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Perioperative hypothermia and stress jeopardize antimetastatic immunity and TLR-9 immune activation: potential mediating mechanisms (experimental studies)

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Background: The perioperative period often involves stress responses and surgery-induced hypothermia, which were suggested to hinder antimetastatic immunity and promote cancer metastasis. During this critical period, immunotherapies are rarely used, given contraindications to surgery. However, recent preclinical studies support the feasibility of perioperative TLR-9 activation using CpG-C.

Materials and methods: Herein, we employed hypothermic-stress and normothermic-stress paradigms to assess their impact on perioperative CpG-C immune stimulation and resistance to experimental hepatic metastasis of CT26 colorectal cancer in BALB/c mice.

Results: Perioperative hypothermic wet-cage stress markedly abrogated CpG-C-induced increase in plasma IL-12 levels, a persistent deleterious effect across different CpG-C doses and administration routes. These effects were not attenuated by blocking glucocorticoids, adrenergic, or opioid signaling, nor by adrenalectomy, suggesting a direct immunosuppressive impact of hypothermia on immunocytes. Indeed, normothermic wet-cage stress, which induced a similar corticosterone response, caused significantly less deleterious effects on IL-12 levels, hepatic NK cell maturation and cytotoxicity, and CT26 metastasis. Additionally, in-vitro exposure of PBMCs to 33°C markedly decreased CpG-C-induced IL-12 production. Last, two normothermic stress paradigms, tilt&light and restraint, did not jeopardize CpG-C-induced IL-12 response nor resistance to CT26 metastases. Interestingly, attenuating glucocorticoid signaling under tilt&light conditions improved CpG-C efficacy.

Conclusions: Overall, these findings suggest that perioperative hypothermic stress can jeopardize antimetastatic immunity and resistance to metastasis, and prevent perioperative response to immune stimulation and its beneficial antimetastatic impacts, effects that are not mediated through classical neuroendocrine stress responses, but potentially through direct hypothermic impact on leukocytes. These findings may have clinical implications in operated cancer patients, many of whom suffer hypothermic stress.

Keywords: metastases, perioperative hypothermia, perioperative immunotherapy, stress, Toll-like receptor 9

Introduction

The immediate perioperative period (IPP), days before and after surgery, is known to profoundly influence long-term cancer outcomes through numerous physiological responses that (i) hinder antimetastatic immunity and/or (ii) directly affect the malignant tissue[1]. Accordingly, effective anticancer immunotherapy during this short timeframe was suggested as a prophylactic means to improve long-term cancer outcomes[2,3]. However, immunotherapies are rarely studied during the short perioperative period, given known and speculated contraindications to surgery, and may be rendered ineffective given the prevalent phenomenon of perioperative immune suppression[2–5].

HIGHLIGHTS

- Perioperative mild hypothermic stress jeopardizes antimetastatic immunity, immune activation, and resistance to cancer metastasis.
- Direct hypothermic impact on immunocytes, rather than HPA, SNS, or opioid signaling, is sufficient to mediate the deleterious in-vivo impact of hypothermic stress conditions.
- Perioperative immunotherapy, using the TLR-9 agonist CpG-C, is highly effective in preventing hepatic metastases, under no stress and normothermic stress conditions.

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Despite limited preclinical research, few antimetastatic immunotherapies were shown to be effective when administered during the IPP, reducing metastasis and improving long-term cancer outcomes[6–10]. However, most of these studies did not adequately simulate the perioperative setting, or employed immunodeficient animal models, and thus their clinical relevance is limited[11]. Clinical studies on antimetastatic immunotherapies during the IPP are scarce[2,3], and published clinical trials are typically small, low-phase, often single-arm, and commonly assess safety profiles and biomarkers rather than long-term cancer outcomes. Furthermore, these studies generally do not systematically address critical aspects of perioperative treatments, such as timing relative to surgery, duration of therapy, and drug combinations, which could mitigate adverse effects manifesting during the IPP. Nonetheless, these studies indicate tolerable safety profiles and promising biomarker results, with few randomized controlled trials assessing long-term cancer outcomes, but mostly underpowered for survival assessments. Specifically, administration of IL-2 during the IPP improved long-term cancer outcomes, including 5-year disease-free and overall survival, in pancreatic, renal cell, and colorectal cancer patients, with no serious adverse events[12–14]. Additionally, immune checkpoint inhibitors used during the IPP were shown to (i) demonstrate tolerable safety profiles[15,16], (ii) increase plasma IL-12 and tumor IFN-gamma levels[16], and (iii) promote early expansion of tumor-specific T cell subsets[15]. Overall, additional research is warranted to study the safety and efficacy of immunotherapy during the IPP to harness the immune system for improved long-term cancer outcomes.

In the last decade, Toll-like receptor (TLR) activation has shown potential as an antimetastatic immune-stimulating approach[17]. TLR-9 agonists are studied as monotherapy or in conjunction with other types of immune-, chemo- and/or radiotherapies against various cancer types[18], and some have reached clinical testing[18] and FDA approval[19]. The TLR-9 agonist CpG-oligodeoxynucleotide 2395 (CpG-C) was shown effective while exerting minimal adverse effects[20], thus suggested as a potential perioperative immunotherapy[2]. Specifically, CpG-C was shown to elevate plasma IL-12 levels[21], and when administered during the IPP to increase NK cytotoxicity, and significantly reduce metastasis in several rodent models[6,9,22,23]. IL-12 is a prominent activator of NK cells[24], which are known to control the development of metastasis[25], and high levels of NK activity are associated with long-term recurrence-free survival in patients harboring various cancers[26,27].

Immunosuppression during and following the IPP is a well-documented and intricate phenomenon[4,5], which may commence while awaiting surgery[28,29]. Cancer patients undergoing surgery are subjected to psychological stress and anxiety[1,30,31], surgical procedures[5], tissue damage[1,5], anesthesia[32,33], hypothermia[34,35], blood loss and/or transfusion[36], and the consequent postoperative resolution of inflammatory processes[1–3,37]. These experiences and physiological responses, which are predominantly characterized by excessive release of catecholamines (CAs) and prostaglandins (PGs), collectively contribute to immunosuppression[2–5], and may also directly accelerate the transformation of minimal residual disease (MRD) into clinically manifested metastatic disease[37,38]. Overall, the IPP encompasses numerous processes that often orchestrate a strong and lasting perioperative immunosuppressive state in cancer patients[2,4,39]. Theoretically, such suppression may accelerate metastasis[1,37]. Importantly, immune-activating agents, such as CpG-C, may counteract these deleterious effects when administered during the IPP[10], potentially improving long-term cancer outcomes[2,3].

Hypothermia is a prevalent side effect of surgery and of anesthetic and analgesic agents, and is defined as a decline in core body temperatures below 36°C[40]. Hypothermia is categorized as mild (36–32°C), moderate (32–28°C), or severe (< 28°C), and even mild hypothermic conditions were shown to have major deleterious effects on patient's health and well-being[40]. Studies have shown that mild hypothermia: (i) increases the incidence of surgical site infection[41]; (ii) escalates intraoperative blood loss and postoperative ischemic myocardial events[42,43]; (iii) hinders CMI, including the suppression of lymphocyte activation and cytokine production[34]; and (iv) prolongs patients' postoperative recovery[44].

