Improving Postoperative Immune Status and Resistance to Cancer Metastasis

A Combined Perioperative Approach of Immunostimulation and Prevention of Excessive Surgical Stress Responses

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Background: Surgical procedures, including primary tumor resection, have been suggested to suppress immune competence and to promote postoperative infections and cancer metastasis. Catecholamines and prostaglandins were recently implicated in these processes, and in directly promoting tumor angiogenesis and invasion.

Objective: To examine the integration of 2 complementary approaches to reduce postoperative immunosuppression and metastatic progression: (1) perioperative immunostimulation with CpG-C and (2) pharmacological blockade of the tumor-promoting and immunosuppressing effects of catecholamines and prostaglandins, using propranolol (P) and etodolac (E), respectively.

Methods: F344 rats were treated before surgery with CpG-C, P+E, both interventions, or vehicles, and were intravenously inoculated with syngeneic MADB106 mammary adenocarcinoma cells. Blood was withdrawn, marginating-pulmonary leukocytes were harvested, and NK activity and lung MADB106 tumor retention were assessed. In addition, C57BL/6 mice were implanted with syngeneic B16F10.9 melanoma cells. When tumors reached 100 mm^3, mice were treated with CpG-C/vehicle, and 24 hours later the tumor was excised along with P+E/vehicle treatment. Recurrence-free survival was monitored thereafter.

Results: Each of the regimens alone, CpG-C or P+E, showed improvement in most indices examined, including improved long-term recurrence-free survival rates. Most importantly, the combined treatment yielded additive or synergistic effects, further improving tumor clearance from the lungs and enhancing NK numbers and cytotoxicity via different, but complimentary, mechanisms.

Conclusions: Treatment aimed at perioperative enhancement of CMI and simultaneous inhibition of excessive catecholamine and prostaglandin responses, employing CpG-C, propranolol, and etodolac, could be successful in limiting postoperative immunosuppression and metastatic progression, more so than each treatment alone.

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Optimal control of minimal residual disease (MRD) following surgery for the removal of a primary tumor is critical for preventing metastatic development and for improving long-term survival rates.[1] Although life-saving and indispensable, the surgical resection of a primary tumor has repeatedly been suggested to increase the incidence of metastatic growth for a number of reasons. First, shedding of tumor cells from the primary tumor is augmented by physical manipulation of the tumor or its vasculature during surgery.[2–5] Second, the abundant release of growth factors after tissue damage has been shown to promote the development of preexisting micrometastases.[6,7] Finally, alterations in the levels of pro- and antiangiogenic factors after tumor excision may further support such metastatic development,[8] as the concentrations of antiangiogenic factors plummet,[9–13] whereas levels of proangiogenic factors are elevated.[14–16]

Another factor suggested to promote postoperative metastatic growth is immune suppression. Specifically, cell-mediated immunity (CMI) is compromised, cytotoxic T lymphocytes and natural killer (NK) cells in particular,[17,18] with the degree of suppression corresponding to the extent of surgical trauma and tissue damage.[19] This immunosuppression has been associated with elevated postoperative infection rates and sepsis, and organ failure.[20–23] We and others have shown that patients awaiting surgery exhibit suppression of NK activity[24] and pro-CMI cytokines, including IFNγ and IL-12p40.[17] Importantly, the suppression of CMI has been reported to contribute to the increased susceptibility of metastatic growth in oncological patients.[25–27]

Various aspects of surgery have been implicated in suppression of CMI.[19,28–37] Of the various bioactive substances that are secreted perioperatively, we believe that catecholamines (CAs)[38,39] and prostaglandins (PGs)[40] are key mediators of postoperative CMI suppression.[41] CA and PG release are brought on by tissue damage, and by physiological perturbations caused by anesthesia, hypothermia, blood loss, and nociception.[41] Some tumors are also known to secrete PGs, presumably to suppress CMI and evade immune destruction.[42,43]

CAs and PGs have been repeatedly shown to directly suppress NK cells and most other aspects of CMI in vitro,[44,45] and are known to suppress T helper type 1 (T_H1) cytokines that are important for maintaining CMI competence.[39] NK cells and other lymphocytes express receptors for these ligands,[39,46–48] and the intracellular cascades triggered by these substances, leading to suppressed activity, have been partly elucidated.[49–51] Recent studies in our laboratory clearly indicate that physiological levels of CAs[52,53] and PGs,[38,54] or the release of these compounds after stress or surgery, suppress NK activity in vivo, and such suppression can compromise resistance to metastasis in several animal models.[55,56] The blockade of these responses was shown to reduce postoperative tumor progression.[55–57]

Immunostimulation using various cytokines and biological response modifiers (BRM) is another approach that has been attempted for the elimination of MRD. However, it has rarely been utilized during the critical perioperative period. Moreover, the relatively few clinical trials that have tested immunotherapy during the perioperative period utilized a single T_H1 (eg, IL-2) or proinflammatory (eg, IFNα) cytokine to boost immunity.[58–61] The use of a synthetic pathogen-associated molecular pattern (PAMP), such as NK-cell activating TLR9 agonist CpG-C oligodeoxynucleotide (ODN),[62] that naturally

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induces an elaborate cytokine response with less side effects, may be preferable.

Overall, it is evident that the perioperative period harbors many risks; however, this period also presents a short window of opportunity to eradicate MRD before it develops and adopts even more elaborate escape mechanisms. Thus, optimal immune function during this critical period may reduce recurrence and increase long-term survival rates, while also decreasing postoperative infection rates and sepsis. In this study, we attempted to optimize perioperative CMI function employing 2 complementary approaches: (1) preoperative immunostimulation with CpG-C and (2) pharmacological blockade of the immunosuppressing factors, CAs and PGs with the commonly prescribed propranolol and etodolac. This integrated approach is also attempted herein given that preoperative immunostimulation with IL-12 alone was reported ineffective, mainly because the surgical procedure and the ensuing immunosuppression nullified its beneficial effects.[63] Therefore, we hypothesized that immunostimulatory treatment alongside immunoprotective pharmacological treatment could be advantageous when administered during the perioperative period.

MATERIALS AND METHODS

Animals and Counterbalancing

Male Fisher 344 (F344) rats and male and female C57BL/6J mice were purchased at the age of 4 to 6 weeks (Harlan Laboratories, Jerusalem, Israel), and housed 3 to 4 per cage in our vivarium with *ad-lib* access to food and water on a 12:12 light:dark cycle at 22 ± 1°C. Animals were acclimated to the vivarium for at least 3 weeks before experimentation and rats were handled daily during the last week before experimentation to reduce potential procedural stress. The number of animals used per experiment is detailed in the brief description of each experiment (see Results). Order of drug administration, surgery, and tumor cell injection were counterbalanced across groups in each experiment. Housing conditions are regularly monitored by the Institutional Animal Care and Use Committee of Tel Aviv University, which also approved all studies described herein.

Drugs and their Administration

CpG ODNs

CpG-C ODN (ODN 2395: 5'-T**CG**T**CG**TTTT**CGG**C**GCG** **CG**C**CG**-3') with a phosphorothioate backbone was used in all experiments. As we have previously compared the effects of CpG-C with both a non-CpG ODN and to phosphate buffered saline (PBS) in rats and found no differences between the 2 controls,[64] in the rat studies described herein we employed only the PBS control. Non-CpG ODN (ODN 2137: 5'-TGCTGCTTTTGTGCTTTTGTGCTT-3') was used as a control as it lacks C-G motifs (in addition to PBS) in the first mouse study. CpG-C was diluted in PBS, was administered at a dose of 330 μg/kg in rats and 10 to 100 μg/mouse, and was injected i.p. unless otherwise noted. Non-CpG was diluted in PBS and was administered at a dose of 100 μg/mouse (i.p.). All ODNs were purchased from Coley Pharmaceuticals Canada (Ottawa, Canada), and contained undetectable levels of endotoxin as measured by the limulus amebocyte lysate assay.

Propranolol

To block β-adrenoceptor stimulation, 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 to 48 hours. One millimeters of the emulsion was administrated to rats subcutaneously 1 hour before laparotomy (1.5 mg/kg). Mice were subcutaneously administered the emulsion immediately before paw amputation (5 mg/kg in a 0.5 mg/mL concentration).

