

Genetic ablation of glutaredoxin-1 causes enhanced resolution of airways hyperresponsiveness and mucus metaplasia in mice with allergic airways disease

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Hoffman SM, Tully JE, Lahue KG, Anathy V, Nolin JD, Guala AS, van der Velden JLJ, Ho Y-S, Aliyeva M, Daphtary N, Lundblad LKA, Irvin CG, Janssen-Heininger YMW. Genetic ablation of glutaredoxin-1 causes enhanced resolution of airways hyperresponsiveness and mucus metaplasia in mice with allergic airways disease. *Am J Physiol Lung Cell Mol Physiol* 303: L528–L538, 2012. First published June 26, 2012; doi:10.1152/ajplung.00167.2012.—Protein-S-glutathionylation (PSSG) is an oxidative modification of reactive cysteines that has emerged as an important player in pathophysiological processes. Under physiological conditions, the thiol transferase, glutaredoxin-1 (Glr1) catalyzes deglutathionylation. Although we previously demonstrated that Glr1 expression is increased in mice with allergic inflammation, the impact of Glr1/PSSG in the development of allergic airways disease remains unknown. In the present study we examined the impact of genetic ablation of *Glr1* in the pathogenesis of allergic inflammation and airway hyperresponsiveness (AHR) in mice. *Glr1*^{−/−} or WT mice were subjected to the antigen, ovalbumin (OVA), and parameters of allergic airways disease were evaluated 48 h after three challenges, and 48 h or 7 days after six challenges with aerosolized antigen. Although no clear increases in PSSG were observed in WT mice in response to OVA, marked increases were detected in lung tissue of mice lacking *Glr1* 48 h following six antigen challenges. Inflammation and expression of proinflammatory mediators were decreased in *Glr1*^{−/−} mice, dependent on the time of analysis. WT and *Glr1*^{−/−} mice demonstrated comparable increases in AHR 48 h after three or six challenges with OVA. However, 7 days postcessation of six challenges, parameters of AHR in *Glr1*^{−/−} mice were resolved to control levels, accompanied by marked decreases in mucus metaplasia and expression of Muc5AC and GOB5. These results demonstrate that the Glr1/S-glutathionylation redox status in mice is a critical regulator of AHR, suggesting that avenues to increase S-glutathionylation of specific target proteins may be beneficial to attenuate AHR.

asthma; lung inflammation; protein S-glutathionylation

CHRONIC INFLAMMATORY DISORDERS of the lung, including asthma, are accompanied by changes in the oxidative environment (7). For instance, oxidative inactivation of the antioxidant enzymes copper zinc containing SOD and manganese SOD, have been demonstrated in patients with asthma (12, 13), and changes in the redox status of the tripeptide antioxidant, GSH have also been reported in patients with asthma compared with healthy controls (17, 37). The exact mechanisms whereby oxidants contribute to disease pathogenesis remain unresolved.

GSH, which is highly abundant and acts as an antioxidant and reducing agent in cells in lung lining fluid, plays a major role in maintenance of overall redox homeostasis. Consequently, agents that cause oxidative stress are known to decrease the ratio of reduced GSH to GSSG (glutathione disulfide) (35). In addition to changes in reduced to oxidized glutathione ratio, under conditions of oxidative stress, glutathione can become conjugated to reactive cysteines in proteins, a posttranslational modification known as protein S-glutathionylation (PSSG; also known as S-glutathiolation, or protein-mixed disulfides). PSSG is a redox-based posttranslational modification of proteins that changes structure and function of proteins in a reversible and tightly regulated manner (21, 26). Mammalian glutaredoxin (Glr) enzymes are members of the thioredoxin family of thiol transferases that, under physiological conditions, specifically catalyze deglutathionylation reactions, leading to the restoration of the reduced sulfhydryl group of protein cysteines (24).

The functional significance of altered regulation of protein function through PSSG in allergic airways disease remains unknown. We recently demonstrated increases in Glr1 expression and increases in Glr activity in lung tissue from mice with antigen-induced allergic inflammation. Increases in Glr1 immunoreactivity occurred predominantly in the bronchiolar epithelium of mice with allergic airways inflammation (39). Furthermore, in mice lacking the Glr1 gene (*Glr1*^{−/−}), LPS-induced inflammation and macrophage activation were attenuated, in association with enhanced S-glutathionylation (4). Primary lung epithelial cells derived from *Glr1*^{−/−} mice also showed markedly attenuated production of inflammatory mediators in response to LPS compared with epithelial cells isolated from wild-type (WT) mice (38). The goal of the present study was to determine the impact of *Glr1* ablation on the development of allergic airways disease, using the well-characterized model of ovalbumin (OVA)-induced Th2 inflammation and airway hyperresponsiveness (AHR). Our results demonstrate that *Glr1*^{−/−} mice showed enhanced resolution of AHR and mucus metaplasia, in association with increases in PSSG, suggesting a role for PSSG in ameliorating AHR.

MATERIALS AND METHODS

Glr1^{−/−} mice were backcrossed for > 10 generations into a BALB/c background (19) (Jackson Laboratories, Bar Harbor, ME) and were housed in the University of Vermont Animal Facility. For all experiments, 8- to 12-wk-old *Glr1*^{−/−} and littermate WT BALB/c control mice were used, as approved by the Institutional Animal Care and Use Committee.

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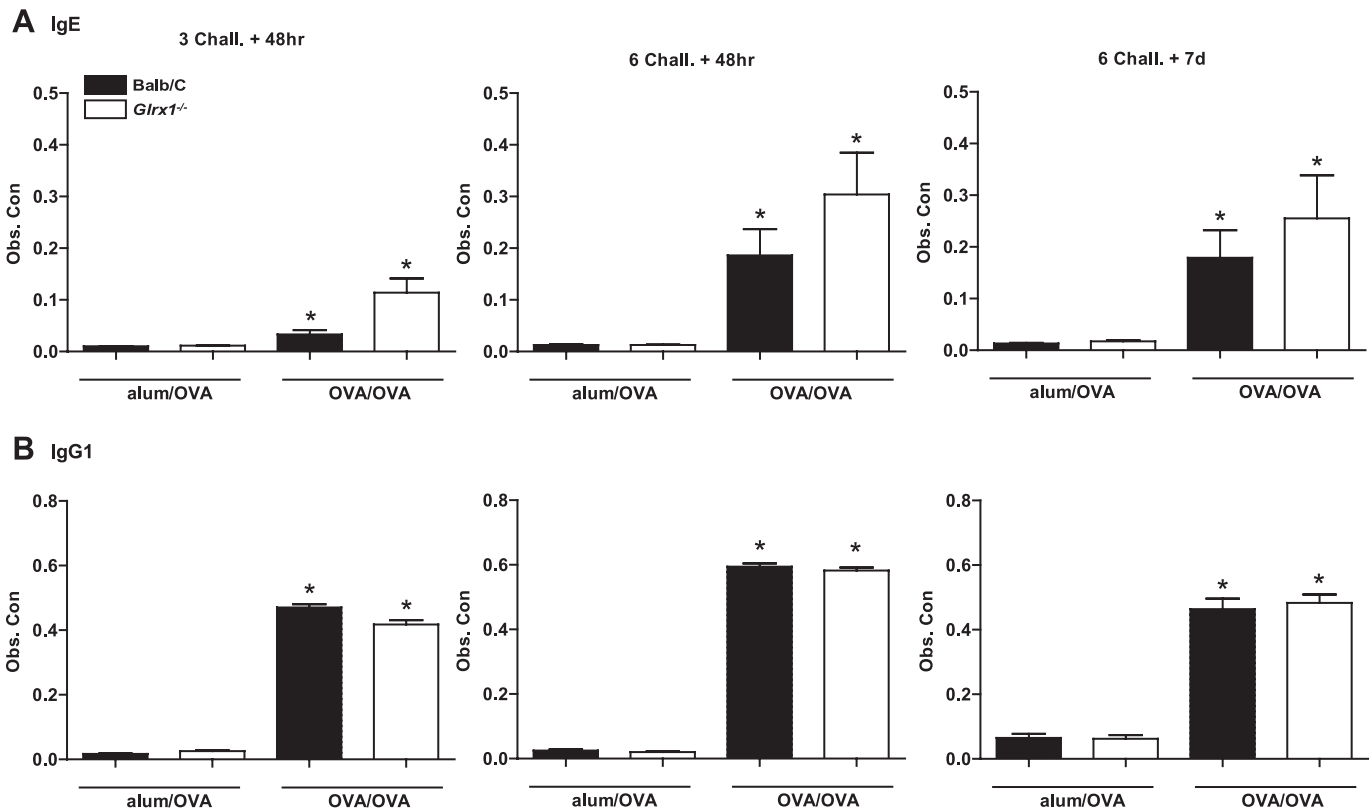


