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IL-10 Deficiency as a Factor in Airway Cellular Responses to Inhaled Sulfur Dioxide

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IL-10 Deficiency as a Factor in Airway Cellular Responses to Inhaled Sulfur Dioxide

By

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Dedication

This work is dedicated to my family, by blood and by choice.

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The University of Texas Medical Branch, 2014

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Abstract: Sulfur dioxide (SO_2) is a problem air pollutant in areas of widespread industrialization, not only in the United States, but also in countries undergoing rapid industrialization, such as China. Thus, air pollution and its effects on the asthmatic condition have become a major focus in environmental research. Asthmatics are known to have: 1) exaggerated bronchospasmic responses in response to SO_2, 2) enhanced airway hyperresponsiveness to reactive oxygen species, and 3) deficient airway IL-10 production. This combination of factors may explain why asthmatics react with greater sensitivity to SO_2 than non-asthmatics. A literature review was undertaken to evaluate the evidence in support of this postulate. While there is indirect support for the postulate, there were few studies which directly tested this possibility and none investigating the role of IL-10 in SO_2-exacerbated asthma. Thus, this study was designed to test the hypothesis that IL-10 deficiency may potentially predispose toward airway SO_2 sensitivity that could manifest itself as increased airway inflammation.

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Chapter 1. INTRODUCTION

Asthma is a pulmonary disease characterized by airway inflammation (AI) and reversible airways obstruction that leads to elevations in airflow resistance and difficulty breathing (Bloemen et al., 2007). In 2005, it was estimated that 300 million people worldwide suffered with asthma with a mortality rate of 250,000 people annually (World Health Organization [WHO], 2007). By 2025, the number of people affected by this disease is expected to grow by more than 100 million, thus reaching approximately 400 million people in total (WHO, 2007).

Previously, asthma was viewed primarily as a disease of airway smooth muscle dysfunction and airway hyperresponsiveness (AHR). However, more recent thinking, investigation, and therapeutic approaches have focused on the significant inflammatory component of this disease. For example, asthmatics are known to be deficient in production of interleukin (IL)-10 (Borish et al., 1996; Calhoun et al., 1996), a major anti-inflammatory cytokine, which may contribute to their inability to resolve AI. However, the consequence of this deficiency in relation to susceptibility to important inhaled environmental asthma triggers, such as sulfur dioxide (SO_2) remains unknown.

One potentially important aspect of the role of IL-10 in the airway response to SO_2 may be its ability to quell inflammation as part of the inflammatory induction and resolution process that may occur with noxious toxicant triggers. This process typically involves an early and late phase immune response, in which pro-inflammatory cytokines are released early, e.g., IL-1β, IL-4, IL-5, IL-13, and TNFα which promotes eosinophilia (Borges et al., 2009; Duramad et al., 2007; Kuo et al., 2001), followed by later release of IL-10, which shuts down the early-phase-dependent inflammation, and decreases eosinophilia (Duramad et al., 2007; Sierra-Filardi et al., 2010). In the case where SO_2 exposure may occur in an asthmatic, it is reasonable to postulate

that a lack of IL-10 production may pre-dispose the asthmatic to a prolonged inflammatory response that does not resolve, and leads to asthma exacerbations. Evidence supporting this idea is presented below, along with experimental data from a number of animal and cellular studies I conducted, to further probe this possibility.

SO_2 as an environmental toxicant

SO_2 is released when sulfur-rich fossil fuel is burned (such as coal or diesel), when metal is extracted from ores, and when gasoline is extracted from oil (Lin et al., 2004). In some locations, a high probability of SO_2 exposure may be confined to the factory area itself and within the vicinity of several square miles. In 2010, the Environmental Protection Agency (EPA) replaced the existing primary SO_2 standards (annual and 24-hour) with a new 1-hour standard set at a level of 75 ppb (**Table 1**).

Year	Averaging Time	Level
1971	24-hr	0.14 ppm
	Annual	0.03 ppm
1996	(Existing 1971 standards retained)	
2010	1-hr	75 ppb
	24-hr	(revoked)
	Annual	(revoked)

Table 1. Evolution of SO_2 primary National Ambient Air Quality Standards, [modified from (US EPA, 2013)]. Averaging time is defined as the "time period established for specific national ambient air quality standards, which must be used when interpreting air quality data." The 1971 standards were revoked in 2010, because they "would not provide additional public health protection, given a 1-hr standard at 75 ppb" (US EPA, 2013).

The National Institute for Occupational Safety and Health (NIOSH) acceptability standards vary from 5 ppm for 15 minutes of SO_2 exposure, to 2 ppm for 10 hours of exposure (**Table 2**). Levels of gaseous SO_2 in polluted urban air can remain as high as 2 ppm, which can still prove to be problematic for those living with asthma. For example, it is known that the odor detection threshold for humans is approximately 2.7 ppm, ranging from 0.33-5 ppm (Brown, 2012; Pohanish, 2004), which means that those suffering from respiratory problems in these industrial areas can live day to day without being aware of their exposure, or knowing about the underlying cause of their lung disease.

Standard	EPA	NIOSH
15 min	-	5 ppm
1 hr	75 ppb	-
10 hr	-	2 ppm
Odor detection threshold: 0.33 – 5 ppm (2.7 ppm avg) Health effects: non-asthmatic (> 2 ppm) : asthmatic (≥ 0.4 – 0.5 ppm)		

Table 2. Comparison of primary National Ambient Air Quality Standards for SO_2, odor detection, and health effects. Standard = recommended time duration; EPA = Environmental Protection Agency; NIOSH = National Institute of Occupational Safety and Health.

Short-term high level exposures to SO_2 gas can cause pulmonary edema, while short-term low level exposures (as low as 0.4-0.5 ppm) can produce bronchoconstriction in asthmatics (Hazardous Substances Data Bank [HSDB], 2012; Lin et al., 2004; Peden, 1997; Schwela, 2000). However, normal (non-asthmatic) humans exposed to an acute, low dose of SO_2 (up to 2.0 ppm), typically do not elicit such a response (Raulf-Heimsoth et al., 2010). Most notably, SO_2 has been

reported to aggravate airway allergic responses to inhaled allergens (D'Amato et al., 2002; Peden, 1997), signifying that it has properties that can be highly detrimental to atopic asthmatics.

Interaction of SO_2 with airborne particles: effect on absorption

SO_2 is highly water-soluble, which means that it typically does not pass the upper respiratory tract, as it is readily absorbed (40-90% of what is inhaled) (WHO, 1979). This inherent "protective" mechanism that filters out SO_2 gas in the upper airway is lost, when the gas comes into contact with fine or ultra-fine particles in the air, and is carried into the airway. For example, in Houston, Texas, the $PM_{2.5}$ (particulate matter with a diameter of 2.5 μm or less) can be up to 40-50% sulfur oxide-based, carried on airborne particulates (Schwela, 2000), which, when inhaled, can be carried deep into the lungs. Upon impact on moist airways, the sulfur-oxide-bearing particulates come into contact with water, producing highly acidic solutions that can deeply penetrate into sensitive areas of the lung, thereby damaging cell membranes and inducing oxidative stress responses. Importantly, environmental pollutant triggers such as SO_2 are known to promote oxidative stress and AI in asthmatics, and in animal models, but there are no data available regarding how SO_2 susceptibility may be promoted by an inability to mount a significant IL-10 response in the airway.

Metabolism of SO_2 in the body

Although the respiratory tract is a primary target for SO_2 gas to exert its toxic effects, other organ systems can also be affected when this gas enters the systemic circulation, via the

bloodstream (National Research Council [NRC], 2002). Due to its high water solubility, hydration of SO_2 results in the formation of sulfite (SO_3^{2-}) and bisulfite (HSO_3^-) anions (Calabrese et al., 1981; Gunnison et al., 1987; NRC, 2002). These ions can then be oxidized in the plasma, forming protein S-sulfonates (NRC, 2002). Elevated protein S-sulfonate levels in plasma, as well as in nasal airway lavage fluid (NALF), have been shown to positively correlate with SO_2 levels in the air, which makes these protein species good indicators of SO_2 exposure (Bechtold et al., 1993; Gunnison and Palmes, 1974; Gunnison and Palmes, 1978).

The mitochondrial enzyme sulfite oxidase detoxifies bisulfite, which is excreted in the urine as inorganic sulfate (Calabrese et al., 1981; NRC, 2002). Studies in sulfite oxidase-deficient animals have proven to be important in deciphering the role that sulfites play in organ toxicity, as well as indicating how cellular defense mechanisms can become overwhelmed. Izgut-Uysal et al., showed that the phagocytic and chemotactic functions of peritoneal macrophages of normal rats were increased following exposure to sulfite, but were greater in those from the sulfite oxidase-deficient rats (Izgüt-Uysal et al., 2005). In 1987, Gunnison et al. observed higher concentrations of sulfite in those rats lacking sulfite oxidase, compared to those animals competent in the enzyme, which did not bio-accumulate sulfite in their plasma, following SO_2 exposure (Gunnison et al., 1987). Given that asthmatics are known to be highly sensitive to SO_2 and therefore, sulfite, one could speculate that they might have a deficiency in the sulfite oxidase detoxification enzyme (Acosta et al., 1989), but this has yet to be proven, and might be an important area of future work.

Animal models of SO_2 exposure

A number of animal studies, notably those of guinea pigs and mice, have investigated the effects of SO_2 on the airways (**Table 3**). A study in guinea pigs found that inhalation of SO_2 (200-300 ppm for 4 hr/day over 4 days) induced AI and enhanced sensitivity to histamine, which could be credited to elevations in ROS (Misawa and Nakano, 1993). Another study in guinea pigs showed an enhancement in the development of allergen-induced asthma following repeated exposures to low levels of SO_2 (0.1 ppm for 5 hr/day over 5 days) (Park et al., 2001). In that study, SO_2-exposed animals had increased enhanced pause (Penh; a measure of enhanced airway responsiveness, *in vivo*), increased BALF eosinophil counts and inflammatory cell infiltration into the lung parenchyma, as well as damage to the bronchiolar epithelium (Park et al., 2001). Inhalation of SO_2 (8.4-42.7 ppm) over a longer period of time in mice (6 hr/day over 7 days) resulted in lipid peroxidation and a decrease in lung anti-oxidant levels (Meng et al., 2003). A later study by the same group indicated that, as a result of SO_2 inhalation, the sulfite content in the lung was higher than that in the heart or brain, for example, which might be explained by the fact that the lung is exposed to the SO_2 first (as a first pass organ), or perhaps the enzymatic action of sulfite oxidase is more efficient in the heart, brain, liver, and kidney, as compared to the lung (Beck-Speier et al., 1985; Cabré et al., 1990; Maier et al., 1999; Meng et al., 2005a; WHO, 2000). Measurement of cytokine levels in the lungs of those SO_2-exposed mice also showed a significant skewing of the pro-inflammatory/anti-inflammatory balance toward pro-inflammatory (Meng et al., 2005b). A fairly recent key study in BALB/c mice (typically considered strong AI responders) investigated the effect of acute induction of AI by inhaled SO_2 (50 ppm for 1 hr/day over 3 days), followed by inhalation of ovalbumin, which resulted in a subsequent induction of chronic allergic AI (Cai et al., 2008). This acute SO_2 exposure model, accompanied by an

allergen trigger, exemplified that the exposure to SO_2 promoted a significant enhancement in the AI response (Cai et al., 2008). A similar finding was reported in another BALB/c model utilizing a house dust mite allergen (Lin et al., 2011). Thus, evidence has shown that allergic AI is highly exacerbated by SO_2 inhalation, and is coupled with changes in ROS levels, pro-inflammatory versus anti-inflammatory balance, and anti-oxidant responses. These findings would presumably have important ramifications for individuals deficient in IL-10, such as asthmatics.

Animal Model	Strain	Study	Observations
Guinea Pig	Hartley, Dunkin-Hartley	Misawa and Nakano 1993	SO_2 induced AI and enhanced sensitivity to histamine due to elevations in ROS
		Park et al. 2001	SO_2 inhalation increased Penh, BALF eosinophil counts, and infiltration of inflammatory cells; damaged epithelium
Mouse	Kungming albino	Meng et al. 2003	SO_2 inhalation induced lipid peroxidation and decreased anti-oxidant levels
		Meng et al. 2005a, Meng et al. 2005b	Sulfite levels were higher in the lung compared to other organs following SO_2 inhalation (lower sulfite oxidase levels?); cytokine levels showed a shift toward pro-inflammatory
	BALB/c	Cai et al. 2008, Lin et al. 2011	Exposure to SO_2 promotes an enhancement in the AI response
Rat	Wistar	Li et al. 2007, Li et al. 2008	OVA compounded with SO_2 enhanced mRNA and protein levels of EGF, EGFR, COX-2, MUC5AC, and ICAM-1 to a greater degree than allergen alone
		Yun et al. 2011	SO_2 exposure increased levels of TNF-α, IL-1β, ICAM-1, and iNOS mRNA
		Xie et al. 2009	SO_2 challenge inhibited expression of p53 and bax, while the expression of bcl-2 was promoted
		Bai and Meng 2005, Yun et al. 2011	SO_2 exposure increased bax mRNA levels, while levels of bcl-2 remained unchanged
		Qin and Meng 2005	SO_2 inhalation suppressed the expression of CYP1A1 and CYP1A2

Table 3. SO_2 experiments in animal models. Penh = enhanced pause, IL-1β = interleukin-1 beta, iNOS = inducible nitric oxide synthase.

Lungs and airways of rats have also been studied to elucidate the effects of SO_2 on gene expression related to asthma and apoptosis, as well as xenobiotic-metabolizing cytochome P450s. For example, studies by Li et al., measured mRNA and protein levels of MUC5AC, ICAM-1, EGF, EGFR, and COX-2 in allergen (OVA)-exposed, SO_2-exposed, and OVA+SO_2-exposed male Wistar rats (Li et al., 2007b; Li et al., 2008). Compared to control rats, OVA alone significantly increased mRNA and protein levels of these asthma-related genes, while OVA compounded with SO_2 (2 ppm for 1 hr/day over 7 days) enhanced the mRNA and protein levels of MUC5AC, ICAM-1, EGF, EGFR, and COX-2 to a greater degree than allergen inhalation by itself (Li et al., 2007b; Li et al., 2008). Yun et al., observed increased levels of TNF-α, IL-1β, ICAM-1 and iNOS mRNA in their male Wistar rat model of SO_2 exposure (2.7-10.7 ppm for 6 hr/day over 7 days) (Yun et al., 2011). Another study using the same strain of rats showed that the expressions of pro-apoptotic genes (p53 and bax) were inhibited by SO_2 challenge (2 ppm for 1 hr/day over 7 days), while the expression of an anti-apoptotic gene (bcl-2) was promoted (Xie et al., 2009). On the other hand, two independent SO_2 exposure studies (encompassing the range of 2.5-20 ppm for 6 hr/day over 7 days) in male Wistar rats illustrated increases in bax mRNA levels in the lung, while bcl-2 mRNA levels remained the same (Bai and Meng, 2005; Yun et al., 2011). The reason for the discrepancy in those two studies could be related to the concentration of SO_2 used, or perhaps due to the fine balancing act that occurs between pro- and anti-apoptotic genes, in a diseased lung versus a non-diseased lung (Abdulamir et al., 2008). Finally, Qin and Meng observed suppression of the expression of CYP1A1 and CYP1A2 in the lungs of rats following SO_2 exposure (5.3-21 ppm for 6 hr/day over 7 days) (Qin and Meng, 2005), suggesting a potential metabolic or oxidative effect that may be important. Taken together, these gene expression data might be indicative of a possible mechanism by which SO_2 encourages and

maintains an inflammatory status in the asthmatic lung, while the cytochome P450 data might indicate a mechanism by which SO_2 attempts to decrease cell damage within the normal lung.

Generation of ROS/RNS following SO_2 exposure

Asthma is an inflammatory disease known to be associated with the generation of ROS as a consequence of ROS-producing leukocytes, most notably eosinophils, neutrophils, and macrophages, recruited to the sites of inflammation and/or injury in the airways (**Figure 1**) (Suzuki et al., 2008).

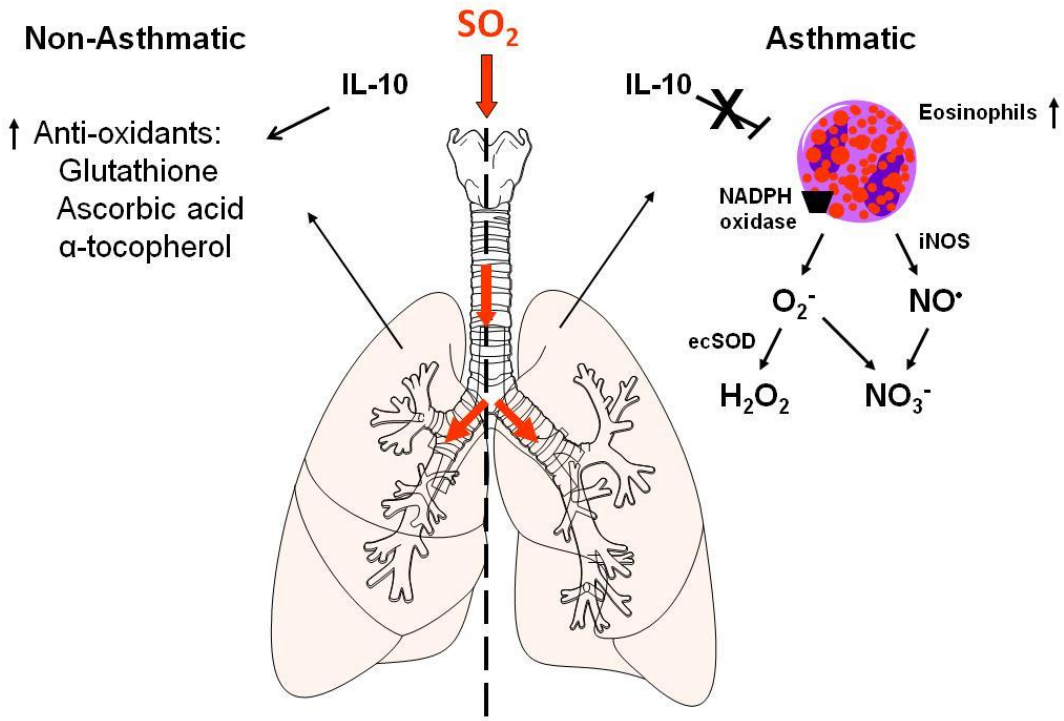

Figure 1. Oxidative stress responses in the lung, [adapted from reference (Bowler, 2004)].

