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CpG-C oligodeoxynucleotides limit the deleterious effects of β -adrenoceptor stimulation on NK cytotoxicity and metastatic dissemination

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Abstract

Suppression of natural killer (NK) cell activity is common following stress, has been reported to predict malignant recurrence in cancer patients, and was shown to underlie metastatic dissemination in animal models. We have previously reported that catecholamines play a major role in NK cell suppression, particularly in the context of physiological stress and surgery. In the current study using F344 rats, we examined the prophylactic use of different regimens of type-C CpG oligodeoxynucleotides (CpG-C ODN) on NK activity and metastatic dissemination in the context of pharmacological stress (using metaproterenol for β -adrenoceptor stimulation). Our results indicated that the beneficial effects of CpG-C ODN were more profound under pharmacological stress than under baseline conditions. A bolus of CpG-C ODN (330 μ g/kg, i.p.) 24 hrs prior to metaproterenol-challenge was most effective at reducing lung tumor retention of an experimental syngeneic mammary adenocarcinoma (MADB106), while having no observable side effects. Depletion of NK cells revealed their key role in improving baseline levels of resistance to metastatic dissemination following CpG-C ODN administration. When NK cell cytotoxicity was assessed in the circulation and the marginating-pulmonary immune compartments we found that CpG-C ODN protected individual NK cells from metaproterenol-induced suppression in both compartments. Moreover, in the critical marginating-pulmonary compartment, CpG-C ODN also elevated baseline cytotoxicity per NK cell against MADB106 tumor cells, and increased NK cell numbers in non-stressed rats. Overall, prophylactic CpG-C ODN treatment can improve immunocompetence and potentially reduce metastatic dissemination, especially in clinical settings characterized by enhanced sympathetic stress responses.

Keywords

CpG-C oligodeoxynucleotides; NK cells; metastatic dissemination; stress; beta-adrenergic stimulation

Introduction

Since their characterization, cytosine-phosphate-guanine (CpG) oligodeoxynucleotides (ODNs) have been studied as immunotherapeutic agents for the treatment of different diseases, including various cancers. The unmethylated C-G motifs in these synthetic ODNs mimic

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bacterial DNA and endow the ODNs with robust immunostimulatory capacity through the engagement of intracellular Toll-like receptor 9 (TLR9).¹ Three distinct families of CpG ODNs have been classified according to the ODNs' sequence, backbone, and the specific immune responses they engender. Members of the CpG-A ODN family are potent natural killer (NK) cell activators, and induce effective responses against NK-sensitive tumors. This class, however, is highly susceptible to nuclease degradation.² CpG-B ODNs are effective activators of B cells, less so of NK cells, and induce a stronger effect when a more elaborate and lengthy immune response needs to be orchestrated.² The novel phosphorothioate-stabilized CpG-C ODN family has more rarely been studied *in vivo*,³⁻⁶ and displays characteristics of both the A and B classes, in addition to unique immunostimulatory characteristics.⁷ CpG-C and CpG-A ODNs induce marked secretion of type I interferons and interleukin (IL)-12 by plasmacytoid dendritic cells (pDCs), which subsequently activate NK cells and other constituents of cell-mediated immunity (CMI), and enhance tumor-specific and non-specific immune responses.⁸⁻¹⁰

CpG ODN immunostimulation has been extensively studied in animal models for the treatment of solid tumors and for the development of cancer vaccines.^{2, 6, 11-13} Unlike treating solid tumors, the efficacy of CpG ODNs in reducing metastatic dissemination^{8, 14, 15} has been studied less frequently. Moreover, their potential benefits have not been examined in the context of physiological stress responses, which characterize various medical procedures.

The majority of cancer patients with solid tumors undergo resection surgery. Although lifesaving, the surgical procedure has also been suggested to render patients more susceptible to the establishment of new metastases.¹⁶ This is due, in part, to the shedding of tumor cells from the primary tumor caused by the physical manipulation of the primary tumor during surgery.¹⁶⁻¹⁸ Furthermore, suppression of CMI, NK cells in particular, has been shown to contribute to this increased susceptibility.¹⁹ Interestingly, augmented secretion of catecholamines (CAs) during the perioperative period has been shown to mediate postoperative suppression of CMI, including NK activity.^{18, 20-23} Such suppression was demonstrated even following preoperative IL-12 treatment, rendering this immunostimulatory approach ineffective in protecting CMI in the context of surgical stress.²⁴ Furthermore, we have previously demonstrated that pharmacological stress (based on β -adrenoceptor stimulation) and surgery can each enhance metastatic dissemination to the lungs and increase the number of actual lung metastases assessed several weeks following tumor cell inoculation.²⁵

In the current study we aimed to develop a regimen of type-C CpG ODN administration to increase the resistance to metastatic dissemination by enhancing NK activity in the context of physiological stress responses. To this end, we employed an *in vivo* NK-sensitive tumor model of experimental metastasis to simulate metastatic dissemination, and assessed NK cell number and activity in the circulation and in the marginating-pulmonary immune compartments. These indices were studied with and without subjecting animals to β -adrenergic stimulation using the pharmacological stressor metaproterenol.

Materials and Methods

Animals and counterbalancing

Male and female Fisher 344 (F344) rats (Harlan Laboratories, Jerusalem, Israel) were housed 4 per cage with free access to food and water on a 12:12 light:dark cycle at 22-24°C. Animals were acclimated to the vivarium for at least 3 weeks prior to experimentation and were handled daily during the last week prior to experimentation to reduce potential procedural stress. Order of drug administration, 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'-TCGTCGTTTTCGGGCGCGGCCG-3') with a phosphorothioate backbone was used in all experiments. In experiment 1 non-CpG ODN (ODN 2137: 5'-TGCTGCTTTTGTGCTTTTGTGCTT-3') was used as a control [in addition to the phosphate buffered saline (PBS) control], as it lacks C-G motifs. ODNs were used in doses ranging from 66 to 660 µg/kg and were injected i.p. unless otherwise noted. All ODNs were purchased from Coley Pharmaceuticals Canada (Ottawa, Canada), and contain undetectable levels of endotoxin as measured by the limulus amebocyte lysate assay.