Fig. 1. Assessment of ovalbumin (OVA)-specific immunoglobulins in wild-type (WT) or *glutaredoxin-1*^{-/-} (*Glxr1*^{-/-}) mice following sensitization and challenge (Chall.) with OVA. Serum immunoglobulin levels were measured by ELISA. Standard curves for OVA-specific IgE (A) and IgG1 (B) were generated from alum/OVA-sensitized BALB/c mouse serum (Obs. Con). For comparisons, values for the most concentrated were set to 10,000 U/ml. Data are expressed as means $\pm$ SE from 8 to 10 mice per group. * $P < 0.05$ (ANOVA) compared with respective alum/OVA control groups. Black bars: BALB/c mice, white bars: *Glxr1*^{-/-} mice.

OVA model of allergic airway disease. WT BALB/c and *Glxr1*^{-/-} mice were administered 20 μ g of the antigen OVA (Grade V; Sigma-Aldrich, St. Louis, MO) with aluminum hydroxide (alum) (Pierce, Rockford, IL) via an intraperitoneal injection on days 0 and 14 as previously described (25, 28). The control group was mock sensitized, receiving intraperitoneal PBS and alum. All groups of mice were challenged using three or six doses of aerosolized 1% OVA in sterile PBS for 30 min on days 21, 22, and 23 (33), or on days 21–26. The animals were harvested 48 h or 7 days postchallenge.

Assessment of AHR. Mice were anesthetized with intraperitoneal pentobarbital sodium (90 mg/kg), tracheotomized, and mechanically

ventilated at 200 breaths/min using a FlexiVent computer-controlled small animal ventilator (SCIREQ, QC, Canada), as previously described (28, 40). Airway responsiveness was quantified by averaging the three highest measurements obtained at each incremental methacholine dose.

Bronchoalveolar lavage and lung processing. Bronchoalveolar lavage (BAL) (800–1,000 μ l) was collected from mice immediately after the completion of measurements of respiratory mechanics. Total and differential cell counts were performed as previously described (28). Briefly, cells were isolated by centrifugation and total cell counts were performed using the Advia 120 automated hematology analyzer

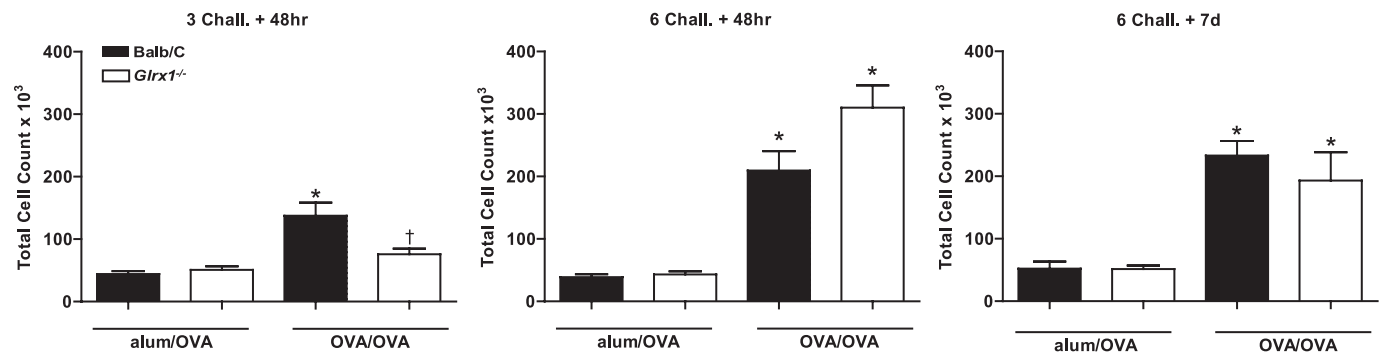


Fig. 2. Total cell counts in bronchoalveolar lavage (BAL) from WT or *Glxr1*^{-/-} mice following sensitization and challenge with OVA. Control mice received an intraperitoneal injection of alum and PBS (alum/OVA) at days 0 and 14. Sensitized mice were administered an intraperitoneal injection of alum and OVA (OVA/OVA) at days 0 and 14. Both groups were nebulized with aerosolized OVA at days 21, 22, and 23, or days 21 through 26. BAL was performed 48 h or 7 days after the last aerosolized OVA challenge. Total cell numbers were determined by Advia, and data are expressed as means $\pm$ SE from 8 to 10 mice per group. * $P < 0.05$ (ANOVA) compared with respective alum/OVA control groups. Black bars: BALB/c mice, white bars: *Glxr1*^{-/-} mice.

Table 1. *Inflammatory cell profiles in bronchoalveolar lavage (BAL) following ovalbumin (OVA) sensitization and challenge of wild-type (WT) or glutaredoxin-1^{-/-} (Glrx1^{-/-}) mice*

Relative Expression	Balb/C		Glrx1 ^{-/-}	
	Alum/OVA	OVA/OVA	Alum/OVA	OVA/OVA
3 Chall. +48 h, 10 ³				
MACS	42.2 ± 5.4	29.5 ± 5.4	52.4 ± 5.4	38.7 ± 4.3
EOS	0.0 ± 0.0	93.2 ± 14.8*	0.9 ± 0.5	27.7 ± 5.4*†
PMN	0.8 ± 0.3	11.7 ± 3.9*	1.2 ± 0.3	7.7 ± 2.1*
LYMPH	0.0 ± 0.0	3.1 ± 0.5*	0.5 ± 0.2	1.7 ± 0.4
6 Chall. +48 h, 10 ³				
MACS	36.1 ± 4.9	32.2 ± 4.8	36.4 ± 4.1	49.1 ± 13.2
EOS	0.2 ± 0.1	150.2 ± 30.7*	0.2 ± 0.1	213.8 ± 27.9*
PMN	1.6 ± 0.6	21.0 ± 9.3*	0.5 ± 0.2	21.4 ± 8.0*
LYMPH	0.9 ± 0.2	6.0 ± 1.4*	0.4 ± 0.2	13.5 ± 5.2*
6 Chall. +7 days, 10 ³				
MACS	49.1 ± 11.1	43.7 ± 7.2	50.3 ± 5.6	44.3 ± 7.2
EOS	0.6 ± 0.3	169.1 ± 21.5*	0.1 ± 0.0	133.0 ± 38.0*
PMN	1.2 ± 0.5	1.3 ± 0.7	3.5 ± 1.5	1.1 ± 0.4
LYMPH	1.3 ± 0.7	6.7 ± 2.5*	10.7 ± 5.4	4.9 ± 3.9*

A minimum of 300 cells were enumerated by 2 independent investigators. Results were averaged and are expressed as means ± SE from 8 to 10 mice per group. Chall., challenged; MACS, macrophages; EOS, eosinophils; PMN, polymorphonuclear neutrophils (leukocytes); LYMPH, lymphocytes. **P* < 0.05 (ANOVA) compared with respective alum/OVA control groups. †*P* < 0.05 (ANOVA) compared with WT OVA/OVA groups.

system. Cytospins were performed and stained using the Hema3 kit (Fisher Scientific, Kalamazoo, MI). Differential cell counts were performed on a minimum of 300 cells. Right lung lobes were flash frozen for RNA and protein isolation. Left lung lobes were fixed with 4% paraformaldehyde, mounted in paraffin, and 5-μm sections were prepared for histopathology as previously described (33).

