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Continuous Stress Disrupts Immunostimulatory Effects of IL-12

Ben Levi, Marganit Benish, Yael Goldfarb, Liat Sorski, Rivka Melamed, Ella Rosenne, and Shamgar Ben-Eliyahu

Neuroimmunology Research Unit, Department of Psychology, Tel-Aviv University, Tel-Aviv 69978, Israel

Abstract

Immune stimulation by biological response modifiers is a common approach in tumor immunotherapy. IL-12 was found effective in various animal studies, but clinical trials showed limited success. However, among other differences, animal models do not simulate psychological or physiological stress while employing IL-12, whereas cancer patients often experience distress while treated with immunostimulants. Thus, in the current study we assessed the impact of continuous stress on the efficacy of IL-12 immunostimulation. F344 rats were subjected to a pharmacological stress paradigm (continuous administration of a β -adrenergic agonist) or to a 20-hr behavioral stress paradigm (wet cage exposure) commencing 2 hrs before IL-12 administration. Twenty-six hr after stress initiation, we studied indices known to reflect IL-12 immunostimulatory impacts, including NK cell numbers and activity in different immune compartments, and *in vivo* resistance to MADB106 lung tumor colonization. The results indicated that both the pharmacological and behavioral stress paradigms significantly reduced the increase in the number and activity of marginating-pulmonary NK cells evident in non-stressed IL-12 treated animals. Additionally, stressed animals exhibited a lower IL-12-induced improvement of MADB106 lung clearance, an *in vivo* index that markedly depends on total marginating-pulmonary NK activity. These deleterious effects of stress were more prominent in males than in females. Overall, the findings demonstrate that prolonged stress exposure can disrupt the efficacy of simultaneous immunostimulatory treatments, irrespective of stress effects on baseline immune measures. Neuroendocrine and cellular mediating mechanisms are yet unknown, but the potential clinical ramifications of these findings warrant consideration in clinical trials employing immunostimulatory agents.

Keywords

stress; IL-12; NK activity; MADB106; metastases

Introduction

Immune stimulation by cytokines or other biological response modifiers (BRMs) is a common approach in the field of tumor immunotherapy. The use of type 1 T-helper (T_H1)

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Corresponding Author: Shamgar Ben-Eliyahu, Dept. of Psychology, Tel-Aviv University, Tel-Aviv, 69978, Israel, Tel: 972-3-640-7266, Fax: 972-3-640-9520, shamgar@post.tau.ac.il.

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and proinflammatory cytokines (e.g. IL-12, IL-2, IFN- γ) was shown to significantly improve T_H1-dependent cellular immunity (Smyth et al., 2004), which is known to play a crucial role in the *in vivo* eradication of tumor cells (Nishimura et al., 2000).

IL-12 is one of the vastly investigated cytokines in this context, being a major T_H1 differentiation and cellular immunity inducer. Its ligation to the IL-12 receptor (IL-12R), primarily expressed by activated T and natural killer (NK) cells (Desai et al., 1992), results in a signal transduction that activates STAT4, which was reported to mediate most of IL-12's biological activities through the production of IFN- γ (Bacon et al., 1995). In concert with other T_H1 cytokines, IL-12 was shown to exert immunostimulatory effects on various cell populations comprising cell-mediated immunity (CMI), including NK cells, dendritic cells (DCs), neutrophils, NKT cells, and cytotoxic T lymphocytes (CTLs) (Collison et al., 1998; Cui et al., 1997; Grohmann et al., 1998; Mehrotra et al., 1993; Robertson et al., 1992). Anti-tumor effects of IL-12 have been reported in various animal models, and have been well established (Colombo and Trinchieri, 2002). For example, intratumoral administration of IL-12 in mice was shown to promote systemic anti-tumor immunity and potentiate host ability to eradicate micrometastases (Rosenberg et al., 1998). Our previous work has utilized IL-12 peri-operatively, and this intervention increased resistance to experimental metastases, which we and others have shown to be promoted by surgery and other immunosuppressive stress paradigms (Schwartz et al., 2008).

However, despite the promising findings from many animal studies regarding the beneficial effects of IL-12 in the context of cancer immunotherapy and surgery, it appears that the use of IL-12 in the clinical setting has yielded rather limited results (Atkins et al., 1997; Colombo and Trinchieri, 2002; Hurteau et al., 2001; Portielje et al., 1999), similar to other therapeutic approaches tested in animal models. Several difficulties in simulating the development of human cancer by animal models were suggested to underlie this discrepancy, most focusing on dissimilarity in the biology of the implanted cancerous tissue and host physiology, including immune susceptibility, compatibility to implanted tissue, and other traits that impact malignant tissue progression in animal models (e.g., (Fidler, 2002)). We have recently suggested an additional factor that may contribute to this discrepancy – the environmental and psychological state that characterizes cancer patients (Ben-Eliyahu, 2003). Specifically, while animals in the experimental setting do not expect IL-12 treatment and do not experience cancer- and treatment-related anxiety, patients may experience a great deal of psychological and physiological stress before and during their treatment with IL-12 or other BRMs. Indeed, as other and we have shown in oncological and/or non-oncological patients awaiting surgery (Bartal et al., 2010), it is likely that cancer patients will exhibit various stress responses (Cohen et al., 2001), including activation of the SNS and HPA axis, as a result of cancer diagnosis or treatment.

