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Identification of novel susceptibility genes in ozone-induced inflammation in mice

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Abstract

Ozone (O_3) remains a prevalent air pollutant and public health concern. *Inf2* is a significant quantitative trait locus (QTL) on murine chromosome 17 that contributes to susceptibility to O_3-induced infiltration of polymorphonuclear leukocytes (PMNs) into the lung, but the mechanisms of susceptibility remain unclear. The study objectives were to confirm and restrict *Inf2*, and to identify and test novel candidate susceptibility gene(s).

Congenic strains of mice that contained overlapping regions of *Inf2* and their controls, and mice deficient in either MHC Class II genes or the *Tnf* cluster were exposed to air or O_3. Lung inflammation and gene expression were assessed.

Inf2 was restricted from 16.42 Mbp to 0.96 Mbp, and bioinformatic analysis identified MHC class II, the *Tnf* cluster, and other genes in this region that contain potentially informative SNPs between the susceptible and resistant mice. Furthermore, O_3-induced inflammation was significantly reduced in mice deficient in MHC class II genes, or the *Tnf* cluster genes, compared to wild-type controls. Gene expression differences were also observed in MHC class II and *Tnf* cluster genes.

This integrative genetic analysis of *Inf2* led to identification of novel O_3 susceptibility genes that may provide important, new therapeutic targets in susceptible individuals.

Introduction

The pollutant ozone (O_3) is a highly toxic principal oxidant found in urban environments throughout industrialized cities worldwide. O_3 exposure has been associated with many adverse health effects, such as exacerbation of asthma [1, 2]. Identification of susceptibility genes involved in O_3-induced pulmonary injury may provide critical information for future risk assessment as well as general international health policies. In 2006, an estimated one-third of U.S. individuals were at an increased risk of adverse effects caused by O_3 and 131 million U.S. residents resided in regions that either approached or exceeded the National Ambient Air Quality Standard of 0.08 parts per million (ppm) O_3 [3].

Significant intersubject differences in pulmonary function and inflammatory responses to O_3 suggest that genetic background contributes to O_3 susceptibility in humans and rodents [4-6]. Furthermore, studies in human subjects have suggested that polymorphisms in oxidant defense genes, such as glutathione S transferase M1, and quinone metabolism genes, such as NADPH quinone oxidoreductase 1, associate with differential responsiveness to O_3 [7, 8]. Activating polymorphisms in inflammatory genes, such as tumor necrosis factor alpha (*TNF*), also enhance susceptibility to O_3 and asthma [9]. A genome-wide linkage analysis of O_3-induced influx of polymorphonuclear leukocytes (PMNs) in an intercross cohort ($B6C3F_2$) derived from susceptible C57BL/6 (B6) and resistant C3H/HeJ (C3) progenitor mouse strains identified a significant susceptibility QTL on chromosome 17 (inflammation 2; *Inf2*) [5]. *Inf2* (33.73 – 50.15 Mbp; D17Mit16 - D17Mit10) contains the *H-2* locus, including major histocompatibility genes and non-MHC genes such as the pro-inflammatory cytokine *Tnf*. Pre-treatment of B6 mice with a monoclonal antibody to TNF-α and deletion of TNF-α receptors 1 and 2 [5, 10] significantly attenuated the inflammatory response to O_3 relative to control mice, supporting the importance of *Tnf* as a candidate gene in this model. However, these studies did not conclusively identify *Tnf* as the susceptibility gene in *Inf2*. In addition, *Inf2* is located in the most gene dense,

polymorphic region of the entire mouse genome, thus making single candidate gene identification difficult [11].

In the current study we sought to confirm the importance of *Inf2* in O_3-induced lung inflammation, to reduce *Inf2*, and to identify candidate susceptibility genes. Using an integrative genomics approach, we utilized congenic mouse strains to limit *Inf2* from 16.42 Mbp to 0.96 Mbp, and bioinformatic analysis which identified MHC class II genes and the entire *Tnf* cluster as candidate susceptibility loci. Functional analyses of these genes confirmed novel roles for modulation of the inflammatory response to O_3 exposure.

Materials and Methods

Mouse strains and O_3 exposure. The following male (6-8 wk) congenic mice were used: B10.A-*H2*^{h2}/(2R)SgSnJ (2R); B10.A-*H2*^{h4}/(4R)SgDvEg (4R), B10.A-*H2*^{i5}H2-*T18*^{a}/(5R)SgSnJ (5R); and C3.SW-H2b/SnJ (C3H-H2^{b}). The congenic region and haplotype for each of the strains are shown in Table 1 and Figure 1. Control strains for the congenic mice were C57BL/10SnJ (B10), A/WySnJ (A), and C3H/HeSnJ (HeSnJ). The location of the congenic region of the 2R, 4R, and 5R mice with respect to the B10 and A/Wy background strains was identified by genotyping (Malhotra and Travis, data not shown). H2^{k} haplotypes are O_3-resistant and the H2^{b} haplotype is O_3-susceptible [5] . Additional strains used for the candidate gene studies were B6.129-H2$^{dlAb1-Ea}$/J (H2-$^{dlAbl-Ea}$; B6 background strain), B6.129S2-*Lta*^{tm1Dch}/J (*Lta*$^{-/-}$, B6 background), B6.129P2-*Ltb/Tnf/Lta*^{tm1Dvk}/J (*Ltb*/*Tnf*/*Lta*$^{-/-}$ mice, B6 background), C57BL/6J (B6) mice, and C3H/HeJ (C3) mice. B6 are O_3-susceptible and are H2^{b}, while C3H/HeJ (C3) mice are O_3-resistant and are H2^{k}. All mice were purchased from Jackson Laboratory (Bar Harbor, ME). All animal use was conducted in facilities accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care and approved by the NIEHS Animal Care and Use Committee and follows the Helsinki convention for the use and care of animals. For air and O_3 exposures, mice were caged in a humidity and temperature-controlled room and provided water and pelleted open-formula rodent diet NIH-07 (Zeigler Brothers, Gardners, PA) *ad libitum* (see O_3 exposure procedures in Supplement Methods).

Bronchoalveolar lavage fluid (BALF) analysis. The procedures used for these techniques have been described previously for right lung lavages and inflammatory cell analysis [5, 10, 12].

Total RNA Isolation and Real-Time Quantitative Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR). Mice were sacrificed immediately after air or O_3 exposure, and lungs of

each animal were snap-frozen in liquid nitrogen. Total RNA isolation, reverse transcription into cDNA and PCR reaction procedures are described in Supplemental Methods.

