

The effect of academic exam stress on mucosal and cellular airway immune markers among healthy and allergic individuals

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Abstract

Research suggests that psychological stress can exacerbate allergies, but relatively little is known about the effect of stress on mucosal immune processes central to allergic pathophysiology. In this study, we quantified vascular endothelial growth factor (VEGF), interferon gamma (IFN- γ), and interleukin-4 concentrations in saliva (S) and exhaled breath condensate (EBC) during final exams and at midsemester among 23 healthy and 21 allergic rhinitis individuals. IFN- γ s decreased during exams for both groups while VEGF_{EBC} increased (and increases in VEGFs were a trend). Elevated negative affect ratings predicted higher VEGF_{EBC} in allergic individuals. IFN- γ _{EBC} increased in healthy individuals early during exams and then decreased, while allergic individuals showed a decrease in IFN- γ _{EBC} throughout final exams. These findings suggest that psychological stress can suppress cellular immune function among allergic individuals while increasing VEGF.

Descriptors: VEGF, IFN- γ , Allergy, Stress, Airway remodeling, Exhaled breath condensate, Saliva

The effect of psychological stress on atopy has long been an area of interest (Wright, Cohen, & Cohen, 2005). Epidemiological studies suggest that there is a link between psychosocial factors and allergies (Mösges & Klimek, 2007) and asthma (e.g., Huovinen, Kaprio, & Koskenvuo, 2001; Kilpeläinen, Koskenvuo, Helenius, & Terho, 2002). Similarly, observational and experimental studies have shown that heightened psychological stress is associated with elevations in inflammatory markers (Chen, Strunk, Bacharier, Chan, & Miller, 2010; Kullowatz et al., 2008; Ritz, Ayala, Trueba, Vance, & Auchus, 2011).

Research on underlying molecular mechanisms has mostly focused on T helper lymphocytes (Th cells, also known as CD4⁺ cells), specifically the Th1 and Th2 subtypes (Marshall et al., 1998). Th1 and Th2 cells mutually suppress one another through the release of their specific cytokines (Elenkov & Chrousos, 1999; Palomares et al., 2010). Thus, the release of Th1 cytokines such as interferon gamma (IFN- γ) leads to a decrease in Th2 cytokines such as interleukin-4 (IL-4), IL-5, or IL-13 and vice versa. Overexpression of Th2 cytokines and suppression of Th1 cells have been thought to be central to allergic pathophysiology (Neurath, Finotto, & Glimcher, 2002). There is evidence that stress can

further exacerbate Th1/Th2 imbalance by suppressing Th1 immunological responses, which in turn leads to an increase in Th2 responses (Höglund et al., 2006; Kang, Coe, McCarthy, Jarjour et al., 1997). Studies that have examined the impact of final examination stress on immune function suggested that this particular stressor decreases the Th1/Th2 ratio and/or natural killer (NK) cell proliferation in atopic individuals (Höglund, et al., 2006; Liu et al., 2002). A cytokine central to Th1 function is IFN- γ , which is a potent initiator of cellular immunity, including the activation of NK cells and macrophages that are crucial to fighting infections caused by viruses (Sadler & Williams, 2008) and intracellular bacteria (Ma et al., 2011). Prior research has shown IFN- γ downregulation in response to stress in animal studies (Curtin, Boyle, Mills, & Connor, 2009; Kang & Weaver, 2010) and human studies of atopy and asthma using the exam stress paradigm (Höglund et al., 2006; Kang & Fox, 2001). Furthermore, asthmatics have reduced levels of IFN- γ collected from exhaled breath condensate (EBC; Shahid, Kharitonov, Wilson, Bush, & Barnes, 2002), and allergic rhinitis individuals have decreased levels of IFN- γ in plasma (Wong et al., 2001) and nasal fluid (Benson, Strannegård, Wennergren, & Strannegård, 2000). However, the effects of psychological stress on IFN- γ expression in the airways in vivo in allergic individuals have not been explored.

In addition, allergic conditions are also closely associated with abnormalities and dysfunctions of several components of the epithelial and mucosal barrier, which constitutes a first line of defense against infectious agents and antigen exposure in the airways (Holmgren & Czerkinsky, 2005; Wang, Bai, Li, Adler, & Wang, 2008). Studies with human stress paradigms have

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demonstrated a susceptibility of innate and mucosal immune parameters to stress including increases and decreases in immunoglobulin A (e.g., Bosch, de Geus, Ring, & Amerongen, 2002; Drummond & Hewson-Bower, 1997; Trueba et al., 2012; Willemssen et al., 1998). However, in the context of allergies and asthma, effects of stress on mucosal immunity have received little attention.