These and other stress responses have been repeatedly shown to modulate various immune responses (Ader, 2007; Elenkov et al., 1999). Therefore, it is feasible that stress hormones may limit the capacity of IL-12 and other BRMs to activate immunocytes, or may prevent activated immunocytes from exerting their beneficial effects. Despite the potential clinical implication, this hypothesis has rarely been addressed by animal or humans studies (Ghanta et al., 1985; Li et al., 1997), and mostly in an indirect manner. Little is known about the specific impact of stress responses on the efficacy of IL-12-induced immune stimulation. Therefore, in the current study we assessed this hypothesis in an animal model of experimental metastasis, and further investigated the involvement of NK cells harvested from different immune compartments in this potential interaction.

Materials and Methods

Animals and counterbalancing

Male and female Fisher 344 (F344) rats (Harlan Laboratories, Jerusalem, Israel), 12-13 weeks old, 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 injection was 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.

The wet cage stress paradigm

Animals were placed in cages that contained 2 cm of room-temperature water, with free access to food and water, for a total period of 20 hrs, with a 2 hr recess period (in the animals' home-cages) that began 9 hr after initiation of the protocol (see diagram 1).

Corticosterone plasma levels assessment

Blood for corticosterone (CORT) plasma levels assessment was drawn by cardiac puncture using heparinized syringes. CORT plasma levels were measured by radioimmunoassay (RIA) (ImmuChem double antibody corticosterone 125I RIA kit, MP Biomedicals, Orangeburg, NY), as per the manufacturers' instructions.

Drugs and their administration

IL-12—Recombinant mouse IL-12 (Cytolab, Israel; specific activity $\geq 10^7$ IU/mg) was dissolved in phosphate buffered saline (PBS) and injected subcutaneously (s.c.) at a dose of 1 μ g/kg.

Metaproterenol—A non-selective β -adrenergic agonist with higher affinity for β_2 than β_1 receptors (Dengler and Hengstmann, 1976). Metaproterenol (Sigma, Rehovot, Israel) administration was used in Exp. 2 and 3 as the continuous pharmacological stress paradigm, and was dissolved in a slow release vehicle and injected to animals s.c. (20 mg/Kg, 0.5 ml/animal). In Exp. 4 and 5 metaproterenol was used to simulate acute stress, and animals were injected with the drug dissolved in PBS simultaneously with tumor inoculation. Males were administered 1 mg/kg s.c., while females were injected with 3mg/kg in order to achieve similar physiological. These doses were based on our previous studies, mimicking the effects caused by other stressors such as surgery or swim stress (Rosenne et al., 2007; Shakhar and Ben-Eliyahu, 1998).

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). Unpublished data from our laboratory indicated that a β -adrenergic antagonist administered in the slow release vehicle exerts its effects 9 and 12 hrs following injection, whereas the same dose of drug administered in saline had no effects at these time points.

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 (Barlozzari et al., 1985). MADB106 tumor cells metastasize only to the lungs (Barlozzari et

al., 1985), and lung tumor retention (LTR), which is highly indicative of the number of metastases that would have developed weeks later, is dependent upon NK cells in this model (Barlozzari et al., 1985; Ben-Eliyahu and Page, 1992; Ben-Eliyahu et al., 1996b; Shakhar and Ben-Eliyahu, 1998). Additionally, because the metastatic process of MADB106 is sensitive to NK activity predominantly in the first 24 hours following inoculation (Barlozzari et al., 1985; Ben-Eliyahu and Page, 1992), LTR is more reflective of *in vivo* NK activity levels than the number of actual metastases (Shakhar and Ben-Eliyahu, 1998). The MADB106 cell line was maintained in monolayer cultures in complete media (RPMI-1640 media supplemented with 10% heat-inactivated fetal calf serum (FCS), 50µg/mL of gentamicin, 2mM of L-glutamine, 0.1mM of non-essential amino-acids, and 1mM of sodium pyruvate, Biological Industries, Kibbutz Biet Haemek, Israel) in 100% humidity, 5% CO₂ at 37°C. Cells were removed from the culture flask with trypsin solution (0.25% in PBS), and were washed with complete media. This cell line was used for both *in vivo* assessment of lung tumor retention and *in vitro* examination of NK cytotoxicity.