Statistics. Data are expressed as group means ± standard error of the mean (S.E.M.). Two-way analysis of variance (ANOVA) was used to evaluate the effects of exposure (air vs O_3) and strain (B10, 2R, 4R, 5R, A, HeSnJ, C3H-H2^b, H2-$^{dlAbl\text{-}Ea^{-/-}}$, H2-$^{dlAbl\text{-}Ea^{+/+}}$, *Lta*$^{-/-}$, *Lta*$^{+/+}$, *Lta*/*Tnf*/*Ltb*$^{-/-}$, *Lta*/*Tnf*/*Ltb*$^{+/+}$, B6, C3) on BAL phenotypes and mRNA expression. The figure legends contain the number of mice used per experiment. Student-Newman-Keuls test was used for *a posteriori* comparisons of means. All analyses were performed using a commercial statistical analysis package (SigmaStat; Jandel Scientific Software, San Rafael, CA). Statistical significance was accepted at $P<0.05$.

Results

Restriction of Inf2

Responses to O_3 in congenic mice. To confirm the significance of *Inf2*, C3H-$H2^b$ mice which contain *Inf2* from a susceptible mouse ($H2^b$) on an O_3-resistant $H2^K$ background [HeSnJ; (see Figure 1)] were exposed to air and O_3. Relative to HeSnJ controls, significant increases in mean BAL protein concentration and numbers of macrophages, PMNs, and epithelial cells were found in C3H-$H2^b$ mice after 48 and 72 h exposure to O_3 (Figure 2); no strain differences were observed in any parameter in the air exposed mice. Results thus confirmed the importance of the region of chromosome 17 encompassed by *Inf2* in susceptibility to O_3-induced inflammation.

To reduce the length of *Inf2*, O_3-induced inflammatory responses in congenic 2R, 4R, and 5R mice were compared to those of similarly exposed O_3-susceptible background B10 and O_3-resistant donor A strains. Each congenic strain contains a different region of the *Inf2* locus from the A strain mouse on a B10 background (see Table 1). O_3 caused significant increases in mean numbers of macrophages and PMNs in B10 but not A mice compared to respective air-exposed animals, and numbers of macrophages and PMNs were greater in B10 mice compared to A mice after O_3 (Figure 3). The mean numbers of PMNs and macrophages in 2R and 5R mice were not different from A strain mice after 48 and 72 h O_3; however, numbers of PMNs and macrophages in 2R and 5R mice were significantly lower compared to B10 mice (Figure 3). In contrast, the numbers of BALF macrophages and PMNs from O_3-exposed 4R mice were not significantly different from those from O_3-exposed B10 mice, and were greater than those from A, 2R, and 5R mice (Figure 3). The mean BALF protein concentrations were significantly reduced in 2R, 4R, and 5R mice compared to B10 mice and significantly greater than A mice after 48 and 72 h O_3. Comparison of the mean numbers of macrophages and PMNs between congenic strains with respective congenic regions thus suggested that the 34.38 – 35.34 Mbp [18.64 – 19.06 centimorgan (cM)] region of chromosome 17 accounted for a major portion of the inflammatory

response to O_3 in these mice (Figure 1). No differences in mean numbers of BALF epithelial cells were found between the congenic, A, or B10 strains after exposure to O_3 (Figure 3C).

Candidate Gene Analysis

Database search for polymorphisms. Genes within the reduced region of *Inf2* (34.38 – 35.34 Mbp) were searched for known exon (non-synonymous), intron (including splice sites) and UTR polymorphisms using the public Mouse Phenome Database [13] (Supplemental Table 1). *H2-Aa* and *H2-Ab1* genes are haplotype $H2^k$ in the 2R and 4R congenic strains, thus excluding these as candidate genes [14]. Additionally, the *H2-Eb1* gene is divided between resistant and susceptible haplotypes and therefore remains a candidate gene, as well as *H2-Eb2* [15]. The genes that contain known non-synonymous polymorphisms between the B6 (susceptible) and C3 (resistant) mice in the reduced *Inf2* in order of location on chromosome 17 are *H2-Eb1, H2-Eb2, Btnl1, Notch4, Fkbpl, Crebl1, Tnxb, C4b, C4a,* and *Stk19.* Two genes (*Hspa1b and Hspa1a*) may have exonic polymorphisms between the B6 and C3, but no details are currently available for the B6 strain at these sites. Genes with intronic polymorphisms between the B6 (susceptible) and C3 (resistant) mice in the reduced *Inf2* are: *H2-Eb1, H2-Eb2, H2-Ea, Btln2, Btln1, Btln7, Notch4, Ppt2, Crebl1, Tnxb, C4b, Cyp21a1, C4a, Stk19, Cfb, C2, Msh5, Ddah2, Ly6g6c, Bat5, Ly6g5c, Csnk2b, Aif1, Tnf,* and *Lta* (Supplemental Table 1). UTR polymorphisms between the B6 and C3 mice were found in *H2-Eb2, Btnl2, Ppt2, C4b, C4a, G6b, Bat5, Ly6g5b,* and *Tnf* (Supplemental Table 1). Information is absent for *Ltb* and *Tnf* UTR and intronic polymorphisms in the C3 strain, thus it is possible that more exist between the B6 and C3 strains.

We then asked if mRNA expression differences between B6 and C3 mice existed for several candidate genes within the reduced *Inf2*, and with either known or possible SNPs (Fig. 4). *H2-Eb1* expression was reduced after 24 h of O_3 exposure in the C3 mice, but unchanged in the B6 mice; *H2-Eb2* expression was significantly increased only in B6 mice at 24 h. Expression of *Hspa1b* and *Btnl1* was significantly increased after 72 h O_3 in B6 mice only. *Tnf* and *Lta*

expression was significantly increased in B6 mice after 24 h O_3 compared to no increases observed in C3 mice.

MHC class II deficient mice. Because of the prevalence of MHC class II genes in *Inf2* and the non-synonymous, intronic, and UTR polymorphisms in *H2-Eb1* and *-Eb2* between the susceptible (B6) and resistant strains (C3) (Supplemental Table 1), we hypothesized that they were candidate susceptibility genes for O_3-induced inflammation. *H2Abl-Ea*$^{-/-}$ mice are deficient in MHC class II genes *H2-Ab1, -Aa, -Eb1, -Eb2,* and *-Ea* compared to the *H2Abl-Ea*$^{+/+}$ mice. The B6 background of these mice is null at *H2-Ea*, therefore *H2Abl-Ea*$^{-/-}$ mice only test the importance of the *H2-Ab1* through *H2-Eb2* MHC class II region. Relative to respective air controls, O_3 caused significant ($P < 0.05$) increases in mean numbers of BALF PMNs and macrophages in *H2Abl-Ea*$^{+/+}$ mice after 24, 48 and 72 h, and after 24 and 48 h O_3 in *H2Abl-Ea*$^{-/-}$ mice (Fig. 5A and Table 2). However, significantly greater numbers of PMNs and macrophages were found in *H2Abl-Ea*$^{+/+}$ mice compared to *H2Abl-Ea*$^{-/-}$ mice after 24-72 h and 48 and 72 h exposures, respectively. Significant strain and O_3 effects on BAL epithelial cells were found after 48 h O_3 (Table 2). Significant strain differences in BAL protein content were also found after 48 h O_3 (Fig. 5A). mRNA expression of *Tnf* and *Lta* was significantly increased in the *H2Abl-Ea*$^{+/+}$ mice after 24 h O_3; however no increase was observed in the *H2Abl-Ea*$^{-/-}$ mice (Fig. 5B).