Airway leukocytes also release a wide range of enzymes involved in inflammation. One enzyme implicated in the formation of ROS in the asthmatic lung following SO_2 exposure is NADPH oxidase. A study conducted by Beck-Speier et al. (1993) examined the effects of low concentrations of sulfite (0.01-1 mM) on human neutrophils and found that NADPH oxidase activity was significantly increased when compared to control cells (Beck-Speier et al., 1993). Neutrophils are known to have an inherently decreased activity of sulfite oxidase, leaving them vulnerable to the effects of sulfite (Beck-Speier et al., 1985). Kienast et al., exposed human alveolar macrophages and peripheral blood mononuclear cells to 0.3-1.5 ppm SO_2 for 30 minutes and 120 minutes and concluded that vast amounts of ROS were produced following activation of these cell types by SO_2 (Kienast et al., 1994). It has been shown that superoxide production can be triggered by sodium sulfite on its own (Labbé et al., 1998), and the increase seen in levels of NADPH oxidase can be circumvented, upon addition of superoxide dismutase (SOD) (Beck-Speier et al., 1993). A study utilizing rat basophilic leukemia cells pretreated with diphenyleneiodinium (DPI; an inhibitor of NADPH oxidase) showed a 50% inhibition of sulfite-induced ROS formation (Collaco et al., 2006). Thus, cellular NADPH oxidase has been implicated as a crucial enzyme responsible for the oxidative response upon challenge with sulfite (Collaco et al., 2006), and may have ramifications for the effects of SO_2 in the asthmatic lung.

Potential relevance of IL-10 in countering SO_2 airway effects

Despite prior investigation into the effects of SO_2, the question remains as to why non-asthmatics are less susceptible to the effects of SO_2, while asthmatics seem to be highly responsive to SO_2 with exacerbations of AI. As shown in **Figure 2**, it is clear that oxidative

stress is an important driver of AI, and that SO_2 promotes ROS production in the lung which can drive AI.

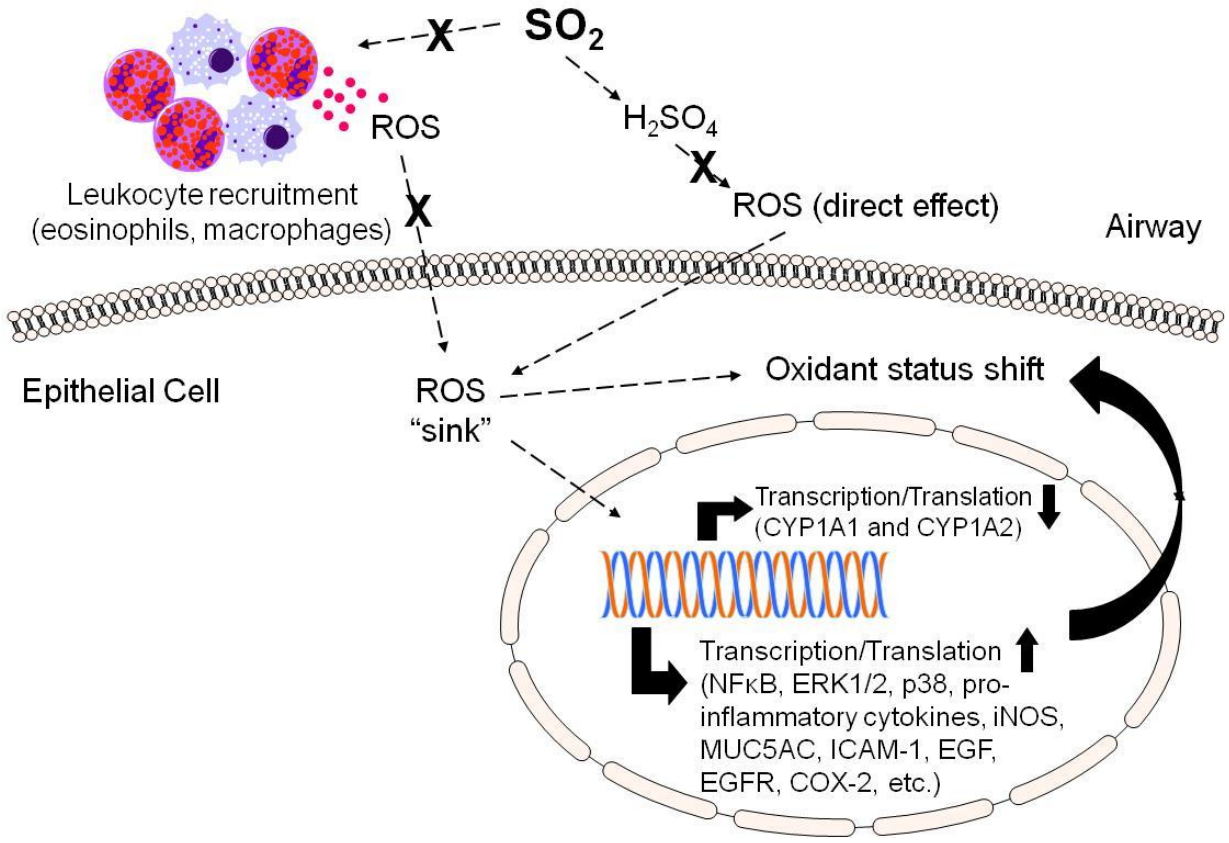

Figure 2. Schematic of SO_2 cellular mechanisms. Effects of leukocyte recruitment in the airway following SO_2 exposure, as well as effects of SO_2 itself, are shown. ROS, as a direct product from SO_2 exposure or via secretion from recruited leukocytes, 1) promotes an oxidant status shift within the epithelial cell and 2) modulates gene and protein levels, which feed back into the oxidant status shift within the epithelial cell. X = site of possible IL-10 inhibitory effects.

While anti-oxidants may afford some treatment or protection from ROS-induced oxidative stress, it is also known that anti-inflammatory treatments that quell AI, such as steroid administration, can elevate levels of IL-10 (Dao Nguyen and Robinson, 2004; Peek et al., 2005; Stelmach et al., 2002), which can contribute to the resolution of AI. This resolution would include reduction of

trafficking leukocytes and particularly, eosinophils, as well as attendant cytokine and chemokine production. The shift of this balance away from pro-inflammatory, and toward anti-inflammatory, is critical to maintaining a homeostatic environment within the lung, following exposure to an environmental toxicant, such as SO_2. However, the role of IL-10 in the airway response to SO_2 remains poorly studied.

Key animal studies cited above have provided some suggestions regarding the importance of inflammatory induction and resolution with exposure to SO_2. However, a common limiting characteristic of the prior studies is that none have been attempted in a model devoid of IL-10, which presumably would be highly relevant to the case of asthma (Borish et al., 1996). While published IL-$10^{-/-}$ mouse studies have identified that a lack of IL-10 results in enhancement of AI (Tournoy et al., 2000; Vissers et al., 2004), which is associated with increased airway iNOS mRNA and iNOS protein (Ameredes et al., 2001), as well as increased IL-4 levels (i.e., a predominance of the Th-2 adaptive immune reaction) (Ameredes et al., 2005; Justice et al., 2001; Mäkelä et al., 2000), none have been performed to determine whether a lack of IL-10 predisposes toward an increased AI and ROS response to SO_2. This shortcoming in our understanding could be important, due to the fact that SO_2 inhalation has been implicated in the production of ROS/RNS within the lung, as outlined above, which is suggestive that some anti-oxidant therapeutic treatment or pre-treatment, or a treatment that enhances IL-10 production, might be beneficial to asthmatics exposed to SO_2.

Some possible hints as to the potential importance of IL-10 in SO_2-exacerbated asthma include that select therapeutic interventions are known to increase endogenous levels of IL-10 (Ogawa et al., 2008). Regulatory T cells (Tregs; $CD^{4+}CD^{25+}$ phenotype), including the inducible type 1 Treg (Tr1), can be utilized for immunotherapy against allergen-sensitivity (Ogawa et al.,

2008). Here, IL-10 production is upregulated via Tr1 cells and immune tolerance is conferred (Lou et al., 2012; Till et al., 2004; Wei et al., 2010). Heme oxygenase, the enzyme that catalyzes the breakdown of heme, is a probable candidate at the center of this phenomenon. The inducible isoform of heme oxygenase (HO-1) is characterized by its ability to protect the cells of the airways from ROS damage via anti-inflammatory and anti-oxidative processes involving increased secretion of IL-10 by Tregs and the overall promotion of Treg cell numbers (Almolki et al., 2004; Ryter and Choi, 2005; Xia et al., 2007, 2006). Therefore, it would seem to be important to retain the functionality of Tregs in the airways, such that specific immunotherapy (SIT) would be a therapeutic possibility to be used as a means to reverse the detrimental effects of SO_2-exacerbated asthma. In all, there appears to be some evidence that asthmatics may be sensitive to SO_2, in part due to their inability to make significant amounts of IL-10, and that therapies targeted toward enhancing or restoring this capability might be beneficial. However, our current state of knowledge of this relationship is minimal, and requires further research.

Summary

This literature review has pointed out a potential role that IL-10 might play in SO_2-exacerbated asthma, but there are few studies of its effects under controlled experimental conditions. Asthmatics are known to have a greater sensitivity to SO_2 than non-asthmatics, but the exact reasons are yet to be fully understood. Preliminary IL-$10^{-/-}$ mouse studies have shown some promise as an investigative tool, in that this model is relevant to the case of asthma, where those people with the disease are deficient in the ability to produce this crucial anti-inflammatory cytokine within the lung. Of relevance, studies have shown that ROS/RNS production within the

lung is increased following SO_2 exposure, which can be reduced with anti-oxidant administration. Consideration of these data suggest that SO_2 may affect asthmatics at lower concentrations than non-asthmatics, due to their inability to produce IL-10 in the airway and to mount a significant counter-regulatory response, e.g., an anti-oxidative response, in reaction to an ROS challenge, secondary to SO_2 exposure. These considerations also support the idea that therapies or treatments that either provide anti-oxidants directly, or bolster the anti-oxidant potential of the airways could be beneficial for those asthmatics exposed to SO_2. However, as a first step to understanding the potential role of IL-10 in the airway response to SO_2, I felt it to be important to determine whether a lack of IL-10 would modify the response to SO_2 in the setting of AI, and therefore conducted the experiments described below.

Hypothesis

Based upon the current knowledge in the field presented above, my central hypothesis is that IL-10 deficiency may potentially predispose toward airway SO_2 sensitivity that could manifest itself as increased airway inflammation. My central hypothesis can be further broken up into several sub-hypotheses, as follows:

1) SO_2 exposure in the setting of AI and IL-10 deficiency enhances the eosinophilic response within the lung, thereby modifying the overall makeup of trafficking airway leukocytes; addition of recombinant IL-10 should decrease this eosinophilia.

2) SO_2 inhalation in the setting of AI and IL-10 deficiency upregulates the production of NO within the airway (measured as nitrite) or the lung (measured as iNOS); addition of recombinant IL-10 should decrease the production of NO within the lung.

3) Exposure to SO_2 in the setting of AI and IL-10 deficiency alters the airway oxidative status toward a relative pro-oxidant milieu, which can be re-established when recombinant IL-10 is added back.

4) SO_2 inhalation in the setting of AI and IL-10 deficiency has a measurable effect on modifying the levels of airway Th2 eosinophil-associated cytokines; addition of recombinant IL-10 reestablishes the airway cytokine signaling.

Chapter 2. EXPERIMENTAL DESIGN AND METHODS

Reagents

Albumin from chicken egg white (grade V), 0.9% endotoxin-free sodium chloride (NaCl) solution, Griess reagents [N-(1-Naphthyl)ethylenediamine dihydrochloride (NED), sodium nitrite, and sulfanilamide], phenylmethanesulfonyl fluoride (PMSF), ethanol, lipopolysaccharides (LPS) from *Escherichia coli* 0111:B4, diethyl pyrocarbonate (DEPC), phosphoric acid (85 wt % solution in water, 99.99% trace metal basis), NaCl, ethylenediaminetetraacetic acid disodium salt dehydrate (EDTA), ethylene glycol-bis(2-aminoethylether)-N,N,N',N'-tetraacetic acid (EGTA), Triton X-100 solution, sodium pyrophosphate, β-glycerolphosphate, sodium orthovanadate, and leupeptin hydrochloride were from Sigma-Aldrich Corp. (St. Louis, MO). Bovine serum albumin (BSA) standard (2.0 mg/mL) and CytoRich Red Collection Fluid were from Thermo Fisher Scientific, Inc. (Rockford, IL). Dulbecco's Modification of Eagle's Medium (DMEM, with 4.5 g/L glucose and sodium pyruvate, but without L-glutamine and phenol red), and Dulbecco's phosphate-buffered saline (PBS) were from Cellgro (Manassas, VA). Fetal bovine serum (FBS), L-glutamine, and penicillin/streptomycin (Pen/Strep) were from Gibco BRL (Grand Island, NY). Camco Stain Pak was from Cambridge Diagnostic Products, Inc. (Fort Lauderdale, FL). Permount Mounting Medium was from Electron Microscopy Sciences (Hatfield, PA). Recombinant mouse interferon-gamma (IFNγ), recombinant mouse IL-10 (rmIL-10), and anti-mouse IL-10 (anti-mIL-10, clone JES052A5) were from R&D Systems, Inc. (Minneapolis, MN). Heparin sodium injection, USP was from Baxter Healthcare Corp. (Deerfield, IL). Tris (hydroxymethyl) aminomethane (Tris) was from IBI Scientific (Peosta, IA). Isoflurane, USP was from Piramal Healthcare Limited (Andhra Pradesh, India). Aluminum hydroxide (alum) was from Acros Organics (Geel,

Belgium). Five, 50, and 500 ppm sulfur dioxide tanks balanced with nitrogen were from Air Liquide (La Porte, TX). Breathing quality air was from UTMB Materials Management (Galveston, TX).

Animals

Male C57BL6 and IL-10 knockout (IL-10 $^{-/-}$) mice were obtained at 6-8 weeks of age (The Jackson Laboratory) and housed in a secure animal facility. Mice were allowed to age to 8-10 weeks and were subjected to experimental protocols described below. All procedures and protocols utilized in these studies were approved by the UTMB Institutional Animal Care and Use Committee (IACUC).

Airway Inflammation

The inflammation protocol used to induce mild/moderate airway inflammation (AI) included two OVA/alum (in saline) sensitizations (0.5 mL, i.p., 50 µg/mL), on days 0 and 7, followed by three intranasal challenges with OVA (10 µL/nostril, 50 mg/mL, one on day 14, one on day 15, and one on day 16), as illustrated in Figure 3. Intranasal challenges were administered to isofluorane-anesthetized mice. Another group of mice had OVA injected i.p., as described above, followed by intranasal challenge with PBS. Response to allergen was assessed in sample subsets of mice (n = 9-10 mice/subset) on day 17 (24 hours after last intranasal challenge).

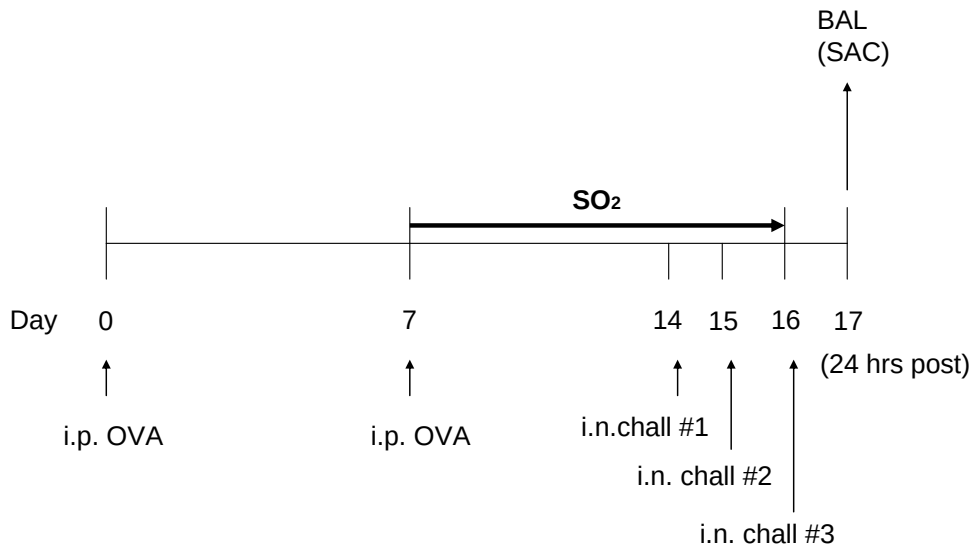

Figure 3: Experimental Timeline.

There are several levels of control for the effects within this model. One is the naïve control, which are mice to which no experimental treatments or allergen have been given; typically these are age-matched with experimental animals (8-15 weeks of age), to keep the effects of aging consistent within the model. The OVA-sensitized, sham-saline intranasal challenge group served as the control, which allowed for the assessment of the effect of intranasal OVA in producing the desired level of AI.

