Published in final edited form as:

Brain Behav Immun. 2010 August ; 24(6): 952–958. doi:10.1016/j.bbi.2010.03.010.

Synergism between immunostimulation and prevention of surgery-induced immune suppression: An approach to reduce postoperative tumor progression

Roi Avraham, Marganit Benish **, Shelly Inbar, Inbal Bartal, Ella Rosenne, and Shamgar Ben-Eliyahu

Department of Psychology, Tel Aviv University, Tel Aviv, Israel

Abstract

Background—A unique opportunity to eradicate cancer is presented immediately after the excision of the primary tumor, but surgical procedures often induce the release of immunosuppressing factors that render cell mediated immunity ineffective. Here we tested the hypothesis that integration of peri-operative immunostimulation and blockade of immunosuppression could synergistically improve postoperative anti-metastatic immunity and long-term survival.

Methods—Two syngeneic tumor models in F344 rats were employed, studying postoperative tumor progression. In the first model, survival following laparotomy and CRNK-16 leukemia was studied. Rats were perioperatively treated with the immuno-stimulant poly I-C (5×0.2mg/kg/inj), with catecholamine- and prostaglandin-blockers (shown to prevent postoperative immunosuppression: 4.5mg/kg nadolol, 4mg/kg indomethacin), with both interventions, or with neither. Long-term survival was assessed thereafter. The second model used the MADB106 mammary adenocarcinoma, assessing its lung tumor retention (LTR) following i.v. inoculation, as well as host marginating-pulmonary NK numbers and activity against this tumor. IL-12 was employed for immunostimulation (4×1.5µg/kg/inj), with and without the above blockers.

Results—Postoperative CRNK-16 survival rates were significantly improved only by the integrated approach of immune stimulation and endocrine blockers. Postoperative MADB106 LTR was additively reduced by the two interventions. Importantly, while IL-12 increased pulmonary NK cytotoxicity against MADB106, surgery markedly suppressed this cytotoxicity in both IL-12 and vehicle treated animals. The blockers prevented this suppression per lung and per single NK cell.

Conclusions—Immunostimulation could be rendered ineffective postoperatively due to immunosuppression; therefore integrating endocrine-blocker therapies into the realm of perioperative immunotherapy could optimize immune control over residual disease, potentially improving clinical outcomes.

Keywords

immunotherapy; cancer; NK cells; endocrinology; surgery; rats

Corresponding Author: Shamgar Ben-Eliyahu, psychoneuroimmunology Research Unit, Dept of Psychology, Tel Aviv University, Tel Aviv, 69978, Israel, Tel: 972-3-640-7266, Fax: 972-3-640-9520/9547, Homepage: <http://freud.tau.ac.il/~shamgar/>.

**The first two authors have contributed equally to this study

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Introduction

The greatest threat to cancer patients is the recurrence of the disease, rather than the damage associated with the often removable primary tumor (Shakhar and Ben-Eliyahu 2003). Metastatic recurrence is most prevalent, and these malignant foci tend to be more aggressive, less reactive to therapy and often inoperable. Postoperative metastases develop from minimal residual disease (MRD), which is comprised of pre-existing micrometastases and scattered tumor cells left after the excision of the primary tumor. Unfortunately, the surgical removal of the primary tumor promotes the release of tumor emboli and induces temporary physiological conditions that favor the survival and progression of MRD (Eschwege et al. 1995; Folkman 1995; Da Costa et al. 1998; Yamaguchi et al. 2000; Lee et al. 2009). To this end, the perioperative period has been suggested to be critical for determining long-term recurrence rates, and presents an opportunity to improve clinical outcomes.

Immune stimulation has been studied in cancer patients for decades, and various approaches have been used to limit side effect and improve efficacy (for review see (Smyth et al. 2001; Del Vecchio et al. 2007)). Among potential obstacles, immunosuppression induced by some tumors was reported to dampen the efficacy of immunostimulation. This has led to the realization that the context of immuno-stimulatory therapy is crucial, and some researchers study immunotherapy for eradicating MRD in the later post-operative context (for review see (Da Costa et al. 1998)).

However, the immediate postoperative period seems most critical for eradicating MRD before uncontrollable metastases are established (Shakhar and Ben-Eliyahu 2003). Unfortunately this period is often characterized by marked immunosuppression caused by the surgical procedure itself. It is our hypothesis that successful peri-operative immunotherapeutic approaches are rendered ineffective by this suppression, further contributing to the limited success of immunostimulatory regimens.

Therefore, in the current study we sought to investigate the feasibility of integrating immunostimulatory therapy with pharmacological interventions that prevent postoperative immunosuppression. We recently reported that pharmacological blockade of catecholamines and prostaglandins (endocrine-blocker therapy), using a non-selective β -blocker (nadolol), and a COX inhibitor (indomethacin), can reduce postoperative immunosuppression and promotion of metastasis induced by surgery (Melamed et al. 2005). In the current study, we assessed the efficacy of immunostimulation (by polyinosate-polycytidylate (poly I-C) or IL-12), alone and in combination with the above endocrine-blocker therapy, in controlling tumor progression after surgery. To this end we employed two syngeneic tumor models in F344 rats; the first is oriented toward assessing the biological significance of such interventions, is based on the CRNK-16 leukemia line, and assess long-term survival rates. Although surgery is clearly not the therapy used to treat leukemia patients, this model was used because it simulates progression of blood-borne malignant cells implanted orthotopically, and assesses survival rates – the most important clinical outcome. This malignancy also constitutes a major cause of natural death in this strain of rats (Reynolds et al. 1984), and is sensitive to NK cells and to other immune functions (Avraham et al. 2006). Last, the model was shown to reflect the effects of stress on survival rates (Avraham et al. 2006; Ben-Eliyahu et al. 2007), as was also suggested to be the case in leukemia patients (Tschuschke et al. 2001; Hoodin and Weber 2003). The second model is used to elucidate mechanisms underlying the potential synergism between immunotherapy and endocrine blockers, including the involvement of NK cells. It is based on the non-immunogenic NK-sensitive MADB106 tumor line, and is used here in two complementary approaches: in vivo, by assessing resistance to experimentally induced lung metastases, and in vitro (ex-vivo) as a target for NK cytotoxicity assays. IL-12 was used as an immunostimulatory agent in this model given its known potency as an NK activator (Trinchieri

1998). Together, these studies offer an insight into host- and immune-tumor interactions in a context of postoperative control of MRD, and demonstrate the potential benefits of the suggested interventions.

