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Metastatic-promoting effects of LPS: Sexual dimorphism and mediation by catecholamines and prostaglandins

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Abstract

Inflammation is implicated in several medical conditions that are sexually dimorphic, including depression, cardiovascular diseases, autoimmunity, and presumably cancer progression. Here we studied the effects of the pro-inflammatory agent, LPS, on MADB106 lung tumor retention (LTR), and sought to elucidate underlying mechanisms and sexual dimorphism. F344 male and female rats were administered with LPS (0.001–1 mg/kg i.v.) simultaneously with tumor cell inoculation, and treated with a β -blocker (nadolol, 0.2–0.3 mg/kg s.c.), a COX-inhibitor (indomethacin, 4 mg/kg s.c.) or both drugs. To study the role of NK cells, numbers and cytotoxicity of marginating-pulmonary NK cells were studied, and selective in vivo NK-depletion was employed. Serum levels of corticosterone, IL-6, and TNF- α were also assessed. The findings indicated that LPS increased LTR in both sexes, but 10-fold higher doses were needed in females to reach the increase evident in males. Additionally, nadolol and indomethacin reduced the effects of LPS, more so in males. In vivo NK-depletion and ex-vivo NK activity studies suggested that LPS affected LTR through both NK-independent and NK-dependent mechanisms, the latter mediated through prostaglandin release in males. Corticosterone, IL-6, and TNF- α responses to LPS were sexually dimorphic, but were not associated with LPS or drugs' impacts on LTR. Overall, our findings demonstrate sexual dimorphism in LPS-induced elevated susceptibility to MADB106 experimental metastasis, and in potential humoral underlying mechanisms. Further studies are needed to elucidate additional immunological and non-immunological mediators of these dimorphisms, as well as to assess their involvement in other sexually dimorphic pathologies that are associated with inflammation.

Keywords

LPS; sex differences; NK; inflammation; metastases

1. Introduction

Several lines of evidence indicate sexual dimorphism in the course of human diseases, specifically in pathologies that are associated with inflammatory immune responses. Men are in a higher risk bracket for cardiovascular and infectious diseases (Klein 2000, Marriott and

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Huet-Hudson 2006, Schroder et al. 1998, Thom et al. 2006, Thorvaldsen et al. 1995), show lower rates of depression and autoimmune diseases (Grigoriadis and Robinson 2007, Kuehner 2003, Whitacre 2001), and a differential rates of allergies, which are age and type specific (Almqvist et al. 2008, DunnGalvin et al. 2006, Schafer et al. 2001). It is noteworthy that inflammatory responses have been proposed to play a role in the etiology, progression, or prognosis of each of these pathological conditions (for cardiovascular and infectious disease Elkind 2006, Lindsberg and Grau 2003, Poulin 1996, Ross 1999, Wang et al. 2007) (for depression and autoimmune diseases Dantzer et al. 2008, Raison et al. 2006, Tincani et al. 2007) (for allergies Averbek et al. 2007, Tsitoura and Tassios 2006). Cancer progression is believed to be associated with inflammation and to show sexual dimorphism, but the evidence is ambiguous (see discussion).

Several immune responses, including inflammation, are believed to be sexually dimorphic in humans. Studies suggested that women mount a stronger immune responses to a variety of antigens (Da Silva 1995, Verthelyi 2001), and others studies demonstrated that men suffer from higher rates of sepsis and septic shock (Moss 2005, Wichmann et al. 2000), including higher circulating levels of the pro-inflammatory TNF- α , and lower levels of the anti-inflammatory IL-10 (Schroder et al. 1998). In agreement with the latter findings, menopause is associated with an increase in the levels of proinflammatory cytokines, including IL-1, IL-6 and TNF- α (Pfeilschifter et al. 2002). Sex-based differences were also shown in vitro following human leukocytes exposure to LPS (Marriott and Huet-Hudson 2006).

LPS, a component of the outer cell wall of gram-negative bacteria, is extensively used in studying systemic inflammatory responses in humans and animals (Fiuza and Suffredini 2001, Lowry 2005, Suffredini et al. 1999). Serum LPS-binding protein (LBP) transfers LPS to the soluble or membrane receptor, CD14, which in turn presents LPS to MD2, a protein associated with Toll-like receptor-4 (TLR4). CD14 and TLR4 are present in several innate immune cells, including monocytes, macrophages and dendritic cells. Their ligation triggers a cascade of signaling events, including activation of the transcription factor NF- κ B and a range of mitogen activated protein kinases, which in turn lead to elevated production and release of various growth factors, inflammatory mediators, and proinflammatory cytokines (reviewed in, Aderem and Ulevitch 2000, Palsson-McDermott and O'Neill 2004, Takeda et al. 2003, Ulevitch and Tobias 1999).

Inflammatory responses have been shown to both activate and suppress several immune functions through direct and indirect mechanisms (e.g., compensatory anti-inflammatory responses) (Laroux 2004, Woiciechowsky et al. 1999). Additionally, studies have demonstrated that systemic inflammation induced by LPS can decrease resistance to experimental metastases (Harmey et al. 2002, Luo et al. 2004, Vidal-Vanaclocha et al. 1996). LPS is known to increase the synthesis of prostaglandins (PGs) (Blatteis 2007, Dubois et al. 1998) and the release of catecholamines (CAs) (Delrue-Perollet et al. 1995, Madden 2003). Various studies using the NK sensitive MADB106 experimental metastasis model in rats (Barlozzari et al. 1983, Barlozzari et al. 1985, Ben-Eliyahu and Page 1992, Ben-Eliyahu et al. 1996b, Shakhar and Ben-Eliyahu 1998) have shown that activation of prostanoids or β -adrenergic receptors can increase susceptibility to metastases via suppressing NK activity (NKA) (Ben-Eliyahu et al. 2000, Shakhar and Ben-Eliyahu 1998, Yakar et al. 2003). Thus, LPS could potentially affect resistance to MADB106 through suppression of NK activity, but other mechanisms may also mediate its potential effects.

Therefore, in the current work, we aimed at studying the effects of LPS on lung clearance of MADB106 tumor cells, as well as identifying potential sexual dimorphism in these effects. We further aimed at elucidating mechanisms underlying the effects of LPS and its sexually

dimorphic impacts, specifically the role of CAs, PGs, NK cells, corticosterone, and proinflammatory cytokines (IL-6 & TNF- α).