In our previous preclinical studies, we have shown that CpG-C can be safely and effectively used during the IPP[6,9,22,23]. Considering the aforementioned deleterious effects of hypothermia and stress, in this study we aimed to assess the efficacy of CpG-C immune stimulation under hypothermic-stress and normothermic-stress conditions, and to study the involvement of specific physiological mechanisms in modulating its efficacy under these conditions. Most cancer patients exhibit perioperative stress-inflammatory responses and/or suffer from perioperative hypothermia. Thus, understanding environmental and physiological factors that jeopardize or improve immunity and perioperative immunotherapy may advance the perioperative use of such approaches and their potential clinical benefits in cancer patients.

Materials and methods

Animals

C57BL/6J olaHSD and BALB/c olaHSD mice (Envigo Laboratories, Israel), 12–16 weeks old, housed four per cage with cotton ball enrichment, and free access to food and water, on a 12:12 light:dark cycle at 22–24°C. Drug administration and tumor inoculation were counterbalanced across groups in each experiment. Housing conditions are regularly monitored by the Institutional Animal Care and Use Committee, which approved all studies described herein (P-12-003, 31/12/2016; 10-18-005, 09/07/2018; 10-18-008, 06/12/2018).

This study has been reported in accordance with the ARRIVE guidelines (Animals in Research: Reporting In Vivo Experiments)[45].

Drugs

CpG-C: CpG-C (Sigma, Israel), a TLR-9 agonist (ODN 2395: 50-TCGTCGTTTTCGGCGCGCGCCG-30), with a phosphorothioate backbone, was dissolved in PBS and administered intraperitoneally (i.p.) at a dose of 50/100 μg per mouse[23].

RU486: RU486 (Sigma, Israel), progesterone and glucocorticoid receptor antagonist, was dissolved in corn oil and administered subcutaneously (s.c.) at a dose of 25 mg/kg.

Propranolol: Propranolol (Sigma, Israel), a non-selective β-adrenergic blocker, was dissolved in a slow-release vehicle (see below) and administered s.c. at a dose of 5 mg/kg.

Slow-release vehicle: an emulsion comprised of four parts PBS, three parts mineral oil (Sigma, Israel), and one part mannide monooleate (Sigma, Israel).

Naltrexone: Naltrexone (Sigma, Israel), a wide-range opioid receptor antagonist, was dissolved in PBS and administrated i.p. at a dose of 2 mg/kg[46].

Tumor cell lines and their maintenance

CT26: murine colon carcinoma cell line chemically-induced undifferentiated carcinoma, syngeneic to the BALB/c strain[47]. Cells were grown in monolayer cultures in complete media 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 1×10^5/ml in PBS-BSA for spleen injection at a volume of 100 µl per animal.

Stress paradigms

Hypothermic wet-cage: Mice were placed in cages filled with room-temperature water to the height of 1 cm, in a fully lighted room and with free access to food and water. This protocol was initiated 2 h after the onset of the dark period.

Normothermic wet-cage: Mice went through the same setup of wet-cage (see above); however, the cages were placed in a heated room (29–30°C) and were filled with heated water (36–37°C).

Tilt&light: Mice cages were placed in a 45° tilted position, in a fully lighted room maintained at 18°C, with free access to food and water, for 12 h. The stress protocol was initiated 3–5 h before the onset of the dark period.

Restraint: Mice were placed in a transparent cylinder with ventilation, with no access to food or water for 8 h. This stress protocol was initiated 3–5 h before the onset of the dark period.

Adrenalectomy and sham operation

This procedure was described elsewhere and adapted herein to mice (1 mg/kg corticosterone per injection to expedite recovery from surgery)[48]. Briefly, mice were anesthetized and both adrenal glands were removed using standard surgical techniques. Sham-operated mice underwent the exact same procedure without removal of the adrenal glands.

CT26 tumor cell intrasplenic inoculation

This procedure was described elsewhere[9]. Briefly, mice were anesthetized and 1×10^4 CT26 tumor cells in 100 µl PBS were injected into the spleen followed by a splenectomy, were the injection not successful, the animal was sacrificed according to ethical constraints.

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 for males and the 25th day for females. 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 bigger than 1 mm in diameter, forming a spherical solid and distinct formation.

Assessment of plasma corticosterone levels

Blood was drawn from the heart into heparinized test tubes. Plasma levels were measured employing ELISA (AssayPro, MO), per the manufacturer's instructions.

Assessment of IL-12 p70 levels using ELISA

IL-12 p70 ELISA kit (eBioscience, Thermo Fischer Scientific, CA) was used to assess IL-12 levels from plasma and supernatants, based on the manufacturer's instructions.

Blood draw and plasma collection

Mice were euthanized with isoflurane, and blood was drawn from the heart, within less than 3 min of approaching the animals, using EDTA-containing syringes (1.8 mg/1ml blood). Blood was then centrifuged for 20 min at 2000*g*, 4°C, for plasma separation, which was collected and stored at – 20°C until assayed for IL-12 and/or CORT levels.

In-vitro CpG-C-induced production of IL-12

Half milliliter of pooled whole blood was washed once with PBS (4-fold dilution, 10 min at 456 g, followed by supernatant removal to restore the original volume) and twice with complete media to discard endogenous IL-12. A 500 µl washed blood aliquot was then added to a well containing 500 µl of complete media with CpG-C, reaching a final concentration of 5 µg CpG-C/ml. Samples were incubated at 100% humidity, 5% CO_2, at 33°C or 37°C for 10 or 20 h. Supernatants were then harvested and stored at – 20°C until assayed for IL-12 levels.

Harvesting of hepatic leukocytes

Mice were sacrificed with an overdose of isoflurane, and hepatic leukocytes were harvested by perfusing the liver with heparinized PBS (30 U/ml), described in detail elsewhere[49].

Assessment of NK cytotoxicity

The standard 4-h 51Cr release assay was used without any cell enrichment procedures, described in detail elsewhere[50].

Flow cytometry

Standard procedures were used to prepare cells for flow cytometry analysis[51]. NK cells were identified as being PE-conjugated anti-mouse NKp46 (clone 195314, R&D), FITC-conjugated CD49b (clone DX5, Peprotech), PC5.5-conjugated CD27 (clone LG3A10, BioLegend), and APC-Alexa Fluor 750-conjugated CD11b (clone M1/70, BioLegend).

Statistical analysis

Prism (version 8.4.2) was used for statistical analysis, and G*power (version 3.1.9.7) software was used for sample size analysis when needed[52]. Levene's *F*-test was used to test homogeneity of variance, and when met, two-way or three-way ANOVA was used, followed by paired Tukey post-hocs to account for multiple comparisons. For data not meeting homogeneity of variance, Welch's corrected unpaired *t*-test for unequal variances was employed to perform $N-1$ (N = number of

experimental groups) independent comparisons. Repeated measures three-way ANOVA was performed, employing the Greenhouse–Geisser adjustment of sphericity, given interconnectedness between dependent variables. Sample size calculations were based on size effects and SD from published and preliminary experiments to ensure 80% power. For instance, the sample sizes for (i) NK cytotoxicity assay (Fig. 4) and (ii) CT26 metastases numbers (Figs. 3 and 6) were calculated based on effect sizes and variance evident in Sorski *et al.*[9]. Specifically, (i) NK cells' cytotoxicity levels and numbers demonstrated an omega-squared of 0.394 ($\omega^2 = \frac{SS_{Treatment} - df_{Treatment} \times MS_{error}}{SS_{Total} + MS_{error}}$), and an effect size of 0.806 ($f_{effect\ size} = \sqrt{\frac{\omega^2}{1-\omega^2}}$), and the required sample size was calculated using G*power to be 4 per group (4–6 per group was chosen herein), (ii) CT26 metastases numbers demonstrated a weighted pooled SD of 43.6 ($SD^*_{Pooled} = \sqrt{\frac{(n_1-1)\times SD_1^2 + (n_2-1)\times SD_2^2}{n_1+n_2-2}}$), and 'Hedges' g' effect size of 2.223 ($g_{effect\ size} = \frac{M1-M2}{SD^*_{Pooled}}$), and the required sample size was calculated using G*power to be 3–4 per group. However, we employed 10–11 per group to enable the recognition of much smaller effect sizes in the context of stress. Omega-squared, pooled SD, and Hedges' g were chosen as they are considered less biased estimators for effect size, especially when the 'n' of the groups are unequal. All statistical tests were two-tailed, and P values smaller than 5% were considered significant.

