

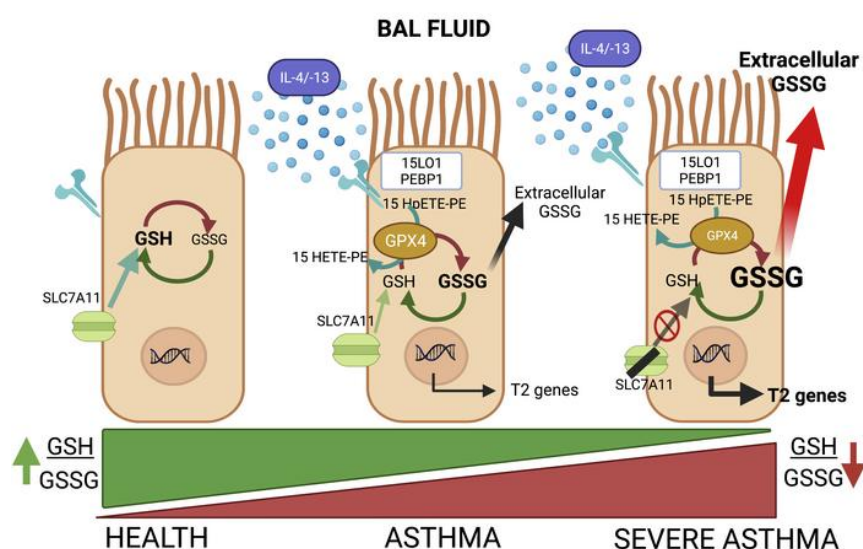
15LO1 dictates glutathione redox changes in asthmatic airway epithelium to worsen type-2 inflammation

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2 inflammation

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31

32 **Competing interests**

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35 have no competing interests.

36

37 **Abstract:** Altered redox biology challenges all cells, with compensatory responses often determining

38 a cell's fate. When 15 lipoxygenase-1 (15LO1), a lipid peroxidizing enzyme abundant in asthmatic

human airway epithelial cells (HAECs), binds phosphatidylethanolamine binding protein-1 (PEBP1), hydroperoxy-phospholipids, which drive ferroptotic cell death, are generated. Peroxidases, including glutathione peroxidase-4 (GPX4), metabolize hydroperoxy-phospholipids to hydroxy derivatives to prevent ferroptotic death, but consume reduced glutathione (GSH). The cystine transporter, SLC7A11, critically restores/maintains intracellular GSH. We hypothesized high 15LO1-PEBP1-GPX4 activity drives abnormal asthmatic redox biology, evidenced by lower bronchoalveolar lavage (BAL) fluid and intraepithelial cell GSH:oxidized GSH (GSSG), to enhance Type-2 (T2) inflammatory responses. GSH, GSSG (enzymatic assays), 15LO1, GPX4, SLC7A11 and T2 biomarkers (western blot and RNAseq) were measured in asthmatic and healthy control (HC) cells/fluids, with siRNA knockdown as appropriate. GSSG was higher and GSH:GSSG lower in asthmatic compared to HC BAL fluid, while intracellular GSH was lower in asthma. *In vitro*, T2 cytokine (IL-13) induced 15LO1 generated hydroperoxy-phospholipids, which lowered intracellular GSH and increased extracellular GSSG. Lowering GSH further by inhibiting SLC7A11 enhanced T2 inflammatory protein expression and ferroptosis. *Ex vivo*, redox imbalances corresponded to 15LO1 and SLC7A11 expression, T2 biomarkers and worsened clinical outcomes. Thus, 15LO1 pathway-induced redox biology perturbations worsen T2 inflammation and asthma control, supporting 15LO1 as a therapeutic target.

56 **Introduction**

57 Among all internal organs, the lung is most consistently exposed to exogenous oxidant threats. Coupled
58 with endogenous reactive oxygen species (ROS) generated as byproducts of various cellular processes,
59 strict regulation of antioxidants is critical to maintaining a balanced reduction/oxidation (redox) state.
60 Reduced glutathione (GSH) is a ubiquitous antioxidant, utilized as a source of reducing equivalents by
61 different GSH peroxidases, which requires constant intracellular GSH synthesis from cysteine,
62 glutamate, and glycine (1, 2). GSH plays a pivotal role in maintaining intracellular redox balance by
63 detoxifying peroxides and other oxidized compounds. Lower GSH levels and GSH to oxidized GSH
64 ratio (GSH:GSSG), as measures of redox balance, have previously been reported in asthmatic lungs (3),
65 although the external or internal immuno-inflammatory pathways driving this dysregulation are
66 unknown. Further, although several previous studies have evaluated GSH levels in various
67 extracellular compartments, including bronchoalveolar lavage (BAL) fluid, these studies generally
68 focused on alterations in GSH/GSSG balance in association with disease, or the consumption of GSH
69 during prostaglandin or leukotriene synthesis, with more modest phenotypic, pathophysiologic or
70 mechanistic information (4, 5).