MATERIALS AND METHODS

Animals

Fischer 344 rats (F344) (Harlan SD, Jerusalem) were housed 4 per cage under a 12:12 hr light/dark cycle, with free access to food and water. Animals were acclimatized to the vivarium for at least 4 weeks prior to the beginning of experimentation and were 12 to 16 weeks old at that time. All experiments were approved by the Institutional Animal Care and Use Committee of Tel Aviv University.

Experimental design

All rats were handled daily for 4 days before the experiment. The order of drug administration, tumor injection and lung collection were counterbalanced across groups.

In the CRNK-16 survival model, animals were allocated to four groups that were treated with (i) poly I-C, (ii) nadolol and indomethacin (endocrine-blocker treatment), (iii) a combination of the two treatments, or (iv) served as controls that were administered with the corresponding vehicles at the same schedule of injection. One hour after the endocrine-blocker treatment, all animals underwent laparotomy. At the end of the surgical procedure, CRNK-16 cells were injected i.v. to all animals. A total of 131 animals were used in the study (see table 1).

In the study employing the MADB106 tumor, a 2×4 design was used in which animals were either operated or not and subjected to one of the following four treatments: immunostimulation with IL-12, endocrine-blocker treatment, a combination of the two treatments, or treated with vehicle (controls). At the end of the surgical procedure, radio-labeled MADB106 cells were injected i.v. to all animals. This study was conducted in three identical replications (each containing all groups) to accumulate a total of 99 animals (n=33, 34, 32 in the three replicas).

Tumor cells

The **CRNK-16** cell line was maintained in complete medium (RPMI 1640 supplemented with 10% heat-inactivated FCS, 50µg/ml gentamicin, 2 mM L-glutamine, 0.1 mM non-essential amino acids and 1 mM sodium pyruvate) at 100% humidity, 5% CO₂ at 37°C. CRNK-16 was administered i.v. at a dose of 60 cells per rat.

MADB106 is a selected variant cell line obtained from a pulmonary metastasis of a mammary adenocarcinoma (MADB100) chemically induced in the inbred F344 rat (Barlozzari et al. 1983; Barlozzari et al. 1985). The number of tumor cells retained in the lungs following i.v. inoculation are highly dependent on NK activity (Barlozzari et al. 1983; Barlozzari et al. 1985; Ben-Eliyahu 1992; Ben-Eliyahu et al. 1996; Shakhar and Ben-Eliyahu 1998). This line also served as target cells in the cytotoxicity assay. The MADB106 cell line was maintained in complete medium (RPMI 1640 medium, Biologic Industries, Beit-Haemek, Israel) at 5% CO₂, 100% humidity, at 37 °C. MADB106 were grown in monolayer cultures and separated from the flask using 0.25% trypsin.

Radiolabeling of MADB106 tumor cells and assessment of lung tumor retention (LTR)—DNA radiolabeling of tumor cells was accomplished by adding 0.5 µCi/ml of ¹²⁵Iododeoxyuridine (¹²⁵IDUR, Danyel Biotech, Rehovot, Israel) to the growing cell culture one day before harvesting the cells for injection. Rats were lightly anesthetized with halothane (Rhone-Poulenc, Bristol, UK), and 2×10⁵ radiolabeled cells in 0.5 ml of PBS

supplemented with 0.1% BSA were injected into their tail vein. Twelve hours later, rats were euthanized with halothane, and their lungs were removed and placed in a γ -counter for assessment of radioactive content. Percentage of tumor cell retention was calculated as the ratio between radioactivity measured in the lungs and the total radioactivity in the injected cell suspension. Our previous studies have indicated that the levels of lung radioactivity reflect the numbers of viable tumor cells in the lungs that are expected to form solid metastasis (Ben-Eliyahu 1992; Ben-Eliyahu et al. 1996; Shakhar and Ben-Eliyahu 1998; Bar-Yosef et al. 2001).

Recording survival following CRNK-16 administration

Rats were inspected for signs of morbidity once or twice daily, until day 80 post tumor inoculation. In our previous studies using this tumor model, we kept animals for three additional months and recorded only 2 cases of mortality in more than 200 animals. Thus, rats that survived this period are unlikely to show CRNK-16 related morbidity later.

Animals were defined as showing signs of morbidity given two or more of the following criterion: more than 10% reduction in body weight, apathy, dehydration, red circles around the eyes, overt inactivity, or paralysis. In such cases, animals were euthanized and inspected to verify the development of metastases. Based on our experience, these symptoms characterize a progressive phase of solid tumors, causing death in the ensuing 24–48 hours. Thus, these animals were included in the mortality report, and considered to have died on the next day. Animals that were euthanized or survived the 80 day period were inspected for solid tumors or irregularity in internal organs (specifically spleen, liver, kidney, all organs in the chest cavity, and for bone softness). None of the survivors showed any signs of illness, while all rats that were euthanized exhibited one or more signs of illness, most commonly solid tumors.

Materials

Poly I-C (Sigma, Rehovot, Israel) is a synthetic double stranded RNA which is recognized by Toll-like receptor-3 and elicits an immune response resembling viral infections. Poly I-C was administered repeatedly (0.2mg/kg i.p.) 5, 3 and 1 days prior to tumor administration, and 1 and 3 days following it.

Lyophilized recombinant murine **IL-12** (rmIL-12) (1×10^7 units/mg, purchased from Cytolab Ltd., Rehovot, Israel) was reconstituted in phosphate-buffered saline (PBS) according to manufacturer's instructions. To achieve stable circulating level, rmIL-12 was administered subcutaneously in a slowly release preparation (emulsion constituting 4 parts of PBS, 3 parts of mineral oil, and 1 part of mannide-monooleate – a non specific surface active emulsifier (all purchased from Sigma, Rehovot, Israel)). Based on our previous examination, using various compounds, this emulsion is absorbed in approximately 36 to 48 hrs. Injections were given every other day for a period of 8 days (0.5 μ g/rat/0.5 ml injection).

