Mild postnatal separation stress reduces repeated stress-induced immunosuppression in adult BALB/c mice

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Abstract

OBJECTIVES: Different inbred mouse strains but also each animal of the same strain show an individually different stress response which is influenced by genetic and environmental factors such as early life experiences. In this study, we investigated consequences of mild postnatal stress exposure on the stress coping style of adult BALB/c mice.

METHODS: We used a model of mild early life stress where neonatal mice were repeatedly separated from the dam staying with their siblings for 1-h each day during the first two postnatal weeks. The environment during maternal separation was adapted to the nest (bedding, 37 °C warm).

RESULTS: Adult female BALB/s mice that underwent the maternal separation protocol or were not isolated from the dam in early life were exposed to combined acoustic and restraint stress in adulthood. Repeated maternal separation which was performed under ambient conditions increased the stress coping ability of mice at the age of 12 weeks when exposed to this psychological stressors. By acoustic and restraint stress-induced alterations such as high corticosterone levels, an anti-inflammatory immune conditioning with an ex vivo hyperinducibility of interleukin-10 of splenocytes and a massive loss of body weight were significantly reduced in the maternally separated group compared with conventionally bred control mice.

CONCLUSIONS: Mild maternal separation in early life modifies the stress coping style of adult female BALB/c mice to a more stress-resistant phenotype which shows reduced repeated stress-induced immune suppression and weight loss and is linked to reduced release of glucocorticoids after stress exposure.

ORIGINAL ARTICLE

Abbreviations :

ACTH	- adrenocorticotropic hormone
ANOVA	- analysis of variance
BW	- body weight
CD	- cluster of differentiation
Co	- control
CRF	- corticotropin releasing factor
DN	- CD4- and CD8-douple negative
DP	- CD4- and CD8-douple positive
FITC	- fluorescein isothiocyanate
GCs	- glucocorticoids
GR	- glucocorticoid receptor
HPA axis	- hypothalamus-pituitary-adrenal axis
IL-10	- interleukin-10
LPS	- lipopolysaccharide
mRNA	- messenger ribonucleotid acid
MS	- maternal separation
PE	- phycoerythrin
PerCP	- peridinin-chlorophyll-protein
SSS	- stress severity score
TNF-α	- tumour necrosis factor-alpha

INTRODUCTION

Neuroendocrine, immunological and behavioral responses to psychosocial stress display striking individual variation. Some individuals tend to develop mood disturbances or even depression or anxiety disorders under chronic stressful conditions whereas others don't show significant alterations due to comparable stressful events (Boyce, 2004; Heim & Nemeroff, 2001; Heim *et al.* 2008; Rutter, 2003; Yu *et al.* 2008). The mechanisms which are responsible for the development of such different coping phenotypes are poorly understood.

Early life experiences essentially modulate the development of central neuronal circuits which translate the behavioral and endocrine response to stressful stimuli through-out the whole life-span. During this process the brain is conditioned to respond to stressful situations. This determines the stress coping abilities during adult life and as a consequence, the susceptibility to stress-associated illnesses, such as cardiovascular disease, diabetes, and depression (Boyce, 2004; Heim & Nemeroff, 2001; Meaney, 2001; Pryce *et al.* 2002).

The influence of maternal separation and maternal care on the development of neuroendocrine and immunological responsiveness was intensively studied in several rodent models. There is high consent that longer periods of maternal deprivation ranging from 1.5–12 hours during the first 7 to 21 postnatal days have deleterious consequences including increased basal glucocorticoid (GC) levels, heightened ACTH and corticosterone responses to stress, impaired gain of body mass and behavioral alterations during adolescence (Colorado *et al.* 2006; Huot *et al.* 2004; Ladd *et al.* 2005; Ryu *et al.* 2008; Slotten *et al.* 2006; Yamazaki *et al.* 2005). In contrast, short term maternal separation seems to be associated with the development of a stress resistant phenotype in adulthood (Macri *et al.* 2007; Meaney, 2001; Pryce *et al.* 2002; Tejedor-Real *et al.* 2007) which essentially depends on the intensity of maternal care. Short-term intermittent separation stimulates maternal licking and grooming after bringing the pups back to the dams. Interestingly, repeated 15–20 min handling periods of pups which included maternal separation, increases maternal care which coincides with a reduced stress-induced hypothalamus-pituitary-adrenal (HPA) axis responsiveness in later life which was associated with diminished behavioral alterations after stress exposure (Liu *et al.* 1997; Meaney, 2001; Pryce *et al.* 2002).