YAC-1—YAC-1 lymphoma is the standard target cell line used for the assessment of rodent *in vitro* NK cytotoxicity. The cell line was maintained in suspension cultures in complete media in 100% humidity, 5% CO₂ at 37°C.

Radiolabeling of MADB106 tumor cells and assessment of lung tumor retention

Tumor cell DNA radiolabeling for assessment of LTR is accomplished by adding 0.5 µCi/ml of ¹²⁵Iododeoxyuridine (¹²⁵IDUR, Danyel Biotech, Rehovot, Israel) to the cell culture for 24 hrs. For tumor cell injection, rats are lightly anesthetized with isoflurane, and 4×10⁵/kg MADB106 tumor cells in 2ml/kg PBS containing 0.1% bovine serum albumin (BSA) were injected into their tail vein. For assessment of LTR, animals were sacrificed with CO₂, their lungs are removed 4 (Exp 3) or 24 hrs (Exp 4 & 5) after inoculation with ¹²⁵IDUR-labeled tumor cells, and placed in a γ-counter to assess percent radioactivity retained in this organ. LTR is calculated using the following formula: (radioactivity count of lung – background radioactivity) ×100/(radioactivity count of the total injected cell suspension – background radioactivity).

***In vitro* assessment of NK 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. One ml of blood was washed once with 3 ml of PBS (400g for 10 min) and twice with 3 ml 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 cytotoxicity—The standard whole blood ⁵¹Cr release assay was used. This procedure assesses anti-tumor NK cell cytotoxicity per ml of effector cells without prior purification of PBMCs or marginating-pulmonary leukocytes. 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 (Ben-Eliyahu et al., 1996a; Ottenhof et al., 1981). The

advantages of this procedure include shorter duration, less interference with the effector cells, and better representation of the original *in vivo* milieu of cell composition.

Six different effector to target (E:T) ratios were formed by serially diluting 150 µl aliquots of the effector-cell preparation in a microtiter plate. Then, 5,000 radiolabeled target cells (MADB106, YAC-1) in 100µl complete media were added to each well on top of the effector-cell preparation. Radiolabeling of the target cells was conducted by incubating them for 1 hour with 100 µCi ^{51}Cr (Rotem Taassiot, Dimona, Israel) in 100µl saline, 100µl FCS, and 50µl complete media. Following this incubation, target cells were washed 3 times (300g for 10 min) in complete media and their concentration adjusted to $5 \times 10^4/\text{ml}$. Spontaneous and maximal releases of radioactivity were determined by substituting effector cells with complete media or Triton-X100 (Sigma, Rehovot, Israel), respectively. Prior to and following a 4 h incubation period (100% humidity, 5% CO_2 at 37°C) plates were centrifuged (400g for 10 min, at 25°C and 4°C, respectively). Finally, 100µl samples of supernatant, which reflect specific target cell lysis, were recovered for the assessment of radioactivity in a γ -counter.

Flow Cytometry

Standard procedures were used to prepare cells for flow cytometric analysis (Melamed et al., In Press). NK cells in both blood and lung perfusate were identified by the FITC-conjugated anti-NKR-P1 mAb (PharMingen, San Diego) 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 [13-15]. T cells were identified using a PE-conjugated anti-CD5 mAb (eBioscience, San Diego) and NKT cells were identified as NKR-P1⁺CD5⁺ lymphocytes. Granulocytes and lymphocytes were identified based on forward and side scatters. Flow cytometry analysis was conducted using a FACScan (Becton Dickinson). To assess the total number of cells per µl of sample (or a specific cell subtype), 300 polystyrene microbeads (20µm, Duke Scientific, Palo Alto) per µl 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, two, or three-way factorial analysis of variance (ANOVA) with a pre-determined significance level of 0.05 were conducted. When NK cytotoxicity was studied, repeated measures ANOVA was conducted (E:T ratios as the repeated measure). 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. Pearson correlations were used for assessing the association between continuous variables (%LTR, % cytotoxicity, number of NK cells).

Procedures and Results

Experiment 1: The “wet cage” stress paradigm elevates corticosterone plasma levels throughout its course

Design and procedure—72 F344 male rats were subjected to the “wet cage” paradigm (see Methods), or served as controls. Corticosterone plasma levels were assessed, once in each rat, at 1, 3, 9, 11, 12, 14, 20, or 22 hrs following the initiation of the paradigm. Blood was drawn by cardiac puncture using heparinized syringes. To control for diurnal changes in corticosterone levels, all samples were collected from the different animals at the same time of the day.

Results—Baseline levels of CORT were 73.5 ng/cc (SEM = 14.8). The wet cage paradigm increased plasma CORT levels 4-6 folds (298.3 to 449 ng/cc, SEM = 16.9 – 46.7) in all

time-points in which animals were within the wet cage (1, 3, 9, 12, 14, and 20 hrs), (Fisher's PLSD, $p < 0.05$ for all time points). At the end of the half-way 2-hr brake (the 11 hrs), as well as 2 hrs after the end of the paradigm (22hrs), animals exhibited CORT levels which were similar to baseline levels (86.5 ± 35 and 71 ± 14.4 ng/cc, respectively) (no figure is provided).