Results

Hypothermic wet-cage stress abolished CpG-C-induced increase in plasma IL-12 levels

We first tested whether the hypothermic wet-cage (HWC – see Methods) stress paradigm affects CpG-C-induced increase in plasma IL-12 levels, when administered in different doses and routes of administration in two separate experiments. C57BL/6J mice were subjected to 8 h of HWC or served as home cage control (HCC). Two hours after the onset of stress, in the first experiment, mice were administered with 100 µg CpG-C, 200 µg CpG-C, or with vehicle (Fig. 1A, $n = 30$); and in the second experiment, were administered with 100 µg CpG-C or with the vehicle either s.c. or i.p. (Fig. 1B, $n = 44$). Plasma IL-12 levels were assessed at the end of stress.

In both experiments, CpG-C administration significantly increased plasma IL-12 levels (main effects, Fig. 1A, $F(2,24) = 12.91$, $P = 0.0002$; Fig. 1B, $F(2,38) = 8.193$, $P = 0.011$) and HWC significantly reduced this effect (A) (main effects, $F(1,24) = 31.65$, $P < 0.0001$), (B) ($F(1,38) = 24.29$, $P < 0.0001$). These findings indicate that CpG-C was significantly less effective in elevating IL-12 levels under hypothermic stress conditions at all doses and administration routes studied.

Beta-adrenergic, glucocorticoid, or opioid signaling did not mediate the effects of hypothermic wet-cage stress on CpG-C-induced increase in plasma IL-12 levels

As HWC abolished CpG-C-induced IL-12 plasma levels, we tested whether beta-adrenergic, opioid, or glucocorticoid signaling are involved in mediating the effects of HWC through blockade of each receptor system (Fig. 2A–C) or by performing adrenalectomy (ADX – Fig. 2D, see Methods). To this end, in four different experiments, C57BL/6J mice were subjected to 8 h of HWC, or served as HCC. In experiments A–C, 30 min prior to the onset of stress, mice were administered with propranolol (Fig. 2A, 5 mg/kg, s.c., $n = 57$); naltrexone (Fig. 2B, 2 mg/kg, s.c., $n = 30$); or with RU486 (Fig. 2C, 25 mg/kg, s.c., $n = 75$), or injected with their respective vehicles. In experiment D, mice underwent ADX or sham surgery 4 weeks prior to stress (Fig. 2D, $n = 91$). In all four experiments, 2 h after the onset of stress, mice were administered with CpG-C (100 µg, i.p.) or a vehicle. Plasma IL-12 (Fig. 2) levels were assessed at the end of stress.

In all four experiments, CpG-C significantly increased plasma IL-12 levels (main effects: Fig. 2A, $F(1,49) = 83.31$, $P < 0.0001$; Fig. 2B, $F(1,24) = 87.89$, $P < 0.0001$; Fig. 2C, $F(1,67) = 123.4$, $P < 0.0001$; and Fig. 2D, $F(1,83) = 59.75$, $P < 0.0001$) and HWC markedly decreased plasma IL-12 levels (main effects: Fig. 2A, $F(1,49) = 22.53$, $P < 0.0001$; Fig. 2B, $F(1,24) = 51.00$, $P < 0.0001$; Fig. 2C$F(1,67) = 81.73$, $P < 0.0001$; and Fig. 2D$F(1,83) = 35.08$, $P < 0.0001$). None of the antagonists significantly altered plasma levels of IL-12, including under HWC conditions. Interestingly,

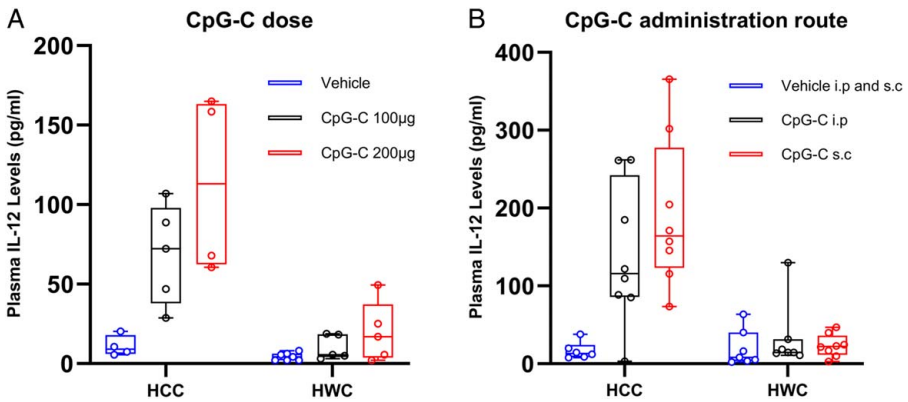

Figure 1. Mice were subjected or not to 8 h of wet-cage stress and were administered with CpG-C or vehicle 2 h after the onset of stress. Plasma IL-12 was assessed at the end of stress. CpG-C elevated IL-12 levels in both experiments (A, $n = 30$, $n = 4$–7 per group, $P = 0.0002$; B, $n = 44$, $n = 6$–8 per group, $P = 0.011$), and hypothermic wet-cage (HWC) stress abolished the CpG-C-induced increase in IL-12 (A, $n = 30$, $P < 0.0001$; B, $n = 44$, $P < 0.0001$). Boxes represent the second and third quartiles, and whiskers show min and max values.