Metaproterenol—A non-selective β -adrenergic agonist with higher affinity to β_2 than β_1 receptors.²⁶ Male rats were subcutaneously administered with 1mg/kg metaproterenol (Sigma, Rehovot, Israel) in PBS, while female rats were injected with 3mg/kg in order to reach the same physiological effect, unless otherwise noted. These doses were based on our previous studies, and were found to induce responses similar in magnitude to those elicited by behavioral and physiological manipulations.^{22, 25}

Slow release vehicle—The slow release vehicle (SRV) is an emulsion used to extend absorption time of drugs, and is based on 4 parts PBS, 3 parts mineral oil (Sigma, Rehovot, Israel), and 1 part mannide-monooleate (a non-specific surface active emulsifier, Sigma, Rehovot, Israel). Following subcutaneous injection (1ml/rat), we found the impact of drugs dissolved in SRV to continue for approximately 36 hrs (unpublished data). In experiment 5 CpG-C ODN was injected s.c. in SRV at a dose of 330 µg/kg.

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.²⁷ MADB106 tumor cells metastasize only to the lungs,²⁷ 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.^{22, 27-29} Additionally, because the metastatic process of MADB106 is sensitive to NK activity predominantly in the first 24 hours following inoculation,^{27, 28} lung tumor retention is more reflective of *in vivo* NK activity levels than the number of actual metastases is.²² Maintenance, radiolabeling and the assessment of lung tumor retention were described elsewhere.²⁴ This cell line was used for both *in vivo* and *in vitro* studies.

YAC-1—YAC-1 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% CO₂ at 37°C.

Experimental laparotomy

The procedure has been described elsewhere.²⁰ Briefly, rats were anesthetized with 2.5% isoflurane and a 4cm midline abdominal incision was performed. The small intestine was externalized, rubbed with a PBS-soaked gauze pad and left hydrated with a PBS-soaked gauze pad for 30 mins. Finally, the intestine was internalized and the abdomen sutured.

Assessment of extrapulmonary MADB106 metastases

Non-labeled MADB106 tumor cells (10⁴ per animal in 0.5ml PBS containing 0.1% bovine serum albumin) were injected into the tail vein of lightly anesthetized rats. Three weeks later all animals were sacrificed and their lungs were removed and placed in Bouin's solution (72% saturated picric acid solution (Sigma, Rehovot, Israel), 23% formaldehyde (37% solution, Sigma, Rehovot, Israel) and 5% acetic acid glacial (Sigma, Rehovot, Israel) for 24hrs. Lungs were then washed in ethanol prior to enumeration of visible extrapulmonary metastases.

Selective *in vivo* depletion 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 above-mentioned dose of the anti-NKR-P1 mAb showed selective and complete abolition of blood and splenic NK cytotoxicity³⁰ and a 200-fold increase in the lung retention and metastatic colonization of MADB106 tumor cells.³¹ 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 in experiment 7 PBS was used as the control.²⁸

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. Three ml of blood were collected from the right ventricle of the heart into heparinized syringes (30U/ml blood). One ml of blood was washed once with 3ml of PBS (400g for 10 min) and twice with 3ml of complete media, and reconstituted to its original volume. Marginating-pulmonary leukocytes were harvested by perfusing the heart with 30U/ml of heparinized PBS. PBS was injected into the right ventricle and perfusate was collected from the left ventricle. The first 3ml of perfusate, which were contaminated with blood, were discarded, and the following 25ml were collected and concentrated to 1ml. This was achieved by centrifuging the perfusate (400g for 10 min), discarding the supernatant, and suspending the pellet in 3ml of complete media, centrifuging the perfusate again (400g for 10 min) and concentrating the perfusate into 1ml.

Assessment of NK cell cytotoxicity—The standard whole blood ⁵¹Cr release assay was used and has been described elsewhere.²² 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 depletion of NK cells abolishes all target-cell killing.^{32, 33} 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 (LU₃₅) per marginating-pulmonary 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 LU₃₅ per the total lung perfusate is the number of aliquots that can be taken from the perfusate, each of which is capable of reaching 35% specific lysis of target cells. Thus, higher LU values reflect greater NK cytotoxicity against target cells. The value of LU₃₅ is mathematically derived from the concentration of effector cells at which 35% specific killing was reached in the standard cytotoxicity assay described above. The regression exponential fit method³⁴ was used to calculate LU₃₅ for each sample, based on percent-specific lysis of YAC-1 target cells by marginating-pulmonary NK cells. To determine cytotoxicity per marginating-pulmonary NK cell, the value of LU₃₅ per lung perfusate was divided by the number of marginating-pulmonary (MP) NK cells per lung perfusate (according to FACS analysis, see below), yielding the index of LU₃₅/MP-NK. This index reflects NK cell cytotoxicity per NK cell in each sample.

Comparing cytotoxicity per marginating-pulmonary NK cell, based on mathematical shifting of cytotoxicity curves—The following is an alternative approach for comparing NK cytotoxicity per NK cell. First, cytotoxicity levels of the 6 effector-cell

concentrations were used for each sample to generate a cytotoxicity curve, based on the regression exponential fit method.³⁴ Then, based on the number of marginating-pulmonary NK cells unique to each sample, each curve was positioned in a cytotoxicity (y) by E:T ratio (x) plot, in a horizontal location based on its individual marginating-pulmonary NK:Target ratio. Thus cytotoxicity in different samples can be compared based on the same number of marginating-pulmonary NK cells. Consequently, mean group cytotoxicity can be compared based on the same marginating-pulmonary NK:Target ratio, which indicates cytotoxicity per marginating-pulmonary NK cell in the context of the whole lung perfusate milieu. In our experience this approach yields a range of at least 4 marginating-pulmonary NK:Target ratios that are common to the great majority of samples in each study.

