Brain, Behavior, and Immunity 58 (2016) 91–98

Contents lists available at ScienceDirect

Brain, Behavior, and Immunity

journal homepage: www.elsevier.com/locate/ybrbi

Full-length Article

Reducing liver metastases of colon cancer in the context of extensive and minor surgeries through β-adrenoceptors blockade and COX2 inhibition

Sorski Liat, Melamed Rivka, Matzner Pini, Lavon Hagar, Shaashua Lee, Rosenne Ella, Ben-Eliyahu Shamgar *

Neuroimmunology Research Unit, Sagol School of Neuroscience, and The School of Psychological Sciences, Tel-Aviv University, Tel-Aviv, Israel

ARTICLE INFO

Article history:
Received 26 December 2015
Received in revised form 16 May 2016
Accepted 24 May 2016
Available online 25 May 2016

Keywords:
CT26 colon-cancer
Liver metastasis
Peri-operative period
Propranolol
Etodolac
β-Adrenergic blocker
COX-2 inhibition
Colorectal cancer

ABSTRACT

Liver metastases are a major cause of colorectal cancer death, and the perioperative period is believed to critically affect the metastatic process. Here we tested whether blocking excess release of catecholamines and prostaglandins during surgical procedures of different extent can reduce experimental liver metastasis of the syngeneic CT26 colon cancer in female and male BALB/c mice. Animals were either treated with the beta-blocker, propranolol, the COX-2 inhibitor, etodolac, both drugs, or vehicle. The role of NK cells in controlling CT26 hepatic metastasis and in mediating the effect of the drugs was assessed by *in vivo* depletion or stimulation of NK cells, using anti-asialo GM1 or CpG-C, respectively. Surgical extent was manipulated by adding laparotomy to small incision, extending surgical duration, and enabling hypothermia. The results indicated that combined administration of propranolol and etodolac, but neither drug alone, significantly improved host resistance to metastasis. These beneficial effects occurred in both minor and extensive surgeries, in both sexes, and in two tumor inoculation approaches. NK cell-mediated anti-CT26 activity is involved in mediating the beneficial effects of the drugs. Specifically, CpG-C treatment, known to profoundly activate mice marginating-hepatic NK cytotoxicity, reduced CT26 hepatic metastases; and NK-depletion increased metastases and prevented the beneficial effects of the drugs. Overall, given prevalent perioperative psychological and physiological stress responses in patients, and ample prostaglandin release by colorectal tumors and injured tissue, propranolol and etodolac could be tested clinically in laparoscopic and open colorectal surgeries, attempting to reduce patients' metastatic disease.

1. Introduction

For decades, surgeries for the excision of primary tumors have been suggested to facilitate the metastatic process, and specific mechanisms mediating deleterious effects of surgery are continuously being unraveled. For example, the surgical manipulation of the malignant tissue and the surrounding blood vessels may disperse tumor cells into the circulation (Eschwege et al., 1995; Yamaguchi et al., 2000), and may induce the release of growth factors by the host, aiming at tissue healing. These processes are believed to promote the initiation of new metastases and the development of pre-existing micrometastases (Fisher et al., 1989; Demicheli et al., 2001; Weitz and Herfarth, 2001). An additional factor is surgery-induced suppression of anti-metastatic cell mediated immunity (CMI) (Ben-Eliyahu, 2003). Such suppression may render the organism susceptible to metastatic progression during the perioperative period, a timeframe which is believed to be critical in determining whether metastatic disease will be arrested or will progress (Shakhar and Ben-Eliyahu, 2003; Neeman and Ben-Eliyahu, 2013; Horowitz et al., 2015). Natural killer cell cytotoxicity (NKCC) is an important aspect of innate immunity. NK cells secrete several prominent pro-CMI cytokines, and exhibit cytotoxicity against a variety of tumor cells, including those that escape adaptive immunity through the elimination of MHC-I molecules (Srivastava et al., 2008; Terunuma et al., 2008).

Surgical procedures were shown to suppress CMI, including NKCC, through various mechanisms, and markedly through access perioperative release of catecholamines (CAs), prostaglandins (PGs), and glucocorticoids (GC) (Shakhar and Ben-Eliyahu, 1998, 2003; Shakhar and Blumenfeld, 2003; Benish et al., 2008; Rosenne et al., 2014). The levels of these hormones increase during the perioperative period as a result of tissue damage, surgical stress responses, and in humans also by psychological distress (Neeman et al., 2012). These immunosuppressive effects were associated or causally linked to a more profound metastatic progression and a greater mortality rate in animal models (Yakar et al., 2003;

* Corresponding author at: Sagol School of Neuroscience & School of Psychological Sciences, Tel-Aviv University, Tel-Aviv 69978, Israel.
E-mail address: shamgar@post.tau.ac.il (S. Ben-Eliyahu).

http://dx.doi.org/10.1016/j.bbi.2016.05.017
0889-1591/

Benish et al., 2008; Glasner et al., 2010; Inbar et al., 2011; Neeman and Ben-Eliyahu, 2013; Rosenne et al., 2014). Additionally, PGs and CAs are known to reduce Th1 cytokine levels (e.g., IFN alpha/beta, and IL-2) and to increase levels of Th2 cytokines, which further suppress various aspects of CMI (Elenkov et al., 2000; Stolina et al., 2000). Last, CAs and PGs, released by the host or the malignant tissue, were shown to promote tumor progression through direct effects on the malignant tissue and its surrounding (Lee et al., 2009; Moreno-Smith et al., 2010; Neeman and Ben-Eliyahu, 2013; Burkholder et al., 2014; Cole et al., 2015; Watkins et al., 2015).

Previous studies conducted in our laboratory demonstrated that the combined use of the β-blocker, propranolol, and the COX-2 inhibitor, etodolac, attenuated immunosuppression following surgery, as indicated by improved postoperative NKCC and reduced postoperative development of NK-sensitive experimental metastases (Melamed et al., 2005; Benish et al., 2008; Inbar et al., 2011). The same combined treatment also reduced postoperative spontaneous metastases and increased survival rates in several tumor models (Glasner et al., 2010; Inbar et al., 2011; Neeman and Ben-Eliyahu, 2013), potentially through various, immunological and non-immunological mechanisms. Importantly, only the combined use of these blockers, but not each drug alone, was found to be effective in attenuating suppression of NKCC (Benish et al., 2008), and in reducing spontaneous metastases and increasing survival rates (Glasner et al., 2010).