Cytokine profiling. BAL samples were thawed and assayed for OVA-specific immunoglobulins and inflammatory cytokine levels as previously described (4). ELISA were utilized to assess cytokine content in undiluted BAL according to manufacturer's instructions (R&D Systems, Minneapolis, MN). For simultaneous quantitation of multiple analytes, undiluted BAL fluid or serum was analyzed using a mouse cytokine 23-plex kit on the Bio-Plex suspension array system (Bio-Rad, Hercules, CA) (8).

mRNA analysis. Total RNA was isolated from pulverized lung tissue using the RNeasy kit (Qiagen, Valencia, CA). One microgram of RNA was used for cDNA synthesis and reverse transcribed for Taqman gene analysis using SYBR green (Bio-Rad). Primers for quantitative RT-PCR include: KC (forward: 5'-GCTGGATTACACCT-CAAGAA-3', reverse: 5'-TGGGGACACCTTTTAGCATC-3'); regulated on activation normal T-expressed and presumably secreted (RANTES) (forward: 5'-ATATGGCTCGGACACCACTC-3', reverse: 5'-TCCTTCGAGTGACAAACACG-3'); IL-6 (forward: 5'-GATGCTGGTGACAAACAC-3', reverse: 5'-CAGAATTGCCATTG-CACAAC-3'); CCL-20 (forward: 5'-AAGACAGATGGCCGATGA-AG-3', reverse: 5'-AGCCCTTTTACCCAGTTCT-3'); Muc5ac (forward: 5'-CAGTGAATTCTGGAGGCCAACAAGGTAGAG-3', reverse: 5'-AGCTAAGCTTAGATCTGGTTGGGACAGCAGC-3'); GOB5 (forward: 5'-ACTAAGGTGGCCTACCTCCAA-3', reverse: 5'-GG-AGGTGACAGTCAAGGTGAGA-3'); and IL-5 (forward: 5'-AT-GGAGATTCCCATGAGCAC-3', reverse: 5'-CCCACGGACAGTTTGATTCT-3'). The fold induction was calculated using the housekeeping gene, *cyclophilin*.

Histopathology. Periodic acid Schiff staining was performed, and mucus metaplasia was evaluated by obtaining three images of small bronchioles (×20 objective) of similar dimensions from each mouse. Images were blinded and ranked by two independent investigators, at a scale from 0 to 3: 0, no reactivity; 1, minimal staining; 2, moderate staining; 3, prominent staining. The cumulative score from each mouse was then averaged according to treatment group.

Biochemical analysis of protein S-glutathionylation in lung tissue. Protein S-glutathionylation in lung tissue was determined using the

glutathione/glutathione reductase/NADPH/5,5'-dithiobis (2-nitrobenzoic acid) recycling assay, according to procedures described previously (36). In brief, pulverized lung tissue stored at -80°C was homogenized in buffer containing 137 mM Tris-HCl, pH 8.0, 130 mM NaCl, and 1% NP-40. Protein content was determined, and samples were equalized for protein content. Then 200 μg of protein was precipitated with acetone. The pellet was resuspended in 0.1% Triton X-100 and 0.6% sulfasalicylic acid containing buffer and

Table 2. *Analysis of mRNA expression of NF-κB-dependent inflammatory cytokines in lung homogenates from WT and Glrx1^{-/-} mice subjected to sensitization and challenge with OVA*

Relative Expression	Balb/C		Glrx1 ^{-/-}	
	Alum/OVA	OVA/OVA	Alum/OVA	OVA/OVA
3 Chall. +48 h				
CCL-20	1.0 ± 0.3	3.8 ± 0.5*	1.4 ± 0.2	1.9 ± 0.3†
IL-6	1.0 ± 0.2	1.5 ± 0.4	0.4 ± 0.1	1.4 ± 0.3
RANTES	1.0 ± 0.1	0.8 ± 0.1	1.0 ± 0.2	1.0 ± 0.1
KC	1.0 ± 0.1	7.6 ± 1.2*	0.9 ± 0.2	3.6 ± 0.9†
IL-5	1.0 ± 0.3	9.4 ± 2.8*	0.9 ± 0.4	1.1 ± 0.5†
6 Chall. +48 h				
CCL-20	1.0 ± 0.3	2.4 ± 0.7*	0.6 ± 0.1	2.4 ± 0.6
IL-6	1.0 ± 0.1	4.8 ± 1.1*	0.6 ± 0.2	2.1 ± 0.7
RANTES	1.0 ± 0.2	9.3 ± 2.2*	1.7 ± 0.5	4.1 ± 0.7
KC	1.0 ± 0.2	2.4 ± 1.2	2.1 ± 0.8	1.3 ± 0.5
IL-5	1.0 ± 0.1	6.2 ± 1.5*	2.1 ± 1.6	9.8 ± 2.0*
6 Chall. +7d				
CCL-20	1.0 ± 0.4	1.8 ± 0.4	0.4 ± 0.1	1.7 ± 0.6*
IL-6	1.0 ± 0.3	0.7 ± 0.3	0.5 ± 0.2	1.4 ± 1.0
RANTES	1.0 ± 0.1	0.4 ± 0.1	0.4 ± 0.1	0.5 ± 0.1
KC	1.0 ± 0.1	2.5 ± 1.1*	0.4 ± 0.1	1.1 ± 0.2
IL-5	1.0 ± 0.2	4.4 ± 1.0*	0.8 ± 0.1	3.8 ± 0.6*

CCL-20, IL-6, RANTES, KC, and IL-5 mRNA from whole lung homogenates of OVA/OVA or alum/OVA mice were analyzed by real-time PCR analyses. Results were normalized to the housekeeping gene *cyclophilin*. CCL-20, chemokine (C-C motif) ligand-20; RANTES, regulated on activation normal T-expressed and presumably secreted; KC, CXCL1 (neutrophil-activating protein). Data are expressed as fold increases in expression compared with WT alum/OVA controls. **P* < 0.05 (ANOVA) compared with respective alum/OVA control groups. †*P* < 0.05 (ANOVA) compared with WT OVA/OVA groups.

Table 3. Analysis of content of NF- κ B-dependent inflammatory cytokines in BAL from WT and *Glrx1*^{-/-} mice subjected to sensitization and challenge with OVA

Relative Expression	Balb/C		<i>Glrx1</i> ^{-/-}	
	Alum/OVA	OVA/OVA	Alum/OVA	OVA/OVA
3 Chall. +48 h, pg/ml				
CCL-20	94.6 $\pm$ 26.6	183.6 $\pm$ 22.9*	55.7 $\pm$ 9.8	83.3 $\pm$ 14.3†
IL-6	2.0 $\pm$ 0.3	2.7 $\pm$ 0.4	2.0 $\pm$ 0.4	6.1 $\pm$ 1.1*
RANTES	16.5 $\pm$ 1.5	21.2 $\pm$ 4.0	17.2 $\pm$ 2.5	20.4 $\pm$ 3.7
KC	44.0 $\pm$ 5.6	249.7 $\pm$ 15.9*	29.1 $\pm$ 4.6	161.7 $\pm$ 27.9*†
6 Chall. +48 h, pg/ml				
CCL-20	40.3 $\pm$ 16.4	95.7 $\pm$ 31.8*	20.2 $\pm$ 5.9	50.1 $\pm$ 16.8*
IL-6	16.8 $\pm$ 3.9	16.2 $\pm$ 2.5	16.1 $\pm$ 3.9	14.2 $\pm$ 2.0
RANTES	79.7 $\pm$ 19.9	67.8 $\pm$ 8.5	76.3 $\pm$ 22.5	63.8 $\pm$ 8.0
KC	142.0 $\pm$ 21.3	677.9 $\pm$ 147.2*	128.6 $\pm$ 30.5	478.7 $\pm$ 104.3*
6 Chall. +7 days, pg/ml				
CCL-20	60.2 $\pm$ 19.3	131.2 $\pm$ 47.8*	86.2 $\pm$ 13.3	40.7 $\pm$ 32.2
IL-6	19.4 $\pm$ 3.7	18.2 $\pm$ 4.4	17.9 $\pm$ 4.5	10.5 $\pm$ 1.7
RANTES	111.1 $\pm$ 16.6	69.9 $\pm$ 12.5	82.2 $\pm$ 14.2	43.0 $\pm$ 9.5
KC	227.9 $\pm$ 80.5	667.3 $\pm$ 164.0*	110.4 $\pm$ 19.8	502.0 $\pm$ 116.9*

BAL fluid was analyzed by ELISA for cytokines: CCL-20, IL-6, RANTES, and KC. Data are expressed as means $\pm$ SE from 8 to 10 mice per group. * P < 0.05 (ANOVA) compared with respective alum/OVA control groups. † P < 0.05 (ANOVA) compared with WT OVA/OVA groups.

