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Lactation modifies stress-induced immune changes in laboratory rats

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ABSTRACT

Lactation and stressor exposure both influence the activity of the immune system, but the interaction of both factors on the immune defense is poorly understood. The aim was therefore to investigate in lactating Long–Evans rats the effect of social stress on aspects of cellular immunity in the blood and mesenteric lymph nodes (MLN). Acute social stress (2 h) was induced in lactating and non-lactating female intruders using a confrontation model that yielded into social defeat and increased plasma corticosterone concentrations. Stress as well as lactation had marked effects on the immune system. Acute social stress caused granulocytosis, reduced lymphocyte proliferation, and cytokine production in the blood, but had no significant effects in MLN. In the blood of lactating rats, increased numbers of granulocytes and enhanced phagocytosis, but decreased B cell numbers and reduced IL-2 production was observed. However, in MLN both lymphocyte proliferation and monocyte numbers were increased in lactating rats. The effect of stress on the immune measures was often similar in lactating and non-lactating females, but a few important differences were evident: Only non-lactating animals showed an increase in blood granulocyte numbers and a decrease in IL-2 production in response to stressor exposure. Thus, during lactation, a neuroendocrine status may exist which impedes stress-induced modulations at least of some immune parameters.

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1. Introduction

The period of lactation takes up a large part of life in many mammalian species, with profound hormonal, morphological, and behavioral changes occurring during that time. Apart from changes related to milk production, alterations in certain brain regions such as the supraoptic nucleus (Mann and Bridges, 2002; Theodosis et al., 1981) and in the HPA system (Carter et al., 2001; Magiakou et al., 1997; Shanks et al., 1999) occur. In addition, the behavior of lactating females is often characterized by reduced anxiety (Hard and Hansen, 1985; Neumann et al., 2000; Toufexis et al., 1999) and increased aggression towards unfamiliar conspecifics (Gammie et al., 2005; Wise, 1974). Whereas in animals lactation is associated with an increased susceptibility to parasitic infections (Barger, 1993; Festa-Bianchet, 1989; Ngwenya, 1977; Shubber et al., 1981), studies in humans show a postpartum relapse of established autoimmune disorders such as rheumatoid arthritis and multiple sclerosis, often described as a flare-up due to the rebound of the immune system after pregnancy (Buchel et al., 2002; Nelson and Ostensen, 1997; van Walderveen et al., 1994). Furthermore, the postpartum period in humans is also associated with the onset of the postpartum autoimmune thyroid syndrome (Amino et al., 1982; Muller et al., 2001).

The immune system of lactating animals was the focus of only a small number of studies with mostly different experimental designs. It appears, however, that some aspects of immune function become suppressed, while others remain unaffected or become enhanced. For example, antibody production after immunization (Jäckel, 2003; Ngwenya, 1977) and IL-2 production in the spleen (Shanks et al., 1997) was found to be suppressed during lactation in rodents. Conversely, increased concentrations of plasma IL-6 or an enhanced proliferative response of lymphocytes from mesenteric lymph nodes (MLN) suggest activation of other immune responses (Shanks et al., 1997). Similarly, in humans, an increase in serum IL-6, TNF-α, IFN-γ, and IL-10 concentrations and higher numbers of blood cytotoxic T cells and B cells were observed during lactation (Groer et al., 2005). For NK cell numbers, conflicting data exist: Groer et al. (2005) reported a decreased NK cell number during lactation, whereas Watanabe and colleagues (1997) show increased or unaltered values.

Stressors have a major impact on the immune system in male (Avitsur et al., 2002; Shurin et al., 1994; Stefanski and Engler, 1998; Stefanski et al., 1996) and female rats (Stefanski and Grüner, 2006). In females, the stress responses can vary considerably depending on the reproductive status. For example, susceptibility to NK cell-dependent tumor metastasis after surgery stress differs

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among oestrous phases (Ben-Eliyahu et al., 2000; Page and Ben-Eliyahu, 1997). So far, however, only a few studies have investigated the effects of stress on the immune system during lactation. In humans, a psychological stressor (public speaking and mental arithmetic) was effective to decrease blood lymphocyte proliferation in response to PWM in the non-postpartum group only. In contrast, PHA-induced lymphocyte proliferation increased in the group of women who were recently parturient and breastfeeding, whereas the non-postpartum group remained unaffected (Redwine et al., 2001). Shanks and colleagues reported differential effects of conditioned stress (electrical shock) on the immune system in lactating and non-lactating Sprague–Dawley rats (Shanks et al., 1997). Stressed lactating rats showed an enhanced proliferation in MLN and higher plasma IL-6 concentrations, but a reduced splenocyte proliferation compared to the stressed non-lactating group. No difference however, was observed with respect to blood lymphocyte proliferation.

The aim of the present study is to investigate the impact of stress on the immune system during lactation using a social confrontation model with high face validity (Stefanski and Engler, 1998; Stefanski and Grüner, 2006; Stefanski et al., 2005). Here, we focus on the effects of an acute (2 h) social stressor on several aspects of cellular immunity in the blood and in MLN. This lymph node region plays a key role during lactation because it provides protection for the offspring against potentially harmful gut pathogens (Head and Seelig, 1983; Lamm et al., 1978; Roux et al., 1977).

