

The Effect of Early-Life Stress on Airway Inflammation in Adult Mice

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Key Words

Asthma • Airway inflammation • Early-life stress •
Maternal separation • Mouse • Psychological stress

Abstract

Background/Aims: Neonatal stress induces permanent physiological changes that may influence the immune system. Early-life stress increases asthma disease severity in children. We investigated the effects of early-life stress on allergic airway inflammation using a murine model of asthma coupled to maternal separation as an early-life stress stimulus. **Methods:** Maternally separated (MS) and unseparated control (CON) mice were sensitized with ovalbumin (OVA) beginning at day 31 after birth. **Results:** Challenging mice with OVA increased airway hyperresponsiveness (AHR) and the number of inflammatory cells recovered in the bronchoalveolar lavage (BAL), compared to saline-challenged mice. Challenging MS mice with OVA resulted in less total inflammatory cells, eosinophils, interferon- γ , and interleukin-4 in BAL compared to CON mice. However, MS mice challenged with OVA exhibited AHR similar to CON mice challenged with OVA. In contrast, an enhanced stress protocol (MS+) involving removal of pups from their home cages following the removal of the dam resulted in inflammatory cell accumulation and cytokine levels in the BAL similar to CON mice and higher than MS mice. **Conclusions:** These findings indicate that the effect of early-life psychological factors on

the development of airway inflammatory diseases such as asthma is very complex and depends on the quality of the psychological stress stimulus.

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Introduction

Epidemiological studies have shown that the mental health of asthmatic children is an important predictor of asthma morbidity [1] and is linked to asthma mortality [2, 3]. Psychological distress in children is associated with asthma that is more difficult to manage [4], with increased frequency and length of hospital admissions [5] and with functional disability [6]. Similar studies linking psychological morbidity to asthma mortality in children have been conducted in New Zealand [3], and the United States [2], and in adults in England [7]. In addition, the presence of chronic stressors leads to more acute and severe asthma attacks [8, 9].

Clinical observations also point towards a detrimental effect of early-life stress on the development of allergy and asthma. Klennert et al. [10] showed that in 150 families with a genetic susceptibility to asthma, only 5% of the children in low-stress households had asthma, but this doubled in high-stress families and increased fivefold in high-stress families with bad parenting. In addition, early-life stress may increase the prevalence of atopy [11].

Higher stress levels in children 6–18 months of age correlated with increased total IgE levels [12] and increased caregiver stress correlated with increased infant wheezing [13]. Compounded with other evidence, this correlation between stress and asthma severity has led to a call for early treatment of childhood depression [14].

Stress early in life can induce permanent changes in the hypothalamic-pituitary-adrenal axis of a child, resulting in chronic increases in circulating corticosteroid levels [15]. Since elevated corticosteroid can inhibit cell and tissue growth, newborns with early-life stress may have lower weights [15–17], which may lead to underdeveloped lungs and incomplete alveolar septation. In addition, since stress early in life appears to cause hypersensitivity to chronic stress later in life [15], those with asthma may also have increased airway inflammation and a worsening of the asthma phenotype.

Neonatal psychological stress has been shown to induce permanent physiological, psychological, and neurological effects [18–21]. One example is a rat model, where the effects of early-life stress include the permanent alteration of colonic epithelial barrier function – resulting in bacterial invasion, increased neutrophil and mast cell infiltration into the gastrointestinal tract, and an increased vulnerability to colitis [18]. Despite such evidence for the gastrointestinal tract, the effects of early-life stress on the respiratory system have not been studied in depth. Well-designed animal studies are needed to better understand the mechanisms through which early-life stress impacts asthma. Animal models that combine early-life stress and asthma or allergic airway inflammation will allow us to better understand the factors that enhance the development and severity of atopy and asthma in children.

In this study, we investigated the effect of early-life stress on allergic airway inflammation using a murine model that combines maternal separation as the early-life stress stimulus and allergic sensitization to chicken egg ovalbumin (OVA).

Materials and Methods

Animal Handling

Pregnant Balb/c mice that were timed to give birth on the same day were obtained from the Health Sciences Laboratory Animal Services breeding facility at the University of Alberta, on day 14 of gestation. Locally bred mice were chosen to minimize the stress caused by shipping pregnant animals. These pregnant dams were housed one per cage in a virus-antibody-free environment, maintained on a 12-hour light/12-hour dark cycle, and supplied with food and water ad libitum. Dams were checked daily until the

birth of the litter. All experiments and procedures were approved by the University of Alberta Animal Care Committee in accordance with the guidelines of the Canadian Council on Animal Care.

Maternal Separation

At birth, litters (with dams) were randomly assigned to one of two groups: maternal separation (MS) or unhandled controls (CON). MS dams were removed from their cages for 3 h daily, at the same time each day (between 1:00 and 4:00 p.m.), for the first 10 days after the birth of the litter. During separation the dams were placed individually in clean cages, in a separate room away from their pups with water and food ad libitum. The original cages with the pups were placed on a circulating water heating pad maintained at a constant 38°C. CON litters were left undisturbed during this period.

In some experiments, an alternate maternal separation protocol (MS+) was used. In this case, immediately after the removal of the dam, the pups were individually removed from their home cage and placed together into a separate clean cage for the duration of the separation. The cages containing the pups were placed on a circulating water heating pad maintained at 38°C. After the 3-hour separation period, the pups were returned individually into their home cage, followed by the return of the dam.

To minimize handling-induced stress, cages of MS, MS+ and CON litters were changed for the first time on day 10 after birth, immediately after the final maternal separation. After weighing and weaning on day 21, all animals received food and water ad libitum, and cages were changed weekly until the end of the experiments.