2. Materials and Methods

2.1 Animals

Fischer 344 male and female rats were raised in our vivarium and housed 2–4 per cage, with free access to food and water on a 12:12 hour light/dark cycle. Animals were handled 3–4 times before experimentation in order to reduce potential procedural stress. Animals were weighed at least once before the experimental procedure, and once after they were sacrificed. The order of drug administration, tumor injection, lung removal, and blood withdrawal or perfusion was counterbalanced across groups in each experiment. LPS and MADB106 administration was always conducted during the first half of the light phase, unless specified otherwise (Exp. 1). All studies were approved by the Institutional Animal Care and Use Committee of Tel Aviv University.

2.2 Drugs and their administration

2.2.1 Indomethacin—A non-selective COX inhibitor was purchased from Sigma, Israel. The drug was first dissolved in dimethyl sulfoxide (DMSO), and then diluted 1:70 in polyethethylene glycol (PEG). The drug was administered subcutaneously at a dose of 4 mg/kg (2 ml/kg) two hours before LPS administration.

2.2.2 Nadolol—A non-selective β -adrenergic blocker was purchased from Sigma, Israel. The drug was diluted in PBS and administered subcutaneously at a dose of 0.2 or 0.3 mg/kg (1 ml/kg) simultaneously with LPS administration.

2.2.3 LPS—Lipopolysaccharide from *Escherichia coli* was purchased from Sigma, Israel. LPS was diluted in PBS and was administered intravenously at a dose of 0.1 mg/kg (unless mentioned otherwise) (1 ml/kg) simultaneously with tumor inoculation (unless mentioned otherwise).

2.2.4 Anti-NKR-P1—A monoclonal antibody (mAb) (originally termed mAb 3.2.3) that binds to a surface antigen (Ag) (NKR-P1) expressed on fresh and IL-2-activated NK cells in rats and, to a much lesser degree, on polymorphonuclear (PMNs) cells (Chambers et al. 1992). In vivo treatment of rats with anti-NKR-P1 selectively depletes NK cells and eliminates NK- and antibody-dependent non-MHC-restricted cell cytotoxicity. T cell function and the percentages of T cells, PBMCs, and PMNs are unaffected (Chambers et al. 1989, van den Brink et al. 1990). We have previously shown that this antibody, but not isotype control antibodies, renders NK cells ineffective in vivo immediately upon administration, and selectively depletes NK cells within a day (Ben-Eliyahu and Page 1992). In addition, we have used other mAbs (R73, W3/25, ED2), mouse serum and saline as controls for the administration of anti-NKR-P1 and found no effects on metastatic colonization, tumor retention, or NK cytotoxicity (Ben-Eliyahu and Page 1992). The antibodies were injected i.v. under light isoflurane anesthesia simultaneously with tumor inoculation. In the ex-vivo experiment, conjugated with FITC, anti-NKR-P1 was used in FACS analysis to identify NK cells.

2.3 Tumor cell lines

2.3.1 MADB106—MADB106 is a selected variant cell line obtained from a pulmonary metastasis of a mammary adenocarcinoma (MADB100) chemically induced in the F344 rat (Barlozzari et al. 1983, Barlozzari et al. 1985). This syngeneic tumor metastasizes to the lungs following i.v. inoculation. The number of tumor cells retained in the lungs 24 h following i.v. inoculation, as well as the consequent metastases enumerated weeks later are highly dependent

on NK activity (Barlozzari et al. 1983, Barlozzari et al. 1985, Ben-Eliyahu and Page 1992, Ben-Eliyahu et al. 1996b, Shakhar and Ben-Eliyahu 1998). The MADB106 line was maintained in 5% CO₂ at 37 °C in monolayer cultures in complete medium, and was used for the in vivo studies, as well as a target cell in the ex-vivo assessment of NK cytotoxicity.

2.3.2 YAC-1—The standard YAC-1 target cell line for assessing rodents NK cytotoxicity in vitro was maintained in 5% CO₂ at 37 °C suspension cultures in complete medium.

2.4 Radiolabeling of MADB106 tumor cells and assessment of lung tumor retention (LTR)

DNA radiolabeling of tumor cells was accomplished by adding 0.5 µCi/ml of ¹²⁵Iododeoxyuridine (¹²⁵IDUR, Danyel Biotech, Rehovot, Israel) to the growing cell culture one day before harvesting the cells for injection. MADB106 cells were removed from the culture flask with trypsin solution (0.25% in PBS), and were washed twice with PBS containing 0.1% BSA. Rats were lightly anesthetized with isoflurane, and 4 × 10⁵ radiolabeled MADB106/kg cells in PBS (supplemented with 0.1% BSA) were injected into their tail vein (2 ml/kg). Following 21 hours, rats were killed with an overdose of isoflurane, and their lungs were removed and placed in a gamma counter for assessment of radioactive content. Percentage of tumor cell retention was calculated as the ratio between radioactivity measured in the lungs and the total radioactivity in the injected tumor cell suspension. Our previous studies have indicated that the levels of lung radioactivity reflect the numbers of viable tumor cells in the lungs that are expected to form solid metastasis (Bar-Yosef et al. 2001, Ben-Eliyahu and Page 1992, Shakhar and Ben-Eliyahu 1998).

2.5 Assessment of NK cytotoxicity

A standard whole blood 4h ⁵¹Cr release assay was used to assess NK-mediated lysis of YAC-1 and of syngeneic MADB106 target cells. Our previous studies indicated that cytotoxicity in this assay depends on NK cells, since their selective depletion nullified all specific killing (Ben-Eliyahu et al. 1996a, Page et al. 1994).

2.5.1 Preparation of marginating pulmonary effector cells—Three hours after LPS administration, rats were overdosed with isoflurane and their thoracic cavities were opened. Adherent lung-capillary (marginating pulmonary) leukocytes were harvested by perfusing this organ with heparinized PBS (30 units/ml): PBS was injected through the right ventricle and 25 ml were collected from the left ventricle. The first 3–5 ml of perfusate were disposed of, as it was contaminated with blood from the lungs and heart. The perfusate was then centrifuged (300g for 10 min), the supernatant removed, and the pellet was washed once again (300g for 10 min) with complete media and concentrated into a volume of 1 ml.