71 Although exogenous factors, including infection and inhaled pollutants could directly
72 oxidize GSH, endogenous disease specific mechanisms could also prime the process. Under Type 2
73 (T2) conditions (IL-13 stimulation), human airway epithelial cells (HAECs) generate oxidized

74 phospholipids under control of the 15 lipoxygenase-1 (15LO1) enzyme and its binding with
75 phosphatidylethanolamine binding protein-1 (PEBP1) (6). These continuously generated electrophilic
76 hydroperoxy-phospholipids are neutralized by a specific GSH dependent peroxidase, glutathione
77 peroxidase 4 (GPX4), which prevents them from damaging membranes and driving ferroptotic cell
78 death (7, 8). In the absence of adequate GPX4, the radical intermediates, readily produced from
79 hydroperoxy-phospholipids, directly oxidize GSH in a one electron reaction to generate a strongly pro-
80 oxidant radical, GSH•. To limit potentially life-threatening depletion of intracellular antioxidant/GSH
81 stores, epithelial cells have developed numerous compensatory pathways, including the cystine-
82 glutamate antiporter (SLC7A11), which critically maintains *de novo* GSH biosynthesis by increasing
83 intracellular cystine levels (9). Thus, under asthmatic conditions, intracellular GSH levels could
84 reflect not only the generation of reactive hydroperoxy-phospholipids by the 15LO1-PEBP1 complex,
85 but also the compensatory levels and activity of GPX4, the availability of newly synthesized and re-
86 generated GSH as well as extracellular transport. While failure of these compensatory mechanisms
87 could ultimately cause ferroptotic cell death, more subtle changes in intracellular redox balance could
88 also enhance signaling pathways, including those associated with T2 inflammation (10).

89 We hypothesized that the observed lower GSH:GSSG in asthmatic lung fluids and cells is at least
90 partially driven by high oxidation of GSH and its export in response to greater intracellular 15LO1-
91 PEBP1 pathway activity. These lower intracellular GSH levels worsen T2 inflammation and asthma
92 severity. To address this hypothesis, we measured BAL fluid and fresh intra-epithelial cell GSH and

93 GSSG levels in well characterized asthmatic and healthy control (HC) participants. To mechanistically
94 determine whether 15LO1 pathway activation alters the intracellular redox balance, particularly under
95 T2 conditions, these *ex vivo* findings were modeled *in vitro* in primary HAECs. Finally, these *in vitro*
96 findings were recapitulated *ex vivo* in two distinct asthma populations and their clinical relationships to
97 asthma severity and control determined.

98

99 **Results**

100 **Characteristics of the study population.**

101 BAL fluid samples were obtained from 23 HC, 32 mild-moderate asthma (M/M), and 43 severe
102 asthma (SA) subjects (Table 1) recruited through either the Severe Asthma Research Program 3
103 (SARP3) (HL-109152) or Immune Mechanisms of Severe Asthma (IMSA) (AI-06684). While the
104 patient groups did not differ by sex, M/M were of slightly lower age relative to both HC and SA and,
105 unsurprisingly, higher fractional exhaled nitric oxide (FeNO) and lower FEV₁ (% predicted) was
106 observed across groups from HC to SA. Bronchoscopically obtained fresh HAECs were analyzed from
107 11 HC, 4 M/M, and 11 SA subjects, a subset of the local IMSA cohort with matching BAL samples
108 (Supplemental Table 1). The epithelial brushing subgroup did not differ from the full IMSA cohort. *In*
109 *vitro* studies were performed on HAECs cultured at air-liquid interface (ALI). Due to limited cell
110 numbers available, the samples from the *in vitro* and *ex vivo* studies did not always overlap.