Lung Morphometry

MS and CON animals were euthanized on day 30 with an intraperitoneal injection of 200 µl of a 50% v/v ketamine (100 mg/ml) and Rompun (20 mg/ml) solution. Dissection of the abdomen was carried out to puncture the diaphragm, which deflates the lungs, and the inferior vena cava was severed to drain blood. A tube was then inserted into the trachea, and a suture was used to form a tight seal. The lungs were filled with 10% formalin under 25 cm H₂O pressure maintained for 5 min to allow fixation. The trachea was tied off, and the lungs were removed from the chest cavity and immersed in 10% formalin for 24–36 h. The left lung was removed, embedded in paraffin, sectioned, and stained with hematoxylin and eosin. Alveolar structures were quantified on a motorized microscope stage using the mean linear intercept (MLI) method [22].

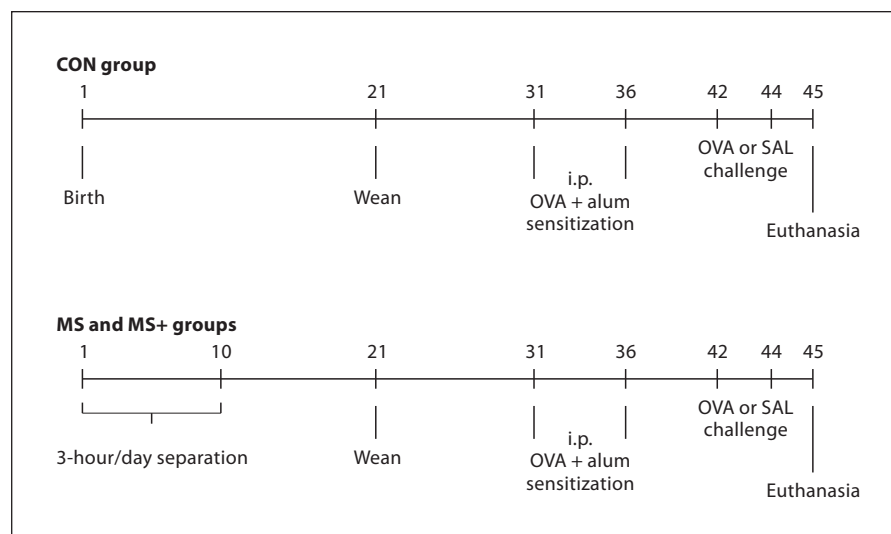
Immunization and Airway Challenge with OVA

On days 31 and 36, animals were sensitized via intraperitoneal injection of OVA (10 µg) and Al(OH)₃ (alum; 2 mg) suspended in 500 µl of 0.9% saline. On days 42 and 44, mice were challenged intranasally with either 50 µg OVA in 25 µl of 0.9% saline (OVA/OVA group) or 25 µl of 0.9% saline (OVA/SAL group). For the intranasal challenge, the mice were anesthetized with an intraperitoneal injection of 200 µl of a solution of ketamine (7.5 mg/ml) and acepromazine (0.25 mg/ml).

Airway Hyperresponsiveness

On day 45 (the day after the final challenge with OVA or SAL), airway hyperresponsiveness (AHR) was determined using the

Fig. 1. Experimental protocols. Litters were assigned to stress or nonstress at birth. Dams (MS) or both dams and pups (MS+) were removed from the cage for 3 h daily during days 1–10 after birth. Unstressed control mice (CON) were left in the cage with the dams, untouched. All litters (CON, MS, and MS+) were weaned at day 21. Animals were sensitized with OVA and aluminum hydroxide (alum) by intraperitoneal injections on days 31 and 36, and then challenged intranasally with OVA or SAL on days 42 and 44. Animals were euthanized on day 45 after measurement of AHR with noninvasive plethysmography.



Buxco whole-body plethysmography (Buxco Electronics, Troy, N.Y., USA) system that calculates a dimensionless variable Penh [23], which correlates with changes in lung function. Increasing doses of methacholine (0–32 mg/ml) were nebulized into the closed chamber system with the mice inside, and a dose-response curve was generated, as previously described [24].

Airway Inflammation

Twenty-four hours after the last allergen challenge, mice were euthanized with an overdose of ketamine and Rompun. After cardiac puncture to remove blood and ensure death, the trachea was exposed via dissection and was catheterized using a polyethylene tube attached to a 21-gauge needle. Bronchoalveolar lavage (BAL) was performed as previously described [24]. Slides were prepared with the cells obtained from BAL using a Cytospin (Thermo Shandon, Pittsburgh, Pa., USA) and stained using DiffQuick (Biochemical Sciences, Swedesboro, N.J., USA), which allowed identification of alveolar macrophages, lymphocytes, neutrophils, and eosinophils based on morphological characteristics. Differential cell counts were performed by counting 100 cells per slide. One slide was prepared for each BAL sample.

Results are presented as ‘total BAL cells’, a number that represents the total number of inflammatory cells recovered in the BAL. In addition, to compensate for interexperimental variability and facilitate statistical analysis, the inflammatory cell numbers recovered from BAL were normalized. To do this, the total number of cells recovered from every mouse was divided by the average number of inflammatory cells recovered from the CON OVA/OVA group from that specific experiment and then multiplied by 100. We present this normalized number as ‘percent of average CON OVA/OVA’.

Cytokine Assays

Interleukin (IL)-4, -5, -6, -9, -13 and interferon (IFN)- γ were measured in the BAL fluid using commercially available ELISA kits (BD Biosciences, San Jose, Calif., USA) as previously described [24, 25]. BAL fluid was kept frozen at -70°C until cytokine analysis.

Serum Corticosterone

Cardiac puncture was performed immediately after the administration of ketamine and Rompun for euthanasia on day 45. Serum obtained from the blood collected by cardiac puncture was used in a radioimmunosorbent assay (MP Biomedicals, Solon, Ohio, USA) to determine corticosterone levels.

OVA-Specific IgE

OVA-specific IgE levels were measured in the same serum samples as the corticosterone assays above, as previously described [26]. The results are shown as absorbance units.