2.5.2 Preparation of target cells—MADB106 cells were removed from the culture flask with trypsin solution (0.25% in PBS), and were washed with complete media. Both MADB106 cells and YAC-1 cells (7 × 10⁶ of each) were incubated for 1 h with 150 µCi ⁵¹Cr (in 150 µl saline, Danyel Biotech, Rehovot, Israel), 100 µl fetal calf serum (FCS) and 300 µl complete media. Following incubation, cells were washed 3 times (300g for 10 min) and adjusted to the concentration of 5 × 10⁴/ml in complete media.

2.5.3 Assessment of cytotoxicity—To assess NK cytotoxicity at three different effector to target (E:T) ratios, effector cells were serially diluted three times beginning at their original concentration, and co-incubated with a fixed number of target cells. Specifically, aliquots of 150 µl of effector cell suspension (from lung perfusate) were placed in wells of a microtiter plate and serially diluted to create the 3 E:T ratios. Five thousand target cells in 100 µl complete media were then added to each well. Spontaneous release was obtained from wells receiving target cells and media only, and maximal release was obtained from wells receiving 1% Triton

X-100. The plates were centrifuged (400g 10 min) and incubated at 37 °C for 4 h. Following incubation, plates were centrifuged again and 100 µl of supernatant were recovered from each well for assessment of radioactivity in a gamma-counter. Percent cytotoxicity was calculated by the following formula: Percent cytotoxicity = $100 \times [(\text{sample release} - \text{spontaneous release}) / (\text{maximal release} - \text{spontaneous release})]$.

2.6 Flow cytometry

FACS analysis was used to assess the number of NK-cells in the lungs. An aliquot of 50 µl lung perfusate was added to 50 µl of PBS++ (PBS supplemented with 2% FCS and 0.1% NaN₃), and 0.1 µg of FITC-conjugated anti-CD161 (anti-NKR-P1) (PharMingen, San-Diego). Samples were kept in the dark at room temperature thereafter. Following a 15 min incubation period, 1 ml of FACS lysing solution (Becton Dickinson, San Jose, CA) was added, and 12 min later samples were centrifuged at 670 g for 5 min and the lysate was aspirated. Cells were rewashed with 1 ml PBS++ (5 min centrifugation, 670 g) and resuspended in 500 µl of PBS++ for flow cytometric analysis using a FACScan (Becton Dickinson). NK cells were identified as CD 161^{bright} lymphocytes. Specifically, the criterion for positive identification of NK cells was defined as being above a level of fluorescence intensity that distinguishes between the non-overlapping bright and dim populations of CD161 positive cells, as described previously by Chambers et al. (1992). Previous studies also demonstrated that CD161 is expressed by 94% of blood large granular lymphocyte (LGL) cells of the rat, and that the NK cytolytic activity was totally contained within the CD161^{bright} cell population. PMN leukocytes were found to express low levels of CD161 and were categorized as dim cells, and macrophages and mast cells were found to be negative (Chambers et al. 1989). Nonspecific binding was assessed using nonspecific IgG1 that consistently yielded 0% of brightly stained cells. To assess the total number of NK cells per µl of effector cells, we added 300 polystyrene microbeads per µl of sample to each sample (20 µm diameter, Duke Scientific, Palo Alto, CA). Following cytometry, the formula: " $\#CD161^{\text{bright}} \times 300 / \# \text{microbeads}$ " was used to calculate the number of NK cells per µl. The coefficient of variation for this method was found in our laboratory to be 6% for identical samples (Ben-Eliyahu et al. 1999).

2.7 Determination of IL-6, TNF-α, and corticosterone levels in serum

Blood samples were collected from the heart by cardiac puncture within 3 minutes from the time the cage was disturbed. The blood was allowed to clot for 40 minutes, centrifuged at 930g for 20 min, and the serum was collected. Levels of IL-6, and TNF-α were determined using enzyme-linked immunosorbent assay (ELISA) kits (R&D systems), as per manufacture instructions. The sensitivity of the assays for IL-6 and TNF-α were 8 pg/ml and 0.7 pg/ml, respectively. Corticosterone levels were evaluated using RIA (DSL laboratories, Rehovot, Israel). The sensitivity of the assay was 2.7 ng/ml. The manufacture reported intra-assay coefficients of variances of 8.8%, 5.1%, and 2.6% for IL-6, TNF-α, and corticosterone, respectively.

2.8 Statistical analyses

One, two, or three-way ANOVA were used for all studies (based on the experimental design). Analysis of NK cytotoxicity was conducted based on the means of the 3 E:T ratios in each sample tested. Provided significant group differences, Fisher's PLSD post hoc comparisons were conducted. In the two studies employing in vivo depletion of NK cells, the variance within NK-depleted animals was markedly and significantly greater than the variance in intact animals, violating the assumption of homogeneity of variances of ANOVA. Thus, we analysed each set of groups (groups containing NK-depleted animals, and groups containing intact animals) separately, as we did not intend to compare across these conditions. Student's t tests were used in Exp. 3 and 4 to compare females and males treated with a combination of LPS

and a specific drug, in which levels of LTR were presented as % of LTR in LPS treated animals (Fig 4c). $p < 0.05$ was considered significant in all studies.

3. Results

3.1 Experiment 1: Time course of the effects of LPS on MADB106 LTR

3.1.1 Design and procedure—Thirty one females 14–15 weeks old were used. Control animals were administered with saline ($n = 4$), and the other groups received LPS (0.1 mg/kg), either together with tumor inoculation (time 0), or 3, 6, or 24 hours before tumor inoculation ($n = 8, 5, 6, 8$, respectively). MADB106 administration was conducted in all animals during the first half of the light phase. Twenty-one hours after MADB106 inoculation, lungs were removed and LTR assessed.

3.1.2 Results—ANOVA revealed significant group differences ($F_{(4,26)} = 22.28, p < 0.05$) (Fig. 1). PLSD analysis indicated that animals injected with LPS simultaneously with the tumor (time 0) or three hours beforehand (–3) showed significantly elevated levels of LTR compared to controls (PLSD, $p < 0.05$). No other time point was significantly different from controls.