The extent of the surgical procedure and tissue damage were suggested to affect the degree of immune suppression and tumor progression, but the evidence is controversial in both animal and human studies (Horowitz et al., 2015). In previous studies we found similar developmental courses of spontaneous melanoma metastases when subjecting animals to mild surgical procedures in comparison to more extensive ones (Glasner et al., 2010). Similarly, human studies in multiple types of cancer indicated that although the short-term immune and inflammatory responses to minimally invasive surgeries (MIS, e.g. laparoscopy) are often lower compared to open surgeries (e.g., laparotomy), there are no significant advantages in long-term cancer outcomes (Horowitz et al., 2015).

Colorectal cancer (CRC) is the third most common cancer in the developed countries, and the second leading cause of cancer-related mortality. Liver metastasis of CRC attracts a particular clinical and scientific interest as a result of their high incidence, poor prognosis, and given the unique immune properties and biological significance of the liver (Siegel et al., 2015).

The current study aimed at testing the use of a β-adrenergic blocker (propranolol) and a COX2 inhibitor (etodolac) against experimental liver metastases of a colon cancer line in the context of surgical procedures of different extents. To this end, syngeneic CT26 colon carcinoma cells were administered to BALB/c mice in the context of surgery via the portal vein or through the spleen (Sorski et al., 2014), and hepatic metastases were studied. Thus, this approach models tumor colonization of a prominent metastatic site of CRC. Employing this model, we addressed potential mediating mechanisms of the deleterious effects of surgeries of different extent, and of the beneficial effects of the drug treatment, specifically focusing on the mediating role of NK cells, of which marginating-hepatic-NK cells were reported to be involved in this model of experimental metastasis (Sorski et al., 2015).

2. Materials and methods

2.1. Animals and counterbalancing

Male and female BALB/c mice were purchased from Harlan laboratories (Jerusalem, Israel) at the age of 4 weeks. Animals were housed 3–4 per cage at 22 ± 1 °C, on a 12:12 light:dark cycle and were allowed ad libitum access to food and water. Animals were used at the age of 8–12 weeks (animals were age-matched between groups within each experiment). The order of tumor inoculation and other experimental procedures were counterbalanced across all experimental groups. The Institutional Animal Care and Use Committee of Tel Aviv University approved all studies.

2.2. Tumor cell lines

2.2.1. The CT26 tumor cell line

The CT26 murine colon carcinoma cell line is a chemically-induced undifferentiated carcinoma, syngeneic to the BALB/c strain (Corbett et al., 1975). Tumor cells were kindly provided by Prof. Eliezer Flesher (Department of Human Microbiology, Faculty of Medicine, Tel-Aviv University). Cells were grown in monolayer cultures in 37 °C, 100% humidity, 5% CO_2, in complete medium (CM) (RPMI 1640, supplemented with 10% heat-inactivated fetal calf serum (FCS), 0.05 mg/ml gentamicin, 2 mM L-glutamine, 1 mM sodium pyruvate and 0.1 mM non-essential amino acids) (Beit Haemek, Israel).

2.3. Preparation of CT26 tumor cells for injection

Cells were removed from the culture flask with a trypsin solution (0.25% 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 supplemented with 0.1% BSA for either portal vein or splenic injection in a volume of 100 μl per animal.

2.4. Drugs and their administration

2.4.1. Propranolol

To block β-adrenoceptor activation, we used the nonselective β-adrenergic blocker, propranolol (Sigma, Rehovot, Israel). The drug was dissolved in PBS and added to a mixture of mineral oil (Sigma, Rehovot, Israel) and mannide monooleate (a nonspecific surface active emulsifier; Sigma), in a 4:3:1 ratio, respectively, to create a slowly absorbed emulsion. Unpublished data from our laboratory have shown that the slow absorbance emulsion is effective for 36–48 h. Mice were subcutaneously administered with the emulsion 30 min prior to the surgical procedure (5 mg/kg in a 0.5 mg/ml concentration).

2.4.2. Etodolac

The semi-selective COX2 inhibitor, etodolac, was kindly donated by Taro, Israel. Etodolac was dissolved in corn oil. The drug was administered subcutaneously 30 min prior to the surgical procedure (50 mg/kg in a 12.5 mg/ml concentration). The T1/2 of this drug in mice was found to be 16 h (Rainsford, 1996).

2.4.3. CpG ODN

A type-C CpG ODN (ODN 2395: 5′-CGTCGTTTTCGGCGCGCGCCG-3′) with a phosphorothioate backbone was used. This ODN has been demonstrated to retain characteristics of both type A and B CpG ODNs by exhibiting potent activation of both innate and adaptive immune responses. CpG was injected i.p. at doses of 12, 25, 50 and 100 μg/animal, in 0.1 ml of PBS supplemented with 0.1% BSA (Sigma-Aldrich, Rehovot, Israel).

2.4.4. Anti-asialo GM1 Ab

Mice were injected i.p. with 30 μl of anti-asialo GM1 (according to manufacturer's instructions, Wako Chemicals, USA, supplied by Enco, Israel), or with PBS as control.

2.5. 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 by radioimmunoassay (RIA) (ImmuChem double antibody corticosterone ^{125}I RIA kit, MP Biomedicals, Orangeburg, NY), per manufacturer's instructions.

2.6. Surgical procedure and tumor inoculation

2.6.1. Hepatic portal vein inoculation

Anesthesia was initiated by subjecting mice to 6% isoflurane in air for 20–30 s. Isoflurane anesthesia was maintained at 1.5–2.5% thereafter, the skin was shaved and rubbed with alcohol pads, and a 1.5 cm midline abdominal incision was conducted. The hepatic portal vein was exposed and 1×10^5 CT26 tumor cells in 100 μl PBS-BSA were injected using a 31G needle. To prevent bleeding, pressure was applied to the injection site with a cotton applicator (Q-tip) for 4 min. Finally, the muscle and skin were sutured using 4/0 blue polypropylene monofilament non-absorbable suture (Johnson & Johnson, Belgium).

2.6.2. Intra-splenic inoculation

Mice were anesthetized as described above. The skin was shaved and rubbed with ethanol pads, and a 0.5-cm abdominal incision was made adjacent to the spleen (a left flank incision approximately 2 cm left of the abdominal midline). For the spleen inoculation approach, tumor cells were injected into the spleen using a 31G needle, which was maintained in the spleen tissue for 2 min. 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. Following the excision of the injected spleen, the peritoneum and skin were sutured with 4/0 non-absorbable filaments. Animals were allowed to recover in their home cages.