Thus, the duration and quality of postnatal stressors as well as the maternal care seem to account for the development of different stress coping phenotypes in later life.

We recently showed that adult female BALB/c mice that were bred under these conditions showed an increased HPA axis activity, hypercatabolism, depression-like behavioral alterations, systemic immunosuppression and impaired antibacterial defense in response to repeated psychological stress compared with non-stressed mice (Depke *et al.* 2008; Depke *et al.* 2009; Kiank *et al.* 2007a; Kiank *et al.* 2006). Interestingly, behavioral alterations measured by an observational stress severity score (SSS) were strongly correlated with the strength of stress-induced HPA axis responsiveness, the degree of immunosuppression indicated by the *ex vivo* inducibility of IL-10 after LPS stimulation of splenocytes, and the bacterial load in blood and liver after experimental infection with *E. coli* (Kiank *et al.* 2006). Although all mice were from the same genetic background and were bred under classical laboratory conditions in the same acclimatized animal breeding facility, we found individual differences of stress susceptibility which have yet to be explained.

Here, we asked whether maternal separation of siblings in an ambient environment for one hour each day during the first 14 postnatal days can alter the stress susceptibility to prolonged intermittent psychological stress in adult BALB/c mice.

MATERIAL AND METHODS

Maternal separation

From postnatal day 1 to day 14 all female and male siblings of the offspring of one BALB/c mother (group size 6–8 mice) maternally separated for 1-h each day between 9:00–11:00 AM. During the separation period, pups stayed within their sibling groups and were placed in bedded bowls layered on a 37 °C warm water bath so that the temperature never declined below the nest temperature of 30–35 °C. After 1-h of isolation, siblings were immediately re-placed to their home cages with their mothers. At postnatal day 21 pups were weaned.

Because of a restricted number of animals allowed to be used and of the fact that most of our previous studies were performed with female mice, we decided to selec-

tively analyze the stress responsiveness of adult female BALB/c also in further experiments. Four weeks prior to stress experiments the female mice were arranged into the experimental groups (9 mice/cage) and adapted to the experimental room where they remained under minimal stressful conditions on a 12:12 light/dark cycle with standardized food and tap water *ad libitum*. All animal procedures were approved by the Ethics Committee for Animal Care, Mecklenburg-Vorpommern, Germany.

Repeated psychological stress model

At the age of 12 weeks the female BALB/c mice were exposed to combined acoustic and restraint stress for 4 successive days, 2-h twice a day (8:00–10:00 AM., 4:00–6:00 PM) and a single morning stress session on day 5 as described previously (Kiank *et al.* 2006). Non-stressed control mice were kept in their familiar environment (n=9/group) without getting acoustic, olfactory and visual contact to stressed animals. All successive analyzes were performed starting at 10 AM after the ninth stress exposure.

Determination of body mass

Body weight was determined between 9:00–11:00 AM at the age of 8, 10 and 12 weeks and after the last stress session.

Harvesting of blood and organ samples

Immediately after the ninth stress session, starting at 10 AM, mice were anesthetized with 75 µg/g BW Ketamin Curamed® (CuraMED Pharma GmbH, Karlsruhe, Germany) and 16 µg/g BW Rompun® (Bayer AG, Leverkusen, Germany). Blood was harvested by orbital puncture in EDTA containing tubes. The spleen and thymus harvested into VLE RPMI 1640 medium (Biochrom KG Berlin, Germany). Single cell suspensions were prepared within 1-h. Blood cells were counted with a hemocounter (Sysmex K-4500, Sysmex GmbH, Norderstedt, Germany).

Measurement of ex vivo inducibility of cytokines

Ex vivo cytokine inducibility was measured as described recently (Kiank *et al.* 2008; Kiank *et al.* 2006). In brief, splenocytes were adjusted to 1×10^7 cells/ml using complete VLE RPMI 1640 medium (Biochrom KG, Berlin, Germany). For *ex vivo* cytokine induction in the splenocyte cultures 1 µg/ml LPS from *S. abortus equi* (Sigma, Steinheim, Germany) or CD3-antibodies (5 µg/ml) and CD28-antibodies (1 µg/ml) – both from BD Pharmingen (Heidelberg, Germany) were used. Cells were incubated for 42-h at 37 °C and 5% CO_2. Supernatant was harvested and immediately stored at –20 °C until further processing. Cytokine concentrations were analyzed by Cytometric Beads Array following the manufacturer's protocol (Mouse-Inflammation-Kit, CBA™, BD Pharmingen). Among other cytokines concentrations of TNF-α and IL-10 were quantified.