3.2 Experiment 2: A dose curve of the effects of LPS on MADB106 LTR in males and females

3.2.1 Design and procedure—Males and females, 14–17 weeks old, received either 0 (control, $n = 7$ & 9), 0.001 mg/kg ($n = 3$ & 4), 0.01 mg/kg ($n = 4$ & 5), 0.1 mg/kg ($n = 12$ & 13), or 1 mg/kg ($n = 8$ & 8) of LPS. LPS was administered simultaneously with tumor inoculation, and 21 hours later animals were euthanized and lungs were harvested to assess LTR.

3.2.2 Results—Two-way ANOVA indicated significant main effects for treatment (LPS dose) ($F_{(4,63)} = 41.86, p < 0.05$) and for sex ($F_{(1,63)} = 32.88, p < 0.05$) on LTR, and a significant interaction ($F_{(4,63)} = 4.38, p < 0.05$). In all LPS doses, except for 0.001 mg/kg, males had higher LTR than females (PLSD, $p < 0.05$), while there was no significant difference between the control groups (Fig. 2a).

With respect to body weight, ANOVA indicated a significant main effect for LPS dose ($F_{(4,63)} = 22.41, p < 0.05$). LPS induced significant weight loss at doses of 0.1 and 1 mg/kg in both sexes (PLSD, $p < 0.05$). None of these doses produced a significant difference between the sexes (Fig. 2b).

3.3 Experiment 3: The effects of LPS and the β -blocker, nadolol, in males and females

3.3.1 Design and procedure—A 2 X 4 design was used, in which 13 week old male and female rats were treated with either LPS (0.1 mg/kg, $n = 7$ & 7), nadolol (0.3 mg/kg, $n = 4$ & 4), LPS and nadolol ($n = 7$ & 8), or served as controls (saline, $n = 6$ & 9). LPS and nadolol were administered simultaneously with tumor inoculation. Twenty one hours after tumor inoculation, animals were euthanized and lungs were harvested to assess MADB106 LTR.

3.3.2 Results—ANOVA revealed significant main effects for treatment (drugs) and for sex ($F_{(3,44)} = 61.3, F_{(1,44)} = 35.74$, respectively, $p < 0.05$), and a significant interaction ($F_{(3,44)} = 10.5, p < 0.05$) (Fig. 3). PLSD analysis indicated that LPS increased LTR in both sexes, but significantly and markedly more so in males ($p < 0.05$). Nadolol significantly decreased the effect of LPS only in males ($p < 0.05$). Nadolol alone significantly increased LTR in males (PLSD, $p < 0.05$), and thus its dose was reduced in the following experiments. To directly test if the effects of nadolol were larger in males, we conducted a 2 X 2 ANOVA (males vs. females by LPS vs. LPS & nadolol) and found two main effects and a significant interaction ($F_{(1,25)} = 38.76, 4.71, 6.51$ respectively, $p < 0.05$), indicating that the effect of nadolol is significantly

greater in males. Additionally, in both males and females treated with LPS & nadolol, we evaluated the relative impact of nadolol by calculating the percent of the respective sex-matched LPS-only levels. This index indicates the percent reduction in LTR caused by nadolol with reference to the respective sex-matched LPS group. An unpaired t test comparing males to females in this index revealed a significant sex difference ($t_{13} = 2.69$, $p < 0.05$) (Fig 4c).

3.4 Experiment 4: The effects of LPS in males and females and its blockade by the β -blocker, nadolol, and the COX inhibitor, indomethacin

3.4.1 Design and procedure—In a 2 X 2 X 4 design, female and male rats (14–24 weeks old), received either saline (control) or LPS (0.1 mg/kg), and were pretreated with either saline, indomethacin (4 mg/kg), nadolol (0.2 mg/kg), or both drugs (group numbers are notated in Table 1). LPS and nadolol were administered simultaneously with tumor inoculation, and indomethacin (or vehicle) two hours beforehand. Twenty one hours after tumor inoculation, animals were euthanized and lungs were harvested to assess MADB106 LTR.

3.4.2 Results—Three-way ANOVA indicated significant main effects for drugs ($F_{(3,132)} = 10.00$, $p < 0.05$), LPS ($F_{(1,132)} = 325.20$, $p < 0.05$), and sex ($F_{(1,132)} = 61.01$, $p < 0.05$), and significant interactions between all pairs of factors (drugs and LPS, drugs and sex ($F_{(3,132)} = 10.99$, 3.01 , respectively, $p < 0.05$), LPS and sex ($F_{(1,132)} = 34.33$, $p < 0.05$)), and a significant three-way interaction ($F_{(3,132)} = 3.44$, $p < 0.05$) (Fig. 4a). PLSD comparisons indicated that males exhibit significantly higher LTR levels than females in all LPS groups ($p < 0.05$), but not in any of the control groups. In males, each of the blockers significantly decreased the effect of LPS, as did the combination of the blockers (PLSD, $p < 0.05$). In females, nadolol alone and nadolol and indomethacin together decreased the effect of LPS (PLSD, $p < 0.05$), but not indomethacin alone. The effect of the drugs' combination did not differ significantly from the effect of each of them alone in either sex.

To directly test whether the effects of nadolol or indomethacin were larger in males, for each drug we conducted a 2 X 2 ANOVA (males vs. females by LPS vs. LPS & drug) and found two main effects and a significant interaction (nadolol, $F_{(1,53)} = 77.03$, 48.55 , 11.19 respectively, $p < 0.05$) (indomethacin, $F_{(1,52)} = 58.67$, 19.82 , 11.14 respectively, $p < 0.05$), indicating that the effect of nadolol and the effect of indomethacin, each is significantly greater in males. Additionally, in both males and females treated with LPS & drug, we evaluated the relative impact of the drug by calculating the percent of respective sex-matched LPS-only levels. This index indicates the percent reduction in LTR caused by the drug with reference to the respective sex-matched LPS group. An unpaired t test for each drug, comparing males to females in this index, revealed a significant sex difference for indomethacin ($t_{21} = 2.57$, $p < 0.05$), and only a trend for nadolol ($t_{22} = 1.59$, $p = 0.12$) (Fig 4c).

With respect to body weights loss, ANOVA indicated significant main effects for drugs ($F_{(3,132)} = 10.06$, $p < 0.05$), and for LPS ($F_{(1,132)} = 64.87$, $p < 0.05$), with no significant effect of sex and no interactions (Fig. 4b). LPS induced significant weight loss (PLSD, $p < 0.05$), nadolol reduced body weight (PLSD, $p < 0.05$), and indomethacin increased it (PLSD, $p < 0.05$).