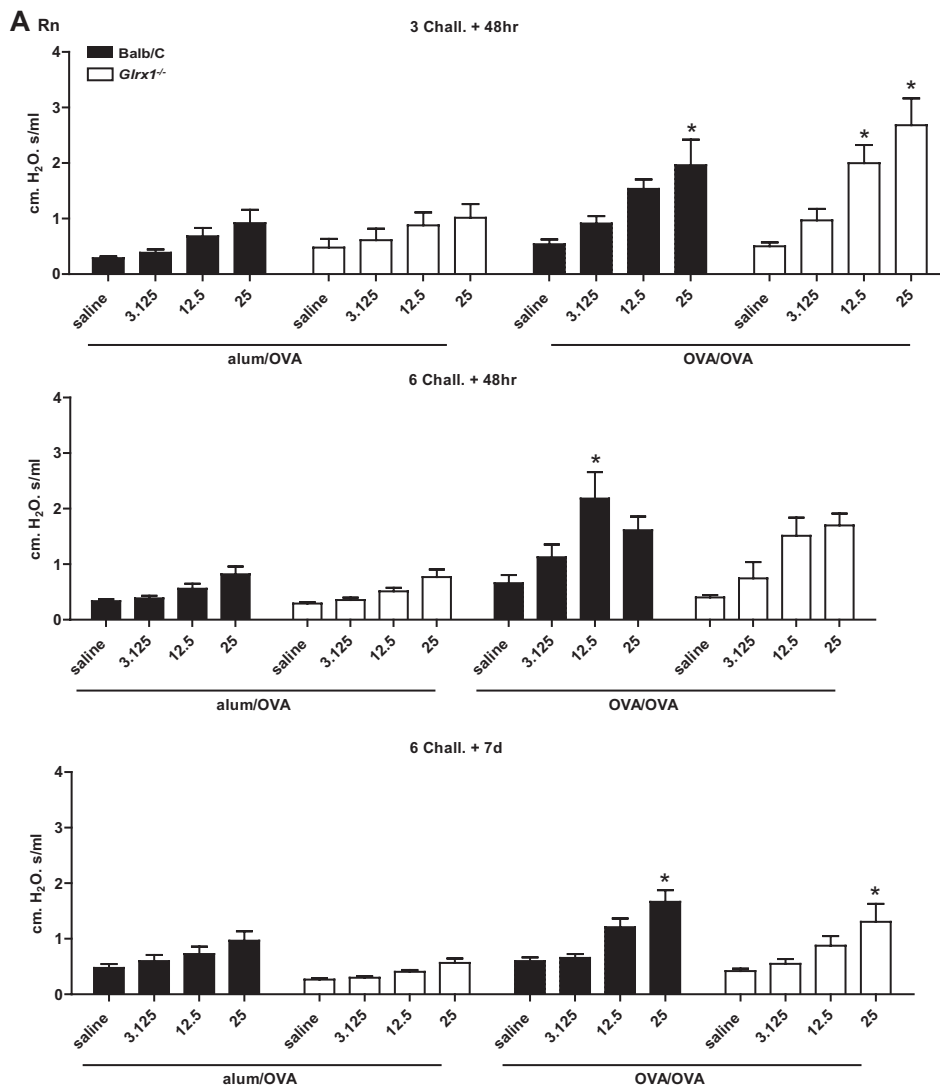


Fig. 3. Assessment of airway hyperresponsiveness (AHR) in WT or *Glrx1*^{-/-} mice following sensitization and challenge with OVA. Changes in respiratory mechanics were analyzed in mock-sensitized (alum/OVA) or OVA-sensitized (OVA/OVA) WT (black bars) or *Glrx1*^{-/-} mice (white bars) 48 h following 3 or 6 challenges with aerosolized OVA, or 7 days following the 6th daily aerosolized OVA challenge. Ascending doses of methacholine were administered to determine airway resistance (R_N; A), tissue dampening (G; B), and elastance/stiffness (H; C) parameters. Data are expressed as % change from baseline measurements $\pm$ SE for each of the parameters measured from 8 to 10 mice per group. * P < 0.05 (ANOVA) compared with respective alum/OVA control groups. † P < 0.05 (ANOVA) compared with WT OVA/OVA groups.

freeze thawed twice. Protein-associated glutathione was released with sodium borohydride, and GSH was determined. Samples that were not treated with sodium borohydride were used as a control. The sodium borohydride sensitive fraction of GSH was calculated and expressed as nanomoles GSH per milligram of protein.

Statistical analyses. Analyses of all data were performed using the Graph Pad Prism software (Graphpad) by ANOVA or Student's *t*-test where appropriate. All experiments were repeated twice, and data from combined experiments are presented means $\pm$ SE. Histological scoring was analyzed using the Kruskal-Wallis test and Dunn's multiple comparison post hoc tests. Analyses with resultant *P* values of < 0.05 were accepted as significant.

RESULTS

Variable impact of *Glrx1* ablation on OVA-induced airways inflammation. To model allergic airways disease, we utilized the well-described model of OVA-induced pulmonary inflammation to produce Th2-dominated allergic airways disease. WT and *Glrx1*^{-/-} mice were immunized with OVA plus alum on days 0 and 14, and challenged with aerosolized OVA on days 21, 22, and 23 and harvested 48 h later (3 Chall.+ 48 h). In addition, separate groups of mice were immunized, and then subjected to six consecutive OVA

challenges and harvested 48 h (6 Chall.+ 48 h) and 7 days (6 Chall.+ 7 days) postfinal challenge. We first assessed OVA-specific immunoglobulins in serum from WT and *Glrx1*^{-/-} mice following sensitization and challenge. Results in Fig. 1 demonstrate comparable increases in levels of IgE (Fig. 1A) and IgG1 (Fig. 1B) in WT and *Glrx1*^{-/-} mice subjected to sensitization and challenge with OVA compared with alum/OVA controls, demonstrating that *Glrx1*^{-/-} mice mounted an equally robust immune response to OVA as WT mice. We next assessed the magnitude of airways inflammation in WT and *Glrx1*^{-/-} mice in response to OVA by enumerating cells obtained via BAL. All mice immunized and challenged with OVA showed a marked influx of cells into the airways (Fig. 2), characterized by increases in eosinophils, and to lesser but significant extent, neutrophils and lymphocytes (Table 1). Inflammation was most robust 48 h after six challenges, and remained strongly elevated, despite 7 days of recovery postfinal challenge. Genetic ablation of *Glrx1*^{-/-} had a variable impact on OVA-induced inflammation. While inflammation was significantly decreased in the *Glrx1*^{-/-} mice 48 h following three challenges with OVA, predominantly due to a lower