Phenotyping of thymocytes

Single cell suspensions of thymocytes (5×10^6 cells/ml) were incubated with monoclonal antibodies: anti-mouse-CD4-FITC, anti-mouse-CD8a-PE or relevant isotype control antibodies; apoptotic cells were detected by AnnexinV-Biotin and streptavidin-PerCP counterstaining. All reagents for cell staining were purchased from BD Pharmingen. Thymocyte populations and apoptotic cells were quantified by flow cytometry (FACScan, BD). FACS-data were analyzed with WinMDI 2.8 (free shareware, internet).

Measurement of corticosterone in the plasma

Plasma corticosterone-levels were quantified by ELISA according to the instructions of the supplier (OCTEIDA Corticosterone EIA, IDS, Boldon, UK).

Statistical analysis

Statistical analysis was carried out with GraphPad Prism Version 5 for Windows (GraphPad Software Inc., San Diego, USA). Differences between samples of stressed and non-stressed mice were analyzed by Mann-Whitney test or by Bonferroni multiple comparison test after one-way ANOVA when multiple groups were compared. The data are depicted as box plots expressing median ± limits, $p<0.05$ was considered statistically significant.

RESULTS

Maternal separation for 1-h each day within the first 14 postnatal days did not cause visible behavioral alterations or signs of illness of the pups during the isolation period or in the home cage. Although we did not performed detailed measurements of the maternal behavior, we noticed increased nursing when placing the pups back into the home cages after separation. This was associated with increased licking, intensive grooming, nest building and heightened arched-back nursing. However, these subjective observations need to be confirmed in further experiments that quantify the maternal behavior.

At 3-month age, postnatally separated mice did not significantly differ in basal corticosterone levels, lymphocyte numbers, and *ex vivo* cytokine inducibility of splenocytes compared with conventionally bred animals (Figure 1–4). All animals gained body weight during adolescence; however, the body weight of maternally separated mice was constantly higher at the age of 8–12 weeks (Figure 1A–C). Moreover, maternally separated animals did not show the typically loss of about 10% of body mass after repeated psychological stress as conventionally bred mice did (Figure 1D).

At 12 weeks age, after narcosis and blood drawn, corticosterone concentrations were by tend reduced in the postnatally separated group (40.6 ± 38.9 ng/ml) compared with conventionally bred mice (136.9 ± 66.9 ng/ml).