The non-selective β -adrenergic blocker, **nadolol** (Sigma, Rehovot, Israel), was used to block β -adrenoceptor stimulation. Nadolol (s.c., 4.5 mg/kg) was administered in a slowly released preparation (see above) 1 h before surgery.

The prostaglandin-synthesis inhibitor, **indomethacin** (Sigma, Rehovot, Israel), was dissolved in propylenglycol and administered i.p. (4 mg/kg) 1 h before surgery.

Experimental laparotomy

Anesthesia was induced by halothane, and maintained at 2–3% halothane concentration using a vaporizer. Rats breathed spontaneously throughout the anesthesia. After hair trimming and scrubbing with alcohol, a four-centimeter midline abdominal incision was performed, and the

small intestines were externalized and rubbed with PBS-soaked gauze at four places. The intestines were then covered with PBS-soaked gauze, and the abdomen remained open for one hour until closure (in one layer) with 4–5 nylon thread sutures. Rats were then removed from the anesthesia and awoke spontaneously in their home cage.

In vitro assessment of cytotoxicity

The standard 4-h ^{51}Cr release assay was used to assess leukocyte anti-tumor cytotoxicity against MADB106 syngeneic tumor cells. Effector cells were serially diluted and co-incubated, at different effector to target (E:T) ratios, with a fixed number of target cells (5000 per well).

Preparation of marginating-pulmonary (MP) effector cells—Rats were euthanized with an overdose of halothane, their thoracic cavities were opened. To harvest leukocytes adhering to the pulmonary endothelium (MP leukocytes), we perfused the lungs by injecting heparinized PBS (30 U/ml) into the right ventricle (approximately 0.5 ml/sec) and collecting 30 ml of perfusate from the left ventricle. To avoid contamination with circulating blood, we discarded the first 3 ml of perfusate. The perfusate was then centrifuged ($300\text{g} \times 10 \text{ min}$), concentrated to approximately 0.5 ml, washed in 4 ml complete media, and concentrated to a final volume of 1 ml. Assessment of NK cytotoxicity was described elsewhere (for details see (Melamed et al. 2005)).

Assessment of cytotoxicity per MP compartment—To create eight different effector to target (E:T) ratios, effector cells were serially diluted five times beginning at their original concentration, and co-incubated with a fixed number of target cells. Specifically, aliquots of 150 μl of effector cell suspension from lung perfusate were placed in wells of a microtiter plate and serially diluted to create the E:T ratios. 5000 target cells in 100 μl complete media were then added to each well. Plates were centrifuged ($400\text{g} \times 10 \text{ min}$) and incubated at 37°C for 4 h. Plates were then centrifuged again and 100 μl of supernatant was removed from each well and counted in a gamma counter to determine experimental release. Spontaneous release was obtained from wells receiving target cells and media only, and total release was obtained from wells receiving 1% Triton X-100. Percent cytotoxicity was calculated by the following formula: Percent cytotoxicity = $100 \cdot [(\text{experimental release} - \text{spontaneous release}) / (\text{total release minus spontaneous release})]$. The results present the average of the four highest E:T ratios.

Assessment of MP cytotoxicity per-NK-cell—In order to attain equivalent NK:target ratios in all samples, lung perfusate samples were diluted with complete medium based on FACSscan enumeration of NK cells per sample. The NK concentration in each sample was brought to the highest of 100, 200, 400 or 800 NK/ μl . Following this procedure, NK cytotoxicity was assessed as described above. The results present the average of the four highest E:T ratios.

Radiolabeling of MADB106 target cells for cytotoxicity assays—MADB106 cells were removed from the culture flask with trypsin solution (0.25% in PBS), and were washed with complete medium. The cells (5×10^6) were incubated for 1.5 h with 100 μCi ^{51}Cr (in 100 μl saline), 100 μl FCS, and 50 μl complete medium. Following incubation, cells were washed three times in complete medium ($300\text{g} \times 10 \text{ min}$) and adjusted to the desired concentration in complete medium.

Flow cytometry

The FACS analysis method was already described elsewhere (Melamed et al. 2005). The FACS (Fluorescence Activated Cell Sorter) analysis was used to identify NK cells. NK cells were

identified as NKR-P1^{bright} (CD161^{bright}) lymphocytes using FITC-conjugated anti-NKR-P1 (Pharmingen, San Diego).

Statistical analysis

For the CRNK-16 study, the survival Kaplan-Meier model was used followed by the Tarone-Ware test for pair-wise group comparisons. For the MADB106 study, results were analyzed using a two-way analysis of variance (ANOVA) with main factors of surgery (operated and non-operated groups) and drug (Vehicle, IL-12, endocrine-blocker treatment, and combined). In the ex-vivo experiments, the average of the three highest E:T ratios was considered for statistical analysis. Provided that ANOVA indicated significant group differences, post-hoc contrasts (Fisher's PLSD) were conducted to compare specific groups. $p < 0.05$ was considered significant in all studies.

Results

The CRNK-16 survival model: Survival rates following laparotomy are improved by the combined immunostimulation-endocrine-blocker therapy

As shown in Figure 1, poly I-C alone and endocrine-blocker therapy alone were not sufficient to cause a significant improvement in survival rates relative to vehicle treatment. However, the combined treatment significantly decreased mortality rate from 65% in the vehicle group to 15% in the combined treatment group (Kaplan-Meier test, Tarone-Ware = 19.784, $p < 0.0001$). The combined treatment group also showed significantly and markedly greater survival rates than the poly I-C group and the endocrine-blocker group (Kaplan-Meier test, Tarone-Ware = 6.461, 10.731, respectively, $p < 0.05$). No difference was evident between the Poly-IC treated group and the endocrine-blocker treated group.