Inhaled SO_2 Exposures

Through the use of the UTMB Inhalation Facility, numerous mice (up to 30) can be simultaneously exposed to desired mixtures of inhaled SO_2 (using a 5, 50, or 500 ppm SO_2 stock cylinder) within a specially-designed cage that fits into a sealed acrylic gas delivery chamber. For the purposes of this dissertation, focus will be on one SO_2 concentration (1 ppm; diluted from stock cylinder with breathing quality air), and on one timing window of SO_2 exposure. The time window explored was that of SO_2 "sensitization/concurrent" exposure, in which mice in the

midst of AI-sensitization inhaled SO_2 starting the second week i.p. OVA injections were administered, and continued inhalation of SO_2 through the intranasal challenge series, subsequently ending with sacrifice of the animals (6-hr exposure/day/10 days; 60 hrs total). This approach allowed us to determine whether an enhancement of sensitization occurred within our model, and more importantly, whether the enhancement was exaggerated in the absence of IL-10 (e.g., in the IL-$10^{-/-}$ mice).

IL-10 Addition and Subtraction

For the IL-10 addition experiment, recombinant murine IL-10, at 0.5 µg/day over 3 days (1.5 µg total concentration), was administered intranasally (10 µL/nare) to IL-$10^{-/-}$ mice concurrently with OVA challenge, to determine whether IL-10 replacement therapy would inhibit the SO_2-exacerbation of AI. This protocol was adapted from a study that utilized intranasal instillation of IL-10 (0.01, 0.03, 0.1 and 0.5 µg) into Balb/c mice to determine if IL-10 regulates antigen-induced cellular infiltration into the airways (Zuany-Amorim et al., 1995).

For the IL-10 subtraction experiment, neutralizing levels of murine IL-10 antibody at 12 µg/day over 3 days (36 µg total concentration for an average 25-gram mouse) were given intranasally (10 µL/nare) to IL-10-sufficient (C57BL6 wild-type) mice, to determine whether a functional knockout of IL-10 would respond in a similar fashion, as those of the genetic IL-$10^{-/-}$. This protocol was adapted after prior IL-10 antibody experiments conducted in our laboratory utilizing mini osmotic pumps (100 µg/kg/day over 14 days) in C57BL6 mice (Ameredes et al., 2005).

Bronchoalveolar Lavage (BAL) Supernatant and Cell Isolation

Mice were anesthetized with isofluorane and killed by rapid cervical dislocation, followed immediately by open-chest BAL using sterile, endotoxin-free normal saline to collect airway cells from both lungs. The volume of saline used was 0.5 mL for the initial BAL, for later assay of proteins, followed by four 1-mL volumes, to insure complete collection of airway leukocytes. The samples were centrifuged (4°C, 1300 rpm, 10 min), the supernatant was collected, aliquoted, and frozen, and the cells were isolated. An aliquot of the initial BAL was taken and cell counts made using differential staining, with counts made on 300 cells/slide. Cell numbers were expressed as number of cells per body weight of mouse, in order to normalize for size differences among mice. BAL macrophage, lymphocyte, neutrophil, and eosinophil numbers were utilized as a marker of AI.

BAL Cell Culture

Twenty-four hours after the last intranasal challenge and/or SO_2 exposure, BAL was performed as described above. Unfractionated BAL cells were used in order to maintain macrophage numbers and viability. This procedure also allowed for the airway cell population to remain in its normal state, thus providing a more relevant physiologic picture of cell signaling and interactions present due to the allergic treatment induced. The cells were immediately centrifuged (4°C, 1300 rpm, 10 min), washed, isolated, and placed in a 96-well polystyrene tissue culture plate (Sarstedt, Inc., Newton, NC) with DMEM, 10% FBS, L-glutamine, and penicillin/streptomycin. Cell culture plating density was targeted to be approximately 300,000-500,000 cells/well in a total volume of 250 µL/well. The plate was then transferred to a humidified incubator (5% CO_2 at 37°C) for 24 hours. The conditioned media was collected after

24 hours, aliquoted, snap-frozen (-80°C), and later assayed. Conditioned media from unstimulated cells, and cells treated with LPS (1000 ng/mL) and IFN-γ (100 U/mL) was collected. All samples were assayed in duplicate.

Griess Assay: Nitric Oxide Measurement

Nitrite (NO_2^-) levels in the initial BAL fluid sample, as well as the collected conditioned media after cell culture, were measured as an index of NOS activity, using a modification of the standard Griess assay (Ameredes et al., 2001; Zamora et al., 1997). The principle of the test was a chemical diazotization reaction utilizing sulfanilamide and NED under acidic conditions, resulting in the detection of nitrite in the sample. The resultant colorimetric reaction was assessed at 550 nm using a Vmax kinetic ELISA microplate reader (Molecular Devices, Sunnyvale, CA) that converted optical density (OD) values to concentrations of nitrite. A standard curve was made for known nitrite concentrations from 0.5 to 64 µM, where the lower limit of reliable detection fell near 0.5 (0.25) µM. Therefore, sample values < 0.5 (0.25) µM were considered to be beneath the limit of detectability of the assay. Final nitrite values were expressed per one million cells, for normalizing BAL nitrite production across mice of varying sizes.

Pro-Oxidant/Anti-Oxidant Balance (PAB) Assay: Oxidative Stress Measurement

The PAB assay for oxidant balance was performed by an assay method modified after one already published (Alamdari et al., 2008). This assay simultaneously measures both pro-oxidants and anti-oxidants using 3,3′,5,5′-tetramethylbenzidine (TMB) and its cation, as a redox indicator participating in two simultaneous reactions. A standard curve was made using varying concentrations of hydrogen peroxide and uric acid, and the results expressed as arbitrary HK

(Hamidi and Koliakos, inventors of the method) units, which are the percentage of hydrogen peroxide on the standard curve. The assay was performed in the samples collected from mice after their AI, SO_2, and IL-10 treatments outlined above.

Cytokine Analysis

MILLIPLEX MAP Kit

Protein levels for the following cytokines were measured in BALF and lung homogenates utilizing the Millipore MILIPLEX MAP kit (Billerica, MA): eotaxin, IL-1β, IFNγ, TNFα, IL-4, IL-5, RANTES, and GM-CSF. The kit relied on Luminex xMAP technology whereby the analytes of choice were captured by fluorescent-coded magnetic beads, subsequently forming a biotinylated detection antibody. Incubation of this reaction mixture with a reporter molecule (Streptavidin-PE conjugate) completed the reaction on the surface of each magnetic bead. The beads were then quickly passed through two lasers, one which excited the dyes within the magnetic beads and the other which excited PE, the fluorescent dye on the reporter molecule. A high throughput processor finally identified each magnetic bead and the results were quantified by fluorescent reporter signals via a standard curve (0.6 to 2,000 pg/mL). The sensitivity, or minimum detectable concentration, of the assay for each analyte was as follows: eotaxin (1.8 pg/mL), IL-1β (5.4 pg/mL), IFNγ (1.1 pg/mL), TNFα (2.3 pg/mL), IL-4 (0.4 pg/mL), IL-5 (1.0 pg/mL), RANTES (2.7 pg/mL), and GM-CSF (10.9 pg/mL). BALF and lung homogenate results were normalized to protein concentration (in pg/mg), as determined by BCA protein assay (Thermo Fisher Scientific, Inc., Rockford, IL).

Statistical Analyses

The number of mice for each experiment typically ranged between 6-12 mice; power analyses (β=0.80 at P<0.05) indicated that numbers of 6-12 mice would allow us to discern statistical differences, if present, in our measured variables (Snedecor and Cochran, 1989). One-way analysis of variance (ANOVA; SigmaPlot version 12.3) was used to assess all measured variables. If the normality test or equal variance test failed, a one-way ANOVA on Ranks was performed. Experimental samples were performed in duplicate. When applicable, *post-hoc* analysis was performed using either the Student-Newman-Keul's (SNK) or Dunn's test, with values of P<0.05 considered significant. For the nitrite BALF and conditioned media data, groups having no detectable levels were set at one-half the detectable level (i.e., 0.25 μM) for subsequent graphical display and statistical comparisons. For the iNOS lung homogenate data, groups having no detectable levels were set to one-half the detectable level (i.e., 0.25 pg/mL) for subsequent graphical display and statistical comparisons. For the Milliplex BALF and lung homogenate data, groups having no detectable levels were set to one-half the detectable level for each cytokine tested for subsequent graphical display and statistical comparisons. These levels were as follows: eotaxin (0.9 pg/mL), IL-1β (2.7 pg/mL), IFNγ (0.55 pg/mL), TNFα (1.15 pg/mL), IL-4 (0.2 pg/mL), IL-5 (0.5 pg/mL), RANTES (1.35 pg/mL), GM-CSF (5.45 pg/mL).

Chapter 3. RESULTS

Leukocyte Differential Shifts

Total Cell Counts. Inhalation of SO_2 by IL-10-sufficient C57BL6 wild-type mice significantly increased total cell counts in the setting of AI (0.86 ± 0.07 vs. 1.10 ± 0.08 cells x10^4/g mouse), but were no different from AI+SO_2 in the IL-$10^{-/-}$ mice (1.09 ± 0.05 cells x10^4/g mouse), as shown in **Table 4**. The replacement of IL-10 in the IL-10-deficient mice did not alter total cell counts, which suggests that either: 1) administered IL-10 cannot modify total cell counts in this experimental scenario, or, possibly more likely, 2) the concentration of IL-10 that I used was unable to overcome the cellular infiltration resulting from AI induction. However, an important set of findings was obtained outside of the setting of AI, in the naïve mice experiments, wherein total BAL cell counts were not increased with SO_2 inhalation in the IL-10-sufficient C57BL6 wild-type mice (0.78 ± 0.04 vs. 0.74 ± 0.03 cells x10^4/g mouse), but were elevated in the IL-10 knockout mice inhaling SO_2 (0.75 ± 0.06 vs. 0.97 ± 0.10 cells x10^4/g mouse; $P<0.05$), and then subsequently significantly decreased by approximately 50% with administration of IL-10 (0.46 ± 0.01 cells x10^4/g mouse; $P<0.05$), back to a level similar to control naïve IL-10 knockout values (0.75 ± 0.06 cells x10^4/g mouse). A drop in total cell number was also observed with administration of IL-10 to IL-10-sufficient C57BL6 wild-type mice (0.46 ± 0.03 cells x10^4/g mouse; $P<0.05$), suggesting a potentially important role for IL-10 in decreasing SO_2-associated leukocyte influx into the airway. In addition to these changes in BAL total cell counts, there were other modifications in specific leukocyte numbers, as described below.

BAL lymphocytes. BAL lymphocytes were significantly increased upon SO_2 inhalation in the setting of AI in IL-10-sufficient C57BL6 wild-type (0.21 ± 0.04 vs. 0.47 ± 0.05 cells x10^4/g mouse; P<0.05), while the opposite effect was seen in the IL-10 knockout mice with AI+SO_2 inhalation (0.68 ± 0.06 vs. 0.36 ± 0.03 cells x10^4/g mouse; ~ 50% reduction; P<0.05 **Table 5**). IL-10 administration to IL-10 knockout mice significantly decreased lymphocyte counts in OVA-challenged mice (0.46 ± 0.03 cells x10^4/g mouse; P<0.05). However, with inhalation of SO_2, lymphocyte counts remained significantly increased when IL-10 was given (0.60 ± 0.04 cells x10^4/g mouse; P<0.05), almost returning to counts measured with AI-alone (0.68 ± 0.06 cells x10^4/g mouse), suggesting that IL-10, at the concentration I used, was unable to negate the SO_2-driven increase in BAL lymphocytes, in the setting of AI. However, as with total cells counts reported above, this suggested that the control of trafficking lymphocytes in response to SO_2 is not mediated by a mechanism that is reversible by IL-10, and likewise suggests that asthmatics lacking IL-10 could experience similar lymphocyte infiltration.

An important set of findings was obtained outside of the setting of AI, in the naïve mice experiments, wherein lymphocyte cell counts were not increased with SO_2 inhalation in the IL-10-sufficient C57BL6 wild-type mice (0.33 ± 0.06 vs. 0.32 ± 0.04 cells x10^4/g mouse), but were elevated in the IL-10 knockout mice inhaling SO_2 (0.11 ± 0.05 vs. 0.16 ± 0.02 cells x10^4/g mouse; P<0.05, although the n=3 was low), and then subsequently significantly decreased by approximately 60% with administration of IL-10 (0.06 ± 0.01 cells x10^4/g mouse), to a level below control naïve IL-10 knockout values (0.11 ± 0.05 cells x10^4/g mouse; P<0.05). A significant decrease in lymphocyte cell numbers was also seen with administration of IL-10 to IL-10-sufficient C57BL6 wild-type mice (0.07 ± 0.0 cells x10^4/g mouse; P<0.05), suggesting a

potentially important role for IL-10 in decreasing SO_2-associated lymphocyte influx into the airway.

BAL neutrophils. Similar to BAL lymphocytes, BAL neutrophils were significantly reduced upon SO_2 inhalation in the setting of AI in IL-10 knockout mice (0.08 ± 0.01 vs. 0.01 ± 0.0 cells x10^4/g mouse, approximately 80% reduction; $P<0.05$, but the n=3 was small); BAL neutrophils in the IL-10-sufficient C57BL6 wild-type mice did not change across all sham AI and AI treatments (range of average values = 0.03 – 0.07 cells x10^4/g mouse), as shown in **Table 6**. IL-10 administration did not change neutrophil counts in OVA-challenged mice. With inhalation of SO_2, neutrophil counts in IL-10 knockout mice were significantly increased when IL-10 was given (0.05 ± 0.01 vs. 0.01 ± 0.0 cells x10^4/g mouse; $P<0.05$), almost returning to counts measured with AI-alone (0.08 ± 0.01 cells x10^4/g mouse), suggesting that IL-10, at the concentration I used, was unable to negate the SO_2-driven decrease in BAL neutrophils. Again, this finding suggested that the control of trafficking leukocytes in response to SO_2 is not mediated by a mechanism that is reversible by IL-10, and suggests that asthmatics deficient in IL-10 could experience similar neutrophil infiltration.

Outside of the setting of AI, in the naïve mice experiments, neutrophil cell counts were not increased with SO_2 inhalation in the IL-10-sufficient C57BL6 wild-type mice (0.02 ± 0.01 vs. 0.01 ± 0.0 cells x10^4/g mouse), but were significantly elevated in the IL-10 knockout mice inhaling SO_2 (0.07 ± 0.01 vs. 0.13 ± 0.02 cells x10^4/g mouse; $P<0.05$), and then subsequently significantly decreased by approximately 80% with administration of IL-10 (0.03 ± 0.0 cells x10^4/g mouse; $P<0.05$), to a level below control naïve IL-10 knockout values (0.07 ± 0.01 cells x10^4/g mouse).

BAL eosinophils. Inhaled SO_2 in the setting of AI had no effect on eosinophils when IL-10 was sufficient, but when IL-10 was absent, SO_2 inhalation significantly increased eosinophils (0.17 ± 0.02 vs. 0.41 ± 0.02 cells $x10^4$/g mouse, approximately double; open bar in second bar set from right vs. open bar in first bar set at right; $P<0.05$ **Figure 4**). This increase in eosinophilia in the IL-10-deficient animals was reversible with replacement of IL-10 via intranasal instillation, which significantly decreased eosinophils by approximately 50% (AI; 0.17 ± 0.02 vs. 0.08 ± 0.01 cells $x10^4$/g mouse), and 66% (AI+SO_2; 0.41 ± 0.02 vs. 0.12 ± 0.01 cells $x10^4$/g mouse), in respective treatment groups (open vs. hatched bars in 2 respective bar sets from rightmost side of figure). These findings suggest a critical role of IL-10, in the eosinophilic response to inhaled SO_2, which may be likewise important in asthmatics, known to have airway IL-10 deficiency (Borish et al., 1996).

An important set of findings was obtained outside of the setting of AI, in the naïve mice experiments, wherein eosinophil cell counts were significantly increased with SO_2 inhalation in the IL-10-sufficient C57BL6 wild-type mice (0.03 ± 0.02 vs. 0.16 ± 0.02 cells $x10^4$/g mouse, approximate five-fold increase, as compared to naïve untreated controls at far left; $P<0.05$), as well as in the IL-10 knockout mice (0.02 ± 0.02 vs. 0.10 ± 0.01 cells $x10^4$/g mouse, approximate five-fold increase; $P<0.05$). IL-10 administration significantly decreased eosinophil cell numbers in both C57BL6 wild-type mice (0.16 ± 0.02 vs. 0.02 ± 0.01 cells $x10^4$/g mouse, approximately 90% reduction; $P<0.05$) and IL-10 knockout mice (0.10 ± 0.01 vs. 0.05 ± 0.0 cells $x10^4$/g mouse, approximate 50% reduction; $P<0.05$) to levels at or near control naïve values (0.03 ± 0.02 and 0.02 ± 0.02 cells $x10^4$/g mouse, respectively). These results suggest a potentially important role for IL-10 in decreasing SO_2-associated eosinophil influx into the airway, and perhaps represents one of the most important findings of my study.

BAL macrophages. As one might expect, a differential shift occurred such that BAL macrophages were significantly decreased with the eosinophilia that occurred with inhalation of SO_2, in the case of IL-10 deficiency (range of average values = 0.21 - 0.37 cells $x10^4$/g mouse), suggesting that macrophages may have been reduced in their role within the airway, with inhalation of SO_2 (**Figure 5**). While it was found that NO within the airway was unaltered (as BAL nitrite levels; **Table 7**), it is known that macrophages are a major source of NO in the airway (Ogawa et al., 2008), and thus it is possible that a lack of airway NO was linked to the decrease in BAL macrophages with SO_2 inhalation. It is important to note here that iNOS upregulation in the lung tissue might be expected to produce NO that can affect the contractile tone of airway smooth muscle, also within the lung tissue, and which may not show up as NO within the airway, particularly at early times like 24 hr (Ameredes et al., 2005).

Outside of the setting of AI, in the naïve mice experiments, macrophage cell counts remained unchanged with SO_2 inhalation in both the IL-10-sufficient C57BL6 wild-type mice and IL-10 knockout mice. IL-10 administration significantly decreased macrophage cell numbers in IL-10 knockout mice (0.58 ± 0.08 vs. 0.32 ± 0.0 cells $x10^4$/g mouse, approximate 50% reduction; $P<0.05$), while IL-10 administration in the C57BL6 wild-type mice did not change macrophage cell numbers. These results suggest a potentially important role for IL-10 in modulating SO_2-associated macrophage influx into the airway.