Experiment 2: Prolonged pharmacological stress disrupts the beneficial effects of IL-12: NK cell numbers and activity in female rats

Design and procedure—Thirty-nine F344 female rats were used in a 2×2 design: a pharmacological stress condition in which rats were injected with metaproterenol (20 mg/Kg, s.c.) dissolved in slow release emulsion, versus control condition (vehicle injection). Rats in each condition were further subdivided and received either immune stimulation with a single IL-12 injection (1 μ g/Kg, s.c.), or served as controls (PBS injection). To simulate the clinical condition, in which the immune stimulation commences when patients are most commonly stressed, rats were injected with IL-12 two hrs after initiation of pharmacological stress. Twenty-four hrs after IL-12 injection, rats were killed and circulating blood and pulmonary leukocytes were harvested for ex-vivo assessment of number and activity of NK cells.

Results

2.1 Circulating NK cell number and activity against YAC-1 target cells: Increased NK activity following IL-12 administration was indicated by a significant main effect for IL-12 treatment ($F_{(1,35)} = 17.563$, $p < 0.05$) (data not shown). No effects for pharmacological stress, or interaction with the effect of IL-12 were evident. With regard to the number of NK cells, no significant effects were revealed by the 2×2 ANOVA.

2.2 Marginating-pulmonary (MP)-NK cell numbers: A two-way ANOVA revealed a significant main effect for IL-12 and for pharmacological stress, as well as a significant interaction ($F_{(1,35)} = 23.028$, $p < 0.05$, $F_{(1,35)} = 9.470$, $p < 0.05$ and $F_{(1,35)} = 4.813$, $p < 0.05$ respectively), (Fig. 1a); Specifically, control animals exhibited a greater increase in MP-NK cell numbers following IL-12 administration compared to animals that were subjected to pharmacological stress. It is important to note that the pharmacological stress had no effect in animals not treated with IL-12 (Fig. 1a).

2.3 MP-NK cell activity against YAC-1 and MADB106 target cells: Activity against both target cell lines showed similar patterns of effects, though greater against the MADB106 line. Against YAC-1 target cells, increased NK activity following IL-12 administration was indicated by a significant main effect for IL-12 treatment ($F_{(1,34)} = 34.641$, $p < 0.05$). Although the pattern of results seems to reflect a trend toward a significant interaction between the effects of IL-12 and pharmacological stress, it did not reach statistical significance (Fig. 1b). However, against the MADB106 target cell, a 2×2 ANOVA indicated a significant main effect for IL-12 and pharmacological stress, and a marginally significant interaction between the effects of IL-12 and pharmacological stress ($F_{(1,35)} = 57.242$, $p < 0.05$, $F_{(1,35)} = 4.621$, $p < 0.05$ and $F_{(1,35)} = 4.018$, $p = 0.0528$ respectively), (Fig. 1c); MP-NK cells of control animals exhibited greater increase in activity following IL-12 administration compared to MP-NK cells from animals subjected to pharmacological stress. It is important to note that the pharmacological stress had no effect in animals not treated with IL-12 (Fig. 1c).

2.4 Correlations between numbers of NK cells and NK activity: There were large and significant correlations between the two variables, as elaborated in section 3.4.

Experiment 3: Pharmacological stress disrupts the beneficial effects of IL-12: MADB106 lung tumor retention (LTR), and NK cell numbers and activity in male rats

Design and procedure—the same experimental design and drug regimen as in Exp. 2 was employed. Thirty nine F344 male rats were used in a 2×2 design: Pharmacological stress vs. control groups, each subdivided to immune stimulation vs. control condition. Unlike Exp. 2, rats were also inoculated intravenously with radiolabeled MADB106 tumor cells 4 hours before being sacrificed for harvesting of blood and MP leukocytes. LTR was assessed in the same lung specimens after harvesting MP-leukocytes. In a previous study we found no significant differences in NK numbers and activity, and in LTR levels, when studying both indices simultaneously (as conducted herein) compared to assessing only one of these indices alone (NK indices or LTR levels) (unpublished data from our laboratory).

Results

3.1 Circulating NK cell numbers and activity against YAC-1 target cells: No significant main effects for IL-12 or of β -adrenergic activation was evident, nor was a significant interaction.

3.2 MP-NK cell numbers: A 2×2 ANOVA indicated a significant main effect for IL-12 ($F_{(1,34)}=28.167, p<0.05$), for pharmacological stress ($F_{(1,34)}=6.867, p<0.05$), and a significant interaction ($F_{(1,34)}=8.909, p<0.05$): In animals subjected to pharmacological stress, IL-12 administration caused a smaller increase in MP-NK cell numbers compared to control animals (Fig. 2a).