The MADB106 experimental metastasis and NK activity model: *In-vivo* and *ex-vivo* results demonstrate a superior effect of the combined immunostimulation-endocrine-blocker therapy in reducing the deleterious effects of surgery

The results are evident in Figure 2A. Two-way ANOVA revealed a main effect for surgery ($F_{1,91}=32.344$, $p<0.0001$), a main effect for drug ($F_{3,91}=6.515$, $p<0.005$) and an interaction ($F_{3,91}=5.408$, $p<0.018$). Post-hoc contrasts revealed that surgery caused a significant 7-fold increase in lung tumor retention (LTR) compared to the no-surgery vehicle (baseline) group. Immunostimulation by IL-12 alone, and endocrine-blocker treatment alone, each significantly reduced this effect to approximately 3-fold ($p<0.019$, $p<0.0003$ respectively). The combined use of the treatments was significantly more efficacious than each treatment alone ($p<0.0227$ compared to IL-12, and $p<0.0002$ compared to endocrine-blocker treatment), reducing the effect of surgery to baseline levels. In non-operated rats, both IL-12 treatments, but not endocrine-blocker treatment alone, reduced LTR to 50% of baseline levels ($p<0.0072$ for IL-12 treatment and $p<0.0138$ for the combined treatment).

As indicated in Figure 2B, the ex-vivo cytotoxicity of the entire lung perfusate against MADB106 target cells is almost an exact mirror image of the above in-vivo results of LTR (Figure 2A). This suggests that NK activity of the MP compartment is a crucial mediator of the in vivo resistance to MADB106 metastasis, as was also supported by our previous studies (Shakhar and Blumenfeld 2003; Melamed et al. 2005). ANOVA revealed main effects for surgery ($F_{1,91}=44.857$, $p<0.0001$) and for drug ($F_{3,91}=6.286$, $p<0.004$). Post-hoc comparisons revealed that surgery caused a significant decrease in cytotoxicity compared to baseline levels ($p<0.0001$). IL-12 alone, and endocrine-blocker treatment alone, each reduced this effect ($p<0.05$, $p<0.035$ accordingly), and the combined use of the two treatments was significantly more efficacious than each of the treatments alone ($p<0.0049$ for IL-12, and $p<0.0029$ for endocrine-blocker treatment), elevating cytotoxicity in the operated groups to baseline levels.

In non-operated rats, only the combined treatment significantly increased NK activity ($p<0.016$).

***In vitro* study of marginating-pulmonary (MP)-NK cytotoxicity: IL-12 and endocrine-blocker therapy act through different but complementary cellular mechanisms in improving postoperative MP-NK activity**

To explore cellular mediating mechanisms, we also assessed the numbers of NK cells in the MP compartment using FACSScan analysis, as well as empirically studied their cytotoxicity per-NK-cell. The latter was accomplished by standardizing the concentration of NK cells in each sample before testing cytotoxicity.

Figure 3A presents cytotoxicity per MP-NK cell against MADB106 target cells. ANOVA revealed main effects for surgery ($F_{1,91}=65.598$, $p<0.0001$) and for drugs ($F_{3,91}=7.594$, $p<0.004$). Post-hoc analysis revealed that in non-operated rats, no treatment had a significant effect on NK cytotoxicity per NK cell. Surgery, on the other hand, significantly decreased cytotoxicity in vehicle treated animals ($p<0.0001$). Importantly, in operated rats, while the IL-12 treatment did not significantly improved cytotoxicity per NK cell compared to vehicle treatment ($p=0.2998$), endocrine-blocker treatment reduced the effects of surgery ($p<0.0001$), and the combined treatment was more efficacious than endocrine-blocker treatment alone ($p<0.0178$), increasing cytotoxicity per NK cell to baseline level ($p=0.4128$ compared to no-surgery vehicle).

Figure 3B presents numbers of MP-NK cells based on FACSScan analysis. ANOVA revealed no main effect for surgery but revealed a main effect for drug ($F_{3,91}=7.139$, $p<0.0002$). Post-hoc tests revealed that in comparison to the control groups, endocrine-blocker treatment did not significantly affect NK numbers ($p=0.26$), whereas the IL-12 treatments (with or without endocrine-blocker treatment) caused a profound 3-fold increase in NK numbers ($p<0.02$ for each of the treatments).

While Fig. 3A indicates cytotoxicity per MP-NK cell, the resistance of the host to MADB106 lung metastasis clearly also depends on the overall number of MP-NK cells. It is thus not a surprise that if one factors the empirical results of cytotoxicity per MP-NK cell (Fig. 3A) by their number per lung (Fig. 3B), the conceptual outcome, which is whole lung cytotoxicity, is very similar to our empirical assessment of this index evident in Fig. 2B. Most important, however, is that these two factorial constituents of whole lung cytotoxicity that we assessed empirically (cytotoxicity per NK cell, and number of NK cells per lung) point at different mechanisms via which each treatment contributes to the overall postoperative preservation of lung resistance to MADB106 tumor cells. While IL-12 acts mainly by increasing numbers of MP-NK cells without affecting their cytotoxicity per cell, endocrine-blocker treatment prevents their suppression on a per-NK-cell basis with minimal impact on their numbers.

Discussion

In the current study we demonstrated the feasibility and the benefits of integrating endocrine-blocker treatment with immunotherapy to improve postoperative immunity and survival rates. In both tumor models employed herein, surgery rendered immunotherapy relatively ineffective during the immediate post-operative period, when we believe it is critical to control MRD (Shakhar and Ben-Eliyahu 2003). The integration of the endocrine-blocker therapy with an immunostimulatory approach increased long-term survival rates in the CRNK-16 model, and completely abolished the deleterious effects of surgery on NK activity and LTR in the MADB106 model.