111

112 **The extracellular and intracellular redox balance, as measured by GSH and GSSG levels differs** 113 **between asthma and controls.**

114 *BAL Fluid.* BAL fluid is an extracellular fluid compartment contributed to by both environmental
115 and cellular factors. GSSG levels were higher in BAL fluid from SA as compared to that from HCs and
116 were marginally elevated in M/M (overall p=0.015). SA patients had higher BAL fluid GSSG than HCs

($p=0.004$). These higher GSSG levels associated with lower ratios of BAL fluid GSH:GSSG (overall $p=0.004$) (Fig. 1A). BAL fluid GSH:GSSG was lower in both M/M ($p=0.020$) and SA ($p=0.001$) compared to HC. Although median levels were numerically lower in SA compared to M/M, there were no differences in BAL fluid GSSG or GSH:GSSG between M/M and SA. Controlling for BMI (significantly different across the participant groups) did not alter the results. GSH levels in BAL fluid did not differ across the 3 groups (overall $p=0.262$). These data are in line with previously published data showing altered redox balance in asthmatic compared to healthy BAL fluid (3). FeNO is a well-recognized marker of T2 inflammation, being elevated in asthma and dramatically lowered by targeted T2/IL-4 receptor alpha antibodies (11, 12).

Intracellular. To determine whether these BAL fluid levels were a reflection of intracellular GSH metabolism, freshly brushed and isolated HAECs were analysed and compared to BAL fluid levels (Fig. 1B). In contrast to BAL fluid, intracellular GSH (overall $p=0.035$) was lower in SA ($p=0.010$) compared to HC patients. Intracellular GSSG levels were low and did not differ between the groups (overall $p=0.440$). However, similar to the BAL fluid, intracellular GSH:GSSG was substantially lower (overall $p<0.001$) in both M/M and SA compared to HC ($p=0.004$ and $p<0.001$, respectively). Neither intracellular GSH nor GSH:GSSG differed between M/M and SA

Relation between the extracellular and intracellular compartments. To determine whether BAL fluid redox measures reflected intracellular (HAEC) levels, overlapping samples were compared ($n=26$). Intracellular GSH levels correlated with BAL fluid GSH levels ($r=0.41$, $p=0.037$). There were no

correlations between intracellular and BAL fluid GSSG or GSH:GSSG (Supplemental Table 2). Of note, enzymatic GSH, GSSG, and GSH:GSSG measurements corresponded well with measures using high-performance liquid chromatography system (Supplemental Fig. 1).

Relationship to FeNO and eosinophils. BAL fluid GSSG and GSH:GSSG correlated with FeNO, indirectly suggesting T2 inflammation may interact with airway redox biology (Table 2 and Fig. 1C). As for BAL fluid, intracellular GSH and GSH:GSSG negatively correlated with FeNO, again supportive of a contribution from T2 inflammatory processes (Table 2 and Fig. 1C) (11, 12). However, neither BAL fluid nor intracellular GSH, GSSG, and GSH:GSSG correlated with BAL eosinophils or any other inflammatory cell, suggesting eosinophilia plays a lesser role in contributing to higher extracellular/BAL fluid GSSG than epithelial cells do (Supplemental Tables 3 and 4).

IL-13 upregulates 15LO1-pathways and lowers intracellular GSH

IL-13 increases the expression and activity of 15LO1, while inducing binding to PEBP1 (6, 7, 10, 13). This binding switches 15LO1's preferred substrate from free arachidonic acid to arachidonic acid conjugated to PE which generates ferroptotic lipids, including the hydroperoxy-phospholipid 15-hydroperoxy-eicosatetraenoic acid-phosphatidylethanolamine (15-HpETE-PE) (6, 7, 10, 13).

Under homeostatic conditions, 15-HpETE-PE is rapidly and specifically metabolized by the GSH-dependent GPX4 to the non-reactive hydroxy derivative 15-HETE-PE (6, 14). This activity could consume GSH and lower intracellular levels of reduced intracellular GSH. Direct oxidation of GSH to GSH• through free radical intermediates formed from 15-HpETE-PE before it is metabolized