Flow Cytometry

Standard procedures were used to prepare cells for FACS analysis.²⁰ NK cells in both 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. This criterion has been shown to exclusively identify more than 95% of cells that exhibit NK activity.³⁵⁻³⁷ T cells were identified using a PE-conjugated anti-CD5 mAb (eBioscience, San Diego, CA) and NKT cells were identified as NKR-P1⁺CD5⁺ lymphocytes. Granulocytes and lymphocytes were identified based on forward and side scatters. To assess the total number of cells per μ l of sample (or a specific cell subtype), 300 polystyrene microbeads (20 μ m, Duke Scientific, Palo Alto, CA) per μ l sample were added to each sample, and the following formula was used: (# of cells in sample/ # of microbeads in sample) $\times$ 300.

Statistical Analysis

One- or two-way factorial analysis of variance (ANOVA) with a pre-determined significance level of 0.05 was conducted. In most *in vivo* studies, the variances evident in groups receiving metaproterenol were larger than in groups receiving vehicle, violating the ANOVA assumption of homogeneity of variance, and thus the ANOVA was conducted on *lan* transformation of all data. Graphs depict group means prior to 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.

Results

CpG-C ODN hinders the development of extrapulmonary metastases

Rats (n = 31 males, 9-12 per group) were administered with CpG-C ODN (330 μ g/kg) either 24 hrs before inoculation with MADB106 tumor cells, or both 24 hrs before and 24 hrs after tumor cell inoculation. The control group was injected with PBS. To control for the effects of the injections themselves, all rats received 2 injections (each containing PBS or CpG-C ODN).

The number of extrapulmonary metastases enumerated 3 weeks later was lower in CpG-C ODN treated animals as revealed by one-way ANOVA [$F_{(2,28)}=5.421$, $p=0.0102$] (Figure 1). Fisher's *post hoc* PLSD indicated that the single injection of CpG-C ODN was of borderline significance from the PBS control group ($p = 0.07$), and 2 injections of CpG-C ODN significantly reduced the number of surface lung metastases ($p=0.0029$). As the metastatic process is complex, and our interest in this study lies in the effect of CpG-C ODN on NK cells that control MADB106 metastasis only during the first 24 hrs after tumor inoculation,²⁸ we limited the rest of our study to this time-frame and assessed lung tumor retention at the 24h time-point.

A single injection of CpG-C ODN is effective in reducing the metastasis-promoting effects of the pharmacological stressor, metaproterenol

Rats (n = 47 males, 4-7 per group) were challenged intraperitoneally (i.p.) with CpG-C ODN (330µg/kg or 66µg/kg), non-CpG ODN (330µg/kg), or PBS 24 hrs prior to intravenous inoculation with radiolabeled MADB106 tumor cells. Simultaneously with tumor inoculation rats from each group were injected with either the pharmacological stressor, metaproterenol (1mg/kg, s.c.), or with PBS. Lung tumor retention was quantified 24 hrs following tumor cell inoculation.

Two-way ANOVA revealed significant main effects for both CpG-C ODN pretreatment and metaproterenol [$F_{(3,39)}=7.12$, $p=0.0006$ and $F_{(1,39)}=270.303$, $p<0.0001$ respectively]. Importantly, Fisher's *post hoc* PLSD indicated that the high dose of CpG-C ODN (330µg/kg) significantly attenuated the increase in lung tumor retention caused by metaproterenol ($p=0.016$, Figure 2), while the low dose of CpG-C ODN (66µg/kg) and non-CpG had no effects. Therefore, in subsequent studies only PBS was used as control. Assessment of the effects of CpG-C ODN on baseline levels of lung tumor retention (no metaproterenol) showed no significant group differences, although a trend towards beneficial effects of the high dose of CpG-C ODN was evident.

Both doses of CpG-C ODN did not cause weight loss in this study (data not shown).

Comparing different routes of CpG-C ODN administration

As Hafner et al.¹⁵ reported that the route of CpG ODN administration affected the potency of its impact on lung metastatic colonization in mice, we compared different administration routes in F344 rats. Animals (n=62 males, 6-10 per group) were lightly anesthetized with isoflurane and injected with CpG-C ODN (330µg/kg) i.p., i.v., or s.c.. Rats not receiving CpG-C ODN were injected with PBS i.v.. Twenty four hrs later, all rats were inoculated with MADB106 tumor cells and were subjected, or not, to metaproterenol (1mg/kg). Lung tumor retention was assessed 24 hrs later as described above.

Two-way ANOVA revealed significant main effects for both CpG-C ODN administration and metaproterenol [$F_{(3,55)}=11.325$, $p<0.0001$ and $F_{(1,55)}=361.375$, $p<0.0001$, respectively]. Importantly, Fisher's *post hoc* PLSD indicated that compared to the PBS control group, all animals treated with CpG-C ODN showed significant reduction in lung tumor retention ($p<0.02$ in all comparisons, Figure 3), and that i.p. administration yielded significantly lower levels of lung tumor retention compared to the other two administration routes ($p<0.02$ in all comparisons).

In this study CpG-C ODN also had an effect on baseline levels of lung tumor retention, as indicated by a main effect of one-way ANOVA conducted on the four groups not receiving metaproterenol [$F_{(3,21)}=3.988$, $p=0.0215$], and followed by PLSD *post hoc*. Specifically, i.p. administration of CpG-C ODN was followed by significantly lower lung tumor retention than PBS and CpG-C ODN administered i.v. ($p=0.0041$ and $p=0.0228$, respectively). Therefore, i.p. was the preferred route of administration in the remainder of our studies.

Time-course of the protective effects of a single CpG-C ODN administration

This study was conducted to confirm the efficacy of CpG-C ODN in both sexes and to establish its time-course of effect. Rats (54 males, 4-5 per group, and 52 females, 4-5 per group) were injected with CpG-C ODN (330µg/kg) at either 4, 12, 24, 48 or 72 hrs prior to inoculation with MADB106 tumor cells. Control animals were injected with PBS at the 4hr time-point. Lung tumor retention was assessed as above with or without subjecting rats to metaproterenol (1mg/kg for both males and females in this study).