Statistical Analysis

Statistics were performed using the Prism 3 software suite (GraphPad Software Inc., La Jolla, Calif., USA). When two groups were compared, Student’s *t* test was used. For comparisons of more than two groups, one-way ANOVA was used. Additionally, two-way ANOVA was used for comparing the effects of stress on total cell inflammation taking into account interexperimental variations. All values are reported as mean ($\pm$ standard error of the mean); a *p* value <0.05 was considered statistically significant.

Results

Maternal Separation Effects on Animal Growth and Lung Development

As mentioned in the Methods section, MS pups were separated from their dams for 3 h a day for the first 10 days of life, while CON litters were left undisturbed. All mice were weaned on day 21. Animals were later sensitized to OVA with intraperitoneal injections of OVA/alum on days 31 and 36, and were challenged with intranasal OVA dissolved in saline on days 42 and 44. Analy-

sis was performed on day 45. The complete experimental protocol is shown in a schematic form in figure 1.

Early-life stress is known to decrease animal growth [27, 28]. To evaluate the effectiveness of maternal separation-induced stress, we measured the weight of the mice at weaning (day 21) and on the completion of the protocol (day 45). In both cases, the weights of MS mice were lower than of CON animals. Across all experiments conducted (3 with male mice for a total of 26 CON and 25 MS animals, and 7 with female mice for a total of 54 CON and 51 MS animals), the average weight of MS mice at weaning was $7.8 \pm 3.2\%$ lower than of CON mice (MS: 12.7 ± 0.9 g; CON: 13.7 ± 0.7 g; $p < 0.05$), and on the day of completion of the protocol, the MS mice remained lighter by $7.8 \pm 1.3\%$ (MS: 18.7 ± 0.4 g; CON: 20.4 ± 0.5 g; $p < 0.01$).

Early-life stress increases circulating levels of corticosterone and other hormones [29]. These hormones may affect lung development, and most importantly, alveolarization. To determine if early-life stress resulted in morphological changes to the lung, we performed lung morphometry in 30-day-old MS and CON mice using MLI as a measure of alveolarization [22]. CON mice showed an MLI of 36.02 ± 0.56 , compared to MS mice that showed an MLI of 37.27 ± 0.6 . Although there was a trend towards higher MLI in MS mice, the difference was not statistically significant. Therefore, our data indicate that early-life stress did not affect lung alveolarization.

Maternal Separation Effects on AHR and Allergic Airway Inflammation

Since lung development in Balb/c mice is histologically complete as of day 30 [30], we initiated allergic sensitization on day 31 of life, which is earlier than in most of the published murine studies. We first verified the potential of our protocol to induce allergic airway inflammation and AHR. OVA-sensitized and -challenged animals exhibited increased AHR and an increased number of total cells in BAL compared to OVA-sensitized SAL-challenged mice (results not shown). The increase in the number of inflammatory cells was primarily the result of an increased number of eosinophils in OVA-challenged compared to SAL-challenged animals (OVA $24,287 \pm 12,296$; SAL 258 ± 125 ; $n = 4$, $p < 0.01$).

To avoid confounding of our data by the effects of the female estrous cycle or the different hormonal environment between males and females, male and female mice were segregated in different rooms after weaning, individual experiments were performed with either male or female mice, and data obtained from each sex were analyzed independently.

We first evaluated the effects of maternal separation on AHR. Both male and female mice challenged with OVA showed increased Penh values compared to SAL-challenged mice but there was no difference between MS and CON mice, challenged with OVA (fig. 2).

BAL fluid obtained from animals sensitized to OVA and challenged with SAL consisted primarily of macrophages and had small numbers of lymphocytes, neutrophils and eosinophils. Early-life stress increased the percentage of macrophages (from $88.55 \pm 1.6\%$ to $94.27 \pm 0.9\%$, $p < 0.01$) and decreased the percentage of lymphocytes (from $1.18 \pm 0.4\%$ to $0.2 \pm 0.2\%$, $p < 0.05$) in the BAL fluid of OVA-sensitized and SAL-challenged mice (fig. 3a). However, we did not detect a statistically significant effect of early-life stress on the absolute number of macrophages, lymphocytes, neutrophils or eosinophils present in the BAL fluid of animals sensitized to OVA and challenged with SAL (data not shown). This was due to the high variability between experiments in the total number of inflammatory cells recovered from the airways.

In general, BAL fluid from OVA-sensitized and -challenged MS animals had lower total numbers of inflammatory cells compared to CON mice. This was seen in female mice (fig. 3b; $p = 0.046$) as well as in male mice, although in male mice the difference did not reach statistical significance (fig. 3c; $p = 0.7$). In both female and male mice, the differences in the cell numbers recovered from the BAL were significantly different when they were normalized to the numbers recovered from CON animals within each individual experiment. In OVA-sensitized and -challenged MS female mice the normalized cell numbers were $67 \pm 7\%$ of the number of cells in CON OVA/OVA female animals ($p = 0.015$), while in MS male mice the normalized number was $58 \pm 11\%$ of the number of cells recovered from CON OVA/OVA male animals ($p = 0.012$) (fig. 3d).