Vascular endothelial growth factor (VEGF) is an immune parameter that plays an important role in the pathophysiology of allergic conditions (Lee et al., 2004). Studies have also implicated VEGF production in asthmatic airway remodeling that is independent of Th2-mediated inflammation (Lee et al., 2004). Much remains to be explored about the potential role of VEGF and other mucosal immune parameters that may also be susceptible to stress (Nowacka & Obuchowicz, 2011; Yang et al., 2009). There is evidence that psychological stress is related to increases in serum VEGF among women with stress and affective disorders (Åsberg et al., 2009). Elevated levels of serum VEGF have also been found in patients with current depression (Nowacka & Obuchowicz, 2011), whereas another study found that higher depression and anxiety ratings were related to a decrease in serum VEGF levels (Katsuura et al., 2011). There is also evidence to suggest that other psychosocial factors are related to VEGF expression. For example, loneliness has been associated in cancer patients with an increase in VEGF expression in tumor tissue (Nausheen et al., 2010), while social support seems to be related to a decrease in serum VEGF (Lutgendorf et al., 2002). Thus, VEGF seems to be highly susceptible to psychosocial influences.

Most studies have examined changes in immune markers and cells in serum, which provides an overall assessment of systemic immune function (Silvestri et al., 2006). However, systemic immune responses may differ from those that are localized, and changes in systemic versus local immune function may have different health implications (Duflo et al., 2002; Wouters, Reynaert, Dentener, & Vernooy, 2009). This is particularly relevant in asthmatics and allergic individuals who have more localized chronic airway inflammation, which may not necessarily be reflected systemically (Wouters et al., 2009). Thus, when relying on systemic immune responses, disease-relevant local processes may be overlooked.

Recently, EBC has been introduced as a method that provides a convenient sampling of a variety of allergic and inflammatory markers localized in the central airways (Horváth et al., 2005). It collects particles from the airway lining fluid in exhaled breath that has been condensed by cooling (Hunt, 2002). Among the methods for sampling immune markers from the airways, EBC has the distinct advantage of being noninvasive compared to induced sputum or bronchoalveolar lavage (Magnussen, Holz, Sterk, & Hargreave, 2000; Ueno et al., 2008). Techniques such as induced sputum have been found to cause coughing, bronchoconstriction, and even to induce inflammatory responses (Baraldi et al., 2003). Also, the collection of EBC is considerably less time consuming. Among the immune markers detected in earlier EBC studies are VEGF (Dalaveris et al., 2009; Leung et al., 2005; Gessner et al., 2010), IFN- γ (Brunetti et al., 2008; Shahid et al., 2002), and IL-4 (Leung et al., 2005; Matsunaga et al., 2006; Tufvesson & Bjermer, 2006).

The aim of this present study was to examine the impact of academic final exam stress on Th cell activity (in this case, IFN- γ and IL-4) and on the immunity of the mucosal epithelia (represented by VEGF) among allergic and healthy individuals. We sought to examine for the first time changes in VEGF, IL-4, and IFN- γ in EBC and saliva from allergic and healthy participants

during final academic exams. By measuring these parameters, both in EBC and in saliva, we were able to control for potential salivary contaminations of EBC measurements (Horváth et al., 2005) and thus explore airway immune changes more selectively. We hypothesized that increases in airway VEGF may provide evidence of dysregulation of mucosal immunity caused by stress. These VEGF increases may be accompanied by decreases in IFN- γ and increases in IL-4, suggesting a shift in Th cell balance towards Th2 cytokine production. We hypothesized that these changes would be more pronounced in the airways of allergic individuals compared to nonallergic controls.

Methods

Study Design

A two-period crossover design was employed, with two visits during the final exam period (early and late exam assessments). The nonstress visit (baseline) was conducted around midterm of either the same term or the subsequent term, at a time when participants had no exams or demanding projects. The midterm assessment was separated from the stress period by 7–8 weeks. Studies that have examined the effect of academic stress on immune makers have varied widely regarding the time points at which samples were collected (see, e.g., Höglund et al., 2006; Jemmott et al., 1983; Jemmott & Magloire, 1988; Liu et al., 2002; Marshall et al., 1998). There is evidence to suggest that stress-induced changes in immune processes can vary in the length of time they take to manifest and recover (Kang, Coe, McCarthy, & Ershler, 1997; Kang, Coe, McCarthy, Jarjour et al., 1997). Hence, we decided to collect samples at two different time points during the final exam period to gain a better understanding of the time frame of stress effects on these immune markers.

During the final exam period, the early and later exam period visits were conducted on days when no exam was scheduled. We included two assessments during the final exam period, one towards the beginning of the final exam period and one towards the end. A break of about 7 days between stress assessments allowed us to expand the window in which we were able to examine the effect of psychological stress on immune parameters. The relative timing of the stress and baseline assessments was counterbalanced, such that half of the participants had their baseline assessment before the stress period and the other half had it after their final exam period in the subsequent term. In addition, half of the individuals participated during the fall and the other half during the spring term. To control for diurnal effects, all assessments were conducted at the same time of day. The protocol was approved by the local Institutional Review Board, and all participants provided informed consent. They received course credit for participation.