2. Methods

2.1. Animals

Adult Long–Evans rats (about 4.5 month old) were housed in male/female pairs in polycarbonate rat cages (26 × 42 × 15 cm) under standard laboratory conditions (20 ± 1 °C, 40 ± 5% humidity). Animals were maintained on a 12:12 h light–dark cycle and had ad libitum access to rat standard diet and water. All experiments were conducted in the dark phase of the cycle (the active period of the animals).

2.2. Social stress procedure

Social stress was induced using an adapted form of the resident–intruder confrontation paradigm as described previously (Stefanski et al., 2005). Briefly, lactating and non-lactating female rats (“intruders”) were introduced to confrontation-experienced resident female rats (Long–Evans) housed in a larger enclosure with chipboard walls (height: 75 cm) and 0.5 m^2 of tiled floor. Intruders were attacked by residents within 5 min. Pre-tests with resident females ensured reliability of attacking intruders. The confrontation lasted 2 h. No evidence of wounding was observed after the confrontation. Non-stressed control animals remained undisturbed in their home cages.

2.3. Synchronization of lactation

All confrontations and samplings were conducted on mid-lactation day 9 ± 1, the time of lactation where milk production is fully established (Morag et al., 1975; Russell, 1980). This sampling time well after end of pregnancy also assured that lactation-associated immune changes were measured, as pregnancy-associated immune changes return to pre-pregnancy values within 5 days after offspring removal (Stefanski et al., 2005). To create comparable experimental conditions in each set of experiments (see below) the time of lactation was synchronized. For synchronization, females were separated from their partners and housed alone for 25 days to exclude pregnancy. Males were re-introduced for 3 days and then removed. For the following periods of pregnancy and lactation, females and their litter were housed alone again. This mating paradigm resulted in an about 50% chance of pregnancy. The non-pregnant rats later served as non-lactating female control group. To exclude possible health abnormalities negatively affecting conception and pregnancy, only females with a previous successful pregnancy were included. (Under the housing in our laboratory, >95% of females became repeatedly pregnant). By taking advantage of the 5 day estrus cycle, this study design therefore did not require sterilized males as mating partners, improving animal welfare.

2.4. Collection of blood and mesenteric lymph nodes

Blood and mesenteric lymph nodes from stressed females were collected immediately after the end of the 2 h confrontation; samples from non-stressed controls were taken at corresponding time points. Up to 2 ml of blood was collected from the lateral tail vein within 5 min as described previously (Stefanski, 2000). Blood collection was conducted without requirement for anesthesia. For corticosterone measurement, time was restricted to 3 min. For functional immune assays, heparinized blood was processed immediately. K_2-EDTA-treated blood was stored at room temperature and analyzed with flow cytometry within 3 h. Untreated blood was centrifuged and serum was stored at −20 °C for future corticosterone measurement. Immediately after blood collection, animals were sacrificed by CO_2 inhalation, mesenteric lymph nodes were collected and transferred to complete RPMI medium (RPMI 1640, Biochrom, supplemented with 10% FCS and 50 μg/ml gentamicin, both Life Technologies). Under sterile conditions, fat tissue was removed and mesenteric lymph nodes were passed through a 40 μm nylon net (BD Biosciences). The cell suspension was centrifuged at 300g for 5 min, resuspended in 0.9% isotonic NaCl, and then treated like blood samples for further analysis.

For technical reasons and in order to keep processing time for each sample to a minimum, the entire study was conducted as a series of experimental sets (repeats) with four sets (12–15 animals each) for MLN analysis and corticosterone measurements and additional four sets (20–25 animals each) for blood immune measures. Limitations due to blood volume (2 ml) did not allow analysis of all blood immune parameters simultaneously. Thus, analysis of blood phagocytic capacity, lymphocyte proliferation (ConA only), and leukocyte subsets was conducted in two sets, blood cytokine concentrations were analyzed in other two sets. Each set consisted of about equal numbers of stress and control as well as lactating and non-lactating animals.

2.5. Behavioral observation

The resident–intruder confrontations were videotaped under infrared light conditions. Agonistic behavior of both intruder and resident female rats was evaluated for the first 30 min of confrontation using “continuous recording” (Martin and Bateson, 1993). The following behavioral elements (Engler and Stefanski, 2003; Stefanski et al., 2005) were analyzed: *attack* (jump at the opponent with physical contact), *submissive posture* (standing with the front legs on the motionless opponent), *chase* (following the opponent at running pace), *sideway* (“lateral threat”, curved broadside orientation in close proximity to the opponent), *approach* (directed movement towards the opponent at walking speed), *upright* (standing on hind legs with ventral body directed towards the opponent), *full defensive posture* (lying motionless on the back with ventral surface exposed to the opponent), *retreat* (directed movement away from the opponent at walking speed), *flight* (like *retreat* but at running pace). A dominance index, DI, ranging from 1 (completely dominant) to 0 (completely defeated) was calculated for each female in a confrontation dyad with the behavioral elements *flight* and *re-*

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treat (Stefanski and Grüner, 2006). Only intruder females with a DI <0.2 were included in the study.

2.6. Corticosterone concentration

Serum corticosterone concentrations were determined with a standard radioimmunoassay (Foster and Dunn, 1974). The specific antibody (Ab 24/6) was kindly provided by Dr. Vescei, Institute of Pharmacology, University of Heidelberg, Germany. [^{3}H]-labeled corticosterone was obtained from Amersham Biosciences. Cross-reactivity of the corticosterone antibody with other relevant steroids was as follows: cortisol 4.4%, cortisone 0.65%, deoxycorticosterone 30.0%, progesterone 35%, deoxycortisol <0.4%, 17 α-OH progesterone <0.4%, testosterone 5.5%, androstendione 3.2%, aldosterone 0.8%. The detection limit of the assay was 5 ng/ml. Intra- and interassay variance was <5% and <10%, respectively.

