



Airway cytokine responses to acute and repeated stress in a murine model of allergic asthma

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ABSTRACT

Although stress is known to exacerbate asthma, the underlying mechanisms are unclear. Using a murine model of allergic asthma, Th1 (interleukin [IL]-2 and gamma-interferon [IFN- γ]) and Th2 (IL-4 and IL-5) airway cytokine responses and Th1:Th2 cytokine ratios to acute and repeated stress were examined in bronchoalveolar lavage fluid (BALF). Asthmatic mice showed significantly higher IL-4 level and Th2 predominance, compared with healthy mice. To acute stress, asthmatic mice significantly increased IL-4 but decreased IFN- γ levels favoring Th2 predominance, whereas healthy mice significantly decreased IL-4 level favoring Th1 predominance in BALF. To repeated stress, asthmatic mice significantly decreased IFN- γ , IL-4 and IL-5 levels overall favoring Th1 predominance, whereas healthy mice basically maintained the same response profile shown to acute stress. These findings suggest that a significant shift toward Th2 predominance in asthmatic mice under acute stress may be a mechanism underlying exacerbation of asthma.

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1. Introduction

Despite the advances in asthma management, the prevalence and severity of asthma have risen significantly over the last two decades (Umetsu et al., 2002). Precise reasons for this rise are unclear. Although environmental factors such as pollution, improved hygiene, and less childhood infections have been blamed, those factors failed to provide full explanation for the rise in asthma (Seaton et al., 1994; Wright et al., 1998). Other factors, such as increasing psychosocial stress and greater adoption of a western life style worldwide, are thought to have additional contributions to rising asthma and allergic diseases (Busse, 2000). In the field of psychoneuroimmunology, stress has been extensively documented to alter various immune and neuroendocrine responses, and alterations in cytokines may be a potential mechanism linking stress and the exacerbation of asthma in some individuals (Glaser, 2005; Tsigos and Chrousos, 2002). This communication across the fields of psychoneuroimmunology and asthma has renewed an interest in the role of psychosocial stress in asthma and underlying mechanisms (Busse et al., 1995; Chen and Miller, 2007; Rietveld et al., 2000; Vig et al., 2006; Wright et al., 1998). Nevertheless, research in this area has remained limited.

Chronic airway inflammation is a central feature in the pathogenesis of asthma, within which T helper (Th) cells play a significant role in the regulation of airway immune responses. Although recently the significance of Th17 cells in inflammation and autoimmune diseases has emerged (Bettelli et al., 2007; Ouyang et al., 2008), the traditional Th1 and Th2 paradigm remains important for understanding the potential mechanisms linking stress and asthma. When stimulated with an appropriate antigen, naïve Th cells proliferate and differentiate into two major subsets of effector cells, Th1 and Th2. These cell clusters are largely characterized by the profile of cytokines produced and by their functions. Th1 cytokines typically include interleukin (IL)-2 and gamma-interferon (IFN- γ) that mostly facilitate the cell-mediated immunity, whereas Th2 cytokines typically include IL-4, IL-5, IL-6 and IL-10 that promote the humoral immunity, including allergic reactions (Mosmann, 1991; Romagnani, 2000). The predominance of a Th2 cytokine profile, particularly increased IL-4 and IL-5 levels, and a shift in the Th1:Th2 cytokine ratio has been associated with the pathogenesis and exacerbation of asthma (Busse and Lemanske, 2001; Kay, 2001).

IL-4 is essential for driving the differentiation of naïve Th0 cells into Th2 cells to develop allergic inflammation (Nakajima and Takatsu, 2007) and is a known B cell stimulating and differentiating factor. IL-4 facilitates the isotype switching and production of immunoglobulin (Ig) E, a hallmark of allergy. IL-5 functions to differentiate, activate, and enhance the viability of eosinophils (Chung and Barnes, 1999; Nakajima and Takatsu, 2007). Activated

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eosinophils release potent proinflammatory mediators that are damaging airway epithelium, and eosinophilic infiltration is an important underlying cause of chronic airway inflammation in asthma (Wardlaw, 1999). On the other hand, Th1 cytokines, in particular IFN- γ exposure has a suppressive effect on Th2 cell proliferation, changing the relative Th1/Th2 balance, and preferentially switches to the IgG2 isotype with a simultaneous reduction in the IgE isotype. Animal studies show that treatment with recombinant IFN- γ or IFN- γ gene therapy reduces airway inflammation and responsiveness and attenuates allergen-induced airway eosinophilia (for review, Lynch et al., 2003). In contrast, mice deficient in IFN- γ receptors show a prolonged airway eosinophilia in response to allergen inhalation (Nakajima and Takatsu, 2007). For IL-2, the IL-2:anti-IL-2 complexes significantly reduce airway inflammation and eosinophilia and suppress IL-5. At the same time, mucus production, airway hyperresponsiveness to methacholine, and airway tissue inflammation decrease dramatically (Wilson et al., 2008). Also, a combination of IL-2 and IL-18, which is known to induce IFN- γ , prevents airway hyperresponsiveness and inflammation (Matsubara et al., 2007). Therefore, we selected IFN- γ , IL-2, IL-4, and IL-5 for the investigation of stress and asthma.

Although stress can induce various changes in immune responses, immune changes may vary depending on the nature and duration of stress (Segerstrom and Miller, 2004; Vig et al., 2006). For example, acute one-time stress can induce immune changes different from those induced by more chronic repeated stress (Cohen et al., 1999; Dhabhar and McEwen, 1997; Stefanski and Engler, 1998). In addition to individual cytokine responses to stress, it is beneficial to understand the overall dynamics of cytokine balance (e.g., Th1:Th2 ratio) for clinical significance (Agarwal and Marshall, 1998; Kang et al., 1997; Marshall et al., 1998). It has been shown that psychosocial stress not only changes individual cytokine responses but also shifts the Th1:Th2 cytokine ratios (Kang et al., 1997; Marshall et al., 1998). However, studies on stress, immune response, and asthma are further hampered by the fact that peripheral blood samples are probably inadequate to reflect the changes occurring in the airways (Kang et al., 1996). However, obtaining airway samples in humans is often difficult and costly, and the sampling procedure itself (i.e., bronchoscopy) can be stressful and confounds the effects of the psychological stress under study. For this reason, we selected a murine model of allergic asthma and stress to directly investigate airway cytokine responses to stress. A well-established murine model of allergic asthma (Krinzman et al., 1996a; Ohkawara et al., 1997) is available, which was validated by demonstrating characteristic cellular and histological features of airway inflammation and hyperresponsiveness of asthma. Similarly, there is a reliable and valid murine model of “open-field” psychological stress; when animals were exposed to this stress model, there was a marked increase in plasma IL-6 levels (LeMay et al., 1990).

Building on these models, the purposes of this study were as follows: (1) examine the effects of two different types of stress (acute one-time stress versus chronic repeated stress) on airway Th1 (IL-2 and IFN- γ) and Th2 cytokine (IL-4 and IL-5) responses and the Th1:Th2 cytokine ratios; and (2) compare these responses between asthmatic and healthy control mice. We hypothesized that both acute and chronic stress would increase Th2 cytokine responses and induce a shift toward a Th2 predominance in the Th1:Th2 cytokine ratios in asthmatic mice, whereas the opposite responses would be observed in healthy control mice.

2. Materials and methods

2.1. Experimental design

Male BALB/c mice, 4–5 weeks old, were purchased from Charles River Laboratories (Wilmington, MA). The strain and age of mice were selected to

Table 1
Experimental groups.