2.7. Assessment of metastatic development

Following tumor inoculation via either the portal vein or the spleen, animals were monitored daily for general wellbeing, and were euthanized with an overdose of isoflurane on the 21th day. Livers were then harvested and weighed, and surface hepatic metastases were counted by an investigator blinded to each animal's experimental group. Colorectal cancer liver metastases (CRLM) were identified as those larger than 1 mm in diameter, forming a spherical solid metastasis with a distinct formation. As the normal (non-inoculated) livers in both male and female mice weigh about 0.9–0.95 g, the scale shown for the liver weight results starts from this value (and not from 0) to better indicate tumor-related increases in liver weight.

2.8. Flow cytometry

2.8.1. Sample preparation and analysis

A known quantity of blood (5–50 μl) or of splenocytes was added to 50 μl of PBS supplemented with 2% FCS and 0.1% NaN_3 (PBS++) containing a set of conjugated antibodies (see below). Samples were kept in the dark at room temperature thereafter. Following a 15 min. incubation period, 1 ml FACS lysing solution (Becton Dickinson) was added. Twelve minutes later, samples were centrifuged (670*g* for 5 min) and the lysate was aspirated. Cells were rewashed twice with 1 ml PBS++ (670*g* for 5 min) and re-suspended in 300 μl of PBS++ for flow cytometry, using a FACScan (Becton Dickinson).

2.8.2. Identification of leukocyte subsets and cellular expression markers

Granulocytes and lymphocytes were identified based on their forward by side scatter position. Within lymphocytes, NK cells were identified as being NKp46^{+} (PE-conjugated anti-mouse NKp46, Peprotech Asia) and CD49b^{++} cells (FITC conjugated CD49b, Peprotech Asia).

2.9. Statistical analysis

Depending on experimental design, we conducted one-, two-, or three-way factorial analyses of variance (ANOVA) with a predetermined significance level of 0.05, to assess the group differences in each of the dependent variables. Specifically, the number of metastases and liver weight, and the number of NK cells were compared between the different groups/conditions. When ANOVA indicated significant group differences, post-hoc contrasts were performed (Fisher's PLSD) based on a-priori hypotheses. Data were always assessed to verify normal distribution and group homogeneity of variance. In the NK-depletion studies, the ANOVA assumption of homogeneity of variance was not met, and thus *t*-tests were conducted to assess the efficacy of the drugs treatment, separately in NK-intact and in NK-depleted animals.

3. Results

3.1. Exp. 1 A&B: The combined administration of propranolol and etodolac reduces the number of surface hepatic metastases and liver weight irrespective of surgical extent

3.1.1. Design and procedure

The intra-splenic injection method was used to evaluate the efficacy of the administration of propranolol and etodolac, alone and in combination, on surface hepatic metastases development and liver weight under two different surgical injection approaches – left abdominal small incision (will be addressed hereafter as "small incision"), or a combination of small incision and additional laparotomy. A 4×2 factorial design was used (drug treatment $\times$ surgical procedure): a total of 57 mice (32 males and 25 females) were injected with propranolol (n = 13), etodolac (n = 16), both drugs (n = 14), or vehicle (n = 14), and 30 min later underwent one of two surgical injection procedures (approximately half from each drug treatment) for the injection of 10^4 CT26 tumor cells into the spleen. Twenty-one days after tumor cells inoculation, mice were sacrificed with an overdose of isoflurane, livers were removed and weighed, and surface hepatic metastases were counted by two experimenters blind to group assignment.

3.1.2. Results

A 4×2 ANOVA indicated a significant main effect for drug treatment ($F(1,49) = 2.913$, $p < 0.05$) on the number of surface hepatic metastases, and a marginal significant effect for surgical procedure ($F(1,49) = 3.135$, $p = 0.0828$) (Fig. 1A). The latter effect indicates a tendency towards higher number of metastases in mice subjected to the more extensive surgical procedure. Only the combined drug treatment, but not each drug alone, significantly reduced the number of surface-hepatic metastases compared to no drug treatment (vehicle) or to each drug alone, in both the mild (small incision) and the extensive surgical procedures (small incision and additional laparotomy) (PLSD $p < 0.05$ in all comparisons).

With respect to liver weight, the exact same pattern of results was observed (Fig. 1B): a significant main effect for drug treatment was evident ($F(1,49) = 2.780$, $p < 0.05$), and a marginal significant effect for the extent of the surgical procedure ($F(1,49) = 3.146$,