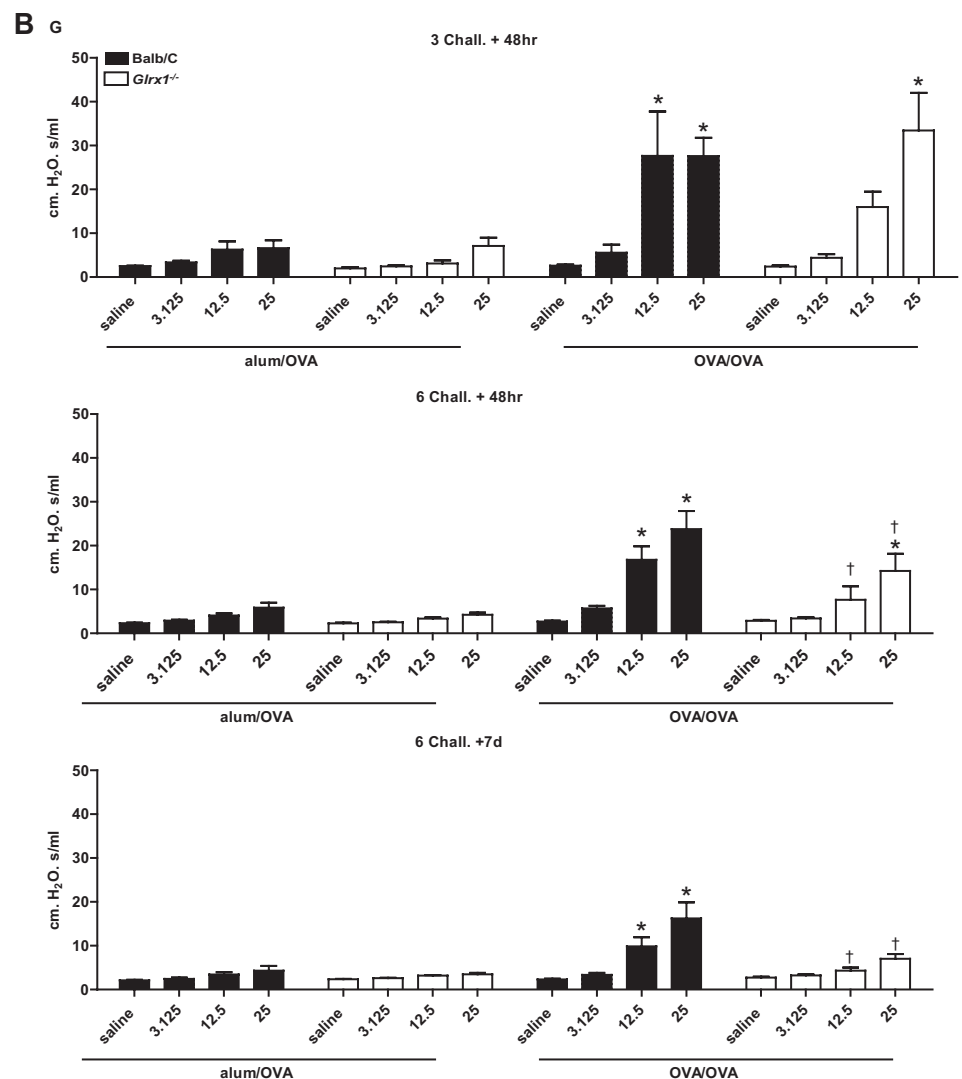


Fig. 3—Continued

influx of eosinophils at the later time points overall airway inflammation was comparable between WT and *Glrx1*^{-/-} mice (Fig. 2 and Table 1).

*OVA-induced expression of proinflammatory mediators is reduced 48 h following 3 challenges in *Glrx1*^{-/-} mice.* We evaluated the expression of proinflammatory mediators by assessment of mRNA in whole lung homogenates, as well as overall content in BAL fluid. Results in Table 2 demonstrate significant increases in mRNA expression of KC, IL-6, RANTES, CCL-20, and IL-5 in WT mice 48 h after three or six OVA challenges, which tended to decrease 7 days following cessation of the six challenge regimen. The content of CCL-20 and KC was increased in BAL in mice immunized and challenged with OVA at all examined time points (Table 3), while BAL levels of RANTES and IL-6 remained widely unchanged. Forty-eight hours after 3 OVA challenges, *Glrx1*^{-/-} mice exhibited significant decreases in mRNA expression of KC and CCL-20 in lung tissue (Table 2, *top*), with accompanying decreases of these chemokines in the BAL (Table 3, *top*), compared with WT mice. Although small increases in mRNA expression of IL-4 and IL-13 were detected in lung tissue in

response to sensitization and challenge with OVA, no clear differences were detected between WT and *Glrx1*^{-/-} mice (data not shown). Collectively, these findings suggest that ablation of the *Glrx1* gene tended to decrease expression of proinflammatory mediators, but did not have a strong impact on overall allergic inflammation, in particular at the later time points that were investigated herein.

*Ablation of *Glrx1* causes enhanced resolution of OVA-induced AHR and mucus metaplasia.* To address the functional consequences of the lack of *Glrx1*, a forced oscillation technique was used to evaluate alterations in respiratory mechanics in response to OVA sensitization and challenge. As expected, in response to OVA sensitization and challenge, airway resistance (Fig. 3A), tissue dampening (Fig. 3B), and elastance/stiffness (Fig. 3C) were significantly increased in WT animals, with the most prominent increases occurring 48 h after three challenges with OVA. It is worth noting that 1 wk postcessation of OVA challenges, all parameters of AHR remained significantly increased in WT animals. At 48 h after 3 challenges, *Glrx1*^{-/-} mice demonstrated comparable increases over respective controls in airway resistance and tissue damp-

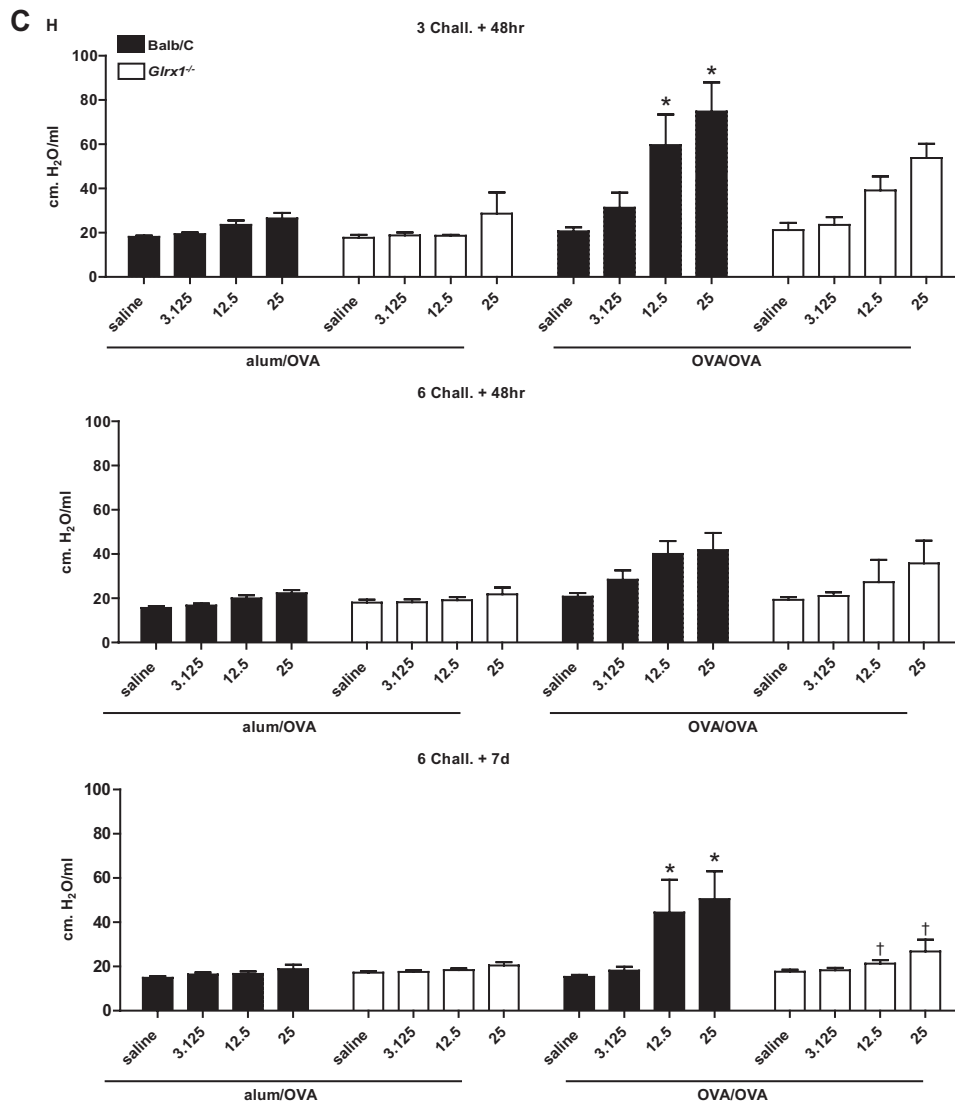


Fig. 3—Continued

ening at 12.5 and 25 mg/ml of Mch. Although tissue elastance tended to increase in *Glrx1*^{-/-} mice in response to sensitization and challenge with OVA, compared with alum/OVA controls, these increases did not reach statistical significance. *Glrx1*^{-/-} mice showed significant attenuation in tissue dampening 48 h after six challenges with OVA, compared with WT controls. Importantly, parameters of AHR were significantly decreased in *Glrx1*^{-/-} mice compared with WT groups 7 days postcessation of the 6-day challenge regimen (Fig. 3, A–C, bottom).