As in our previous studies,³⁸ metaproterenol had a larger effect on males than on females, and thus the data were analyzed separately. For males, two-way ANOVA demonstrated a significant main effect of CpG-C ODN and of metaproterenol [$F_{(5,42)}=5.707$, $p=0.0004$ and $F_{(1,42)}=222.76$, $p<0.0001$, respectively]. Notably, Fisher's *post hoc* PLSD analysis showed that administration of CpG-C ODN 12 and 24 hrs prior to tumor inoculation provided maximal protection in this context, bringing about a reduction of up to 68% in lung tumor retention (Figure 4a, $p<0.05$ in all statistically significant comparisons).

As for the females, the same kinetics of CpG-C ODN protection were evident, and the same statistical analysis yielded significant effects [CpG-C ODN administration - $F_{(5,40)}=4.266$, $p=0.0033$, and metaproterenol - $F_{(1,40)}=78.649$, $p<0.0001$, Figure 4b]. In this experiment CpG-C ODN did not significantly affect baseline levels of lung tumor retention in either males or females, although a trend toward such an effect was evident.

A bolus of CpG-C ODN is more effective than continuous slow administration

We compared acute administration of CpG-C ODN as administered above in PBS, to a regimen employing a slow release vehicle (SRV) that prolongs absorption to approximately 36 hrs. Rats ($n = 74$ males, 7-10 per metaproterenol group, 3-6 per saline group) were injected with CpG-C ODN (330 μ g/kg) in either PBS or SRV, or received vehicle injections of either PBS or SRV. CpG-C ODN was injected 24 or 48 hrs prior to MADB106 tumor cell inoculation, while all control rats received the injection at the 24hr time-point. To control for the timing and number of injections, all rats in the study received two injections (no more than one SRV injection). At the time of tumor cell inoculation rats were exposed to the pharmacological stressor metaproterenol (1 mg/kg) or not. Twenty four hours later all animals were sacrificed and lung tumor retention was assessed.

Two-way ANOVA indicated significant main effects for CpG-C ODN treatment and metaproterenol [$F_{(5,63)}=16.402$, $p<0.0001$ and $F_{(1,63)}=653.911$, $p<0.0001$, respectively]. CpG-C ODN administered both in PBS and SRV was effective at reducing lung tumor retention at both time-points, compared to both control groups (PLSD $p<0.0004$ in all comparisons, Figure 5). When two-way ANOVA was conducted on the 4 groups treated with CpG-C ODN and metaproterenol, CpG-C ODN administered in PBS was more effective at reducing lung tumor retention than CpG-C ODN in SRV, and pre-treatment 24 hrs prior to tumor cell inoculation was more effective than the 48 hr interval [$F_{(1,29)}=18.953$, $p=0.0002$ for PBS vs. SRV, and $F_{(1,29)}=23.229$, $p<0.0001$ for 24 vs. 48 hrs]. In this study CpG-C ODN also caused a significant reduction in baseline levels of lung tumor retention [$F_{(5,17)}=7.03$, $p=0.001$]. Body weight was measured throughout the experiment every 24 hrs. As evident in the previous studies, CpG-C ODN administered in PBS did not cause weight loss. However, when administered in SRV, a significant reduction in body weight was evident 24 hrs following challenge [$F_{(3,70)}=4.469$, $p=0.0063$] compared to all other groups (PLSD *post hoc*, $p<0.02$ in all comparisons). Therefore, as administration of CpG-C ODN in PBS was more effective in reducing lung tumor retention under pharmacological stress, while having less side effects, it was used in the following studies.

A single dose of CpG-C ODN is as effective at improving lung tumor retention as repeated exposure

In order to determine whether repeated exposure to CpG-C ODN increases its efficacy in reducing lung tumor retention following pharmacological stress, rats (72 male and 77 female, 9-10 per metaproterenol group and 3-9 per saline group) were injected with CpG-C ODN or PBS once, twice, or three times in 24 hr intervals, and CpG-C ODN was employed in 3 different doses (0, 165, 330, 660 μ g/kg). For control purposes, all rats received a total of 3 daily injections (vehicle or CpG-C ODN). Twenty four hrs following the final administration of CpG-C ODN,

all rats were inoculated with MADB106 tumor cells with or without being subjected to metaproterenol (males 1mg/kg, females 3mg/kg). Lung tumor retention was assessed as described above.

The data showed very similar trends in males and females, as well as comparable tumor retention levels, and were thus analyzed together. CpG-C ODN and metaproterenol showed significant main effects [$F_{(9,129)}=6.897$, $p<0.0001$ and $F_{(1,129)}=411.35$, $p<0.0001$, respectively, Figure 6], and Fisher's *post hoc* PLSD contrasts indicated that within the metaproterenol groups all CpG-C ODN regimens significantly lowered lung tumor retention compared to PBS ($p<0.01$ in all comparisons).

Comparison of the different groups receiving both metaproterenol and CpG-C ODN yielded no significant effect for the number of CpG-C ODN injections on lung tumor retention levels. However, a main effect of CpG-C ODN dose was revealed [$F_{(2,80)}=3.366$, $p=0.0395$], with the groups receiving the lowest dose (165 μ g/kg) having significantly higher lung tumor retention than the groups receiving 330 μ g/kg (PLSD *post hoc* $p=0.0116$). As there was no difference between lung tumor retention levels in rats receiving 330 μ g/kg or 660 μ g/kg, in the following studies CpG-C ODN was administered in a single dose (acutely) of 330 μ g/kg.

As in some of the previous experiments, CpG-C ODN also brought about a reduction in baseline levels of lung tumor retention in animals not treated with metaproterenol [$F_{(9,40)}=3.385$, $p=0.0036$] in some of the CpG-C ODN regimens (PLSD *post hoc* $p<0.01$).

The role of NK cells in mediating the beneficial effects of CpG-C ODN *in vivo*

Here we tested whether NK cells are involved in the aforementioned beneficial *in vivo* effects of CpG-C ODN, by employing functional *in vivo* depletion of NK cells using anti-NKR-P1 mAb. Rats ($n = 156$ males, 8-11 per NK-intact group and 11-19 per NK-depleted group; data were collected in 2 replications in which all groups were represented) were challenged with CpG-C ODN (330 μ g/kg) or injected with PBS. Twenty four hrs later, rats were assigned to receive metaproterenol (1mg/kg), to undergo surgical stress (laparotomy), or to serve as no-stress controls, and were injected with MADB106 tumor cells for the assessment of 24 hr lung tumor retention. Simultaneously with MADB106 administration, half of the rats from each group were injected with the anti-NKR-P1 mAb and the other half with vehicle. We chose to administer the NK-depleting mAb simultaneously with MADB106 (and not days earlier) in order to prevent interference with the effects of CpG-C ODN that was administered 24 hrs prior to MADB106 administration.