2.7. Leukocyte counts and subpopulations

Total leukocytes were counted with an automatic cell counter (Coulter Counter Z2, Coulter Electronics Ltd.). Cells were then incubated for 20 min at room temperature in buffer (2% FBS, 0.1% NaN_3 in PBS) with anti-rat CD45LCA: PE (clone OX-1, 20 μg/ml), anti-rat CD172a: FITC (clone ED9, 10 μg/ml, Serotec Ltd., Düsseldorf, Germany), anti-rat CD3: PE (clone G4.18, 12,5 μg/ml), anti-rat CD4: FITC (clone OX-38, 50 μg/ml), anti-rat CD8b: FITC (clone 341, 50 μg/ml), or anti-rat NKR-P1A: FITC (clone 10/78, 50 μg/ml). Unless otherwise stated, all antibodies were obtained from BD PharMingen (Heidelberg, Germany). Following incubation, erythrocytes were lysed with FACS lysing solution (BD Immunocytometry Systems) and the percentage of leukocyte and lymphocyte subpopulations was determined on a flow cytometer (FACSCalibur, BD Immunocytometry Systems). Ten thousand cells were analyzed for each sample. Leukocyte subpopulations were identified by forward and sidescatter characteristics and by differences in CD45 and ED9 expression. Lymphocyte subpopulations were identified by characteristic expression of the surface markers $CD3^+/CD4^+$ (T helper cells), $CD3^+/CD8b^+$ (cytotoxic T cells) and $CD3^-/NKR\text{-}P1A^+$ (NK cells). The percentage of B cells was obtained by subtracting all other subpopulations from the total lymphocytes.

2.8. Proliferative response

Peripheral blood mononuclear cells (PBMC) or mesenteric lymph node cells (MLNC) were isolated by Nycoprep density gradient (Nycoprep™ 1.077A, Axis-Shield). After washing, cells were adjusted to 1.5×10^6 (PBMC) or 2×10^6 (MLNC)/ml in complete PRMI medium. One hundred microliters of this cell suspension were then transferred to each well of a 96-well round-bottomed tissue culture plate. For mitogenic stimulation of lymphocytes, 100 μl of ConA or PWM (both Sigma–Aldrich) in complete RPMI medium were added yielding final concentrations of 0.625 μg/ml (ConA or PWM, MLNC) and 5 μg/ml (ConA, PBMC). Due to limited amounts of blood, no stimulation of PBMC with PWM could be conducted. ConA primarily stimulates T cells and PWM primarily stimulates B cells. Unstimulated controls were treated with complete RPMI medium. All stimulations were set up in triplicates. Cells were incubated at 37 °C, 5% CO_2 for 48 h, pulsed with 0.5 μCi Methyl-[^{3}H]-thymidine (NEN, Boston) and incubated for an additional 24 h. Cells were harvested on glass filters (Filtermats W/Binding, Molecular Devices) and radioactivity was measured as counts per minute (cpm).

2.9. Phagocytosis

Phagocytic activity was determined in whole blood samples. Twenty microliters of heparinized blood were incubated in 460 μl medium with 10 μl of lucigenin solution (5.1 mg/ml PBS, Sigma) at 37 °C, 5% CO_2 for 30 min. After addition of 10 μl Zymosan A (12.5 mg/ml PBS, Sigma), chemiluminescence was measured for 30 min at 37 °C on a luminometer (Multi Bioluminat LB 9505 C, Berthold, Germany). Results are expressed as total counts per 30 min. Preliminary tests revealed no detectable phagocytic activity for isolates from MLN.

2.10. Cytokine production

For the determination of cytokine production in cell culture supernatants, cells were isolated by Nycroprep density gradient, adjusted to 2.5×10^6 (PBMC) or 3×10^6 cells/ml (MLNC) and cultured in 96-well plates as described above. Cells were stimulated with ConA at final concentrations of 5 μg/ml (PBMC) or 1.25 μg/ml (MLNC) for 24 h. Supernatants were centrifuged and stored at −80 °C for later analysis.

IL-2 and IFN-γ concentrations in supernatants were determined with a standard ELISA procedure. All antibodies and standards were obtained from Biolegend, San Diego. A 96-well high affinity-protein binding ELISA plate was coated with 2 μg/ml of anti-rat IL-2 (clone: BL-7015) or anti-rat IFN-γ (clone: DB-1) in sodium carbonate buffer and incubated at 4 °C overnight. Nonspecific binding was blocked (10% FCS in PBS) for 1 h at room temperature. One hundred microliters of samples or standard (recombinant rat IL-2 or recombinant rat IFN-γ, source *Escherichia coli*, in blocking solution) were applied and incubated for 3 h at room temperature. The standard curve (20 ng/ml to 40 pg/ml) was analyzed in triplicates, each sample in duplicates. Then, 0.5 μg/ml biotinylated anti-rat IL-2 (clone BL-7030) or anti-rat IFN-γ (clone Poly5109) in blocking solution were applied and incubated for 1 h at room temperature, followed by incubation with streptavidin–horseradish peroxidase (Southern Biotech, 1:6000 in blocking solution) for 30 min at room temperature. Finally, 50 μl of TMB substrate solution (Biomeda) were applied for 8–12 min, the color reaction was stopped with 0.5 M H_2SO_4, and the absorption measured at 450 nm. Cytokine concentrations were calculated from standard curves created in Origin 7G SR4 for Windows. In the present study, IL-4 concentrations were measured in supernatants from stimulated cell cultures as well, with an IL-4 ELISA using anti-rat IL-4 (clone BL-7045) for capture and biotinylated anti-rat IL-4 (clone BL-7060) for detection. In all supernatants, IL-4 concentrations were below the detection limit of the assay (40 pg/ml).