could also occur. In line with these metabolic possibilities, we observed that previously described increases in 15LO1 pathway proteins and activity were accompanied by decreases in both intracellular GSH ($p < 0.001$) and GSH:GSSG ($p = 0.004$) (Fig. 2A). Although there were only marginal increases in intracellular GSSG levels ($p = 0.064$), IL-13 increased extracellular (apical supernatant) GSSG ($p = 0.009$), while lowering both GSH ($p = 0.012$) and GSH:GSSG ($p = 0.006$) (Fig. 2B), supportive of intracellular compensatory mechanisms which mirror the *ex vivo* (BAL and fresh HAEC) findings. In this regard, as 15LO1 increased, GPX4 expression concomitantly increased to balance the high 15LO1 activity (Fig. 2C). In addition, SLC7A11, the antiporter providing intracellular cystine (necessary to maintain intracellular GSH levels) also increased (Fig. 2C and Supplemental Fig. 2A). These data support an effect of IL-13 to decrease total and reduced GSH through high 15LO-PEBP1-GPX4 activity which is partially compensated by 1) increased expression/activity of SLC7A11 to enhance new intracellular GSH production and 2) increased export of GSSG.

15LO1 increases intracellular oxidative potential by lowering GSH and increasing extracellular GSSG

To confirm that 15LO1-PEBP1 complex activity alters IL-13 induced intracellular GSH levels, we modelled these relationships *in vitro* by stimulating cultured HAECs (at ALI) with IL-13 for 7-10 days in the presence and absence of 15LO1 protein [dicer siRNA knockdown (KD) of ALOX15 gene] or enzymatic activity (specific 15LO1 inhibitor BLX2477) (14, 15). ALOX15 KD normalized the

intracellular GSH ($p=0.003$), while chemical inhibition of 15LO1 had a marginal, but insignificant effect ($p=0.066$). GSH:GSSG increased (both $p<0.05$) to levels seen in control wells. Intracellular GSSG was marginally decreased by KD of ALOX15 ($p=0.069$), but not by chemical inhibition ($p=0.203$) (Fig. 3A and 3B). These small differences in efficacy of BLX2477 may be explained by the 3- log higher IC₅₀ of BLX2477 for free vs PE-esterified eicosatetraenoic acid (ETE) (Supplemental Fig. 3). ALOX15/15LO1 KD, did however, lead to decreased extracellular GSSG ($p=0.021$) with marginal but insignificant increases in extracellular GSH:GSSG ($p=0.083$). It did not impact extracellular GSH ($p=0.260$) (Fig. 3C). Of note, KD of ALOX15 and inhibition of 15LO1 decreased GPX4 protein expression, consistent with less need for peroxidase activity. There were inconsistent and non-significant reductions in SLC7A11 (Fig. 3D, 3E and Supplemental Fig. 2B and 2C).

We previously reported that IL-13 increases intracellular 15-HpETE-PE via 15LO1 *in vitro* in modest molar amounts (see also Supplemental Fig. 4A) (7). These picomolar levels of 15-HpETE-PE alone (Supplemental Fig. 4A, generated under IL-13+arachidonic acid conditions) would be insufficient to directly oxidize the nanomolar changes caused by oxidation of GSH to GSSG observed in response to IL-13 (or the change with inhibition of 15LO1 or KD of ALOX15/15LO1). Given that GPX4 can reduce hydroperoxy-derivatives of different lipids, we analyzed the amounts of all major hydroperoxy- and hydroxy-phospholipids impacted by IL-13 by LC-MS/MS (See methods). As seen in Supplemental Fig. 4B, the sum of total intracellular hydroperoxy- and hydroxy-phospholipids following IL-13 stimulation is ~2 nmoles/mg protein; that is, sufficient amounts to quantifiably impact the change

in intracellular GSH (and the increases in GSSG) under these two conditions as shown in Supplemental Fig. 4C under similar conditions. Thus, the loss of intracellular GSH in the presence of active generation of hydroperoxy-phospholipids could occur through several active mechanisms, including formation of GSH•, export of GSSG, inadequate replenishment of GSH or insufficient levels of GSSG reductase, each directly or indirectly dependent on the generation of hydroperoxy-phospholipids. It should be also noted that the actual amounts of hydroperoxy-phospholipids formed after IL-13 treatment may be even greater if one accounts for the electrophilic secondary products of hydroperoxy-phospholipid decay that attack the nucleophilic sites of proteins and form covalent adducts.