3.3 MP-NK cell activity against YAC-1 and MADB106 target cells: Activity against both target cell lines showed similar patterns of effects, though greater in the MADB106 line. A 2×2 ANOVA indicated a significant main effect for IL-12 (YAC-1: $F_{(1,33)}=127.740, p<0.05$, MADB106 $F_{(1,34)}=63.314, p<0.05$), for pharmacological stress (YAC-1: $F_{(1,33)}=36.081, p<0.05$, MADB106 $F_{(1,34)}=27.954, p<0.05$), and a significant interaction between the two effects (YAC-1: $F_{(1,33)}=5.703, p<0.05$, MADB106 $F_{(1,34)}=16.724, p<0.05$): In animals subjected to pharmacological stress, IL-12 administration caused a smaller increase in MP-NK cell activity compared to control animals (Figs. 2b and 2c).

3.4 Correlations between activity and numbers of NK cells in Exps. 2 and 3: The following relates to both Exp. 2 and 3 (each experiment separately). Across all groups (all animals in a given study), significant and large positive correlations were evident between the number of NK cells/ml and their activity per ml tested for cytotoxicity. As indicated in Table 1, these high correlations were evident in both the circulation and the MP compartments, and with respect to both target cell lines. These high correlations suggest that alteration in NK cell numbers were at least partially responsible for alteration in NK activity levels in both compartments. Within each group, the correlation was commonly positive and significant, and increased as did the variance in the numbers of NK cells (for example see Fig 2d).

3.5 % Lung Tumor Retention (LTR): Animals subjected to pharmacological stress showed markedly higher levels of LTR (2.5-5 fold). Therefore, to assess the efficacy of IL-12 in reducing LTR in animals subjected to pharmacological stress and in those that were not, all levels of LTR were transformed to percent of their respective non-IL-12 group. In our view, the ratio in LTR between the different groups is a more indicative and biologically relevant index than absolute differences (provided that there are no ceiling or floor effects). As evident in Fig 3, IL-12 caused a significantly smaller decrease in LTR in animal subjected to pharmacological stress, as indicated by a significant interaction between the effects of IL-12 and pharmacological stress ($F_{(1,34)}=5.663, p<0.05$). These findings cannot

be explained by a floor effect, as baseline levels in the pharmacological stress group were actually higher, leaving a greater margin for reducing LTR by IL-12. Absolute LTR levels of the non-IL-12 groups (presented as 100 percent in Fig. 3) are 24.4% for the pharmacological stress and 8.3% for vehicle group.

3.6 Correlation between MP-NK activity against MADB106 and % MADB106 LTR:

As we previously shown (Melamed et al., 2005), MADB106 target cells are sensitive to MP-NK activity both *in vivo* and *in vitro*. MADB106 LTR levels were inversely and non-linearly correlated with MP-NK activity against MADB106 target cells, as can be expected, and as seen in Fig. 4. The correlation, however, is asymptotic to both axes, indicating that below a certain level of LTR and below a certain level of NK activity, no relations are evident between these two indices, seemingly as a result of technical or biological floor effects. Therefore, in our following experiments employing LTR as our main outcome, we aimed at achieving sufficiently high *in vivo* levels of LTR that would be within the linear range of the correlation, and thus would reflect differences in effects of IL-12 on NK activity levels. To this end, in Exps. 4 and 5 we treated half of the animals with an acute adrenergic challenge (that is known to elevate levels of LTR (Shakhar and Ben-Eliyahu, 1998)) simultaneously with MADB106 inoculation. Additionally, we replaced the pharmacological stress paradigm with the behavioral stress paradigm that ended 6 hrs before MADB106 inoculation. These modifications aimed at resolving or reducing the leftover effects of the pharmacological stress paradigm.

Experiment 4: Effects of prolonged behavioral stress on the beneficial effects of IL-12 as indicated in an *in vivo* index of LTR in males following acute adrenergic challenge

Design and procedure—In the following experiments we employed an *in vivo* index of resistance to experimental metastasis that is sensitive to NK activity, studying MADB106 lung tumor retention. We also replaced the prolonged pharmacological paradigm with a more natural behavioral stress paradigm. Based on the aforementioned floor effect in LTR at high NK activity/low LTR levels (see Fig. 4) it is unclear whether it would be possible to detect further increases in NK activity or decreases in LTR when such effects are expected. Therefore, we also tested the beneficial effects of IL-12, and their attenuation by prolonged behavioral stress, in the context of an acute adrenergic challenge, which is known to suppress NK activity and elevate LTR.