Mice deficient in Lta or the Tnf cluster. Based on the existing evidence to support a role for the *Tnf* cluster in the O_3 response [10], as well as the known intronic and UTR polymorphisms in *Tnf* and *Lta* genes between the B6 and HeJ mice, we tested the role of the *Tnf* cluster in the model. *Lta*$^{-/-}$, *Lta*/*Tnf*/*Ltb*$^{-/-}$, and wildtype mice were exposed continuously to air or 0.3 ppm O_3 for 24, 48 or 72 h. PMNs were significantly elevated in the *Lta*$^{+/+}$ and *Lta*/*Tnf*/*Ltb*$^{+/+}$ mice compared to mice deficient in *Lta* or the *Lta*/*Tnf*/*Ltb* cluster following 24 and 48 h O_3 for both strains, and 72 h exposure for *Lta*/*Tnf*/*Ltb*$^{-/-}$ mice compared to air controls ($p<0.05$; Fig. 6A, B). Mean numbers

of BALF macrophages were also significantly elevated in wildtype mice compared to *Lta*$^{-/-}$ and *Lta*/*Tnf*/*Ltb*$^{-/-}$ mice following 24-72 h O_3 compared to air controls ($p<0.05$; Table 2). Epithelial cell numbers were not different between exposure or strains (Table 2). The BAL protein content was not affected by these genes, except for 72 h exposure in the *Lta*$^{-/-}$ mice, supporting the hypothesis that different genes regulate these specific phenotypes [16] (Fig. 6A, B).

MHC class II gene expression was also significantly different between the strains deficient in the *Tnf* cluster. *H2-Eb1* mRNA was unchanged in B6 wildtype mice, however, this gene was significantly decreased after 48 h O_3 in the *Lta*$^{-/-}$ and *Lta*/*Tnf*/*Ltb*$^{-/-}$ mice compared to controls (Fig. 6C). *H2-Eb2* mRNA was significantly increased in B6 mice after 24 and 48 h O_3, and was significantly greater after O_3 than mRNA expression in *Lta*$^{-/-}$ and *Lta*/*Tnf*/*Ltb*$^{-/-}$ mice at these time points.

Discussion

The overall objective of this investigation was two-fold. We first sought to restrict the length of *Inf2* to elucidate candidate susceptibility genes for O_3-induced inflammation. The second objective was to validate the role(s) of identified gene candidates. Significant differences in O_3-induced inflammation between C3.SW-H2b/SJ and C3H/HeSnJ strains confirmed the role of *Inf2* inasmuch as the only difference between these two strains is a congenic region of chromosome 17 that encompasses *Inf2*. Comparison of inflammatory responses in 2R, 4R, and 5R congenic mice with respective background strains further confirmed the importance of *Inf2,* and more importantly reduced *Inf2* to 0.96 Mbp. The reduced *Inf2* includes MHC class I, II, and III genes, and non-MHC genes, some of which have previously been identified as candidate genes in other lung injury models (e.g. *C4a*; [17]). In the current study, gene expression and sequence analyses of the reduced *Inf2* suggested that MHC class II genes and the *Tnf* cluster may be important in O_3-induced inflammation, and significantly different phenotypes between O_3-exposed *H2Abl-Ea*$^{+/+}$ and *H2Abl-Ea*$^{-/-}$ mice and the mice deficient in *Lta* alone or the entire *Tnf* cluster confirmed a role for these genes. To our knowledge, these are the first studies to conclusively demonstrate a role for MHC class II genes and the entire *Tnf* cluster in oxidant-induced lung inflammation and provide evidence supporting a susceptibility "superlocus".

Genetic association studies have implicated several gene categories, such as pro-inflammatory cytokine genes [9], metabolism genes [18], and innate immunity genes in responses to environmental stimuli in human populations [19]. In inbred mouse models, positional cloning approaches identified QTLs for a number of lung diseases [20]. However, while identification of QTLs is an important initial step to understanding the genetic determinants of disease, QTLs can contain hundreds of genes. It is therefore necessary to refine the disease QTLs to a limited set of genes that can be evaluated [21]. In our integrative genomics approach to reduce *Inf2* and identify candidate genes, we used congenic strains that were developed initially for histo-incompatiblity studies by repeated backcrossing of regions of

chromosome 17 from the donor strain onto the recipient background strain [22]**.** One advantage of these strains is that the allelic designations are well characterized and, after phenotyping these strains for their inflammatory response to O_3, it enabled restriction of the previously described *Inf2* QTL to a limited chromosomal interval to identify gene candidates.

We queried the Mouse Phenome Database to identify genes located within the narrowed *Inf2* that are polymorphic between susceptible B6 and resistant C3 mice. Several of the MHC class II and III genes (and those non-MHC genes located within these regions) have non-synonymous polymorphisms in exons, many have intronic polymorphisms, and several have UTR polymorphisms. However, not all of the genes have been assessed for polymorphisms between B6 and C3 mice (e.g. *Hspa1b*). It is important to note that functional polymorphisms may also exist in intronic regions, such as with *K-ras* and mouse lung cancer [23]. Future studies will delineate the functionality of polymorphic regions between the B6 and C3 mice in those genes in which the functionality is unknown, such as *Ltb*.

Organization of the H2 locus. Genes in the mouse *H-2 locus* and the homologous human *MHC* locus are organized in the same relative gene order [24] and span several Mbp. The *H2* locus contains class I, class II, and class III genes. Class I and class II genes are important in fulfilling immunologic functions. Class I molecules are expressed by most cells, and present endogenous antigens (cytosolic-derived) to $CD8^+$ cytotoxic T cells. Class II molecules (e.g. *H2-Eb2*, *Btnl1*) are expressed in antigen presenting cells (APC) such as dendritic cells, and present exogenous, endocytically derived antigens to $CD4^+$ helper T-cells [25]. Class III genes are diverse in functionality, are located between the class I and class II genes and include the complement component genes (*C2* and *C4*), heat shock proteins (*Hspa1a*, *Hspa1b*, and *Hsc70t*), and the *Tnf* cluster.

Candidate gene analysis. Deficiency in MHC class II genes and the *Tnf* cluster decreased the O_3-induced PMN and macrophage phenotypes, similar to those reductions seen in the 2R and 5R

mice. Previous studies as well as the data presented here support a clear role for the *Tnf* cluster in these O_3-induced phenotypes. The *H2-Ea* gene (which is deficient in B6 mice), *H2-Ab1*, and *H2-Aa* (as described earlier) can be excluded as candidate susceptibility genes.