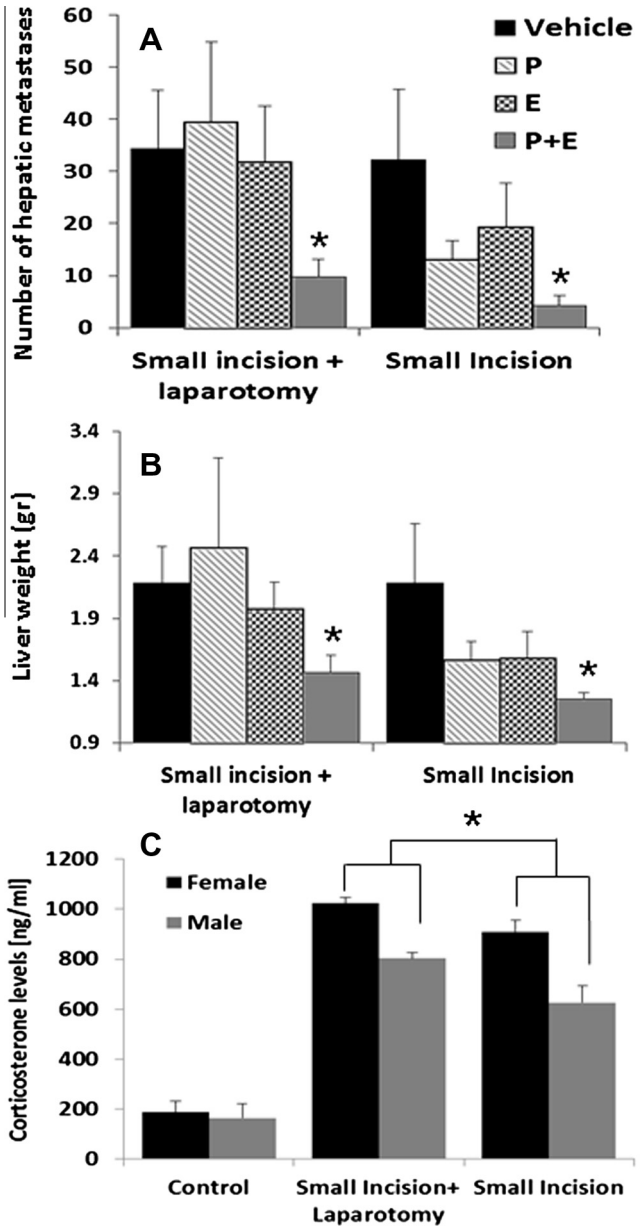

Fig. 1. (A&B) The beneficial effects of propranolol, etodolac, and their combined administration on the number of surface hepatic metastases and liver weight: A total of 57 mice (32 males and 25 females) were injected with propranolol (n = 13), etodolac (n = 16), both drugs (n = 14), or vehicles (n = 14), and 30 min later underwent one of two surgical procedures (small incision with or without laparotomy, approximately half from each drug treatment) for the injection of CT26 tumor cells into the spleen. Twenty-one days later, surface hepatic metastases were counted and livers were weighted. (A) Only the combined drug treatment, but not each drug alone, significantly reduced the number of surface-hepatic metastases compared to no drug treatment (vehicle), as indicated by an *, in both surgical procedures (PLSD $p < 0.05$ in all comparisons). (B) Liver weight showed the exact same pattern of results, and livers without tumors weigh ~1 g. (C) Comparison of corticosterone levels between the two surgical procedures: 14 females and 10 males were subjected to either a small incision or to laparotomy and small incision, and 6 h later blood corticosterone levels were assessed. Significantly lower levels of corticosterone were evident in mice undergoing the minor surgical procedure compared to mice undergoing the more extensive surgical procedure. Data are presented as mean ± SEM.

$p = 0.0823$). Again, only the combined treatment, but not each drug alone, significantly reduced liver weight (PLSD $p < 0.05$) compared to control (vehicle) levels, irrespective of the extent of the surgical procedure.

A significant correlation between liver weight and the number of surface-hepatic metastases was evident ($r = 0.83$, $p < 0.05$) (data not shown).

3.2. Exp. 1C: Adding laparotomy to small incision further increase the corticosterone response

3.2.1. Design and procedure

To compare the two aforementioned surgical procedures (of different surgical extent) in terms of stress responses, we subjected 14 females and 10 males to either a small incision or to small incision and laparotomy. Six hours later mice were sacrificed and cardiac blood withdrawn for the assessment of corticosterone levels using RIA.

3.2.2. Results

Two way ANOVA revealed significant group differences, with significantly lower levels of corticosterone in mice subjected to the minor surgical procedure compared to mice undergoing the extensive procedure ($F(2,18) = 144.792$, $p < 0.0001$) (Fig. 1C).

3.3. Exp. 2. The effects of sex and of the combined administration of propranolol and etodolac on the number of surface hepatic metastases

In Exp. 1, sex differences were observed, but the number of animals within each group (always males and females) was too small to address sex as a factor in this study. Thus, in the current study we addressed this factor and its potential interaction with the effects of the combined drug treatment.

3.3.1. Design and procedure

The intra-splenic injection method was used to evaluate the efficacy of sex and of the combined administration of propranolol and etodolac on surface hepatic metastases development. A 2×2 factorial design was used (males or females $\times$ drug treatment or vehicle): a total of 47 males and 41 females. Mice were injected with propranolol and etodolac (n = 42), or with vehicle (n = 46), and 30 min later underwent the small incision surgical procedure for the injection of 10^4 CT26 tumor cells into the spleen. Twenty-one days later, mice were sacrificed with an overdose of isoflurane, livers were removed and weighed, and surface hepatic metastases were counted by two researchers blinded to group assignment.

3.3.2. Results

A two way ANOVA (sex by drug treatment) indicated a significant main effect for sex and for drug treatment on the number of surface hepatic metastases ($F(1,84) = 48.878$, $p < 0.0001$, $F(1,84) = 8.450$, $p = 0.0047$, respectively). Specifically, drug treatment significantly reduced the number of surface-hepatic metastases compared to control (vehicle) levels, and females presented markedly fewer metastases than males (Fig. 2). Fisher PLSD indicated that in each sex category the drug treatment reduced the number of hepatic metastases ($p < 0.05$). As in Exp. 1, the index of liver weight showed a similar pattern of effects, and a significant correlation with the number of hepatic metastases (data not shown).

3.4. Exp. 3. The effects of surgical extent and of combined administration of propranolol and etodolac on the establishment of hepatic metastases – using the portal vein inoculation approach

In the previous two experiments all animals underwent splenectomy. This experiment employed the hepatic-portal vein inoculation approach that spares the spleen, but is (i) technically more challenging, (ii) always involves laparotomy, and (iii) bears a greater risk of bleeding as a result of puncturing the portal vein.

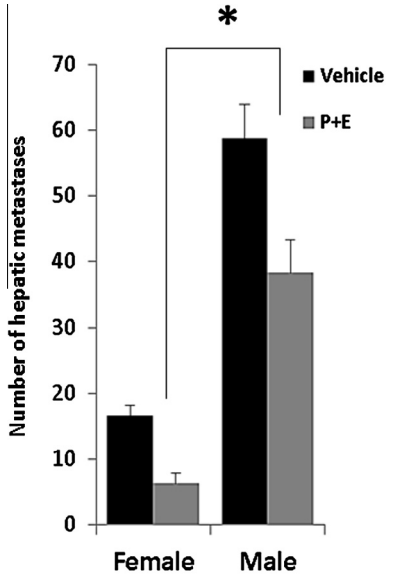

Fig. 2. Females present markedly fewer metastases than males, and drug administration is effective in both sexes: A total of 47 males and 41 females were injected with propranolol and etodolac (n = 42), or with vehicles (n = 46), and 30 min later underwent the minor incision surgical procedure for the injection of CT26 tumor cells into the spleen. Twenty-one days later, mice were sacrificed with an overdose of isoflurane, livers were removed and weighed, and surface hepatic metastases were counted. Drug treatment significantly reduced the number of surface-hepatic metastases compared to control (vehicle) levels in both sex categories ($p < 0.05$ for both), and females presented markedly fewer metastases than males, as indicated by * ($p < 0.05$). Data are presented as mean ± SEM.