Group	Group name	Sensitized	Antigen challenged	Stressed (days)
1	Control			
2	Ag-Control		x	
3	Asthma	x	x	
4	Acute Stress-Control		x	x
5	Acute Stress-Asthma	x	x	x
6	Repeated Stress-Control		x	xxxx
7	Repeated Stress-Asthma	x	x	xxxx

Ag: antigen challenge. Sensitized: an intraperitoneal injection of 8 μ g of chicken ovalbumin (OVA) adsorbed overnight at 4 °C to 4 mg aluminum hydroxide in 0.5 ml phosphate-buffered saline on Day 0 and Day 5. Antigen challenged: aerosolized antigen (6% OVA) for 25 min via a Bennet nebulizer at a flow rate of 8 l/min from Day 15 to Day 21 for 7 days. Stressed: exposure to open-field stress for 60 min per day.

closely follow the established protocols of a murine model of asthma with airway inflammation and hyperresponsiveness (Krinzman et al., 1996a,b; Ohkawara et al., 1997). Male mice were used to control for potential influence of the estrus-cycle-dependent sex hormones in female mice. Mice were maintained under conventional animal housing conditions following a 12-h light–dark cycle and had free access to water and food. Mice were acclimated for 1 week before any procedure was performed.

As shown in Table 1, a total of 70 mice were randomly divided into seven groups ($n = 10$ per group): G1—no manipulation (Control); G2—antigen challenged only control (Ag-Control); G3—sensitized and antigen challenged (Asthma); G4—antigen challenged and stressed one time (Acute Stress-Control); G5—sensitized, antigen challenged, and stressed one time (Acute Stress-Asthma); G6—antigen challenged and stressed for 4 consecutive days (Repeated Stress-Control); and G7—sensitized, antigen challenged, and stressed for 4 consecutive days (Repeated Stress-Asthma). The Ag-Control group was designed to control for potential changes from antigen exposure itself but was found to be similar to the responses of the no-manipulation control group. Three mice (one each from G4, G5, and G6) were lost during the initial acclimation period for unknown reasons. The protocol was approved by the Institutional Animal Care and Use Committee of the university.

2.2. Sensitization and antigen challenge

The sensitization and antigen challenge protocols were followed as previously described with minor modifications (Krinzman et al., 1996a,b; Ohkawara et al., 1997). Briefly, after animals were acclimated, mice were sensitized on Day 0 by an intraperitoneal injection of 8 μ g of chicken ovalbumin (OVA) (Sigma Chemical, St. Louis, MO) adsorbed overnight at 4 °C to 4 mg aluminum hydroxide (Aldrich Chemical, Milwaukee, WI) in 0.5 ml phosphate-buffered saline (PBS). A booster dose of the same intraperitoneal injection was repeated on Day 5 following the established protocols. Antigen challenge was performed as follows: on Day 15, mice were placed in a Plexiglas chamber (25 cm $\times$ 15 cm $\times$ 10 cm) and were exposed to aerosolized antigen (6% OVA) for 25 min per day for 7 consecutive days using a Bennet nebulizer at a flow rate of 8 l/min.

When a murine model of asthma was developed (Krinzman et al., 1996a,b; Ohkawara et al., 1997), untreated mice, saline-treated control mice, and OVA antigen challenged control mice without sensitization did not show any significant difference in their airway responsiveness to any dose of methacholine. However, mice both sensitized and challenged with OVA developed asthma-like features by showing increased pulmonary resistance, decreased dynamic compliance, increased airway responsiveness to methacholine, as well as increases in neutrophils and eosinophils in bronchoalveolar lavage fluid (BALF) and goblet cell metaplasia in lung tissues. Accordingly, mice sensitized and antigen challenged were treated as mice with asthma in this study, whereas mice antigen challenged only were treated as controls.

2.3. Stress paradigm

Animals were exposed to “open-field” stress (LeMay et al., 1990) combined with mild rotation. A 60-min exposure to open-field stress was found to increase plasma IL-6 levels more than 7 times higher than the basal levels (LeMay et al., 1990), and this model has been used as a stress paradigm in many murine studies (Dubovicky and Jezova, 2004; Wrona et al., 2004). Briefly, a mouse was placed alone in a 2 ft $\times$ 2 ft $\times$ 2 ft brightly illuminated, white-finished box on top of a rotator at 50 rpm for 60 min. For chronic repeated stress, animals were exposed to the same stress daily for 60 min for 4 consecutive days at different times of the day during the light cycle to minimize the potential habituation to the ‘time’ of the day. A 4-day chronic repeated stress model was selected based on habituation behaviors to stress in animal studies; male rats exposed to open-field stress significantly decreased the food and water intake from Day 2 up to Days 8–10, significantly

Table 2
Experimental protocol schedule.

Protocol	Day																					
	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
Sensitization	x					x																
Antigen challenge																x	x	x	x	x		x
Acute one-time stress																						x
Chronic repeated stress																			x	x	x	x

Day 0 starts following 1-week of acclimation in the cage. On Day 21, immediately following the last procedure, animals were sacrificed for sample (bronchoalveolar lavage fluid) collection. Sensitization: an intraperitoneal injection of 8 µg of chicken ovalbumin (OVA) adsorbed overnight at 4 °C to 4 mg aluminum hydroxide in 0.5 ml phosphate-buffered saline on Day 0 and Day 5. Antigen challenge: aerosolized antigen (6% OVA) for 25 min via a Bennet nebulizer at a flow rate of 8 l/min from Day 15 to Day 21 for 7 days. Acute one-time stress: exposure to open-field stress for 60 min per day for 1 day. Chronic repeated stress: exposure to open-field stress for 60 min per day for 4 consecutive days.

decreasing body weight (Dubovicky and Jezova, 2004); and male rats exposed to a moderate intensity noise stress (98 dB) daily for 4 days decreased mean corticosterone responses nearly half of the Day 1 responses (Sasse et al., 2008).

2.4. Sensitization, antigen challenge, and stress schedule

The challenge schedule is presented in Table 2 and briefly summarized below: following acclimation, Day 0 and Day 5—sensitization (original dose and a booster dose); Days 15–21—antigen challenge; Day 21—acute one-time stress; and Days 18–21—chronic repeated stress. Mice assigned to the stress groups were exposed to open-field stress either on the last 1 day or 4 days of antigen challenge.

2.5. Collection of airway samples

Immediately following the last session of stress, animals were placed in a CO₂ chamber until died. Immediately after death, BALF was collected by cannulating the intact trachea with 22 g needles and syringes. Using 0.7 ml of phosphate-buffered saline (PBS), the airway was gently lavaged twice, consistently recovering 80–90% of PBS. After centrifugation of BALF at 2000 rpm for 5 min, supernatants were aliquoted and stored at –80 °C until assayed.

2.6. Cytokine measurement

Cytokine levels were determined in duplicate by enzyme-linked immunosorbent assay (ELISA) using mouse cytokine kits (Biosource, Camarillo, CA) and reading optical density with a spectrophotometer (Tecan Spectra Rainbow, Research

Triangle Park, NC). To control for inter-assay variations, all samples were batch-processed at the same time. Intra-assay precision was reported to be <6% for all cytokines (Biosource, Camarillo, CA). The sensitivity of IFN-γ, IL-2, IL-4 and IL-5 in this study was 0.1, 1.5, 4.2 and 1.4 pg/ml.

2.7. Data analyses

The data presented two challenges for analysis: (1) some values were left-censored, meaning that values were below the level of detection (LOD) for the ELISA technique employed; and (2) those values at or above the LOD were not normally distributed. While statistical techniques are available to adequately deal with right-censored, non-normally distributed data, no such techniques are available for left-censored values. However, Zhang et al. (2009) described a method to convert left-censored distributions to right-censored by “flipping” the values. Using Monte Carlo simulations, applying nonparametric methods, such as the Peto-Peto test, to those flipped values was found to be superior to naïve (e.g., substituting the minimum detection level) or parametric (e.g., Tobit) approaches. Acceptable performance for the Peto-Peto test was observed even with samples as small as 10 per group and censoring rates as high as 54%.