Participants

Two groups of participants (18–22 years old) were recruited from psychology undergraduate courses: (1) allergic individuals, and (2) healthy individuals. The inclusion criterion required a minimum of three examinations during the final exam period (including large final projects) and the ability to perform the appropriate exhaled breath condensate and saliva sampling. Exclusion criteria were the following: self-reported physical conditions: angina, myocardial infarction, congestive heart failure, transient ischemic attacks, or cerebrovascular accidents (no potential participant was excluded for any of these reasons). Exclusion criteria also included the intake of antibiotics or oral or injected corticosteroids within the past 6

weeks. None of the participants were current or past smokers. Allergic participants were those who reported a clinical diagnosis of allergic rhinitis with or without additional asthma by a physician, and had rhinitis-specific symptoms ("hay fever or allergic nasal symptoms as from pollen or animals," "recurrent or chronic nasal symptoms apart from respiratory infection") for more than 1 year (Kilpeläinen, Terho, Helenius, & Koskenvuo, 2001).

Physiological Measures

Saliva collection and analysis. Saliva samples were collected using cotton swabs (Salivettes; Sarstedt, Inc., Newton, NC), which participants placed in their mouth for 2 min. The samples were frozen at -80°C until they were analyzed. Collected saliva volumes were recorded to account for changes in salivary flow, which could affect protein concentrations across assessments.

Exhaled breath condensate collection and analysis. Samples were collected with the R-tube (Respiratory Research, Inc., Austin, TX) equipment. To collect the sample, participants breathed through a plastic tube for 10 min. The tube was enclosed by a frozen metal cylinder that cooled the exhaled air. The assessment protocol adhered to currently proposed standards (Horváth et al., 2005). EBC volumes were also recorded, and samples were stored at -80°C until they were analyzed.

Enzyme immunoassay (EIA). Concentrations of VEGF, IFN- γ , and IL-4 in saliva and EBC were measured using commercial EIA kits (Enzo Life Science, Plymouth Meeting, PA). Samples were analyzed in duplicate following manufacturer's protocol. IFN- γ required 50 μL and VEGF 100 μL of saliva or EBC fluid. The sensitivity of these assays was 14.04 pg/mL for VEGF and < 2 pg/mL for IFN- γ and IL-4. The inter- and intra-assay coefficients of variation were less than 10%.

Spirometry. For diagnostic characterization, we used the forced expiratory volume in the first second (FEV_1) from baseline lung function assessments (Jaeger AM2; CareFusion, Höchberg, Germany).

Self-Report Questionnaires

Perceived stress scale (PSS). The PSS is a 10-item self-report measure of perceived stress in the past month. It has adequate psychometric properties (Cohen, Kamarck, & Mermelstein, 1983).

Asthma-related questionnaires. An ad hoc instrument was used to collect information about control, severity, and manifestation of asthma, as well as medication and health care use. Patients with asthma also completed the Asthma Control Test questionnaire (ACT; Schatz et al., 2006). The average ACT score among asthmatic participants in this sample was 21.35, and the range was 17–25.

Allergic rhinitis questionnaire. This measure contains three questions on nasal symptoms and physician's diagnosis of hay fever or nasal allergy. Items on this questionnaire have been shown to be highly related to allergic rhinitis rendered from allergy tests (positive predictive value: 0.76, specificity: 0.87, and sensitivity: 0.87; Kilpeläinen et al., 2001). The questionnaire also explored if the patient had been previously diagnosed with allergic rhinitis by their physician, one of the three gold standards in allergic rhinitis diagnosis (Gendo & Larson, 2004).

Positive and negative affect schedule (PANAS). The PANAS measures current positive and negative affect. The Negative Affect scale used in the current study has 10 items and adequate psychometric properties (Watson, Clark, & Tellegen, 1988).

Current stress levels. A unipolar ad hoc 10-point item (1 = *not at all*, 10 = *extremely*) was used to measure current stress levels experienced by participants.

Aeroallergen counts. We used archive data of aeroallergen counts from trees, grasses, weeds, and molds (per cubic meter) obtained from the American Academy of Allergy, Asthma and Immunology certified counting stations in the area to quantify for each participant the average of personally relevant allergens present on the assessment day (available online at <http://www.entdocs.com/pollencountarchives.htm>). Counts for specific allergens were used whenever participants mentioned being allergic to these; otherwise, if no specific allergens were reported and only pollen allergens as a global category, the average local average allergen count was used instead.

Wisconsin upper respiratory symptom survey 44 (WURSS; Barrett et al., 2005). The WURSS-44 measures symptoms linked to upper respiratory symptom infections. It includes 32 items to assess symptom severity, which we used to control for possible cold symptoms that could account for changes in immune markers across time.

Procedure

Participants were invited for three laboratory assessments of approximately 1-h duration each. They were asked to refrain from eating, drinking (except for water), or exercising for 2 h prior to each session. Participants were instructed to continue their medication as usual, except for the assessment days, for which participants with asthma were asked to abstain from using their short-term beta-adrenergic bronchodilator for 8 h prior to the assessment and to not take any morning dose of inhaled corticosteroids and/or long-term beta-adrenergic bronchodilators. However, if they experienced symptoms, they were encouraged to take these medications and to reschedule their appointment. None of the participants felt compelled to reschedule. Upon arrival at the laboratory, participants were provided with a consent form and the experimenter answered any questions. Participants then filled out questionnaires. The experimenter asked participants to place the cotton swab from the salivette in their mouth for 2 min and then asked for a second sample. Then, participants' EBCs were collected, for which the experimenter gave them the R-tube and a nose clip to ensure that they were breathing only through the mouth. Participants breathed into the tube for 10 min. At the end of the session, lung function was measured with the handheld spirometer.