As expected, functional depletion of NK cells caused a dramatic increase in lung tumor retention, validating the significant role of NK cells in controlling MADB106 lung tumor retention (Figure 7) [$F_{(1,144)}=2531.362$, $p<0.0001$]. CpG-C ODN treatment and the stressors applied also had main effects on lung tumor retention [$F_{(1,144)}=73.337$, $p<0.0001$ and $F_{(1,144)}=81.517$, $p<0.0001$, respectively]. Importantly, CpG-C ODN significantly reduced lung tumor retention in each of the NK-intact groups, but did not improve tumor clearance in non-stressed NK-depleted animals. Therefore it can be concluded that the beneficial effects of CpG-C ODN under the non-stress condition are mediated by NK cells [$F_{(1,53)}=22.428$, $p<0.0001$, interaction between NK depletion $\times$ CpG treatment, within the no-stress groups]. On the other hand, lung tumor retention levels in stressed animals (metaproterenol and laparotomy) were improved by CpG-C ODN treatment even following NK depletion, and thus it can be concluded that NK-independent mechanisms are involved in the beneficial effects of CpG-C ODN following surgery and metaproterenol, alongside the NK-dependent mechanisms (see next experiment).

With respect to the relative impact of metaproterenol and surgery: Whereas in NK-intact animals metaproterenol caused a significantly larger effect than surgery, in NK-depleted animals surgery caused a markedly and significantly larger effect than metaproterenol. This indicates that surgery has a greater non-NK mediated factor than metaproterenol. The beneficial effects of CpG-C ODN in metaproterenol-treated animals seem to depend mainly on NK cells, as they are evident in animals with intact NK cells, but to a much lesser extent in NK-depleted animals. Overall, it can be concluded that the beneficial effects of CpG-C ODN in operated animals are mediated not solely by NK cells, whereas such beneficial effects in metaproterenol treated animals are mediated mainly by NK cells (also see ²²), and in no-stress animals only through NK cells.

The effects of CpG-C ODN and metaproterenol on *ex-vivo* NK cell activity

As the effect of CpG-C ODN on *in vivo* resistance to metastasis in the context of metaproterenol seemed to be mediated to a large extent via NK cells, the following *ex-vivo* study was conducted to examine alterations within the NK cell population that may underlie the *in vivo* findings. Rats (n=39 males, 9-10 per group) were challenged with CpG-C ODN (330µg/kg) or vehicle, and 23 hrs later were assigned to receive either metaproterenol (1mg/kg) or vehicle. One hour later, blood and lung perfusate were collected from all animals for quantification of NK cytolytic activity and FACS analysis of cell composition.

Blood NK cell activity against YAC-1 target cells—In the circulation, metaproterenol caused significant suppression of NK activity [$F_{(1,35)}=8.368$, $p=0.0065$] (Figure 8a). CpG-C ODN did not affect baseline levels of NK activity, but completely protected the animals from suppression by metaproterenol [$F_{(1,35)}=8.239$, $p=0.0069$]. Both the effects of metaproterenol and CpG-C ODN seem to occur on a per-NK cell basis, as the numbers of NK cells did not change due to metaproterenol or CpG-C ODN treatment (Figure 8a). NK activity against MADB106 was not studied in the blood, as we have previously demonstrated that circulating NK cells are incapable of specific lysis of MADB106 target cells. ²⁰

Marginating-pulmonary NK cell activity against YAC-1 target cells—In the lung perfusate, both CpG-C ODN and metaproterenol caused marked effects on the numbers of marginating-pulmonary NK cells (Figure 8b, $F_{(1,34)}=13.577$, $p=0.0008$ for CpG-C ODN x metaproterenol interaction), and on their activity. When examining NK activity of the entire marginating-pulmonary NK population, it is evident that metaproterenol caused significant suppression of its cytolytic activity, and that independently of this suppression CpG-C ODN caused a significant elevation of NK activity [$F_{(1,34)}=132.325$, $p<0.0001$, and $F_{(1,34)}=47.352$, $p<0.0001$, respectively] (Figure 8b).

Examination of marginating-pulmonary NK activity per NK cell (rather than per the entire marginating-pulmonary NK population) was conducted based on calculation of LU₃₅/MP-NK# (see Methods). Figure 8c shows that while metaproterenol decreases NK activity per marginating-pulmonary NK cell, CpG-C ODN completely prevented this effect, without increasing baseline NK activity levels, as also indicated by a significant interaction between CpG-C ODN and metaproterenol [$F_{(1,34)}=9.494$, $p=0.0041$].

Marginating-pulmonary NK activity against MADB106 target cells—Similarly to marginating-pulmonary NK activity against YAC-1 target cells, CpG-C ODN increased total marginating-pulmonary NK activity against MADB106, and metaproterenol suppressed it (Figure 9a, $F_{(1,34)}=27.433$, $p<0.0001$ for CpG-C ODN, $F_{(1,34)}=41.996$, $p<0.0001$ for metaproterenol). Notably, the rise in NK cell number caused by CpG-C ODN within the marginating-pulmonary population was approximately 1.5-fold, which is not sufficient to explain the approximately 4-fold increase in marginating-pulmonary NK activity in this group.

Therefore, we compared marginating-pulmonary NK activity per NK cell between groups by shifting lysis curves based on sample-specific NK cell numbers, as described above (see Methods). As expected, CpG-C ODN elevated MADB106 cytotoxicity per marginating-pulmonary NK cell in both metaproterenol-treated and in control animals (Figure 9b $F_{(1,27)}=33.548$, $p<0.0001$ for CpG-C ODN, $F_{(1,27)}=12.938$, $p=0.0013$ for metaproterenol).