Nitrite Production

Lung homogenate. **Figure 6** indicates that iNOS in lung tissue was significantly upregulated with SO_2 inhalation in the early (24 hr) response to AI in the IL-10-sufficient

C57Bl6 wild-type mice (85.8 ± 6.2 vs. 192.2 ± 19.7 ng/mg total protein, approximately double; $P<0.05$; 3rd black bar from left vs. 4th black bar from left), but was not upregulated in the IL-10-deficient case (3rd and 4th open bars from left, respectively). Although my study did not show an increase in BALF nitrite at 24 hours post-AI-induction, which would be consistent with this idea, it might indicate that nitrite release (or spill-over from the tissue) into the airway occurs at a later time point, e.g. 48 or 72 hours. Previously published BALF nitrite data from the Ameredes lab exemplified this notion that nitrite levels are maximum at 48 hours post-challenge with OVA aerosol for IL-10-sufficient C57BL6 wild-type mice (Ameredes et al., 2005). Replacement of IL-10 via intranasal instillation to IL-10 knockout mice significantly decreased iNOS levels by approximately 50% (AI; 116.7 ± 14.1 vs. 49.8 ± 0.7 ng/mg total protein, approximate 66% reduction; $P<0.05$), and 66% (AI+SO_2; 117.7 ± 18.3 vs. 64.1 ± 4.9 ng/mg total protein, approximate 50% reduction; $P<0.05$), in respective treatment groups, indicating a strong ability of IL-10 to regulate lung iNOS in the setting of AI and AI+SO_2.

Cell culture-conditioned media. As seen in **Table 8**, the significant increases in nitrite levels in cytokine stimulated (LPS and IFN-γ) mouse BAL leukocyte cell cultures versus spontaneous (no cytokine stimulus added) cell cultures indicate the relative AI-induced "priming" of those airway cells, in mice that had received OVA challenge [C57BL6 (10 ± 0.8 vs. 86.7 ± 11.4 μM NO_2^-/million cells; $P<0.05$); IL-10 knockout (12.8 ± 0.7 vs. 75.4 ± 8.8 μM NO_2^-/million cells; $P<0.05$)]. Inhalation of SO_2 in the setting of AI and cytokine stimulation did not further modulate NO production in cultured airway leukocytes from both IL-10-sufficient and IL-10-deficient mice [C57BL6 (86.7 ± 11.4 vs. 68.8 ± 9.1 μM NO_2^-/million cells); IL-10 knockout (75.4 ± 8.8 vs. 57.6 ± 7.8 μM NO_2^-/million cells)], potentially further suggesting no specific effects of SO_2 on leukocyte-associated NO-production within the airway. While these

results indicate that the airway leukocytes are potentially primed to make NO as a result of the induction of AI, the lack of differences in BALF nitrite levels suggest that this mechanism is somehow inhibited, or not active, as a function of SO_2 inhalation. Replacement of IL-10 via intranasal instillation to IL-10 knockout mice did not further alter nitrite levels in conditioned media from spontaneous or stimulated BAL leukocyte cell cultures, which corroborates with the BAL data mentioned above.

Oxidant Balance

As seen in **Figure 7**, ROS assessment indicated that SO_2 inhalation significantly upregulated the relative early (24 hr) pro-oxidant potential of the airway in the IL-10-sufficient C57BL6 wild-type mice with AI (64.0 ± 1.1 vs. 86.9 ± 1.3 HK units/saline control; $P<0.05$; 2nd black bar from right vs. far right black bar), suggesting that SO_2 either increased ROS directly, or it increased the ROS signal from other endogenous sources in the normal airway. However, in the IL-10-deficient IL-10 knockout mice, SO_2 inhalation had the opposite effect, significantly decreasing the relative pro-oxidant status of the airway (82.1 ± 2.9 vs. 65.3 ± 2.8 HK units/saline control; $P<0.05$; 2nd open bar from right vs. far right open bar), which was re-established when recombinant IL-10 was added (65.3 ± 2.8 vs. 121.9 ± 2.3 HK units/saline control; $P<0.05$). IL-10 is implicated in the involvement of the generation of decreased pro-oxidant responses, since removal appeared to allow more pro-oxidants to exist at baseline (far left bars: 74.7 ± 1.5 vs. 121.8 ± 1.7 HK units/saline control**; Figure 7**). However, after a mild inflammatory stimulus, alternative anti-oxidant mechanisms appeared to shift the balance further away from pro-oxidant [C57BL6 (74.7 ± 1.5 vs. 64.0 ± 1.1 HK units/saline control); IL-10 knockout (121.8 ± 1.7 vs.

82.1 ± 2.9 HK units/saline control)], which became even less pro-oxidant when SO_2 was inhaled (similar to levels observed without AI). These findings suggest that SO_2 inhalation may upregulate an ROS signal in the IL-10-sufficient animals, which is absent when IL-10 is lacking, and may likewise be responsible for the difference in response of asthmatics to SO_2 inhalation. Thus, an ROS "driving" signal, which may induce subsequent anti-oxidant protective mechanisms that can detoxify excess oxidant species in the normal case, appears to be lost when IL-10 is absent, and re-established when IL-10 is added. This observation suggests that the oxidant balance in the lung may be critical in the determination of the eosinophilia response to inhaled SO_2 with the IL-10 deficiency in IL-10 knockout mice, and potentially in IL-10 deficient asthmatics.

Modulation of Th-2 Eosinophil-Associated Cytokines: Adaptive Immunity

IL-5. Modulation of IL-5 may be the important AI-driven early signal for the recruitment of eosinophils in my model. As seen in **Figure 8**, IL-5 in lung homogenate can be significantly downregulated by SO_2 when IL-10 is sufficient (16.0 ± 3.3 vs. 5.6 ± 2.2 pg/mg total protein; $P<0.05$), possibly leading to no further eosinophilia in the case of the C57BL6 mice. In the case of IL-10 deficiency, this eosinophilic regulation was somehow overcome, since we observed that SO_2 inhalation significantly increased eosinophils over that with AI alone. This argues for an IL-5-independent mechanism of SO_2-driven eosinophilia, in the IL-10 deficient case, suggesting that inhaled SO_2 may likewise act to increase eosinophilia in asthmatics in an IL-5-independent fashion. As expected, IL-10 administration significantly reduced the AI-induced IL-5 levels in IL-10 knockouts (7.5 ± 0.8 vs. 2.1 ± 0.9 pg/mg total protein; $P<0.05$). However, in the case of

SO_2 inhalation, while IL-10 replacement suppressed BALF eosinophils (**Figure 4**), IL-10 replacement did not significantly alter BALF IL-5 levels (**Figure 9**), further arguing for an IL-5-independent mechanism of SO_2-associated eosinophilia.

Changes in BALF IL-5 appeared to track lung homogenate IL-5, in the experimental groups with induction of AI (**Figures 8 and 9**). In the IL-10-sufficient case, AI significantly increased BALF IL-5 levels (367 ± 75 vs. 6173 ± 1226 pg/mg total protein/kg body weight; **Figure 9**), which was significantly reduced upon SO_2 inhalation (6173 ± 1226 vs. 1550 ± 284 pg/mg total protein/kg body weight). In the IL-10-deficient mice, SO_2 inhalation in the setting of AI significantly decreased BAL IL-5 levels (2769 ± 768 vs. 720 ± 215 pg/mg total protein/kg body weight). IL-10 administration also significantly reduced the AI-induced IL-5 levels in IL-10 knockouts (2769 ± 768 vs. 499 ± 124 pg/mg total protein/kg body weight).

IL-4. Changes in lung homogenate IL-4 appeared to track changes in BALF IL-4 in the C57BL6 mice (**Figures 10 and 11**). AI significantly increased IL-4 levels versus naïve [lung homogenate (0.2 ± 0.0 vs. 3.1 ± 0.8 pg/mg total protein; $P<0.05$; **Figure 10**); BALF (82 ± 15 vs. 243 ± 40 pg/mg total protein/kg body weight; $P<0.05$; **Figure 11**)]; SO_2 inhalation decreased IL-4 levels, but not significantly. Changes seen in lung homogenate IL-4 from IL-$10^{-/-}$ mice did not appear to track similarly to changes in BAL IL-4. In the IL-10-deficient mice, SO_2 inhalation in the setting of AI was not different from AI-alone in the lung, but IL-10 administration did significantly increase IL-4 levels (0.2 ± 0.0 vs. 1.7 ± 0.5 pg/mg total protein; $P<0.05$).

Eotaxin and RANTES. BALF eotaxin in both C57BL6 and IL-$10^{-/-}$ mice did not track changes seen in lung homogenate eotaxin (**Figures 12 and 13**). BALF RANTES in both C57BL6 and IL-$10^{-/-}$ animals tracked changes seen in lung homogenate RANTES (**Figures 14**

and 15) [AI increased RANTES, but only reaching significance in the IL-10$^{-/-}$ mice (BALF: 1489 ± 161 vs. 2543 ± 219 pg/mg total protein/kg body weight; lung homogenate: 9.6 ± 1.0 vs. 28.5 ± 7.5 pg/mg total protein; P<0.05); inhalation of SO_2 decreased RANTES levels, but only reaching statistical significance in the BALF of IL-10$^{-/-}$ mice (2543 ± 219 vs. 630 ± 87 pg/mg total protein/kg body weight; P<0.05)]. BALF eotaxin in both C57BL6 and IL-10$^{-/-}$ animals tracked changes seen in BALF RANTES [AI increased both cytokines, but only to significance for eotaxin in C57BL6 (529 ± 57 vs. 1669 ± 323 pg/mg total protein/kg body weight; P<0.05) and RANTES in IL-10$^{-/-}$ (1489 ± 161 vs. 2543 ± 219 pg/mg total protein/kg body weight; P<0.05); inhalation of SO_2 decreased these levels, but only to significance in the IL-10$^{-/-}$ animals (eotaxin: 2477 ± 627 vs. 763 ± 85 pg/mg total protein/kg body weight; P<0.05; RANTES: 2543 ± 219 vs. 630 ± 87 pg/mg total protein/kg body weigh; P<0.05)], but this was not the case for both cytokines in lung homogenate. Aspects of eotaxin and RANTES tracked changes in IL-5 and IL-4, which agrees with the fact that all are Th2 eosinophil-associated cytokines. In BALF, C57s tracked changes across all treatments for all Th2 cytokines, while eotaxin tracked changes in IL-5 and RANTES tracked changes in IL-4 in IL-10$^{-/-}$. In lung homogenate, RANTES tracked changes seen in IL-5 in C57s, while RANTES tracked changes seen in IL-5 and IL-4 in IL-10$^{-/-}$ animals. Eotaxin did not track changes seen in any other Th2 cytokine in lung homogenate.

These findings all seem to indicate that SO_2 has a measurable effect on modulating Th2 eosinophil-associated cytokines. However, in considering IL-5 as a model Th2 cytokine, the Th2 cytokines do not appear to be the early (by 24 hr) modulators of the SO_2-associated increase in eosinophilia.

Lack of Modulation of Th-1 Cytokines: Innate Immunity

As seen in **Table 9**, there were no significant changes observed in lung homogenate or BALF levels of IL-1β and GM-CSF in IL-10-sufficient C57BL6 and IL-$10^{-/-}$ mice exposed to SO_2 in the setting of AI. IFNγ and TNFα are Th1 cytokines typically associated with macrophage infiltration (Ogawa et al., 2008), and thus it might be expected that their levels might be decreased, given that I saw significant decreases in macrophages with the development of eosinophilia due to SO_2 inhalation in the C57BL6 mice. This was indeed the case for TNFα in BALF from C57BL6 mice (1653 ± 197 vs. 319 ± 25 pg/mg total protein/kg body weight; $P<0.05$). The opposite was found for IFNγ in lung homogenate in IL-$10^{-/-}$ mice (22.9 ± 3.6 vs. 1.0 ± 0.4 pg/mg total protein; $P<0.05$). There is a possibility that the majority of Th1 signals may be increased earlier than 24 hr, when I collected my samples. Thus, within the limits of my model, SO_2 inhalation did not seem to measurably modify cytokines associated with innate immunity, at least in the early time point (at 24 hr) of the inflammatory response. Overall, it appears that innate immunity may be less important in the responses I studied, as it is non-antigen-specific, and may not be upregulated with the acute exposures that I studied.

IL-10 Antibody Administration

The experiments involving IL-10 antibody administration to the IL-10-sufficient C57BL6 animals resulted in no differences, therefore the single dose I tested was ineffective. These experiments were considered failed, and future studies will need to be performed to optimize the dose in order to test the effects of the antibody with certainty.

Tables and Graphs

Total Cells in BALF	C57BL6	IL-10 Knockout
Naïve	0.78±0.04 (6)	0.75±0.06 (4)
Naïve+SO_2	0.74±0.03 (5)	**0.97±0.1 (3)[d,^]**
Naïve+SO_2+IL-10	**0.46±0.03 (5)[a,b]**	**0.46±0.01 (4)[d,e]**
O/S	0.81±0.04 (10)	0.81±0.05 (16)
O/S+IL-10	-	0.71±0.01 (3)
O/S+SO_2	0.99±0.07 (16)	0.87±0.05 (17)
O/S+SO_2+IL-10	-	0.72±0.08 (3)
O/O	0.86±0.07 (12)	**1.14±0.06 (16)[d,f,^]**
O/O+IL-10	-	0.95±0.05 (6)
O/O+SO_2	**1.10±0.08 (14)[c]**	1.09±0.05 (18)
O/O+SO_2+IL-10	-	**1.05±0.05 (5)[g]**

Table 4. Total cells in BALF, C57BL6 (C57) vs. IL-10 Knockouts (k/o). All values are means±SE in WBCs x 10^4/g mouse. a=*P*<0.05 vs. naive C57; b=*P*<0.05 vs. naïve+SO_2 C57; c=*P*<0.05 vs. O/O C57; d=*P*<0.05 vs. naïve k/o; e=*P*<0.05 vs. naïve+SO_2 k/o; f=*P*<0.05 vs. O/S k/o; g=*P*<0.05 vs. naïve+SO_2+IL-10 k/o; ^=*P*<0.05 vs. matched C57; n in parentheses; significant findings are in bold (SNK or Dunn's post-hoc analysis).

Lymphocytes in BALF	C57BL6	IL-10 Knockout
Naïve	0.33±0.06 (6)	0.11±0.05 (4)
Naïve+SO_2	0.32±0.04 (5)	**0.16±0.02 (3)[^]**
Naïve+SO_2+IL-10	**0.07±0.0 (5)[a,b]**	**0.06±0.01 (4)[e,f]**
O/S	0.33±0.02 (10)	**0.38±0.05 (16)[e]**
O/S+IL-10	-	0.15±0.05 (3)
O/S+SO_2	0.38±0.09 (16)	0.45±0.04 (17)
O/S+SO_2+IL-10	-	**0.23±0.1 (3)[i]**
O/O	**0.21±0.04 (12)[c]**	**0.68±0.06 (16)[e,g,^]**
O/O+IL-10	-	**0.46±0.03 (6)[h]**
O/O+SO_2	**0.47±0.05 (14)[d]**	**0.36±0.03 (18)[h]**
O/O+SO_2+IL-10	-	**0.60±0.04 (5)[j,k]**

Table 5. Lymphocytes in BALF, C57BL6 vs. IL-10 Knockouts (k/o). All values are means±SE in Cells x 10^4/g mouse. a=*P*<0.05 vs. naive C57; b=*P*<0.05 vs. naive+SO_2 C57; c=*P*<0.05 vs. O/S C57; d=*P*<0.05 vs. O/O C57; e=*P*<0.05 vs. naive k/o; f=*P*<0.05 vs. naive+SO_2 k/o; g=*P*<0.05 vs. O/S k/o; h=*P*<0.05 vs. O/O k/o; i=*P*<0.05 vs. O/S+SO_2 k/o; j=*P*<0.05 vs. O/O+SO_2 k/o; k=*P*<0.05 vs. naïve+SO_2+IL-10 k/o; ^=*P*<0.05 vs. matched C57; n in parentheses; significant findings are in bold (SNK or Dunn's post-hoc analysis).

Neutrophils in BALF	C57BL6	IL-10 Knockout
Naïve	0.02±0.01 (6)	**0.07±0.01 (4)^**
Naïve+SO_2	0.01±0.0 (5)	**0.13±0.02 (3)c,^**
Naïve+SO_2+IL-10	**0.07±0.01 (5)a,b**	**0.03±0.0 (4)c,d,^**
O/S	0.04±0.01 (10)	0.05±0.01 (16)
O/S+IL-10	-	0.07±0.01 (3)
O/S+SO_2	0.07±0.02 (16)	**0.02±0.0 (17)e,^**
O/S+SO_2+IL-10	-	**0.07±0.0 (3)f,i**
O/O	0.06±0.01 (12)	0.08±0.01 (16)
O/O+IL-10	-	0.08±0.01 (6)
O/O+SO_2	0.03±0.01 (14)	**0.01±0.0 (18)g**
O/O+SO_2+IL-10	-	**0.05±0.01 (5)h**

Table 6. Neutrophils in BALF in C57BL6 vs. IL-10 Knockouts. All values are means±SE in Cells x 10^4/g mouse. a=*P*<0.05 vs. naive C57; b=*P*<0.05 vs. naïve+SO_2 C57; c=*P*<0.05 vs. naive k/o; d=*P*<0.05 vs. naïve+SO_2+IL-10 k/o; e=*P*<0.05 vs. O/S k/o; f=*P*<0.05 vs. O/S+SO_2 k/o; g=*P*<0.05 vs. O/O k/o; h=*P*<0.05 vs. O/O+SO_2 k/o; i=*P*<0.05 vs. naive+SO_2+IL-10 k/o; ^=*P*<0.05 vs. matched C57; n in parentheses; significant findings are in bold (SNK or Dunn's post-hoc analysis).