Data Analysis

Mixed effects analysis of variance (ANOVA) using SPSS software was used for analysis because it allowed for inclusion of all participants even if they had missing data. Missing data in this study resulted mainly from missed assessments or sporadic small or zero volume samples. The analysis also allowed the inclusion of time-varying predictors. EBC and saliva parameters were log-transformed to reduce skewness.

We used the same model to test the effect of the final exam period on most of our dependent variables (including immune

markers, self-reported negative affect, and perceived stress). The predictors in this model included group (allergic vs. healthy individuals), time, and the Group $\times$ Time interaction. The time variable was comprised of three levels: baseline, the beginning of the exam period (early exam period), and near the end of the exam period (late exam period). Dependent variables were IFN- γ and VEGF concentrations in EBC (denoted as IFN- γ_{EBC} and VEGF $_{\text{EBC}}$) and in saliva (S, denoted as IFN- γ_s and VEGF $_s$), stress level, and negative affect. IL-4 was not analyzed as the concentrations were below the detection limit. We also included gender as a covariate and the Gender $\times$ Time interaction in all our models. When the Gender $\times$ Time interaction was nonsignificant in the initial analysis, it was dropped and the models were recomputed to reduce complexity, as gender was not part of the primary hypotheses.

In the models for the EBC and salivary markers, we controlled for VEGF $_s$ and IFN- γ_s as time-varying predictors when analyzing VEGF $_{\text{EBC}}$ and IFN- γ_{EBC} , respectively, and vice versa. This allowed us to examine each immune parameter from a specific source, EBC or saliva, while controlling for potential cross-contamination by the other source (e.g., VEGF $_{\text{EBC}}$ being impacted by contamination from VEGF $_s$).

We also explored the effect of negative affect as a predictor of immune parameter changes across assessments. For this, we included negative affect as a time-varying predictor of the immune parameters in the above analyses. This allowed us to assess the degree to which changes in immune parameters over time were related to changes in negative affect over time.

To appropriately specify the covariance matrix of the errors of the repeated measures over time, we selected the covariance structure with the fewest parameters for which the deviance statistic ($-2 \log$ likelihood) was not significantly worse than the full unstructured covariance matrix (Heck, Thomas, & Tabatha, 2010). In this case, Toeplitz with heterogeneous variances over time was the best fit for our models examining the effects of academic stress on immune parameters, in which $-2 \log$ likelihood for Toeplitz heterogeneous did not differ significantly from the unstructured covariance matrix for VEGF $_{\text{EBC}}$, $\chi^2(1) = 2.00$, $p = .168$; for IFN- γ_s , $\chi^2(1) = 0.23$, $p = .630$; for VEGF $_s$, $\chi^2(1) = 0.63$, $p = .430$; for IFN- γ_{EBC} , $\chi^2(1) = 0.28$, $p = .599$. Compound symmetry with heterogeneous variances over time was the best fit for negative affect, $\chi^2(2) = 0.22$, $p = .894$, and for stress level, $\chi^2(2) = 1.48$, $p = .477$.

Results

Participant Characteristics

The final sample was comprised of 44 participants. Twenty-one had allergic rhinitis, either with asthma ($n = 8$) or without ($n = 13$), and 23 were healthy individuals. Their ages ranged from 18–21, and the majority were female (75%). The overall racial composition was 81.8% White non-Hispanic, 9.1% Hispanic White, 4.5% African American, and 4.5% Asian. There were no significant differences between groups in age, gender, body mass index, or FEV $_1$ (Table 1). Of the 8 asthma participants, one had “poorly controlled” asthma, one had “not well controlled” asthma, and 6 had “well controlled” asthma (NHLBI/NAEP, 2007). Two participants with asthma were taking inhaled corticosteroids in combination with long-acting beta-adrenergic bronchodilators, and the remaining 6 used short-acting beta-adrenergic bronchodilators only. Among asthmatic participants, 87% had received a diagnosis before the age

Table 1. Comparison of Basic Characteristics between Healthy and Allergic Participants

	Allergic participants ($n = 21$)	Healthy participants ($n = 23$)	p level for sample differences
White %	90.48	91.31	0.924
Non-Hispanic %	100	80.50	0.060
Females %	71.40	73.91	0.740
Age, mean (SD)	19.8 (0.75)	19.75 (0.85)	0.923
BMI, mean (SD)	22.93 (2.81)	23.83 (3.43)	0.349
Height, mean (SD), in m	1.72 (0.11)	1.70 (0.09)	0.572
Weight, mean (SD), in kg	67.52 (8.91)	70.41 (9.62)	0.308
FEV $_{1\%}$ of predicted at baseline, mean (SD)	99.21 (2.43)	96.40 (2.32)	0.390
Corticosteroid medication use %	9.5	NA	NA
Specific allergies			
Tree and grasses %	15.38	NA	NA
Cedar %	7.69	NA	NA
Nonspecific pollen %	23.08	NA	NA
Nonspecific seasonal %	53.85	NA	NA

Note. BMI = body mass index; NA = not applicable.

of 12, and all allergic participants received a diagnosis of allergic rhinitis after the age of 12.