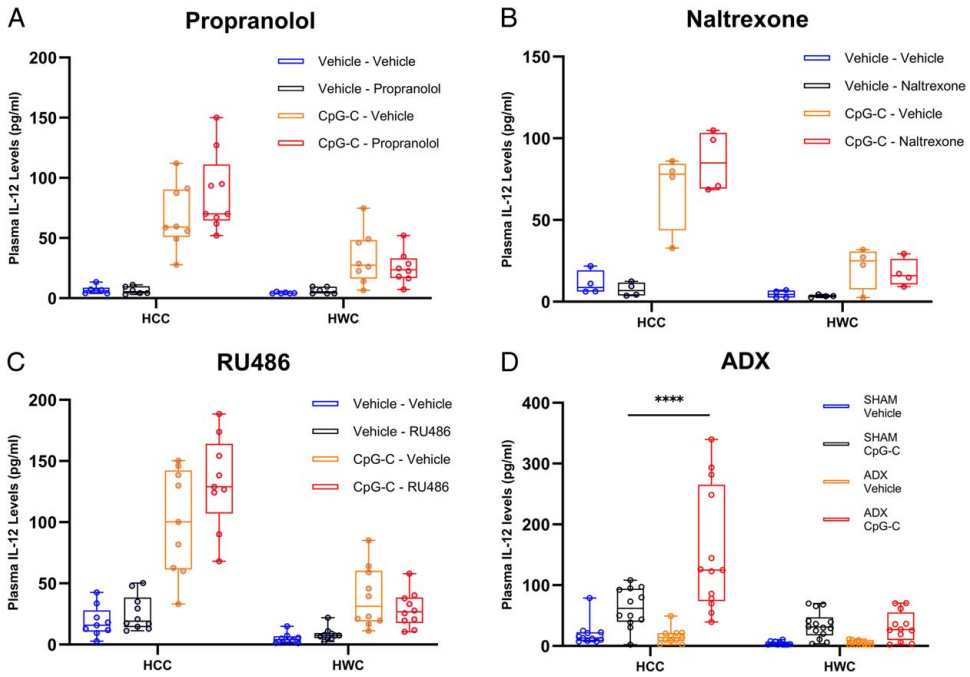

Figure 2. In experiments A–C, 30 min prior to the onset of stress, mice were administered with propranolol (A), RU486 (B), or naltrexone (C), or with vehicle. In experiment D, mice were adrenalectomized (ADX) or underwent sham surgery 4 weeks before stress. In all four experiments, mice were subjected not to 8 h of hypothermic wet-cage (HWC) stress and administered with CpG-C or vehicle 2 h after the onset of stress. IL-12 plasma levels were assessed at the end of stress. CpG-C significantly elevated plasma IL-12 levels (A, $n=57$, $n=6$–9 per group; B, $n=32$, $n=4$ per group; C, $n=75$, $n=8$–10 per group, $P<0.0001$; D, $n=91$, $n=11$–14, $P<0.0001$) and wet-cage markedly attenuated CpG-C-induced increase in plasma IL-12 (A, $P<0.0001$; B, $P<0.0001$; C, $P<0.0001$; and D, $P<0.0001$). Adrenalectomy improved CpG-C-induced IL-12 levels, but only under HCC conditions (****$P<0.0001$). Boxes represent the second and third quartiles, and whiskers show min and max values.

ADX significantly modulated plasma IL-12 levels compared to sham operation, but only in nonstressed animals administered with CpG-C ($P<0.0001$). Thus, it appears that none of these three neuroendocrine mechanisms mediate the IL-12-reducing effects of hypothermic stress.

Hypothermic and normothermic wet-cage stressors differentially affected core body temperatures, plasma IL-12 levels, and CT26 metastasis, but not corticosterone levels

To start elucidating the specific impact of hypothermia in HWC, we developed a normothermic counterpart variation (NWC – see Methods) and conducted three experiments in which we compared the effects of the two paradigms on the efficacy of CpG-C.

In experiment 1 (Fig. 3A, $n=45$), BALB/c mice were subjected to 8 h of HWC or NWC. Mice core temperatures were measured at the onset of stress and 4 and 8 h after its initiation. HWC decreased mice core body temperatures to nearly 33°C (main effect, Fig. 3A, $F(1,13)=201.328$, $P<0.0001$). Additionally, HWC significantly decreased core temperatures at 4 h ($P=0.0003$) and 8 h ($P<0.0001$) compared to NWC, which did not affect core body temperatures.

In experiment 2 ($n=43$), we compared the impact of the two paradigms on plasma corticosterone (Fig. 3B) and IL-12 (Fig. 3C) levels. In experiment 3 ($n=67$), we compared the impact of the two paradigms on CT26 metastasis (Fig. 3D). In both experiments, 2 h after the onset of stress, BALB/c mice were administered with CpG-C (100 μg, i.p.) or vehicle. In experiment 2, plasma IL-12 and corticosterone levels were assessed at the end of stress. In experiment 3, mice underwent CT26 tumor cell inoculation (see Methods) at stress cessation, and 20 days later hepatic metastases were enumerated. Both HWC and NWC significantly elevated corticosterone levels (main effect, Fig. 3B, $F(2,36)=73.09$, $P<0.0001$) in a similar manner (no significant difference between the two paradigms $P=0.6042$) and CpG-C did not affect corticosterone levels. CpG-C administration significantly (i) increased IL-12 levels (Fig. 3C, $F(1,37)=100.1$, $P<0.0001$) and (ii) markedly reduced the number of hepatic metastases (Fig. 3D, $F(1,61)=135.1$, $P<0.0001$). The stress paradigms significantly (i) decreased plasma IL-12 levels (Fig. 3C, $F(2,37)=23.02$, $P<0.0001$) and (ii) increased the number of metastases (Fig. 3D, $F(2,61)=13.44$, $P<0.0001$). Most importantly, in mice that were administered with CpG-C, NWC markedly (i) decreased IL-12 levels (Fig. 3C, $Wt(8.681)=3.592$, $P<0.0001$) and (ii) significantly increased the number of metastases (Fig. 3D, $Wt(10.75)=2.989$, $P=0.0126$) compared to HCC, and HWC further reduced plasma IL-12 levels (Fig. 3C, $Wt(11.81)=3.138$, $P=0.0087$) and further increased metastases (Fig. 3D, $Wt(12.06)=3.599$, $P=0.0036$) compared to NWC. Overall, while the HWC and NWC stress paradigms caused a very similar corticosterone response, the HWC paradigm had significantly more deleterious effects on IL-12 and metastasis than the NWC paradigm.

Hypothermic and normothermic wet-cage stress paradigms differentially affected CpG-C-induced liver NK cell maturation and cytotoxicity

Our previous studies indicated that CpG-C reduces the development of CT26 metastases through increasing hepatic NK cell numbers and cytotoxicity[49]. Therefore, we compared HWC to NWC with respect to their impact on hepatic NK cells. To this