OVA challenge of OVA-sensitized mice induced, as expected, a very large increase in the number and percent of eosinophils in the BAL compared to SAL-challenged mice in both CON and MS male and female mice. MS mice sensitized and challenged with OVA (male and female together) showed a small reduction in the percentage of eosinophils in BAL compared to CON mice sensitized and challenged with OVA (CON OVA/OVA: $75.2 \pm 2.2\%$, MS OVA/OVA: $59.5 \pm 7.4\%$; $n = 11$, $p < 0.05$; fig. 3a). The absolute numbers of eosinophils showed a trend to lower numbers in MS mice (fig. 3e; $n = 22$ for CON and $n = 19$ for MS, $p = 0.1$). This lack of statistical significance was due to the high variability in total cells and total eo-

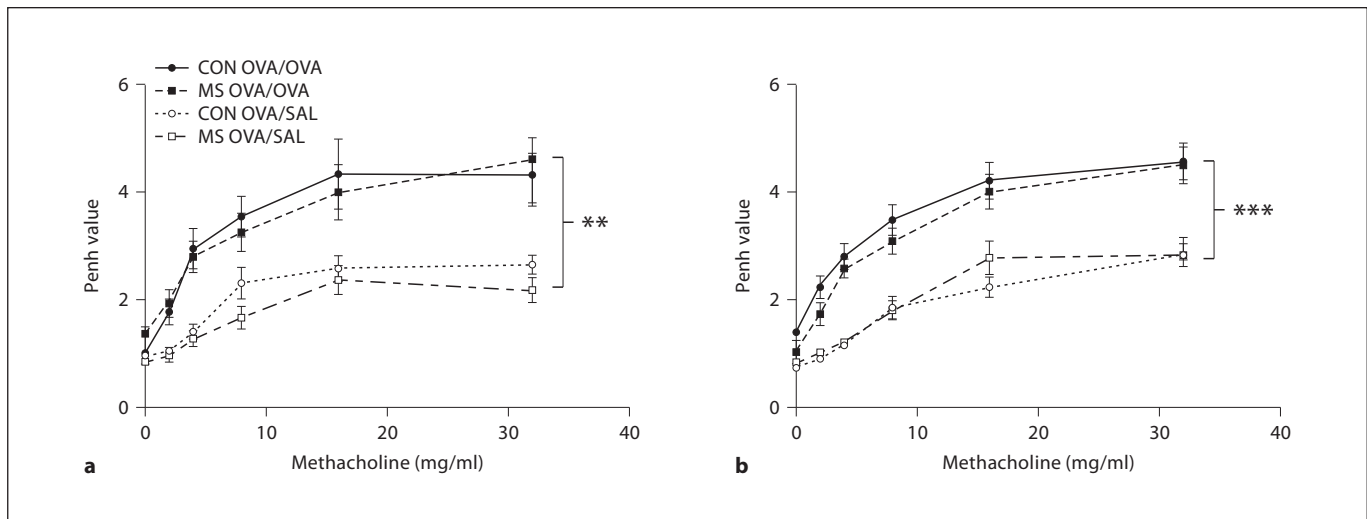


Fig. 2. Effect of MS on AHR Penh values from male (a) and female (b) MS and CON mice sensitized to OVA and challenged with OVA or SAL. Penh in response to increasing levels of methacholine was measured with noninvasive plethysmography. Each point represents a mean $\pm$ SEM ($n = 15$). ** $p < 0.01$, *** $p < 0.001$.

sinophils recovered from mice in different experiments. When eosinophil numbers were analyzed in individual experiments, some of these experiments showed a statistically significant decrease in total eosinophils recovered in the BAL fluid (data not shown).

Measures of Allergic Sensitization and Inflammation

To determine if the decreased accumulation of inflammatory cells could be the result of decreased intensity of allergic sensitization, the levels of OVA-specific IgE were compared between MS and CON OVA-sensitized and -challenged mice. Our results showed no significant difference in OVA-specific IgE levels between CON (0.234 ± 0.007 OD₄₀₅) and MS (0.237 ± 0.005 OD₄₀₅) mice sensitized and challenged with OVA ($n = 14$, $p = 0.784$).

To better understand the mechanism that leads to decreased numbers of inflammatory cells in the airways following maternal separation, we also measured the concentration of several cytokines in the BAL fluid of MS and CON animals. We focused primarily on cytokines that could indicate a shift in the T_H1 vs. T_H2 profile of the immune response in the lung, which may be associated with the reduction in eosinophil numbers recovered in the BAL. There was no difference in BAL cytokine levels between CON OVA/SAL and MS OVA/SAL mice (data not shown). OVA/OVA mice had higher levels of cytokines in the BAL than OVA/SAL mice in both CON and MS groups (data not shown). There was a significant drop

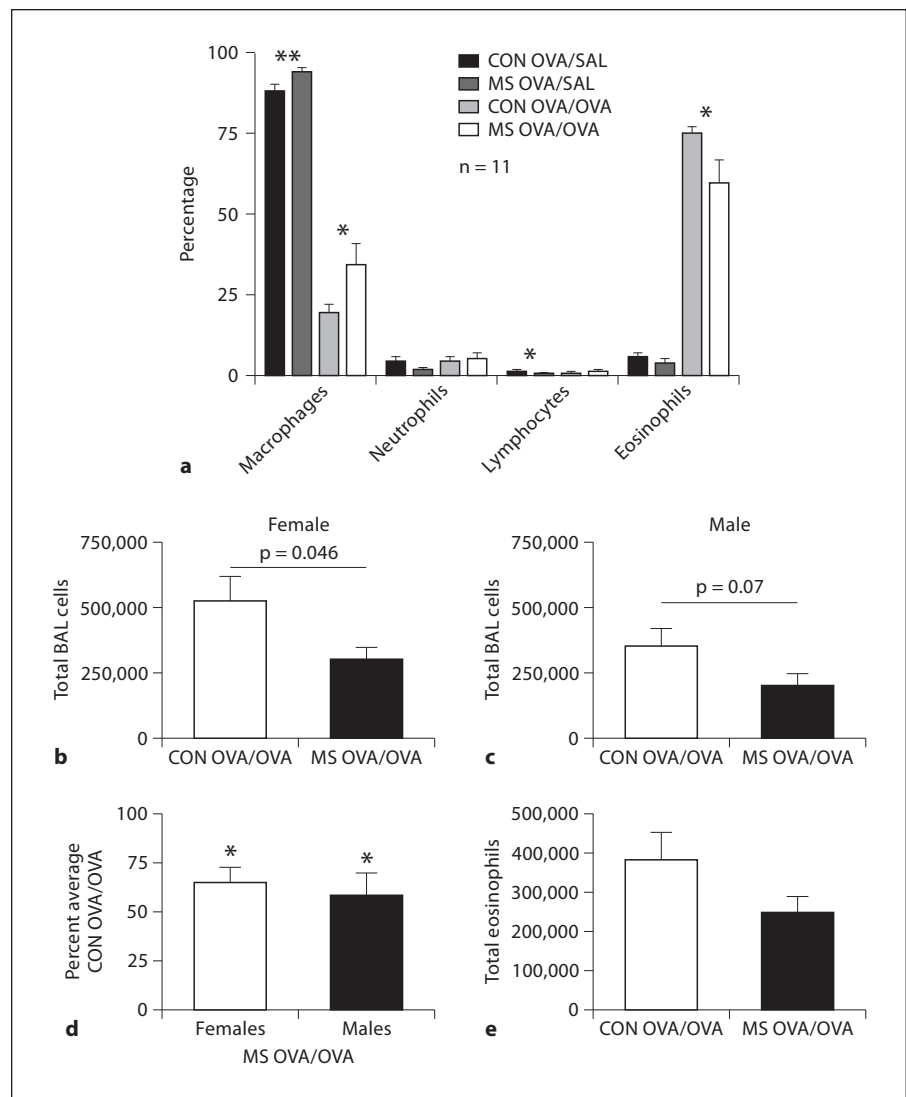
in IL-4 and IFN- γ levels in the BAL of OVA-sensitized and -challenged MS OVA/OVA mice compared to CON OVA/OVA mice ($p = 0.05$), but no difference in IL-5, -6, -9, and -13 levels between the two groups (fig. 4).