If an individual cytokine value was below the LOD, a value of .09 was assigned, and the value was marked as censored. Zhang et al.’s method was applied by subtracting individual cytokine values from a constant, producing right-censored distributions. The resulting differences were analyzed using the Peto-Peto test as implemented in SAS PROC LIFETEST (SAS Institute, 2004). Parameter estimates (e.g., median, Q2, Q3, and mean) were converted back to the original scale by subtracting the estimate from the constant used to “flip” those values.

Table 3
Individual cytokine responses and group comparisons.

Cytokine	Group (n)	Censored (%)	Q2	Median (mean, SEM)	Q3	Peto-Peto test χ^2 (DF)	p value
IL-2	Antigen-Control (10)	2 (20%)	2.1	22.25 (22.66, 6.26)	38.8	17.35 (5) ^a	.004
	Asthma (10)	0 (0%)	5.3	10.45 (11.29, 2.30)	18.0	1.14 (1)	.285
	Acute Stress-Control (9)	0 (0%)	24.2	33.2 (40.4, 9.04)	43.2	2.21 (1)	.137
	Acute Stress-Asthma (9)	0 (0%)	14.1	17.0 (18.01, 3.13)	26.4	1.98 (1)	.159
	Repeated Stress-Control (9)	2 (22%)	4.9	55.2 (40.3, 11.50)	63.2	1.00 (1)	.318
	Repeated Stress-Asthma (10)	4 (40%)	NA ^b	8.3 (10.55, 1.36)	15.2	1.46 (1)	.226
IFN-γ	Antigen-Control (10)	0 (0%)	1.7	2.45 (3.94, 1.33)	3.7	17.99 (5) ^a	.003
	Asthma (10)	0 (0%)	2.2	2.7 (3.45, 0.61)	4.4	.330 (1)	.566
	Acute Stress-Control (9)	0 (0%)	0.8	1.5 (2.06, 0.65)	2.6	1.92 (1)	.165
	Acute Stress-Asthma (9)	4 (44%)	NA ^b	0.3 (1.63, 0.74)	1.7	5.90 (1)	.015
	Repeated Stress-Control (8)	4 (50%)	NA ^b	NA ^b (2.34, 0.32)	2.8	2.18 (1)	.140
	Repeated Stress-Asthma (10)	2 (20%)	0.3	1.05 (1.11, 0.24)	1.5	13.0 (1)	<.001
IL-4	Antigen-Control (10)	0 (0%)	15.3	16.2 (16.03, 0.38)	17.0	66.36 (5) ^a	<.001
	Asthma (10)	0 (0%)	22.8	33.4 (31.7, 2.61)	39.1	18.2 (1)	<.001
	Acute Stress-Control (9)	9 (100%)	NA ^b	NA ^b	NA ^b	16.4 (1)	<.001
	Acute Stress-Asthma (9)	1 (11%)	49.1	98.4 (149.7, 56.3)	120.0	4.72 (1)	.030
	Repeated Stress-Control (9)	9 (100%)	NA ^b	NA ^b	NA ^b	16.4 (1)	<.001
	Repeated Stress-Asthma (10)	7 (70%)	NA ^b	NA ^b (5.31, 0.89)	4.2	18.2 (1)	<.001
IL-5	Antigen-Control (10)	2 (20%)	3.1	11.9 (24.0, 8.05)	41.5	16.4 (5) ^a	.006
	Asthma (10)	0 (0%)	23.2	38.0 (39.6, 7.71)	51.9	2.83 (1)	.092
	Acute Stress-Control (9)	2 (22%)	14.0	30.1 (24.0, 3.11)	31.4	0.06 (1)	.806
	Acute Stress-Asthma (9)	2 (22%)	9.7	21.4 (32.9, 11.74)	30.0	1.50 (1)	.220
	Repeated Stress-Control (9)	4 (44%)	NA ^b	9.1 (22.1, 7.54)	27.7	0.33 (1)	.565
	Repeated Stress-Asthma (10)	8 (80%)	NA ^b	NA ^b (7.2, 5.22)	NA ^b	13.8 (1)	<.001

IL-2: interleukin 2, IFN-γ: interferon gamma, IL-4: interleukin 4, and IL-5: interleukin 5.

Group comparisons: the Asthma group was compared with the Antigen-Control group; the Acute Stress-Control and Repeated Stress-Control groups were compared with the Antigen-Control group; and the Acute Stress-Asthma and Repeated Stress-Asthma groups were compared with the Asthma group.

Censored: the number of censored indicates the number of values below the level of detection. Q2: 25th percentile; Q3: 75th percentile; SEM: standard error measurement.

^a Overall group difference across all groups.

^b NA: estimate is not available due to censoring. One sample was lost during the IFN-γ assay.

Table 4

Th2/Th1 cytokine ratios and group comparisons.

	Group (n)	Censored (%)	Q2	Median (mean, SEM)	Q3	Peto-Peto test χ^2 (DF)	p value
IL-4/IL-2	Antigen-Control (8)	0 (0%)	0.41	0.60 (1.61, 0.94)	1.25	48.3 (5) ^a	<.001
	Asthma (10)	0 (0%)	1.69	3.26 (5.49, 2.00)	5.84	6.96 (1)	.008
	Acute Stress-Control (9)	9 (100%)	NA ^b	NA ^b	NA ^b	16.0 (1)	<.001
	Acute Stress-Asthma (9)	1 (11%)	3.59	7.27 (8.74, 2.79)	8.25	0.97 (1)	.325
	Repeated Stress-Control (7)	7 (100%)	NA ^b	NA ^b	NA ^b	12.4 (1)	<.001
	Repeated Stress-Asthma (6)	6 (100%)	NA ^b	NA ^b	NA ^b	10.9 (1)	.001
IL-4/IFN- γ	Antigen-Control (10)	0 (0%)	4.20	6.33 (6.50, 1.05)	9.93	77.7 (5) ^a	<.001
	Asthma (10)	0 (0%)	5.70	11.4 (11.6, 1.92)	16.5	3.38 (1)	.066
	Acute Stress-Control (9)	9 (100%)	NA ^b	NA ^b	NA ^b	16.4 (1)	<.001
	Acute Stress-Asthma (5)	0 (0%)	29.8	32.7 (378.9, 304.1)	231.8	9.80 (1)	.002
	Repeated Stress-Control (4)	4 (100%)	NA ^b	NA ^b	NA ^b	7.27 (1)	.007
	Repeated Stress-Asthma (8)	7 (88%)	NA ^b	NA ^b (5.78, NA ^b)	NA ^b	12.0 (1)	<.001
IL-5/IL-2	Antigen-Control (8)	0 (0%)	0.57	1.24 (1.22, 0.31)	1.46	42.6 (5) ^a	<.001
	Asthma (10)	0 (0%)	2.66	3.14 (4.64, 1.17)	4.24	10.5 (1)	.001
	Acute Stress-Control (9)	2 (22%)	0.15	0.47 (0.84, 0.30)	0.97	1.35 (1)	.245
	Acute Stress-Asthma (9)	2 (22%)	0.64	0.95 (1.70, 0.65)	1.30	9.64 (1)	.002
	Repeated Stress-Control (7)	2 (29%)	NA ^b	0.37 (0.67, 0.26)	1.20	2.26 (1)	.133
	Repeated Stress-Asthma (6)	4 (67%)	NA ^b	NA ^b (0.83, 0.49)	0.49	7.86 (1)	.005
IL-5/IFN- γ	Antigen-Control (10)	2 (20%)	2.21	4.49 (5.53, 1.13)	9.33	16.4 (5) ^a	.006
	Asthma (10)	0 (0%)	9.67	11.6 (12.3, 1.74)	17.6	7.69 (1)	.006
	Acute Stress-Control (9)	2 (22%)	2.46	10.5 (46.4, 34.1)	30.0	1.56 (1)	.211
	Acute Stress-Asthma (5)	0 (0%)	6.92	20.0 (88.3, 71.3)	39.0	0.85 (1)	.357
	Repeated Stress-Control (4)	0 (0%)	7.64	10.7 (12.9, 4.09)	18.2	5.41 (1)	.020
	Repeated Stress-Asthma (8)	6 (75%)	NA ^b	NA ^b (4.18, 2.16)	NA ^b	9.21 (1)	.002