Forty F344 male rats were used in a 2×2×2 design: a “prolonged behavioral stress” condition, in which rats were subjected to the wet cage stress paradigm (see Methods) for 20 hrs, or remained at their home cage (HC) condition. Two hours after the onset of the stress paradigm, rats in each condition were subdivided and received either immune stimulation with a single IL-12 injection (1 µg/Kg, s.c.), or served as controls (PBS injection). Twenty-four hrs following IL-12 injection (6 hrs after the end of the behavioral stress paradigm), all animal were inoculated with radiolabeled MADB106 tumor cells, and half of the animals in each of the above 4 conditions were subjected to an acute adrenergic challenge (received metaproterenol in PBS, 1 mg/Kg, s.c.). Twenty-four hrs after MADB106 inoculation, rats were sacrificed for the assessment of LTR.

LTR levels—Three-way ANOVA revealed significant main effects of prolonged behavioral stress, IL-12, and metaproterenol ($F_{(1,31)}=37.164$, $p<0.05$, $F_{(1,31)}=5.912$, $p<0.05$, and $F_{(1,31)}=255.792$, $p<0.05$ respectively), as well as a significant interaction between prolonged behavioral stress and IL-12 ($F_{(1,31)}=5.407$, $p<0.05$). The triple interaction was marginally significant ($F_{(1,31)}=3.909$, $p=0.057$), suggesting that the prolonged stress by IL-12 interaction was larger in the acute metaproterenol condition, as we had predicted.

As LTR levels of animals that did not receive acute metaproterenol seemed too low, we also conducted a separate 2×2 ANOVA only including groups that received acute metaproterenol. This analysis revealed a significant interaction between the effects of IL-12 and chronic stress ($F_{(1,16)}=5.337, p<0.05$). IL-12 caused a significantly smaller decrease in LTR in animal subjected to prolonged behavioral stress compared to HC animals. The same analysis in rats that were not subjected to acute adrenergic challenge did not reveal any significant effect (Fig. 5a).

Experiment 5: Effects of prolonged behavioral stress on the beneficial effects of IL-12 as indicated in an *in vivo* index of LTR in females following acute adrenergic challenge

Design and procedure—The exact same experimental design as in Exp. 4 was employed, except that females were used rather than males.

LTR levels—The pattern of the results was similar to the one that was evident in the previous experiment in males. However, the interaction between prolonged behavioral stress and IL-12 did not reach statistical significance, neither in the three-way ANOVA nor in 2×2 ANOVA including only females subjected to acute challenge (Fig. 5b).

Discussion

In this study we showed that continuous stress alongside immunostimulatory treatment can disrupt the beneficial effects of immunostimulation. Specifically, exposure to 20 hr of behavioral stress, commencing 2 hrs before IL-12 administration, diminished the increase in NK number and activity caused by IL-12 in non-stressed animals. This interaction between the effects of continuous stress and IL-12 immunostimulation was evident in the marginating-pulmonary immune compartment, which we found critical for resisting lung metastases (Melamed et al., 2010; Melamed et al., 2005), but not in the circulation. In addition, we employed the MADB106 experimental metastases model and assessed lung tumor retention, an index which reflects *in vivo* NK activity and reliably predicts lung metastases (Barlozzari et al., 1985; Ben-Eliyahu et al., 1996b; Shakhar and Ben-Eliyahu, 1998). We observed similar interactions - while IL-12 administration efficiently improved MADB106 lung clearance, continuous stress alongside IL-12 treatment markedly reduced these beneficial effects. It is important to note that all the above indices were assessed six hrs after the behavioral stress has ended. Thus, and as elaborated below, we propose that the continuous stress affected the ability of IL-12 to cause immunostimulation, irrespective of affecting baseline levels of the measured outcome. Last, all *ex-vivo* and *in vivo* indices were studied both in males and females. The stress-immunostimulation interaction, although evident in both sexes in some indices, seemed more profound in males than in females in most measures.

It is critical to distinguish between the effects of continuous stress on the efficacy of IL-12 treatment, and the effects of continuous stress on baseline levels of the measured outcomes. We used two stress paradigms that exert prolonged stress responses. The behavioral “wet cage” paradigm was shown here to induce an endogenous and prolonged stress response, as reflected by elevated plasma CORT levels throughout the paradigm. The pharmacological stress paradigm used was based on a slow release preparation of the adrenergic agonist metaproterenol, which is known to activate β -adrenoceptors and to induce a marked rise in CORT levels (Ben-Eliyahu et al., 2010). It was expected that these stress paradigms would affect the baseline levels of our immune indices, at least to some extent. Indeed, although immune measures were taken 6 hr following the cessation of the behavioral stress paradigm (and 4 hrs after CORT levels had returned to baseline), stressed animals that were not treated with IL-12 still exhibited higher LTR levels than their non-stressed counterparts.