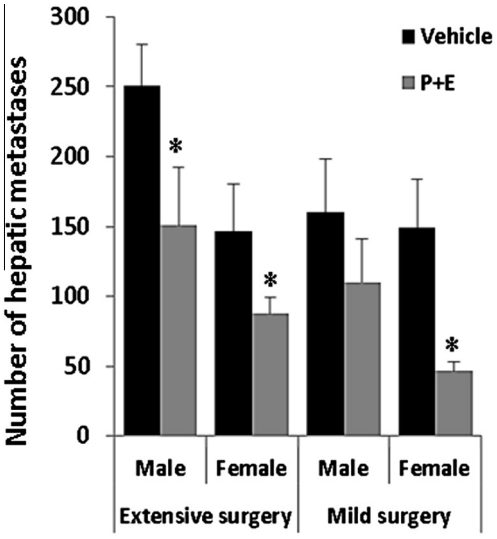

Fig. 3. Using the portal vein inoculation approach – Drugs treatment significantly reduced the number of surface-hepatic metastases, and females presented fewer metastases than males: CT26 tumor cells were injected through the hepatic portal vein to male (n = 34) or female (n = 28) mice without removing the spleen. 30 min before tumor injection, mice were injected with propranolol and etodolac, or with vehicles. Twenty-one days later, mice were sacrificed, livers were removed and surface hepatic metastases were enumerated. Drug treatment significantly reduced the number of surface-hepatic metastases compared to control (vehicle) levels, and females presented significantly fewer metastases than males (ANOVA main effects). * indicate a significant effect for drug separately in each group. Data are presented as mean ± SEM.

3.4.1. Design and procedure

A 2 × 2 × 2 factorial design was used – male (n = 34) or female (n = 28) mice were injected with either the combination of propranolol and etodolac (n = 27), or with vehicle (n = 35), and 30 min later underwent either laparotomy (n = 40), or an extensive laparotomy (n = 22) procedure where we prolonged the duration of surgery (from 10 min to 45 min), added a rubbing of the colon with gauze, and maintained a lower room temperature during operation (15 °C instead of 22 °C). CT26 tumor cells were injected through the hepatic portal vein in a dose of 2×10^4/mouse. Twenty-one days later, mice were sacrificed with an overdose of isoflurane, livers were removed and surface hepatic metastases were enumerated by two researchers blinded to group assignment.

3.4.2. Results

A three way ANOVA (sex by drug treatment by surgical procedure) indicated a significant main effect for drug treatment, and for sex on the number of surface hepatic metastases ($F(1,54) = 7.758$, $p = 0.0074$), ($F(1,54) = 4.667$, $p = 0.0352$ respectively). Specifically, drug treatment significantly reduced the number of surface-hepatic metastases ($p < 0.05$ in all comparisons) compared to control (vehicle) levels, and females presented fewer metastases than males (Fig. 3). Although mice in the extensive surgical procedure exhibit more metastases than those subjected to the milder form, this difference did not reach statistical significance ($p = 0.134$), and the beneficial effects of drug treatment were similar in both surgical approaches (no interactions were evident).

3.5. Exp. 4: A critical role for NK cells in controlling CT26 hepatic metastasis

In this study we aimed at assessing the role of NK cells in controlling CT26 hepatic metastasis. To this end, we compared naïve mice to mice subjected to an established procedure of NK cell depletion or NK cell activation. These procedures are based on the administration of anti-asialo GM-1 (Kasai et al., 1981) or CpG-C (Goldfarb et al., 2011), respectively.

3.5.1. Design and procedure

A total of 27 female mice were injected with either anti-asialo GM1 (n = 10) (known to deplete NK cells in BALB/c mice), CpG-C (n = 7) (known to activate NK cells) or vehicle (n = 10). Anti-asialo GM1 and CpG-C were injected i.p. in doses of 30 µl (in 100 µl PBS), and 100 µg/mouse, respectively. Twenty-four hours later, all mice were inoculated through the spleen with 2×10^4 CT26 tumor cells. Twenty-one days later, all mice were sacrificed, their livers were removed and weighed, and surface-hepatic metastases were counted by two researcher blinded to group assignment.

To verify the capacity of anti-asialo GM1 to deplete NK cells, a separate study compared the number of circulating and splenic NK cells in mice treated with anti-asialo GM1 or vehicle control (rabbit serum or PBS). Anti-asialo GM1 significantly eliminated NK cells ($CD49b^{++}$ $NKp46^{+}$ lymphocytes) as seen in (Fig. 4A).

3.5.2. Results

One-way ANOVA revealed significant group differences $F(2,24) = 11.634$, $p = 0.0003$). As indicated by Fisher PLSD, NK depletion significantly increased the number of hepatic metastases by approximately 7 fold ($p < 0.05$) (Fig. 4B). CpG-C reduced the number of metastases from an average of 10 in the vehicle treated group to 0.1 (0 in all mice except for one animal that exhibit one metastasis). Given significant differences in the variance of these 2 groups, a student's *t*-test for inequality of variance was used and showed significant differences between the two groups ($p = 0.044$). Similar effects were evident in liver weight (not shown). Overall, these findings strongly suggest that NK cells are significant in controlling CT26 hepatic metastasis.