Etodolac

The semiselective COX2 inhibitor, etodolac, was kindly donated by Taro, Israel. Etodolac was dissolved in corn oil. The drug was administered subcutaneously 1 hour before surgery in rats (12.5 mg/kg). The $T_{1/2}$ of this drug in rats was found to be 18 hours.[65] In mice, the drug was administered subcutaneously immediately before paw amputation (50 mg/kg in a 12.5 mg/mL concentration). The $T_{1/2}$ of this drug in mice was found to be 16 hours.[66]

The combined use of propranolol and etodolac will be referred to as pharmacological blockade.

Tumor Cell Lines and Their Maintenance

MADB106

MADB106 is a selected variant cell line obtained from a pulmonary metastasis of a chemically induced mammary adenocarcinoma (MADB100) in the F344 rat.[67] MADB106 tumor cells injected into the tail vein of F334 rats migrate to the lungs and 100% of the cells are found in the lungs 5 minutes after cell inoculation.[68] These cells form metastases only in the lungs,[67] and lung tumor retention, which is highly indicative of the number of metastases that would have developed weeks later, is dependent upon NK cells in this model.[67–70] In addition, because the metastatic process of MADB106 is sensitive to NK activity predominantly in the first 24 hours after inoculation,[67,68] lung tumor retention is more reflective of in vivo NK activity levels than the number of actual metastases is.[69] The MADB106 line was maintained in 5% CO_2 at 37°C in monolayer cultures in complete medium. This cell line was used for both in vivo and in vitro studies.

YAC-1

YAC-1 murine T-cell lymphoma is the standard target cell line used for the assessment of rodent NK cytotoxicity in vitro. The cell line was maintained in suspension cultures in complete media in 100% humidity, 5% CO_2 at 37°C.

B16F10.9

B16F10.9 (B16) melanoma cells, syngeneic to the C57BL/6J mouse strain, were kindly provided by Prof. Amiram Raz (Department of Biochemistry, Faculty of Life Sciences, Tel-Aviv University, obtained from Drs. M.L. Kripke and I.J. Fidler from the University of Texas—M.D. Anderson Hospital, Houston, TX). Cells were grown in cultures in 5% CO_2, 100% humidity, 37°C, in CM.

Radiolabeling of MADB106 Tumor Cells and Assessment of Lung Tumor Retention

For assessment of MADB106 lung tumor retention, DNA radiolabeling of tumor cells was accomplished by adding 0.5 μCi/mL of ^{125}iododeoxyyuridine (125-IDUR, Danyel Biotech, Rehovot, Israel) to the growing cell culture 24 hours before harvesting the cells for injection. For tumor cell injection, rats were lightly anesthetized with isoflurane, and 10^5 radiolabeled cells in 0.5 mL of PBS (supplemented with 0.1% BSA) were injected into their tail vein (approximately 4 × 10^5/kg). Two hours later, rats were sacrificed with an overdose of isoflurane, blood was collected via cardiac puncture and lung perfusate was collected as described later. Then, the lungs were removed and placed in a gamma counter for assessment of radioactive content. The 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.[28,68–70]

Experimental Laparotomy

The procedure has been described elsewhere.[38] Briefly, rats were anesthetized with 2.5% isoflurane and a 4-cm midline abdominal incision was performed. The small intestine was externalized, rubbed with a PBS-soaked gauze pad, and left hydrated with a PBS-soaked gauze pad for 30 minutes. Finally, the intestine was internalized and the abdomen sutured.

Selective In Vivo Inhibition of NK Cells in Rats

Approximately 1.5 mg/kg of mouse anti-rat NKR-P1 monoclonal antibody (mAb) was injected i.v. under isoflurane anesthesia. Previous studies using the abovementioned dose of the anti-NKR-P1 mAb showed immediate, selective, and complete abolition of blood and splenic NK cytotoxicity[71] and up to 200-fold increase in the lung retention and metastatic colonization of MADB106 tumor cells.[39,69] Our previous studies using control mAbs (R73, W3/25, and ED2), mouse serum, or saline as controls for anti-NKR-P1 administration, have shown that neither had an effect on NK-cell function and metastatic dissemination and therefore PBS was used as the control.[68]

In Vitro Assessment of NK Cell Cytotoxicity

Harvesting and Preparing Circulating Leukocytes and Marginating-Pulmonary Leukocytes for Assessment of NK Cytotoxicity

Rats were sacrificed with an overdose of isoflurane and the peritoneal and chest cavities opened. One milliliter of blood was collected into an EDTA-coated tube, then 3 mL of blood were collected from the right ventricle of the heart into heparinized syringes (30 U/mL of blood). One milliliter of heparinized blood was washed once with 3 mL of PBS (400*g* for 10 minutes) and twice with 3 mL of complete media, and reconstituted to its original volume. Marginating-pulmonary (MP) leukocytes were harvested by perfusing the heart with 30 U/mL of heparinized PBS. PBS was injected into the right ventricle and perfusate was collected from the left ventricle. The first 3 mL of perfusate, which were contaminated with blood, were discarded, and the next 25 mL were collected and concentrated to 1 mL. This was achieved by centrifuging the perfusate (400*g* for 10 minutes), discarding the supernatant, and suspending the pellet in 3 mL of complete media, centrifuging the perfusate again (400*g* for 10 min) and concentrating the perfusate into 1 mL.

Assessment of NK-Cell Cytotoxicity

The standard whole blood ^{51}Cr release assay was used and has been described elsewhere.[69] Earlier studies have indicated that cytotoxicity measured using this procedure is attributable to NK cells, rather than other cell types or soluble factors, as the selective inhibition of NK cells abolishes all target-cell killing.[72,73] The advantages of this procedure include shorter duration, less interference with the effector cells, and better representation of the original in vivo cell composition milieu.

Calculation of Lytic Unit (LU_{50}) Per MP-NK Cell

Transformation of cytotoxicity curves to LU is a standard approach to extract one index that represents the killing efficacy of NK cells depicted by specific lysis in the multiple effector:target (E:T) ratios. This transformation should be conducted only when the cytotoxicity curves (E:T ratios by % specific killing) of the different groups are parallel, as is the case in this study regarding lung perfusate cytotoxicity against YAC-1 target cells. The value denoted by LU_{50} per the total lung perfusate is the number of aliquots that can be taken from the perfusate, each of which is capable of reaching 50% specific lysis of target cells. Thus, higher LU values reflect greater NK cytotoxicity against target cells. The value of LU_{50} is mathematically derived from the concentration of effector cells at which 50% specific killing was reached in the standard cytotoxicity assay described earlier. The regression exponential fit method[74] was used to calculate LU_{50} for each sample, based on percent-specific lysis of YAC-1 target cells by MP-NK cells. To determine cytotoxicity per MP-NK cell, the value of LU_{50} per lung perfusate was divided by the number of MP-NK cells per lung perfusate (according to FACS analysis), yielding the index of LU_{50}/MP-NK. This index reflects NK-cell cytotoxicity per NK cell in each sample.

FLOW CYTOMETRY

Standard procedures were used to prepare cells for FACS analysis.[38] NK cells in both rat blood and lung perfusate were identified by the FITC-conjugated anti-NKR-P1 mAb (PharMingen, San Diego, CA) as being NKR-P1^{bright} (CD161^{bright}) cells. Mouse NK cells were identified by FITC-conjugated NK1.1 mAb (eBioscience, San Diego, CA). This criterion has been shown to exclusively identify more then 95% of cells that exhibit NK activity.[71,75,76] T cells were identified using a PE-conjugated anti-CD5 mAb (eBioscience) and NKT cells were identified as NKR-P1$^+$CD5$^+$ lymphocytes. CD11a$^+$ leukocytes were identified by a biotin-conjugated mAb (Biolegend, San Diego, CA) followed by PE-Cy5 conjugated streptavidin (eBioscience). Granulocytes and lymphocytes were identified based on forward and side scatters. To assess the total number of cells per microliter of sample (or a specific cell subtype), 300 polystyrene microbeads (20 μm, Duke Scientific, Palo Alto, CA) per microliter sample were added to each sample, and the following formula was used: (number of cells in sample/number of microbeads in sample) $\times$ 300.

IL-12p70+p40 ELISA

One milliliter of blood harvested into EDTA-coated tubes was centrifuged for 20 minutes at 2000 rpm to enable plasma collection. EDTA plasma was stored at -80°C until sample assay. The anti-rat IL-12p70+p40 ELISA was conducted as per the manufacturer's instructions (BioSource International, Camarillo, CA).