Individual experiments exhibited a high degree of variability in total cell numbers recovered from the OVA/OVA groups. In addition, the differences in recovered cells observed between CON OVA/OVA and MS OVA/OVA groups were also highly variable. However, in all experiments airway inflammation was different between CON and MS groups, as was the weaning weight of MS and CON animals. The change in inflammation between MS OVA/OVA and CON OVA/OVA groups (percent change inflammation) observed in individual experiments correlated with the change in weaning weight in the same experiments (fig. 5; $r^2 = 0.5512$, $p = 0.0349$). This result may indicate that the same mechanisms that lead to decreased body weight in stressed mice are also responsible for decreased allergic airway inflammation.

Enhanced Maternal Separation and Airway Inflammation

To study the effect of the quality and intensity of stress on the maternal separation-induced changes in allergic inflammation, we used the MS+ model. To increase the intensity of stress, we removed the pups from their familial environment by transferring them to a new cage during the maternal separation period. MS+ mice, like MS mice above, also exhibited a statistically significant drop

Fig. 3. Effect of MS on inflammatory cell accumulation in the BAL fluid. **a** The combined differential cell count of the BAL fluid of both males and females showed a relative increase in macrophages and decrease in lymphocytes in MS OVA/SAL animals compared to CON OVA/SAL animals, and an increase in macrophages with a decrease in relative eosinophils in MS OVA/OVA animals compared to CON OVA/OVA animals. Female (**b**) and male (**c**) MS animals showed decreased absolute inflammatory cell numbers recovered in the BAL 24 h after OVA challenge compared to CON animals. **d** Relative numbers of inflammatory cells significantly decreased in both female and male mice. **e** Total eosinophil numbers in the BAL fluid of CON OVA/OVA and MS OVA/OVA mice. Each bar represents the mean $\pm$ SEM (males: $n = 12$; females: $n = 26$). * $p < 0.05$, ** $p < 0.01$.



in weaning weight (CON mice 11.34 ± 0.19 g; MS+ mice 10.54 ± 0.22 g; $p = 0.013$; $n = 19$) indicating the presence of an adequate stress response.

We compared the effects of MS and MS+ on allergic airway inflammation and AHR. These experiments were performed with 3 groups of animals (MS, MS+ and CON) in parallel. OVA-sensitized and -challenged animals showed a significant increase in total cells in all groups compared to OVA-sensitized animals challenged with SAL (fig. 6a), which was primarily eosinophilic. Maternal separation stress, as expected from our previous data, significantly reduced normalized inflammatory cell numbers in the BAL fluid of OVA-sensitized and -challenged mice. In contrast, MS+ mice showed no difference in inflammatory cell recruitment to the airways com-

pared to CON mice (fig. 6a). Normalized inflammatory cell numbers in OVA-sensitized and -challenged MS mice were $60 \pm 10\%$ of the values in CON animals ($p < 0.05$, $n = 8$), whereas normalized inflammatory cell numbers in OVA-sensitized and -challenged MS+ mice were $108 \pm 23\%$ of the values in CON animals (no significant difference, $n = 8$). Similarly to what we have shown above for MS, MS+ had no effect on AHR compared to CON animals (fig. 6b).

Furthermore, OVA-sensitized and -challenged MS+ mice showed no significant difference in BAL cytokine levels compared to CON animals that received the same treatment (data not shown). The lowered level of IL-4 and IFN- γ in the BAL fluid of MS mice as compared to CON mice (shown in fig. 4) was not evident in MS+ mice.