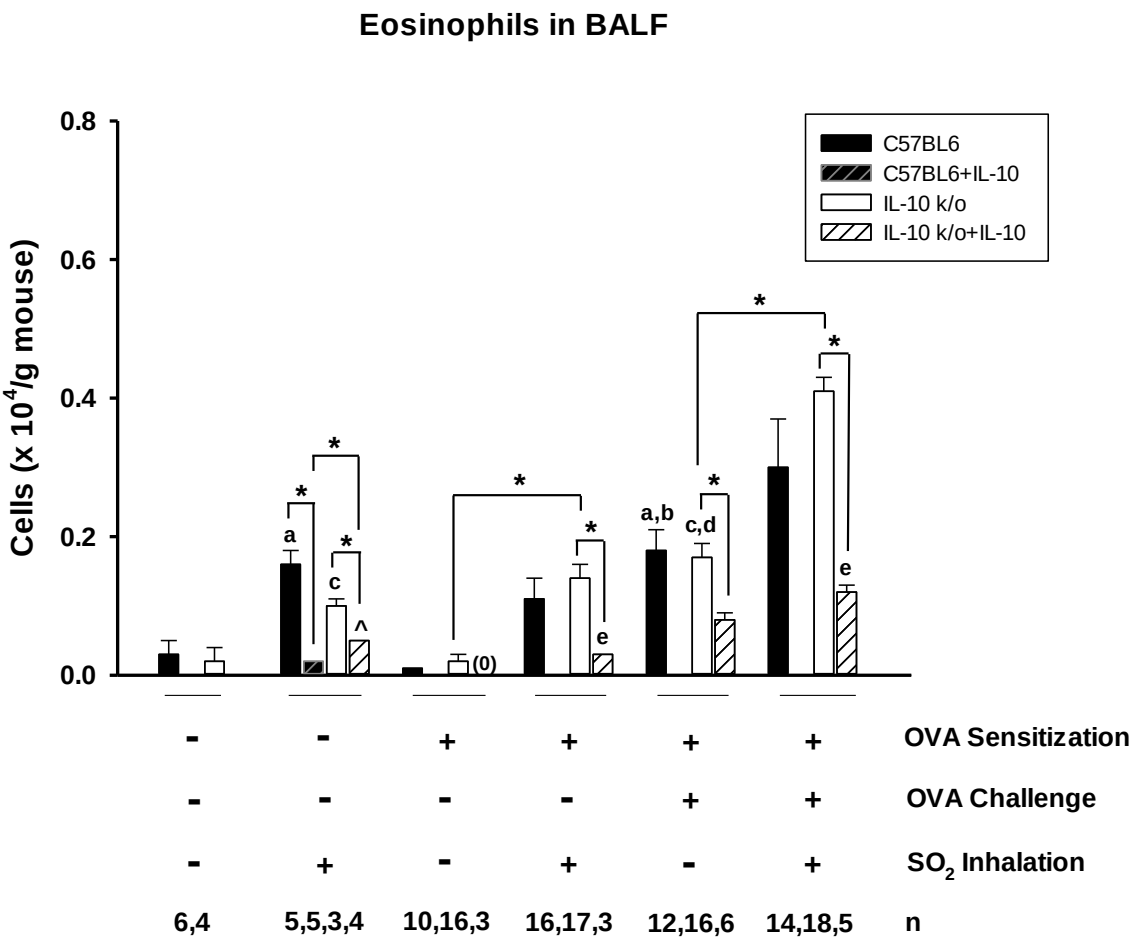

Figure 4. Eosinophils in BALF, C57BL6 vs. IL-10 Knockouts (k/o). All values are means ± SE in Cells x 10^4/g mouse. a=*P*<0.05 vs. naïve C57; b=*P*<0.05 vs. O/S C57; c=*P*<0.05 vs. naïve k/o; d=*P*<0.05 vs. O/S k/o; e=*P*<0.05 vs. naïve+SO_2+IL-10 k/o; ^=*P*<0.05 vs. matched C57; significant findings via SNK or Dunn's post-hoc analysis.

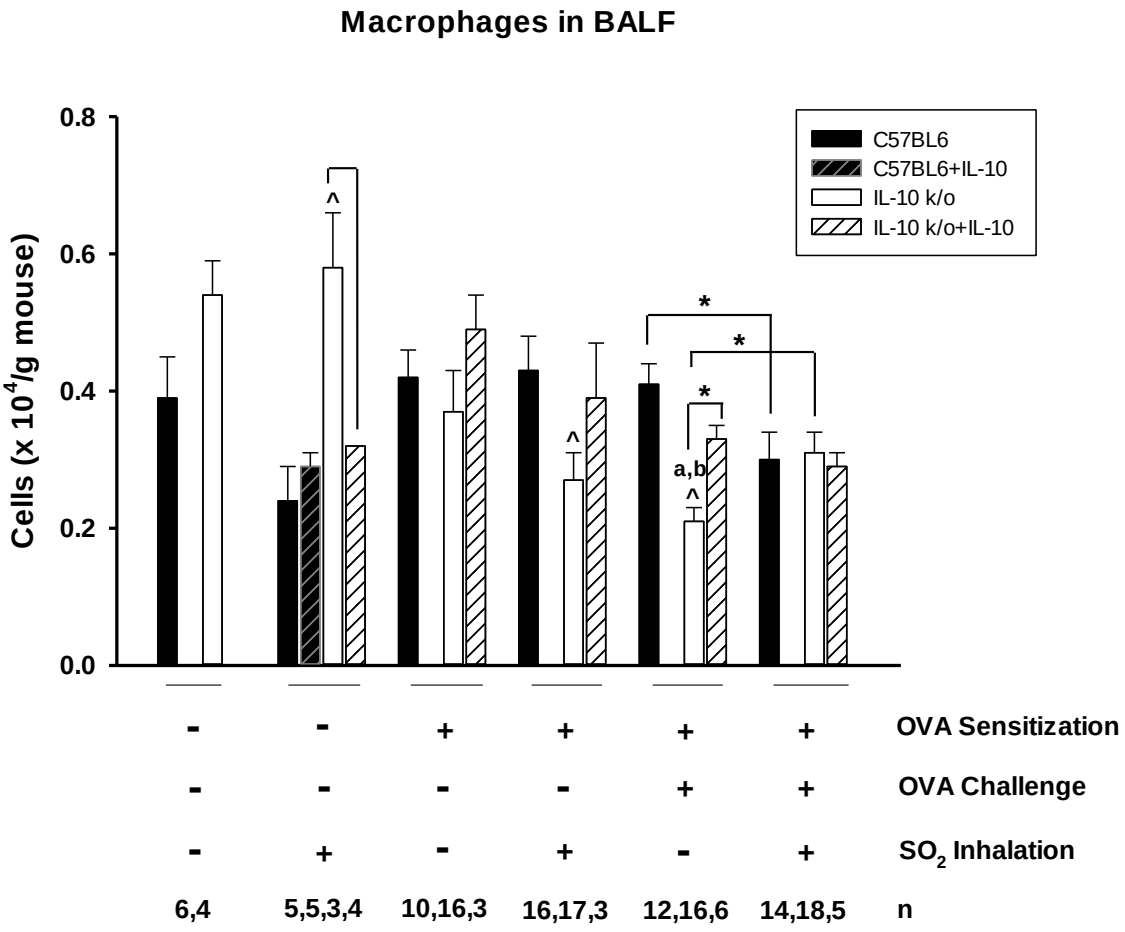

Figure 5. Macrophages in BALF, C57BL6 vs. IL-10 Knockouts (k/o). All values are means ± SE in Cells x 10^4/g mouse. a=*P*<0.05 vs. naïve k/o; b=*P*<0.05 vs. O/S k/o; ^=*P*<0.05 vs. matched C57; significant findings via SNK or Dunn's post-hoc analysis.

Nitrite in BALF	C57BL6	IL-10 Knockout
Naïve	47.7±1.9 (6)	42.8±1.5 (3)
Naïve+SO_2	55.4±3.7 (11)	41.9±3.5 (4)
O/S	59±6.5 (23)	57.1±5.6 (16)
O/S+IL-10	-	40.4±4.1 (3)
O/S+SO_2	66.2±4.8 (22)	62.6±5.2 (21)
O/S+SO_2+IL-10	-	64.6±1.6 (3)
O/O	48.3±2.7 (23)	66.6±9.3 (16)
O/O+IL-10	-	38.4±6.8 (6)
O/O+SO_2	54±2.9 (26)	61.6±6.6 (24)
O/O+SO_2+IL-10	-	36.9±3.5 (6)

Table 7. NO/Nitrite in BALF at 24 hours after AI induction, C57BL6 vs. IL-10 Knockouts. All values are means ± SE in µM NO_2^-/kg body weight. All n.s.; n in parentheses.

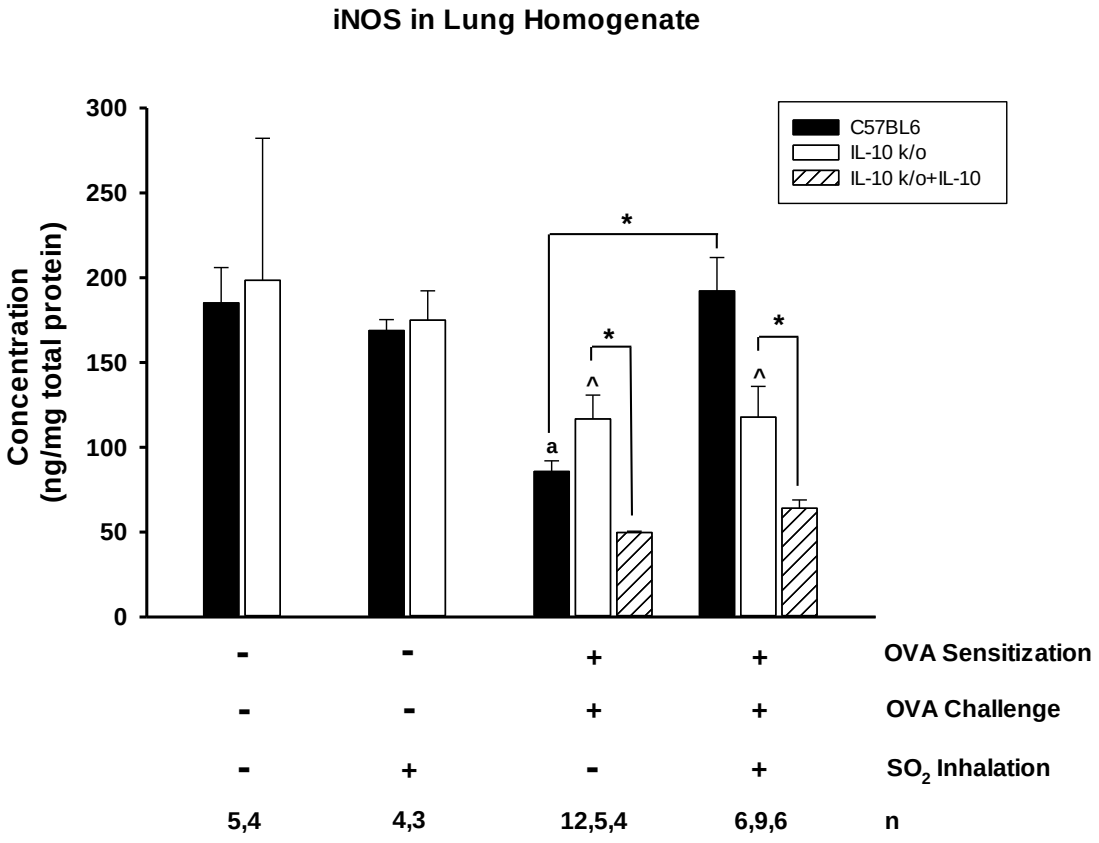

Figure 6. iNOS in lung homogenate, C57BL6 vs. IL-10 Knockouts (k/o). All values are means ± SE in ng/mg total protein. a=*P*<0.05 vs. naïve C57; ^=*P*<0.05 vs. matched C57; significant findings via SNK or Dunn's post-hoc analysis.

Nitrite in Conditioned Media	Cell Treatment	C57BL6	IL-10 Knockout
Naive	**Spontaneous**	15.1±0.8 (5)	12.9±1.1 (3)
	Stimulated	**33.3±1.6 (5)$^{\#}$**	**35.6±6.9 (3)$^{\#}$**
Naive+SO_2	**Spontaneous**	21.1±2.4 (10)	**10.7±1.4 (4)$^{\wedge}$**
	Stimulated	**39.1±5.1 (10)$^{\#}$**	**49.7±8.5 (4)$^{\#}$**
O/S	**Spontaneous**	**9.8±0.7 (21)^{a}**	**15.3±1.4 (15)$^{\wedge}$**
	Stimulated	**34±3.4 (21)$^{\#}$**	**40.7±4.6 (15)$^{\#}$**
O/S+IL-10	**Spontaneous**	-	18.3±0.7 (3)
	Stimulated	-	**28.6±2.5 (3)$^{\#}$**
O/S+SO_2	**Spontaneous**	10.6±0.9 (21)	**14.4±0.8 (19)$^{\wedge}$**
	Stimulated	**32.1±3.2 (21)$^{\#}$**	**28.4±2.5 (19)$^{c,\#}$**
O/S+SO_2+IL-10	**Spontaneous**	-	18.7±2.1 (3)
	Stimulated	-	29.5±3.3 (3)
O/O	**Spontaneous**	**10±0.8 (23)^{a}**	**12.8±0.7 (16)$^{\wedge}$**
	Stimulated	**86.7±11.4 (23)$^{b,\#}$**	**75.4±8.8 (16)$^{c,\#}$**
O/O+IL-10	**Spontaneous**	-	12±0.9 (6)
	Stimulated	-	**82.2±10.8 (6)$^{\#}$**
O/O+SO_2	**Spontaneous**	9.5±0.7 (24)	**15.2±0.9 (24)$^{\wedge}$**
	Stimulated	**68.8±9.1 (24)$^{\#}$**	**57.6±7.8 (24)$^{\#}$**
O/O+SO_2+IL-10	**Spontaneous**	-	15.7±3.0 (6)
	Stimulated	-	**66.5±15.2 (6)$^{\#}$**

Table 8. NO/Nitrite in cell culture-conditioned media, C57BL6 vs. IL-10 Knockouts (k/o). All values are means ± SE in µM NO_2^-/million cells. a=*P*<0.05 vs. naive C57 spontaneous; b=*P*<0.05 vs. O/S C57 stimulated; c=*P*<0.05 vs. O/S k/o stimulated; ^=*P*<0.05 vs. matched C57 spontaneous; #=*P*<0.05 vs. matched spontaneous group; significant findings are in bold (SNK or Dunn's post-hoc analysis); n in parentheses.

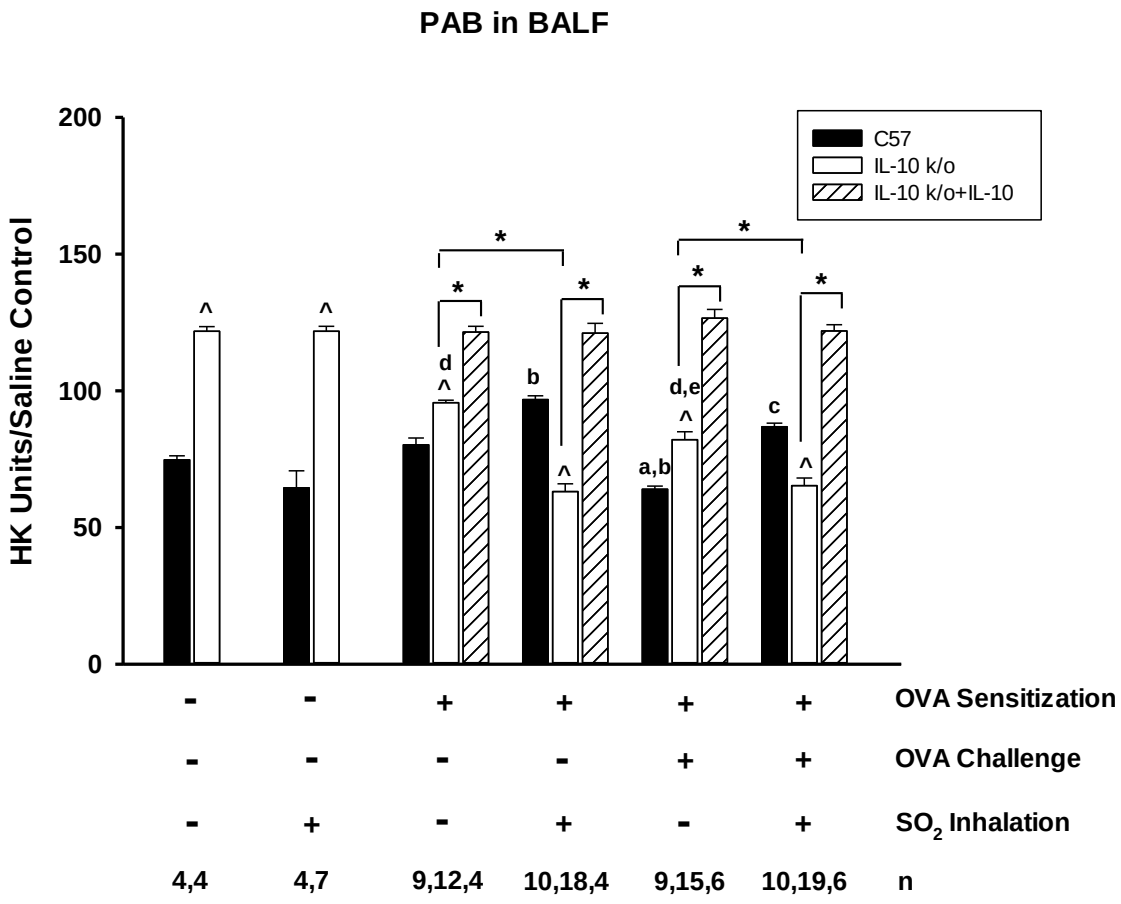

Figure 7. PAB in BALF, C57BL6 vs. IL-10 Knockouts (k/o). All values are means ± SE in HK units/saline control. a=*P*<0.05 vs. naive C57; b=*P*<0.05 vs. O/S C57; c=*P*<0.05 vs. O/O C57; d=*P*<0.05 vs. naive k/o; e=*P*<0.05 vs. O/S k/o; ^=*P*<0.05 vs. matched C57; significant findings via SNK or Dunn's post-hoc analysis.