However, the interaction between the effects of continuous stress and efficacy of immunostimulation, rather than the effects of continuous stress on baseline levels, was the focus of our attention, as it specifically reflects the deleterious effects of stress responses on the beneficial effects of IL-12. In our results, effects of stress on baseline levels were evident in some indices, but not others, while the interaction was evident irrespective of these long-lasting effects of continuous stress. It is also important to note that in the clinical setting medical procedures that pose risk to the patient would most likely be associated with profound stress responses, and some levels of stress responses would be expected to continuously persist as a result of the physiological response to the malignant tissue, as well as the psychological state of having cancer (Cohen et al., 2001; Stanton and Snider, 1993). Control over the initiation or secession of stress responses can not be expected in patients, unless specific pharmacological interventions will be employed. Thus, the distinction between the effects of stress on the efficacy of IL-12, versus its effects on baseline immune measures, cannot be easily appreciated in patients, and at best the cumulative effects of stress and immunostimulatory treatment could be observed.

The immunological and endocrinological mechanisms through which continuous stress disrupts immunostimulatory treatment are important to understand. We have previously shown that IL-12 administration elevated NK cell numbers in the marginating compartment of the lungs, which accounted for most of the increase in cytotoxicity induced in this compartment by IL-12 (Schwartz et al., 2008). Similarly, in the current study the levels of NK cytotoxicity per lung, as measured by specific lysis of YAC-1 and MADB106 target cells in all experimental conditions, highly correlated with lung NK cell numbers ($r = 0.7-0.9$). Most importantly, the same pattern of interaction between continuous stress and IL-12 efficacy was evident with respect to NK cell numbers and their activity in this compartment. These results suggest that alterations in NK cell numbers underlie both the increase in NK activity following IL-12 treatment, as well as the deleterious effect of continuous stress on this index.

The neuroendocrine mechanisms involved in the observed interaction are also critical to understand. Based on the stress paradigms that were employed in this study, CORT and/or CAs are primary candidates in mediating the effects of continuous stress on immunostimulation. The “wet cage” paradigm was shown herein to exert prolonged CORT secretion, and studies from our laboratory indicated a profound sympathetic activation by this paradigm, as a β -adrenergic blocker abolished many of its effects (Ben-Eliyahu et al., 2010). The pharmacologic stress paradigm is based on continuous secretion of a β -adrenoceptor agonist that was also reported to increase systemic CORT levels (unpublished data from our laboratory). Further support for the potential role of CORT in this interaction can be derived from *in vitro* studies. Specifically, CORT was shown to down regulate IL-12 receptors on NK and T cells, thus lowering their responsiveness to IL-12 and inhibiting IFN- γ production (Wu et al., 1998). An additional mechanism may relate to the T_H1/T_H2 cytokine balance. A pivotal mechanism through which IL-12 exerts its immunostimulatory effects is by inducing pro-inflammatory/ T_H1 cytokine secretion, including IFN- γ , which in concert with IL-12 potentiate cellular immunity (Trinchieri, 2003; Trinchieri and Scott, 1995). Induction of a shift towards T_H2 cytokine secretion by stress hormones, on the other hand, inhibits T_H1 cytokine production, as the two types of the responses (T_H1 and T_H2) are often mutually inhibitory (Elenkov et al., 2000). CAs and CORT are known to affect the immune system and inhibit CMI through different mechanisms, including the induction of a T_H2 dominant cytokine balance. Thus, stress may disrupt the efficacy of IL-12 by mounting a T_H2 response before the beginning of IL-12 treatment, and consequently hindering the ability of IL-12 to induce an effective T_H1 response, which is needed for activation of CMI. Nevertheless, it was beyond the scope of this study to systematically assess the involvement

of each of these hormones, and clearly other stress related immunomodulators may be involved (e.g. opioids).

An additional mechanism underlying the interaction between stress and the efficacy of IL-12 may involve brain-IL-12 interactions. Other proinflammatory cytokines (e.g., IL- β and others) were reported to induce neuroendocrine responses through central (Kelley et al., 2003). Stressors preceding such effects may modulate these central responses, and thus hypothetically may affect centrally-mediated impacts of IL-12 on peripheral physiological indices.

The interaction between the effects of continuous stress and IL-12 with respect to NK activity was not evident in the circulation, but was significant in the MP immune compartment. These findings, in conjunction with the homologous findings regarding MADB106 lung clearance, suggest the significance of the MP-NK compartment to resisting circulating malignant cells, and point at the unique impact of immunostimulation on this immune compartment, as was also indicated by our previous studies (Melamed et al., 2010; Melamed et al., 2005).