B16 Melanoma Tumor Cell Inoculation, Tumor Development, and Amputation

Mice were orthotopically injected with 10^5 B16 melanoma cells into the right footpad subcutaneous space. Developing tumors were visually inspected daily thereafter. Once a tumor reached a 100 mm^3 volume (tumor length $\times$ width $\times$ height), the mouse underwent tumor excision by paw amputation. For the amputation, mice were anesthetized with 3% isoflurane, and the tumor-bearing hindpaw was amputated with surgical scissors 2 mm above the ankle joint. The wound was treated with an antiseptic paste (10% povidone iodine). Mice were subsequently monitored for morbidity signs on a daily basis for an 80-day period (more than 3 weeks after the last morbidity incidence). Mice showing sickness behavior or manifesting cancer recurrence above the amputation site were overdosed with isoflurane and autopsied to determine malignant foci. Sickness behavior was defined by slow body movements, irresponsiveness to environmental stimuli, tremor, or loss of more than 10% body weight.

STATISTICAL ANALYSES

One-, 2- or 3-way factorial analysis of variance (ANOVA) with a predetermined significance level of 0.05 was conducted. In some in vivo studies, the variances evident in groups undergoing surgery

were larger than in groups receiving vehicle, violating the ANOVA assumption of homogeneity of variance, and thus the ANOVA was conducted on ln transformation of all data. Graphs depict group means before transformation. When NK cytotoxicity was studied, repeated measures ANOVA was conducted (repeated measures of E:T ratios). Provided significant group differences were found, Fisher's protected least significant differences (Fisher's PLSD) contrasts were performed to compare specific pairs of groups, based on a priori hypotheses. In the survival study, the Kaplan-Meier survival model was employed followed by the Tarone-Ware test for pair-wise group comparisons. All statistical analyses were conducted using StatView software (SAS Institute, San Francisco, CA).

RESULTS

Combined Treatment With CpG-C, Propranolol, and Etodolac (Pharmacological Blockers) Eliminates the Deleterious Effects of Surgery on Tumor Cell Clearance and NK Cytotoxicity

Rats received either a single CpG-C or PBS injection. Twenty-three hours later, each group was further subdivided and received either propranolol and etodolac or their respective vehicles. Immediately thereafter, each of the 4 groups was further subdivided to undergo laparotomy or to serve as home cage controls. Immediately after the surgical procedure, all animals were injected with radiolabeled MADB106 tumor cells and 2 hours later all rats were sacrificed. This experiment was run in 2 replicates, in which all groups were represented (total n = 65, males, 6–9 per group).

Pulmonary Tumor Clearance and MP-NK Activity Against MADB106 Target Cells

With respect to LTR, 3-way ANOVA revealed that surgery significantly increased LTR levels ($F_{(1,55)} = 245.2$, $P < 0.0001$), whereas both CpG-C ($F_{(1,55)} = 115.401$, $P < 0.0001$) and the pharmacological blocker ($F_{(1,55)} = 45.861$, $P < 0.0001$) treatments each significantly reduced these levels, without interaction (Fig. 1A). Importantly, the combination of immunostimulation and pharmacological blockade was most effective, decreasing the levels of LTR to the levels found in nonoperated controls. As indicated by our previous studies,[38] NK activity in the MP compartment is of grave importance to pulmonary tumor clearance at the time-point examined. Therefore, the activity of harvested MP-NK cells was examined ex vivo against the same MADB106 tumor cells. Interestingly, we found that MP-NK activity is an exact mirror image of LTR levels. Specifically, surgery suppressed MP-NK activity per the entire MP compartment ($F_{(1,53)} = 42.175$, $P < 0.0001$), whereas both CpG-C ($F_{(1,53)} = 37.616$, $P < 0.0001$) and pharmacological blockade treatments ($F_{(1,53)} = 9.042$, $P = 0.004$) improved specific lysis of MADB106 tumor cells (Figs. 1B and 2B), and together completely prevented this suppression compared with nonoperated control animals. In nonoperated animals CpG-C treatment elevated MP-NK activity and reduced LTR compared with baseline levels, and the addition of the pharmacological

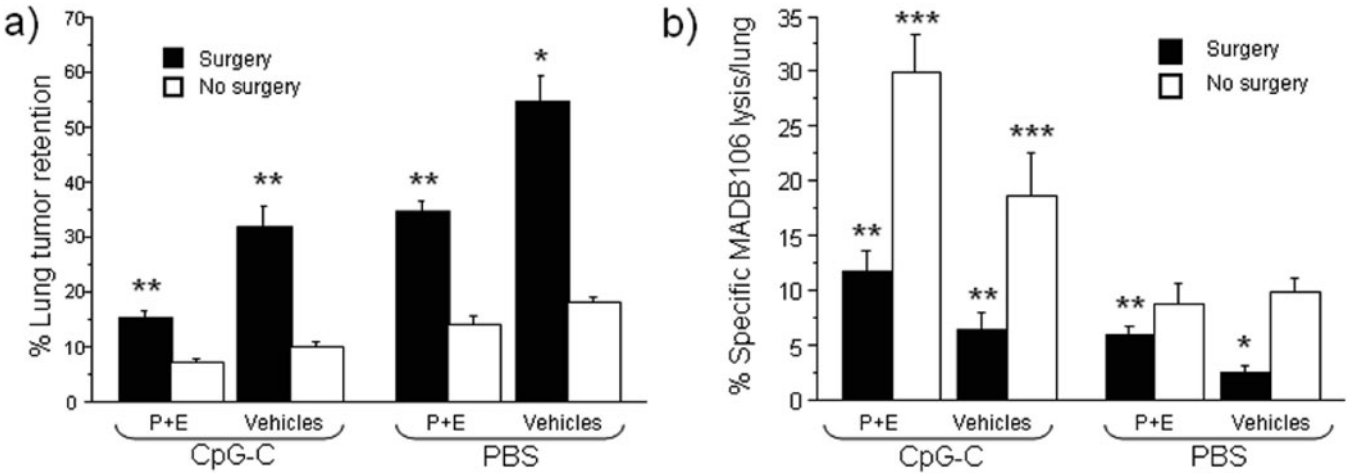

FIGURE 1. Combined treatment with CpG-C and pharmacological blockers abolished the deleterious effects of surgery on tumor cell clearance and NK cytotoxicity. Rats received either a single CpG-C or PBS injection. Twenty-three hours later, each group was further subdivided and received either propranolol and etodolac (P+E) or their respective vehicles. Immediately thereafter, each of the 4 groups was further subdivided to undergo laparotomy (Surgery) or to serve as home cage controls (No surgery). Immediately after the surgical procedure all animals were injected with radiolabeled MADB106 tumor cells and 2 hours later all rats were sacrificed. A, Surgery significantly increased lung tumor retention levels (*, compared with nonoperated controls), and both CpG-C and the pharmacological blocker (P+E) treatments each significantly reduced the deleterious effects of surgery (**, compared with the nontreated surgery group), with the combination of CpG-C and P+E being most effective. B, Marginating pulmonary (MP) NK-cell cytotoxic activity against syngeneic MADB106 target cells was an exact mirror image of lung tumor retention levels (average of the 2 highest effector:target [E:T] ratios). Surgery suppressed MP-NK activity per lung (*), whereas both CpG-C and P+E treatments improved MADB106 specific lysis (**), and together completely prevented this suppression compared with that in nonoperated control animals. In nonoperated animals, CpG-C treatment elevated MP-NK activity compared with baseline levels (***), and the addition of the pharmacological blockers further augmented these effects. Data are presented as mean + SEM, $P < 0.05$.