Potential cellular mechanisms underlying these beneficial interactions are suggested by the ex-vivo studies, targeting the MP-immune compartment. This compartment is a site where metastases of various cancer types are established, as the tight vasculature dictates a slow circulation and an opportunity for extravasation. Specifically relevant to the current study, our previous research indicated that MP-leukocytes exhibit a significant NK cytotoxicity against the syngeneic MADB106 tumor, whereas circulating leukocytes fail to show such activity (Melamed et al. 2005; Benish et al. 2008). Similarly, activated leukocytes are found in other immune compartments, including Pit cells in the liver that guard yet another common organ for metastases. Such immunocytes may be affected by surgery and the interventions presented in this study similarly to MP leukocytes. Immunostimulation by IL-12 increased numbers of MP-NK cells in both operated and control rats, leading to enhanced total lung NK activity. However, surgery markedly suppressed MP-NK cytotoxicity per lung and per-NK-cell to a similar degree in IL-12 treated and non-treated rats. The endocrine-blocker therapy markedly protected individual NK cells from immunosuppression, enabling the beneficial impacts of IL-12 to materialize. These findings are sufficient to explain the in vivo synergism between IL-12-immunostimulation and endocrine-blocker therapy, as seen in the LTR index.

The mechanisms via which surgery causes suppression of cell mediated immunity (CMI), NK cells in particular, include alterations in various cytokines and other soluble factors. As demonstrated by our current and previous studies (Melamed et al. 2005; Benish et al. 2008), catecholamines (CAs) and prostaglandins (PGs) are key in vivo mediators of these complex processes. Studies have shown that the ligation of CA- and PG-membrane receptors on cytotoxic NK and T cells increases intracellular cAMP levels (Whalen and Bankhurst 1990; Malygin et al. 1993). This, in turn, was shown to inhibit perforin- and FasL-mediated mechanisms of NK cytotoxicity (Raskovalova et al. 2005), and to induce the expression of inhibitory CD94/NKG2A NK receptors (Zeddou et al. 2005). Additionally, CAs and PGs were reported to shift the cytokine balance toward a T_H2 dominance, leading to suppression of cellular immunity (Woiciechowsky et al. 1998; Woiciechowsky et al. 1999).

Our current results and previous studies (Schwartz et al. 2007) clearly indicate that in vivo immunostimulation with IL-12 does not protect individual NK cell cytotoxicity against inhibition by surgery. Interestingly, our previous studies showed that the regimen of poly I-C used herein increased the numbers of MP-NK cells, as well as reduced the suppression of MP-NK caused by surgery (Rosenne et al. 2007; Shakhari et al. 2007). Nevertheless, in the current study the integration of the endocrine blockers with the poly I-C regimen still had an additional beneficial value that was evident in terms of survival rates following CRNK-16 challenge. Thus, integration of endocrine-blocker treatments into various immunotherapeutic approaches may hold benefits in clinical settings that potentially relay on additional mechanisms that were not directly explored in the current study. For example, Sood et al. reported that an adrenergic blocker reduced vascular endothelial growth factor (VEGF) secretion by malignant human cells, as well as their tissue invasiveness and growth (Sood et al. 2006; Thaker et al. 2006; Lee et al. 2009).

Several aspects of immunotherapy, which are employed in contexts other than surgery, can also be complemented by and benefit from the proposed endocrine-blocker treatment. First, the ongoing proliferative and secretory impact of immunostimulation on immunocytes depends on intracellular processes that could be inhibited by CAs and PGs. Specifically, PGs were shown to inhibit the production of IL-12 by immunocytes following LPS stimulation (van der Pouw Kraan et al. 1995). CAs and PGs were also shown to reduce the production of $IFN\gamma$ by NK cells (Lala et al. 1986; Elenkov et al. 2000; Walker and Rotondo 2004; Kim et al. 2005), which is a key mediator of several immunostimulatory approaches (Billiau et al. 1998) and the proliferation of NK cells (Walzer et al. 2005). Both CAs and PGs are abundant in cancer patients undergoing immunotherapy due to their secretion by tumor cells (Mitsuhashi et al.

2004) or sympathetic physiological and psychological stress responses. Thus, the endocrine-blocker therapy used in this study may potentiate the efficacy of immunotherapy along its course and in the presence of a primary tumor.

Second, immunostimulatory agents are known to cause endocrine responses, including corticosteroid and CA release (Harbour-McMenamin et al. 1985; Smith and Blalock 1988; Hasko et al. 1995; Pruett et al. 2003), which can eventually cause immunosuppression. This could represent a self-limiting mechanism of immunostimulation. Indeed, the current study demonstrated that IL-12, which is known to enhance NK activity in vitro (Chehimi et al. 1993), had no beneficial effect on total MP-NK cytotoxicity in non-operated rats (see Fig. 2B) despite a 3-fold increase in numbers of MP-NK cells. Clearly, subjecting NK cells to immunostimulation in the in vivo milieu can involve different and additional processes not occurring in vitro. However, the addition of endocrine-blocker treatments to IL-12 treatment in non-operated rats, albeit during the last 12 hr of immunotherapy, significantly increased baseline levels of cytotoxicity (Fig. 2B). This might be due to the reduction of self-limiting impacts of IL-12 that are mediated by IL-12-induced secretion of CAs or PGs. This hypothesis may also explain the apparently more than additive effect of the combined treatment in operated rats with respect to cytotoxicity per-NK-cell (see Fig. 3A).

Third, although this study focused on the effects of IL-12 treatment on NK cells, other effector cells (e.g. B, T and NKT cells (Smyth et al. 2001)) were also shown to be suppressed following surgery (Faist et al. 1996; Elenkov et al. 2000) and in response to CAs and PGs (Elenkov et al. 2000). Thus, integrating endocrine-blocker treatment with other immunotherapeutic agents and approaches might prove beneficial.

Each of the two tumor models (MADB106 and CRNK-16) used in this study demonstrated the beneficial effects of the proposed combination of immunotherapy and endocrine blockage. However, one clearly cannot infer about mechanisms involved in one model based on mechanisms in the other model. Specifically, while the MADB106 LTR and ex-vivo MP-NK activity levels are highly related to each other, they cannot be used to infer about mechanisms relevant to the CRNK-16 tumor model. Although the latter was found to be sensitive to NK cells, it is also sensitive to other immune functions, and has a different time course of in vivo sensitivity and organ-target specificity compared to the MADB106 model, to name only few differences between the models. As specified in the Introduction, the advantage of the CRNK-16 model is its ability to indicate the biological significance of the findings in terms of the most important clinical outcome, namely survival rates. Its obvious shortcoming with respect to the clinical generalizability of the findings is that leukemia patients commonly do not undergo surgery. Thus, further studies are warranted to strengthen the clinical relevance of our approach, as would be the case for any tumor model.