IL-2: interleukin 2, IFN- γ : interferon gamma, IL-4: interleukin 4, and IL-5: interleukin 5; IL-4/IL-2: interleukin 4:interleukin 2 ratio, IL-4/IFN- γ : interleukin 4:interferon gamma ratio, IL-5/IL-2: interleukin 5:interleukin 2 ratio, and IL-5/IFN- γ : interleukin 5:interferon gamma ratio.

Group comparisons: the Asthma group was compared with the Antigen-Control group; the Acute Stress-Control and Repeated Stress-Control groups were compared with the Antigen-Control group; and the Acute Stress-Asthma and Repeated Stress-Asthma groups were compared with the Asthma group.

Censored: the number of censored indicates the number of values below the level of detection. Q2: 25th percentile; Q3: 75th percentile; SEM: standard error measurement.

^a Overall group difference across all groups.

^b NA: estimate is not available due to censoring.

The balance between Th1 and Th2 cytokines was examined by calculating Th2:Th1 ratios, which would be the reverse of Th1:Th2 ratios. The Th2 cytokine levels were divided by the Th1 cytokine levels. This approach of Th2:Th1 ratio was employed to maximize data available for analysis because Th2 cytokines had a high number of censored values. A technique similar to that used for individual cytokine values was used to “flip” cytokine ratios by subtracting a constant. Cytokine ratios having a censored value in the numerator were marked as censored. However, since ratios with a denominator below LOD are, in essence, right-censored, they could not be flipped, and were set to missing.

When overall group differences were present, two-group comparisons (between asthma and healthy control and between acute one-time stress and repeated stress, etc.) were performed. Ideally, Bonferroni or modified Bonferroni adjustments would be applied to adjust for the number of comparisons present, but sample sizes made such adjustments impractical. Limiting paired comparisons to only those cytokines where the overall test was statistically significant provides partial protection for inflation of Type I error rate.

3. Results

Details are presented in Table 3 for individual cytokine responses and Table 4 for Th2:Th1 cytokine ratios. As indicated

in Section 2.7, IL-4 had the largest number of values below the LOD. Therefore, using Th2 cytokines as numerator was the most useful approach to assess the balance between Th1 and Th2 cytokines.

3.1. Cytokine responses to stress

Cytokine responses to stress were examined by comparing responses between the stressed group and the non-stressed control group for healthy mice and asthmatic mice separately. At the initial step, we compared differences between the two control groups, the Control group and the Ag-Control (sham control) group, and found no significant group differences in any cytokine responses (Table 5). We therefore used the Ag-Control group as the non-stressed comparison group for healthy control mice, because the antigen exposure would better reflect realistic environmental settings. For asthmatic mice, stress responses of the acute one-time stress (Acute Stress-Asthma) and chronic repeated stress groups (Repeated Stress-Asthma) were compared with those of the Asthma group without stress (Asthma).

Table 5

Comparison of cytokine responses between the Control and Antigen-Control Groups.

Cytokine	Group (n)	Censored (%)	Q2	Median (mean, SEM)	Q3	Peto-Peto test χ^2 (DF)	p value
IL-2	Control (10)	0 (0%)	14.6	33.8 (35.14, 8.76)	39.6	1.28 (1)	.257
	Antigen-Control (10)	2 (20%)	2.1	22.25 (22.66, 6.26)	38.8		
IFN- γ	Control (10)	0 (0%)	2.1	2.6 (3.26, 0.63)	4.2	0.02 (1)	.883
	Antigen-Control (10)	0 (0%)	1.7	2.45 (3.94, 1.33)	3.7		
IL-4	Control (10)	0 (0%)	14.0	17.1 (17.7, 1.33)	20.6	0.18 (1)	.667
	Antigen-Control (10)	0 (0%)	15.3	16.2 (16.03, 0.38)	17.0		
IL-5	Control (10)	0 (0%)	20.3	28.7 (29.7, 6.27)	41.4	0.69 (1)	.406
	Antigen-Control (10)	2 (20%)	3.1	11.9 (24.0, 8.05)	41.5		

IL-2: interleukin 2, IFN- γ : interferon gamma, IL-4: interleukin 4, and IL-5: interleukin 5.

Censored: the number of censored indicates the number of values below the level of detection. Q2: 25th percentile; Q3: 75th percentile; SEM: standard error measurement.

The overall statistical significance for group differences indicated the presence of differences for each individual cytokine and the Th2/Th1 ratios (Tables 3 and 4). In healthy control mice, both the Acute Stress-Control and Repeated Stress-Control groups showed significantly lower IL-4 responses and lower IL-4:IL-2 and IL-4:IFN- γ ratios than those of the Ag-Control group without stress (Tables 3 and 4). In addition, the Repeated Stress-Control group showed a significantly higher IL-5: IFN- γ ratio than that of the Ag-Control group. In asthmatic mice, both the Acute Stress-Asthma and Repeated Stress-Asthma groups showed significantly lower IFN- γ responses and IL-5:IL-2 ratio than those of the Asthma group without stress (Tables 3 and 4). Furthermore, acute and repeated stress induced significant changes in IL-4 responses and IL-4:IFN- γ ratio, but the direction of change was opposite: the Acute Stress-Asthma group showed significantly higher IL-4 responses and IL-4:IFN- γ ratio than those of the Asthma group without stress, whereas the Repeated Stress-Asthma group showed significantly lower IL-4 responses and IL-4:IFN- γ ratio than those of the Asthma group without stress (Table 4). The Repeated Stress-Asthma group also showed significant decreases in IL-5 responses and IL-4:IL-2 and IL-5:IFN- γ ratios than those responses of the Asthma group without stress.

These findings indicated that healthy control mice showed predominantly a profile of lower Th2 (higher Th1) balance to both types of stress, whereas asthmatic mice showed a mixed profile of cytokine responses to acute versus repeated stress. To acute stress, asthmatic mice predominantly decreased Th1 but increased IL-4 cytokine and IL-4 related Th2 balance, whereas, to repeated stress, asthmatic mice decreased both Th1 and Th2 responses shifting the balance toward lower Th2 (higher Th1) balance. Therefore, the hypothesis is supported in healthy control mice but only partially supported in asthmatic mice.

3.2. Acute one-time stress versus chronic repeated stress

When cytokine responses to acute one-time versus chronic repeated stress were directly compared within each healthy control and asthmatic mice (Table 6), healthy control mice showed no significant differences in the response pattern between the two types of stress. In contrast, asthmatic mice showed significant

Table 6

Comparison between acute and repeated stress responses in healthy and asthmatic mice.