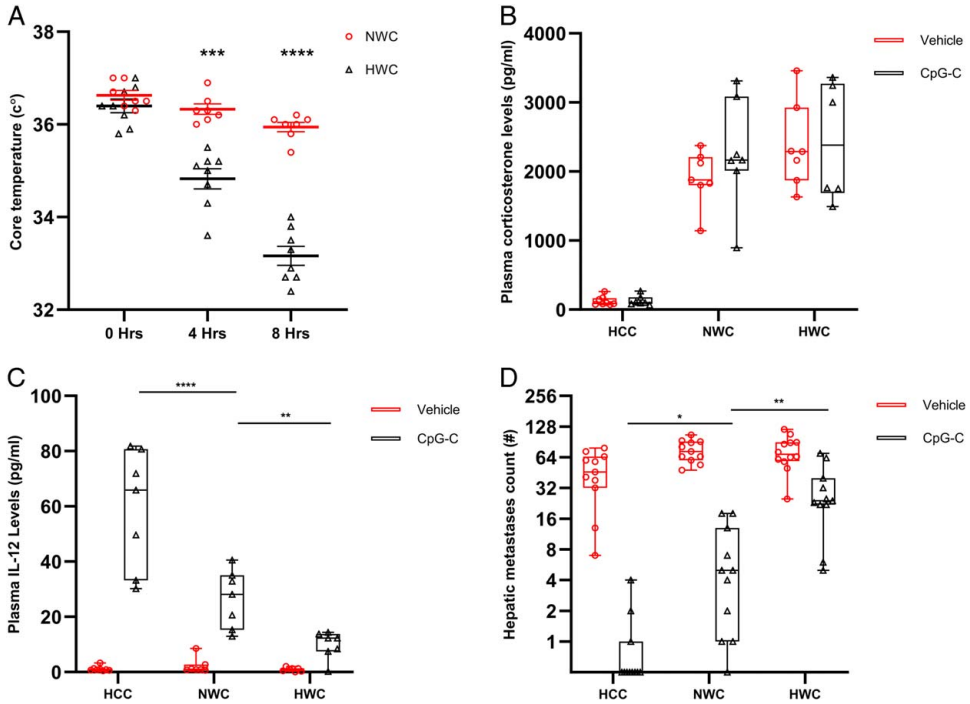

Figure 3. Comparison between the hypothermic and normothermic stress paradigms. (A) Mice were subjected to hypothermic (HWC) or normothermic (NWC) wet-cage stress, and core temperatures were measured at 0, 4, and 8 h following stress initiation. (B–D) Mice were subjected to 8 h of HWC, NWC, or served as HCC, and were administered with CpG-C or vehicle 2 h after the onset of stress. Visible hepatic metastases were counted 20 days later for males and 25 days later for females. (A) HWC significantly reduced core body temperatures at 4 h ($n = 15$, $n = 7$–8 per group, $P = 0.003$) and at 8 h ($P < 0.0001$) compared to NWC. (B) Both HWC and NWC significantly elevated plasma corticosterone levels to a similar degree (main effect – $n = 43$, $n = 7$–8 per group, $P < 0.0001$, Tukey – $P = 0.6024$, $P = 0.9935$). (C) CpG-C significantly increased plasma IL-12 levels (main effect – $n = 43$, $n = 7$–8 per group, $P < 0.0001$), and in animals administered with CpG-C, NWC decreased plasma IL-12 levels compared to HCC ($P < 0.0001$), and HWC decreased it significantly more ($P = 0.0087$). Similarly, CpG-C significantly reduced the number of hepatic metastases (D) (main effect – $n = 67$, $n = 11$–12 per group, $P < 0.0001$), and in animals administered with CpG-C NWC increased the number of hepatic metastases compared to HCC ($P = 0.0126$), and HWC elevated this number significantly more ($P = 0.0036$) (*$P < 0.05$; **$P < 0.001$, ***$P < 0.001$; ****$P < 0.0001$). (A) Graphs represent mean ± SEM. (B–D) Boxes represent the second and third quartiles, and whiskers show min and max values.

end, BALB/c mice ($n = 31$) were subjected to 8 h of HWC or NWC, or served as HCC. Two hours after the onset of stress, mice were administered with CpG-C (100 μg, i.p.) or vehicle. Hepatic leukocytes harvested (see Methods) at the end of stress, and studied for maturation and numbers using flow cytometry and assessed for NK cells cytotoxicity (see Methods).

CpG-C administration markedly (i) elevated NK cells cytotoxicity (main effect, Fig. 4B, $F(1,25) = 32.16$, $P < 0.0001$); (ii) increased NK cell numbers (main effect, Fig. 4C, $F(1,24) = 11.17$, $P = 0.0027$); and (iii) increased percentages of mature NK cells ($CD11b^+$ $CD27^-$) of the entire NK cell population (main effect, Fig. 4D, $F(1,24) = 12.36$, $P = 0.0018$). Both stress paradigms significantly (i) reduced NK cells cytotoxicity (main effect, Fig. 4B, $F(2,25) = 3.954$, $P = 0.0322$); (ii) reduced NK cell numbers (main effect, Fig. 4C, $F(2,24) = 3.468$, $P = 0.0475$) and (ii) decreased the percentage of mature NK cells (main effect, Fig. 4D, $F(2,24) = 6.736$, $P = 0.0048$). Most importantly, in animals that were administered with CpG-C, HWC but not NWC significantly reduced NK cells cytotoxicity (Fig. 4B, Tukey, $P = 0.033$) and numbers (Fig. 4C, $Wt(6.608) = 2.753$, $P = 0.03$) compared to HCC. Additionally, both HWC and NWC significantly reduced the percentage of mature NK cells compared to HCC (Fig. 4D, $Wt(5.006) = 2.963$, $P = 0.031$; $Wt(3.64) = 3.557$, $P = 0.027$).

In-vitro PBMCs subjected to 33°C exhibited a significantly attenuated CpG-C-induced IL-12 production compared to 37°C

To assess the direct effects of hypothermic conditions on PBMCs *in vitro* (see Methods), PBMCs were incubated with CpG-C or not, for 10 or 20 h at 33°C or 37°C (Fig. 5). Supernatants were collected in duplicates from each well at the end of each incubation period. Hypothermic conditions significantly decreased ($F(1,8) = 580.367$, $P < 0.0001$) the production of IL-12 by PBMCs, and CpG-C elevated it ($F(1,8) = 3470.541$, $P < 0.0001$). Additionally, hypothermic conditions attenuated CpG-C-induced elevation of IL-12 at 10 h ($P < 0.0001$) and at 20 h ($P < 0.0001$). These results indicate that in-vitro IL-12 production by leukocytes, with or without CpG-C is modulated by temperature. Additionally, these findings suggest that the in-vivo effects of hypothermic stress we found in the previous experiments are at least partially mediated by direct effects of temperature on leukocyte activities.

Restraint and tilt&light normothermic stress paradigms did not affect CpG-C-induced elevation of plasma IL-12 levels

To further study the unique impact of hypothermic stress, we employed two additional normothermic stress paradigms in C57BL/6J mice, which were subjected to 8 h of the restraint stress

