, employing the TLR-4 agonist GLA-SE or the TLR-9 agonist CpG-C in conjunction with several behavioral stress paradigms. GLA-SE and CpG-C were used as both were shown to bear anti-metastatic effects, while having minimal to no adverse effects in animal and clinical studies, thus constituting candidates for clinical perioperative use (Goldfarb et al., 2011; Matzner et al., 2016; Behzad et al., 2012; Krieg, 2012; Vignali and Kuchroo, 2012). Various outcomes were examined, including *in vitro* cytokine production, circulating leukocyte composition, corticosterone and IL-12 plasma levels, as well as resistance to experimental metastasis of the mammary adenocarcinoma MADB106 in rats and the colorectal carcinoma CT-26 in mice.

We found that different stress paradigms (with psychological and physiological aspects) can abolish or reduce the efficacy of immune stimulation. Also, through a glucocorticoid-dependent mechanism, pre-operative prolonged stress has worsened post-operative outcomes, and abolished the efficacy of pre-operative immune stimulation. An integrated perioperative intervention, based on (i) antagonizing the impacts of glucocorticoids before surgery, (ii) activating anti-metastatic immunity, and (iii) blocking excessive operative and post-operative adrenergic and inflammatory responses, successfully abolished the deleterious effects of pre-operative psychological stress (tilt-light paradigm) and of surgery on immune stimulation and on post-operative resistance to tumor metastasis, reaching or exceeding resistant levels evident in naïve non-stressed non-operated animals. As discussed below, such an integrated approach may bear clinical implications in cancer patients, circumventing some deleterious processes that drive post-operative metastatic disease.

The perioperative period in cancer patients is complex and involves several unavoidable processes. Psychological distress often commences with diagnosis, days or weeks before surgery, and its levels are rising as surgery approaches (Montgomery et al., 2010; Neeman et al., 2012). As shown herein, several stress paradigms can each alone harm the efficacy of immune stimulation, depending on known and unknown characteristics of the stress paradigm, including activation of the HPA axis that can be reduced pre-operatively and restore immune activation. Also, in the clinical setting of surgery, preceding distress may worsen post-operative resistance to metastasis, as evident herein in several measures and animal models of metastasis. Importantly, some of these effects were also mitigated herein through pre-operative blockade of the glucocorticoid pathway.

Surgery is a cornerstone intervention in the curative treatment of solid tumors. Unfortunately, stress and inflammatory responses, due to having cancer and the surgical trauma, harm host anti-metastatic immunity and drive the malignant tissue and its microenvironment toward becoming pro-metastatic. In our previous studies we have shown that these effects are at least partially mediated through the intra- and post-operative secretion of CAs and prostaglandins (Glasner et al., 2010), as was also shown herein, where the administration of the β-adrenergic receptor antagonist propranolol and the COX-2 inhibitor etodolac 30 min prior to surgery partially attenuated its deleterious effects on immune competence and resistance to metastasis (Neeman and Ben-Eliyahu, 2013; Horowitz, 2015; Holte and Kehlet, 2002). Interestingly, the blockade of glucocorticoid response just before surgery was markedly less effective (Rosenne et al., 2014). However, psychological distress, here and in the clinical setting, commence hours or days before surgery. Therefore, it is expected that the blockade of the adrenergic-inflammatory response initiated with surgery would not suffice to eliminate the effects of such prolonged pre-operative stress on pre-operative immune stimulation, whereas the addition of the glucocorticoid receptor antagonist RU-486 prior to pre-operative stress and immune stimulation would result in improved effects, as was indeed evident herein. Importantly, the integrated use of all three approaches ((i) blockade of pre-operative stress by RU-486, (ii) pre-operative immune activation by GLA-SE, and (iii) blockade of the stress-inflammatory response to surgery by propranolol and etodolac) improved resistance to metastases two-fold higher than each of the interventions



B

Pre-surgical treatment		Surgery		No Surgery	
		Propranolol & Etodolac	Vehicle	Propranolol & Etodolac	Vehicle
GLA-SE	Tilt-light stress				
	Home cage				
RU-486 & GLA-SE	Tilt-light stress				
	Home cage				
Vehicle	Tilt-light stress				
	Home cage				