	Groups compared	Peto-Peto test χ^2 (DF)	p value
IL-2	Control	0.16 (1)	.690
	Asthma	5.93 (1)	.015
IFN- γ	Control	0.92 (1)	.336
	Asthma	0.06 (1)	.804
IL-4	Control	NA	NA
	Asthma	12.7 (1)	.004
IL-5	Control	0.90 (1)	.343
	Asthma	6.49 (1)	.011
IL-4/IL-2	Control	NA	NA
	Asthma	8.73 (1)	.003
IL-4/IFN- γ	Control	NA	NA
	Asthma	13.3 (1)	<.001
IL-5/IL-2	Control	0.07 (1)	.792
	Asthma	2.44 (1)	.118
IL-5/IFN- γ	Control	0.02 (1)	.880
	Asthma	9.74 (1)	.002

NA: estimate is not available due to censoring. IL-2: interleukin 2, IFN- γ : interferon gamma, IL-4: interleukin 4, and IL-5: interleukin 5; IL-4/IL-2: interleukin 4:interleukin 2 ratio, IL-4/IFN- γ : interleukin 4:interferon gamma ratio, IL-5/IL-2: interleukin 5:interleukin 2 ratio, IL-5/IFN- γ : interleukin 5:interferon gamma ratio.

Table 7

Comparisons between healthy and asthmatic mice for their responses to acute and repeated stress.

	Stress group	Peto-Peto test χ^2 (DF)	p value
IL-2	Acute stress	5.66 (1)	.017
	Repeated stress	2.33 (1)	.127
IFN- γ	Acute stress	1.34 (1)	.247
	Repeated stress	0.03 (1)	.871
IL-4	Acute stress	13.1 (1)	.003
	Repeated stress	3.0 (1)	.083
IL-5	Acute stress	0.16 (1)	.691
	Repeated stress	2.96 (1)	.085
IL-4/IL-2	Acute stress	13.1 (1)	<.001
	Repeated stress	NA	NA
IL-4/IFN- γ	Acute stress	15.0 (1)	<.001
	Repeated stress	0.50 (1)	.480
IL-5/IL-2	Acute stress	0.71 (1)	.400
	Repeated stress	0.80 (1)	.371
IL-5/IFN- γ	Acute stress	0.55 (1)	.457
	Repeated stress	6.90 (1)	.009

NA: estimate is not available due to censoring. IL-2: interleukin 2, IFN- γ : interferon gamma, IL-4: interleukin 4, and IL-5: interleukin 5; IL-4/IL-2: interleukin 4:interleukin 2 ratio, IL-4/IFN- γ : interleukin 4:interferon gamma ratio, IL-5/IL-2: interleukin 5:interleukin 2 ratio, IL-5/IFN- γ : interleukin 5:interferon gamma ratio.

differences in several cytokine responses and their ratios. Asthmatic mice showed significantly higher IL-2, IL-4 and IL-5 responses as well as significantly higher IL-4:IL-2, IL-4:IFN- γ and IL-5:IFN- γ ratios to acute one-time stress than they did to chronic repeated stress (Table 6).

3.3. Comparison of cytokine responses between healthy control and asthmatic mice

Asthmatic mice had significantly higher IL-4 responses and IL-4:IL-2, IL-5:IL-2, and IL-5:IFN- γ ratios than did healthy control mice. In response to stress, differences between healthy and asthmatic mice were mostly apparent in their responses to acute one-time stress (Table 7). Healthy mice showed significantly higher IL-2 responses to acute stress, whereas asthmatic mice showed significantly higher IL-4 responses and IL-4:IL-2 and IL-4:IFN- γ ratios to acute stress than did their healthy counterparts. In response to chronic repeated stress, the two groups did not differ significantly, except that healthy mice showed a significantly higher IL-5:IFN- γ ratio than that of asthmatic mice.

4. Discussion

The airway cytokine responses to acute one-time and chronic repeated stress indicated that stress induced significant changes in Th1 and Th2 cytokine production and Th1:Th2 cytokine ratios in healthy and asthmatic mice. The profiles of cytokine responses to stress, however, differed between healthy and asthmatic mice and between acute one-time stress and chronic repeated stress.

In response to acute one-time stress, asthmatic mice significantly increased the IL-4 levels but significantly decreased the IFN- γ levels, thereby increasing the IL-4:IFN- γ ratio and indicating a shift toward Th2 predominance. In contrast, healthy mice showed an opposite profile of cytokine responses with a significant decrease in the IL-4 levels and a shift toward Th1 predominance. These differences are interesting, potentially offering an explanation for the relationship between stress and exacerbation of asthma.

The pathogenesis and exacerbation of asthma have been associated with predominant Th2 cytokine profiles (Ackerman

et al., 1994; Barnes, 2008; Chung and Barnes, 1999; Kang et al., 1997; Marshall and Agarwal, 2000). Previous studies indicated that individuals with asthma had significantly higher levels of IL-4, IL-5, and the IL-4:IFN- γ ratio in BALF, a greater production of IL-5 from stimulated peripheral blood cells, and higher expression of IL-5 mRNA in sputum than did non-asthmatic counterparts (Kang et al., 1997; Till et al., 1998; Truyen et al., 2006; Walker et al., 1992). Intracellular staining of sputum CD4+ and CD8+ cells also indicated significantly higher frequencies of IL-4, IL-5, and IFN- γ producing sputum cells in asthmatic individuals than in healthy controls, which were inversely related to airway hyperresponsiveness (Cho et al., 2005). Rhinovirus infection was found to cause significantly greater lower respiratory symptoms, greater airway hyperresponsiveness, and impaired lung function in asthmatics than in normal controls, and these clinical features were strongly associated with lower production of IFN- γ and higher production of IL-4 and IL-5 (Message et al., 2008). An animal study also provided a similar support in that transgenic mice carrying large segments of IL-4, IL-5, and IL-13 genes and overexpressing Th2 cytokines spontaneously developed asthma-like airway inflammation against environmental allergens (Lee and Flavell, 2004). These findings together clearly indicate Th2 predominance in the pathogenesis and the exacerbation of asthma. Our findings in asthmatic mice for their responses to acute one-time stress are consistent with this profile and suggest that such cytokine changes are a potential mechanism underlying stress-induced exacerbation of asthma.

The profiles of cytokine responses to chronic repeated stress, however, differed from those to acute one-time stress. Asthmatic mice exposed to chronic repeated stress showed overall decreases in both Th1 and Th2 cytokine responses but overall shifts toward Th1 predominance. These cytokine profiles closely resembled those shown by healthy control mice exposed to chronic repeated stress, which included significant decreases in IL-4 responses, IL-4:IL-2 and IL-4:IFN- γ ratios favoring Th1 predominance. In addition, healthy mice showed an increased IL-5:IFN- γ ratio in response to repeated stress, but its clinical significance was not clear. Compared with the responses to acute one-time stress, it was interesting to find that the overall magnitude of cytokine responses to chronic repeated stress in asthmatic mice was significantly lower, suggesting a down-regulation or adaptation of cytokine reactivity with repeated stress. This is consistent with a previous reporting on the lower magnitude of immune cell changes to chronic repeated stress than to acute stress (Stefanski and Engler, 1998). Differential patterns of physiological responses to acute versus chronic repeated stress have been observed in other studies too. Nociception responses as well as immune cell numbers and functions to acute and chronic repeated stress have differed significantly, often showing opposite responses (Forsythe et al., 2004; Gamaro et al., 1998; Okuyama et al., 2007; Stefanski and Engler, 1998).