Defining features of asthma include excess mucus secretion, goblet cell hyperplasia and submucosal gland hypertrophy (27, 40), which have been shown to contribute to airway closure and hyperresponsiveness (14, 41). mRNA analysis of the mucus genes MUC5ac and GOB5, revealed similar increases in expression in response to OVA in WT and *Glrx1*^{-/-} mice 48 h after six challenges with OVA (Fig. 4, A and B). Decreases in MUC5ac mRNA were apparent in *Glrx1*^{-/-} compared with WT mice 7 days after the 6-day OVA challenge regimen (Fig. 4A). IL-13 levels in BAL fluid recovered from WT mice sensitized and challenged with OVA, were significantly elevated over alum/OVA controls in WT mice 48 h after 3 challenges (Fig. 5A). Although no clear increases in IL-13 content in the BAL occurred in OVA-sensitized and -challenged mice compared with the alum/OVA group, at the later time points, IL-13 content in BAL from *Glrx1*^{-/-} mice was slightly decreased 48 h after six challenges, and significantly decreased after 7 days compared with WT groups (Fig. 5A,

middle and right). Consistent with these observations, histopathological analysis of lungs from WT and *Glrx1*^{-/-} mice revealed comparable mucus metaplasia 48 h after three and six aerosolized OVA challenges (Fig. 5B). Although significant increases in mucus metaplasia remained 7 days following cessation of six challenges in WT mice, PAS positivity was significantly reduced in *Glrx1*^{-/-} mice (Fig. 5B, right, Fig. 5C). In aggregate, these findings demonstrate that *Glrx1*^{-/-} mice display enhanced resolution of AHR and mucus metaplasia, compared with WT counterparts.

Absence of Glrx1 leads to increases in protein S-glutathionylation in response to sensitization and challenge with OVA. Under physiological conditions, the main function of Glrx1 is to deglutathionylate proteins restoring protein sulfhydryl groups. In response to sensitization and challenge with OVA, increases in endogenous Glrx1 content occurred in WT mice over time (Fig. 6A). In WT mice, no clear increases in overall PSSG content occurred in response to OVA, compared with alum/OVA controls at any of the time points investigated. We next assessed whether alterations in PSSG content occurred in lung tissue from mice lacking *Glrx1* compared with WT animals. Ablation of *Glrx1* resulted in slight increases in PSSG content, in the alum/OVA group compared with WT mice. Slight, but significant increases in PSSG content were apparent 48 h after 3 challenges in *Glrx1*^{-/-} mice, with striking increases occurring 48 h after six challenges (Fig. 6B). Surprisingly, increases in PSSG were no longer apparent in *Glrx1*^{-/-} mice, 1 wk postcessation of OVA challenges.

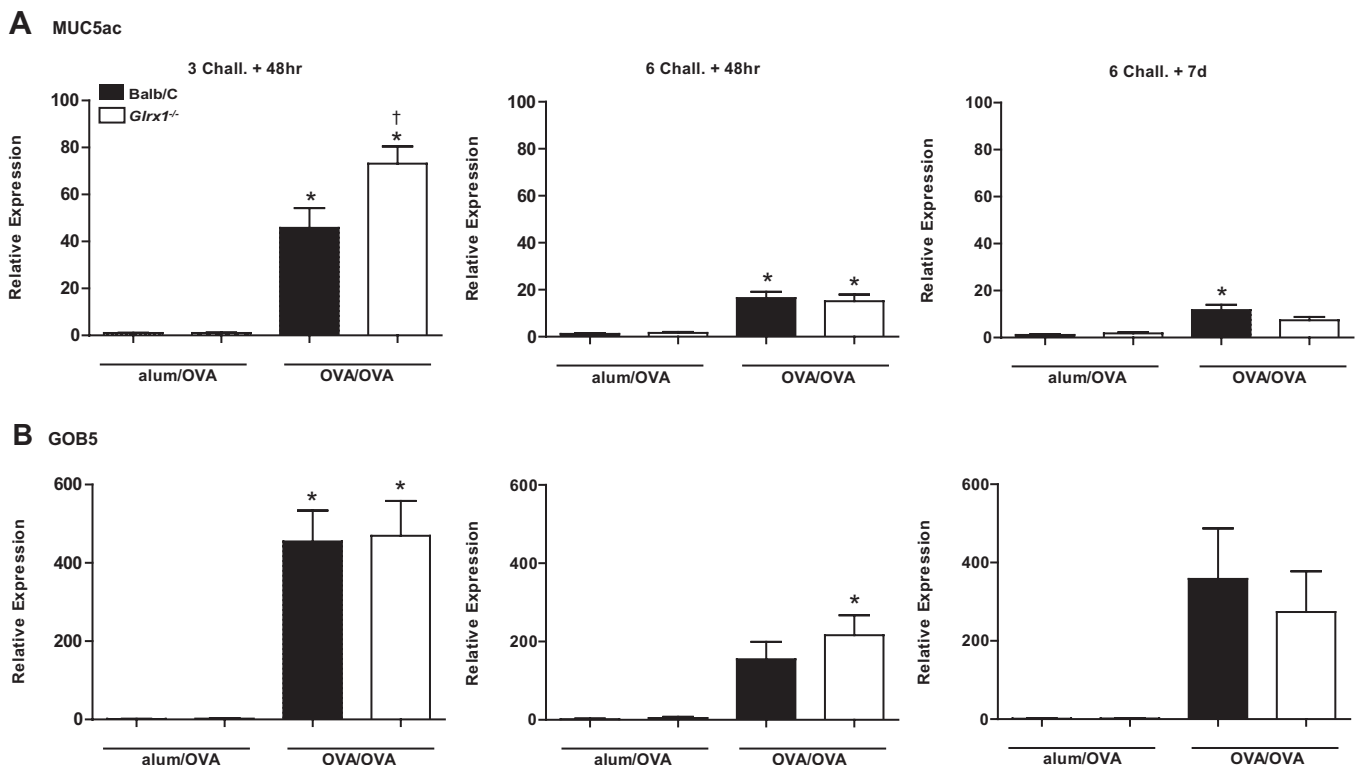


Fig. 4. Evaluation of GOB5, MUC5ac, and IL-13 gene expression in WT and *Glrx1*^{-/-} mice subjected to sensitization and challenge with OVA. RNA from lung homogenates of OVA-sensitized and challenged mice (OVA/OVA) or mock-sensitized mice (alum/OVA) was analyzed by real-time PCR, and results were normalized to the housekeeping gene cyclophilin. Data are expressed as fold increases in expression compared with WT alum/OVA controls. **P* < 0.05 (ANOVA) compared with respective alum/OVA control groups. †*P* < 0.05 (ANOVA) compared with WT OVA/OVA groups. Black bars: BALB/c mice, white bars: *Glrx1*^{-/-} mice.