These *in vitro* mechanisms were recapitulated *ex vivo* as observed by the strong correlations of *ex vivo* 15LO1 protein with intracellular GSH ($r=-0.68$, $p=0.005$) and GSH:GSSG ($r=-0.62$, $p=0.014$) in freshly brushed HAECs (Fig. 3F). Because SLC7A11 regulates GSH synthesis (through intracellular cystine availability) and 15LO1 decreases intracellular GSH, a high SLC7A11 to 15LO1 protein ratio likely supports high/normal intracellular GSH. Indeed, intracellular GSH ($r=0.78$, $p=0.003$) and GSH:GSSG ($r=0.60$, $p=0.038$) positively correlated with the SLC7A11:15LO1 protein ratio, without relation to intracellular GSSG. This suggests that under T2 “stressed” conditions, increases in SLC7A11 activity help maintain intracellular redox balance (Fig. 3G). Thus, we identify 15LO1 pathway activity *in vitro* as a prime driver of lower intracellular reduced GSH levels and export of GSSG. Under steady state conditions, this GSSG is rapidly exported to extracellular fluid, potentially contributing to the

higher levels of GSSG measured in asthmatic BAL fluid, while also contributing to an altered intracellular redox balance.

Further alterations in intracellular redox balance driven by lower GSH availability induce cell death and enhance T2 signature protein expression

We previously demonstrated that 15LO1-PEBP1 complex activation consistently upregulates the expression of T2-signature gene/protein expression, including MUC5AC, periostin/POSTN and CCL26 (6, 10, 14). Although the mechanism for this upregulation is not fully characterized, altered redox states are known to enhance expression of numerous genes/proteins. To determine whether increases in intracellular GSH directly enhance expression of T2-signature proteins in HAECs, we lowered intracellular GSH levels by inhibiting SLC7A11 using the chemical inhibitor erastin (16). As anticipated, chemical inhibition of SLC7A11 by erastin lowered intracellular GSH and GSH:GSSG (Fig. 4A). Interestingly, these changes were accompanied by marginal increases in 15LO1 ($p=0.082$), potentially further contributing to GSH oxidation (Fig. 4B). Physiologically, this lowering of GSH led to increased LDH release ($p=0.031$), consistent with redox activation of ferroptosis and cell death (Fig. 4C) (8, 17). Worsening intracellular oxidative potential was accompanied by increases in intracellular expression of downstream T2 signature proteins, such as periostin (POSTN) and CCL26, with non-significant increases in inducible NO synthase (iNOS/NOS2) (Fig. 4D and 4E). In addition to enhancement of intracellular expression, basal secretion of POSTN and CCL26 increased in parallel

(Fig. 4D and 4F). iNOS is not a secreted protein and was not evaluated for secretion. Apical protein secretion of MUC5AC (by ELISA) (Fig. 4F) was also increased with erastin, as compared to IL-13 alone. These results confirm that decreases in intracellular GSH (and associated increased oxidative stress) worsen T2 inflammatory protein expression and secretion in HAECs, while increasing their susceptibility to ferroptotic death.

BAL fluid and intracellular GSH pathways correlate with HAEC T2-associated gene expression

To determine whether these *in vitro* findings were reflected *ex vivo*, BAL fluid GSSG levels were compared to fresh HAEC expression of T2-related genes from 2 different RNAseq databases (SARP3 and IMSA), which were harmonized and merged as outlined in the Methods (Table 3). BAL fluid GSSG strongly and positively correlated with ALOX15 expression with weaker relationships to POSTN and NOS2 expression. Importantly, ALOX15 expression also correlated strongly with a T2 inflammatory gene signature (Supplemental Table 5), supporting the link between the GSH pathway, T2 inflammation and 15LO1. Perhaps not surprisingly, intracellular GSH levels more strongly correlated with fresh HAEC expression of ALOX15 (Table 3). These results support the hypothesis that T2-induced 15LO1 pathway induced alteration of intracellular redox balance enhances T2 inflammatory responses *ex vivo*.

Altered redox balance ex vivo correlates with worsened clinical and physiologic outcomes

BAL Fluid. Both BAL fluid GSSG and GSH:GSSG were (differentially) correlated with FEV₁ (Table 2 and Fig. 5A). Similarly, patients who experienced asthma exacerbations in the past year had lower BAL fluid GSH:GSSG than those who did not (Fig. 5A). Asthma Quality of Life Questionnaire (AQLQ) score correlated with BAL fluid GSH:GSSG as well ($r=0.33$, $p=0.005$). These data suggest a relationship of worsening airway obstruction and asthma control with higher GSH-related oxidative stress measures.