Discussion

Physiological stress responses, which were partly simulated by the administration of metaproterenol in the current study, involve the release of CAs, and have been shown to profoundly affect immune function by impairing CMI, NK cells in particular. In fact, NK cells are believed to be greatly affected due to their high density of β_2 -adrenoceptors compared to other lymphocytes.³⁹ The effects of NK cell suppression could also be of clinical relevance during the surgical excision of a primary tumor, as suppression of NK cytotoxicity during the perioperative period has been correlated with increased outbreak of postoperative metastasis in cancer patients.⁴⁰⁻⁴² Amongst other pro-metastatic effects attributed to surgery are enhanced angiogenesis, pain, anesthesia, hypothermia, pre-operative distress, and blood transfusion.¹⁸ 43, 44 Importantly, studies in our laboratory clearly indicate that administration of physiological levels of β -adrenergic agonists, or the endogenous release of CAs following various stressors, including surgical stress, suppresses NK activity *in vivo*, and this suppression compromised resistance to metastasis and leukemia.^{20, 23, 31, 45, 46} Therefore, it is important to understand the efficacy of immune stimulation in the context of stress and surgery.

In the current study we found that CpG-C ODN reduced metastatic development in F344 rats. In order to evaluate the role NK cells play in this dynamic process, and how their function may be impacted by stress and CpG-C ODN, we focused our study on the 24 hr period following tumor cell inoculation, as this time frame has been determined to be most important in NK activity against metastatic development.²⁸ Thus, we simulated dissemination of tumor cells, which commonly occurs during resection surgery, by intravenously inoculating rats with MADB106 tumor cells. The results indicated improved tumor clearance following CpG-C ODN treatment. Most importantly, as elaborated below, these beneficial effects were more profound under pharmacological stress conditions (metaproterenol) than under no-stress (baseline) conditions.

In the current study, a single intraperitoneal bolus of CpG-C ODN (330 μ g/kg) administered 24 hrs prior to tumor challenge was most effective at reducing the deleterious effects of the pharmacological stressor metaproterenol on *in vivo* resistance to metastatic dissemination. The approximately 60% attenuation of MADB106 tumor retention from the lungs induced by CpG-C ODN is attributable to the presence of unmethylated C-G motifs within the ODN, as rats treated with non-CpG ODN (which does not contain C-G motifs) did not differ from vehicle-treated animals.

Independently of the beneficial effects of CpG-C ODN, a difference in the magnitude of the effects of metaproterenol was evident between males and females, and is attributable to a diminished response to adrenoceptor stimulation in female F344 rats.³⁸ Therefore, in subsequent studies a higher dose of metaproterenol (3mg/kg) was used in females in order to equate its effects in both sexes. The beneficial effects afforded by this CpG-C ODN bolus were maximal (in both sexes) at 12 and 24 hours post-administration, diminishing thereafter and reaching baseline levels at 72 hrs post-administration. Surprisingly, unlike IL-12 administration,²⁴ repeated CpG-C ODN administration was not advantageous to its acute administration, nor was a higher dose. This could be attributed to a self-limiting effect of TLR9 activation, or a different homeostatic mechanism of immune regulation in response to CpG-C ODN. Perhaps related, an important merit of the chosen CpG-C ODN regimen is the lack of

observable side effects. Specifically, no differences in body weight were evident after treatment with a bolus of CpG-C ODN. In contrast, extended absorption of the same dose of CpG-C ODN (using a slow release vehicle) brought about significant weight loss without improving resistance to the malignancy.

As in the current study, CpG ODNs have been reported to elevate baseline NK activity and to improve tumor cell lysis.^{2, 47, 48} In rodents this effect is indirect, as NK cells do not express TLR9 receptors, but are activated by cell-to-cell contact with activated pDCs and by type I interferons secreted by the pDCs.^{49, 50} Sivori et al.⁵¹ reported that human NK cells express functional TLR9, and following *in vitro* exposure to CpG ODN human NK cells express cell-activation markers such as CD69 and CD25. Murine and human TLR9 systems differ in other aspects as well, as they are each optimally activated by different CpG sequences and produce differential immune responses.^{52, 53} Moreover, cells of myeloid lineage in rodents, but not humans, express functional TLR9.⁵³⁻⁵⁵

Previous studies have also indicated that CpG-C ODN can induce different immune responses in different immune compartments.⁵⁶ In the current study we evaluated the effect of CpG-C ODN pretreatment on NK activity in both the circulation and the marginating-pulmonary compartment, as we recently found a qualitative difference between these two NK cell populations.²⁰ Marginating-pulmonary NK cells, which adhere to the lung vasculature, contain a higher percentage of large NK cells, show greater cytolytic activity than circulating NK cells, and were the only NK cell population to exhibit significant *in vitro* cytotoxicity against the syngeneic MADB106 tumor line.^{20, 23} CpG-C ODN pretreatment positively affected NK cell activity in both immune compartments, having a greater impact in the context of the pharmacological stressor than under no-stress conditions. This is demonstrated by elevated lysis capacity of individual circulating NK cells against YAC-1 targets, and of individual marginating-pulmonary NK cells against both YAC-1 and MADB106 targets. The differences seen in the beneficial effects between marginating-pulmonary NK cell lysis of the xenogeneic YAC-1 and the syngeneic MADB106 tumor lines may be related to differential cytotoxicity mechanisms utilized by marginating-pulmonary NK cells against each tumor line. Also, it is worth noting that total marginating-pulmonary NK cytotoxicity against MADB106 tumor cells is a mirror image of the *in vivo* index of MADB106 lung tumor retention, suggesting a causal relationship. Furthermore, in the lungs, as opposed to the circulation, both CpG-C ODN treatment and the pharmacological stressor had marked effects on numbers of marginating-pulmonary NK cells. Interestingly, CpG-C ODN increased marginating-pulmonary NK cell numbers in non-stressed animals, but did not prevent the drop in their numbers following pharmacological stress, despite its aforementioned ability to protect individual marginating-pulmonary NK cells from immunosuppression. This suggests that metaproterenol-induced redistribution of marginating-pulmonary NK cells and its impact on their cytotoxicity rely on different molecular mechanisms or exhibit different kinetics.