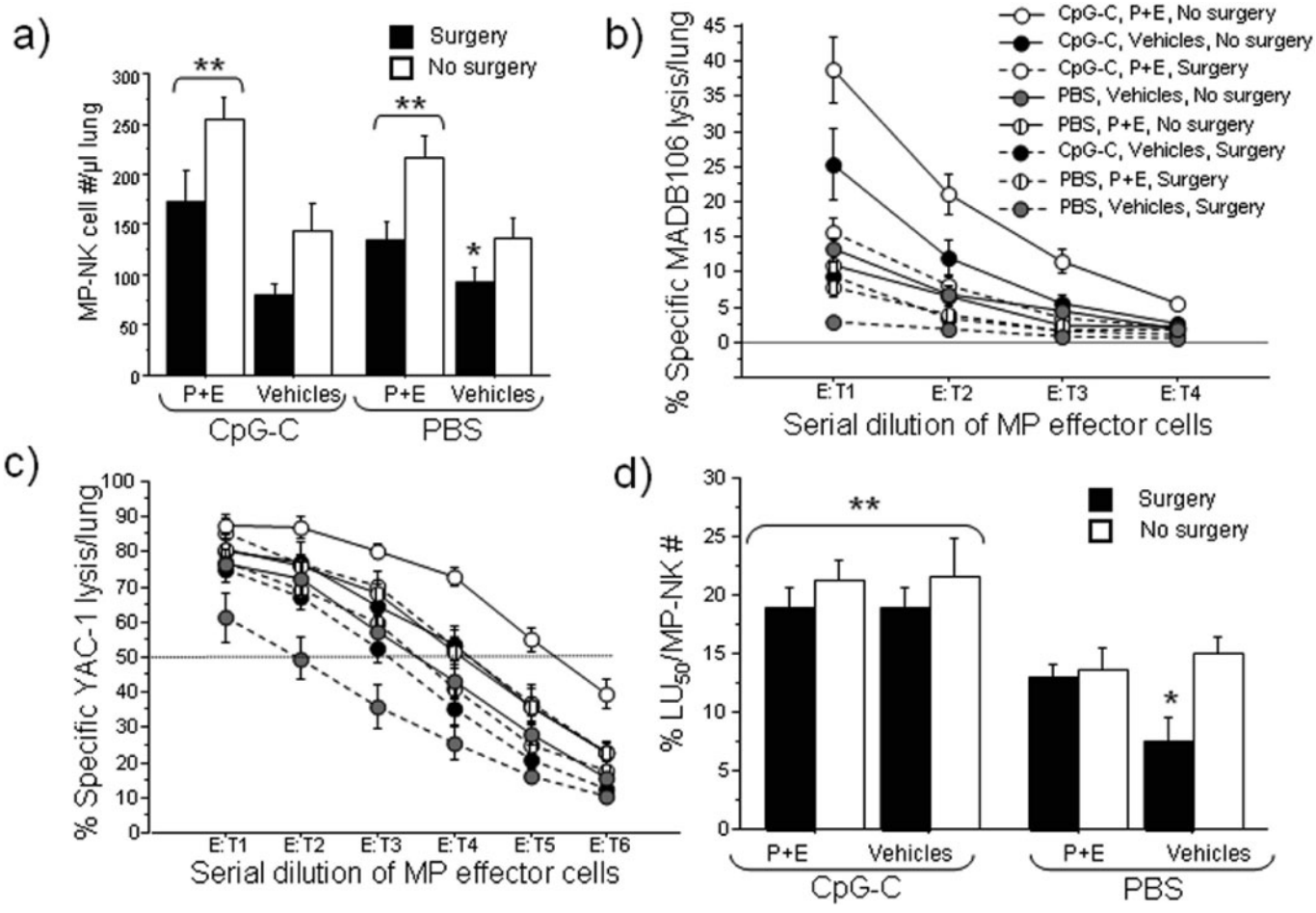

FIGURE 2. Combined treatment with CpG-C and pharmacological blockers elevated specific tumor cell lysis in the lungs by enhancing marginating-pulmonary (MP) NK cell activity per cell. Rats received either a single CpG-C or PBS injection. Twenty-three hours later, each group was further subdivided and received either propranolol and etodolac (P+E) or their respective vehicles. Immediately thereafter, each of the 4 groups was further subdivided to undergo laparotomy or to serve as home cage controls. MP leukocytes were harvested 2 hours later. A, The number of MP-NK cells was significantly reduced by surgery (*), and P+E, but not CpG-C, elevated MP-NK cell numbers (**). B,C, Surgery suppressed MP-NK activity per the entire MP compartment against both MADB106 and YAC-1 target cells, whereas CpG-C and P+E treatments increased cytotoxicity. Lytic unit (LU) calculation cannot be conducted on the MADB106 cytotoxicity curves as they are not parallel, therefore only an estimation of individual MP-NK cell activity against MADB106 target cells can be inferred from the relationship between MP-NK cytotoxicity per lung and MP-NK cell numbers derived from this compartment. Individual MP-NK cell activity was elevated, at least within the CpG-C treated groups. D, Examination of MP-NK activity per NK cell against YAC-1 target cells was conducted based on calculation of LU_{50}/MP-NK# derived from the cytotoxicity curves in panel (c) and the MP-NK numbers in panel (a). Surgery decreased NK activity per MP-NK cell (*) and P+E prevented this effect without affecting baseline levels. CpG-C increased baseline levels and prevented suppression by surgery (**). Data are presented as mean ± SEM, $P < 0.05$.

blockers further augmented these effects. The pharmacological blockers alone did not affect these indices in nonoperated animals, suggesting synergistic effects between these interventions in these animals.

MP-NK Numbers and Activity Per MP-NK Cell Against MADB106 and YAC-1 Target Cells

MP-NK Cell Numbers

The number of MP-NK cells was significantly reduced by surgery ($F_{(1,57)} = 17.509$, $P < 0.0001$) (Fig. 2), the pharmacological blockers had an apparent effect elevating MP-NK cell numbers ($F_{(1,57)} = 26.147$, $P < 0.0001$) (Fig. 2A), and CpG-C treatment did not affect MP-NK cell numbers. No interaction between the effect of surgery and either treatment was evident.

Individual MP-NK Cell Lysis of MADB106 Cells

Calculation of lytic units cannot be conducted on the specific lysis of MADB106 tumor cells in the lungs as the cytotoxicity curves in the different groups are not parallel. However, an estimation of individual MP-NK cell activity can be inferred from the relationship between MP-NK cytotoxicity per lung and MP-NK cell numbers derived from this compartment. Specifically, improved MP-NK cytolytic ability described above can be attributed to an elevation in individual

MP-NK cell activity, at least within the CpG-C treated groups, as the numbers of NK cells were not elevated by CpG-C in operated and nonoperated animals. Changes in activity per MP-NK cell in groups treated with the pharmacological blockers may be attributed both to increased activity per cell and to the elevation in MP-NK cell numbers (Fig. 2B).

MP-NK Cell Activity Against YAC-1 Target Cells

When MP-NK activity per the entire MP compartment was assessed against the standard YAC-1 tumor cell line, the same pattern of results reported above against MADB106 target cells was apparent (main suppressing effect of surgery [$F_{(1,53)} = 23.459$, $P < 0.0001$], and main effects of CpG-C [$F_{(1,53)} = 17.646$, $P = 0.0001$] and pharmacological blockers [$F_{(1,53)} = 26.118$, $P < 0.0001$] increasing cytotoxicity) (Fig. 2C). Examination of MP-NK activity per NK cell was conducted based on calculation of LU_{50}/MP-NK (see Materials and Methods), as the data permit this analysis. Figure 2D shows that whereas surgery decreased NK activity per MP-NK cell [$F_{(1,56)} = 6.104$, $P = 0.0166$], the pharmacological blockers prevented this effect without affecting baseline levels. CpG-C increased baseline levels and prevented suppression by surgery.

Circulating NK Numbers and Activity Against YAC-1 Target Cells

NK-Cell Numbers

The pattern apparent in MP-NK cell numbers is also apparent in the circulation. Surgery reduced circulating NK-cell numbers ($F_{(1,57)} = 8.883$, $P = 0.0042$) (Fig. 3A), whereas the pharmacological blockers brought about an elevation in these numbers ($F_{(1,57)} = 6.614$, $p = 0.0127$). CpG-C had no effect.

Circulating NK-Cell Activity Against YAC-1 Target Cells

Surgery severely diminished target cell lysis ($F_{(1,57)} = 17.918$, $P < 0.0001$). Treatment with CpG-C restored this capacity ($F_{(1,57)} = 28.839$, $P < 0.0001$) and the combined use of both CpG-C and the pharmacological blockers further elevated target cell lysis (Figs. 3B and C). Lytic unit calculations cannot be conducted as the cytotoxocity curves are not parallel. Specifically, improved NK cytolytic ability described earlier can be attributed to an elevation in individual NK-cell activity, at least within the CpG-C treated groups, as the numbers of NK cells were not elevated by CpG-C in operated and nonoperated animals. Changes in activity per NK cell in groups treated with the pharmacological blockers may be attributed both to increased activity per cell, and to the elevation in NK-cell numbers.