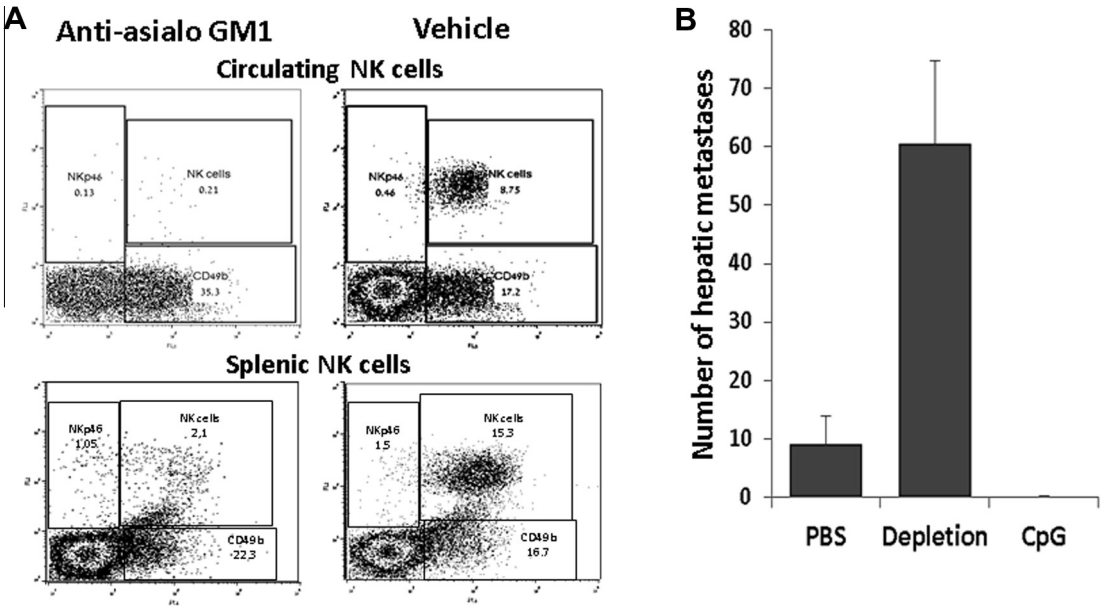

Fig. 4. NK cells are significant in controlling CT26 hepatic metastasis: To verify the capacity of anti-asialo GM1 to deplete NK cells, a separate study compared the number of circulating and splenic NK cells in mice treated with anti-asialo GM1 or vehicle control (rabbit serum or PBS). (A) Anti-asialo GM1 significantly eliminated NK cells. (B) A total of 27 female mice were injected with either anti-asialo GM1 (n = 10), CpG-C (n = 7) (known to activate NK cells), or vehicle (n = 10). Twenty-four hours later, all mice were inoculated through the spleen with CT26 tumor cells. Twenty-one days later, all mice were sacrificed, their livers were removed and weighed, and surface-hepatic metastases were counted. CpG-C reduced the number of metastases from an average of 10 in the vehicle treated group to 0.1 (0 in all mice except for one animal that exhibit one metastasis) ($p = 0.044$). Anti-asialo GM1 caused an 8-fold significant increase in the number of metastases. Similar effects were evident in liver weight (not shown). Data are presented as mean ± SEM.

3.6. Exp. 5: NK cells are significantly involved in the beneficial effects of the combined drug treatment

To study the involvement of NK cells in mediating the impact of the combined drug treatment on CT26 metastases *in vivo*, we compared naïve mice to mice subjected to an established procedure of NK cell depletion, as also used and verified in Exp. 4. In both conditions, mice were administered either with the combination of propranolol and etodolac, or with their vehicles. This study was conducted twice, and in the second study the depletion procedure was also verified through FACS enumeration of circulating NK cells.

3.6.1. Design and procedure

3.6.1.1. First study. A total of 44 male mice were employed using a 2×2 factorial design. Mice were injected with either anti-asialo GM1 (30 μl i.p. in 100 μl PBS) (n = 20), or vehicle (n = 24). Twenty-four hours later, each group was further sub-divided to receive either propranolol and etodolac, or vehicles. Thirty minutes later, all mice were inoculated through the spleen with 10^4 CT26 tumor cells. Twenty-one days later, all mice were sacrificed, their livers were removed and weighed, and surface-hepatic metastases were counted by two researchers blinded to group assignment.

3.6.1.2. Second study. The same design and procedure were used (a total of 33 male mice), with the exception that during the tumor cell injection procedure, axillary blood was withdrawn through a heparinized capillary (10–20 μl) for assessment of circulating NK cells.

3.6.2. Results

Two-way ANOVA revealed a significant main effect for depletion in both studies (First study – $F(1,40) = 17.239$, $p = 0.0002$; Second study – $F(1,29) = 40.097$, $p < 0.0001$). This indicates the effect of NK-depletion across drug treatment (Fig. 5A&B). However, given significant differences in the variance between the NK-depleted and NK-intact groups (violating an ANOVA assumption of homogeneity of variance), we assesses the effects of the drug treatment separately within each depletion category using t-tests. In both studies, drug treatment significantly reduced the number of CT26 metastases in NK-intact animals (First study – $p = 0.0093$, Fig. 5A; Second study – $p = 0.00873$, Fig. 5B), but no drug-treatment effects were evident in NK-depleted animals. In the Second study, FACS analysis revealed marked and significant group differences in the percentage of NK cells (within the lymphocyte population) between mice that were NK-depleted and those that remained NK-intact ($p = 0.0018$).

4. Discussion

In the United States alone, approximately 133,000 patients are annually diagnosed with colon or rectal cancer (CRC), and approximately 50,000 die, mostly due to metastatic disease (Siegel et al., 2015). The liver is the main target organ for metastases of CRC, and roughly two-thirds of affected patients have extrahepatic spread of metastases.

This study is the first to show the beneficial effects of the beta blocker, propranolol, and the COX-2 inhibitor, etodolac, in reducing experimental hepatic metastasis of colon cancer in the context of surgery. These findings generalize our previous findings regarding similarly beneficial effects of these drugs for lung metastasis of a mammary adenocarcinoma (Melamed et al., 2005; Benish et al., 2008), and regarding spontaneous metastasis and survival following surgical excision of primary tumors in mice bearing syngeneic orthotopic melanoma or lung carcinoma (Glasner et al., 2010).

The beneficial effects of the drugs in this study seem to be NK cells mediated. Supporting this claim are our findings that CT26 metastases are controlled by NK cells, and that NK-depletion eliminates the beneficial effects of our drug treatment. Specifically, anti-asialo GM1, which was shown herein (and elsewhere (Sorski et al., 2015)) to deplete NK cells, markedly increased CT26

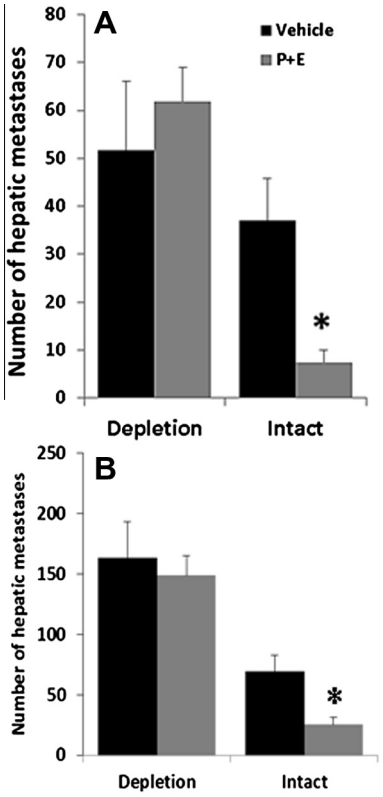

Fig. 5. NK cells are significantly involved in the beneficial effects of the combined drug treatment: (A) A total of 44 male mice were injected with either anti-asialo GM1 (n = 20), or vehicle (n = 24). Twenty-four hours later, each group was further sub-divided to receive either propranolol and etodolac, or vehicles. Thirty minutes later, all mice were inoculated through the spleen with CT26 tumor cells. Twenty-one days later, all mice were sacrificed, their livers were removed and weighed, and surface-hepatic metastases were counted. (B) The same design and procedure were used (a total of 33 male mice), with the exception that during tumor injection procedure, axillary blood was withdrawn through a heparinized capillary (10–20 ml) for assessment of circulating NK cell number. (A&B) A significant main effect for depletion was evident in both studies, which indicates the effect of NK-depletion across drug treatment. Drug treatment significantly reduced the number of CT26 metastases in NK-intact animals (*t*-test), as indicated by *, but no effects for drug treatment were evident in NK-depleted animals. Data are presented as mean ± SEM.

metastasis. CpG-C immune stimulation, which we recently shown to increase marginating-hepatic (MH)-NK cytotoxicity against the CT26 tumor line (Sorski et al., 2015), markedly reduced the number of CT26 hepatic metastases in this study. Additionally, in a recent study we showed that CpG-C exert its anti-CT26 effects in naïve, but not in NK-depleted mice. These findings strongly suggest a role for NK cells in controlling CT26 liver metastasis. Most importantly, in NK-depleted mice, our drug treatment had no beneficial effects, in contrast to their marked metastases-reducing effect in animals with intact NK cells, thus implicating NK cells as a significant mediating mechanism of the beneficial effects of these drugs.