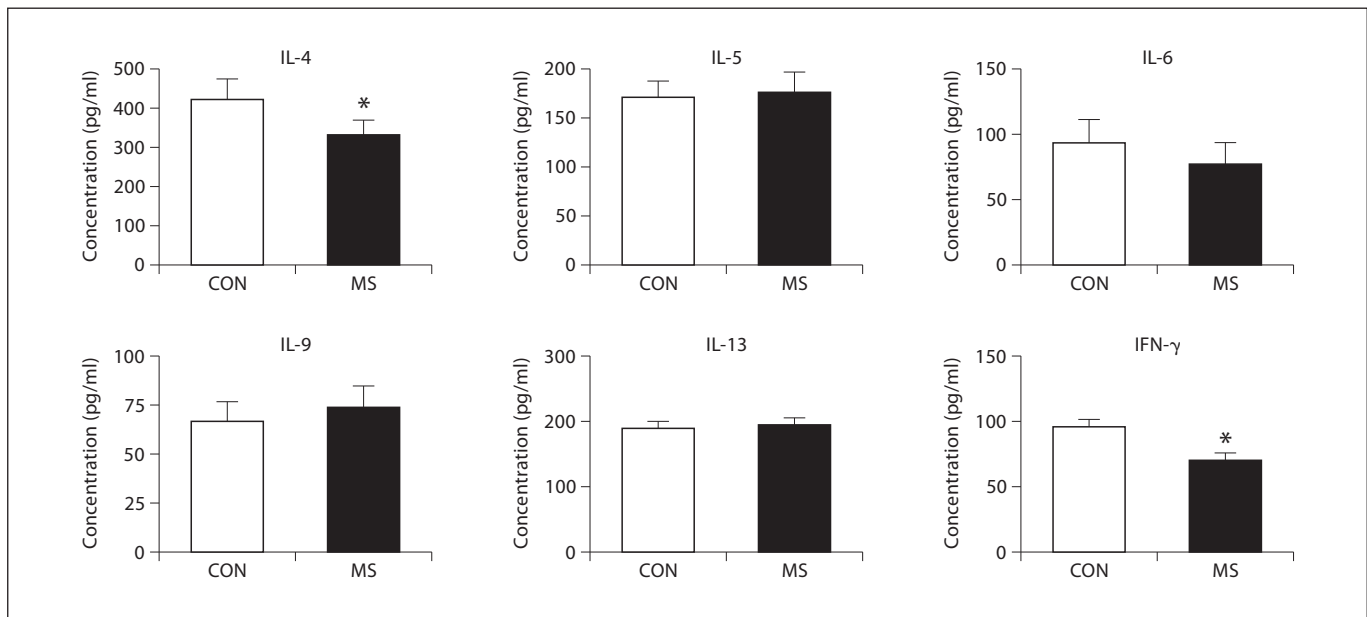


Fig. 4. Effect of MS on BAL cytokine levels. Cytokine levels in the BAL of OVA-sensitized and -challenged CON and MS animals. Bars represent mean values $\pm$ SEM ($n = 14$, pooled male and female mice). * $p < 0.05$.

Maternal Separation and Corticosteroid Levels

Corticosterone levels in the serum were quantified using a radioimmunoassay. In both CON and MS groups, corticosterone levels were higher in mice challenged with OVA relative to those challenged with SAL (fig. 7). Additionally, there was a tendency towards higher corticosterone levels in MS OVA mice than CON OVA mice, although this increase was not statistically significant (fig. 7a). In MS+ mice, there was a tendency towards lower corticosterone levels (fig. 7b).

Discussion

In the majority of cases of human asthma, early-life stress appears to worsen asthma [8]. In clinical studies, these chronic early-life stressors include parent-child interactions, caregiver stress, and lower socioeconomic status [10, 12, 13]. In general, stress is thought to mediate a reduction in T_H1 type inflammation, but an increase in T_H2 type inflammation, which is involved in asthma [31].

However, the primary finding of this study is that the effect of maternal separation on allergic airway inflammation depends on the quality and/or intensity of the early-life stress stimulus. Airway inflammation in MS animals was lower compared to nonstressed mice, while airway inflammation in MS+ animals was comparable to

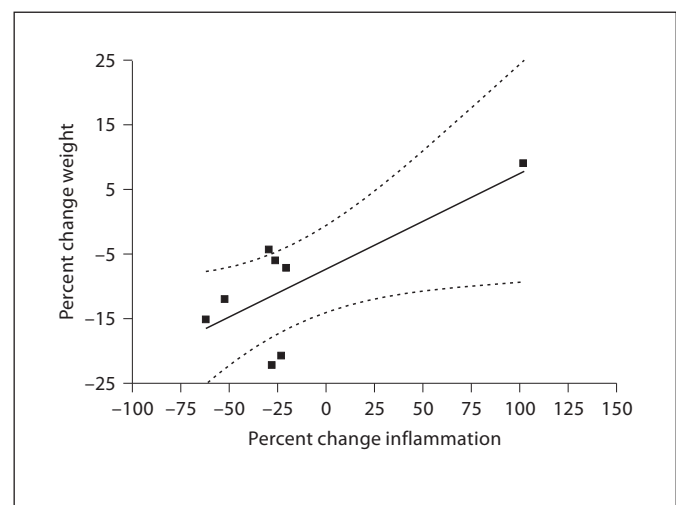


Fig. 5. Correlation between 'percent change inflammation' between MS OVA/OVA and CON OVA/OVA groups and 'percent change weight' in 8 independent experiments with male and female mice.

inflammation of unstressed CON mice. These results indicate that under certain conditions, early-life stress reduced allergic airway inflammation in adult animals, while early-life stress stimulus of a different quality did not have this effect. In contrast, AHR showed little change between CON and either of the MS groups. Our previous