Chen et al. [26] demonstrated that inhibition of the $CD4^+$ T-cell population using a monoclonal CD4 antibody *in vivo* greatly reduced the O_3-induced phenotypes (including PMNs, lymphocytes, and epithelial cells) in B6 mice after 72 h continuous O_3 exposure. These studies support our findings in the *H2Abl-Ea*$^{-/-}$ mice and suggest that in O_3 susceptible strains, CD4+ T-cells are critical to O_3-induced inflammation and injury. A recent study in human airway monocytes isolated from exposed individuals suggests that O_3 primes the airway monocytes for innate immune responses, increases the capacity of the monocytes to present antigen to the $CD4^+$ T-cells, and increases the overall population of antigen presenting cells in the lungs [19].

Candidate susceptibility genes in Inf2 and human disease. The MHC class II genes and the *Tnf* cluster in the reduced *Inf2* are all located in close proximity on chromosome 17 in mice and chromosome 6 in humans (Fig. 1, Supplement Table 1). Several of these homologous genes have been implicated in human association studies. For example, polymorphisms in *HLA-DRB1* (*H2-Eb1*), *TNF*, and *LTA* have been associated with sarcoidosis [27], a chronic granulomatous disease of unknown etiology, supporting a role for a susceptibility "superlocus". In addition, *TNF* is a susceptibility gene for O_3-induced changes in lung function in humans [28]. Thus, it is possible that a cluster of genes in the MHC class II region (*H2-Eb1* and *H2-Eb2*) interacts with the *Tnf* cluster (*Tnf*, *Lta* and *Ltb*) to promote O_3-induced lung inflammation and injury. In support of this notion, the MHC (specifically HLA genes) has the strongest influence on susceptibility to human autoimmune diseases, but recent evidence suggests that *TNF* may also be involved in autoimmune diseases [29].

In summary, this integrative genomics investigation utilized congenic mouse lines to narrow the chromosome 17 QTL for susceptibility to O_3-induced inflammation in the mouse. We used sequence analysis to identify candidate genes in the narrowed QTL, which were then

tested for proof of concept. The novel role of MHC Class II genes and the *Tnf* cluster in susceptibility to O_3-induced inflammation provides unique insight to the mechanisms of O_3 effects in the lung and may lead to alternative means to prevent oxidant injury to lung tissues.

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Table 1. Nomenclature and characteristics of the congenic and control strains used for investigation.

		Congenic Region		
Strain Name	**Abbreviation**	**Mbp**	**cM**	**H2 Haplotype****
Congenic strains				
B10.A-*H2*^{h2}/(2R)SgSnJ	2R	33.74 – 35.34	18.20– 19.06	H2^{h2}
B10.A-*H2*^{h4}/(4R)SgDvEgJ	4R	29.09 – 34.38	16.30 – 18.64	H2^{h4}
B10.A-*H2*^{i5} *H2-T18*^{a}/(5R)SgSnJ	5R	34.45 – 36.35	18.70 – 19.14	H2^{i5}
C3.SW-*H2*^{b}/SnJ	C3H-H2^{b}	33.74 – 43.80	18.20 – 23.30	H2^{bc}
Control strains				
C57BL/10SnJ†	B10	---	---	H2^{b}
A/WySnJ ‡	A	---	---	H2^{a}
C3H/HeSnJ^{++}	HeSnJ	---	---	H2^{k}

* Mbp and cM were determined using the MGI website:http://www.informatics.jax.org/searches/marker and genotyped by Malhotra and Travis, unpublished).

** MHC H2 Haplotypes according to Appendix 2: MHC H2 haplotypes (www.jax.org/jaxmice/search).

†, background strain for 2R, 4R, and 5R mice , ‡donor strain for 2R, 4R, and 5R mice., $^{++}$ background strain for C3H-H2^{b} mice.

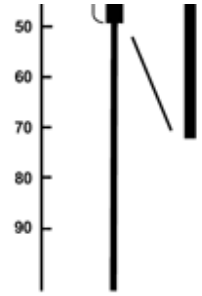
Table 2: Effect of deletion of *H2-Abl-Ea*, *Lta*, or *Lta*/*Tnf*/*Ltb* on macrophages and epithelial cells recovered from BAL following O_3 exposure.

		No. of cells (X 10^3 ml/BAL)			
			O_3 exposed		
Cell type	**Genotype**	**Air***	**24 h**	**48 h**	**72 h**
Macrophages	*H2Abl-Ea*$^{+/+}$	19.6 ± 0.1	33.6 ± 3.9**	48.1 ± 0.4**§	37.9 ± 3.7**§
	H2Abl-Ea$^{-/-}$	19.0 ±0.1	38.5 ± 6.3**	33.2 ± 0.4**	22.5 ± 1.3
	Lta$^{+/+}$	12.1 ± 1.2	22.2 ± 3.9**§	29.7 ± 7.7**§	37.9 ± 3.7**
	Lta$^{-/-}$	13.8 ±1.9	7.6 ± 2.2	21.2 ±2.7	32.0 ± 2.5**
	Lta/*Tnf*/*Ltb*$^{+/+}$	18.3 ± 3.4	26.7 ± 3.0	36.8 ± 3.9**§	38.5 ± 3.4**§
	Lta/*Tnf*/*Ltb*$^{-/-}$	26.6 ± 3.2	32.7 ± 3.7	25.2 ± 3.0	24.4 ± 3.9
Epithelial cells	MHC$^{+/+}$	1.4 ± 0.3	1.0 ± 0.2	2.6 ± 0.7**§	1.0 ± 0.3
	MHC$^{-/-}$	0.1 ± 0.2	1.8 ± 0.6	0.9 ± 0.1	0.9 ± 0.2
	Lta$^{+/+}$	0.7 ± 0.2	0.85 ± 0.3	1.4 ± 0.2	1.0 ± 0.3
	Lta$^{-/-}$	0.4 ± 0.2	0.5 ± 0.2	1.2 ± 0.3	1.7 ± 0.2
	**Lta*/*Tnf*/*Ltb*$^{+/+}$	1.0 ± 0.5	0.6 ± 0.04	1.5 ±0.3§	1.8 ± 1.5
	**Lta*/*Tnf*/*Ltb*$^{-/-}$	1.8 ± 1.5	1.0 ± 0.3	0.6 ± 0.05	1.7 ± 0.3

Data are presented as mean ± SEM, n = 3 – 10 mice per treatment group. * Air controls shown are for the 72 hr time point except for the *Tnf*$^{+/+}$ and *Tnf*$^{-/-}$ mice (48hr time point shown). Air controls for each time point were not significantly different from one another. ** $p < 0.05$, significantly elevated numbers of cells compared to air controls. § $p < 0.05$, significantly elevated numbers of cells compared to the transgenic strain.