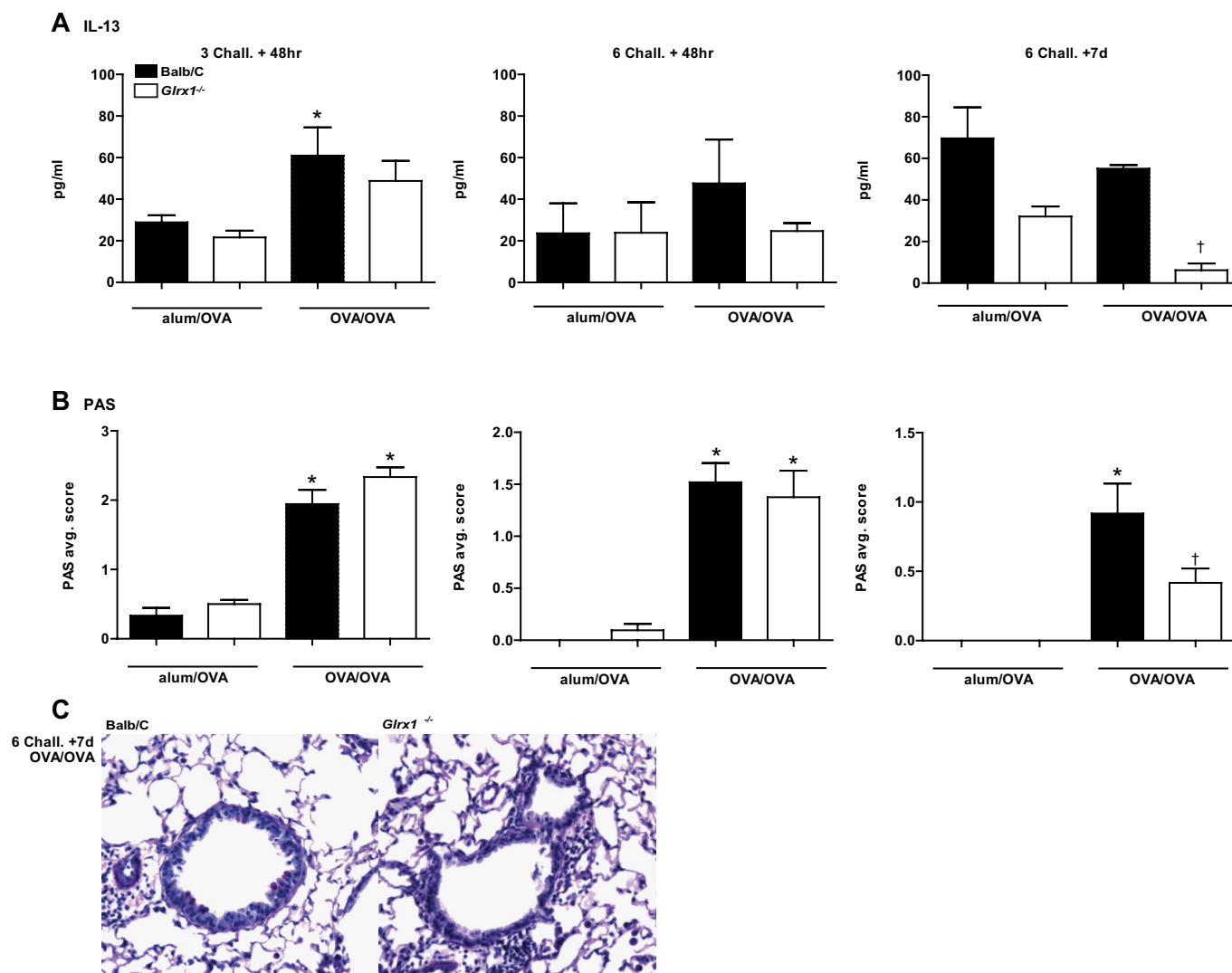


Fig. 5. Evaluation of IL-13 content in BAL and mucus metaplasia in WT and *Glrx1*^{-/-} mice subjected to sensitization and challenge with OVA. **A:** IL-13 content in the BAL was determined by ELISA. Data are expressed as means $\pm$ SE from 8 to 10 mice per group. * P < 0.05 (ANOVA) compared with respective alum/OVA control groups. † P < 0.05 (ANOVA) compared with WT OVA/OVA groups. **B:** mucus metaplasia was assessed in periodic acid Schiff-stained (PAS) bronchiolar epithelium from WT and *Glrx1*^{-/-} mice sensitized and challenged with OVA (OVA/OVA). Mucus metaplasia was evaluated by obtaining 3 images of small bronchioles ($\times 20$ objective) of similar dimensions from each mouse. Average scores of 2 independent evaluations were determined. Data are expressed as means $\pm$ SE from 8 to 10 mice per group. * P < 0.05 (Kruskal-Wallis) compared with respective alum/OVA control groups. † P < 0.05 (Kruskal-Wallis) compared with WT OVA/OVA groups. Black bars: BALB/c mice, white bars: *Glrx1*^{-/-} mice.

DISCUSSION

PSSG has emerged as an archetypal, redox-dependent post-translational modification of target protein cysteines. PSSG not only has the potential to affect protein structure and function, it also protects proteins from irreversible thiol oxidations. The impact of protein *S*-glutathionylation depends upon the target protein, and activation (1, 2), gain of function (6), and inhibition of various proteins (10, 31, 38) has been observed (21). The biological significance of PSSG is bolstered by the evolution of glutaredoxins, thioltransferases that catalyse reversible *S*-glutathionylation, and deglutathionylation reactions. Under physiological conditions, cytosolic Glrx1 preferentially catalyses deglutathionylation reactions via the NH₂-terminal active site cysteine. This results in *S*-glutathionylation of Glrx1 itself, which is subsequently reduced by a second GSH molecule (21, 26). Pathophysiological functions for Glrx1 are

emerging that include roles in the regulation of apoptosis, transcription, tissue inflammation, and remodeling (26).

In the present study, we addressed the impact of ablation of *Glrx1* on OVA-induced allergic airways disease. Our results demonstrate clear increases in the content of PSSG reactivity in lung tissues from *Glrx1*^{-/-} mice after sensitization and repeated challenges with OVA, compared with WT mice, consistent with its role in catalysis of deglutathionylation reactions in physiological settings. Surprisingly, 7 days after six challenges with antigen, PSSG content decreased in the lungs of *Glrx1*^{-/-} mice (Fig. 6B). A previous study reported no evidence for compensation of various antioxidant enzymes or Glrx2 content in the lungs of *Glrx1*^{-/-} mice, which also lacked detectable deglutathionylation activity (19). However, our data indicate the presence of other deglutathionylating events or enzymes in the lung in the setting of allergic inflammation. In