Changes in other cell population numbers in both the circulation and the MP compartment are summarized in Table 1.

Expression of CD11a on NK Cells in the Circulation and MP Compartments

The adhesion and costimulatory molecule CD11a is an important factor determining NK cytotoxicity.[77,78] Surgery reduced expression levels of CD11a on circulating and MP-NK cells (blood: $F_{(1,57)} = 28.265$, $P < 0.0001$; lungs: $F_{(1,57)} = 23.789$, $P < 0.0001$) (Fig. 4A and B). In contrast, after CpG-C treatment CD11a expression levels were elevated on NK cells, again in both compartments (blood: $F_{(1,57)} = 44.294$, $P < 0.0001$; lungs: $F_{(1,57)} = 30.374$, $P < 0.0001$). No effects of the pharmacological blockers, or interactions between the effects of CpG-C and surgery were evident in either compartment.

Levels of Plasma IL-12p70+p40

IL-12 is a potent stimulator of NK-cell cytotoxicity, and its production is known to be increased after CpG-C immunostimulation.[79]

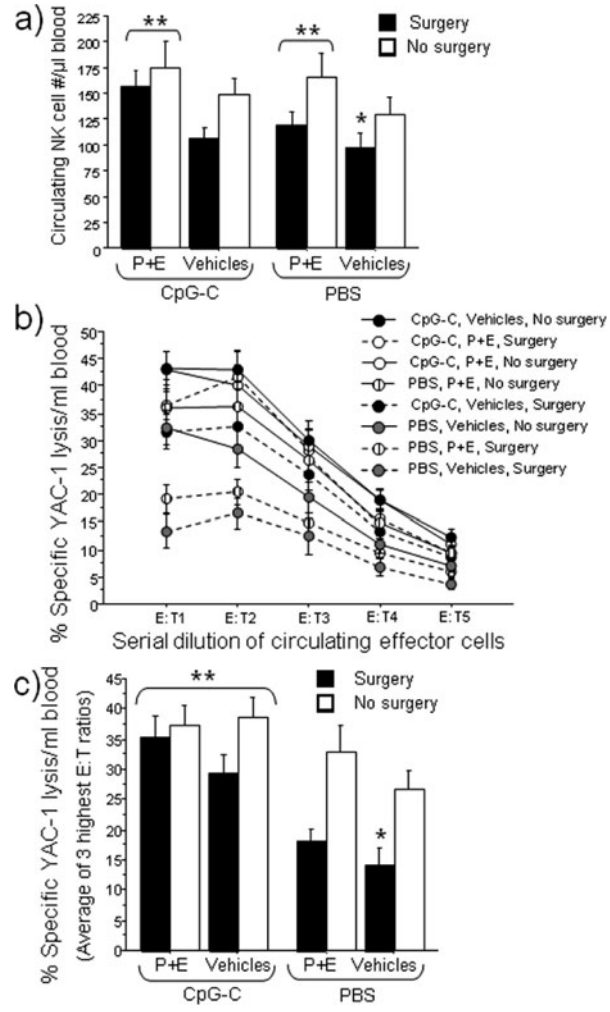

FIGURE 3. Combined treatment with CpG-C and pharmacological blockers prevented surgery-induced suppression of circulating NK-cell cytotoxicity. Rats received either a single CpG-C or PBS injection. Twenty-three hours later, each group was further subdivided and received either propranolol and etodolac (P+E) or their respective vehicles. Immediately thereafter, each of the 4 groups was further subdivided to undergo laparotomy or to serve as home cage controls. Blood was withdrawn from all rats 2 hours later. A, Across all drug treatments, the number of NK cells was significantly reduced by surgery (*), and P+E, but not CpG-C, elevated NK-cell numbers (**). B,C, Surgery severely diminished YAC-1 target cell lysis (*), although treatment with CpG-C significantly restored this capacity (**) and the combined use of both CpG-C and the pharmacological blockers was most effective. Data are presented as mean ± SEM, $P < 0.05$.

We thus assessed its plasma levels 2 hours after surgery, which occurred 26 hours after CpG-C administration. We found that surgery caused a diminution in IL-12p70+p40 (IL-12) levels ($F_{(1,56)} = 6.995$, $P = 0.0106$) (Table 2), however, no effects for CpG-C were apparent. In addition, no effects were found for the pharmacological blocker treatment.

TABLE 1. Numbers of Leukocyte Subpopulations (Per μL Blood/Lung Perfusate) in the Circulation and the Marginating-Pulmonary (MP) Compartment

Treatment			Cell Population							
			Granulocytes		Lymphocytes		T Cells		NKT Cells	
			Circulation	MP Compartment	Circulation	MP Compartment	Circulation	MP Compartment	Circulation	MP Compartment
CpG-C	P+E	Surgery	2629.3 ± 257.9*	3157.0 ± 517.2†	1583.1 ± 179.9*†	1391.3 ± 299.1*†	893.1 ± 124.7*	466.4 ± 109.3*†	22.5 ± 4.5*†‡	19.8 ± 4.5*†‡
		No surgery	2569.5 ± 368.7	2991.5 ± 317.7†	2329.5 ± 170.7†	1730.4 ± 221.0†	1258.8 ± 105.8	512.4 ± 97.9†	34.5 ± 1.8†‡	29.9 ± 1.5†‡
	Vehicles	Surgery	3117.1 ± 508.8*	2112.4 ± 375.7	1153.2 ± 136.7*	549.3 ± 58.3*	634.5 ± 103.7*	187.1 ± 32.4*	12.0 ± 0.8*‡	10.0 ± 1.2*‡
		No surgery	1709.4 ± 178.6	1860.3 ± 411.8	2271.3 ± 113.2	1366.8 ± 448.1	1327.6 ± 86.3	361.7 ± 94.8	26.5 ± 1.7‡	22.5 ± 2.2‡
PBS	P+E	Surgery	2867.1 ± 194.9*	3090.2 ± 396.2†	1595.0 ± 110.5*†	1143.4 ± 162.1*†	851.5 ± 60.5*	355.5 ± 48.3*†	20.7 ± 2.0*†	19.2 ± 1.8*†
		No surgery	3012.6 ± 282.1	2963.5 ± 405.6†	2576.5 ± 163.2†	1696.4 ± 211.0†	1392.9 ± 73.3	620.3 ± 95.8†	46.6 ± 4.7†	43.9 ± 4.9†
	Vehicles	Surgery	3231.0 ± 478.6*	3061.4 ± 572.5	1383.5 ± 142.7*	917.9 ± 151.0*	779.9 ± 96.5*	371.6 ± 76.9*	21.5 ± 3.0*	21.5 ± 3.2*
		No surgery	1587.2 ± 223.8	1798.3 ± 368.1	2419.9 ± 117.4	1048.6 ± 99.8	1382.4 ± 70.0	365.3 ± 43.4	36.9 ± 3.3	29.0 ±2.6

*Denotes a significant difference ($P < 0.05$) between the surgery and no surgery groups.
†Denotes a significant difference ($P < 0.05$) between the P+E (propranolol and etodolac) treated groups and the groups treated with vehicles.
‡Denotes a significant difference ($P < 0.05$) between the CpG-C treated groups and those treated with PBS.

Both NK-Dependent and Independent Mechanisms Play a Role in the Effects of CpG-C and the Pharmacological Blockers on Pulmonary Tumor Clearance

To determine the contribution of NK cells to the beneficial in vivo effects of CpG-C and the pharmacological blockers on pulmonary tumor clearance, we functionally inhibited NK cells in vivo using the anti-NKR-P1 mAb. As elaborated in "Materials and Methods," this approach selectively inhibits NK cells and their activity. Rats (n = 53 males, 4–8 per NK-inhibited group) were administered CpG-C or PBS. Twenty-three hours later, rats received either a combination of propranolol and etodolac, or their respective vehicles, and 1 hour later were further subdivided to undergo laparotomy, or to serve as home cage controls. Immediately after the surgical procedure all animals were injected with MADB106 tumor cells for the assessment of 21-hour LTR. Simultaneously with MADB106 administration, rats were injected with the anti-NKR-P1 mAb. We chose to administer the NK-inhibiting mAb simultaneously with MADB106 tumor cells (and not days earlier) to prevent interference with the effects of CpG-C that was administered 24 hours before the surgical procedure. Four additional naive animals that did not undergo NK-inhibition or surgery were included as a reference group to indicate the effects of NK-inhibition.