Figure Legends

Figure 1. Schematic of the chromosome 17 congenic segments for 2R, 4R, 5R, and C3H-H2^{b} mice that were used to reduce inflammation 2 (*Inf2*). The congenic segments are shown with respect to *Inf2* (on left). The red rectangle represents the reduced *Inf2*. Mouse genes and human homologues and their chromosomal locations within the reduced *Inf2* are shown at right. While most of the genes are in the same order in both species, some are not. These locations were identified using the MGI website:http://www.informatics.jax.org/searches/marker website and NCBI. The yellow highlighted genes are major histocompatibility complex (MHC) class II genes and *Tnf* cluster genes identified as candidate genes using proof of concept experiments (see Figs. 5 and 6). Abbreviations:*Ager*, advanced glycosylation end product-specific receptor; *Agpat1;* 1-acylglycerol-3-phosphate O-acyltransferase 1; *Aif1*, allograft inflammatory factor 1; *Atf6b*, activating transcription factor 6 beta; *Bat5*, HLA-B associated transcript 5; *Btnl2*, butyrophilin-like 2; *Btnl1*, butyrophilin-like 1; *C2*, complement component 2; *C4a*, complement component 4A; *C4b*, complement component 4B; *Cfb*, complement factor B; CREBL1, cAMP responsive element binding protein-like 1; *Cyp21a1*, cytochrome P450, family 21, subfamily a, polypeptide 1; *Cyp21a2-ps*, cytochrome P450, family 21, subfamily a, polypeptide 2 pseudogene; *Ddah*, dimethylarginine dimethylaminohydrolase 2;*Egfl8*, EGF-like domain 8; *Ehmt2*, euchromatic histone-lysine N-methyltransferase 2; *Fkbpl*, FK506 binding protein-like; *Gpsm3*, G-protein signalling modulator 3*; H2-Ab1*, class II antigen A, beta 1; *H2-Aa*, class II antigen A, alpha; *H2-Eb1*, class II antigen E beta; *H2-Eb2*, class II antigen E beta 2; *H2-Ea*, class II antigen E alpha; *H2-ob*, O region beta locus; *Hspa1b*, heat shock 70kDa protein 1B; *Hspa1a*, heat shock 70kDa protein 1A; *Lsm2*, LSM2 homolog; *Lta*, lymphotoxin a; *Ltb*, lymphotoxin b; *Ly6g6c*, lymphocyte antigen 6 complex, locus G6C; *Ly6g5c*, lymphocyte antigen 6 complex, locus G5C; *Msh5*, mutS homolog 5;*Neu1*, neuraminidase1; *Notch4*, notch gene homolog 4; *Pbx2*, pre B-cell leukemia transcription factor 2; *Ppt2*, palmitoyl-protein thioesterase 2; *Prrt1*, proline-rich transmembrane protein 1; *Psmb8*, proteosome subunit, beta type 8; *Rdbp*,



C4a	34.865	C4A	32.091
Cyp21a2-ps	34.859	CYP21A2	32.114
Cyp21a1	34.937	CYP21A1	32.114
Stk19	34.959	STK19	32.048
Rdbp	34.986	RDBP	32.028
Cfb	34.991	CFB	32.022
C2	34.998	C2	32.003
Zbtb12	35.030	ZBTB12	31.975
Ehmt2	35.035	EHMT2	31.956
Slc44a4	35.050	SLC44A4	31.934
Neu1	35.066	NEU1	31.935
Hspa1b	35.093	HSPA1B	31.904
Hspa1a	35.094	HSPA1A	31.891
Hspa1l	35.110	HSPA1L	31.885
Lsm2	35.119	LSM2	31.873
Vars	35.136	VARS	31.853
Msh5	35.166	MSH5	31.816
Ddah	35.196	DDAH	31.803
Ly6g6c	35.204	LY6G6C	31.794
Bat5	35.226	BAT5	31.771
Ly6g5c	35.245	LY6G5C	31.754
Aif1	35.308	AIF1	31.691
Ltb	**35.331**	**LTB**	**31.657**
Tnf	**35.336**	**TNF**	**31.653**
Lta	**35.340**	**LTA**	**31.648**

carrier family 44, member
ing cassette, sub-family B
B (MDR/TAP); *Tnf*, tumor
b12, zinc finger and BTB

Figure 2. Bronchoalveolar lavage fluid (BALF) inflammatory parameters in the C3H-H2b and HeSnJ mice exposed continuously to 0.3 ppm O_3 or air for 48 or 72 h. A. Macrophages; B. Polymorphonuclear leukocytes (PMNs); C. Epithelial cells; D. Total BALF protein. Data are presented as means ± SEM (n=3-5 per experimental group). *, Significantly different from air exposed mice ($P < 0.05$). $^{+}$, Significantly different from HeSnJ mice ($P < 0.05$).

Figure 3. Bronchoalveolar lavage fluid (BALF) inflammatory parameters in B10, 4R, 5R, 2R, and A/Wy mice exposed continuously to 0.3 ppm O_3 or air for 48 or 72 h. A. Macrophages; B. Polymorphonuclear leukocytes (PMNs); C. Epithelial cells; D. Total BALF protein. Data are presented as means ± SEM (n=3-12 per experimental group). *, Significantly different from air exposed mice ($P < 0.05$). $^{+}$, Significantly different from B10 mice ($P < 0.05$).

Figure 4. Gene expression for candidate genes identified within the reduced *Inf2* region of B6 (susceptible) and C3 (resistant) mice. Six genes were analyzed using quantitative RT-PCR (*H2Eb1*, *H2Eb2*, *Hspa1b*, *Btnl1*, *Tnf*, *Lta*). Data are presented as means ± SEM (n=3-7 per experimental group) and were determined using the comparative C_T method (see Supplemental Materials and Methods section). *Y-axis* represents the gene of interest normalized first to 18S

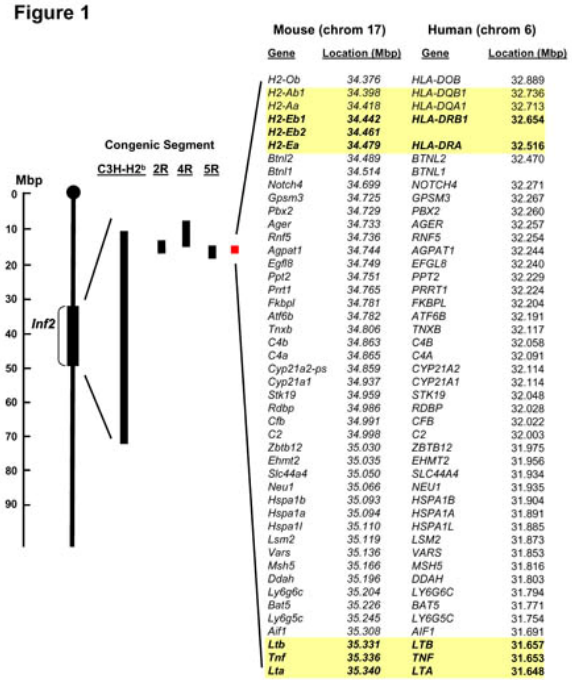
followed by determination of the fold-change relative to the average air control value for each strain. *, Significantly different from air exposed mice ($P < 0.05$). $^{+}$, Significantly different from B6 mice ($P < 0.05$).