2.11. Statistics

Behavioral data were analyzed using the Mann–Whitney *U*-test. Immunological and hormone data were analyzed with two-way ANOVA for the factors *stress* and *lactation*. Shapiro Wilk testing for normal distribution and Levene testing for homogeneity of variance were performed prior to ANOVA. Standard transformations were applied to achieve normal distribution and homogeneity of variance. Student's *t*-test was used for *post hoc* analysis. Benjamini–Hochberg corrections of *p*-values were applied to adjust for multiple comparisons. A *p*-value of <0.05 was considered significant. Relations of immune parameters with corticosterone were analyzed with Pearson's correlation. All statistics were calculated using SPSS 12.0 for Windows.

3. Results

3.1. Agonistic behavior of resident and intruder rats

Females from both intruder groups were clearly defeated (DI $\leqslant$0.2). However, lactating intruders showed less frequently defen-

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sive behavior (Fig. 1A) and were less frequently attacked by the residents (Fig. 1B).

3.2. Serum corticosterone concentrations

Serum corticosterone concentrations were about twofold higher in confronted rats as compared to controls (non-lactating females: 455 ± 34 (confrontation) vs. 227 ± 27 ng/ml (control); lactating: 411 ± 29 vs. 258 ± 33 ng/ml) [*stress*: $F_{1,41} = 36.98$; $p < 0.001$]. Neither a difference between lactating and non-lactating rats [*lactation*: $F_{1,41} = 0.041$; n.s.] nor interaction between factors *stress* × *lactation* [$F_{1,41} = 1.44$; n.s.] was observed.

3.3. Stress- and lactation-induced changes in leukocyte and lymphocyte subsets

Several leukocyte and lymphocyte subsets were affected by either stress or lactation (Table 1). In general, more changes were observed in the blood than in the MLN.

Lymphocyte subsets: Blood lymphocyte numbers after confrontation were about 40% lower in both lactating and non-lactating rats [*stress*: $F_{1,41} = 16.73$; $p < 0.001$]. This is predominantly due to a reduction in the numbers of B cells [*stress*: $F_{1,40} = 54.04$; $p < 0.001$] and, to a lesser extent, of NK cells [*stress*: $F_{1,40} = 4.17$; $p < 0.05$]. Generally, lactating rats had lower blood B cell numbers [*lactation*: $F_{1,40} = 17.14$; $p < 0.001$]. In addition, in MLN a tendency towards higher NK cell numbers in lactating animals [*lactation*: $F_{1,48} = 3.22$; $p = 0.079$] was observed. T cell populations in both blood and MLN were neither affected by stress nor lactation. No significant interactions between the factors *stress* and *lactation* were observed.

Table 1
Effects of lactation and confrontation on numbers of immune cells in blood and MLN.

	Control		Confrontation	
	No lactation	Lactation	No lactation	Lactation
Blood cell populations [N/μl]				
Number of animals	10	13	10	12
Leukocytes	6555 ± 215	7354 ± 367	6780 ± 1075	5804 ± 612
Lymphocytes	4392 ± 351	3453 ± 411	2641 ± 384[a]	2215 ± 341[a]
Monocytes	886 ± 106	959 ± 83	887 ± 111	877 ± 73
Granulocytes	1277 ± 186[b,c]	2942 ± 252	3252 ± 605	2714 ± 296
T helper cells	1391 ± 194	1241 ± 191	1028 ± 196	1063 ± 217
T cytotoxic cells	432 ± 68	358 ± 55	316 ± 74	299 ± 80
NK cells	221 ± 16	221 ± 24	206 ± 43	157 ± 27
B cells	2348 ± 228	1634 ± 205[d]	1091 ± 133[a]	637 ± 84[a,d]
MLN cell populations [N/MLN]				
Number of animals	16	14	13	13
Leukocytes × 10^6	126 ± 15	109 ± 15	108 ± 12	133 ± 17
Lymphocytes × 10^6	117 ± 14	104 ± 12	106 ± 12	111 ± 13
Monocytes × 10^3	1637 ± 286	2424 ± 333[d]	1066 ± 142	2555 ± 426[d]
Granulocytes × 10^3	640 ± 106	536 ± 89	466 ± 107	562 ± 97
T helper cells × 10^3	36212 ± 5354	29938 ± 4190	35742 ± 6260	32442 ± 5045
T cytotoxic cells × 10^3	13560 ± 2653	12776 ± 2538	12446 ± 2557	11906 ± 2350
NK cells × 10^3	700 ± 100	1652 ± 670	588 ± 77	960 ± 218
B cells × 10^3	66110 ± 7498	59671 ± 6534	57624 ± 5320	65396 ± 7445

Data are given as mean ± SEM. Statistics: *t*-test with Benjamini–Hochberg correction; $p < 0.05$.
[a] Significantly different (at least $p < 0.05$) from control.
[b] Significantly different (at least $p < 0.05$) from all other groups.
[c] Interaction stress × lactation (at least $p < 0.056$).
[d] Significantly different (at least $p < 0.05$) from non-lactating group.