As in our previous studies,[64] in this study NK inhibition dramatically raised LTR levels by approximately 50-fold, indicating the significant role of NK cells in MADB106 LTR (Fig. 5). Surgery further increased these levels in NK inhibited animals ($F_{(1,45)} = 26.441$, $P < 0.0001$), indicating that other, NK-independent mechanisms, are also involved in elevating LTR levels after surgery. Neither CpG-C nor the pharmacological blockers affected LTR levels in nonoperated animals, indicating that their beneficial effects in NK-intact nonoperated animals are NK dependent, as was also found in a previous study.[64] Most importantly, in operated animals we found that the pharmacological blockers significantly reduced the effects of surgery ($F_{(1,45)} = 12.935$, $P = 0.0008$, for pharmacological blockers × surgery interaction), indicating that they also block non-NK mechanisms affecting LTR (in addition to their effect on NK cells indicated earlier). CpG-C alone did not significantly reduce LTR levels in operated animals ($P = 0.156$), but its combination with the pharmacological blockers was most effective, completely abolishing all increases caused by surgery, and significantly more so than the blockers alone ($F_{(1,45)} = 9.343$, $P = 0.0038$). We can therefore infer that the majority of the beneficial effects of CpG-C on LTR levels in NK-intact animals may be attributed to activity of NK cells, and that in the absence of functional NK cells, other mechanisms are capable of clearing tumor cells from the lungs through mechanisms affected by the pharmacological blockers and their combination with CpG-C. This, of course, does not exclude the involvement of NK cells in the process taking place in NK-intact animals.

CpG-C Elevated NK Activity in Mice in a Dose-Dependent Manner

In our subsequent studies we utilized a model of spontaneous metastases in C57BL/6 mice, and therefore we first tested the efficacy of CpG-C treatment on NK activity in these animals. Mice (n = 18, females, 4–5 per group) were injected with 10, 30, or 100 μg CpG-C, 100 μg non-CpG, or PBS. Twenty-eight hours later all mice were sacrificed and blood was withdrawn.

Circulating NK-Cell Numbers and Activity Against YAC-1 Target Cells

CpG-C augmented target cell lysis in a dose-dependent manner [$F_{(4,14)} = 17.028$, $P < 0.0001$] (Fig. 6), whereas NK-cell numbers

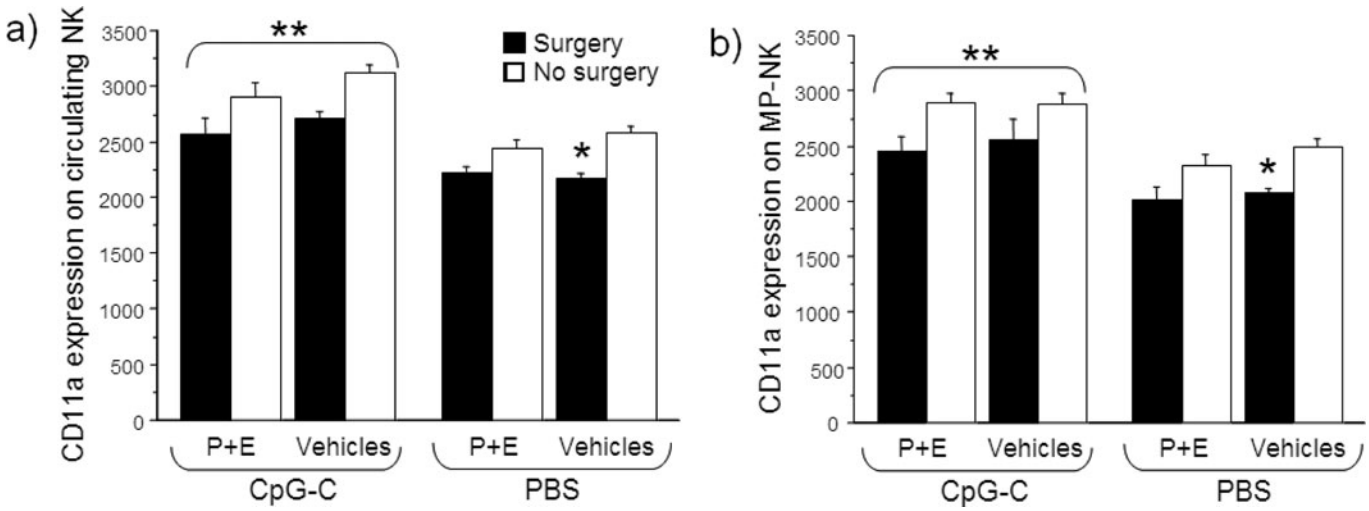

FIGURE 4. Expression of CD11a is enhanced on NK cells in both the circulation and marginating pulmonary (MP) compartment after CpG-C treatment. Rats received either a single CpG-C or PBS injection. Twenty-three hours later, each group was further subdivided and received either propranolol and etodolac (P+E) or their respective vehicles. Immediately thereafter, each of the 4 groups was further subdivided to undergo laparotomy or to serve as home cage controls. Blood and MP leukocytes were harvested from all animals 2 hours later. Across all drug treatments, surgery reduced expression levels of CD11a on circulating (A) and MP-NK cells (B) (*), and CpG-C treatment, but not P+E treatment, elevated CD11a expression levels on these cells, again in both compartments (**). Data are presented as mean + SEM, $P < 0.05$.

TABLE 2. IL-12p70+p40 Plasma Levels (pg/mL)

Treatment	Surgery	No surgery
CpG-C		
P+E	115.2 ± 30.8*	179.8 ± 46.1
Vehicles	127.7 ± 55.7*	147.8 ± 43.6
PBS		
P+E	136.1 ± 82.6*	152.6 ± 55.1
Vehicles	101.7 ± 23.4*	133.5 ± 32.4

*Denotes a significant difference ($P < 0.05$) between the surgery and no surgery groups.
P+E, propranolol and etodolac treatment.

remained unchanged (data not shown). Therefore, it can be inferred that NK activity was elevated per NK cell. As both the dose of 30 and 100 μg were highly effective, but not significantly different from each other, in the following study we chose an intermediate dose of 50 μg/mouse.

The Effects of Pharmacological Blockade and CpG-C on Postoperative Survival Rates After Removal of a Spontaneously Metastasizing Tumor

As the previous studies focused on the short-term effects of our treatment regimens on immune status and clearance of tumor cells, the following experiment sought to examine a more clinically relevant index, long-term survival in a model of spontaneous metastasis, in which B16 melanoma is implanted orthotopically. This model allowed for the evaluation of long-term survival in mice that underwent surgery for the removal of a spontaneously metastasizing primary tumor and received either treatment regimens or their combination. Mice (n = 229, 56–58 per group) were injected with B16 tumor cells into the right hindpaw and were inspected daily. When tumors reached a volume of $\geq$100 mm^3, mice were administered with CpG-C (50 μg/mouse) or PBS. Twenty-four hours later the pharmacological blockers or their vehicles were administered, and the tumor-bearing hindpaw was amputated. A day after the amputation, the animals received another course of CpG-C/PBS. Long-term survival was assessed for 80 days thereafter and at least 3 weeks after the last case of morbidity.

Assessment of changes in tumor volume and body weight between the first CpG-C treatment and the day of amputation revealed no main effects for CpG-C. When overall survival was assessed using the Kaplan-Meier model (Tarone-Ware test) we found a significant elevation in recurrence-free survival rates in animals treated with the pharmacological blockers, irrespective of CpG-C treatment [$\chi^2_{(3,N=229)} = 17.629$, $P = 0.0005$] (Fig. 7). CpG-C treatment had only a marginal effect on recurrence-free survival levels in this study (irrespective of the pharmacological blockers) ($\chi^2_{(1,N=229)} = 3.483$, $P = 0.062$).

DISCUSSION

In this study we addressed the common and critical postoperative complications of immunosuppression and accelerated tumor progression, and attempted to overcome them by simultaneously employing 2 strategies: (i) attenuating excessive neuroendocrine CA and PG responses that promote tumor progression by directly impacting the malignant tissue and by causing immunosuppression and (ii) inducing perioperative immunostimulation employing CpG-C. The integrated use of these therapies during the critical perioperative period improved the depressed immune profile observed after laparotomy, as indicated by augmented postoperative NK-cell numbers and cytotoxicity, improved tumor cell clearance from the lungs, and increased long-term recurrence-free survival after primary tumor excision. These beneficial outcomes were the result of additive and/or synergistic impacts of the 2 approaches.