Our studies in NK-depleted animals provide further evidence that the above alterations in marginating-pulmonary NK activity may explain the effects of CpG-C ODN and metaproterenol on the *in vivo* resistance to MADB106 retention in the lungs. NK cells have been previously²² and currently shown to markedly impact *in vivo* levels of MADB106 lung tumor retention in the first 24 hrs following tumor inoculation, as indicated by more than a 100-fold increase in this index following selective NK-depletion. The improved tumor clearance induced by CpG-C ODN in NK-intact animals could be explained by the evident increase in total lung marginating-pulmonary NK activity (assessed against both the MADB106 and the YAC-1 target cells), as it was completely absent in NK-depleted animals. The 6.5-fold increase in lung tumor retention caused by metaproterenol is, by and large, also attributable to the suppression in NK activity, as a much smaller increase (only 1.2-fold) was evident in NK-depleted animals. This smaller effect of metaproterenol in NK-depleted animals

cannot be attributed to a ceiling effect, as surgical stress caused a greater effect than metaproterenol in NK-depleted animals, though having a lesser effect in NK-intact animals. Thus, suppression of NK cell activity is the predominant mediator of the metaproterenol-induced rise in MADB106 lung tumor retention in NK-intact animals, and it can be further inferred that the beneficial effects of CpG-C ODN in metaproterenol-treated animals are mediated, at least partly, through the evident increase in marginating-pulmonary NK cell activity. With regard to the effect of CpG-C ODN on lung tumor retention in operated animals, it is clearly evident that NK cells do not account for the entire effect of CpG-C ODN: In NK-depleted animals surgery caused a marked increase in lung tumor retention, and CpG-C ODN pre-treatment significantly reduced it by approximately 50%. These interesting and important NK-independent mediated effects are the focus of our ongoing studies.

Overall, our findings regarding NK activity suggest that the mechanisms by which CpG-C ODN improve *in vivo* resistance to MADB106 metastasis are two-fold. On the one hand, in non-stressed rats CpG-C ODN significantly improve baseline levels of lung tumor retention through increased number and activity of marginating-pulmonary NK cells. On the other hand, CpG-C ODN treatment prevented or attenuated the metaproterenol-induced suppression of circulating- and marginating-pulmonary NK cells, conserving their lytic ability per-NK cell. CpG-C ODN's dual mechanism of action encompasses characteristics of other immunotherapeutic approaches. As we have previously reported, treatment with the TLR3 agonist, poly I:C, protected NK cells from metaproterenol-induced suppression without affecting baseline levels of lung tumor retention,²⁵ whereas treatment with the T helper 1 (Th1) cytokine, IL-12, improved baseline levels of lung tumor retention without protecting NK cells from suppression by metaproterenol, stress, or surgery.²⁴ Thus, the current CpG-C ODN regimen has advantages of each of these approaches.

In summary, CpG-C ODN treatment in pharmacologically stressed rats is shown to markedly improve resistance to metastatic dissemination, at least partly by enhancing NK cytolytic activity and preventing their suppression under physiological stress, but also by other mechanisms. The distinct effects of CpG-C ODN on NK cell number and activity in the marginating-pulmonary compartment versus the circulation warrant further characterization of its effects in different immune compartments in order to optimize treatment efficacy and outcome. Furthermore, as human and rodent TLR9 systems react somewhat differently it is crucial that pre-clinical finding in rodents be closely examined and modified prior to the commencement of clinical trials. Our findings that CpG-C ODN treatment is most beneficial in the context of physiological stress responses justify its further examination during the critical perioperative period with the aim of restricting postoperative metastatic recurrence.

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Figure 1. CpG-C ODN hinders the development of extrapulmonary metastases

Male rats were administered with CpG-C ODN (330µg/kg) either 24 hrs before inoculation with MADB106 tumor cells, or both 24 hrs before and after tumor cell inoculation. The control group was injected twice with PBS. Three weeks later extrapulmonary metastases were enumerated. Data are expressed as mean + SEM. * indicates a borderline significant difference from the PBS group ($p = 0.07$), and ** indicates a significant difference from the PBS group ($p = 0.0029$).

Figure 2. CpG-C ODN is effective at reducing lung tumor retention (LTR) in metaproterenol-treated animals when administered at a dose of 330 µg/kg

Male rats pre-treated with CpG-C ODN, non-CpG ODN or PBS (vehicle), were inoculated with MADB106 tumor cells. Simultaneously with tumor inoculation rats from each group were injected with either the pharmacological stressor, metaproterenol, or with saline. LTR was assessed 24 hrs following tumor cell inoculation. Control (PBS+saline group) levels of radioactivity retained in the lungs were 0.945% of total injected radioactivity. Data are expressed as mean + SEM. * indicates a significant difference from the PBS-metaproterenol group ($p < 0.05$).

Figure 3. Intraperitoneal administration of CpG-C ODN is most effective at reducing lung tumor retention (LTR)

Male rats were injected with CpG-C ODN i.p., i.v., or s.c. or with PBS (i.v.). Twenty four hours later, all rats were inoculated with MADB106 tumor cells and were subjected, or not, to the pharmacological stressor metaproterenol. LTR was assessed and control (PBS IV+saline group) levels of radioactivity retained in the lungs were 1.485% of total injected radioactivity. Data are expressed as mean + SEM. * indicates a significant difference from the PBS-IV-metaproterenol group ($p < 0.05$). ¥ $p < 0.05$ compared to CpG-C ODN IV-metaproterenol and § $p < 0.05$ compared to CpG-C ODN IV-saline and PBS-IV-saline groups.

Figure 4. A single administration of CpG-C ODN protects against the deleterious effects of metaproterenol on lung tumor retention (LTR) when given 12 or 24 hrs prior to tumor cell inoculation in both sexes

(a) Male and (b) female rats were injected with CpG-C ODN at either 4, 12, 24, 48 or 72 hrs prior to inoculation with MADB106 tumor cells. Control rats were administered with PBS at the 4hr time-point. In all rats LTR was assessed 24 hrs after tumor inoculation with or without subjection to metaproterenol (1 mg/kg, both sexes). Control (PBS+saline group) levels of radioactivity retained in the lungs were 1.031% of total injected radioactivity in males and 0.585% in females. Data are expressed as mean + SEM. * indicates a significant difference from the respective PBS-metaproterenol group ($p < 0.05$, in both sexes). Notice the greater effects of metaproterenol in males.