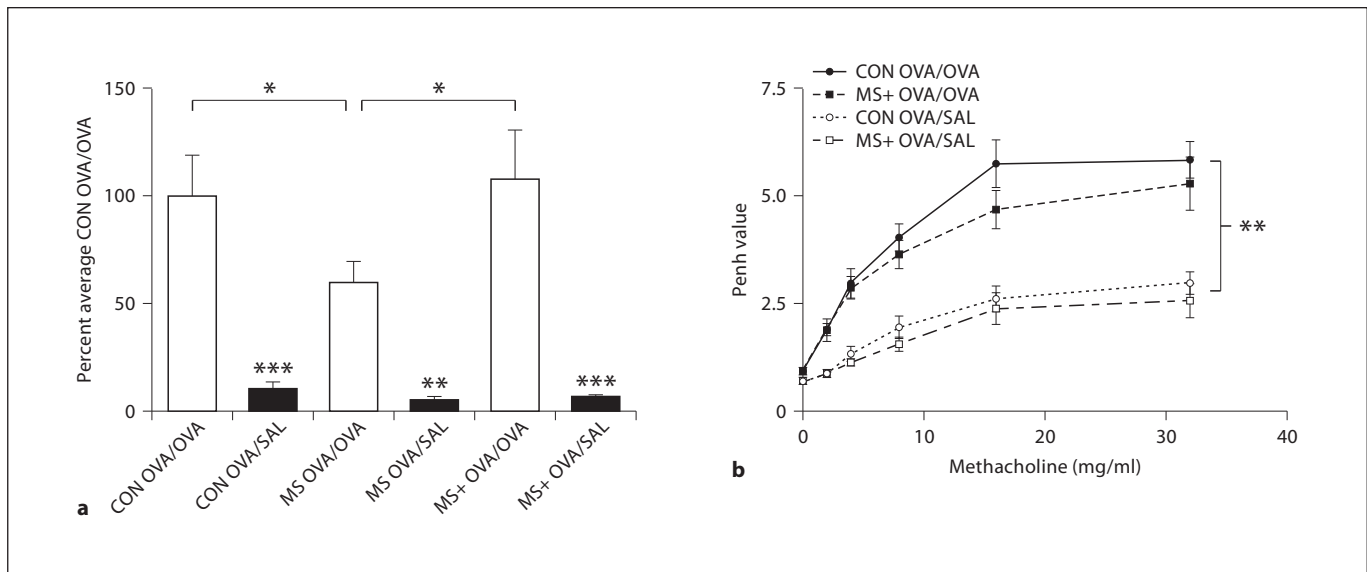


Fig. 6. Effect of MS+ on airway inflammation and AHR. The effect of enhanced early-life stress (MS+) on normalized total BAL cells (**a**) and AHR (**b**) are shown. **a** Each bar represents the mean $\pm$ SEM (challenged with OVA: $n = 8$; SAL: $n = 6$). * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. **b** Each point represents the mean $\pm$ SEM ($n = 14$, 7 males and 7 females). ** $p < 0.01$.

stress studies performed in adult mice also showed significant effects of stress on airway inflammation, but did not show differences in AHR [24]. Other investigators have shown stress-induced AHR alterations using different mouse strains [32]. It is known that different rodent strains show differential responses to stress stimuli [33, 34] and therefore the effects we describe may be specific for this genetic background.

Few studies have investigated the role of early-life stress in murine models of asthma, and in general they have shown that early-life stress increases airway inflammation [35, 36]. None of those studies, however, have directly stressed young pups as we have done in this study. A recent study using a prolonged 24-hour prenatal restraint stress of the dam showed increased inflammatory cell accumulation in the airways, increased AHR, and increased serum IgE levels in prenatally stressed versus nonstressed mice [35]. Since the stress stimulus was given prenatally, that study represents a more complex model of both an early physical and a more delayed psychological stress stimulus to the offspring. Another study showed that early-life stress increased airway inflammation, especially eosinophil influx into the lungs, due to activation of the hypothalamic-pituitary-adrenal axis [36], though the stress stimulus in this study was given during the fourth week of life, at the tail end of lung development and while the immune system was relatively mature and

much later than the stress stimulus in our study. These stresses may therefore not allow for permanent developmental changes to occur which would affect the nervous or immune systems later in life. Finally, one other study showed increased allergic airway inflammation in rats that have undergone maternal separation [37], but the authors do not describe the exact protocol used, rendering direct comparisons difficult. In addition, the animals underwent behavioral testing before allergic sensitization and were sensitized at a more advanced age than the mice in our experiments. These examples, along with our data, are an illustration of the disparities that exist between stress protocols, which differ in timing and quality, and of how these distinct protocols may generate different results. A study comparing all of these protocols in many different strains of mice or rats could offer great insight into the mechanisms of stress-induced alterations in allergic airway inflammation.

Early-life stress has multiple immediate and delayed psychological and physiological effects on the offspring [38]. One of these effects is a decrease in weaning weight, which has been shown to be the result of failure to thrive in maternally separated mice [39]. Maternal separation for periods of over 2 h results in decreased growth hormone levels [40] and prolonged maternal separation can also increase thyroid hormone levels [41]. In addition stress induces increased gut permeability [18, 42], which