As mentioned above, all experiments were conducted in both males and females, as there is an acknowledged sexual dimorphism in stress as well as immune responses (Barker et al., 2005; Bouman et al., 2005; Da Silva, 1995; Naor et al., 2009; Uji et al., 2007). Although the patterns of results in females resembled those evident in males, the disruption of IL-12 treatment by continuous stress in females often did not reach statistical significance and was much smaller. In accordance, previous and ongoing studies in our laboratory indicate that females show smaller impacts on NK activity and smaller elevations in LTR following behavioral or surgical stress compared to males, and 2-3 fold higher doses of adrenergic agonists and of CORT are needed in females to cause the same effect on MADB106 LTR levels (Ben-Eliyahu et al., 2010; Goldfarb et al., 2009; Page and Ben-Eliyahu, 1997; Page et al., 2008). However the mechanisms underlying these and other sexual dimorphisms in immunomodulation are hardly understood. Although females are known to have higher levels of ACTH and CORT than males, both in basal conditions and in response to stress conditions (Kant et al., 1983; Le Mevel et al., 1978), they also exhibit higher levels of circulating corticosterone-binding globulin that could buffer the response to elevated CORT secretion (Mataradze et al., 1992). In addition, sex hormones were shown to modulate immune status. Perhaps directly related to the current study, estradiol and progesterone were shown to impact expression levels of β -adrenergic receptors, and to modulate physiological responses that rely on β -adrenergic receptor activation, including asthma attacks (Ben-Eliyahu et al., 2000; Tan et al., 1997).

Last, our findings may have significant clinical implications. Patients treated with immunostimulatory agents often experience psychological and physiological stress due to illness and treatment related factors. Stress responses in patients may include a continuous systemic elevation of stress hormones that, as shown herein, may disrupt the efficacy of the immunostimulatory treatment. Ongoing research in our laboratory is focused on identifying specific stress hormones that contribute to this interaction, and aim at elucidating their specific cellular and molecular mediating mechanisms. Gaining such insights may lead to potential interventions that could prevent the disruption to immunostimulatory treatment, and reduce the doses needed and side effects experienced.

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Fig. 1. MP-NK cell numbers, and MP-NK cell activity against YAC-1 and MADB106 target cells in females

(a) Total MP-NK cell number (presented as 1/1000 of total lung perfusate). A significant interaction between the effects of pharmacological stress and IL-12 was evident. (b and c) MP-NK cell activity against YAC-1 and MADB106 target cells. IL-12 increased MP-NK activity, a trend toward an interaction between the effects of IL-12 and pharmacological stress was evident only regarding MADB106 killing ($p = 0.0528$). Data is shown as mean $\pm$ SEM.

Fig. 2. MP-NK cell numbers and activity against YAC-1 and MADB106 target cells in males, and the correlation between these indices

(a) Total MP-NK cell number (presented as 1/1000 of total lung perfusate). A significant interaction between the effects of pharmacological stress and IL-12 was evident. **(b and c)** MP-NK cell activity against YAC-1 and MADB106 target cells. A significant interaction between the effects of pharmacological stress and IL-12 was evident. Data is shown as mean $\pm$ SEM. **(d)** Correlation between MP-NK cell number and activity against MADB106 target cells. A significant positive correlation between MP-NK cell number and activity was evident.

Fig. 3. Pharmacological stress attenuated the reduction of lung tumor retention (LTR) caused by IL-12

IL-12 reduced LTR levels in both pharmacological stress and control conditions. However, IL-12 caused a significantly smaller reduction in LTR in animals subjected to pharmacological stress, as indicated by a significant interaction between the effects of IL-12 and pharmacological stress. All levels of LTR were transformed and presented as percent of their respective non-IL-12 group. Absolute %LTR levels in the non-IL-12 groups (presented as 100 percent) were 24.40 for the pharmacological stress and 8.32 for vehicle group. Data is shown as mean $\pm$ SEM.

Fig. 4. The relation between MP-NK activity levels against MADB106 tumor cells, and in vivo levels of LTR

MADB106 LTR levels were inversely and non-linearly correlated with MP-NK activity against MADB106 target cells. Below a certain level of LTR, no relations are evident between these two indices (Indicated by the circle).

Fig. 5. Prolonged behavioral stress abolished the beneficial effects of IL-12 on lung tumor retention (LTR) levels

(a) Males: A significant interaction between the effects of IL-12 and behavioral stress (wet cage exposure) was evident in rats subjected to acute adrenergic challenge simultaneously with tumor challenge (*). No such significant interaction was evident in animals that were not subjected to acute adrenergic challenge. **(b) Females:** Although the pattern of effects is similar to that evident in males, the interaction did not reach statistical significance. Data is shown as mean $\pm$ SEM.

Diagram 1.

Schematic representation of the behavioral stress paradigm and experimental procedures.

Table 1

Pearson correlations between number and activity of MP-NK cell against each target cell across all groups in each experiment

Sex and target cell line	r	p
Female MP-NK# - % YAC-1 lysis (exp. 2)	r = 0.730	p < 0.05
Female MP-NK# - % MADB106 lysis (exp. 2)	r = 0.806	p < 0.05
Male MP-NK# - % YAC-1 lysis (exp. 3)	r = 0.897	p < 0.05
Male MP-NK# - % MADB106 lysis (exp. 3)	r = 0.892	p < 0.05