An ongoing challenge in stress and asthma is a need to sort out the role of acute versus chronic stress in the exacerbation of asthma and lung function. Because definitions of “acute or short-term” versus “chronic or long-term” stress have not been standardized, terms have been used variably by the investigators to represent a wide range of stress duration, further adding to the difficulty in differentiating the two sets of the effect. Duration of acute stress, for example, ranges from experimental stress challenges lasting less than 10 min (Laube et al., 2003) to stressful events over 3 months in a naturalistic setting (Marin et al., 2009). A recent review reports the mixed findings for the impact of acute experimental stress on lung function. While an induction of negative emotion typically demonstrates bronchoconstriction leading to a small but significant decline in lung function, an induction of acute experimental stress shows mixed responses: lung function declines in some studies or improves or does not

change in the others (Ritz and Kullowatz, 2005). Because of many methodological issues, including variable techniques in measuring lung function, variations in capturing stress experience, and inadequate experimental protocols, a clear conclusion cannot be reached (Ritz and Kullowatz, 2005). In a naturalistic setting, a group of investigators followed children with verified asthma closely for 18 months and found that major stressful life events had acute effects on exacerbation of asthma. The risk of asthma exacerbation rose by 4.7 times within 1–2 days following stressful life event. This risk then dropped but rose again 4 weeks after in a lower magnitude (Sandberg et al., 2004), indicating other temporal complexity involved in dynamics of stress and asthma.

Similarly, a review of long-term stress in animal models of asthma also shows mixed findings for changes in inflammatory cells, cytokines, and airway responsiveness (Vig et al., 2006). A 7-day long-term restraint stress significantly increased the number of inflammatory cells in BALF, but there were no changes in cytokines or airway responsiveness to methacholine (Forsythe et al., 2004). In contrast, 24-h ultrasonic sound stress, termed long-term stress by some other investigators, increased both leukocytes in BALF and airway responsiveness in an animal model of allergic asthma (Joachim et al., 2003). In humans, stressful family and social environments serving as an indicator of chronic stress were linked to greater asthma symptoms and poorer lung function (Chen et al., 2007). However, daily hassles over the past 3 months were associated with increases in lung function and decreases in airway inflammation (Kullowatz et al., 2008), showing opposite effects. Furthermore, an 18-month follow-up with lung function and asthma symptom diaries of 90 asthmatic children indicated that chronic stress had no independent effects on asthma attacks (Sandberg et al., 2000). Therefore, it is again difficult to reach any clear conclusion about the impact of chronic stress in exacerbation of asthma and lung function.

One alternative view is that stress alone is not capable of modifying immune functions sufficient to trigger asthmatic symptoms, but stress may intensify airway inflammatory responses to environmental triggers to exacerbate asthmatic symptoms (Chen and Miller, 2007). In a similar vein, the significance of interactions between acute and chronic stress in asthma has been noted. When cytokine responses to acute and chronic stress were examined, only those children who were under higher chronic family stress responded to acute stress by significantly increasing the production of IL-4, IL-5, and IFN- γ from stimulated peripheral mononuclear cells. In a subgroup of children with moderate-to-severe asthma, changes in IL-4 and IL-5 levels were positively correlated with asthma symptoms (Marin et al., 2009). The risk of new asthma attacks was significantly increased when severe stressful events were added onto high chronic stress (Sandberg et al., 2000). These findings clearly indicate the need for paying greater attention to interactions between acute and chronic stress in exacerbation of asthma.

The consensus is that stress affects asthma symptoms and lung function at least in a subset of individuals with asthma. What remains unclear is the mechanisms underlying these changes. The suggested mechanisms include immune responses, an imbalance in the autonomic nervous system input, and the roles of catecholamines and glucocorticoids, which continued to be explored (Bailey et al., 2009; Barnes, 1996; Carr et al., 1996; Lehrer et al., 1996; Marshall and Agarwal, 2000; Vig et al., 2006; Wright et al., 1998).

Now that asthma is considered as a chronic inflammatory disease of the airways, the immune assessment of Th1/Th2 cytokines as well as type and extent of inflammatory cells would have provided a more comprehensive view on potential differences among individuals (Hamid and Tulic, 2009). Most typically, T lymphocytes, eosinophils, and neutrophils are major players of

airway cytokine production in asthma. A lack of cell assessment is a limitation of this study. However, the findings on cell traffics in relation to asthma exacerbation and severity have been quite consistent and well studied, showing the influx of these cells into the airways. Sputum eosinophils were significantly increased at 6 and 24 h following airway antigen challenge, and these responses were enhanced during the stressful period of final examination in college students with mild allergic asthma (Liu et al., 2002). The higher levels of IL-4 and IL-5 in individuals with allergic asthma were significantly correlated with the numbers of activated T cells and eosinophils in BALF (Till et al., 1998; Walker et al., 1992); the mRNA expression levels of IL-4 and IL-5 in sputum were correlated with the percentage of eosinophils (Truyen et al., 2006); and lower levels of family support were associated with greater nighttime symptoms, lower morning pulmonary function, higher eosinophil count and IL-4 (Chen et al., 2007). In addition, impaired lung function was significantly associated with neutrophilic and eosinophilic lower airway inflammation in asthmatic individuals with rhinovirus infection and lower respiratory symptoms (Message et al., 2008).

Similarly, in animal studies, stress-enhanced airway reactivity or paw edema to an antigen challenge was accompanied by increased eosinophil numbers and T lymphocytes in BALF of asthmatic mice (Datti et al., 2002; Joachim et al., 2003). The cell changes, however, differed based on duration of stress: generally, short-term stress (6 h to 3 days) induced a significant decrease in the number of leukocytes (e.g., eosinophils) while increasing the levels of cytokines (e.g., IL-6, IL-9, and IL-13) in BALF, but long-term stress (6–7 days) induced a significant increase in the total number of cells without changing cytokine levels in BALF (Forsythe et al., 2004; Okuyama et al., 2007). Although some variability was noted based on strain of mice, the type and direction of cell changes were mostly consistent. As such, the use of the murine model of asthma has provided many avenues for increasing our understanding on complex pathophysiology of asthma. However, the similarity of the murine asthma model with asthma in humans is not undisputed, calling for continued refinement of the animal models of asthma (Kumar and Foster, 2001; Kumar et al., 2001; Nials and Uddin, 2008).

In conclusion, considering the ubiquitous presence of stress and the rising prevalence of asthma, stress and asthma will remain as an important area of research. A shift toward Th2 predominance in airway cytokine responses to acute stress supports a potential mechanism underlying stress and asthma exacerbation. However, concurrent assessments of other neuroendocrine and immune responses and their potential interactions will further facilitate the elucidation of the mechanisms involved in acute and chronic stress on exacerbation of asthma and lung function. At the same time, investigators should consider individual differences in stress response and its sources, differential responses to different nature of stress, a need for close monitoring of asthma symptoms and lung function over time for temporal kinetics, and comparisons of the responses between the airways and peripheral circulation to gain greater insights to this complex relationship between stress and asthma. A greater understanding of stress and asthma would lead to strategies for a better management of asthma.