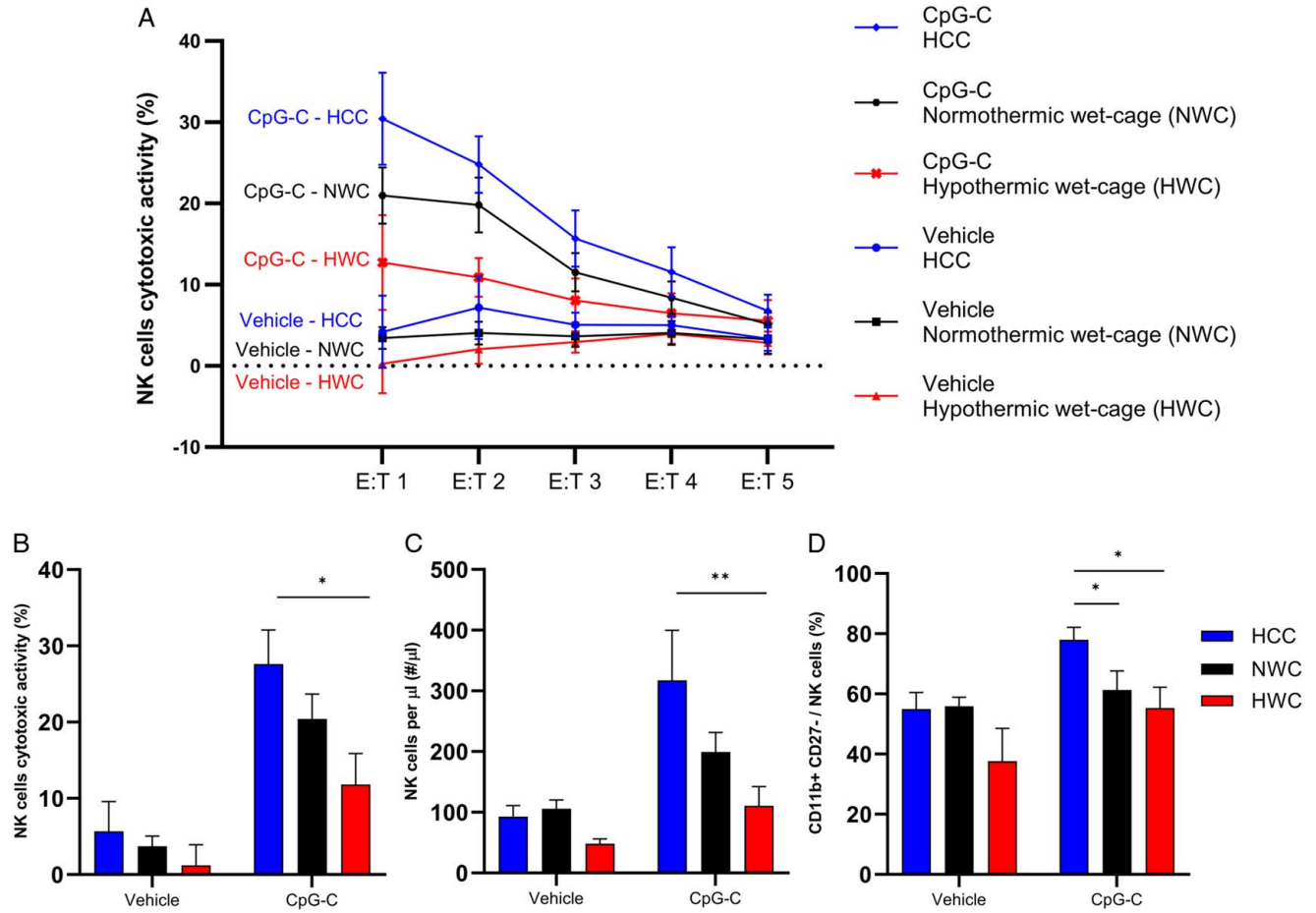

Figure 4. Comparison between the effects of hypothermic (HWC) and normothermic (NWC) stress paradigm on hepatic NK cells activity, numbers, and maturation ($n = 31$, $n = 4$–6 per group). Mice were subjected to 8 h of HWC, NWC, or served as HCC and were administered with CpG-C or vehicle 2 h after the onset of stress. Hepatic leukocytes were harvested at the end of stress. (A) Percentage of cytotoxic activity against CT26 cells in different effector-to-target (E:T) ratios. (B) Average of E:T 1 and 2 in each animal. (C) NK cells count per microliter of liver perfusate. (D) $CD11b^+$ $CD27^-$ NK cell percentages of the entire NK cell population. CpG-C significantly (B) elevated NK cells cytotoxicity (main effect, $P < 0.0001$); (C) increased NK cell numbers (main effect, $P = 0.0027$); and (D) increased $CD11b^+$ $CD27^-$ NK cell percentages of the entire NK cell population (main effect, $P = 0.0018$). In animals that were administered with CpG-C, HWC but not NWC significantly reduced (B) NK cell cytotoxicity ($P = 0.033$) and (C) numbers ($P = 0.03$) compared to HCC. Additionally, (D) both HWC ($P = 0.031$) and NWC ($P = 0.027$) significantly reduced the percentages of $CD11b^+$ $CD27^-$ NK cells compared to HCC (*$P < 0.05$; **$P < 0.001$). Graphs represent mean ± SEM.

paradigm (Fig. 6A, $n = 31$, see Methods) or 12 h of the tilt&light stress paradigm (Fig. 6B, $n = 36$, see Methods), each compared to HCC condition. Two hours after the onset of the stress paradigms, mice were administered with CpG-C (50 µg, i.p.) or vehicle. Plasma IL-12 and corticosterone levels were assessed at the end of stress.

CpG-C significantly increased plasma IL-12 levels under both stress conditions (Fig. 6A, $F(1,27) = 23.27$, $P < 0.0001$; Fig. 6B, $F(1,32) = 40.48$, $P < 0.0001$). As opposed to the HWC paradigms (Figs 1–4), restraint and tilt&light paradigms did not significantly interfere with CpG-C-induced elevation in plasma IL-12 levels.

Tilt&light stress reduced IL-12 levels and increased the number of CT26 metastases but did not prevent the beneficial effects of CpG-C: limited mediation by glucocorticoids

Previous studies indicated that glucocorticoids, but not catecholamines or opioids, modulate plasma IL-12 levels. Thus, in the context of tilt&light stress, we tested whether attenuation of glucocorticoid signaling affects plasma IL-12 levels and CT26 metastasis and whether it interacts with CpG-C-induced immune stimulation. To this end we conducted two studies with the same design in BALB/c mice, one assessing IL-12 levels ($n = 78$, Fig. 6C) and the second studying CT26 metastases ($n = 97$, Fig. 6D). Mice were subjected to the tilt&light stress paradigm for 12 h or served as HCC, and were injected with RU486 or vehicle 30 min before stress initiation. Two hours after the onset of the stress, mice were administered with CpG-C (50 µg, i.p.) or vehicle. Plasma IL-12 levels were assessed at the end of stress (experiment 1, Fig. 6C), or mice were inoculated with CT26 tumor cells at the end of stress, and 20 days later, the number of hepatic metastases was assessed (experiment 2, Fig. 6D).

Tilt&light stress significantly decreased plasma IL-12 levels (main effect – Fig. 6C, $F(1,70) = 6.237$, $P = 0.0149$), and increased numbers of CT26 metastases (main effect – Fig. 6D, $F(1,89) = 6.170$, $P = 0.0149$). CpG-C administration significantly elevated plasma IL-12 levels (main effect – Fig. 6C, F

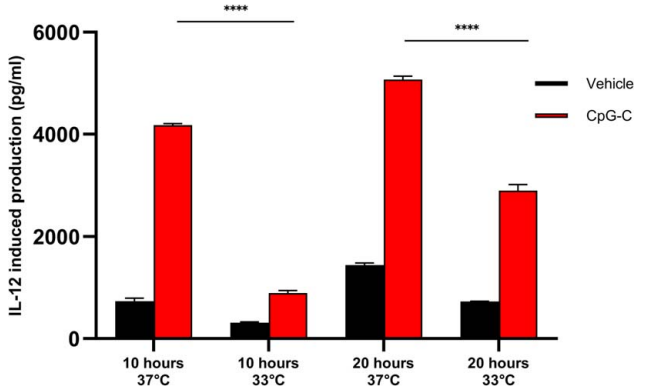

Figure 5. Comparison between hypothermic and normothermic conditions on CpG-C-induced IL-12 production. PBMCs were incubated for 10 or 20 h, at 33°C or 37°C. The study was conducted in duplicates, and supernatants were collected in duplicates from each well at the end of each incubation period. Hypothermic conditions significantly decreased (main effect, $P < 0.0001$) the production of IL-12 by PBMCs, and CpG-C elevated it (main effect, $P < 0.0001$). Hypothermic conditions attenuated CpG-C-induced elevation of IL-12 at 10 h ($P < 0.0001$) and at 20 h ($P < 0.0001$) (****$P < 0.0001$). The graph represents mean ± SEM.

(1,70) = 28.40, $P < 0.0001$) and markedly reduced numbers of CT26 metastasis (main effect – Fig. 6D, $F(1,89) = 403.9$, $P < 0.0001$). RU486 did not negate the deleterious effects of stress on IL-12 levels but did improve the antimetastatic effects of CpG-C under stress conditions (Wt(12.94) = 2.993, $P = 0.0104$).

Discussion

In this study, we explored how hypothermic and normothermic stress conditions modulate (i) immune responses and resistance to cancer metastasis, (ii) the beneficial effects of perioperative CpG-C immunotherapy on these indices, and (iii) the involvement of specific neuroendocrine mechanisms in these modulations. The results indicate that perioperative hypothermic stress can decrease (i) both baseline IL-12 levels and their elevation following CpG-C treatment, (ii) hepatic NK cell cytotoxicity, and (iii) resistance to CT26 hepatic metastasis. Additionally, blockade of glucocorticoids, β-adrenergic, or opioid receptors, or adrenalectomy did not overcome these deleterious effects of hypothermic stress.