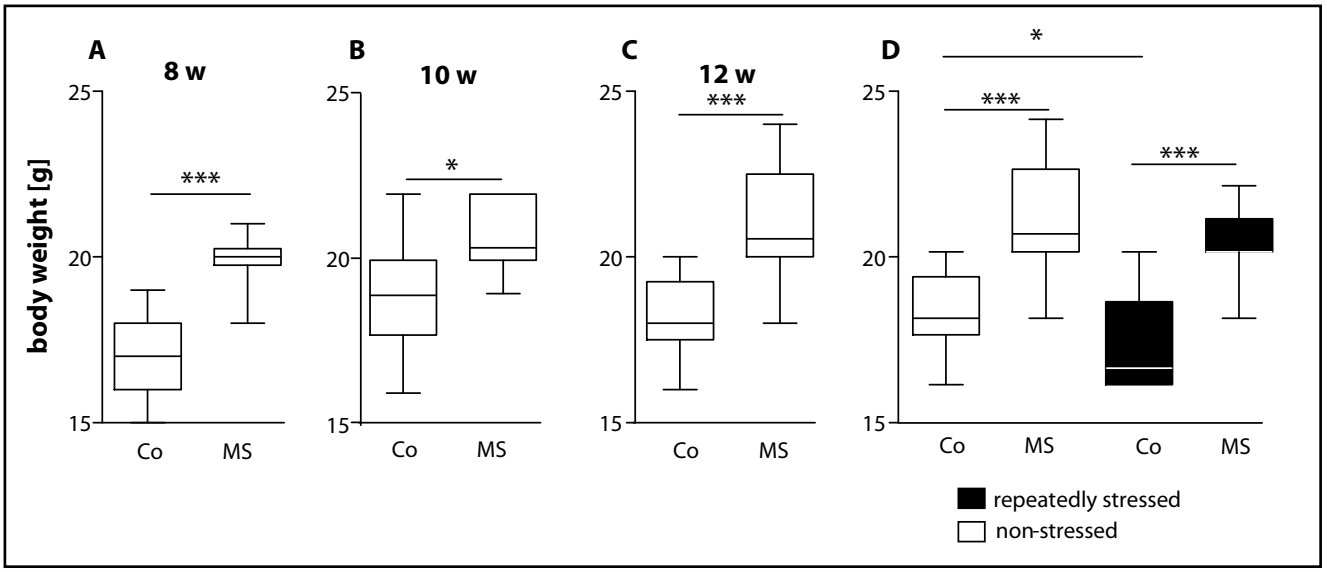

Fig. 1. Changes of body weight during adolescence and psychological stress. A–C. Body mass of maternally separated (MS) and conventionally bred controls (Co) at the age of 8 (A), 10 (B), and 12 weeks (C) and D. prior to starting the stress experiments (white box plots) and immediately after 4.5-days of stress exposure (black box plots), n=12 mice/group, summary of two independent experiments giving comparable results. *p<0.05, ** p<0.01, *** p<0.001 A–C, comparison by Mann-Whitney test, D, comparison by one-way ANOVA and Bonferroni correction.

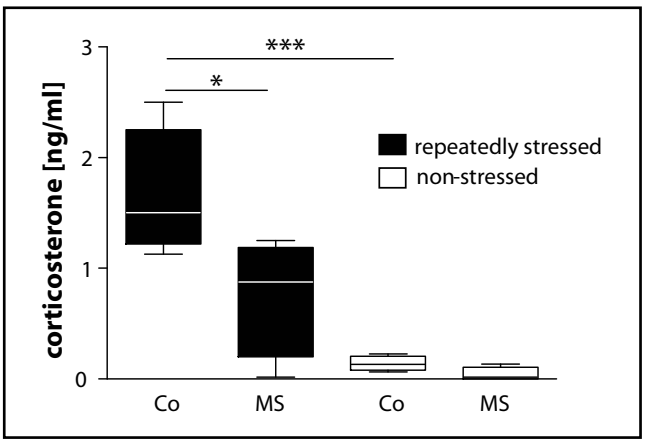

Fig. 2. Effects of mild separation on glucocorticoid response during repeated psychological stress. Plasma corticosterone levels of mice that were exposed to maternal separation stress (MS) and of controls (Co) in early life and that underwent stress exposure (black box plots) or remained untreated (white box plots) at age of 12 weeks., n=6 mice/group, one representative of two experiments is shown. *p<0.05, ** p<0.01, *** p<0.001 by Bonferroni multiple comparison test after one-way ANOVA.

As in previous studies, we found increased plasma corticosterone levels mediating thymocyte apoptosis and altered lymphocyte differentiation in conventionally bred mice that were exposed to repeated combined acoustic and restraint stress (Kiank *et al.* 2006). Postnatally separated mice, instead, showed an attenuated stress-induced HPA axis response in adulthood (Figure 2) and consistently, reduced stress-induced disturbances of thymocyte development (Figure 3A,B) and diminished $CD4^+$ thymocyte apoptosis after the 9^{th} acoustic and restraint stress session (Figure 3C).

In addition, it is known that conventionally bred BALB/c mice display an anti-inflammatory immune conditioning when they are repeatedly stressed (Depke *et al.* 2009; Kiank *et al.* 2007a; Kiank *et al.* 2006). This may also be influenced by mild postnatal stress and was analyzed in this study.

First, splenocytes were e*x vivo* stimulated with CD3/CD28 antibodies. Both postnatally separated and non-separated mice developed a stress-induced anti-inflammatory cytokine bias with a by trend reduced stress-induced IL-10 hyperinducibility in the deprived group (data not shown). This created a TNF-α /IL-10 ratio of 0.7268 ± 0.1251 in stressed maternally separated mice, of 0.4125 ± 0.2798 in stressed conventionally bred mice compared with 1.051 ± 0.3961 in maternally separated and 1.605 ± 0.9196 and control bred mice that were not stress (1-way ANOVA: p=0.0195, F=4.708, n=6 mice/group). Only the non-separated stressed group showed a significant reduced TNF-α /IL-10 ratio compared to the conventionally bred controls (p<0.05, in Bonferroni post-testing).