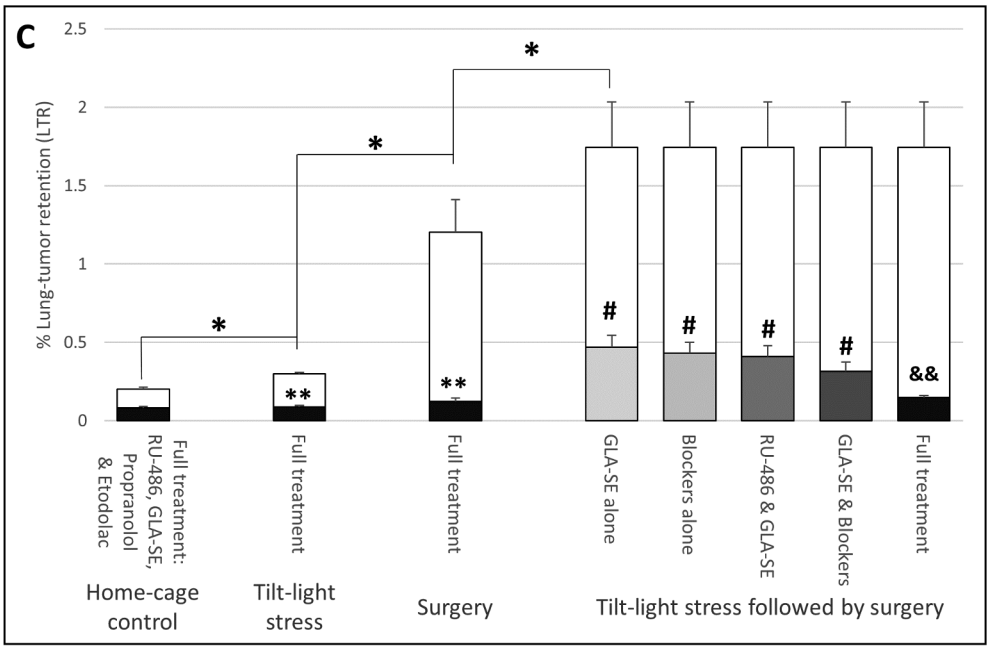


Fig. 7. Simulation of the entire clinical setting of the perioperative period, and the beneficial effects of different treatments. (A) A schematic presentation of the timing of each drug treatment and stress manipulation. (B) Of the 24 possible experimental groups, we studied 12 conditions that best reflect the clinical settings and their respective controls (highlighted). (C) The effects of pre-operative stress and/or surgery are depicted by the white empty bars, and the beneficial reduction of each treatment is portrayed by different grayscale within this bar (n = 8–17 per group). Compared to home-cage control, tilt-light stress and surgery each significantly elevated LTR levels (*, $p < 0.01$ for each), and together had a significant additive effect (*) (white bars). The integrated treatment completely abolished the effects of stress and of surgery when employed alone (**). In the 6 groups that underwent both pre-operative stress and surgery, each of the three elements of the integrated treatment studied significantly lowered LTR levels, which was still significantly higher than LTR levels in treated home-cage controls (#). The integrated treatment was significantly more effective than any partial treatment (&&) (add a sign to the figure), and the only one that reduced LTR levels to those evident in treated home-cage controls.

alone or any combination of the two interventions, stressing the potential benefits of employing all of the perioperative approaches described herein.

However, the use of glucocorticoid receptor antagonists in proximity to surgery might be clinically challenging due to the importance of glucocorticoids in various host anti-inflammatory and immune-modulating mechanisms involved in post-surgical recovery (Larson et al., 2000). Thus, the use of pharmacological modulation of the HPA axis would become less feasible as surgery approaches, and it is important to find substitutes for reducing pre-operative distress levels. Indeed, several researchers have reported that addressing pre-operative distress by employing group treatments before surgery for discussing concerns (Kuchler et al., 2007; Cohen et al., 2011) or learning coping skills and relaxation techniques for stress management (such as diaphragmatic breathing and guided imagery) (Huang et al., 2008), led to elevation in post-operative NK cell cytotoxicity (Huang et al., 2008), blockade of IFN-γ suppression (Kuchler et al., 2007), and, in a single study, also to a significant improvement in 10-year overall survival rates (Cohen et al., 2011).

Numerous pre-clinical studies employing immune stimulation have shown promising results, but these have rarely been translated into significant clinical improvements (Mak et al., 2014). Here, for the first time while simulating the perioperative context, we identify pre-surgical psychological stress as a major component in jeopardizing the efficacy of immune stimulation. Given that the perioperative period is non-proportionately significant in determining long-term cancer outcomes (Horowitz, 2015), and that thus immune stimulation during this critical period might be advantageous over later use, it is important to address pre-surgical stress to improve pre- or perioperative immune stimulation, as implemented herein through the use of RU-486. Interestingly, various immune stimulating approaches activate stress responses (Denicoff et al., 1989; Baker et al., 1989; Haldar et al., 2018), as

is also evident herein with GLA-SE, which might serve as a negative regulator for over immune activation in such unnatural settings, and in natural settings. Therefore, inhibition of HPA axis responses should be considered cautiously.

Last, we have recently reported encouraging findings from a phase II biomarker clinical trials employing a perioperative intervention with the β-adrenergic receptor antagonist propranolol and the COX-2 inhibitor etodolac (used in the current study) initiated five days prior to surgery (Shaashua et al., 2017; Haldar et al., 2017; 2018). This pharmacological intervention reduced pre-operative stress-inflammatory responses, including the complete reversal of pre-operative elevated IL-6 and CRP levels. In the excised tumor this intervention improved molecular markers of immunity, inflammation, and several pro-metastatic indices. Thus, this approach also seems efficient in reducing deleterious effects of pre-operative events. We believe that employing additional pharmacological and/or psychological interventions for reducing pre-operative stress, and simultaneously initiating pre-operative immune stimulation, would further benefit this approach in cancer patients.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbi.2019.03.005.

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