3.5 Experiment 5: The effects of NK depletion on MADB106 LTR in males and females

3.5.1 Design and procedure—Since LTR is highly dependent on NK activity, we studied potential sexual dimorphism in the effects of NK depletion on MADB106 LTR. Female and male rats, 13 weeks old, either received saline ($n = 9$ & 6) or underwent selective depletion of NK cells by administration of anti-NKR-P1 mAb in one of 3 different doses (6 , 3 , 1.5 mg/kg) ($n = 5$ & 4 in each group). Rats were administered with the mAb simultaneously with tumor

inoculation, and 21 h later animals were sacrificed and lungs were harvested to assess MADB106 LTR.

3.5.2 Results—Selective depletion of NK cells increased LTR levels in both males and females by more than 55-folds (Fig. 5). ANOVA was conducted separately on LTR levels of NK-intact rats and NK-depleted rats given markedly different variances between these conditions. No significant sex differences were evident in NK-intact or NK-depleted animals, and the 3 doses of anti-NKR-P1 seemed similarly effective.

3.6 Experiment 6: The effects of LPS and indomethacin in NK-depleted males and females

3.6.1 Design and procedure—This study was conducted to test whether the effects of LPS evident in NK-intact animals (Exp. 1–4), could also be seen in NK-depleted animals, indicating the involvement of NK-independent mechanisms in the effects of LPS. Based on Exp. 5 we used a depletion paradigm that was similarly effective in both sexes (a dose of anti-NKR-P1 mAb that was within a ceiling effect of depletion). Female and male rats, 14–16 weeks old, were randomly assigned to one of five groups. One group did not undergo NK-depletion for baseline references ($n = 8$ & 9). NK-depleted animals (treated with anti-NKR-P1 mAb) were either injected with saline (control, $n = 10$ & 9), indomethacin ($n = 4$ & 5), LPS ($n = 10$ & 10), or a combination of LPS and indomethacin ($n = 10$ & 10). Indomethacin (4 mg/kg) was administered 2 h before tumor inoculation, while the rest of the animals were administered with vehicle. LPS (0.1 mg/kg) and anti-NKR-P1 mAb (1.5 mg/kg) were administered simultaneously with tumor inoculation. Twenty one hours after tumor inoculation, animals were sacrificed and lungs were harvested to assess MADB106 tumor retention. The experiment included only indomethacin as a blocker for the effects of LPS, as our preliminary studies indicated that administration of nadolol and LPS in NK-depleted animals caused marked morbidity.

3.6.2 Results—ANOVA was conducted separately in NK-intact animals and in NK-depleted rats, given markedly different variances. In rats with functional NK cells, males showed significantly higher levels of LTR ($F_{(1,15)} = 6.40$, $p < 0.05$) (Fig. 6). In NK-depleted animals ANOVA indicated significant main effects for drugs and for sex ($F_{(3,60)} = 11.71$, $F_{(1,60)} = 9.08$, respectively, $p < 0.05$), without interaction. Similarly to NK-intact rats, in the NK-depleted rats administered with saline, males showed significantly higher LTR levels than females (PLSD, $p < 0.05$). Most importantly, PLSD comparisons showed that LPS significantly increased LTR in the NK-depleted rats in both sexes (PLSD, $p < 0.05$). Indomethacin had no effect within saline or LPS in both males and females, and did not reduce the effect of LPS.

3.7 Experiment 7: The effects of LPS and its blockade by nadolol and indomethacin on numbers and activity of marginating-pulmonary NK cells in males and females

3.7.1 Design and procedure—We studied NK cell numbers and cytotoxicity in the marginating-pulmonary immune compartment, as previous studies indicated that NK activity levels in this compartment against MADB106 target cells is most predictive regarding in vivo MADB106 LTR (Benish et al. 2008, Melamed et al. 2005). Male and female rats, 12–19 weeks old, were randomly assigned to receive either saline (control), LPS (0.1 mg/kg), blockers (combination of nadolol 0.2 mg/kg and indomethacin 4 mg/kg), or LPS together with blockers ($n = 4$ – 6 in each group). Indomethacin was administered 2 h before LPS, and nadolol was injected simultaneously with LPS. Three hours after LPS administration animals were overdosed with isoflurane and lungs were perfused to collect marginating-pulmonary leukocytes. Lung perfusate was assessed for number and activity of NK cells against YAC-1 and MADB106 target cells.

A 3 hour time-point for assessing the levels of NK numbers and activity was chosen as the index of MADB106 LTR is most sensitive to anti-metastatic NK activity during the first hours after MADB106 administration. Specifically, LTR is an index that cumulatively reflects the processes that occurred between MADB106 administration and the sacrifice of the animal. Previous studies indicated that levels of NK activity most profoundly impact LTR during the first hours following MADB106 administration, while completely lose their impact at 24 h post MADB106 administration (Ben-Eliyahu and Page 1992). LPS is known to immediately cause perturbations in cytokine and stress hormones, some of which dissipate within few hours (Harden et al. 2006, Johnson et al. 2003). Thus, the 3h time point is appropriate to reflect the effects of LPS on mechanisms that can affect NK indices within the timeframe that could substantially impact LTR.

3.7.2 Results—The patterns of results were very similar in the two target cell lines (YAC-1 & MADB106) (Fig. 7b & 7c), and therefore are described only for the MADB106 line given its higher relevance to the in vivo index of MADB106 LTR. Analysis of cytotoxicity was conducted on the mean of the 3 E:T ratios within each animal as these repeated measures showed the exact same pattern of effects (and one outcome per animal enables clearer presentation of the eight groups). ANOVA indicated a significant main effect of treatment ($F_{(3,32)} = 5.03, p < 0.05$), but not for sex nor an interaction. PLSD indicated that both LPS and the blockers, each significantly increased NK cytotoxicity per lung ($p < 0.05$). Notably, the blockers had no effect on the impact of LPS. These results argue against the hypothesis that LPS-induced alterations in NK activity underlie the in vivo effects of LPS on LTR.

With respect to numbers of marginating-pulmonary NK cells: ANOVA indicated a significant main effect for treatment ($F_{(3,31)} = 5.50, p < 0.05$), but not for sex nor an interaction (Fig. 7a). PLSD comparisons showed that the combination of blockers and LPS significantly elevated NK cell numbers (PLSD, $p < 0.05$).