Figure 5. Deficiency in MHC class II genes significantly reduced O_3-induced responses. A. Bronchoalveolar lavage fluid (BALF) polymorphonuclear leukocytes (PMNs) and total protein in *H2Ab1-Ea*$^{-/-}$ and *H2Ab1-Ea*$^{+/+}$ mice exposed continuously to 0.3 ppm O_3 or air for 48 or 72 h. Data are presented as means ± SEM (n=3-10 per experimental group). *, Significantly different from air exposed mice ($P < 0.05$). $^{+}$, Significantly different from *H2Ab1-Ea*$^{+/+}$ mice ($P < 0.05$). B. Gene expression for *Lta* and *Tnf* genes in *H2Ab1-Ea* deficient mice compared to wildtype mice. Data are presented as means ± SEM (n=3-6 per experimental group) and were determined using the comparative C_T method (see Supplemental Materials and Methods section). *Y-axis* represents either *Lta* or *Tnf* normalized first to 18S followed by determination of the fold-change relative to the average air control value for each strain. *, Significantly different from air exposed mice ($P < 0.05$). $^{+}$, Significantly different from *H2Ab1-Ea*$^{+/+}$ mice ($P < 0.05$).

Figure 6. O_3-induced responses in mice deficient in *Lta* or the *Tnf* cluster (*Lta, Tnf, Ltb*) genes. A) BALF PMNs and total protein for mice deficient in *Lta* and their wildtype controls. Data are presented as means ± SEM (n=3-10 per experimental group). *, Significantly different from air exposed mice ($P < 0.05$). $^{+}$, Significantly different from *Lta*$^{+/+}$ mice ($P < 0.05$). B. BALF inflammatory parameters for the *Lta*/*Tnf*/*Ltb* deficient mice in response to O_3 compared to wildtype mice. Data are presented as means ± SEM (n=3-10 per experimental group). *, Significantly different from air exposed mice ($P < 0.05$). $^{+}$, Significantly different from *Lta/Tnf/Ltb*$^{+/+}$ mice ($P < 0.05$). C) Gene expression for the MHC class II genes (*H2-Eb1* and *H2-Eb2*) in the mice deficient in either *Lta* or *Lta*/*Tnf*/*Ltb* compared to the wildtype mice. B6 are the wildtype for both strains and the numbers were combined since they were not significantly different from one another. No 24 h samples for *Lta*/*Tnf*/*Ltb*$^{-/-}$ mice were done for gene

expression analysis. Data are presented as means ± SEM (n=3-6 per experimental group) and were determined using the comparative C_T method (see Supplemental Materials and Methods section). *Y-axis* represents either *H2-Eb1* or *H2-Eb2* normalized first to 18S followed by determination of the fold-change relative to the average air control value for each strain. *, Significantly different from air exposed mice ($P < 0.05$). $^{+}$, Significantly different from wildtype mice ($P < 0.05$).

Figure 1



Mouse (chrom 17)		Human (chrom 6)	
Gene	Location (Mbp)	Gene	Location (Mbp)
H2-Ob	*34.376*	*HLA-DOB*	32.889
H2-Ab1	*34.398*	*HLA-DQB1*	32.736
H2-Aa	*34.418*	*HLA-DQA1*	32.713
H2-Eb1	***34.442***	***HLA-DRB1***	**32.654**
H2-Eb2	***34.461***		
H2-Ea	***34.479***	***HLA-DRA***	**32.516**
Btnl2	*34.489*	*BTNL2*	32.470
Btnl1	*34.514*	*BTNL1*	
Notch4	*34.699*	*NOTCH4*	32.271
Gpsm3	*34.725*	*GPSM3*	32.267
Pbx2	*34.729*	*PBX2*	32.260
Ager	*34.733*	*AGER*	32.257
Rnf5	*34.736*	*RNF5*	32.254
Agpat1	*34.744*	*AGPAT1*	32.244
Egfl8	*34.749*	*EFGL8*	32.240
Ppt2	*34.751*	*PPT2*	32.229
Prrt1	*34.765*	*PRRT1*	32.224
Fkbpl	*34.781*	*FKBPL*	32.204
Atf6b	*34.782*	*ATF6B*	32.191
Tnxb	*34.806*	*TNXB*	32.117
C4b	*34.863*	*C4B*	32.058
C4a	*34.865*	*C4A*	32.091
Cyp21a2-ps	*34.859*	*CYP21A2*	32.114
Cyp21a1	*34.937*	*CYP21A1*	32.114
Stk19	*34.959*	*STK19*	32.048
Rdbp	*34.986*	*RDBP*	32.028
Cfb	*34.991*	*CFB*	32.022
C2	*34.998*	*C2*	32.003
Zbtb12	*35.030*	*ZBTB12*	31.975
Ehmt2	*35.035*	*EHMT2*	31.956
Slc44a4	*35.050*	*SLC44A4*	31.934
Neu1	*35.066*	*NEU1*	31.935
Hspa1b	*35.093*	*HSPA1B*	31.904
Hspa1a	*35.094*	*HSPA1A*	31.891
Hspa1l	*35.110*	*HSPA1L*	31.885
Lsm2	*35.119*	*LSM2*	31.873
Vars	*35.136*	*VARS*	31.853
Msh5	*35.166*	*MSH5*	31.816
Ddah	*35.196*	*DDAH*	31.803
Ly6g6c	*35.204*	*LY6G6C*	31.794
Bat5	*35.226*	*BAT5*	31.771
Ly6g5c	*35.245*	*LY6G5C*	31.754
Aif1	*35.308*	*AIF1*	31.691
Ltb	***35.331***	***LTB***	**31.657**
Tnf	***35.336***	***TNF***	**31.653**
Lta	***35.340***	***LTA***	**31.648**