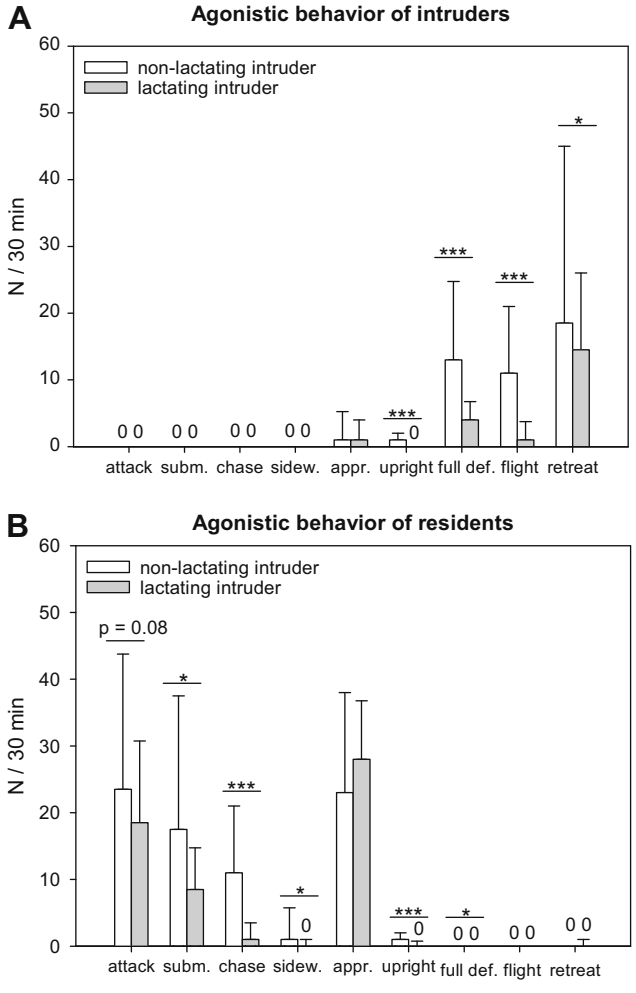

Fig. 1. Agonistic behavior of female resident and intruder rats. Non-lactating and lactating rats were exposed to resident–intruder confrontations for 2 h. Behavior was monitored for the first 30 min of confrontation. Results are given as median ± interquartile range. *subm.*, submissive; *sidew.*, sideway; *appr*, approach; *full def.*, full defense; $n = 34$ for each group; Statistics: Mann–Whitney *U*-test; $^{*}p < 0.05$; $^{***}p < 0.001$.

Granulocytes: In non-lactating animals, blood granulocyte numbers were more than 100% higher in stressed animals than in non-stressed controls [*stress*: $F_{1,41} = 6.04$; $p < 0.05$]. In contrast, lactating control animals had very high granulocyte numbers even before confrontation, but stress had no additional effect. This asymmetry resulted in a strong interaction of the factors *stress* and *lactation* [$F_{1,41} = 9.6$; $p < 0.01$]. The effect of *lactation* [$F_{1,41} = 2.51$; $p = 0.12$] alone did not reach the level of significance. There was no effect of *stress* and *lactation* on MLN granulocytes.

Monocytes: Stress had no effect on monocyte numbers in both the blood and the MLN. However, an up to twofold higher monocyte cell number was observed in MLN of lactating rats [$F_{1,48} = 31.65$; $p < 0.001$], while blood monocytes remained unaffected. A significant interaction of factors *stress* and *lactation* was not observed.

3.4. Effects of stress and lactation on lymphocyte proliferation in blood and MLN

Stress had a negative impact on ConA-induced proliferation of blood lymphocytes [$F_{1,32} = 9.42$; $p < 0.01$], resulting in an up to 60% decrease (Fig. 2A). In MLN, a slightly negative effect of stress on PWM induced lymphocyte proliferation (a decrease of about 10%) [$F_{1,48} = 5.57$; $p < 0.05$] (Fig. 2C) was evident. The effect on ConA-induced proliferation did not reach level of significance [$F_{1,48} = 2.43$; $p = 0.125$] (Fig. 2B and C).

Lactation had no effect on mitogen-induced proliferation of blood lymphocytes [$F_{1,32} = 0$; n.s.]. However, proliferation of MLN