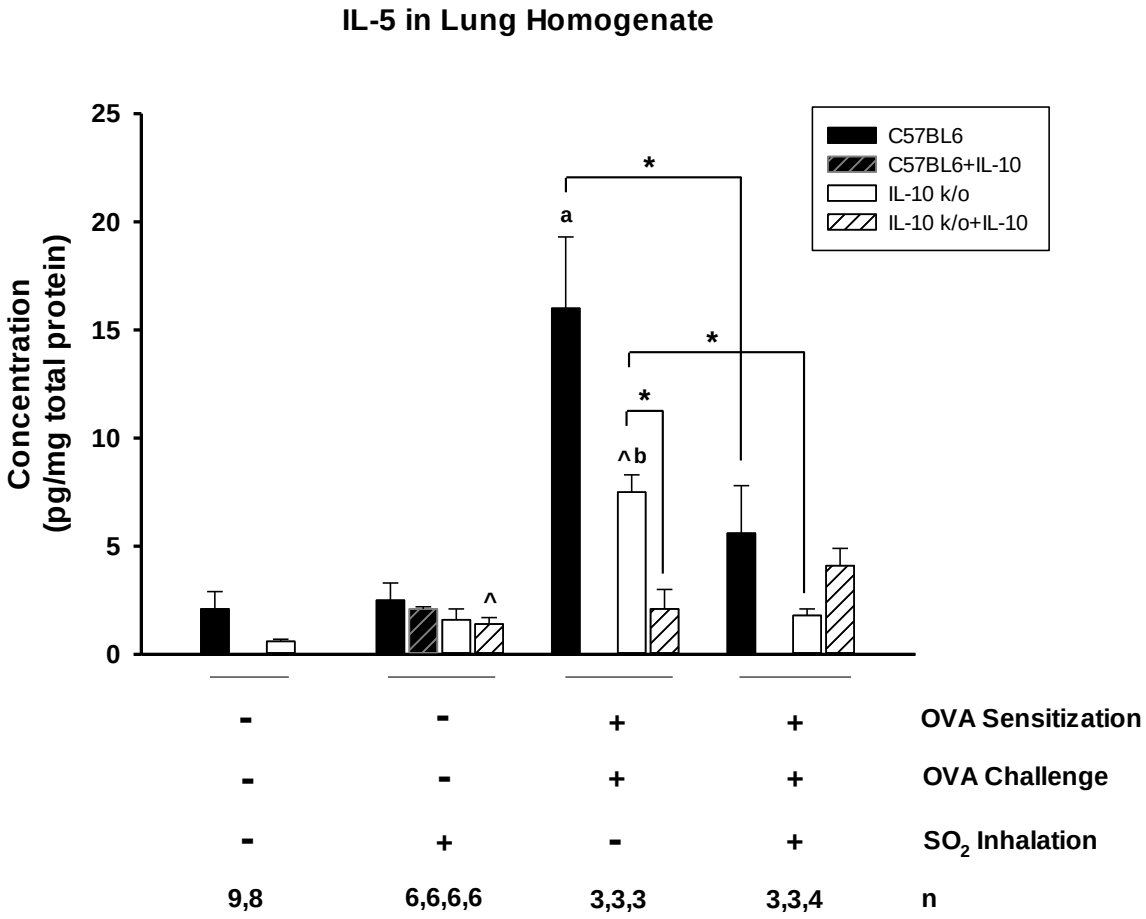

Figure 8. IL-5 in lung homogenate, C57BL6 vs. IL-10 Knockouts (k/o). All values are means ± SE in pg/mg total protein. a=*P*<0.05 vs. naive C57; b=*P*<0.05 vs. naïve k/o; ^=*P*<0.05 vs. matched C57; significant findings via SNK or Dunn's post-hoc analysis.

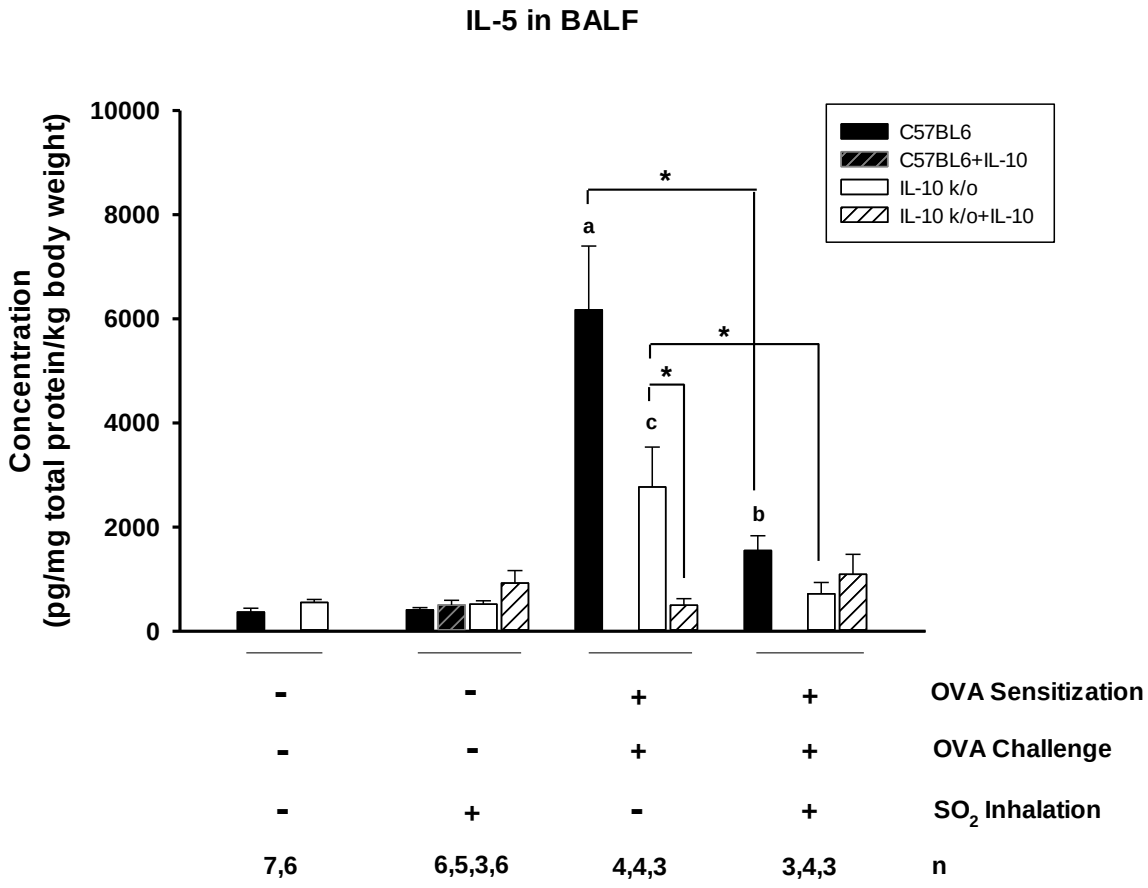

Figure 9. IL-5 in BALF, C57BL6 vs. IL-10 Knockouts (k/o). All values are means ± SE in pg/mg total protein/kg body weight. a=*P*<0.05 vs. naïve C57; b=*P*<0.05 vs. naïve+SO_2 C57; c=*P*<0.05 vs. naïve k/o; significant findings via SNK or Dunn's post-hoc analysis.

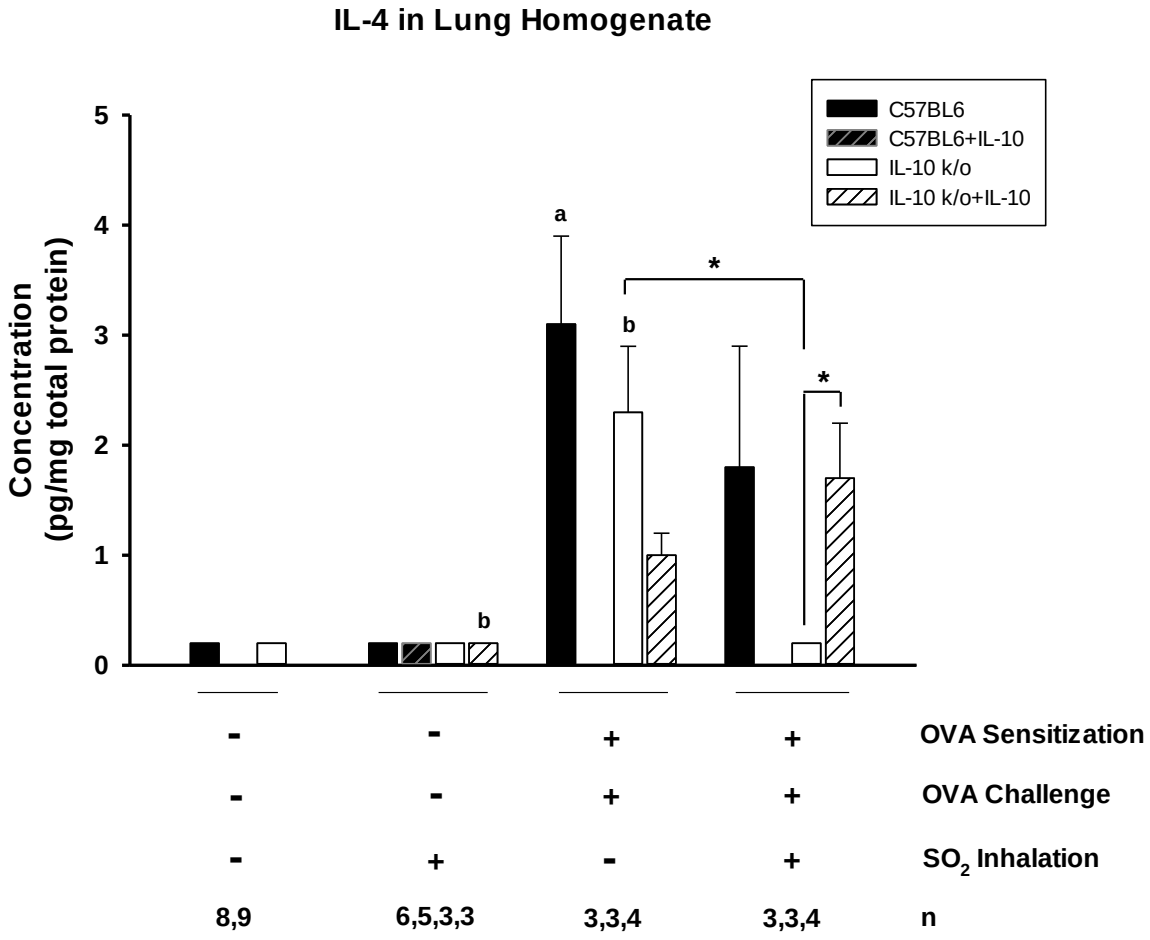

Figure 10. IL-4 in lung homogenate, C57BL6 vs. IL-10 Knockouts (k/o). All values are means ± SE in pg/mg total protein. a=*P*<0.05 vs. naïve C57; b=*P*<0.05 vs. naïve k/o; significant findings via SNK or Dunn's post-hoc analysis.

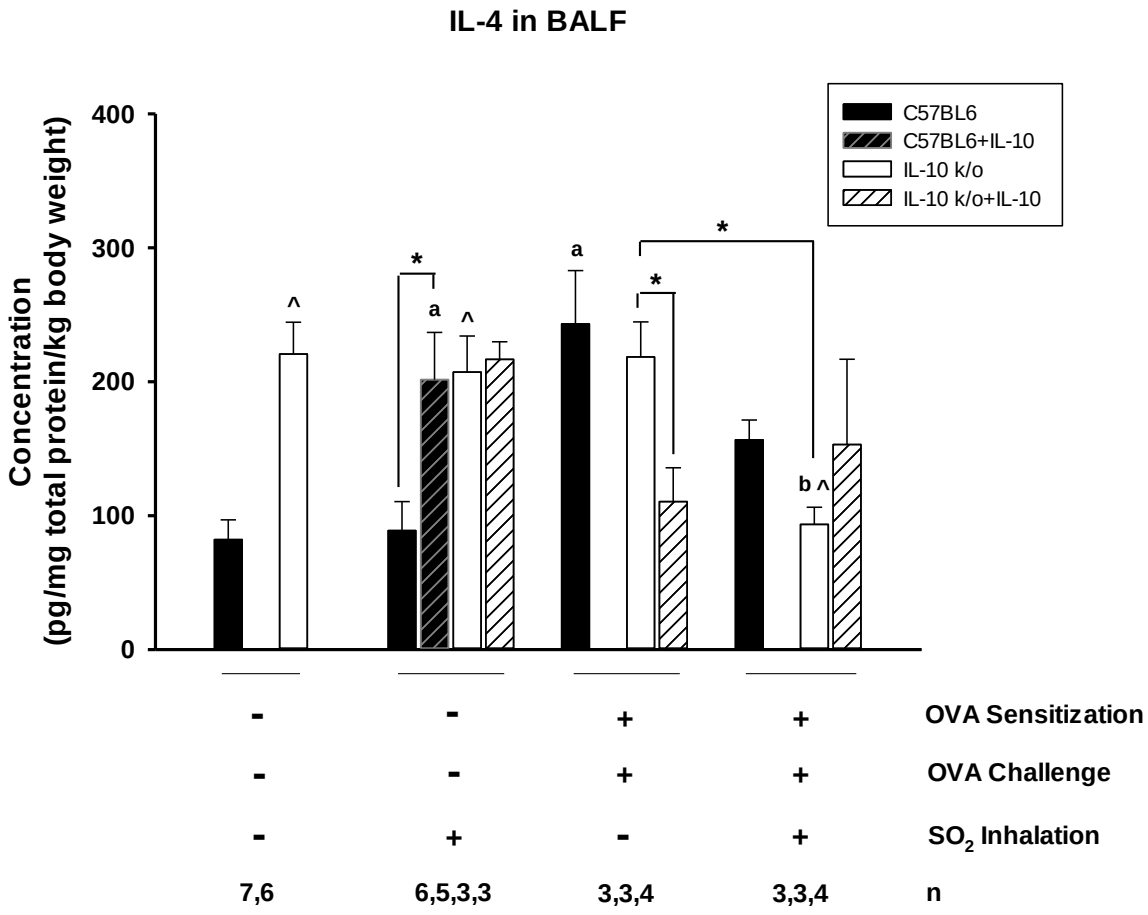

Figure 11. IL-4 in BALF, C57BL6 vs. IL-10 Knockouts (k/o). All values are means ± SE in pg/mg total protein/kg body weight. a=*P*<0.05 vs. naïve C57; b=*P*<0.05 vs. naïve+SO_2 k/o; ^=*P*<0.05 vs. matched C57; significant findings via SNK or Dunn's post-hoc analysis.

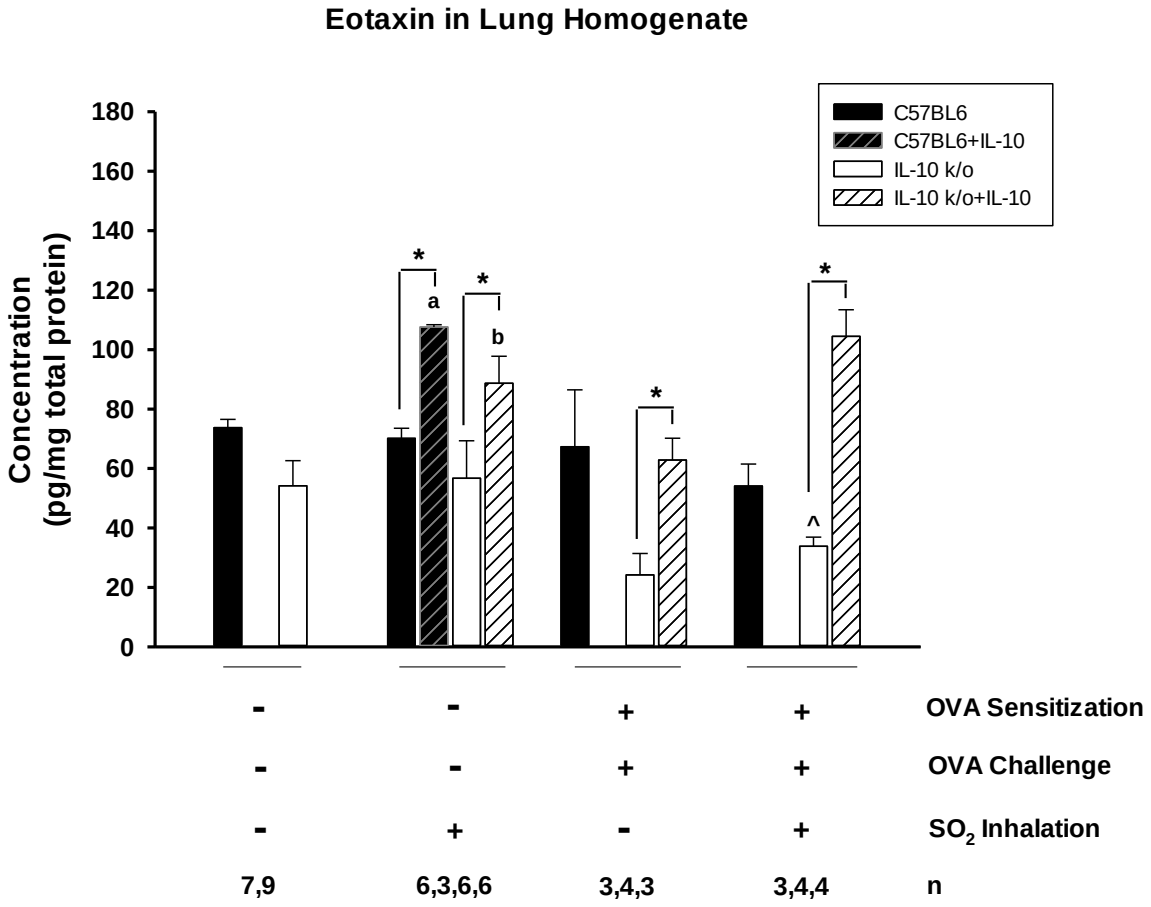

Figure 12. Eotaxin in lung homogenate, C57BL6 vs. IL-10 Knockouts (k/o). All values are means ± SE in pg/mg total protein. a=*P*<0.05 vs. naïve C57; b=*P*<0.05 vs. naïve k/o; ^=*P*<0.05 vs. matched C57; significant findings via SNK or Dunn's post-hoc analysis.