Figure 2

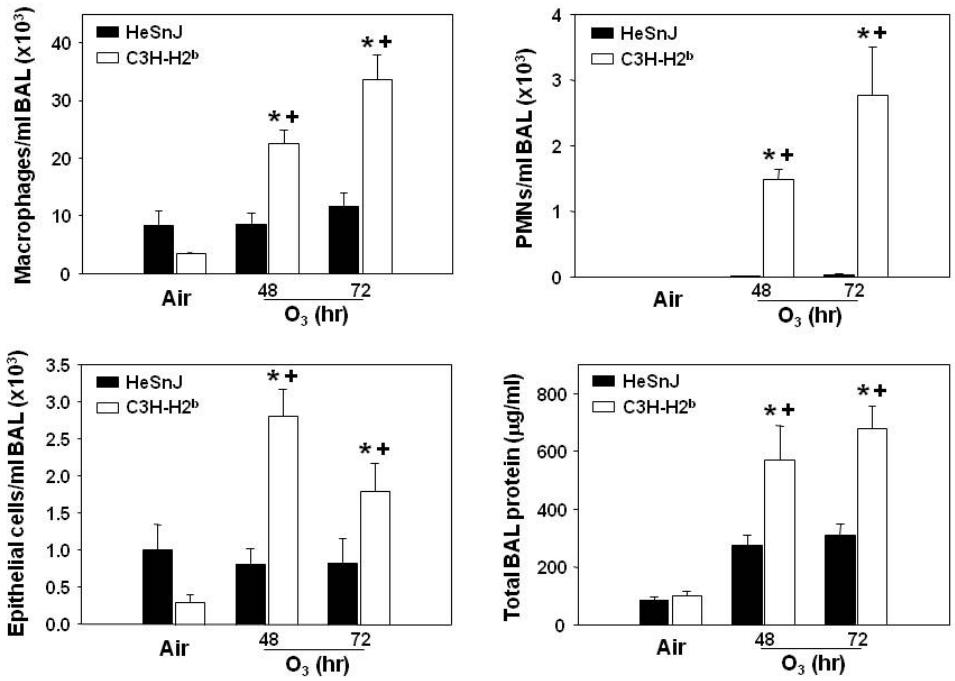


Figure 3

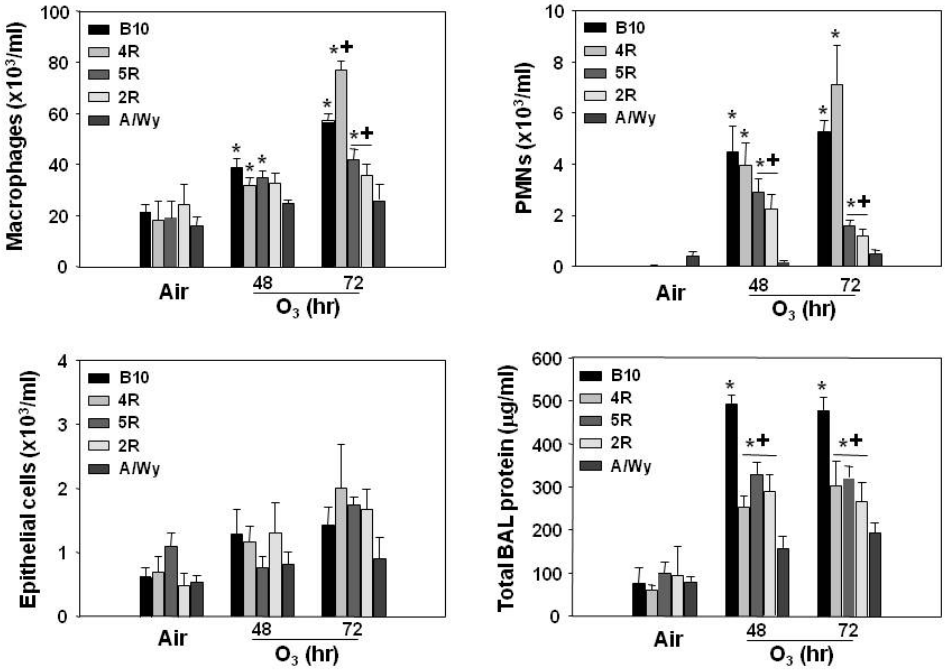

Macrophages (x10³/ml)
PMNs (x10³/ml)
Epithelial cells (x10³/ml)
Total BAL protein (µg/ml)
B10
4R
5R
2R
A/Wy
Air
48
72
O3 (hr)

Figure 4

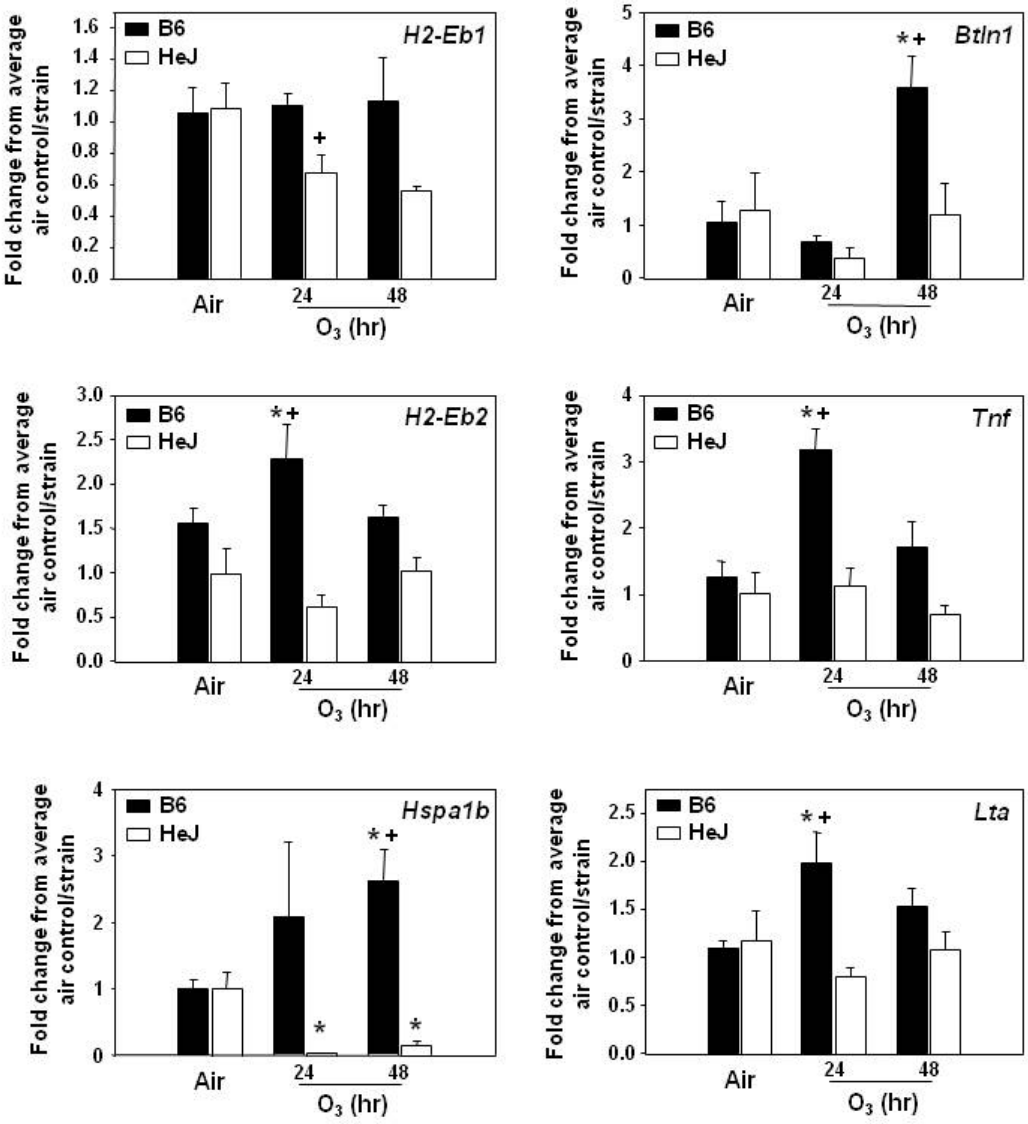

B6
HeJ
H2-Eb1
Btln1
H2-Eb2
Tnf
Hspa1b
Lta
Fold change from average air control/strain
Air
24
48
O3 (hr)
*
+