3.8 Experiment 8: The effects of LPS, nadolol, and indomethacin on levels of IL-6, TNF- α , and corticosterone in males and females

3.8.1 Design and procedure—In a previous study we thoroughly evaluated baseline levels (no LPS) of corticosterone in the two sexes and found that in female F344 rats corticosterone levels significantly depend on the estrous cycle, while across the cycle male-female levels were comparable, reaching approximately 50 ng/ml at the day period samples were taken in the current study (Haim et al. 2003). In a pilot study we assessed levels of IL-6, and TNF- α in rats not treated with LPS, and as expected, these levels were below detection threshold. Thus, in this experiment, baseline levels of all 3 factors were not assessed. LPS (0.1 mg/kg) was administered to all female and male rats (12–19 weeks old). In addition, rats received either vehicle ($n = 5$ & 3), indomethacin (4 mg/kg, $n = 3$ & 5), nadolol (0.2 mg/kg, $n = 5$ & 5), or both indomethacin and nadolol ($n = 3$ & 5). Indomethacin or its vehicle was administered 2 h before LPS, and nadolol was injected simultaneously with LPS. Three hours after LPS administration (mid-light phase) animals were anesthetized and 1ml of blood was drawn from the heart by cardiac puncture within less than 3 min of disturbing the animals.

The 3 hour time-point for assessing the levels of these factors was chosen as the index of MADB106 LTR is most sensitive to various impacts during the first hours after MADB106 administration (see above, Exp. 7). This time-point also coincides with the time frame in which we expected these indices to be affected by LPS (Harden et al. 2006, Johnson et al. 2003).

3.8.2 Results—For IL-6 levels, ANOVA indicated significant main effects for drug treatment and for sex ($F_{(3,26)} = 24.84, F_{(1,26)} = 13.11$, respectively $p < 0.05$) (Fig. 8a), without interaction. Males exhibited significantly higher levels of IL-6 (PLSD, $p < 0.05$). Indomethacin reduced

the levels of IL-6 in both sexes (PLSD, $p < 0.05$), and nadolol increased it, but significantly so only in males (PLSD, $p < 0.05$). For TNF- α , ANOVA revealed a significant main effect only for drug treatment ($F_{(3,26)} = 52.38, p < 0.05$) (Fig. 8b). TNF- α levels were increased by nadolol (with or without indomethacin) in both sexes (PLSD, $p < 0.05$), but were not affected by indomethacin. In corticosterone, ANOVA indicated a significant main effect only for sex ($F_{(1,26)} = 73.68, p < 0.05$) (Fig. 8c). Females had significantly higher corticosterone levels than males in all drug conditions.

None of the patterns evident in these 3 humoral factors are in congruence with the in vivo results of MADB106 LTR.

4. Discussion

Our goals in this study were to examine the potential effect of LPS on MADB106 LTR, to explore sexual dimorphism in this outcome, and to start elucidating mediating mechanisms. While LPS increased LTR in both sexes when administered simultaneously with tumor cells, the results demonstrated marked sex differences in the effects of LPS; males consistently exhibit higher LPS-induced increases in LTR compared to females. In females approximately 10-fold higher doses of LPS were needed to reach the same response as in males. Furthermore, the effects of LPS on LTR were attenuated in a dimorphic manner by nadolol, a β -blocker, and by indomethacin, a COX inhibitor. Specifically, the attenuating effects of nadolol were greater in males, and indomethacin reduced the effects of LPS only in males.

In some, but not other experiments, we observed higher baseline levels of LTR in males. However, the marked and significant dimorphism in the effects of LPS was observed in all experiments, including those in which the two sexes had very similar baseline levels (see Exp. 3, 4). As LTR of the MADB106 is highly sensitive to NK activity in vivo (Barlozzari et al. 1983, Barlozzari et al. 1985, Ben-Eliyahu and Page 1992, Ben-Eliyahu et al. 1996b, Shakhbar and Ben-Eliyahu 1998), NK cells can potentially mediate these baseline differences. However, in the first NK-depletion experiment, very similar male-female baseline levels were observed in both NK-intact and NK-depleted rats, and in the second NK-depletion study, a higher baseline levels in NK-intact males was also evident in NK-depleted males. Thus, baseline differences in LTR, when appearing, seem not to be related to NK activity, and the sexually dimorphic effects of LPS are independent of these potential baseline differences.

β adrenergic receptors are expressed on most resting and activated immune cells, including lymphocytes, NK cells, neutrophils and macrophages (Benschop et al. 1996, Elenkov et al. 2000). β -adrenergic agonists carry anti-inflammatory effects, which include increased IL-10 and reduced TNF- α and IL-1 β production (Hasko and Szabo 1998, Van der Poll and Lowry 1997). Sex differences have been reported to exist in β -adrenergic mechanisms and in its effects on the inflammatory response (Barker et al. 2005, de Coupade et al. 2004), but no generalization seems to be warranted. In the current study the β -blocker nadolol was more effective in males, attenuating the impacts of LPS on MADB106 LTR. Compatible with this observation, in a recent study we reported that administration of the same dose of a β -adrenergic agonist caused a greater increase in MADB106 LTR in males than in females (Page et al. 2008). Taken together, it could be suggested that in response to LPS females and males exhibit similar neuroendocrine sympathetic responses, but these responses may bear lesser impact at the cellular levels in females.

COX-2, a protein induced by various proinflammatory agents, including LPS, cytokines, and growth factors (Lee et al. 1992), is primarily responsible for the synthesis of the inflammatory mediator prostaglandin E2 (PGE₂) (Hinz and Brune 2002, Kam and See 2000). PGE₂ has profound immunosuppressive effects on CMI, and was suggested to promote the release of IL-4 and IL-10, and to attenuate the production of TNF- α , IL-1, and IL-6 by LPS (Ayala et al.

1994, Dubois et al. 1998, Strassmann et al. 1994). The biological actions of PGE₂ are mediated by E prostanoïd (EP) receptors which have different influences in males and females (Audoly et al. 1999), as is elaborated below.