Secondly, we used LPS to stimulated cytokine secretion in splenocyte cultures. In line with our previous data, repeated stress exposure reduced the *ex vivo* inducibility of TNF-α but enhanced the release of IL-10 in conventionelly bred mice (Figure 4A,B). However, animals that underwent the postnatal mild separation protocol did not show this stress-induced inhibition of TNF-α release (Figure 4A) and no hyperinducibility of IL-10 (Figure 4B). Thus, repeatedly separated offspring became resistant to stress-induced loss of immune cells and anti-inflammatory conditioning of the immune response in adulthood.

DISCUSSION

In mammalian species the interaction of the primary caregiver and the offspring is important for a healthy development. Many studies showed that a disruption in this relationship alters HPA axis activity, impairs growth as well as cognitive development and memory formation. This may induce behavioral and emotional pathologies and thus, alter the vulnerability for stress-associated diseases (Francis *et al.* 2002; Ladd *et al.* 2005; Liu *et al.* 1997; Meaney, 2001). This is supported by several investigations in mammals showing that chronic stressful experiences in early life such as repeated maternal deprivation for more then 3-h a day or severe social stressors alter the development of appropriate stress coping skills and, in consequence, heighten the risk of health problems in adulthood (Avitsur *et al.* 2006; Colorado *et al.* 2006; Slotten *et al.* 2006; Ward *et al.* 1998).

In our experiments, repeated maternal separation, however, improved coping with repeated psychological stress in adulthood: It resulted in reduced GC levels and diminished immune suppression compared with conventionally bred female BALB/c mice after multiple acoustic and restraint stress sessions which opposes several other studies (Francis *et al.* 2002; Ladd *et al.* 2005; Levine & Mody, 2003; Slotten *et al.* 2006; Yamazaki *et al.* 2005).

Environmental conditions during the separation period seem to determine the maternal separation-induced conditioning of later life stress responsiveness. In our experiments, the daily 1-h maternal isolation time was relatively short. Pups remained in their sibling groups during the separation and were put into a warm ambient environment mimicking the temperature of the nest. Our findings are supported by Yamazaki *et al.* who showed that daily maternal deprivation for 3 or 12-h performed during the first postnatal week at a temperature at 37 °C and additional dummy littering during the separation period effectively prevented stress-induced hypercortisolism in adult Wistar rats (Yamazaki *et al.* 2005). In addition, Huot *et al.* demonstrated that foster littering during daily 3-h maternal deprivation for 14 days after birth attenuated HPA axis responsiveness in adult Evans Long rats (Huot *et al.* 2004).

Under laboratory experimental conditions investigators aim to standardized environmental factors such as temperature, humidity, ventilation or social structures in the home cage to reduce variability between animals of the same experimental group and between different studies (Castelhano-Carlos & Baumans, 2009). In nature, however, there are physiological stressors. One of these natural stressors is short-term separation of the offspring from their parents which is necessary because the dam needs foraging to replenish energy reservoirs (Johnson *et al.* 2001; Macri *et al.* 2007; Ward