Saliva volumes were small for some assessments in some participants, which led to missing data points. As a result, 3.6% (8 samples) for IFN- γ and 5.9% (13 samples) for VEGF were missing. All samples in which the concentrations of a specific immune parameter were below detection limit were given a value of zero in the analyses of that specific immune parameter. All of the VEGF $_s$ samples were above the detection limit. For baseline, eight VEGF $_{\text{EBC}}$ samples, one IFN- γ_{EBC} sample, and one IFN- γ_s were below detection limit. In the early final exam assessment, three VEGF $_{\text{EBC}}$ samples, two IFN- γ_{EBC} samples, and one IFN- γ_s sample were below detection limit. In the late final exam period, four VEGF $_{\text{EBC}}$ samples, one IFN- γ_{EBC} sample, and one IFN- γ_s sample were below detection limit.

Baseline Assessment of VEGF and IFN- γ

IFN- γ and VEGF concentrations levels were not significantly different between allergic and healthy individuals at the baseline assessment in either EBC or saliva ($ts < 1$). Concentrations of IFN- γ_s were not significantly correlated with IFN- γ_{EBC} in either healthy or allergic individuals (Table 2). Similarly, VEGF $_s$ and VEGF $_{\text{EBC}}$ were not significantly correlated for either group. IFN- γ_s and VEGF $_s$ showed a significant positive association in healthy individuals.

Effects of Academic Stress on Self-Report Measures

There was a significant main effect for time (baseline, early exam period, late exam period) for all the self-report measures (Table 3). Regardless of allergic or nonallergic condition, participants reported higher perceived stress, $F(2,62) = 29.55$, $p < .001$, higher negative affect, $F(2,70) = 7.57$, $p < .001$, and more momentary stress levels, $F(2,53) = 112.74$, $p < .001$, during the academic stress period than at the baseline assessment. There were no significant effects for gender ($ps > .100$), for Gender $\times$ Time ($ps > .100$), for group ($ps > .100$), nor for the Group $\times$ Time interaction ($ps > .100$) for any of the self-report measures.

Table 2. Correlations for VEGF and IFN- γ between Saliva and EBC at Baseline for Healthy and Allergic Participants

		Healthy participants			
		<i>n</i> = 23			
		IFN- γ_s	IFN- γ_{EBC}	VEGF _s	VEGF _{EBC}
Allergic participants <i>n</i> = 21	IFN- γ_s		−0.111 <i>n</i> = 22	0.524* <i>n</i> = 20	0.080 <i>n</i> = 22
	IFN- γ_{EBC}	0.241 <i>n</i> = 20		−0.239 <i>n</i> = 21	0.167 <i>n</i> = 23
	VEGF _s	−0.112 <i>n</i> = 19	0.236 <i>n</i> = 20		−0.233 <i>n</i> = 21
	VEGF _{EBC}	−0.076 <i>n</i> = 20	0.157 <i>n</i> = 21	0.123 <i>n</i> = 20	

**p* ≤ .050.

Academic Stress Effects on Immune Parameters in EBC and Saliva

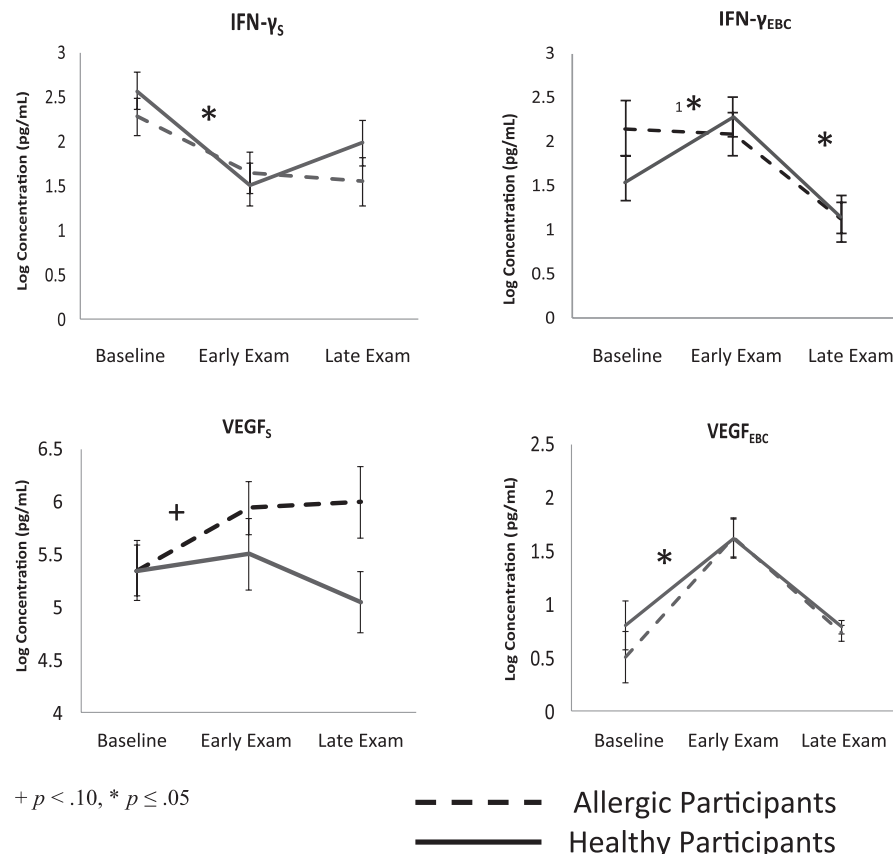
IFN- γ . Regardless of atopic status, IFN- γ_s concentrations changed significantly over time, $F(2,74) = 12.28$, $p < .001$. IFN- γ_s was significantly lower at early and late exam periods compared to baseline, $\beta = -1.03$, $t(46) = -4.11$, $p < .050$, and $\beta = -0.51$, $t(57) = -2.08$, $p < .050$ (Figure 1, upper left panel). For IFN- γ_{EBC} , there was a main effect of time, $F(2,44) = 41.76$, $p < .001$, and a Group $\times$ Time interaction, $F(2,42) = 4.30$, $p < .050$. Healthy par-