The current study and current literature offer the following insights regarding immunostimulatory therapy in the short peri-operative clinical setting: a) for effective eradication of MRD, immunotherapy should precede surgery, as the immediate aftermath of surgery is critical for elimination of MRD (Avraham et al. 2004; Avraham et al. 2006); b) Because immunotherapy is often efficient against the primary tumor and isolated malignant cells even before surgery, it most likely increases the selection pressure of the immune system on the malignant tissue, rendering postoperative MRD more resistant to immune control (Dunn et al. 2002). Thus, in order to minimize the establishment of new tumor escape mechanisms caused by immunostimulation, the pre-operative immune intervention should be initiated in proximity to the surgical excision of the primary tumor; c) many studies have tried to overcome the limited efficacy of immunostimulatory agents by increasing their amounts or prolonging their administration (Colombo and Trinchieri 2002), approaches that have often lead to dangerous toxicity (Cohen 1995). As suggested herein, the integration of endocrine-blocker

treatment into the context of immunotherapy presents yet another approach to improve immunotherapy without exacerbating side effects.

We conclude that in the perioperative setting, immunotherapy in and of itself may not protect against suppression caused by surgery, and may be rendered ineffective by such immunosuppression. Combining endocrine-blocker therapy with immunotherapeutic agents is expected to attenuate postoperative immunosuppression, thus enabling potentially effective immunotherapies to promote the eradication of MRD. The use of endocrine-blocker therapy may also benefit immunotherapy by counteracting self-limiting mechanisms and attenuating the impact of various immunosuppressive factors before surgery. Overall, it seems that the fields of immunotherapy and neuro-endocrine regulations of immunity have matured to a state in which their reciprocal interactions should be considered clinically to advance cancer therapy in various stages of the disease (also see (Antoni et al. 2006)), specifically during the critical peri-operative period.

Bibliography

- Antoni MH, Lutgendorf SK, Cole SW, Dhabhar FS, Sephton SE, McDonald PG, Stefanek M, Sood AK. The influence of bio-behavioural factors on tumour biology: pathways and mechanisms. *Nat Rev Cancer* 2006;6(3):240–8. [PubMed: 16498446]
- Avraham R, Inbar S, Rosenne E, Ben-Eliyahu S. Autologous control of a highly malignant syngeneic CRNK-16 leukemia in the rat: a role for NK cells. *Cancer Immunol Immunother* 2006;55(11):1348–57. [PubMed: 16465528]
- Avraham, R.; Schwartz, Y.; Rosenne, E.; Melamed, R.; Ben-Eliyahu, S. Psychoneuroimmunology Research Society. Titisee, Germany: Elsevier; 2004. IL-12-based immunotherapy reduces post-surgery immunosuppression and metastasis, and increases survival from experimental leukemia.
- Barlozzari T, Leonhardt J, Wiltout RH, Herberman RB, Reynolds CW. Direct evidence for the role of LGL in the inhibition of experimental tumor metastases. *Journal of Immunology* 1985;134(4):2783–9.
- Barlozzari T, Reynolds CW, Herberman RB. In vivo role of natural killer cells: involvement of large granular lymphocytes in the clearance of tumor cells in anti-asialo GM1-treated rats. *Journal of Immunology* 1983;131(2):1024–7.
- Bar-Yosef S, Melamed R, Page GG, Shakh G, Shakh K, Ben-Eliyahu S. Attenuation of the tumor-promoting effect of surgery by spinal blockade in rats. *Anesthesiology* 2001;94(6):1066–73. [PubMed: 11465599]
- Ben-Eliyahu S, Page GG, Schleifer SJ. Stress, NK cells, and cancer: Still a promissory note. *Brain Behav Immun.* 2007
- Ben-Eliyahu S, Page GG, Yirmiya R, Taylor AN. Acute alcohol intoxication suppresses natural killer cell activity and promotes tumor metastasis. *Nature Medicine* 1996;2(4):457–60.
- Ben-Eliyahu S, Page GG. In vivo assessment of natural killer cell activity in rats. *Progress in Neuroendocrineimmunology* 1992;5:199–214.
- Benish M, Bartal I, Goldfarb Y, Levi B, Avraham R, Raz A, Ben-Eliyahu S. Perioperative use of beta-blockers and COX-2 inhibitors may improve immune competence and reduce the risk of tumor metastasis. *Ann Surg Oncol* 2008;15(7):2042–52. [PubMed: 18398660]
- Billiau A, Heremans H, Vermeire K, Matthys P. Immunomodulatory properties of interferon-gamma. An update. *Ann N Y Acad Sci* 1998;856:22–32. [PubMed: 9917861]
- Chehimi J, Valiante NM, D'Andrea A, Rengaraju M, Rosado Z, Kobayashi M, Perussia B, Wolf SF, Starr SE, Trinchieri G. Enhancing effect of natural killer cell stimulatory factor (NKSF/interleukin-12) on cell-mediated cytotoxicity against tumor-derived and virus-infected cells. *Eur J Immunol* 1993;23(8):1826–30. [PubMed: 8102101]
- Cohen J. IL-12 deaths: explanation and a puzzle. *Science* 1995;270(5238):908. [PubMed: 7481785]
- Colombo MP, Trinchieri G. Interleukin-12 in anti-tumor immunity and immunotherapy. *Cytokine Growth Factor Rev* 2002;13(2):155–68. [PubMed: 11900991]