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References

Ackerman, V., Marini, M., Vittori, E., Bellini, A., Vassali, G., Mattoli, S., 1994. Detection of cytokines and their cell sources in bronchial biopsy specimens

- from asthmatic patients. Relationship to atopic status, symptoms, and level of airway hyperresponsiveness. *Chest* 105 (3), 687–696.
- Agarwal, S.K., Marshall Jr., G.D., 1998. Glucocorticoid-induced type 1/type 2 cytokine alterations in humans: a model for stress-related immune dysfunction. *Journal of Interferon & Cytokine Research* 18 (12), 1059–1068.
- Bailey, M.T., Kierstein, S., Sharma, S., Spaitis, M., Kinsey, S.G., Tliba, O., et al., 2009. Social stress enhances allergen-induced airway inflammation in mice and inhibits corticosteroid responsiveness of cytokine production. *Journal of Immunology* 182 (12), 7888–7896.
- Barnes, P.J., 1996. Neuroeffector mechanisms: the interface between inflammation and neuronal responses. *Journal of Allergy & Clinical Immunology* 98 (5 (Pt 2)), S73–S81 (discussion S81–83).
- Barnes, P.J., 2008. The cytokine network in asthma and chronic obstructive pulmonary disease. *Journal of Clinical Investigation* 118 (11), 3546–3556.
- Bettelli, E., Oukka, M., Kuchroo, V.K., 2007. T(H)-17 cells in the circle of immunity and autoimmunity. *Nature Immunology* 8 (4), 345–350.
- Busse, W.W., 2000. Mechanisms and advances in allergic diseases. *Journal of Allergy & Clinical Immunology* 105 (6 (Pt 2)), S593–S598.
- Busse, W.W., Kiecolt-Glaser, J.K., Coe, C., Martin, R.J., Weiss, S.T., Parker, S.R., 1995. NHLBI workshop summary. Stress and asthma. *American Journal of Respiratory & Critical Care Medicine* 151 (1), 249–252.
- Busse, W.W., Lemanske Jr., R.F., 2001. Asthma. *New England Journal of Medicine* 344 (5), 350–362.
- Carr, R.E., Lehrer, P.M., Hochron, S.M., Jackson, A., 1996. Effect of psychological stress on airway impedance in individuals with asthma and panic disorder. *Journal of Abnormal Psychology* 105 (1), 137–141.
- Chen, E., Chim, L.S., Strunk, R.C., Miller, G.E., 2007. The role of the social environment in children and adolescents with asthma. *American Journal of Respiratory and Critical Care Medicine* 176 (7), 644–649.
- Chen, E., Miller, G.E., 2007. Stress and inflammation in exacerbations of asthma. *Brain Behavior, and Immunity* 21 (8), 993–999.
- Cho, S.H., Stanciu, L.A., Holgate, S.T., Johnston, S.L., 2005. Increased interleukin-4, interleukin-5, and interferon-gamma in airway CD4+ and CD8+ T cells in atopic asthma. *American Journal of Respiratory and Critical Care Medicine* 171 (3), 224–230.
- Chung, K.F., Barnes, P.J., 1999. Cytokines in asthma. *Thorax* 54 (9), 825–857.
- Cohen, F., Kearney, K.A., Zegans, L.S., Kemeny, M.E., Neuhaus, J.M., Stites, D.P., 1999. Differential immune system changes with acute and persistent stress for optimists vs pessimists. *Brain, Behavior, and Immunity* 13 (2), 155–174.
- Datti, F., Datti, M., Antunes, E., Teixeira, N.A., 2002. Influence of chronic unpredictable stress on the allergic responses in rats. *Physiology & Behavior* 77 (1), 79–83.
- Dhabhar, F.S., McEwen, B.S., 1997. Acute stress enhances while chronic stress suppresses cell-mediated immunity in vivo: a potential role for leukocyte trafficking. *Brain, Behavior, and Immunity* 11 (4), 286–306.
- Dubovicky, M., Jezova, D., 2004. Effect of chronic emotional stress on habituation processes in open field in adult rats. *Annals of New York Academy Science* 1018, 199–206.
- Forsythe, P., Ebeling, C., Gordon, J.R., Befus, A.D., Vliagoftis, H., 2004. Opposing effects of short- and long-term stress on airway inflammation. *American Journal of Respiratory & Critical Care Medicine* 169 (2), 220–226.
- Gamaro, G.D., Xavier, M.H., Denardin, J.D., Pilger, J.A., Ely, D.R., Ferreira, M.B., et al., 1998. The effects of acute and repeated restraint stress on the nociceptive response in rats. *Physiology & Behavior* 63 (4), 693–697.
- Glaser, R., 2005. Stress-associated immune dysregulation and its importance for human health: a personal history of psychoneuroimmunology. *Brain, Behavior, and Immunity* 19 (1), 3–11.
- Hamid, Q., Tulic, M., 2009. Immunobiology of asthma. *Annual Review of Physiology* 71, 489–507.
- Joachim, R.A., Quarcoo, D., Arck, P.C., Herz, U., Renz, H., Klapp, B.F., 2003. Stress enhances airway reactivity and airway inflammation in an animal model of allergic bronchial asthma. *Psychosomatic Medicine* 65 (5), 811–815.
- Kang, D.H., Coe, C.L., McCarthy, D.O., 1996. Academic examinations significantly impact immune responses, but not lung function, in healthy and well-managed asthmatic adolescents. *Brain, Behavior, and Immunity* 10 (2), 164–181.
- Kang, D.H., Coe, C.L., McCarthy, D.O., Jarjour, N.N., Kelly, E.A., Rodriguez, R.R., et al., 1997. Cytokine profiles of stimulated blood lymphocytes in asthmatic and healthy adolescents across the school year. *Journal of Interferon & Cytokine Research* 17 (8), 481–487.
- Kay, A.B., 2001. Allergy and allergic diseases. First of two parts. *New England Journal of Medicine* 344 (1), 30–37.
- Krinzman, S.J., De Sanctis, G.T., Cernadas, M., Kobzik, L., Listman, J.A., Christiani, D.C., et al., 1996a. T cell activation in a murine model of asthma. *American Journal of Physiology* 271 (3 (Pt 1)), L476–L483.
- Krinzman, S.J., De Sanctis, G.T., Cernadas, M., Mark, D., Wang, Y., Listman, J., et al., 1996b. Inhibition of T cell costimulation abrogates airway hyperresponsiveness in a murine model. *Journal of Clinical Investigation* 98 (12), 2693–2699.
- Kullowatz, A., Rosenfield, D., Dahme, B., Magnussen, H., Kannies, F., Ritz, T., 2008. Stress effects on lung function in asthma are mediated by changes in airway inflammation. *Psychosomatic Medicine* 70 (4), 468–475.
- Kumar, R.K., Foster, P.S., 2001. Murine model of chronic human asthma. *Immunology and Cell Biology* 79, 141–144.
- Kumar, R.K., Herbert, C., Foster, P.S., 2001. The “classical” ovalbumin challenge model of asthma in mice. *Current Drug Targets* 9, 485–494.
- Laube, B.L., Curbow, B.A., Fitzgerald, S.T., Spratt, K., 2003. Early pulmonary response to allergen is attenuated during acute emotional stress in females with asthma. *European Respiratory Journal* 22 (4), 613–618.