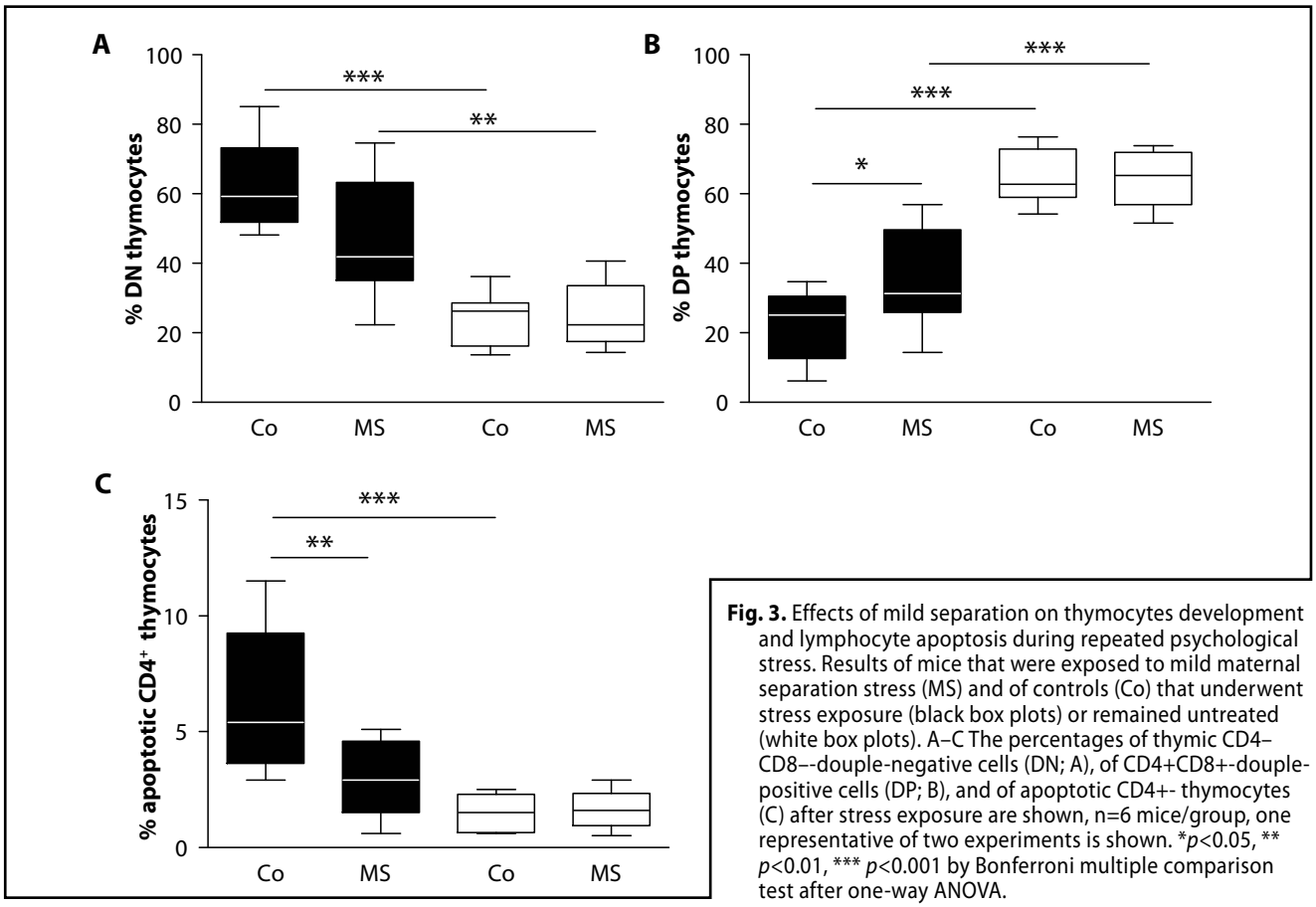

Fig. 3. Effects of mild separation on thymocytes development and lymphocyte apoptosis during repeated psychological stress. Results of mice that were exposed to mild maternal separation stress (MS) and of controls (Co) that underwent stress exposure (black box plots) or remained untreated (white box plots). A–C The percentages of thymic CD4–CD8–-douple-negative cells (DN; A), of CD4+CD8+-douple-positive cells (DP; B), and of apoptotic CD4+- thymocytes (C) after stress exposure are shown, n=6 mice/group, one representative of two experiments is shown. *$p<0.05$, ** $p<0.01$, *** $p<0.001$ by Bonferroni multiple comparison test after one-way ANOVA.