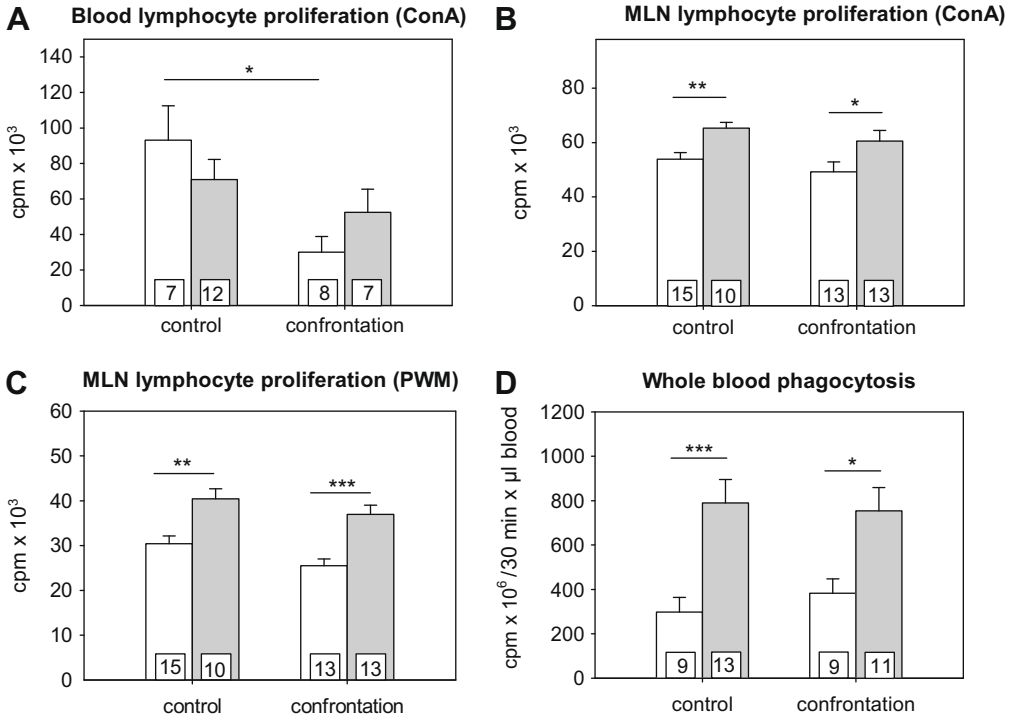

Fig. 2. Effects of lactation and confrontation on lymphocyte proliferation in blood and MLN and phagocytosis in whole blood. White bars, no lactation; gray bars, lactation. Data are given as mean ± SEM. Numbers of animals are indicated at the bottom of the bars. Statistics: *t*-test with Benjamini–Hochberg correction; $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

lymphocytes was higher in lactating animals in response to both PWM [$F_{1,48} = 32.49$; $p < 0.001$] and ConA [$F_{1,48} = 12.12$; $p < 0.01$] (Fig. 2B and C). No significant *stress* × *lactation* interaction was observed.

3.5. Stress- and lactation-induced effects on whole blood phagocytosis

No significant effect of stress on whole blood phagocytosis was observed [$F_{1,38} = 0.15$; $p = 0.7$] (Fig. 2D). Both control and stressed lactating animals had a substantially higher phagocytic capacity [$F_{1,38} = 22.88$; $p < 0.001$], resulting in up to more than twofold increase. No significant *stress* × *lactation* interaction was observed.

3.6. Effects of stress and lactation on cytokine production

IFN-γ production of blood lymphocytes was strongly reduced in stressed animals [*stress*: $F_{1,30} = 16.08$; $p < 0.001$] (Fig. 3A), reaching only one fourth of control levels. Lactation had no significant effect on IFN-γ-production, and no *stress* × *lactation* interaction was observed.

In contrast, an effect of stress on blood lymphocyte IL-2 production [*stress*: $F_{1,30} = 14.34$; $p < 0.01$] (Fig. 3B) could only be detected in non-lactating females [*stress* × *lactation*: $F_{1,30} = 4.97$; $p < 0.05$; *lactation*: $F_{1,30} = 2.7$; $p = 0.11$]. Lactating females had a very low IL-2 production, and no additional reduction in IL-2 production occurred in response to stressor exposure (Fig. 3B). In MLN, neither an effect of stress nor of lactation on cytokine production was noted (Fig. 3C and D).

3.7. Relationship between MLN immune parameters and corticosterone

Experimental sets involving MLN included both immune and endocrine measurements from the same individuals, allowing correlation analyses between corticosterone and the 12 MLN immune parameters determined in each of the four experimental groups. No significant relationships between serum corticosterone concentrations and MLN immune parameters were detected.

4. Discussion

In this study we demonstrate that lactation strongly affects the immune system in laboratory rats, but the impact on blood and MLN varies. In blood, a reduced mitogen-induced IL-2 production and lower B cell numbers were observed, while an enhanced lymphocyte proliferation was found in MLN. The present findings complement a previous study by Shanks et al. (1997) who reported reduced IL-2 production of splenocytes and increased proliferation of MLN lymphocytes during lactation. A low number of B cells and a decreased IL-2 production in blood and spleen correspond with clinical observations such as a reduced ability to mount antibody response or an increased susceptibility to infection (Drazen et al., 2003; Jäckel, 2003). However, based on the present data, one should not draw the conclusion that the immune system is generally suppressed during lactation: first, functionally active immune cells from the blood or spleen might migrate into other body compartments in lactating females. Thus, it cannot be ruled out that a decreased IL-2 production may be assessed in the blood while simultaneously IL-2 production in other body parts such as the intestines, breasts, or the reproductive tract is increased. Second, the increased number of phagocytes in the blood suggests an enhancement of at least some aspects of the innate immunity in the circulation. Third, lymphocyte activity is increased in MLN. During lactation, this enhanced activity appears to be especially beneficial in order to assure an adequate maternal–offspring antibody transfer. B cells from MLN travel to the breast, producing antibodies specific to gut pathogens (Head and Seelig, 1983; Roux et al., 1977). These antibodies (particularly IgA) enter the milk and