Sex-based differences observed in immune responses have been generally assumed to be a consequence of reproductive hormones, which have been shown to induce immunomodulation through their respective receptors on a variety of immunocytes and through additional mechanisms (reviewed in Angele et al. 2000). It has been suggested that estrogen suppress inflammation and has immunoprotective effects, while testosterone augments inflammatory responses and has immunosuppressive effects, as exemplified by testosterone-induced exacerbation of cerebrovascular inflammation (Razmara et al. 2005). Although the mechanisms have not been elucidated, it was suggested that androgens target the NF- κ B pathway to increase expression of inflammatory mediators (Death et al. 2004). In females, 17 β -estradiol inhibits NF- κ B activation and exerts anti-inflammatory activity, including reduced cytokine and COX-2 production (Baker et al. 2004, Deshpande et al. 1997, Evans et al. 2001, Ospina et al. 2004). This anti-inflammatory activity exhibited in females may provide an explanation of our finding that indomethacin attenuated the effects of LPS only in males. On the other hand, following LPS treatment, male-derived macrophages were reported to produce lower amounts of the PGE₂ than female-derived macrophages (Marriott et al. 2006).

The combination of nadolol and indomethacin was not more effective than each drug alone in reducing the promotion of LTR by LPS. A complex relationship exists between CAs and PGs. There are reports that high CA levels can cause the release of PGs (Ueda et al. 1994), and that PGs can induce the release of CAs (Yokotani et al. 1995). In addition, it was found that pretreatment with indomethacin suppressed the increase in CAs induced by LPS (Sakata et al. 1994). If such serial relationships mediate the effects of LPS in the current study, blocking one of these factors may suffice, rendering the other blocker redundant.

LPS administration caused weight loss in all experiments. This finding is consistent with the sickness response to endotoxin which includes anorexia, hypothermia, fever, cachexia and decrease in behavioral arousal. These symptoms seem to be triggered by the proinflammatory cytokine response to LPS (reviewed in Dantzer 2001). However, as opposed to the effect of LPS on LTR, weight loss as % of body weight did not exhibit sexual dimorphism in any of the studies. Interestingly, nadolol, which reduced the tumor promoting effects of LPS on LTR did not reduce LPS effects on weight loss, but rather significantly worsen it. Indomethacin, which reduced the effects of LPS on LTR only in males, reduced weight loss in both sexes. Thus, it seems that the effects of LPS on LTR and on body weight are not causally related.

The role of TNF- α , IL-6, and corticosterone in mediating the effects of LPS and the blockers on MADB106 LTR seems unresolved. All three factors dramatically increased following LPS administration, and thus may mediate some of the effects of LPS on MADB106 LTR. However, corticosterone reached markedly higher levels in females, which showed lower effects of LPS on MADB106 LTR. This suggests that corticosterone is unlikely to constitute a fundamental mediating mechanism. Consistent with this suggestion, studies conducted in our laboratory indicated that administration of corticosterone that induced high physiological levels did not increase MADB106 LTR (unpublished data), and other studies also refute a significant effect of corticosterone on MADB106 LTR (Shakhar and Ben-Eliyahu 2003). The two inflammatory cytokines could have mediated some of the effects of LPS on MADB106 LTR, but not the sexually dimorphic effects of LPS, nor the effects of the two blockers – the sex-differences and/or the impacts of the blockers on the cytokine levels did not coincide with the effects of LPS on MADB106 LTR. For example, following LPS challenge the β -blocker nadolol increased levels of both IL-6 and TNF- α , which is expected given the reports that CAs antagonize some inflammatory responses (Hasko et al. 1998, Hasko and Szabo 1998, Van der

Poll and Lowry 1997). However, this increase is contrary to the effects of nadolol on LTR, which actually reduced the effects of LPS.

Trying to elucidate the role of NK cells in our results, we conducted in vivo NK-depletion experiments. Additionally, we assessed NK cell numbers and cytotoxicity in the marginating-pulmonary compartment, which seems most causally related with respect to MADB106 LTR (Melamed et al. 2005). The data yielded suggests that NK cells are not the major mechanism underlying the effects of LPS on MADB106 LTR, although the dimorphic effects of indomethacin seem to depend on NK activity. Specifically, LPS increased LTR in both NK-intact and in NK-depleted rats of both sexes. Thus, there is a significant non-NK mechanism mediating the effects of LPS. On the other hand, indomethacin reduced the effects of LPS in NK-intact males, but not in NK-depleted males, and had no impact in control rats. Thus, the beneficial effects of indomethacin in the context of LPS are mediated through an NK-dependent mechanism, as is an aspect of the LTR-increasing impact of LPS in males. Unfortunately, we were unable to study the effects of nadolol in NK-depleted animals, as our preliminary studies indicated that nadolol caused marked morbidity in NK-depleted animals treated with LPS. Thus, a sympathetic NK-mediated aspect of the LTR-increasing effects of LPS is unclear. The ex vivo studies support the conclusion that a NK-independent mechanism exists in the MADB106 LTR-increasing effects of LPS. As elaborated upon in Exp. 7, the time-point for assessing NK indices was chosen to maximize our ability to identify alterations in NK activity that can potentially impact LTR. However, rats treated with LPS actually showed increased total lung marginating-pulmonary NK activity, rather than a decrease that would be expected if NK activity would be the sole mediator of the effects of LPS.

Numerous non-NK related mechanisms activated by systemic administration of LPS can induce enhanced MADB106 LTR. LPS is known to induce a wide range of cytokines, growth factors, and other mediators, which can create permissive environment for metastasis progression. LPS and inflammation have been associated with key determinants of metastasis, including increased vascular permeability, increasing tumor cell invasion and migration, and enhanced angiogenesis (Arias et al. 2005, Balkwill and Mantovani 2001, Coussens and Werb 2002, Harnsey et al. 2002). Some studies suggested that NF- κ B may be a link between inflammation and tumorigenesis (Baldwin 2001, Balkwill and Coussens 2004, Karin et al. 2002). In support of a link between inflammation and tumor progression, accumulating data suggest that anti-inflammatory agents, including non-steroidal anti-inflammatory drugs (NSAIDs), inhibit both tumorigenesis and growth of colon and mammary tumors (reviewed in Hinz and Brune 2002, Shiff et al. 2003). NSAIDs were also shown to reduce the promotion of metastasis by surgery (Benish et al. 2008, Melamed et al. 2005, Page and Ben-Eliyahu 2002, Yakar et al. 2003).