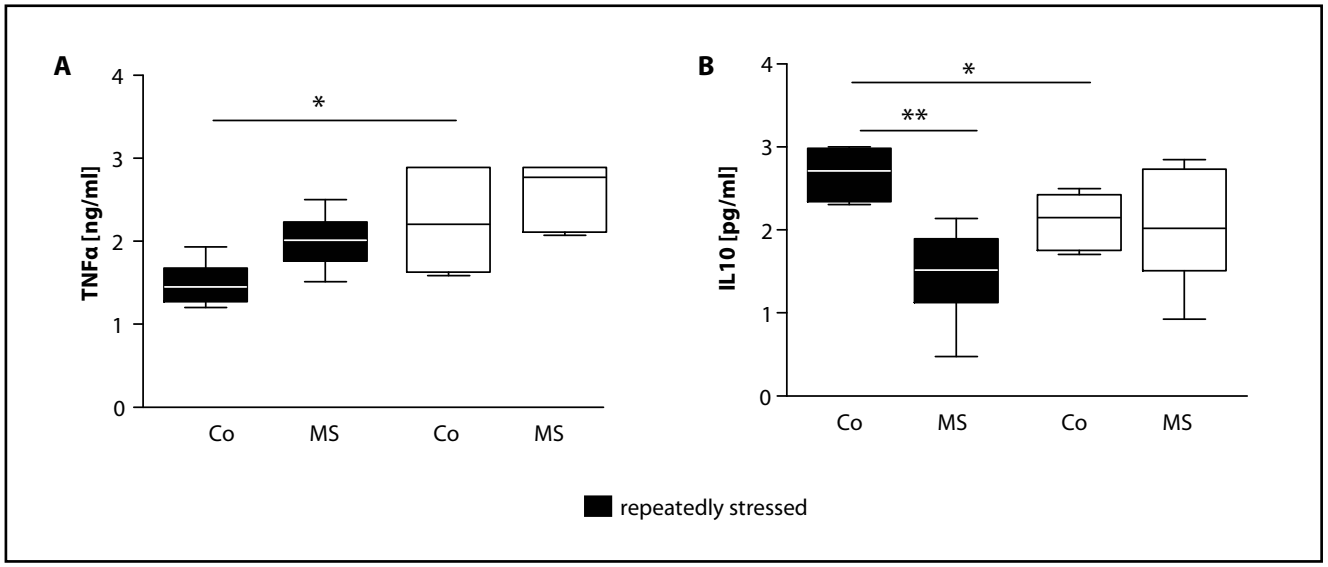

Fig. 4. *Ex vivo* cytokine inducibility after repeated psychological stress. A,B. Cytokine concentrations in the supernatant of LPS-stimulated splenocyte cultures derived from mice that underwent maternal separation stress in the postnatal period (MS) and non-deprived controls (Co) that were repeatedly stressed (black box plots) or non-stressed (white box plots). The concentration of tumour necrosis factor-alpha (A), and interleukin-10 (B) is shown. n=6 mice/group, one representative of two experiments is shown. *$p<0.05$, ** $p<0.01$, *** $p<0.001$ by Bonferroni multiple comparison test after one-way ANOVA.

et al. 1998). Laboratory animals which are bred under constant environmental conditions with supply of food and water do not need to pause care for their offspring (Balcombe *et al.* 2004).

Thus, our results suggest that conventional breeding of laboratory animals is a non-physiological process lacking environmental challenges which creates a high stress susceptible phenotype in adulthood which is blunted by early life mild stress of maternal separation.

The idea that mild and short-term stress in the 3-week postnatal period is protective rather than detrimental is supported by findings that repeated brief handling which includes short-term maternal separation lasting for 10–15 min/day dampens the stress sensitivity of the HPA axis, reduces pain perception, diminishes behavioral alterations such as novelty preference, and increases resistance to pathogen infection in several rodent models (Balcombe *et al.* 2004; Escorihuela *et al.* 1995; Francis *et al.* 2002; Macri *et al.* 2007; Meaney, 2001; Morley-Fletcher *et al.* 2003; Tejedor-Real *et al.* 2007).

This is confirmed in studies in rats where it was shown that environmental enrichment such changing spaces in the cage, several types of stairs, ropes, tunnels and exercise wheels, novel objects from weaning to adulthood, reduced the gain body weight during adolescence, lowered corticosterone levels in response to mild-stress acute exposure, enhanced exploration and novelty seeking behaviors, improved learning of complex tasks and enhanced long-term memory (Pena *et al.* 2009). In addition, housing under such enrichment conditions with toys after weaning compensated the effects of the maternal deprivation on stress reactivity as shown in rats that were maternally separated for 3-h a day during the first 15 postnatal days and enrichment housing prevented that a HPA axis hyper-responsiveness to 20-min restraint stress developed compared with daily 15-min neonatal handled rats (Francis *et al.* 2002; Pena *et al.* 2009). Interestingly, the deprived animals that stayed in the enriched environment showed sustained increased CRFmRNA but reduced GRmRNA expression in the brain as typically found after maternal deprivation indicating that other systems then the central HPA axis reactivity are also important in determining the stress reactivity in adulthood. Finally, communal nesting of mouse pups as a model of early social enrichment has beneficial effects on the development on adult social competence, higher grooming, increased offensive behavior, improved learning and memory building (D'Andrea *et al.* 2007).