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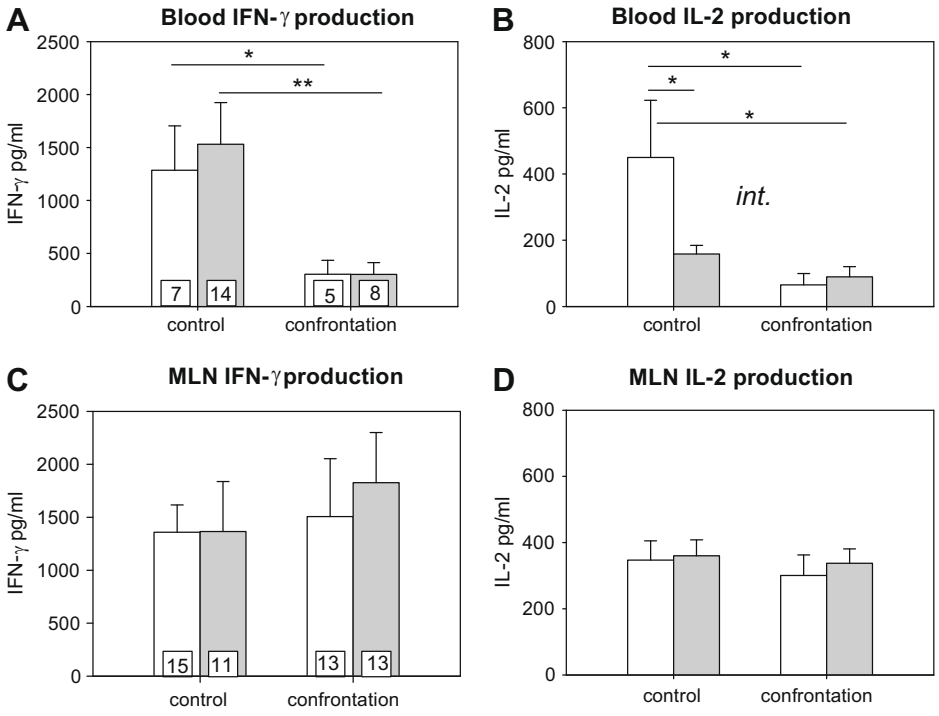

Fig. 3. Effects of lactation and confrontation on ConA-induced cytokine production in lymphocytes from blood and MLN. IFN-γ and IL-2 concentrations in cell culture supernatants. White bars, no lactation; gray bars, lactation. Data are given as mean ± SEM. Numbers of animals are indicated at the bottom of the bars. Statistics: *t*-test with Benjamini–Hochberg correction; $^{*}p < 0.05$; $^{**}p < 0.01$; *int.*, interaction *stress* × *lactation* ($p < 0.05$).

protect the offspring against gut pathogen-associated diseases (Lamm et al., 1978; Shanks et al., 1997).

It has been hypothesized in eco-immunological literature that a trade-off may exist between reproduction and immune activity (reviewed in Harshman and Zera, 2007). According to this concept, particularly during the energy demanding process of lactation (Hansen and Ferreira, 1986; Papworth and Clubb, 1995), a reallocation of resources from the immune system may be required to meet the costs of lactation (Coop and Kyriazakis, 1999; Demas et al., 1997; Jäckel, 2003). One problem with this concept is that maintenance of activity levels of resting immune cells does apparently not consume much energy (Krauss et al., 2001; Maciver et al., 2008). Moreover, the present data show that rather than being immunosuppressed, the immune system appears to be readjusted during lactation. This involves both reducing and enhancing effects in different body parts. More studies on the migration pattern of immune cells during lactation and the assessment of their activity levels in various body compartments are thus required before more generalized conclusions can be made.

Although the physiological mechanisms underlying the effects of lactation on the immune system were not the focus of this study, it should be mentioned that the diverse effects on immune cells in the blood and in the MLN might be caused by local concentration differences of hormones. Prolactin would be a potential candidate, as this hormone is known to inhibit lymphocyte proliferation at high concentrations while having enhancing effects at lower concentrations (Matera et al., 1992). High plasma prolactin concentrations during lactation (Arbogast and Voogt, 1996; Grattan, 2001) might therefore affect lymphocytes in circulation quite strongly. In addition, several studies describe a differential expression of prolactin receptor isoforms during lactation in lymphoid organs (Feng et al., 1998; Gunes and Mastro, 1997), which possibly also contributes to the discrepancies observed here.

Acute social stress in non-lactating female rats causes a well known pattern of changes in blood immune cells, which is characterized by granulocytosis, reduction of B cell numbers, and decreased lymphocyte proliferation (Stefanski, 1998; Stefanski and Engler, 1998; Stefanski and Grüner, 2006; Stefanski et al., 1996). The present study extends our limited knowledge on stress-induced alterations in female blood cytokine production. Reduced mitogen-induced IL-2 and IFN-γ production in the blood in concert with previous findings from spleen and popliteal lymph nodes (Brenner and Moynihan, 1997; Iwakabe et al., 1998; Shanks et al., 1997) suggest a shift from a T_h1 to a T_h2 cytokine response as observed during pregnancy (Saito et al., 2007). Glucocorticoids and catecholamines have been identified as key players in mediating stress-induced effects on blood immune cells. It is likely that the same basic hormonal mechanisms are also involved in affecting functional capacity, cytokine production, migration, and adhesion, as well as apoptosis of immune cells in lactating and non-lactating female rats (Dhabhar, 2002; Elenkov and Chrousos, 2002; Moynihan, 2003).