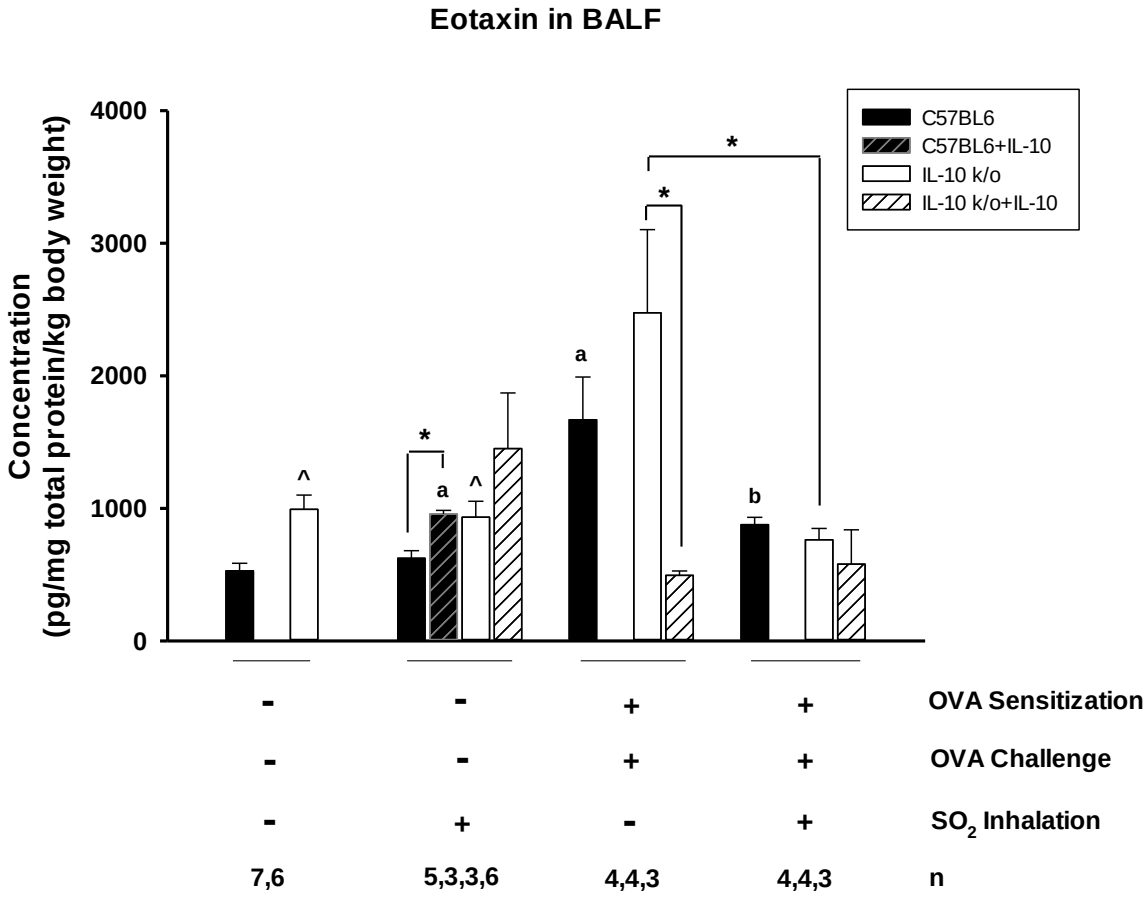

Figure 13. Eotaxin in BALF, C57BL6 vs. IL-10 Knockouts (k/o). All values are means ± SE in pg/mg total protein/kg body weight. a=*P*<0.05 vs. naïve C57; b=*P*<0.05 vs. naïve+SO_2 C57; ^=*P*<0.05 vs. matched C57; significant findings via SNK or Dunn's post-hoc analysis.

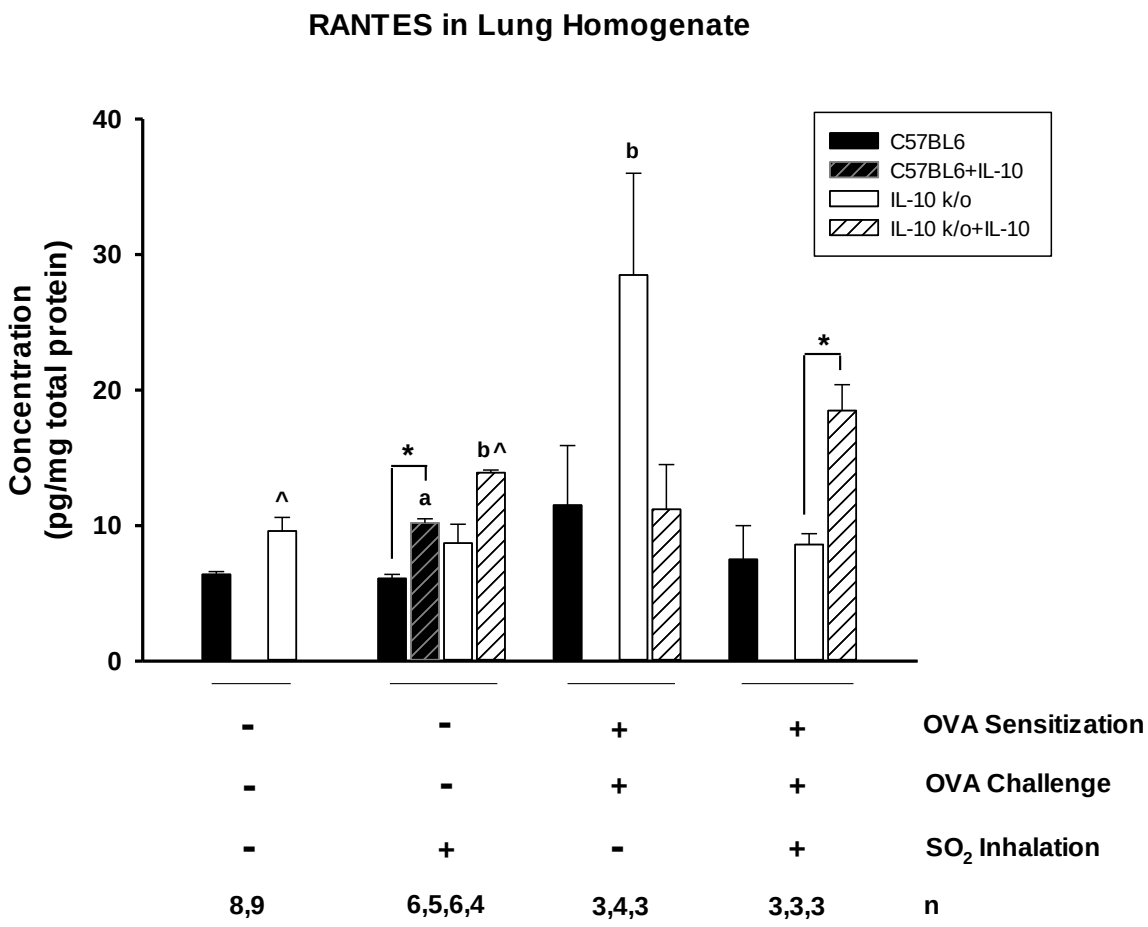

Figure 14. RANTES in lung homogenate, C57BL6 vs. IL-10 Knockouts (k/o). All values are means ± SE in pg/mg total protein. a=*P*<0.05 vs. naïve C57; b=*P*<0.05 vs. naïve k/o; significant findings via SNK or Dunn's post-hoc analysis.

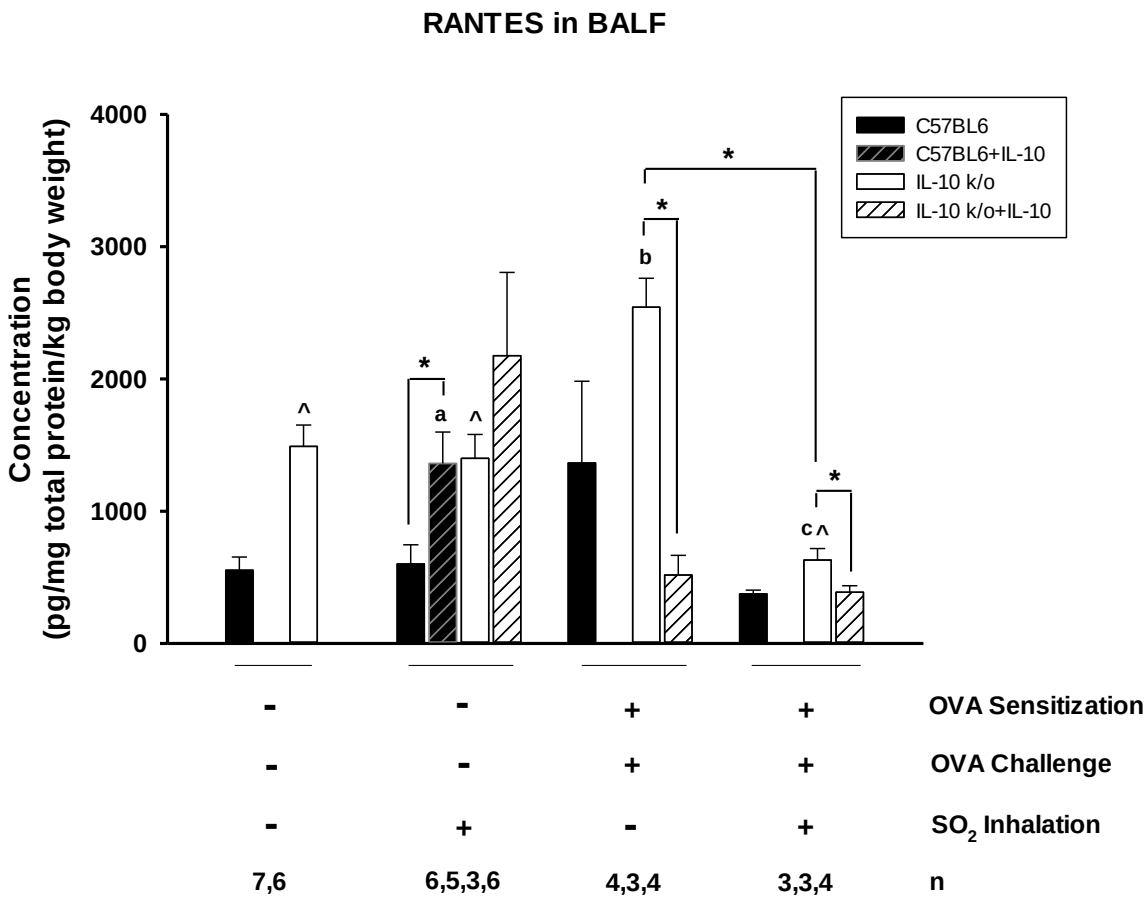

Figure 15. RANTES in BALF, C57BL6 vs. IL-10 Knockouts (k/o). All values are means ± SE in pg/mg total protein/kg body weight. a=*P*<0.05 vs. naïve C57; b=*P*<0.05 vs. naïve k/o; c=*P*<0.05 vs. naïve+SO_2 k/o; ^=*P*<0.05 vs. matched C57; significant findings via SNK or Dunn's post-hoc analysis.

		C57BL6					IL-10$^{-/-}$						
Th1 Cytokine	**Sample**	**Naive**	**Naïve+ SO_2**	**Naïve+ SO_2+ IL-10**	**O/O**	**O/O+ SO_2**	**Naive**	**Naïve+ SO_2**	**Naïve+ SO_2+ IL-10**	**O/O**	**O/O+ IL10**	**O/O+SO_2**	**O/O+ SO_2+ IL10**
IL1β	**BALF**	1333±106 (7)	1562± 266 (6)	**2722±476 (5)[a]**	1521± 405 (4)	841±100 (3)	**2979± 322 (6)^**	**2798± 363 (3)^**	4352±1261 (6)	**1001±50 (3)[b]**	1069±315 (3)	**1260±173 (3)[c]**	794±135 (3)
	LH	29.9±3.1 (9)	35.2±5.0 (6)	37.1±3.4 (6)	**52.4±4.9 (3)[a]**	38.9±6.9 (3)	26.2±2.5 (9)	31.9±4.5 (6)	**27.4±2.1 (6)^**	**19.8±6.0 (4)^**	27.6±4.3 (3)	29.2±2.0 (3)	24.1±3.0 (4)
IFNγ	**BALF**	280±29 (5)	520±121 (6)	**554±97 (5)[a]**	**6352± 1745 (4)[a]**	**2178±640 (4)[b]**	**700±74 (5)^**	570±74 (3)	887±257 (16)	**15875± 5482 (3)[c]**	1249±387 (4)	1020±344 (3)	1321±95 (3)
	LH	3.6±0.6 (9)	2.4±0.7 (6)	**7.2±0.9 (6)[a,b]**	8.1±4.1 (3)	2.2±1.6 (3)	2.6±0.7 (8)	4.4±1.2 (6)	**6.6±0.4 (6)[c]**	**22.9±3.6 (3)[c]**	**1.9±1.5 (3)[d]**	**1.0±0.4 (3)[d]**	**5.6±1.4 (4)[e]**
TNFα	**BALF**	472±85 (7)	623±137 (6)	**1159±203 (5)[a,b]**	**1653± 197 (3)[a]**	**319±25 (3)[c]**	**1269± 137 (6)^**	**1192± 154 (3)^**	1854±537 (6)	**512±100 (3)[d,]^**	441±126 (4)	**537±74 (3)[e,]^**	**330±42 (4)[f]**
	LH	1.0±0.0 (9)	1.1±0.1 (6)	1.0±0.1 (5)	2.7±1.6 (3)	2.1±0.8 (3)	**1.3±0.1 (9)^**	1.1±0.1 (6)	0.9±0.0 (5)	4.0±1.7 (3)	0.8±0.1 (3)	1.3±0.1 (4)	**0.8±0.1 (4)[a]**
GM-CSF	**BALF**	2777±288 (5)	3605± 559 (3)	**5851±577 (4)[a,b]**	-	-	**6069± 356 (4)^**	30272± 24274 (3)	5904±361 (3)	9072±5884 (3)[^]	-	2544±349 (3)[^]	-
	LH	4.4±0.0 (3)	4.4±0.2 (3)	12.9±4.1 (6)	-	-	5.0±0.2 (6)	6.4±2.2 (3)	13.6±3.5 (6)	12.7±4.3 (3)	-	6.0±0.3 (4)	-

Table 9. Th1 cytokines in BALF and lung homogenate, C57BL6 vs. IL-10 Knockouts (k/o). All values are means ± SE in pg/mg total protein/kg body weight (BALF) or pg/mg total protein (lung homogenate, LH); significant findings are in bold (SNK or Dunn's post-hoc analysis); n in parentheses. IL-1β: BALF, a=P<0.05 vs. naive C57; b=P<0.05 vs. naive k/o; c=P<0.05 vs. naive+SO_2 k/o; ^=P<0.05 vs. matched C57; LH, a=P<0.05 vs. naïve C57; ^=P<0.05 vs. matched C57; IFNγ: BALF, a=P<0.05 vs. naive C57; b=P<0.05 vs. naive+SO_2 C57; c=P<0.05 vs. naive k/o; ^=P<0.05 vs. matched C57; LH, a=P<0.05 vs. naive C57; b=P<0.05 vs. naïve+SO_2 C57; c=P<0.05 vs. naive k/o; d=P<0.05 vs. O/O k/o; e=P<0.05 vs. O/O+SO_2 k/o; TNFα: BALF, a=P<0.05 vs. naive C57; b=P<0.05 vs. naïve+SO_2 C57; c=P<0.05 vs. O/O C57; d=P<0.05 vs. naive k/o; e=P<0.05 vs. naïve+SO_2 k/o; f=P<0.05 vs. O/O+SO_2 k/o; ^=P<0.05 vs. matched C57; LH, a=P<0.05 vs. O/O+SO_2 k/o; ^=P<0.05 vs. matched C57; GM-CSF: BALF, a=P<0.05 vs. naive C57; b=P<0.05 vs. naïve+SO_2 C57; ^=P<0.05 vs. matched C57; LH, all n.s.

Chapter 4. DISCUSSION

1. Experimental Overview

I hypothesized that IL-10 may be a critical modulator of SO_2-exacerbated airway inflammation (AI), potentially via its effect on early changes (24 hr.) in cytokines, iNOS, and ROS. To test this hypothesis, I utilized a mouse model of inhaled SO_2 in the setting of AI, using IL-10-sufficient C57BL6 mice as controls representing non-asthmatics, and IL-10 knockout (-/-) mice as an experimental group, representing asthmatics, who are deficient in IL-10 production (Borish et al., 1996). I further tested the importance of IL-10 by the administration of IL-10 to the IL-$10^{-/-}$ mice, to determine whether changes seen in the absence of IL-10 could be reversed, and possibly made similar to measurements made in the IL-10-sufficient C57BL6 controls. In these experiments, AI was induced with sensitization and intranasal challenge using ovalbumin, with the induction of BAL eosinophilia considered to be evidence of its success. These results corroborate previously published data from the Ameredes laboratory, which utilized an OVA aerosol protocol to induce inflammation within the lung (Ameredes et al., 2005). Conversely, reduction in BAL eosinophilia was considered to be evidence of reduction in AI, due to the experimental treatments I tested. A schematic figure of the relative changes in significantly modified variables is shown in **Figure 16**, focusing on the early (24 hr.) changes in eosinophilia, IL-5, iNOS and NO, and ROS status in the lung, due to AI, SO_2, and IL-10 administration.