- Lee, G.R., Flavell, R.A., 2004. Transgenic mice which overproduce Th2 cytokines develop spontaneous atopic dermatitis and asthma. *International Immunology* 16 (8), 1155–1160.
- Lehrer, P.M., Hochron, S., Carr, R., Edelberg, R., Hamer, R., Jackson, A., et al., 1996. Behavioral task-induced bronchodilation in asthma during active and passive tasks: a possible cholinergic link to psychologically induced airway changes. *Psychosomatic Medicine* 58 (5), 413–422.
- LeMay, L.G., Vander, A.J., Kluger, M.J., 1990. The effects of psychological stress on plasma interleukin-6 activity in rats. *Physiology & Behavior* 47 (5), 957–961.
- Lynch, E.L., Little, F.F., Wilson, K.C., Center, D.M., Cruikshank, W.W., 2003. Immunomodulatory cytokines in asthmatic inflammation. *Cytokine & Growth Factor Reviews* 14, 489–502.
- Liu, L.Y., Coe, C.L., Swenson, C.A., Kelly, E.A., Kita, H., Busse, W.W., 2002. School examinations enhance airway inflammation to antigen challenge. *American Journal of Respiratory & Critical Care Medicine* 165 (8), 1062–1067.
- Marin, T.J., Chen, E., Munch, J.A., Miller, G.E., 2009. Double-exposure to acute stress and chronic family stress is associated with immune changes in children with asthma. *Psychosomatic Medicine* 71 (4), 378–384.
- Marshall Jr., G.D., Agarwal, S.K., 2000. Stress, immune regulation, and immunity: applications for asthma. *Allergy Asthma Proceedings* 21 (4), 241–246.
- Marshall Jr., G.D., Agarwal, S.K., Lloyd, C., Cohen, L., Henninger, E.M., Morris, G.J., 1998. Cytokine dysregulation associated with exam stress in healthy medical students. *Brain, Behavior, and Immunity* 12 (4), 297–307.
- Matsubara, S., Takeda, K., Kodama, T., Joetham, A., Miyahara, N., Koya, T., et al., 2007. IL-2 and IL-18 attenuation of airway hyperresponsiveness requires STAT4, IFN- γ , and natural killer cells. *American Journal of Respiratory Cell & Molecular Biology* 36 (3), 324–332.
- Message, S.D., Laza-Stanca, V., Mallia, P., Parker, H.L., Zhu, J., Keadze, T., et al., 2008. Rhinovirus-induced lower respiratory illness is increased in asthma and related to virus load and Th1/2 cytokine and IL-10 production. *Proceedings of National Academy of Science United States of America* 105 (36), 13562–13567.
- Mosmann, T.R., 1991. Cytokine secretion phenotypes of TH cells: how many subsets, how much regulation? *Research in Immunology* 142 (1), 9–13.
- Nakajima, H., Takatsu, K., 2007. Role of cytokines in allergic airway inflammation. *International Archives of Allergy & Immunology* 142 (4), 265–273.
- Nials, A.T., Uddin, S., 2008. Mouse models of allergic asthma: acute and chronic allergen challenge. *Disease Models & Mechanisms* 1, 213–220.
- Ohkawara, Y., Lei, X.F., Stampfli, M.R., Marshall, J.S., Xing, Z., Jordana, M., 1997. Cytokine and eosinophil responses in the lung, peripheral blood, and bone marrow compartments in a murine model of allergen-induced airways inflammation. *American Journal of Respiratory Cell & Molecular Biology* 16 (5), 510–520 (see comments).
- Okuyama, K., Ohwada, K., Sakurada, S., Sato, N., Sora, I., Tamura, G., et al., 2007. The distinctive effects of acute and chronic psychological stress on airway inflammation in a murine model of allergic asthma. *Allergology International* 56 (1), 29–35.
- Ouyang, W., Kolls, J.K., Zheng, Y., 2008. The biological functions of T helper 17 cell effector cytokines in inflammation. *Immunity* 28 (4), 454–467.
- Rietveld, S., Everaerd, W., Creer, T.L., 2000. Stress-induced asthma: a review of research and potential mechanisms. *Clinical & Experimental Allergy* 30 (8), 1058–1066.
- Ritz, T., Kullowatz, A., 2005. Effects of emotion and stress on lung function in health and asthma. *Current Respiratory Medicine Reviews* 1, 209–218.
- Romagnani, S., 2000. T-cell subsets (Th1 versus Th2). *Annals of Allergy, Asthma & Immunology* 85 (1), 9–18 quiz 18, 21.
- Sandberg, S., Jarvenpaa, S., Penttinen, A., Paton, J.Y., McCann, D.C., 2004. Asthma exacerbations in children immediately following stressful life events: a Cox's hierarchical regression. *Thorax* 59 (12), 1046–1051.
- Sandberg, S., Paton, J.Y., Ahola, S., McCann, D.C., McGuinness, D., Hillary, C.R., et al., 2000. The role of acute and chronic stress in asthma attacks in children. *Lancet* 356 (9234), 982–987.
- SAS Institute, I., 2004. SAS/Stat 9.1. User's Guide. SAS Institute, Inc., Cary, NC.
- Sasse, S.K., Greenwood, B.N., Masini, C.V., Nyhuis, T.J., Fleshner, M., Day, H.E., et al., 2008. Chronic voluntary wheel running facilitates corticosterone response habituation to repeated audiogenic stress exposure in male rats. *Stress* 11 (6), 425–437.
- Seaton, A., Godden, D.J., Brown, K., 1994. Increase in asthma: a more toxic environment or a more susceptible population? *Thorax* 49 (2), 171–174.
- Segerstrom, S.C., Miller, G.E., 2004. Psychological stress and the human immune system: a meta-analytic study of 30 years of inquiry. *Psychological Bulletin* 130 (4), 601–630.
- Stefanski, V., Engler, H., 1998. Effects of acute and chronic social stress on blood cellular immunity in rats. *Physiology & Behavior* 64 (5), 733–741.
- Till, S.J., Durham, S.R., Rajakulasingam, K., Humbert, M., Huston, D., Dickason, R., et al., 1998. Allergen-induced proliferation and interleukin-5 production by bronchoalveolar lavage and blood T cells after segmental allergen challenge. *American Journal of Respiratory & Critical Care Medicine* 158 (2), 404–411.
- Truyen, E., Coteur, L., Dilissen, E., Overbergh, L., Dupont, L.J., Ceuppens, J.L., et al., 2006. Evaluation of airway inflammation by quantitative Th1/Th2 cytokine mRNA measurement in sputum of asthma patients. *Thorax* 61 (3), 202–208.
- Tsigos, C., Chrousos, G.P., 2002. Hypothalamic–pituitary–adrenal axis, neuroendocrine factors and stress. *Journal of Psychosomatic Research* 53 (4), 865–871.
- Umetsu, D.T., McIntire, J.J., Akbari, O., Macaubas, C., DeKruyff, R.H., 2002. Asthma: an epidemic of dysregulated immunity. *Nature Immunology* 3 (8), 715–720.
- Vig, R.S., Forsythe, P., Vliagoftis, H., 2006. The role of stress in asthma: insight from studies on the effect of acute and chronic stressors in models of airway inflammation. *Annals of New York Academy of Science* 1088, 65–77.
- Walker, C., Bode, E., Boer, L., Hansel, T.T., Blaser, K., Virchow, J.-C., 1992. Allergic and nonallergic asthmatics have distinct patterns of T-cell activation and cytokine production in peripheral blood and bronchoalveolar lavage. *American Review of Respiratory Disease* 146, 109–115.
- Wardlaw, A.J., 1999. Molecular basis for selective eosinophil trafficking in asthma: a multistep paradigm. *Journal of Allergy & Clinical Immunology* 104 (5), 917–926.
- Wilson, M.S., Pesce, J.T., Ramalingam, T.R., Thompson, R.W., Cheever, A., Wynn, T.A., 2008. Suppression of murine allergic airway disease by IL-2: anti-IL-2 monoclonal antibody-induced regulatory T cells. *Journal of Immunology* 181 (10), 6942–6954.
- Wright, R.J., Rodriguez, M., Cohen, S., 1998. Review of psychosocial stress and asthma: an integrated biopsychosocial approach. *Thorax* 53 (12), 1066–1074.
- Wrona, D., Jurkowski, M.K., Tokarski, J., 2004. Blood and spleen natural killer cell cytotoxicity after exposure to open field stress in rats: the effect of spontaneous locomotor activity. *Journal of Neuroimmunology* 150 (1–2), 88–97.
- Zhang, D., Fan, C., Zhang, J., Zhang, C.H., 2009. Nonparametric methods for measurements below detection limit. *Statistics in Medicine* 28 (4), 700–715.