- Da Costa ML, Redmond HP, Finnegan N, Flynn M, Bouchier-Hayes D. Laparotomy and laparoscopy differentially accelerate experimental flank tumour growth. *Br J Surg* 1998;85(10):1439–42. [PubMed: 9782033]
- Da Costa ML, Redmond P, Bouchier-Hayes DJ. The effect of laparotomy and laparoscopy on the establishment of spontaneous tumor metastases. *Surgery* 1998;124(3):516–25. [PubMed: 9736904]
- Del Vecchio M, Bajetta E, Canova S, Lotze MT, Wesa A, Parmiani G, Anichini A. Interleukin-12: biological properties and clinical application. *Clin Cancer Res* 2007;13(16):4677–85. [PubMed: 17699845]
- Dunn GP, Bruce AT, Ikeda H, Old LJ, Schreiber RD. Cancer immunoediting: from immunosurveillance to tumor escape. *Nat Immunol* 2002;3(11):991–8. [PubMed: 12407406]
- Elenkov IJ, Wilder RL, Chrousos GP, Vizi ES. The sympathetic nerve--an integrative interface between two supersystems: the brain and the immune system. *Pharmacol Rev* 2000;52(4):595–638. [PubMed: 11121511]
- Eschwege P, Dumas F, Blanchet P, Le Maire V, Benoit G, Jardin A, Lacour B, Loric S. Haematogenous dissemination of prostatic epithelial cells during radical prostatectomy. *Lancet* 1995;346(8989):1528–30. [PubMed: 7491049]
- Faist E, Schinkel C, Zimmer S. Update on the mechanisms of immune suppression of injury and immune modulation. *World J Surg* 1996;20(4):454–9. [PubMed: 8662134]
- Folkman, J. Tumor Angiogenesis. In: Medelsohn, PMHJ.; Israel, MA.; Liotta, LA.; Saunders, WB., editors. *The Molecular Basis of Cancer*. 1995.
- Harbour-McMenamin D, Smith EM, Blalock JE. Bacterial lipopolysaccharide induction of leukocyte-derived corticotropin and endorphins. *Infect Immun* 1985;48(3):813–7. [PubMed: 2987131]
- Hasko G, Elenkov IJ, Kvetan V, Vizi ES. Differential effect of selective block of alpha 2-adrenoreceptors on plasma levels of tumour necrosis factor-alpha, interleukin-6 and corticosterone induced by bacterial lipopolysaccharide in mice. *J Endocrinol* 1995;144(3):457–62. [PubMed: 7738470]
- Hoodin F, Weber S. A systematic review of psychosocial factors affecting survival after bone marrow transplantation. *Psychosomatics* 2003;44(3):181–95. [PubMed: 12724499]
- Kim R, Emi M, Tanabe K. Cancer cell immune escape and tumor progression by exploitation of anti-inflammatory and pro-inflammatory responses. *Cancer Biol Ther* 2005;4(9):924–33. [PubMed: 16177562]
- Lala PK, Parhar RS, Singh P. Indomethacin therapy abrogates the prostaglandin-mediated suppression of natural killer activity in tumor-bearing mice and prevents tumor metastasis. *Cell Immunol* 1986;99(1):108–18. [PubMed: 2944621]
- Lee JW, Shahzad MM, Lin YG, Armaiz-Pena G, Mangala LS, Han HD, Kim HS, Nam EJ, Jennings NB, Halder J, Nick AM, Stone RL, Lu C, Lutgendorf SK, Cole SW, Lokshin AE, Sood AK. Surgical stress promotes tumor growth in ovarian carcinoma. *Clin Cancer Res* 2009;15(8):2695–702. [PubMed: 19351748]
- Malygin AM, Meri S, Timonen T. Regulation of natural killer cell activity by transforming growth factor-beta and prostaglandin E2. *Scand J Immunol* 1993;37(1):71–6. [PubMed: 8380302]
- Melamed R, Rosenne E, Shakhar K, Schwartz Y, Abudarham N, Ben-Eliyahu S. 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* 2005;19(2):114–26. [PubMed: 15664784]
- Mitsuhashi M, Liu J, Cao S, Shi X, Ma X. Regulation of interleukin-12 gene expression and its anti-tumor activities by prostaglandin E2 derived from mammary carcinomas. *J Leukoc Biol* 2004;76(2):322–32. [PubMed: 15123779]
- Pruett SB, Fan R, Zheng Q. Acute ethanol administration profoundly alters poly I:C-induced cytokine expression in mice by a mechanism that is not dependent on corticosterone. *Life Sci* 2003;72(16):1825–39. [PubMed: 12586220]
- Raskovalova T, Huang X, Sitkovsky M, Zacharia LC, Jackson EK, Gorelik E. Gs protein-coupled adenosine receptor signaling and lytic function of activated NK cells. *J Immunol* 2005;175(7):4383–91. [PubMed: 16177079]