Although anti-asialo GM1 was also reported to affect basophils and certain cell populations in the brain (Nishikado et al., 2011), it seems unlikely that its acute use herein, which led to detrimental impacts on hepatic metastases, is mediated through these mechanisms, rather than through NK cells. No study has reported a significant role for basophils in controlling metastasis.

We have already reported in a previous study that females exhibit markedly fewer CT26 metastases than males, irrespective of (i) different injection procedures used, (ii) the load of CT26 cells challenge, and (iii) the magnitude of the surgical procedure (Sorski et al., 2014). We speculated that, among other mechanisms, males may be more prone to the impact of surgery while being administered with tumor cells, thus exhibiting more metastases. However, in the current study we found that the combined use of the blockers, which we also reported to have immune-protective effects, had a similar beneficial effect in both sexes. This findings suggest that despite potential sexual dimorphism in neuroendocrine and paracrine responses to surgery, our therapeutic approach is efficacious in both sexes and in two tumor inoculation approaches, and should thus be studied in both women and men in the clinical setting.

As surgery simultaneously increases both catecholamines (CAs) and prostaglandins (PGs), and as each of these factors can suppress immunity and promote metastasis through other mechanisms (directly or indirectly affecting the malignant tissue), we believe that the combined use of propranolol and etodolac, rather than each alone, should be employed in the clinical setting, as is evident herein and in our previous preclinical studies (Benish et al., 2008; Glasner et al., 2010; Inbar et al., 2011). Specifically, either CAs or PGs can suppress NK activity through activating their respective membrane receptors on NK cells and elevating intra-cellular cAMP levels (Whalen and Bankhurst, 1990; Torgersen et al., 1997). Thus, only the simultaneous blockade of these receptors during the perioperative period would be efficacious in preventing the suppression of NK activity, as was indeed evident in a previous study in the context of surgery (Benish et al., 2008). It is worthy to note that the combined use of these drugs is considered safe and plausible in the clinical setting of oncological surgeries in patients without contraindications. We have recently initiated phase II pilot clinical studies employing propranolol and etodolac perioperatively in breast and colon cancer patients, and observed no adverse effects (Zmora et al., 2016).

Additional findings of the current study are that (i) mild surgical procedures and more extensive ones induce similar susceptibility to metastasis, and that (ii) the combined drugs treatment improves host resistance to metastasis similarly in both the mild and the extensive procedures. Specifically, in one experiment we added laparotomy to minimal incision, and in a second experiment extended the duration of laparotomy, added physical manipulations to the colon, and maintained lower room temperature during operation, all of which were reported to exacerbate the deleterious effects of surgery on various short term outcomes (Shakhar and Ben-Eliyahu, 2003). No significant differences in metastatic development were evident between mild and extensive procedures, and the drug treatment was similarly efficacious in both conditions. In previous studies we also found similar developmental courses of experimental liver metastases in this tumor model employing surgical procedures of different extent (Sorski et al., 2014). Clinical studies comparing laparotomy to laparoscopy in colorectal cancer patients also did not show significant differences in long-term cancer outcomes (Jayne et al., 2010), despite many short-term advantages to minimal invasive surgeries (Biondi et al., 2013), as was also evident herein by lower levels of corticosterone in animals subjected to small incision. Last, in a study assessing spontaneous metastatic spread and mortality rates, the blockade of catecholamines and prostaglandins during the immediate perioperative period had similar beneficial effects in mild and in more extensive surgeries (Glasner et al., 2010). Taken together, the clinical practice should consider the potential use of such prophylactic measures, also when employing minimal invasive surgeries.

Overall, the combined use of propranolol and etodolac in the perioperative context of colorectal surgeries may be a safe, inexpensive, and efficient approach to reduce long-term recurrence rates. Clinical studies employing these or similar drugs should be conducted in both laparoscopy and open-surgeries of various metastatic cancers, may be especially efficacious against colorectal liver metastasis, and may also be accompanied by a perioperative approach of immune-stimulation when feasible.

Acknowledgments

This work was supported by NIH/NCI grant # CA125456 and CA172138 (SBE) and by the Israel-USA bi-national Science Foundation # 2005331 (SBE & GGP).