Figure 5

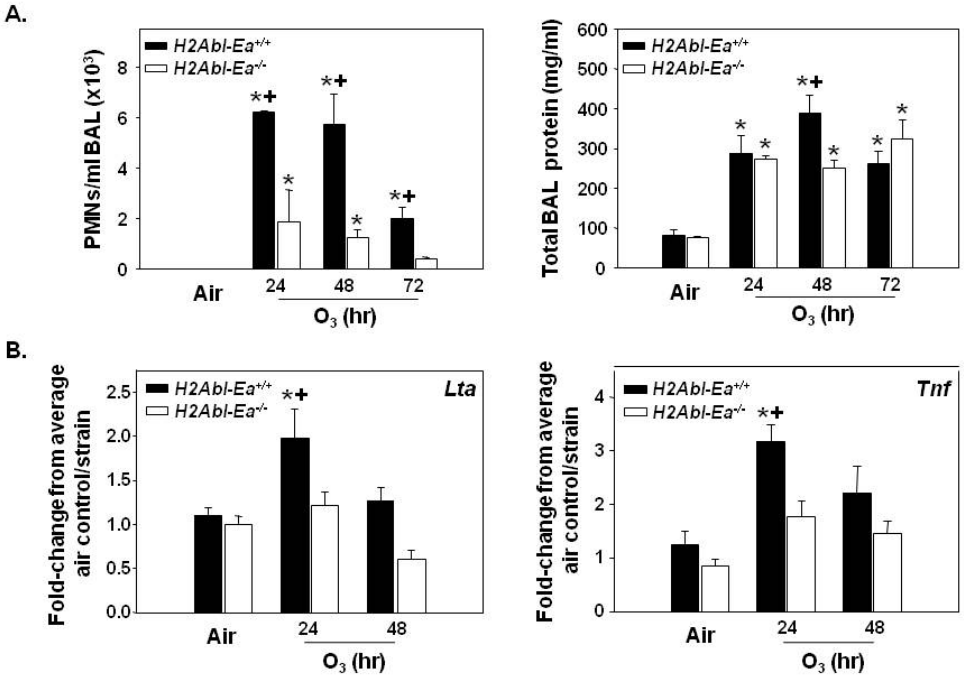
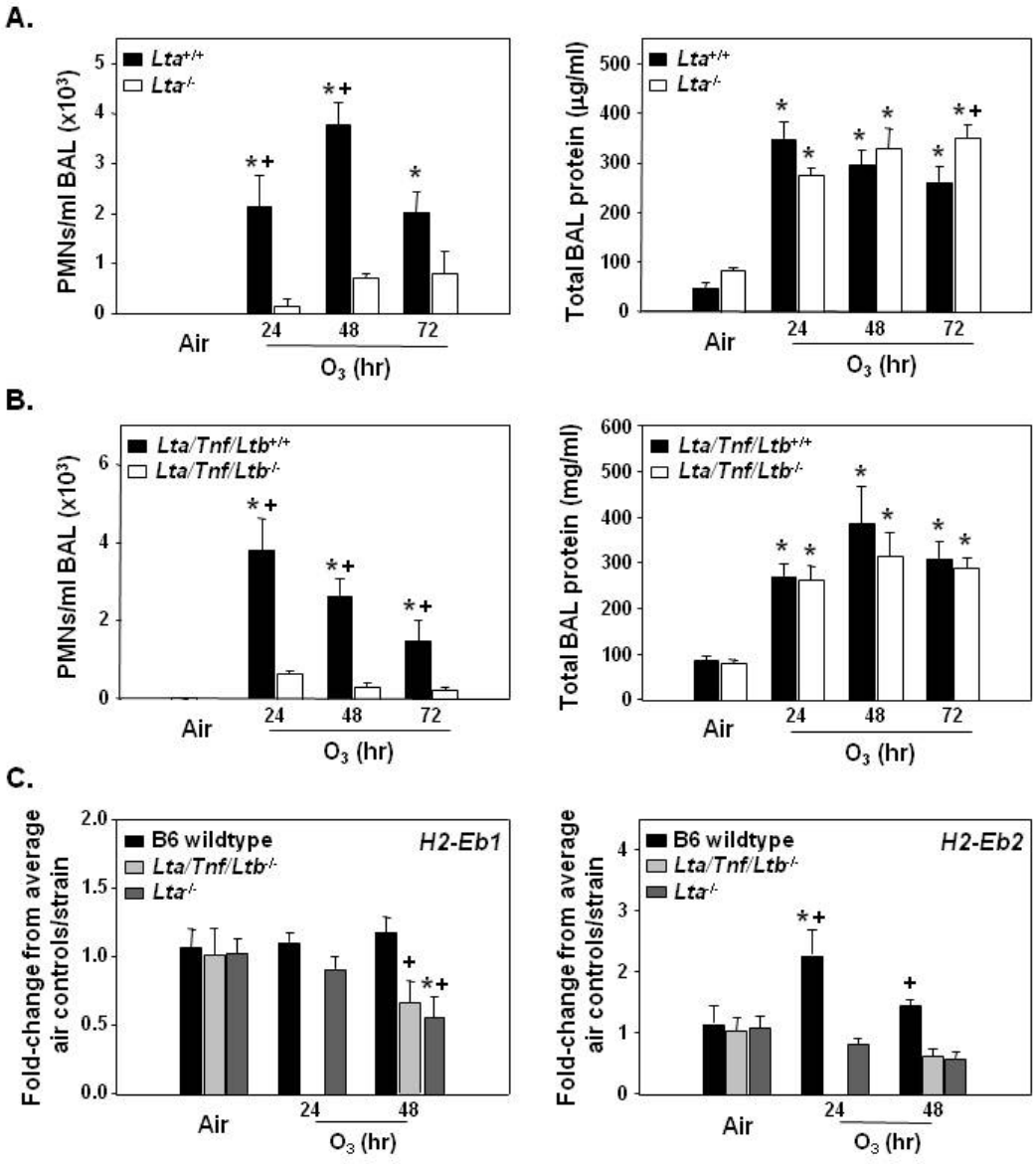

A.
PMNs/ml BAL (x10³)
H2Abl-Ea+/+
H2Abl-Ea-/-
Air
24
48
72
O3 (hr)
Total BAL protein (mg/ml)
B.
Fold-change from average air control/strain
Lta
Tnf

Figure 6


A.
PMNs/ml BAL (x10³)
Lta+/+
Lta-/-
Air
24
48
72
O3 (hr)
Total BAL protein (µg/ml)
B.
Lta/Tnf/Ltb+/+
Lta/Tnf/Ltb-/-
Total BAL protein (mg/ml)
C.
Fold-change from average air controls/strain
B6 wildtype
Lta/Tnf/Ltb-/-
Lta-/-
H2-Eb1
H2-Eb2