2. Major Findings

The major findings of this project regarding the effects of SO_2 inhalation 24 hours after the induction of AI were: 1) significant AI-associated increases in BAL eosinophilia in both C57BL6 and IL-$10^{-/-}$ mice, with no further change in eosinophils with SO_2 inhalation in IL-10-sufficient C57s, in contrast to a significant increase in eosinophils with SO_2 inhalation in IL-$10^{-/-}$

mice, which was reversed with administration of recombinant IL-10, 2) significant AI-associated increases in both BALF and lung IL-5 in both C57BL6 and IL-$10^{-/-}$ mice, indicating upregulation of a potential signal supporting eosinophilia, which was significantly decreased with administration of recombinant IL-10 to IL-$10^{-/-}$ mice; but, IL-5 was also unexpectedly decreased with SO_2 inhalation in both C57BL6 and IL-$10^{-/-}$ mice, 3) early downregulation of iNOS with AI in the lungs of both C57BL6 and IL-$10^{-/-}$ mice, which was increased with SO_2 inhalation in IL-10-sufficient C57s, and was unchanged in IL-$10^{-/-}$ mice, but decreased with IL-10 administration to IL-$10^{-/-}$ mice that had inhaled SO_2, and 4) the early relative airway pro-oxidant status was increased with SO_2 inhalation in IL-10-sufficient C57 mice, and unexpectedly decreased in IL-$10^{-/-}$ mice, but was increased with IL-10 administration to IL-$10^{-/-}$ mice in both air-only and SO_2 inhalation groups. Furthermore, in all cases tested, airway NO, as measured by BAL nitrite levels, was not significantly different across all treatment groups, suggesting no changes in NO within the airway at the early time point of 24 hours.

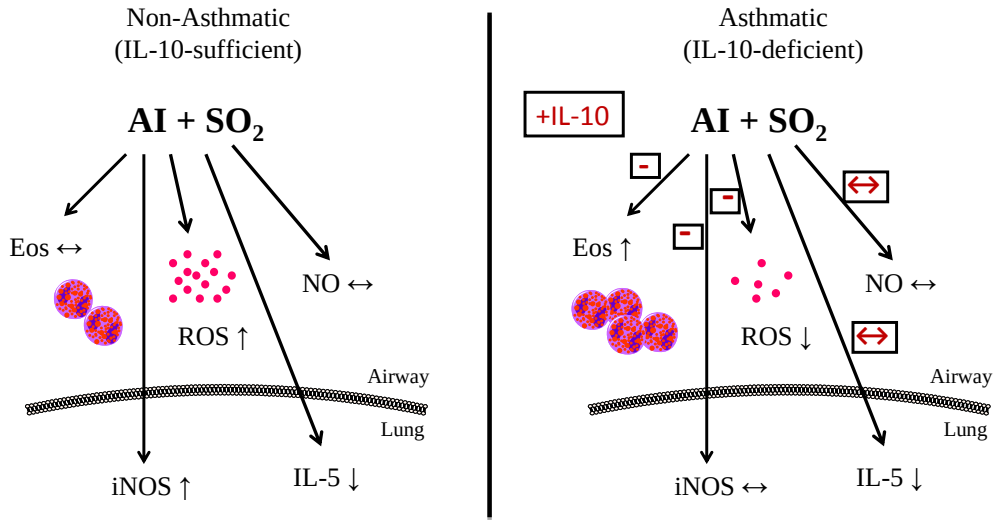

Figure 16. Relative changes in significantly modified variables in the airway and lung, C57BL6 vs. IL-10 Knockouts. Effects of IL-10 administration are indicated by rectangular boxes. It was noted that inhaled SO_2 in the setting of IL-10 deficiency resulted in increased BAL eosinophils which was not associated with changes in BALF IL-5 levels.

3. Comparison with prior studies.

Comparison of my results to the literature outlined in the Introduction has revealed some consistencies, as well as some inconsistencies in terms of the endpoints measured in my model of airway inflammation and SO_2 exposure. This work represents some of the first in the area, and there are relatively few studies with which to compare my results as prior studies have not been conducted in animal models devoid of IL-10.

Airway inflammation and oxidant responses. Animal models of SO_2 exposure have illustrated increases in AI induction, as well as increases in ROS (Cai et al., 2008; Lin et al., 2011; Meng, 2003; Misawa and Nakano, 1993; Park et al., 2001). In the setting of AI in my studies, SO_2 increased eosinophilia in mice lacking IL-10, while significantly decreasing the pro-oxidant status in these animals; SO_2, under the same conditions, had no effect on eosinophilia in mice sufficient in IL-10, but did significantly increase the pro-oxidant status in the lung. Therefore, the development of AI in my model of SO_2 exposure in the IL-10 knockout mice corroborates the findings in the literature, despite the fact that an increase in pro-oxidant status was not observed in these mice. Rather, the ROS results I obtained for the IL-10-sufficient C57BL6 mice fit in nicely with the literature. Thus, an ROS "driving" signal, which may induce subsequent anti-oxidant protective mechanisms that can detoxify excess oxidant species in the normal case, appears to be lost when IL-10 is absent. This observation suggests that the oxidant balance in the lung may be critical in the determination of the eosinophilic response to inhaled SO_2 with IL-10 deficiency in the knockouts, and potentially IL-10 deficient asthmatics.

Nitrite production. Previously published animal studies have illustrated the importance of nitrite production in the lung (Ameredes et al., 2005; Yun et al., 2011). SO_2 exposure in rats

showed increased levels of iNOS mRNA (Yun et al., 2011), which supports my observation in IL-10-sufficient C57BL6 mice with AI, but not IL-10-deficient mice. This finding might suggest that SO_2 may induce upregulation of iNOS in the epithelia and other lung cells in non-asthmatics, in order to protect the airway with bronchodilation by NO. As mentioned before, previous work conducted in my laboratory showed a maximal BALF nitrite response at 48 hours post-OVA challenge (Ameredes et al., 2005); my results at 24 hours post-challenge did not reveal any differences. While it must be kept in mind that the prior study utilized aerosolized OVA, as compared with my intranasal OVA challenge, my data leads me to speculate that asthmatics may lack a protective inducible bronchodilatory mechanism within the lung, mediated perhaps through NO, which may be fully functional in IL-10-sufficient non-asthmatics. One might wonder how this could be so, given that exhaled NO levels are known to be high in untreated asthmatics (Kharitonov and Barnes, 2000); however, we argue here that, lacking IL-10, their iNOS may be maximized to the point that no further induction of iNOS can occur, when challenged with SO_2, leading them to have problems mounting a bronchoprotective response.

Cytokine production. Animal models of SO_2 exposure have illustrated modulatory effects on pro-inflammatory cytokine production in the lung (Meng et al., 2005b; Yun et al., 2011). My results also showed that SO_2 has a measurable effect on modulating Th2 cytokines, including IL-4, IL-5, eotaxin, and RANTES. Inhaled SO_2 modified the release of cytokines associated with adaptive immunity, suggesting that exposure to this environmental pollutant exacerbates an asthmatic's response to allergic disease.

4. Potential for an SO_2-associated neurogenic inflammatory mechanism.

Given that I saw a robust increase in eosinophilia in both naïve C57BL6 and IL-$10^{-/-}$ animals exposed to SO_2 without the induction of AI, I explored non-allergy-mediated mechanisms that might be playing a role. As outlined above, oxidative stress associated with ROS generation may be important in promoting the inflammatory and physiological effects of inhaled SO_2 and inhaled particulate-borne sulfates within the airway. One possible mechanism through which ROS may act to produce exacerbations in asthmatics is through the inherent "irritant" properties of SO_2, and subsequent induction of neurogenic inflammation. Neurogenic inflammation is defined as inflammation stemming from the nervous system following stimulation of chemical irritant receptors on sensory nerves (Meggs, 1993). Neuropeptide mediators such as substance P and neurokinin A are released from sensory nerves and stimulate effector cells to initiate an inflammatory response (Meggs, 1993). Furthermore, neuropeptide production resulting from neurogenic inflammation can mimic the pathology of asthma that is seen in the case of immune system-induced inflammation (Meggs, 1993); however, these inflammatory responses have been found to be removed from the typical allergen-induced inflammation regulated by the immune system.

In the presence of a moist environment, such as in the nasal passages and airways, SO_2 converts into sulfuric acid, which can activate chemical irritant receptors and potentially set into motion subsequent non-allergic-associated neurogenic inflammatory responses (Pawelek-Krombholz et al., 1985). Airway acidification is a strong inducer of bronchospasm, and low EBC pH has been associated with acute exacerbations of asthma and poor asthma control (Ricciardolo et al., 2004; Wood et al., 2013). The role of chemical irritants, such as SO_2, and their association with neurogenic inflammation has been studied in animal models of asthma (Barnes, 2001;

Fujimaki et al., 2004; Lin et al., 2009; Meggs, 1993). For example, formaldehyde, a chemical widely present in the environment in household products, cigarette smoke, and industrial exhaust (US EPA, 2003; WHO, 2006, 1999), was found to promote a neurogenic inflammatory response that was separate from an allergic immunological response, in a mouse model of inflammation (Fujimaki et al., 2004). Plasma levels of Substance P were significantly increased in animals exposed to inhaled formaldehyde at the level of 2000 ppb (2 ppm), providing strong evidence for stimulation of pulmonary C-fibers resulting in a non-allergen-associated inflammatory response induced by the nervous system (Fujimaki et al., 2004). Similarly, a study of airway injury with low-level inhaled SO_2 [400 ppb (0.4 ppm) for 6 hours/day over 3 days] in rats implicated neurogenic inflammation as a "critical pathophysiological mechanism" due to the observations of significantly elevated levels of Substance P in plasma, and positive staining for Substance P in C-fibers within the lung tissue (Lin et al., 2009). Given that the study by Lin et al. (2009) was the only research article found after searching for the terms "SO_2 and neurogenic inflammation" illustrates the need to possibly shift our thinking toward the potential that the nervous system may play a significant role in the inflammatory response associated with SO_2 inhalation and exacerbations of asthma.

Chapter 5. CONCLUSION

To date, asthma, a disease characterized by airway constriction and inflammation, is a major medical problem within the United States, as well as worldwide. Asthmatics struggle daily to manage their symptoms, which have proven more difficult when living in oil industry-rich areas of the country (Schwela, 2000). The Houston-Galveston area is a perfect example of this type of environment; oil refineries are highly abundant and emissions are monitored daily. SO_2 is one of many major gaseous components that make up pollution near the refinery zone. Because a deficiency in the anti-inflammatory cytokine IL-10 has been implicated as a potentially key factor behind asthma's characteristic phenotype of persistent airway inflammation, I tested whether a deficiency in IL-10 might also indicate why asthmatics have a heightening sensitivity to the effects of SO_2 in the airway. I found that there may be modulation of several early endpoints, including nitrite production, oxidant balance, and cytokine release, associated with the difference in eosinophil response, when IL-10 is lacking, in a mouse model of allergic AI.

In conclusion, the absence of IL-10 in my mouse model of airway inflammation was associated with increased eosinophilia with inhalation of SO_2, which was not seen when IL-10 was present, either in the case of IL-10-sufficiency, or with IL-10 replacement. This finding may explain why asthmatics, who are deficient in IL-10, have exacerbation of asthma symptoms with inhalation of SO_2. The SO_2-associated eosinophilia with a lack of IL-10 also appeared to be unlinked from the modulation of IL-5, and possibly other eosinophil-associated cytokines, suggesting that SO_2 may act to increase eosinophils possibly by a non-Th2-associated mechanism, that may involve ROS. The fact that SO_2 upregulates lung iNOS when IL-10 is present suggests another potential protective mechanism that non-asthmatics may have to defend airway caliber with induction of a protein that produces NO, a bronchodilator, via relaxation

effects on airway smooth muscle. Because lung iNOS was unchanged with SO_2 inhalation in the absence of IL-10, there is a suggestion that asthmatics may not be able call on that mechanism of bronchodilation when exposed to SO_2, at least in the early timeframe of AI development (24 hr). Finally, the relative increase in the pro-oxidant status of the normal airway with SO_2 inhalation may be an early ROS signal (Bowler, 2004) important in deflecting subsequent SO_2-associated AI or airway spasm responses, which is absent in the case of IL-10 deficiency, and may also contribute to the heightened airway responses in asthmatics with inhalation of SO_2. My data are provocative, but also indicate that further research is necessary to clarify the mid- and late-inflammatory responses (e.g., at 48 and 72 hr) of these and other pathways, that may occur after the early 24-hr response, with inhalation of SO_2.

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Summer 2014	Graduate Teaching Assistant Course Leader University of Texas Medical Branch Galveston, TX Course: Medical School Matriculation Program (MSMP) Research Papers
Spring 2008 – 2012	Graduate Teaching Assistant Course Leader (2011) Texas A&M University Galveston, TX Course: MARB414 Fundamentals of Toxicology: Toxic Responses of the Respiratory System I/II
Summer 2008	Graduate Teaching Assistant Department of Internal Medicine, APICS Division University of Texas Medical Branch Galveston, TX Course: Griess Assay: Nitric Oxide Detection, Research Mini Course
Spring – Summer 2005	Graduate Teaching Assistant Department of Pathology University of Texas Medical Branch Galveston, TX Courses: Pathobiology & Host Defense, MS-1 (3 laboratory sessions); Introduction to Parasitology, CLS (2 lectures)
Summer 2002	Research Assistant Summer Medical & Research Training Program Department of Biochemistry & Molecular Biology Baylor College of Medicine Houston, TX

PUBLICATIONS – IN PRESS:

A. ARTICLES IN PEER-REVIEWED JOURNALS:

1. Madison AL, Perez ZA, To P, Maisonet T, Rios EV, Trejo Y, Ochoa-Paniagua C, **Reno A**, Stemp ED. Dependence of DNA-protein cross-linking via guanine oxidation upon local DNA sequence as studied by restriction endonuclease inhibition. Biochemistry. Vol. 51(1): 362-369, 2012.

SUBMITTED FOR PEER REVIEW:

A. REVIEW ARTICLE

1. **Reno AL**, Brooks EG, Ameredes BT. Mechanisms of heightened airway sensitivity and responses to SO_2 in asthmatics. Inhalation Toxicology. Resubmitted July 2014.

B. ABSTRACTS

1. Duru EA, **Reno AL**, Parks JL, Panettieri, Jr. RA, Calhoun WJ, Ameredes BT. (S)-albuterol associated modulation of p38 activation as a function of serum stimulation in human airway smooth muscle cells. Am. J. Resp. Crit. Care Med. Vol. 177:A494, 2008.
2. Duru EA, **Reno AL**, Parks IV JL, Panettieri, Jr. RA, Calhoun WJ, Ameredes BT. Enantiomer-associated p38 activation as a function of stimulation intensity and duration in human airway smooth muscle cells. J. Allergy Clin. Immunol. Vol. 123(2):S120, 2009.
3. Duru EA, **Reno AL**, Parks IV JL, Panettieri, Jr. RA, Calhoun WJ, Ameredes BT. Modulation of ERK1/2 levels by enantiomers of albuterol in stimulated human airway smooth muscle cells. Am. J. Resp. Crit. Care Med. Vol. 179:A3914, 2009.
4. **Reno AL**, Duru EA, Parks IV JL, Aloi FP, Choi AMK, Calhoun WJ, Ameredes BT. IL-12p40 subunit expression in lungs of mice administered low-dose carbon monoxide. Am. J. Resp. Crit. Care Med. Vol. 179:A5448, 2009.
5. Hasan RJ, **Reno AL**, Duru EA, Parks IV JL, Panettieri, Jr. RA, Calhoun WJ, Ameredes BT. Modulation of ERK1/2 activation by enantiomers of formoterol in human airway smooth muscle cells. J. Allergy Clin. Immunol. Vol. 125(2):AB45, 2010.
6. **Reno AL**, Parks IV JL, Calhoun WJ, Ameredes BT. Modulation of nitric oxide production in airway leukocytes of mice with mild airway inflammation. Am. J. Resp. Crit. Care Med. Vol. 181:A4251, 2010.
7. Hasan RJ, **Reno AL**, Duru EA, Parks IV JL, Panettieri, Jr. RA, Calhoun WJ, Ameredes BT. Modulation of p38 by enantiomers of formoterol in human airway smooth muscle cells. Am. J. Resp. Crit. Care Med. Vol. 181:A5658, 2010.
8. **Reno AL**, Hallberg LM, Parks IV JL, Brooks EG, Calhoun WJ, Ameredes BT. Modulatory effects of sulfur dioxide on the production of nitric oxide in murine airway inflammation. Am. J. Resp. Crit. Care Med. Vol.183: A1423, 2011.
9. Hasan RJ, **Reno AL**, Parks IV JL, Panettieri, Jr. RA, Calhoun WJ, Ameredes BT. Modulation of NF-κb (p65) activation by enantiomers of formoterol in human airway smooth muscle cells. Am. J. Resp. Crit. Care Med. Vol.183: A4490, 2011.

10. **Reno AL**, Hallberg LM, Spear WC, Parks IV JL, Principe MF, Brooks EG, Ameredes BT. Sulfur dioxide promotes a pro-oxidant shift in IL-10-deficient mice with airway inflammation. The Toxicologist. Vol. 126(1): 258, 2012.
11. **Reno AL**, Hallberg LM, Spear WC, Parks IV JL, Principe MF, Brooks EG, Ameredes BT. Sulfur dioxide promotes a pro-oxidant shift in IL-10-deficient mice with airway inflammation. Lung Research Day - Gulf Coast Consortia for Quantitative Biomedical Sciences, published in printed program only, not online.
12. **Reno AL**, Hallberg LM, Spear WC, Parks IV JL, Chaparro AP, Brooks EG, Ameredes BT. Sulfur dioxide modulates oxidative stress responses in IL-10-deficient mice with airway inflammation. The Toxicologist. Vol. 132(1): 431, 2013.

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