Thus, mild stressors during adolescence under laboratory conditions are not essentially detrimental but can support the individual development of social and for stress coping abilities. In our study, 1-h isolation together with the siblings may have copied "natural" maternal separation stress which seems to be beneficial for the development of a more stress-resistant phenotype showing reduced alterations after repeated stress exposure.

Another important factor for the development of the more stress-resistant phenotype in our experiments may be due to the fact that dams of deprived offspring showed rich nursing behavior after separation. Unfortunately, we did not quantify these changes in maternal behavior so that our observations remain to be subjective and need confirmation in further experiments.

However, other authors showed that the quality of maternal care can profoundly modulate HPA regulatory circuits until adulthood. Liu *et al.* recently demonstrated that repeated short-term maternal separation of Norway rats was followed by increased maternal care when pups were brought back into the home cage. Importantly, increased frequency of maternal licking and grooming of deprived pups was correlated with lowered HPA axis responsiveness to stressors in adulthood (Liu *et al.* 1997). This was mediated by a reduced corticotropin-releasing factor (CRF) and increased glucocorticoid receptor (GR) mRNA expression in the brain, indicating a strengthened feedback-regulation of the HPA axis. Glucocorticoids (GCs) have a high potency to induce apoptosis and immunosuppression, thereby modulating the immune response (Lundberg, 2005; McEwen, 2004). Thus, a diminished HPA axis activation after repeated stress exposure may be accountable for reduced stress-induced alterations of immune cell functions. Indeed, in our study, repeated mild separation stress which was associated with lower stress-induced GC levels also attenuated psychological stress-induced impaired thymocyte development and lymphocyte apoptosis in adulthood. Moreover, separated animals did not respond with an increased anti-inflammatory cytokine bias which has been typically found in conventionally bred stressed BALB/c mice (Kiank *et al.* 2008; Kiank *et al.* 2007a; Kiank *et al.* 2006, Kiank *et al.* 2007b).

Finally, we showed that the reduced HPA axis responsiveness in maternally separated mice was associated with increased gain of body weight during growth. BALB/c mice that are highly susceptible to stress typically lose body weight due to GC-induced hypercatabolism (Depke *et al.* 2008). Recently, it was shown that social isolation during maternal separation of daily 180 min during the first two weeks after birth promoted hyperphagia and gain of body weight during adolescence but maternal separation alone did not affect food intake (Ryu *et al.* 2008). Others show that the consumption of food of female rats during rebound hyperphagia increases when the animals were maternally separated in the postnatal period whereas male rats did not show an altered food intake (Iwasaki *et al.* 2000) which indicates a significant role of gender. The underlying mechanisms of such eating behavioral alterations need further investigations.

Finally, besides many rodent studies, it was shown that also primates develop different stress coping phenotypes due to stress in early life. As in mice, moderate stress exposure in childhood seems to increase the stress resistance in squirrel monkeys which is characterized by lower basal ACTH levels, reduced stress-induced corticotropin and cortisol release, increased food intake as well as with reduced anxiety and reduced exploratory behavior in novel environments (Parker *et al.* 2004; Pryce *et al.* 2002). Another study using chimpanzees showed that early childhood trauma by separating young animals from their mothers in Africa and transportation to Europe for pharmacological testing induced general "helplessness" in adulthood which is characterized by reduced explorative behavior, aggressiveness, less social abilities and showed a higher stress response due to transportation, during habituation and resocialization (Reimers *et al.* 2007). Thus, the effect of maternal deprivation seems to be comprehensive for many mammalian species and very pronounced in primates.

Overall, our data indicate that moderate stress such as short lasting repeated separation of offspring from the dam which occurs typically in the wild results in an increased coping ability compared with conventionally bred laboratory animals. We conclude that conventional breeding conditions increases the stress vulnerability in laboratory animals compared to wild species. Mild stress in the early postnatal period seems not be essentially harmful, but rather adjust the offspring to stressful challenges by conditioning the HPA axis responsiveness towards a more stress resistant phenotype. This protects them from the development of adverse stress effects such as immunosuppression and depression by preventing an exaggerated release of glucocorticoids.

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