- Reynolds CW, Bere EW Jr, Ward JM. Natural killer activity in the rat. III. Characterization of transplantable large granular lymphocyte (LGL) leukemias in the F344 rat. *J Immunol* 1984;132(1):534–40. [PubMed: 6197459]
- Rosenne E, Shakhar G, Melamed R, Schwartz Y, Erdreich-Epstein A, Ben-Eliyahu S. Inducing a mode of NK-resistance to suppression by stress and surgery: a potential approach based on low dose of poly I-C to reduce postoperative cancer metastasis. *Brain Behav Immun* 2007;21(4):395–408. [PubMed: 17240115]
- Schwartz Y, Avraham R, Benish M, Rosenne E, Ben-Eliyahu S. Prophylactic IL-12 treatment reduces postoperative metastasis: mediation by increased numbers but not cytotoxicity of NK cells. *Breast Cancer Res Treat*. 2007
- Shakhar G, Abudarham N, Melamed R, Schwartz Y, Rosenne E, Ben-Eliyahu S. Amelioration of operation-induced suppression of marginating pulmonary NK activity using poly IC: a potential approach to reduce postoperative metastasis. *Ann Surg Oncol* 2007;14(2):841–52. [PubMed: 17091332]
- Shakhar G, Ben-Eliyahu S. In vivo beta-adrenergic stimulation suppresses natural killer activity and compromises resistance to tumor metastasis in rats. *Journal of Immunology* 1998;160(7):3251–8.
- Shakhar G, Ben-Eliyahu S. Potential prophylactic measures against postoperative immunosuppression: could they reduce recurrence rates in oncological patients? *Ann Surg Oncol* 2003;10(8):972–92. [PubMed: 14527919]
- Shakhar G, Blumenfeld B. Glucocorticoid involvement in suppression of NK activity following surgery in rats. *J Neuroimmunol* 2003;138(1–2):83–91. [PubMed: 12742657]
- Smith EM, Blalock JE. A molecular basis for interactions between the immune and neuroendocrine systems. *Int J Neurosci* 1988;38(3–4):455–64. [PubMed: 3286560]
- Smyth MJ, Godfrey DI, Trapani JA. A fresh look at tumor immunosurveillance and immunotherapy. *Nat Immunol* 2001;2(4):293–9. [PubMed: 11276199]
- Sood AK, Bhatti R, Kamat AA, Landen CN, Han L, Thaker PH, Li Y, Gershenson DM, Lutgendorf S, Cole SW. Stress hormone-mediated invasion of ovarian cancer cells. *Clin Cancer Res* 2006;12(2):369–75. [PubMed: 16428474]
- Thaker PH, Han LY, Kamat AA, Arevalo JM, Takahashi R, Lu C, Jennings NB, Armaiz-Pena G, Bankson JA, Ravoori M, Merritt WM, Lin YG, Mangala LS, Kim TJ, Coleman RL, Landen CN, Li Y, Felix E, Sanguino AM, Newman RA, Lloyd M, Gershenson DM, Kundra V, Lopez-Berestein G, Lutgendorf SK, Cole SW, Sood AK. Chronic stress promotes tumor growth and angiogenesis in a mouse model of ovarian carcinoma. *Nat Med* 2006;12(8):939–44. [PubMed: 16862152]
- Trinchieri G. Immunobiology of interleukin-12. *Immunol Res* 1998;17(1–2):269–78. [PubMed: 9479588]
- Tschuschke V, Hertenstein B, Arnold R, Bunjes D, Denzinger R, Kaechele H. Associations between coping and survival time of adult leukemia patients receiving allogeneic bone marrow transplantation: results of a prospective study. *J Psychosom Res* 2001;50(5):277–85. [PubMed: 11399286]
- van der Pouw Kraan TC, Boeije LC, Smeenk RJ, Wijdenes J, Aarden LA. Prostaglandin-E2 is a potent inhibitor of human interleukin 12 production. *J Exp Med* 1995;181(2):775–9. [PubMed: 7836930]
- Walker W, Rotondo D. Prostaglandin E2 is a potent regulator of interleukin-12- and interleukin-18-induced natural killer cell interferon-gamma synthesis. *Immunology* 2004;111(3):298–305. [PubMed: 15009430]
- Walzer T, Dalod M, Vivier E, Zitvogel L. Natural killer cell-dendritic cell crosstalk in the initiation of immune responses. *Expert Opin Biol Ther* 2005;5(Suppl 1):S49–59. [PubMed: 16187940]
- Whalen MM, Bankhurst AD. Effects of beta-adrenergic receptor activation, cholera toxin and forskolin on human natural killer cell function. *Biochemical Journal* 1990;272(2):327–31. [PubMed: 2176460]
- Woiciechowsky C, Asadullah K, Nestler D, Eberhardt B, Platzer C, Schoning B, Glockner F, Lanksch WR, Volk HD, Docke WD. Sympathetic activation triggers systemic interleukin-10 release in immunodepression induced by brain injury. *Nat Med* 1998;4(7):808–13. [PubMed: 9662372]
- Woiciechowsky C, Schoning B, Lanksch WR, Volk HD, Docke WD. Mechanisms of brain-mediated systemic anti-inflammatory syndrome causing immunodepression. *J Mol Med* 1999;77(11):769–80. [PubMed: 10619437]

- Yamaguchi K, Takagi Y, Aoki S, Futamura M, Saji S. Significant detection of circulating cancer cells in the blood by reverse transcriptase-polymerase chain reaction during colorectal cancer resection. *Ann Surg* 2000;232(1):58–65. [PubMed: 10862196]
- Zeddou M, Greimers R, de Valensart N, Nayjib B, Tasken K, Boniver J, Moutschen M, Rahmouni S. Prostaglandin E2 induces the expression of functional inhibitory CD94/NKG2A receptors in human CD8+ T lymphocytes by a cAMP-dependent protein kinase A type I pathway. *Biochem Pharmacol* 2005;70(5):714–24. [PubMed: 15978547]

Figure 1.

Neither treatment alone (poly I-C, or endocrine-blocker) improved survival rates following surgery, but the combined treatment caused a marked and significant increase in survival rates.

Figure 2.

(A) Only treatment with IL-12 improved baseline (control) levels of tumor resistance (LTR) (indicated by #). Each of the two treatments reduced the effect of surgery when used alone (* indicates the significant effect of surgery from baseline, ** indicates a significant attenuation of the effects of surgery), and when combined were significantly more efficacious than each treatment alone (***), nullifying the effects of surgery. (B) NK activity of the entire lung perfusate (marginating pulmonary compartment) yielded results that mirrored well the in vivo findings seen in A. Results presented as mean+SE

Figure 3.

(A) Studying MP-NK activity on a per-NK-cell basis, endocrine-blocker treatment, but not IL-12, significantly reduced the effects of surgery (* indicates the suppressive effects of surgery in the vehicle and IL-12 treated groups, and ** indicate its attenuation). The combined use of the two approaches was more efficacious than each treatment alone in protecting from suppression by surgery on a per NK cell basis (***). (B) Studying total MP-NK numbers, there was no significant difference between the operated and non-operated groups in all drug treatments. On the other hand, each group treated with IL-12 showed significant increase in the total numbers of MP-NK cells, compared to the vehicle group (*). Results presented as mean+SE.

Table 1

Experimental groups and total number of animals

Cell line/model studied	condition	Vehicle	Immuno-stimulation	Endocrine blocker therapy	Combined
MADB106	Control	12	12	12	11
	Surgery	13	13	13	13
CRNK-16	Surgery	45	32	25	29