These results emphasize the potential detrimental effects of hypothermic stress, and to a lesser extent of normothermic stress, in the clinical context of oncological surgery. Operated cancer patients often experience psychological and physiological stress during the IPP, along with inflammatory responses, all of which may inhibit antimetastatic immunity and immune-based therapies and promote metastatic properties of MRD[2,3]. These stressors are mainly characterized by excessive release of CAs and PGs, which were shown to specifically promote immunosuppression and the progression of MRD. Preclinical studies have demonstrated that CAs and PGs can facilitate suppression of antitumor immunity by reducing the number and activity of $CD8^+$ and $CD4^+$ effector T cells[53], impairing checkpoint inhibitor therapy[53], enhancing the activity of regulatory T cells and

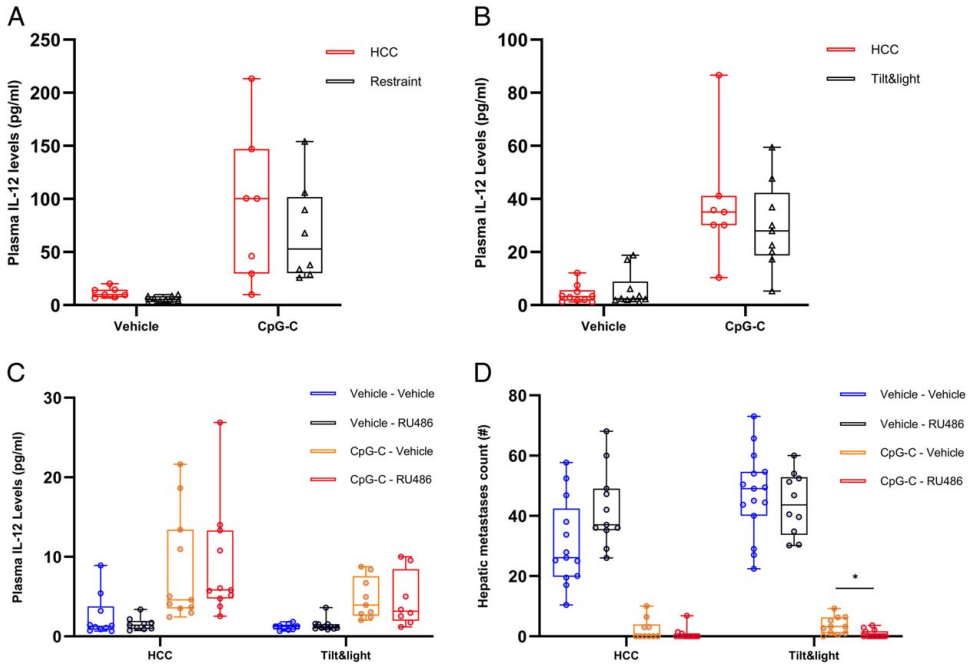

Figure 6. In four different experiments, mice were subjected to 8 h of restraint stress (A) or 12 h of tilt&light (B–D) stress. In experiments C and D, mice were administered with RU486 or vehicle 30 min prior to the onset of stress. In all experiments (A–D) mice were administered with CpG-C or vehicle 2 h after the onset of stress. (A–C) Plasma IL-12 levels were assessed at the end of each stress paradigm. (D) Visible hepatic metastases were counted 20 days later. (A, B) Restraint ($n = 31$, $n = 7$–9 per group) and tilt&light ($n = 36$, $n = 7$–10 per group) stress paradigms did not interrupt the CpG-C-induced elevation in plasma IL-12 levels. (C) Tilt&light stress significantly decreased plasma IL-12 levels (main effect, $n = 78$, $n = 9$–11 per group, $P = 0.0149$) and (D) increased numbers of CT26 metastases (main effect, $n = 97$, $n = 10$–15 per group, $P = 0.0149$), but did not prevent the beneficial effects of CpG-C on these indices: (C) increased IL-12 levels (main effect, $P < 0.0001$, and (D) decreased CT26 metastases, main effect, $P < 0.0001$). RU486 (C) did not negate the deleterious effects of stress on IL-12 levels, but (D) did improve the antimetastatic effects of CpG-C under stress conditions ($P = 0.0104$) (*$P < 0.05$). Boxes represent the second and third quartiles, and whiskers show min and max values.

myeloid-derived suppressor cells[54–56], diminishing natural killer (NK) cell cytotoxicity[50,57], promoting M2 macrophage polarization[58,59], and shifting the TH1/TH2 balance toward TH2 dominance. Notably, clinical studies suggest that cancer patients may experience both preoperative and postoperative perturbations of inflammatory and immune-suppressive factors. Stress and inflammatory responses are promoting each other[31], and in cancer patients, preoperative psychological stress was reported to elevate systemic inflammatory status, and various tumors are known to secrete prostaglandins. For example, patients with breast or ovarian cancer have exhibited elevated plasma levels of cortisol, IL-6, and CRP prior to surgery[28,60]. Additionally, decreased levels of interferon-gamma (IFN-γ) and IL-12 production were reported in breast cancer patients the morning following surgery[28]. In lung cancer patients, postoperative increases in PD-1/PD-L1 expression on $CD4^+$ and $CD8^+$ T cells and NK cells, along with reduced T and NK cell counts, have been reported[61]. Our results indicate that while the beneficial effects of CpG-C were significantly attenuated by hypothermic stress, CpG-C demonstrated resilience under normothermic stress conditions, maintaining its efficacy despite various stressors, including surgery. These and additional findings point to CpG-C as a promising candidate for IPP treatment to reduce cancer metastasis under normothermic stress conditions.

Perioperative hypothermia is a common complication of anesthesia and surgery. Mild hypothermia (36–32°C), as in the current study, is known to occur in ~70% of patients undergoing surgery[62–64]. In a prospective study of patients, Sari *et al*.[63] indicated that when the operation was longer than 2 h, all patients suffered some degree of hypothermia, and 24.4% of the patients exhibited a core temperature below 35°C. The great majority of these patients were subjected to passive intraoperative warming, as opposed to active warming. Active warming was shown to be more effective in reducing intraoperative hypothermia, although hypothermia may still persist even with active warming[65]. Additionally, cancer patients may be subjected to cold stress irrespective of surgery (elaborated in[66,67]), and such conditions in awake animals were shown to dampen anticancer immunity and promote tumorgenesis[68]. Specifically, Repasky *et al*. have shown that the common vivarium condition (22°C) subject animals to chronic cold stress, promoting beta-adrenergic signaling[53], which in turn can lead to: increased resistance to cytotoxic therapies[69]; enlarged primary tumors[70]; decreased numbers of antigen-specific $CD8^+$ T lymphocytes and active $CD8^+$ T cells in the tumor microenvironment[53,70]; elevated numbers of regulatory T cells and MDSCs[53,70]; and decreased non-plasmacytoid DC activation[71].