References

Ben-Eliyahu, S., 2003. The promotion of tumor metastasis by surgery and stress: immunological basis and implications for psychoneuroimmunology. Brain Behav. Immun. 17 (Suppl. 1), S27–36.
Benish, M., Bartal, I., et al., 2008. Perioperative use of beta-blockers and COX-2 inhibitors may improve immune competence and reduce the risk of tumor metastasis. Ann. Surg. Oncol. 15 (7), 2042–2052.
Biondi, A., Grosso, G., et al., 2013. Laparoscopic vs. open approach for colorectal cancer: evolution over time of minimal invasive surgery. BMC Surg. 13 (Suppl. 2), S12.
Burkholder, B., Huang, R.Y., et al., 2014. Tumor-induced perturbations of cytokines and immune cell networks. Biochim. Biophys. Acta 1845 (2), 182–201.
Cole, S.W., Nagaraja, A.S., et al., 2015. Sympathetic nervous system regulation of the tumour microenvironment. Nat. Rev. Cancer 15 (9), 563–572.
Corbett, T.H., Griswold Jr., D.P., et al., 1975. Tumor induction relationships in development of transplantable cancers of the colon in mice for chemotherapy assays, with a note on carcinogen structure. Cancer Res. 35 (9), 2434–2439.
Demicheli, R., Valagussa, P., et al., 2001. Does surgery modify growth kinetics of breast cancer micrometastases? Br. J. Cancer 85 (4), 490–492.
Elenkov, I.J., Wilder, R.L., et al., 2000. The sympathetic nerve–an integrative interface between two supersystems: the brain and the immune system. Pharmacol. Rev. 52 (4), 595–638.
Eschwege, P., Dumas, F., et al., 1995. Haematogenous dissemination of prostatic epithelial cells during radical prostatectomy. Lancet 346 (8989), 1528–1530.
Fisher, B., Gunduz, N., et al., 1989. Presence of a growth-stimulating factor in serum following primary tumor removal in mice. Cancer Res. 49 (8), 1996–2001.
Glasner, A., Avraham, R., et al., 2010. Improving survival rates in two models of spontaneous postoperative metastasis in mice by combined administration of a beta-adrenergic antagonist and a cyclooxygenase-2 inhibitor. J. Immunol. 184 (5), 2449–2457.
Goldfarb, Y., Sorski, L., et al., 2011. Improving postoperative immune status and resistance to cancer metastasis: a combined perioperative approach of immunostimulation and prevention of excessive surgical stress responses. Ann. Surg. 253 (4), 798–810.
Horowitz, M., Neeman, E., et al., 2015. Exploiting the critical perioperative period to improve long-term cancer outcomes. Nat. Rev. Clin. Oncol.
Inbar, S., Neeman, E., et al., 2011. Do stress responses promote leukemia progression? An animal study suggesting a role for epinephrine and prostaglandin-E(2) through reduced NK activity. PLoS ONE 6 (4), e19246.
Jayne, D.G., Thorpe, H.C., et al., 2010. Five-year follow-up of the Medical Research Council CLASICC trial of laparoscopically assisted versus open surgery for colorectal cancer. Br. J. Surg. 97 (11), 1638–1645.
Kasai, M., Yoneda, T., et al., 1981. In vivo effect of anti-asialo GM1 antibody on natural killer activity. Nature 291 (5813), 334–335.
Lee, J.W., Shahzad, M.M., et al., 2009. Surgical stress promotes tumor growth in ovarian carcinoma. Clin. Cancer Res. 15 (8), 2695–2702.
Melamed, R., Rosenne, E., et al., 2005. Marginating pulmonary-NK activity and resistance to experimental tumor metastasis: suppression by surgery and the prophylactic use of a beta-adrenergic antagonist and a prostaglandin synthesis inhibitor. Brain Behav. Immun. 19 (2), 114–126.
Moreno-Smith, M., Lutgendorf, S.K., et al., 2010. Impact of stress on cancer metastasis. Future Oncol. 6 (12), 1863–1881.
Neeman, E., Ben-Eliyahu, S., 2013. Surgery and stress promote cancer metastasis: new outlooks on perioperative mediating mechanisms and immune involvement. Brain Behav. Immun. 30 (Suppl.), S32–40.
Neeman, E., Zmora, O., et al., 2012. A new approach to reducing postsurgical cancer recurrence: perioperative targeting of catecholamines and prostaglandins. Clin. Cancer Res. 18 (18), 4895–4902.
Nishikado, H., Mukai, K., et al., 2011. NK cell-depleting anti-asialo GM1 antibody exhibits a lethal off-target effect on basophils in vivo. J. Immunol. 186 (10), 5766–5771.
Rainsford, K., 1996. Advances in Anti-Rheumatic Therapy. CRC Press.
Rosenne, E., Sorski, L., et al., 2014. In vivo suppression of NK cell cytotoxicity by stress and surgery: glucocorticoids have a minor role compared to catecholamines and prostaglandins. Brain Behav. Immun. 37, 207–219.
Shakhar, G., Ben-Eliyahu, S., 1998. In vivo beta-adrenergic stimulation suppresses natural killer activity and compromises resistance to tumor metastasis in rats. J. Immunol. 160 (7), 3251–3258.
Shakhar, G., Ben-Eliyahu, S., 2003. Potential prophylactic measures against postoperative immunosuppression: could they reduce recurrence rates in oncological patients? Ann. Surg. Oncol. 10 (8), 972–992.
Shakhar, G., Blumenfeld, B., 2003. Glucocorticoid involvement in suppression of NK activity following surgery in rats. J. Neuroimmunol. 138 (1–2), 83–91.
Siegel, R.L., Miller, K.D., et al., 2015. Cancer statistics, 2015. CA Cancer J. Clin. 65 (1), 5–29.
Sorski, L., Levi, B., et al., 2014. The impact of surgical extent and sex on the hepatic metastasis of colon cancer. Surg. Today 44 (10), 1925–1934.
Sorski, L., Melamed, R., Lavon, H., Matzner, P., Rosenne, E., Ben-Eliyahu, S., 2015. Acute and innocuous CpG-C immune stimulation potentiates host resistance to hepatic metastases of colon cancer and protects against immunosuppressive effects of surgery. In: PNIRS Annual Scientific Meeting. Seattle.
Srivastava, S., Lundqvist, A., et al., 2008. Natural killer cell immunotherapy for cancer: a new hope. Cytotherapy 10 (8), 775–783.
Stolina, M., Sharma, S., et al., 2000. Specific inhibition of cyclooxygenase 2 restores antitumor reactivity by altering the balance of IL-10 and IL-12 synthesis. J. Immunol. 164 (1), 361–370.
Terunuma, H., Deng, X., et al., 2008. Potential role of NK cells in the induction of immune responses: implications for NK cell-based immunotherapy for cancers and viral infections. Int. Rev. Immunol. 27 (3), 93–110.
Torgersen, K.M., Vaage, J.T., et al., 1997. Selective activation of cAMP-dependent protein kinase type I inhibits rat natural killer cell cytotoxicity. J. Biol. Chem. 272 (9), 5495–5500.
Watkins, J.L., Thaker, P.H., et al., 2015. Clinical impact of selective and nonselective beta-blockers on survival in patients with ovarian cancer. Cancer 121 (19), 3444–3451.
Weitz, J., Herfarth, C., 2001. Surgical strategies and minimal residual disease detection. Semin. Surg. Oncol. 20 (4), 329–333.
Whalen, M.M., Bankhurst, A.D., 1990. Effects of beta-adrenergic receptor activation, cholera toxin and forskolin on human natural killer cell function. Biochem. J. 272 (2), 327–331.
Yakar, I., Melamed, R., et al., 2003. Prostaglandin e(2) suppresses NK activity in vivo and promotes postoperative tumor metastasis in rats. Ann. Surg. Oncol. 10 (4), 469–479.
Yamaguchi, K., Takagi, Y., et al., 2000. Significant detection of circulating cancer cells in the blood by reverse transcriptase-polymerase chain reaction during colorectal cancer resection. Ann. Surg. 232 (1), 58–65.
Zmora, O., Shaashua, L., 2016. The perioperative use of a beta-adrenergic blocker and a COX-2 inhibitor in colorectal cancer patients for the prevention of cancer recurrence. A preliminary study assessing feasibility and safety. 23rd Annual PNIRS Scientific Meeting, Brighton, UK, BBI.