Table 3. Mean and Standard Deviations (in Parentheses) of Self-Reported Variables

Variable	Baseline	Early exam period	Late exam period
Perceived stress	15.1 (0.7)	19.7 (0.7)	19.7 (0.7)
Negative affect	9.8 (1.0)	13.3 (1.2)	13.8 (0.9)
Stress level	0.3 (0.1)	4.1 (0.4)	4.1 (0.3)

ticipants showed a significant increase from baseline to the early exam period, $\beta = 0.84$, $t(45) = 3.90$, $p < .001$, followed by a significant decrease from the baseline to the late exam period, $\beta = -0.38$, $t(44) = -1.98$, $p < .050$, whereas allergic individuals showed no increase, but only a decrease in the late exam period, $\beta = -0.88$, $t(40) = -4.03$, $p < .001$ (Figure 1, upper right panel). No other significant effects were found.

VEGF. VEGF_{EBC} concentrations significantly varied over assessments, $F(2,41) = 5.92$, $p < .001$, with a significant increase from baseline to early exam period, $\beta = 0.87$, $t(37) = 2.46$, $p < .050$. Overall changes in VEGF_s concentrations across assessments were seen as a trend ($p = .091$). There was also a trend for the Group $\times$ Time interaction, $F(2,66) = 2.50$, $p = .090$, such that allergic individuals showed pronounced VEGF_s increases throughout the final exam period, whereas healthy individuals showed little change in VEGF_s over assessments (Figure 1, lower left and right panel). No other significant effects were found.

**Figure 1.** IFN- γ and VEGF mean log transformed concentrations in saliva and EBC across final exam stress (early and late in the final exam week) and baseline periods in allergic and healthy participants. †Significant only for healthy participants.

Sample volume. Changes in saliva and EBC volumes across assessments could potentially influence the concentrations of the immune markers, but volumes did not change systematically between baseline and early and late exam periods, $F_s < 1.00$. When saliva and EBC volumes were added as time-varying predictors in the statistical models to control for the effect of fluctuation volumes on immune parameter concentrations, our results remained the same.

Negative affect as a predictor of concentrations of immune parameters. Changes in negative affect within subjects over time were positively related to changes in $VEGF_{EBC}$ concentrations within individuals over time, but only for allergic individuals, $\beta = 0.08$, $t(113) = 3.00$, $p < .010$, and not among healthy individuals, $\beta = -0.00$, $t(113) = -0.09$, $p = .933$.

Exploratory Analyses of Potential Confounding Variables

We repeated the analyses statistically controlling for cold symptoms (by adding cold symptoms as a covariate in the model) and found that respiratory symptoms did not predict changes in immune parameters. In order to control potential effects of antihistamine, inhaled corticosteroids and Singulair (Merck & Co. Inc.) use, we repeated the analyses with maintenance medication use as a covariate (coded 1 for participants using these medications and 0 for participants not using medication) and found no substantial change in the results. Similarly, excluding two participants that used β -adrenergic inhalers regularly ($> once a week$) did not change the results substantially.

To control for fluctuations of aeroallergen counts in the environment across time, we added the average pollen count variable as a time-varying predictor of outcomes in the mixed effects models. In these models, we analyzed allergic individuals only. Changes in pollen counts across assessments were not related to changes in EBC or saliva parameters in the allergic group, over time. We also examined whether there were differences between asthmatic versus nonasthmatic participants (both asthmatics and nonasthmatics were present in our allergic group) in any of our analyses and found no significant main effects or interactions involving asthmatic status, $ps > .300$. In further exploratory analyses, we found that our results were the same regardless of whether we removed or included $VEGF_s$ and $IFN-\gamma_s$ as time-varying predictors when analyzing $VEGF_{EBC}$ and $IFN-\gamma_{EBC}$, respectively, and vice versa.