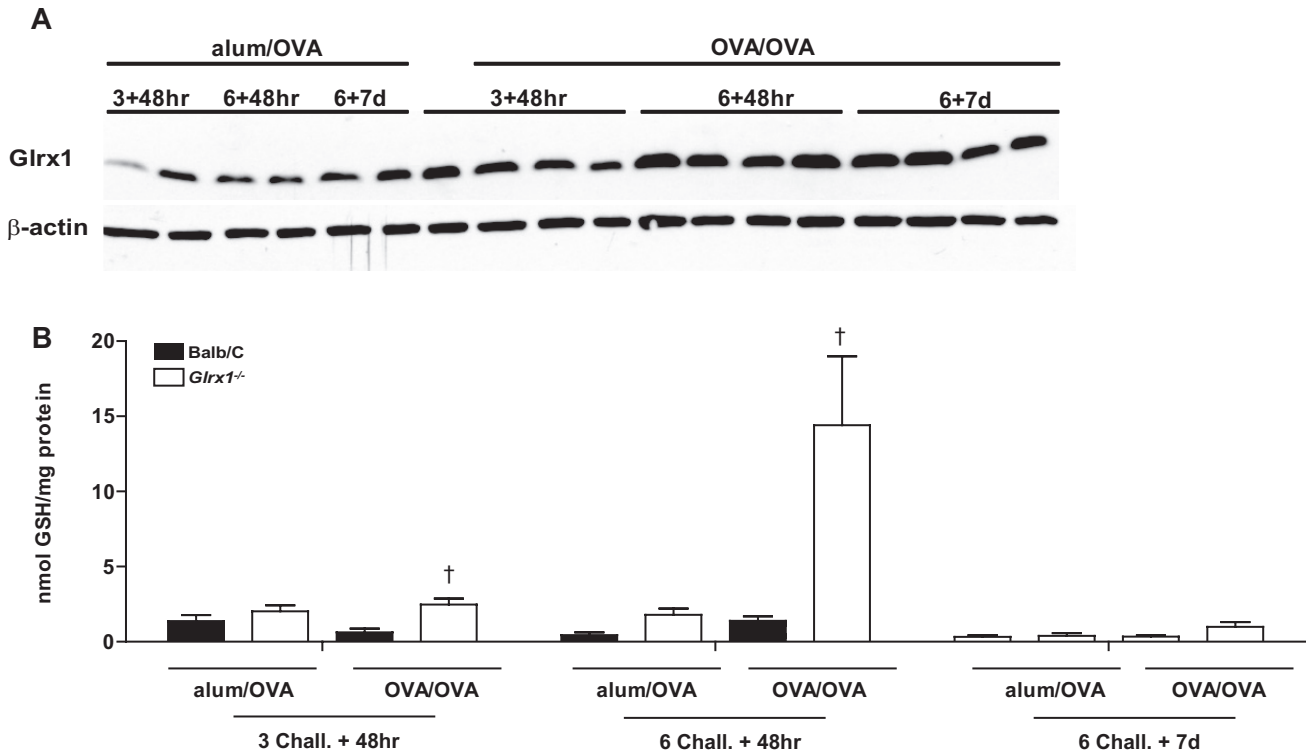


Fig. 6. Assessment of protein S-glutathionylation and Glrx1 expression in WT and *Glrx1*^{-/-} mice following sensitization and challenge with OVA. **A**: assessment of Glrx1 content in lung tissue from WT mice at different time points following sensitization and challenge with OVA. Lung tissues were homogenized, and Glrx1 content assessed in via Western blot analyses/β-actin is shown as a loading control. Results from 2 independent mice are shown per time point in the alum/OVA groups, whereas 4 independent mice were analyzed per time point in the OVA/OVA groups. **B**: biochemical analysis of protein S-glutathionylation in homogenized lung tissues of WT (black bars) and *Glrx1*^{-/-} mice (white bars) 48 h and 7 days following the 6th daily aerosolized OVA challenge. Homogenized lung tissue was prepared, proteins were precipitated, and glutathione was released using Na-borohydride and quantified using the glutathione disulfide reductase recycling assay, with 5,5'-dithiobis-(2-nitrobenzoic acid) as substrate. Na-borohydride-dependent formation of 5'-thio-2-nitrobenzoic acid was calculated and normalized to protein content. Data are expressed as nanomoles tripeptide GSH per milligram protein. Data are expressed as means ± SE from 8 to 10 mice per group. **P* < 0.05 (ANOVA) compared with respective alum/OVA control groups. †*P* < 0.05 (ANOVA) compared with WT OVA/OVA groups.

this regard, putative roles for the related oxidoreductase, thioredoxin (18) as well as sulfiredoxin (15, 29) have been suggested, which could account for decreases in PSSG content over time in lungs from *Glrx1*^{-/-} mice. Additional analyses that were beyond the scope of the present study will be required to determine the source of the deglutathionylating activity in lungs of *Glrx1*^{-/-} mice. In the present study we also observed slight increases in PSSG in lung tissues from mock-sensitized (alum/OVA) *Glrx1*^{-/-} mice compared WT alum/OVA groups, findings that are in apparent contrast to previous reports indicating that the systemic lack of *Glrx1* does not affect baseline PSSG (4, 19). It is plausible that aerosolized antigen, in the absence of previous sensitization, causes a mild perturbation of the redox status, which could be more apparent in the absence of *Glrx1*.

Our laboratory has previously demonstrated a causal role for activation of the transcription factor NF-κB within the bronchial epithelium in orchestrating allergic inflammation (8, 28, 32). The transcription factor NF-κB has been shown to be inhibited via S-glutathionylation of IKKβ, RelA, and p50 (5, 31, 34, 38). Genetic ablation or siRNA-mediated knockdown of *Glrx1* resulted in enhanced S-glutathionylation of IKKβ, and attenuated production of proinflammatory mediators by epithelial cells following stimulation with LPS. Conversely, overexpression of Glrx1 prolonged LPS-induced proinflammatory responses in lung epithelial cells in association with

decreases in glutathionylated IKKβ and prolonged activation of NF-κB (5, 38). It is therefore surprising that despite increases in the overall content of PSSG in lung tissue the overall extent of airway inflammation was only transiently diminished in *Glrx1*^{-/-} mice after sensitization and challenge with OVA compared with WT mice. Nonetheless, expression of proinflammatory mediators was consistently decreased in *Glrx1*^{-/-} mice compared with WT animals. It is possible that Glrx1 affects the resolution of airway inflammation. Since inflammatory cells in the airways are still prominent 7 days after cessation of the six-challenge regimen, more extended time points will be required to determine the impact of Glrx1 on the resolution of allergic disease. Within the time frame of investigation of the present study we failed to demonstrate clear differences in nuclear content of RelA in lung homogenates from WT and *Glrx1*^{-/-} mice (data not shown). Future studies will be required to elucidate whether S-glutathionylation of NF-κB family members within the lung epithelium are affected in *Glrx1*^{-/-} mice. Such analyses were beyond the scope of the present study, and will require dissection of bronchiolar epithelium, the compartment wherein NF-κB activation is predominant. It is also plausible that the systemic ablation of *Glrx1* may have obscured the contribution of Glrx1, specifically in airway epithelium towards NF-κB activation, and subsequent inflammatory responses. Therefore, elucidation of the exact role of Glrx1 in orchestrating inflammation awaits

additional studies aimed at conditional manipulation of *Glrx1* during specific times and specifically within the bronchial epithelium during allergen-driven inflammation.

One of the most striking phenotypes of *Glrx1*^{-/-} mice in the present study was the enhanced resolution of AHR, compared with WT mice. Despite the sustained presence of OVA-induced inflammatory cells in the lungs at the time of assessment of respiratory mechanics, *Glrx1*^{-/-} animals exhibited marked reductions in hyperresponsiveness to methacholine compared with WT mice. Notably, the elastance and tissue dampening parameters in *Glrx1*^{-/-} mice sensitized to OVA were significantly reduced 7 days following OVA challenge, whereas increases in Newtonian resistance, reflective of conducting airways, still occurred in *Glrx1*^{-/-} mice, albeit to a slightly lesser extent than WT mice. These findings suggest a more pronounced impact of *Glrx1* deficiency in the lung periphery. Additionally, *Glrx1*^{-/-} mice demonstrated significant decreases in gene expression of MUC5ac and mucus metaplasia 7 days postcessation of OVA challenges, compared with WT mice (Figs. 4 and 5). These findings suggest that the enhanced resolution of AHR in *Glrx1*^{-/-} mice may be functionally linked to altered regulation of mucin gene expression, mucin glycoprotein processing, and/or secretion. A firm link between oxidants and mucus metaplasia has been established; oxidants can increase expression of Muc5AC mRNA (3, 16, 20, 22, 42) and a role for oxidants in the transcriptional upregulation of the IL-13 gene has been shown (9). In keratinocytes stimulated with IL-4 and IL-13, activation of dual specificity oxidase-1 was reported to be critical in the activation of STAT6, following oxidative inactivation of protein tyrosine phosphatase 1B. These collective studies demonstrate that changes in redox homeostasis are important in the development of mucus metaplasia. However, it is unknown to date whether the signals that regulate mucin gene expression or intracellular processing of mucins is regulated via *Glrx1*-controlled *S*-glutathionylation.

S-glutathionylation reflects an oxidative event that can be triggered via multiple biochemical mechanisms, including oxidant production via activation of NADPH oxidases (21, 26). Oxidative events have been strongly implicated in the pathogenesis of allergic airways disease (11, 30, 37). It is therefore perhaps counterintuitive that in mice lacking *Glrx1*, wherein PSSG (an oxidative event) is increased, AHR, mucus metaplasia, and expression of proinflammatory mediators are decreased. These findings suggest putative beneficial function for *S*-glutathionylation in the pathophysiology of allergic airways disease. The exact mechanisms, whereby *S*-glutathionylation exerts protective effects during the pathogenesis of allergic airways disease, awaits additional investigation into the proteins that are targeted via *S*-glutathionylation, and the impact of *S*-glutathionylation on the regulation of their structure and function. It is plausible that *S*-glutathionylation exerts its protective function by preventing irreversible protein oxidations. In this regard, irreversible protein oxidation has been detected in lungs from mice exposed to cigarette smoke in association with loss of *S*-glutathionylation (23).

In conclusion, the present study demonstrates that a systemic loss of *Glrx1* attenuates airways hyperresponsiveness, in association with decreased mucus metaplasia in antigen-induced allergic airways disease. Our results highlight the potential functional importance of the *Glrx1*/PSSG redox module in the

resolution of AHR in allergic asthma, findings that could hold clinical relevance and offer potential therapeutic opportunities.

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DISCLOSURES

No conflicts of interest, financial or otherwise are declared by the author(s).

AUTHOR CONTRIBUTIONS

Author contributions: S.M.H. and Y.M.W.J.-H. conception and design of research; S.M.H., J.E.T., K.G.L., J.D.N., M.A., and N.D. performed experiments; S.M.H., J.E.T., V.A., A.S.G., and N.D. analyzed data; S.M.H., L.K.L., and C.G.I. interpreted results of experiments; S.M.H. prepared figures; S.M.H. drafted manuscript; S.M.H., J.E.T., K.G.L., J.D.N., A.S.G., J.L.J.v.d.V., Y.-S.H., C.G.I., and Y.M.W.J.-H. edited and revised manuscript; S.M.H., Y.-S.H., and Y.M.W.J.-H. approved final version of manuscript.

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