In this study, we sought to determine the unique impact of perioperative hypothermic stress vs. normothermic stress on cancer metastasis, and mediating immune mechanisms. The hypothermic wet-cage stress paradigm gradually decreased core temperatures to ~33°C within 8 h, whereas the normothermic variation of this paradigm maintained body temperature above 36°C. Notably, the two paradigms induced a similar elevation in glucocorticoid release. In the context of CpG-C immune stimulation, the hypothermic stress paradigm reduced plasma IL-12 and hepatic NK cell maturation and cytotoxicity levels, and increased metastasis, significantly and markedly more than the normothermic paradigm. Additionally, *in vitro*, hypothermic conditions (33°C) significantly reduced IL-12 production by PBMCs, compared to normothermic (37°C) conditions, with or without CpG-C. Last, we employed two additional stress paradigms that do not involve hypothermia – the restraint and the tilt&light paradigms. Neither paradigm attenuated CpG-C-induced IL-12 secretion nor its beneficial effects on CT26 metastases. NK cell cytotoxicity is known to be potentiated by IL-12[24], and our previous studies showed that the reduction in CT26 metastases induced by CpG-C administration is mediated by elevated NK cell cytotoxicity[49]. Thus, we suggest that in this study the deleterious impact of hypothermia is mediated, at least partly, through reduced IL-12 and hepatic NK maturation and cytotoxicity levels (observed herein), which in turn jeopardize the host capacity to eliminate liver metastases.

When stress conditions do not involve hypothermia, the efficacy of CpG-C may be further enhanced if specific stress responses are restricted. In our previous studies, we found that endogenous levels of glucocorticoids and prostaglandins-E_2, but not catecholamines or opioids, mediate the effects of stress in reducing plasma IL-12 levels[48]. Additionally, in-vivo pharmacological administration of catecholamines or opioids, as may also occur clinically, was shown to decrease IL-12 levels through endogenous glucocorticoid response rather than directly[48]. Thus, it appears that glucocorticoids are the main modulator of IL-12 secretion[48,72]. Additionally, high levels of glucocorticoids have been shown to directly induce pDC apoptosis, suppress IFNα production, suppress pDCs induction of high plasma IL-12 levels, and downregulate co-stimulatory molecules[73–75], all associated with reduced antimetastatic immunity. Considering the above, we assessed the efficacy of CpG-C treatment and a glucocorticoid antagonist on IL-12 levels and CT26 metastasis in the context of tilt&light normothermic stress. Stress alone significantly elevated the numbers of hepatic CT26 metastases but did not dampen the beneficial effects of CpG-C on IL-12 levels and the number of metastases. Importantly, under stress conditions, the combined treatment of CpG-C and glucocorticoid blockade decreased the number of CT26 metastases compared to CpG-C treatment alone, suggesting a synergistic effect of CpG-C and attenuation of glucocorticoid signaling in the context of non-hypothermic stress.

While preclinical studies of immunotherapy show great potential in the treatment of cancer metastasis, clinical outcomes are clearly less promising[2,76,77]. Herein, we suggest that one important difference that may contribute to such discrepancies is the prevalence of physiological and psychological stressors in cancer patients[31], including hypothermic conditions[62], which are prevalent clinically but are rarely simulated in preclinical studies. We herein simulated hypothermic and normothermic stress conditions and studied their effects on TLR-9 immune activation during the IPP. While the wet-cage stress paradigm enabled us to specifically isolate the effects of hypothermia by comparing it to its normothermic counterpart, the robustness of this paradigm may mask more subtle stress responses that could occur clinically in cancer patients[31]. Additionally, despite their significant potential in reducing metastatic burden in preclinical studies, TLR-9 agonists have rarely demonstrated similar success in clinical research[78,79]. Such inefficacies may be due to the delayed administration of these agents in the clinical setting[80], which typically begins at least a month postoperatively. This timing may reduce the effectiveness of these agents due to surgery-induced and/or chemotherapy-induced immunosuppression, in contrast to the perioperative use of CpG-C explored in this study.

Additionally, clinical inefficacy may also be attributed to a different distribution of TLR-9 on immunocytes in humans compared to rodents[81,82]. The difference in the distribution of TLR-9 may lead to an altered immune response in humans, potentially indicating CpG-C therapy to be less effective clinically. Lastly, herein we employed an experimental metastasis model (CT26), which did not involve the removal of a primary tumor, and CpG-C was administered before CT26 was introduced to the mice, thus distancing the model from clinical context. This further warrants the testing of CpG-C in tumor-bearing animals, under various perioperative stress conditions. Notably, we have previously shown the efficacy of CpG-C in tumor-bearing animals but not in the broader clinical perioperative context[6].

Considering the above, we suggest several approaches to promote the use of immunotherapy during the IPP, and specifically CpG-C, to reduce metastatic development in cancer patients. First, the use or the development of new psychological stress paradigms that better mimic stress responses of cancer patients during the IPP will enable better simulation of the clinical context, and potentially reduce the efficacy gap between preclinical and clinical studies regarding immunotherapies. Additionally, although tumor cell inoculation in the CT26 model was conducted in the context of a surgical procedure, future studies should employ several types of cancer models, including models of spontaneous metastasis, to promote the generalization of the impact of CpG-Cs and the simulation of the clinical oncological context. Additionally, translational research of CpG-C is warranted in models that better mimic human TLR-9 distribution, such as TLR-9 partial knockouts or humanized mouse models and human tissues. Herein, plasma IL-12 and hepatic NK cytotoxicity and maturation levels were shown to at least partially mediate the detrimental effects of hypothermia on hepatic metastasis. Future studies should evaluate the predictive value and causal effects of IL-12 and of NK cytotoxicity and maturation levels under both hypothermic and normothermic stress conditions during the IPP in various metastasis models and organs. If found to predict long-term cancer outcomes, perioperative levels of IL-12 could be used as a clinical biomarker for perioperative NK activity and hepatic metastases risk, and IL-12 replacement therapy may be tested.

Conclusions

This study indicates that perioperative stressors, hypothermic and normothermic, can modulate perioperative immune function, dampen resistance to experimental metastasis, and jeopardize the efficacy of CpG-C immunotherapy. Mild hypothermia, which is prevalent clinically, can lead to potent suppression of immunity and reduced resistance to cancer metastasis, which herein was not mediated by common neuroendocrine stress responses but apparently through direct effects of low temperature on immunocytes. These results emphasize the importance of routine thermoregulation monitoring and maintenance in cancer patients throughout the IPP. Patients undergoing surgery with general anesthesia are typically warmed (actively or passively) only during the surgical procedure itself, but not during critical time points in the IPP, including induction of anesthesia and postoperative awakening and recovery periods. Notably, current practices of thermoregulation were found insufficient to prevent hypothermia even during the procedure itself[65,83]. The current study also suggests potential strategies to enhance the beneficial effects of CpG-C immunotherapy under non-hypothermic conditions. Pharmacological reduction of stress responses or behavioral stress management may effectively complement various immunotherapies during the IPP, thereby reducing metastasis and improving long-term cancer outcomes[2,3]. These findings have significant clinical implications for oncological surgery, including the potential implementation of immune-stimulating approaches in cancer patients undergoing surgery and more effective and comprehensive prevention of hypothermia throughout the IPP.

Ethical approval

Housing conditions are regularly monitored by the Institutional Animal Care and Use Committee of Tel Aviv University, which also approved all studies described herein. P-12-003, 31/12/2016; 10-18-005, 09/07/2018; 10-18-008, 06/12/2018.

Consent

Not applicable.

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Author contribution

E.S.: conceptualization, data curation, formal analysis, investigation, methodology, project administration, validation, visualization, writing – original draft, and writing – review and editing; P.M.: conceptualization, investigation, and validation; A.E.: investigation, methodology, validation, and writing – review and editing; L.S.: investigation and writing – review and editing; E.R.: investigation; I.N.: writing – review and editing; S.B.-E.: conceptualization, formal analysis, funding acquisition, methodology, resources, supervision, and writing – review and editing.

Conflicts of interest disclosure

The authors have no conflicts of interest.

Research registration unique identifying number (UIN)

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Data availability statement

The data generated during this study will be available upon request.

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