Discussion

The present study sought to explore the differential effect of allergy on stress-induced changes in mucosal and cellular immune function. Prior research has found that stress is related to changes in cellular immune function, specifically to a decrease in $IFN-\gamma$ concentrations (Kang & Fox, 2001) and NK cell proliferation (Locke et al., 1984). However, little was known about the effects of psychological stress on other aspects of immune function such as VEGF, which can alter susceptibility to infection and lead to remodeling of the airways among allergic individuals (Lee et al., 2004). To the best of our knowledge, this is the first study to examine the effect of psychological stress and negative affect on VEGF concentrations in the airways in vivo. We found that VEGF concentrations increased from baseline to early in the final exam period, and that $IFN-\gamma$ concentrations decreased from baseline to late during the final exam period.

A candidate pathway for the release of VEGF in stress is the corticotrophin-releasing hormone that is peripherally secreted from

sympathetic nerves, which activates mast cells to selectively release VEGF (Cao et al., 2005; Webster, Torpy, Elenkov, & Chrousos, 1998). In cancer, tumor cells exposed to norepinephrine also produce more VEGF (Yang et al., 2009). However, the role of stress on VEGF in other body compartments such as the airways had not been explored before. Beyond systemic VEGF changes associated with psychological factors, as observed in earlier research (Katsuura et al., 2011; Nowacka & Obuchowicz, 2011; Yang et al., 2009), we were able to demonstrate that VEGF in the central airways and the oral cavity is also sensitive to naturally occurring stress. We also found evidence that allergy may be linked to a stronger negative affect-induced expression of VEGF. The demonstration of such associations would help elucidate a proposed association between stress, infections, and asthma or allergy exacerbations (Wright, Rodriguez, & Cohen, 1998).

Consistent with previous research, we observed a significant decrease in $IFN-\gamma_s$ concentrations during academic stress (Kang & Fox, 2001; Liu et al., 2002; Marshall et al., 1998). Expanding earlier findings with this paradigm, we measured these changes in vivo without stimulating lymphocytes (in vitro or ex vivo). The disadvantage of ex vivo or in vitro methods is that it may not reflect the actual mobilization of immune defenses in an ecologically valid context. Supporting our hypothesis, changes in $IFN-\gamma_{EBC}$ concentrations were different among allergic and healthy individuals. Healthy individuals experienced an initial increase in $IFN-\gamma_{EBC}$ during the early exam period and then concentrations decreased. In contrast, only allergic participants experienced a drop in $IFN-\gamma_{EBC}$ at the early exam period, which was sustained throughout the final exam stress period. These findings suggest a suppression of the Th1 immune processes more consistently among allergic individuals. Low levels of $IFN-\gamma$ may be a possible mediator of stress-induced increased susceptibility to respiratory infections, especially among atopic individuals.

Previous studies have also reported reduced overall concentrations of $IFN-\gamma$ in the airways (measured from EBC) of asthmatic children (Shahid et al., 2002). Although we found a difference in the stress-induced changes in $IFN-\gamma_{EBC}$ concentrations between allergic and healthy individuals, our allergic and asthmatic participants did not have overall lower $IFN-\gamma_s$ concentrations, and $IFN-\gamma_s$ levels were reduced during the final exam period for both groups. This suggests that stress effects on immune function in healthy versus allergic individuals may vary by the compartment in which samples were collected. Beyond that, the Th1/Th2 paradigm also has its limits (e.g., Salvi, Babu, & Holgate, 2001). Marin, Chen, Munch, and Miller (2009) found elevated $IFN-\gamma$ levels in asthmatic children suffering from a combination of chronic family stress and acute stressors. More recent research has shown the antiinflammatory role of T regulatory (T reg) cells and their release of the IL-10 in atopy (Lloyd & Hawrylowicz, 2009). Stress hormones can reduce T regulatory cell activity and thus lead to increased allergic exacerbation (Theoharides et al., 2012).

This study adds to the literature on psychosocial factors and immune function by capturing the impact of stress on permeability of the airway epithelia. Specifically, we quantified VEGF concentrations in saliva and EBC and observed an increase in $VEGF_{EBC}$ concentrations early in the final exam period across both allergic and healthy participants. Differential changes in $VEGF_s$ concentrations between allergic and healthy individuals were only observed in tendency. $VEGF_s$ increased during the final exam period and remained high across the early and late exam period in allergic individuals and less so among healthy individuals. In addition, we found that only among allergic individuals, negative affect

was positively related to VEGF expression in the airways, suggesting a unique vulnerability in this population. In contrast to negative affect, we did not find that current stress levels were directly related to VEGF_{EBC}. Negative affect captures a range of unpleasant emotional states (including but not limited to stress) that may be more closely related to VEGF than stress, given previous cross-sectional findings of an association of VEGF with depression (Katsuura et al., 2011; Nowacka & Obuchowicz, 2011; Sharma, Greenman, Sharp, Walker, & Monson, 2008) and loneliness or lack of social support (Lutgendorf et al., 2002; Nausheen et al., 2010).

The differential effect of negative affect on VEGF_{EBC} in allergic and healthy individuals may be due to the fact that allergic individuals have elevated mast cell concentrations in their airways (Brightling et al., 2002; Naclerio, 1997). VEGF expression at elevated levels can lead to problematic inflammation and airway remodeling, thus contributing to the pathophysiology of asthma and allergies (Barnes, 2008; Chetta et al., 2005). The observed increases in VEGF in the airways during final examination psychological stress could confer harmful consequences for airway atopy, as they are already burdened by chronic inflammation and hyperpermeability of the airway epithelium (Buckle & Cohen, 1975).