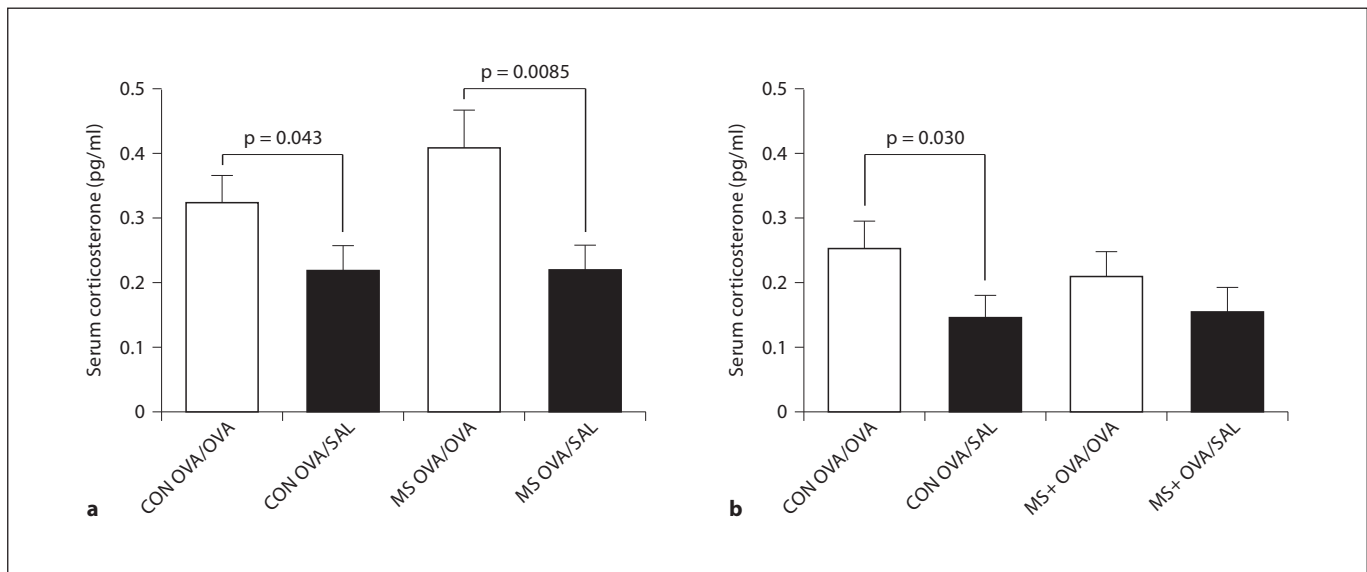


Fig. 7. Effect of MS and OVA challenge on serum corticosterone levels. Corticosterone concentration in the serum of CON and MS (a) or CON and MS+ (b) mice sensitized to OVA and challenged with OVA or SAL. Data bars represent the mean $\pm$ SEM of male and female mice ($n = 30$).

may also affect body weight. All these mechanisms may mediate in part the decreased weight seen in maternally separated mice. Our data showed that the more pronounced the effect of maternal separation on weaning weight, the more pronounced the decrease in inflammation was. There was a good correlation between these two variables over 8 separate experiments with both male and female mice. It will be interesting to speculate that similar mechanisms regulate early-life stress-induced failure to thrive and decreased inflammation later in life. However, the exact mechanism of these effects is not clear.

Although there have been many studies on the effects of various stress stimuli on allergic airway inflammation, there is very little information in the literature on direct comparisons between different stress protocols. In our study, we compared two protocols with different stress intensity: the MS+ protocol has been shown to induce more fear and anxiety behavior in mice compared to the MS protocol [20]. In our study, both protocols led to maternal separation-induced changes in body weight; however, the first maternal separation protocol (MS) showed a general decrease of inflammatory cell accumulation and some of the cytokines in the BAL, while mice from the more intense maternal separation protocol (MS+) showed no differences in inflammation compared to CON mice. An important characteristic of maternal separation stress is stress hypersensitivity, namely its ability

to increase sensitivity to stress and increase the severity of stress reactions [20, 34, 43]. Therefore, this stress hypersensitivity in MS mice would induce a corticosteroid response to the acute stress of allergen challenge, which is significantly greater and more sustained. Since acute stress can depress airway inflammation in adult Balb/c mice [24, 44], stress hypersensitivity could explain the reduced airway inflammation in our MS model. We believe that MS+ mice, in addition to this resetting of the stress response, may also develop other stress-induced chronic changes that are pro-inflammatory. The result is therefore that these mice show levels of inflammation similar to CON mice, which is the average of anti- and pro-inflammatory effects of early-life stress. Future studies should be able to answer this question by evaluating other pathways activated by stress, such as peripheral corticotropin-releasing hormone release. Corticotropin-releasing hormone has a number of pro-inflammatory effects in general [45] and can mediate the pro-inflammatory effects of stress [46, 47].

Our murine model did not reproduce the effects of early-life stress that were expected based on the results from human epidemiological studies. However, the problem with murine models is that mice live in a very controlled environment compared to humans. A recent review of the evolutionary role of the stress phenotype suggests that the environment surrounding an adult or-

ganism plays an important role in the stability or plasticity of the phenotype resulting from early-life stress [34]. This observation may indicate that to elicit a phenotype resulting from exposure to early-life stress the adult animals would require repeated and/or frequent stress stimuli throughout life. Therefore, more appropriate models, where early-life stress is accompanied by repeated exposures to stress in adult life, may be needed to reproduce the observations we have from longitudinal human studies.

Since serum levels of corticosteroids can mirror the organism's response to stress, we measured serum corticosterone levels in MS and CON mice. Our data show that corticosteroid levels in allergen-sensitized animals increase as a result of allergen challenge compared to challenge with saline. These data indicate that allergen challenge is a stressor for an allergen-sensitized mouse, even though mice usually do not appear to develop respiratory symptoms or suffer in any other way during allergen challenges.

However, we detected no difference in serum corticosteroid levels between MS and CON animals, although there was a trend towards higher corticosteroid levels in OVA-challenged MS mice. This inability to notice differences between MS and CON animals may be a result of the timing of obtaining serum, since laboratory stresses increase corticosteroid levels with a peak at approximately 20–40 min after the onset of the stress. In all cases, we measured corticosteroid levels in serum obtained during euthanasia 24 h after the final allergen or saline challenge. It is possible that, had we assessed serum corticosteroid levels 30 min after the allergen challenge, we may

have been able to show differences between MS and CON groups.

Our results show no significant differences of the effects of early-life stress between male and female mice. Overall, throughout our experiments, we found that although the total BAL cell counts varied between males and females (females consistently had higher cell numbers than males), stress-dependent effects occurred equally in males and females. Differential responses to stress between males and females have been shown before, both for maternal separation [48] and for other models of both chronic and acute stress [49]. Our results may also indicate that stress does not interact with sex hormones in the context of our protocol, although increased airway inflammation as a result of estrogens has been shown previously [50].

In conclusion, we have shown that early-life stress induces significant changes in the response to an allergen challenge. These responses depend on the quality of the stressor, as in our hands a change in protocol that was expected to affect the intensity of stress resulted in drastically different results. These studies may allow us to start understanding how early-life stress affects the development of airway inflammatory diseases.

Acknowledgments

This work was supported by grants from SickKids Foundation, Alberta Heritage Foundation for Medical Research (AHFMR) and Canadian Institutes of Health Research (CIHR). H.V. is an AHFMR Scholar.

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