Our previous studies have found PGs and CAs to have a profound deleterious impact on perioperative immunity. Furthermore, intervention studies conducted in our laboratory have demonstrated

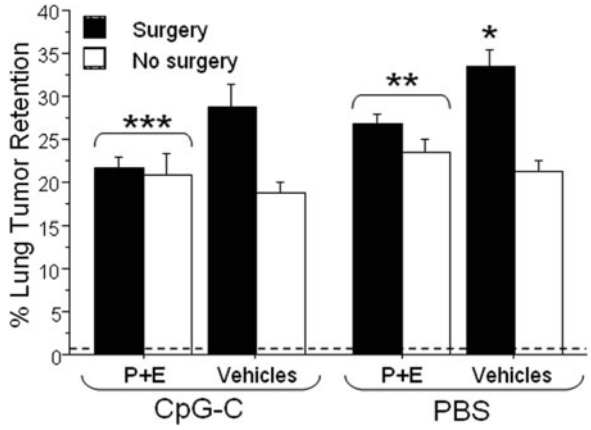

FIGURE 5. Both NK-dependent and independent mechanisms play a role in the effects of CpG-C and the pharmacological blockers on pulmonary tumor clearance. Rats were administered CpG-C or PBS. Twenty-three hours later, rats received either a combination of propranolol and etodolac (P+E), or their respective vehicles, and 1 hour later were further subdivided to undergo laparotomy, or to serve as home cage controls. Immediately after the surgical procedure all animals were injected with MADB106 tumor cells for the assessment of 21-hour lung tumor retention. Simultaneously with MADB106 administration, rats were injected with the anti-NKR-P1 mAb for the functional inhibition of NK cells. NK inhibition dramatically raised lung tumor retention levels by approximately 50-fold, compared with animals that did not undergo NK-inhibition and had an average lung tumor retention of 0.47% (see dashed line). Surgery significantly increased tumor retention levels in NK-inhibited animals compared with nonoperated controls (*), indicating non-NK mediated effects of surgery. A significant interaction between the effects of surgery and P+E treatment (**) indicated that the blockers reduced the non-NK mediated effects of surgery. No such interaction was evident with CpG-C, indicating that its beneficial effects are mostly mediated through NK cells. The combination of CpG-C and the pharmacological blockers was most effective, completely abolishing all increases caused by surgery, and significantly more so than the blockers alone (***). Data are presented as mean + SEM, $P < 0.05$.

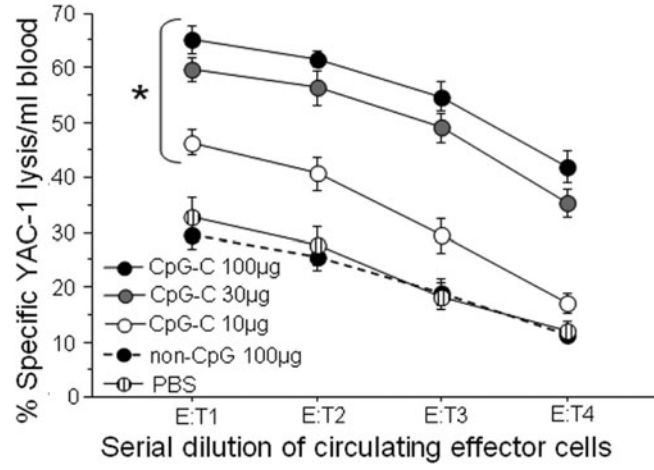

FIGURE 6. CpG-C elevated NK activity in mice in a dose-dependent manner. Mice were injected with 10, 30, or 100 μg CpG-C, 100 μg non-CpG, or PBS. Twenty-eight hours later all mice were sacrificed and blood was withdrawn. Only CpG-C augmented YAC-1 target cell lysis in a dose-dependent manner (*), while leaving NK-cell numbers unchanged (not shown). Data are presented as mean ± SEM, $P < 0.05$.

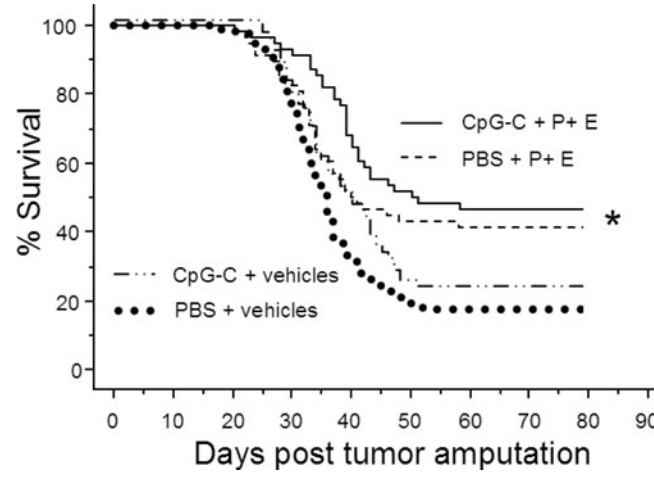

FIGURE 7. The effects of pharmacological blockade and CpG-C on postoperative survival rates after removal of a spontaneously metastasizing tumor. Mice were orthotopically implanted with syngeneic B16 melanoma tumor cells into the right hindpaw. When tumors reached a volume of ≥100 mm^3, mice were administered CpG-C or PBS. Twenty-four hours later the pharmacological blockers (P+E) or their vehicles were administered, and the tumor-bearing hindpaw was amputated. A day after the amputation, the animals received another course of CpG-C/PBS. Long-term survival was assessed for 80 days thereafter. Significant elevation in recurrence-free survival levels was found in animals treated with P+E, irrespective of CpG-C treatment (*$P < 0.05$). Although CpG-C treatment increased recurrence-free survival rates, these effects were only marginally significant ($P = 0.062$).

the advantage of simultaneous preoperative administration of propranolol (β-blocker) and etodolac (COX2 inhibitor), as opposed to either drug alone. Specifically, only their combined administration attenuated postoperative immune suppression in rats,[80] and similarly only their coadministration improved long-term survival rates after excision of a primary B16 melanoma.[55] Therefore, in this study only the combined use of propranolol and etodolac was employed. It is noteworthy that use of these drugs during the perioperative period has additional benefits, as propranolol reduces the need for anesthetic and analgesic agents during the perioperative period, in addition to having neuroprotective effects after stroke, and limiting perioperative cardiac complications.[81] Premedication with etodolac has been reported to reduce postoperative cortisol levels, and decrease postoperative pain scores,[82] although use of other selective COX2 inhibitors preoperatively has also shown reduced postoperative opioid requirements.[83] Moreover, preventing postoperative immune suppression with these drugs could reduce the incidence of infection, sepsis, and organ failure, postoperative complications that are often fatal.

The use of propranolol and etodolac is also rationalized by the fact that their effects on metastatic progression are achieved via both immune-dependent and independent mechanisms. Immune-mediated mechanisms are exemplified, in part, by their abovementioned effects on NK-cell activity, but also by the previously reported amplified postoperative levels of Fas receptor (CD95) on NK cells,[55] and restored levels of tumor necrosis factor alpha (TNFα).[84] Furthermore, tumor cells have been demonstrated to secrete PGs that inhibit $CD4^+$ T-cell signaling,[43] thus evading immune detection and destruction. Immune-independent mechanisms include the prevention of tumor angiogenesis and increased tumor cell apoptosis by COX2 inhibition,[85] and reduced tumor cell secretion of vascular endothelial growth factor (VEGF)[57] and matrix metalloproteinase 9 (MMP9)[86] by propranolol. In the context of tumor development, propranolol has also been shown to limit the stress-induced augmentation of B16 melanoma primary tumor growth in C57BL/6 mice,[87] and the selective COX2 inhibitor NS-398 combined with IFNγ (but not alone) was effective in reducing B16 development.[88]