The MADB106 experimental metastasis model we used has very limited clinical implications to cancer progression. However, the current findings could trigger and guide more translational studies regarding sex differences in inflammation and tumor metastasis, as well as clinical studies. It is noteworthy that we chose to conduct our studies focusing on acute administration of LPS simultaneously with tumor cells, as these studies have potential clinical implications to cancer patients undergoing surgical excision of a primary tumor. At this critical perioperative period, the metastatic process is exacerbated and bears long-term consequences, and patients are exposed to inflammatory agents and inflammatory processes (Shakhar and Ben-Eliyahu 2003). It has been reported that bacterial contamination during surgery, or inflammation during the postoperative period, can increase metastatic tumor growth in mice (Pidgeon et al. 1999) and in cancer patients (Taketomi et al. 1997). Given the sexual dimorphisms reported in the current study, it is interesting to examine whether sexual dimorphisms also exist with respect to tumor development and metastasis progression in cancer patients. Indeed, several clinical studies reported that men are more susceptible to metastatic development and exhibit reduced

survival rates. In melanoma patients, although the primary tumor seems to occur independently of sex (or in greater proportion in women), females show prolonged survival and higher survival rates (Apte et al. 2006, Miller and Mac Neil 1997, Rampen 1980, Rampen 1984, Scoggins et al. 2006). In small cell lung cancer, males exhibit reduced survival rates (Sahmoun et al. 2005). Lastly, in colorectal cancer, men were suggested to be more prone to liver metastasis than females (Zhang et al. 2002). These sexual dimorphisms warrant further studies and may be related to sex differences in inflammatory process or to other dimorphic mechanisms that impact the metastatic process.

In conclusion, our study demonstrates that LPS can augment MADB106 LTR, markedly more so in male than in female F344 rats. These effects were attenuated, but not abolished, by blockade of CAs and PGs, and the magnitude of blockade was greater in males, but not sufficient to underlie the entire sexual dimorphism. Although NK cells mediated the PGs-dependent effects of LPS in males, the greater portion of the effects of LPS are yet unexplained and warrant further studies, including the role of sex hormones in the observed dimorphisms. The abundance of LPS in our environment, and the suspected clinical impact of inflammation on various medical conditions, including cancer metastases, cardiovascular diseases, depression, autoimmunity, and infectious diseases, suggest the importance of conducting further studies of these effects and their sex-dependencies. Understanding the mechanisms that underlie the sexual dimorphism of the inflammatory response and their blockade may provide additional insight into the complex and multifaceted effects of inflammation, and could suggest therapeutic strategies.

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Fig. 1.

Time-course of the effect of LPS on MADB106 LTR (mean + SEM). Animals injected with LPS together with tumor cells (0) or 3 hrs beforehand (-3) showed significantly elevated levels of LTR compared to controls (indicated by *).

Fig. 2.

(a) Sex differences in the effects of different doses of LPS on MADB106 LTR (mean + SEM). LTR was significantly higher in males than females in LPS doses of 0.01, 0.1, and 1 mg/kg. * indicates a significant sex difference in the respective dose. (b) Body weight loss as a result of different doses of LPS (mean + SEM). LPS decreased body weight in a dose-dependent manner. There were no significant sex differences in any of the doses. # indicates significant difference from the respective sex-matched control group.

Fig. 3.

Sex differences in the effects of LPS on MADB106 LTR and its blockade by nadolol (mean + SEM). LPS significantly increased LTR in both sexes compared to the respective control (*), but significantly more so in males compared to females (¥). Nadolol decreased this effect of LPS on LTR only in males (#).

Fig. 4.

(a) The effects of LPS on MADB106 LTR and its blockade by nadolol and indomethacin (indo) in males and females (mean + SEM). LPS significantly increased LTR in males more than in females in all conditions. In males, each of the drugs alone, and their combination, decreased the effects of LPS (*). In females only nadolol was effective (**) (with or without indomethacin). The drug combination was not more effective than each drug alone in either sex. (b) The degree of body weight loss in the above study (mean + SEM). Across all other conditions, LPS decreased body weight, nadolol caused weight loss, and indomethacin increased body weight. No significant sex differences were evident, nor were interactions of sex with LPS or drugs. (c) The effects of nadolol and indomethacin in LPS-treated animals,

presented as % of the respective sex-matched levels of LTR in animals treated only with LPS.
* indicates significant sex difference within each drug.

Fig. 5. Effects of NK-depletion in male and female rats on MADB106 LTR (mean + SEM). NK-depletion increased LTR by approximately 55-fold. No sex differences were evident in NK-intact or in NK-depleted rats. All antibody doses were similarly effective. Notice that LTR is presented in a logarithmic scale.

Fig. 6.

Effects of NK-depletion in male and female rats treated with LPS and indomethacin, as indicated by MADB106 LTR (mean + SEM). Males exhibited higher LTR than females in intact and in NK-depleted rats (*). LPS significantly increased LTR in NK-depleted rats in both sexes (#), and indomethacin had no effect (did not attenuate the effects of LPS). Notice that different scales are used for LTR in NK-depleted animals and NK-intact animals.

Fig. 7.

The effects of LPS and their blockade by nadolol and indomethacin (blockers) on (a) the numbers of marginating-pulmonary NK cells and their activity against (b) YAC-1 and (c) MADB106 target cells (mean $\pm$ SEM). No sex differences were evident. Across sex, the combination of LPS and blockers increased NK numbers (a), each of these factors alone increased NKA against the MADB106 tumor line (c), and together no additional effects were evident on NKA. * indicates a significant difference from the saline control.

Fig. 8.

Serum levels of IL-6 (a), TNF- α (b), and corticosterone (c) in male and female rats treated with LPS (mean $\pm$ SEM). Rats were subjected to nadolol, indomethacin (indo), both drugs, or no drug (saline), and blood withdrawal 3 h after LPS administration. * indicate significant differences compare to the sex-matched control. ** indicate significant sex differences within a specific drug condition. Levels of IL-6 were significantly higher in males, but only across all conditions.

Table 1

Animal numbers in Experiment 4.

	Saline		Indo		Nadolol		Indo & Nadolol	
	Female	Male	Female	Male	Female	Male	Female	Male
Control	12	11	5	5	3	7	4	4
LPS	16	17	12	11	12	12	8	9