Figure 5. CpG-C ODN is more effective at reducing lung tumor retention (LTR) in metaproterenol-treated rats when given in a bolus rather than in a slow release vehicle (SRV)

Rats were injected with CpG-C ODN in either PBS or SRV, or received control (vehicle) injections of either PBS or SRV. CpG-C ODN was injected 24 or 48 hrs prior to MADB106 tumor cell inoculation. Simultaneously with tumor inoculation, rats were subjected, or not, to metaproterenol. Twenty four hrs later LTR was assessed. Control levels of radioactivity retained in the lungs were 0.765% of total injected radioactivity for the PBS+saline group and 0.945% in the SRV+saline group. Data are expressed as mean + SEM. * $p < 0.05$ compared to controls-metaproterenol. § $p < 0.05$ for a bolus of CpG-C ODN in PBS vs CpG-C ODN in SRV. ¥ $p < 0.05$ for CpG-C ODN 24 hrs vs 48 hrs in both the bolus and SRV conditions. ♂ $p < 0.05$ compared to controls-saline.

Figure 6. A single dose of CpG-C ODN is as effective in reducing lung tumor retention (LTR) as are repeated administrations

All rats received 3 consecutive daily injections each containing CpG-C ODN or PBS. Rats were injected with CpG-C ODN once, twice, or three times, the last injection given 24h before MADB106 tumor inoculation. Simultaneously with the tumor inoculation, rats were subjected, or not, to metaproterenol, and LTR was assessed. Control (PBS+saline group) levels of radioactivity retained in the lungs were 1.565% of total injected radioactivity. Data are expressed as mean + SEM. * $p < 0.05$ compared to PBS-metaproterenol. ¥ $p < 0.05$ compared to PBS-saline.

Figure 7. The role of NK cells in the beneficial effects of CpG-C ODN on lung tumor retention (LTR)

Male rats were injected with CpG-C ODN or PBS. Twenty four hrs later, rats were assigned to receive metaproterenol (meta.), to undergo surgical stress (laparotomy), or to serve as non-stress controls (saline), and were injected with MADB106 for the assessment of 24hr LTR. Simultaneously with MADB106 administration, half the rats from each group were injected with the anti-NKR-P1 mAb that functionally depletes NK activity, and the other half were injected with vehicle. Data are presented as mean + SEM. Control levels of radioactivity retained in the lungs were 1.197% of total injected radioactivity in the NK-intact PBS-no-stress group (40.909% in the NK-depleted PBS-no-stress group). NK-depletion dramatically elevated LTR levels (** $p < 0.05$). CpG-C ODN treatment reduced LTR levels in each of the NK-intact groups, but in NK-depleted rats only in those that underwent surgery or were administered metaproterenol (* $p < 0.05$). Thus, the beneficial effects of CpG-C ODN under no-stress conditions are mediated by NK cells. Also, as CpG-C ODN reduced LTR in operated and metaproterenol-treated animals that were NK-depleted it can be inferred that the beneficial effects of CpG-C ODN under these conditions are mediated, at least in part, by NK-independent mechanisms. Lastly, as LTR levels in operated animals were smaller compared to metaproterenol-treated animals in the NK-intact animals, but were higher in the NK-depleted animals, it can be inferred that the NK-independent factors affecting operated animals are greater than those affecting metaproterenol treated animals.

Figure 8. NK cell number and activity against YAC-1 target cells following CpG-C ODN pretreatment and metaproterenol

Male rats were challenged with CpG-C ODN or PBS, and 23 hrs later were assigned to receive either metaproterenol or saline. One hour later all animals were sacrificed and NK number and activity tested in serial dilutions of whole blood and lung perfusate. **(a)** Cytotoxicity per ml of whole blood: Metaproterenol significantly suppressed NK activity without impacting NK cell numbers, and CpG-C ODN prevented suppression of NK cytotoxicity. **(b)** Cytotoxicity per ml of whole lung perfusate (entire marginating-pulmonary (MP) compartment): Metaproterenol suppressed both MP-NK cell numbers and total lung activity. CpG-C ODN elevated cytotoxicity in both metaproterenol and saline groups, and elevated numbers of MP-NK cells

only in the saline groups. (c) To elucidate changes in individual NK cytotoxicity against the YAC-1 target cells per MP-NK cell, we calculated LU₃₅ per MP-NK cell and found that CpG-C ODN protected against the metaproterenol-induced suppression of MP-NK cytotoxicity per MP-NK cell. Data are presented as mean $\pm$ SEM. * $p < 0.05$ indicates a significant difference between metaproterenol and the respective saline group. # $p < 0.05$ indicates a significant difference between CpG-C ODN and the respective PBS group. ET represents the effector cell to target cell ratio achieved by serial dilution of whole blood or whole lung perfusate.

Figure 9. Marginating-pulmonary (MP)-NK cell numbers and cytotoxicity against MADB106 target cells

Male rats were administered with CpG-C ODN or PBS, and 23 hrs later received either metaprotegeron or saline. One hour later all animals were sacrificed. **(a)** Per lung, metaprotegeron reduced MP-NK cell numbers and suppressed MP-NK cytotoxicity. CpG-C ODN elevated cytotoxicity in both saline- and metaprotegeron-treated animals, but did not prevent the reduction in MP-NK cell numbers. ET represents the effector cell to target cell ratio achieved by serial dilution of whole lung perfusate. **(b)** MP-NK cytotoxicity per MP-NK cell: CpG-C ODN significantly elevated it and metaprotegeron independently reduced it. Data are presented as mean $\pm$ SEM. * $p < 0.05$ indicates a significant difference between metaprotegeron and the respective saline group. # $p < 0.05$ indicates a significant difference between CpG-C ODN and the respective PBS group.