In contrast to blood, MLN were only marginally affected by acute social stress. This relative resistance of MLN lymphocytes to stress is in agreement with the literature, indicating that the effects in MLN are often less intense than the effects in blood or the spleen (Moraska et al., 2002; Nguyen et al., 2000; Shanks et al., 1997). Possibly, blood leukocytes are more directly exposed to glucocorticoids or catecholamines in the circulation, whereas effective concentrations could be lower in MLN. Furthermore, glucocorticoid receptor expression levels are known to differ between tissues (Miller et al., 1998) and may thus also be lower in MLN. However, to the best of our knowledge, there is no study on local hormone concentrations in MLN. Considering the relative resistance of MLN to stressor action it is also not surprising that correlations between serum corticosterone concentrations and measures of MLN immunity were absent in the present study.

Many effects of stress on the immune system are similar in non-lactating and lactating animals, but the present study also reveals a few important differences. One is that stress has no further impact

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on high granulocyte numbers and low blood IL-2 production in lactating females. It appears that there is a mechanism preventing a strong activation or suppression of some key immune functions. Certainly, a minimum capacity of lymphocytes to produce IL-2 is essential to maintain basic immune functions. The "ceiling effect" on granulocytes is not exclusive to lactation (Stefanski et al., 2005) and can also be observed in pregnant rats. Here it was argued that a limitation of neutrophile release into circulation prevents a self-destructive activation of innate immunity. One additional difference between non-lactating and lactating animals is a dampened effect of stress on ConA-induced T cell proliferation. A possible mechanism could be a resistance of lymphocytes to glucocorticoids, which has also been described in other contexts before (Sheridan et al., 2000).

In a report particularly relevant for the present study, Shanks et al. (1997) investigated the effect of conditioned stress on immune function in lactating Sprague–Dawley rats. Interestingly, although the present and previous studies indicate that lactation alters the effects of stress on immune function, the pattern of change and the parameter affected was not always uniform. In contrast to the aforementioned study we found a stress-buffering effect of lactation on blood lymphocyte proliferation, but no evidence for an increase in MLN lymphocyte proliferation in stressed lactating rats. Further discrepancies also exist with respect to corticosterone secretion. Plasma corticosterone concentrations in response to social stressors were similar in both lactating and non-lactating rats in the present study. This finding does not agree with several previous studies that observed a buffering effect of lactation on HPA responsiveness (Neumann et al., 1998; Shanks et al., 1997, 1999; Sibolboro Mezzacappa et al., 2003; Stern et al., 1973; Torner et al., 2002; Windle et al., 1997). Since only corticosterone concentrations were assessed in the present report, the possibility cannot be ruled out that other indices of HPA axis activity such as ACTH concentrations differ in lactating females. On the other hand, our findings of a similar corticosterone response in lactating and non-lactating rats do correspond well with a study in humans involving a psychosocial stressor (Trier social stress test), which also does not report an attenuated HPA response (cortisol and ACTH) during lactation (Altemus et al., 2001). The reasons for these discrepancies are not clear, but might be caused by methodological differences such as the nature and duration of stressor, the last time point of suckling, or the phase of lactation. It is also worthy of note that the condition of the pups (especially when stressed) may diminish the buffering of the corticosterone response in lactating rats (Smotherman et al., 1976, 1977). However, this sensitizing effect is unlikely to play a role in the present paradigm because mothers were not exposed to their pups during or after the acute social stressor exposure.

Lactating females less frequently displayed defensive behavior as compared to non-lactating intruders, which may lead to the assumption that the differential effect of social stress on the immune system in lactating females is related to behavioral differences. The reduced amount of defensive behavior in lactating animals may be due to reduced anxiety levels, as previously reported in other behavioral settings during lactation (Hansen and Ferreira, 1986; Hard and Hansen, 1985; Sibolboro Mezzacappa et al., 2003; Torner et al., 2002; Toufexis et al., 1999; Windle et al., 1997). Alternatively, a dampened resident aggressiveness may be caused by the specific odor of lactating and pregnant females aimed to protect themselves and the litter from conspecifics' aggression (Clegg and Williams, 1983; Kilpatrick et al., 1983; Mennella and Moltz, 1989; Moltz and Lee, 1981). In any case, lactating females may have perceived social conflict in confrontations as less threatening. This, however, was not reflected in reduced CORT secretion, but may have been associated with lowered sympathetic activation. Since catecholamines have major immuno-modulatory properties (Engler et al., 2004; Schedlowski et al., 1996; Stevenson et al., 2001), a specific role of these sympathetic mediators in lactating females should be addressed in the future.

In our study we used a social stressor that allows active coping behavior of the intruder and therefore represents a realistic picture of stressor-induced changes also occurring in natural environments. Lactation- and stress-induced changes of the immune system do not only affect the mother but might also directly influence the offspring via an altered transfer of maternal antibodies, which emphasizes the importance of lactation. In future studies, measurements of prolactin and IgA and also the study of other immune compartments such as spleen, lymph node areas other than MLN, or breast and reproductive organs would provide further useful information. In the present study, we investigated a time of lactation during which milk production is already well established and quite energy demanding for the mother. Nevertheless, a time course over the whole period of lactation would be of interest, since lactation-associated effects might differ at the beginning and towards the end of lactation (normal weaning starts at about day 20). Since the effects of acute and prolonged stressor exposure differ considerably, chronic social stress situations should also be taken into account. The present study nevertheless clearly demonstrates that an acute social stressor significantly alters both behavior and immune system during lactation. The findings contribute to an understanding of the complex interactions between the female reproduction system and a stressor.

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