Saliva assessment of hormones and immune markers is a well-established, noninvasive technique in psychoneuroimmunology. It has been employed successfully for the study of stress effects on humoral markers (e.g., Bosch et al., 2001; Gallagher et al., 2008; Hucklebridge, Clow, & Evans, 1998; Matos-Gomes et al., 2010; Phillips et al., 2006; Ring et al., 1999; Strahler, Mueller, Rosenlocher, Kirschbaum, & Rohleder, 2010; Willemsen et al., 1998) and disease processes including asthma, cancer, and autoimmune diseases (e.g., Lee & Wong, 2009; Wong, 2006; Zhang, Xiao, & Wong, 2009). At this point, it is difficult to determine the origin of the salivary IFN- γ or VEGF identified in our study. It is possible that changes in these salivary markers reflect oral inflammation. Salivary biomarkers have been thought to partially reflect systemic immune processes, as blood and saliva immune markers have been found to be significantly correlated (Nagler, Hershkovich, Lischinsky, Diamond, & Reznick, 2002). The lack of substantial correlations between EBC and saliva markers in our study may argue against saliva capturing the same processes as EBC. Future studies are needed to further elucidate origins of VEGF and IFN- γ identified in different tissue compartments.

The findings of our study should be interpreted in the light of a number of limitations. First, we did not determine atopy by skin testing or allergen-specific IgE analysis. However, although somatic markers of atopy would have added to the characterization of participants, the utilized self-report measure of allergic rhinitis has been shown to have high sensitivity and specificity (Kilpeläinen et al., 2001). Other studies have also found good agreement between self-reported allergic rhinitis and physicians' diagnoses (Barbee, Kaltenborn, Lebowitz, & Burrows, 1987; Bauchau & Durham, 2004). Self-report of allergic rhinitis has also been the instrument of choice for many epidemiological studies (Bachert, van Cauwenberge, Olbrecht, & van Schoor, 2006;

Bauchau & Durham, 2004; Jones, Smith, Carney, & Davis, 1998; Malone, Lawson, Smith, Arrighi, & Battista, 1997; Nathan, Meltzer, Seiner, & Storms, 1997; Olsson, Berglund, Bellander, & Stjärne, 2003; Price, Zhang, Koccar, Yin, & Thomas, 2005). Nevertheless, employment of multiple criteria for atopic diagnosis including detailed allergy history and identification of sensitization to individual allergens would strengthen future studies. Second, our assays were not able to detect IL-4 in EBC. For quantification of IL-4 concentrations, more sensitive detection methods may be needed. Other studies have relied on concentrating samples (Corradi, Zinelli, & Caffarelli, 2007; Tufvesson & Björner, 2006) or the use of modified assays to increase sensitivity (Robroeks et al., 2007), flow cytometry (Robroeks et al., 2006), or protein array methods (Matsunaga et al., 2006) for improved detection. Overall, success in detecting IL-4 in EBC has been mixed in the past (Matsunaga et al., 2006; Robroeks et al., 2006; Romieu et al., 2008; Shahid et al., 2002; Tufvesson & Björner, 2006). Third, we used salivettes as a method to collect saliva. In order to control for salivary flow, participants were asked to hold the cotton swab for 2 min. This allowed us to control for the effect of saliva volume on cytokine concentrations. A possible problem with this method is that the cotton swabs may become saturated. However, passive drooling and salivettes can detect similar stress-induced changes in immune markers across time such as alpha amylase (Rohleder, Wolf, Maldonado, & Kirschbaum, 2006), and saliva volumes collected using a cotton pledget continue to increase after 3 min (Beltzer et al., 2010).

Another potential limitation of our study is that we did not record alcohol use and changes in diet that may have occurred during the final exam period compared to the low stress baseline. In addition, it was not possible to monitor potential changes in oral hygiene and sources of oral inflammation (gingivitis), but these factors would not have affected EBC parameters, especially after controlling for salivary levels of these parameters. Finally, given the naturalistic nature and the time-sensitive stressors, some participants may have had concomitant colds that could account for the changes in immune parameters. We did not verify infections diagnostically by isolating viruses in nasal washing or by measuring virus-specific HI antibody titers in serum (Cohen, Doyle, & Skoner, 1999), because infection was not the major focus of this study. However, we assessed cold symptoms with a psychometrically validated questionnaire, and controlling for symptoms did not change results substantially.

Despite these limitations, our findings can add to the knowledge on stress-induced immune changes in the airways. Using EBC may provide more intimate insights about immune dynamics in the epithelia of the airways. Future research should expand these findings to antiinflammatory processes such as T reg cells and cytokines such as IL-10 that are also susceptible to stress (Theoharides et al., 2012). More research should explore mucosal immune processes such as VEGF expression in individuals experiencing psychological stress, as elevations in VEGF can lead to the deterioration of many health conditions including atopy and asthma.

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