As treatment with either CpG-C immunostimulation or pharmacological blockade was effective in limiting stress and surgery-induced immunosuppression and tumor progression, we integrated these treatments to achieve optimal effects. The improved short-term outcomes of the integrated treatment were exemplified throughout the study. Notably, although both CpG-C and the pharmacological blockers improved postoperative tumor clearance from the lungs, only their combination resulted in nullification of the detrimental effects of surgery. Examination of NK cytotoxicity levels revealed the same pattern of beneficial effects in both the circulation and the MP compartment. It is noteworthy that these beneficial effects occurred on a per NK cell basis, rather than resulting from redistribution of NK cells. Importantly, although CpG-C was mostly effective through increasing NK activity, the pharmacological blockers acted mostly by preventing suppression of MP-NK cytotoxicity. The elevated NK cytotoxicity in both immune compartments may be partly attributed to the enhanced expression of CD11a (LFA-1, lymphocyte function-associated antigen 1) on NK cells after treatment with CpG-C, as CD11a plays a crucial role, not only in NK-cell mobilization, but also in phosphorylation-dependent NK-cell activation.[77,78]

The superior effects and the complementary nature of the combined treatment, compared with each treatment alone, may be accounted for through the different cellular mechanisms targeted by each approach: CpG-C binds to TLR9 and activates a robust proinflammatory immune response that includes augmented NK activity,[79] whereas propranolol and etodolac prevent CAs and PGs (respectively) from activating their cell surface receptors on NK cells, thus preventing the intracellular rise of cAMP, known to suppress NK cytotoxicity.[51,89,90] The differences in mechanisms of action of the 2 approaches were also reflected in our study by the different profiles of LTR that emerged after functional NK-cell inhibition. Our findings showed that although the pharmacological blockers lowered LTR levels by both NK-dependent and -independent mechanisms, CpG-C functioned mostly through a NK-mediated mechanism. Taken together, it is clear that the integrated perioperative use of CpG-C, which increased immune activity, and the pharmacological blockers, which maintained immunocompetence, enabled the improvement of surgery-induced immunosuppression, and enhanced the animals' capacity to battle MRD.

A treatment regimen that results in sustained IL-12 levels may be clinically beneficial, as decreased levels of IL-12 have also been documented in patients after surgery,[17] and suppressed pre- and postoperative levels of IL-12 have been associated with an increased risk of postoperative sepsis.[22,23] CpG is know to increase IL-12 levels few hours after its administration,[91] but in this study we were unable to observe such an increase, probably as we assessed IL-12 levels 26 hours after CpG-C immunostimulation. The evident elevation in NK cytotoxicity, however, supports its preceding increase. Postoperatively, neither CpG-C immunotherapy nor the pharmacological blockers protected the animals from a drop in circulating IL-12 levels, when tested 2 hours after surgery. Future studies should attempt inhibition of corticosterone, as it has been shown to dampen the ability of dendritic cells to produce IL-12 upon antigen uptake,[92] and it is associated with postoperative immunosuppression.[93] Furthermore, recent studies in our laboratory have shown that after preoperative treatment with propranolol and etodolac, the elevated postoperative levels of corticosterone drop considerably at 12 hours, but not at 2 hours after surgery.[55] Thus, the surgery-induced reduction in IL-12 levels evident 2 hours postoperatively may be attenuated at a later time-point, with or without the additional use of a steroid antagonist.

The short-term effects of the combined treatment regimen are promising, and may also be reflected in reduced postoperative complications. However, with respect to tumor development, the MADB106 experimental metastasis model used is of limited clinical relevance. Therefore, we also used the combined regimen in a model of spontaneous metastasis in which B16 melanoma was implanted orthotopically, and long-term survival rates were assessed after excision of the primary tumor. Our findings showed that a single preoperative administration of the pharmacological blockers was effective, more than doubling the long-term recurrence-free survival rates in mice bearing this solid, weakly immunogenic, metastasizing, primary tumor. On the other hand, at the dose and time given, the effects of CpG-C administration (the day before and the day after tumor removal) were marginal ($P = 0.062$), and survival rates increased only by 5%. The efficacy of the pharmacological blockade replicates our pervious findings,[55] however, the marginal CpG-C effect is surprising, as it has repeatedly been reported to be effective in regressing tumors at the given, or similar, doses.[94,95] Moreover, NK cells, which were demonstrated herein to be predominantly responsible for the short-term effects of CpG-C on tumor retention, have also been shown to play a significant role in the control of B16 metastatic colonization.[96] Nonetheless, the marginal success of CpG-C in preventing metastasis in this model may be due to a limited ability of CpG-C to activate NK cells in tumor bearing mice, or to limited sensitivity of the B16 spontaneous-metastasis process to NK cytotoxicity. On the other hand, B16 tumor cells and their spontaneous metastasis may be sensitive to other biological mechanisms, specifically those that are repressed by anti-PG and anti–β-adrenergic pharmacologic agents, which may include changes in angiogenesis, inflammatory cytokine/growth factor signaling, macrophage contributions to metastasis, etc.[97] Another possible explanation for CpG-C's limited efficiency in the B16 model may be attributed to elevated PG levels reported to result from CpG administration.[98–101] As the CpG-C injections in the study were delivered either 24 hours before or after the administration of the COX2 inhibitor, CpG-C–induced secretion of PGs may have had self-limiting effects. Moreover, B16 melanoma tumors themselves have been shown to induce secretion of PGE2 from macrophages, therefore the addition of CpG-C–induced PGE2 may have further exacerbated the conditions in the B16 model. Thus, future studies should examine the effects of simultaneous administration of CpG-C and a COX2 inhibitor in this model. Finally, various cytokines and chemokines induced by immunostimulators are known to promote tumor proliferation,[102] thus CpG-C and other BRMs may be less effective in the presence of a primary tumor and micrometastases, than in models of experimental metastasis (eg, MADB106).

The use of paw amputation is a radical approach for tumor excision but unavoidable in the current B16 model. In cancer patients, less aggressive approaches are commonly used as surgeons try to preserve as much viable tissue as possible. However, the current use of the B16 melanoma can model the majority of resection procedures in patients in terms of tumor dissemination, as it has been widely documented

that during all types of surgical procedures, including laproscopic and some "no touch" procedures, tumor cells disseminate into the bloodstream[3,103,104] and have the potential to form metastases. Also noteworthy are the reports that surgical procedures that are considered less severe can result in similar future metastatic development. For example, it has been reported that port-site metastasis formation, which is clearly the result of the surgical procedure, is found at a rate comparable to the metastatic rate documented after open procedures.[105]

Several limitations of the study are noteworthy. Although our combined treatment regimen was highly effective in reducing postoperative immunosuppression and improving MADB106 lung tumor retention, the generalizablity to the treatment of MRD in humans after resection surgery is yet unclear. The metastatic process of MADB106 has been shown to be sensitive to NK activity predominantly in the first 24 hours after inoculation,[67,68] and therefore the index of lung tumor retention is clearly reflective of in vivo NK activity levels, but additional processes most likely affect the number of metastases that the animals develop weeks later.[69] Therefore, the findings from the MADB106 model strongly support a short-term role for NK cells in mediating the benefits of the combined perioperative therapeutic approach. However, the role of NK cells in mediating the beneficial effects of pharmacological blockade on survival in the B16 melanoma model remains unclear and requires further investigation, although some studies suggested a role for NK cells in this model.[106] Finally, in clinical trials, the significance of NK-cell activity for survival after resection surgery has not yet reached a consensus, as findings both supporting and refuting the role of NK cells in the mediation of cancer recurrence and metastatic control have been reported.[107–109]

In sum, metastatic outbreak is common after resection surgery, and is the major cause of cancer-related deaths that are attributable to metastatic damage to vital organs.[1,41] Thus, it is essential to minimize postoperative progression of MRD. Removal of the primary tumor eliminates part to the immune suppression apparent before surgery,[110] however, the majority of patients suffer from surgery-induced immunosuppression during the postoperative period. The perioperative treatment regimen tested in this study, employing CpG-C immunotherapy along with propranolol and etodolac, was efficient in counteracting postoperative immunosuppression, increasing the animals' ability to clear tumor cells from the lungs, and elevating long-term disease-free survival rates. These were achieved by enhancing immunocompetence, providing protection against immunosuppression, and potentially restricting nonimmunological tumor-promoting processes by the pharmacological blockade. Further studies are warranted in humans to determine whether the treatment regimen can improve short-term postoperative susceptibility for infections, and long-term outcomes in patients undergoing oncological surgery.

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