Intracellular. Intracellular GSH:GSSG also correlated with FEV₁ (% predicted), with a numerically stronger correlation than observed in BAL fluid (Fig. 5B). Intracellular GSH also positively correlated with AQLQ score (Table 2). Intracellular GSH:GSSG did not differ by history of asthma exacerbations, perhaps due to the smaller sample size (Fig. 5B).

Discussion

Variation in redox state, as represented by the intracellular GSH:GSSG can have profound clinical implications, enhancing cell signalling and even cell death. The studies outlined here identify a specific pro-oxidant pathway which dramatically impacts both intra and extracellular redox states, enhances T2 inflammation and cell death and is associated with worsening asthma control. Almost all inflammatory diseases exhibit evidence of enhanced oxidative stress (18). Yet, treatment of disease with anti-oxidant approaches has nearly uniformly failed (19, 20), perhaps partly because oxidative stress is often compartmentalized or driven by multiple non-specific (chemical) and specific (enzymatic) pathways. Here, we confirm previously reported evidence of redox abnormalities in asthma (3), but greatly expand on these findings to identify a specific intracellular mechanism regulating these levels. We identify high HAEC 15LO1 activity *in vitro* as a prime driver of worsening (lower) intracellular (epithelial) GSH:GSSG observed in association with T2-Hi asthma ex vivo. Loss of intracellular GSH is likely multifactorial including generation of high levels of hydroperoxy-phospholipids (which directly or indirectly oxidize GSH and limit GSSG recycling by reductases) as well as by active export of GSSG generated by high GPX4 activity (7, 8, 21). In this setting, any perturbation which lowers intracellular GSH to an as yet unknown threshold, could eventually induce cell death and, in all likelihood, ferroptosis, but also significantly increase expression and secretion of numerous T2-associated proteins.

276 A consistent imbalance in GSH and GSSG has been observed in asthma, however, the
277 mechanisms for this imbalance have been unclear (22). Although studies have suggested a role for
278 superoxide dismutase (23), the relationships with redox changes and clinical parameters have generally
279 been weak, supporting involvement of other pathways. However, no studies to date have reported
280 intracellular GSH levels in relation to asthma or specific lower airway cell type. Epithelial cells, the
281 lining cells of the lung, by both position and abundance, are likely to contribute substantially to BAL
282 fluid GSH:GSSG, through increasing export of GSSG under conditions of endogenous (or exogenous)
283 redox changes. While significantly lower GSH:GSSG ratios were not seen using traditionally-defined
284 severity phenotypes, lower ratios corresponded to a greater history of exacerbations, lower lung
285 function, and higher FeNO. In line with the intracellular contributions to these clinical findings,
286 correlation of intraepithelial cell GSH levels to FEV1 was considerably stronger than the relationship
287 in BAL fluid, supporting the overall importance of *endogenous* epithelial cell responses to clinical
288 outcomes.

289 Ferroptosis is strikingly driven by worsening redox imbalance which generates and maintains high
290 levels of hydroperoxy-phospholipids (7, 24). In IL-13 stimulated HAECs, it is specifically driven by
291 15LO1-PEBP1 complex-catalyzed generation of 15-HpETE-PE (7). Under steady state conditions,
292 these hydroperoxy-phospholipids are metabolized to hydroxy-phospholipids by GPX4, consuming
293 large quantities of GSH and generating GSSG (25). Normally, this GSSG is recycled by reductases to
294 maintain sufficient intracellular levels of reduced GSH. However, these events are not without redox

consequences, as shown here. Under IL-13 conditions, intracellular GSH levels (and hence GSH:GSSG) fall through 15LO1-PEBP1 dependent reactions. This loss of GSH occurs through several mechanisms, including direct enzymatic GSH oxidation to GSSG by GPX4 during reduction of hydroperoxy-phospholipids, export of GSSG and dependence on the continuous availability of newly formed GSH. It is also possible that radical intermediates non-enzymatically produced from hydroperoxy-PLs directly react with GSH leading to the formation of a highly-pro-oxidant GS• radical. Finally, GSH could also be consumed under conditions of oxidative stress by a process known as S-glutathionylation (26). This process was reported to also be active in epithelial cells under T2 conditions, in association with 15LO1 (27). However, its relative impact on overall intraepithelial cell levels of GSH, or activation of other pro-inflammatory mediators, is unclear and requires further study. When GSH regeneration is further limited by inhibition of SLC7A11 (with erastin), cell death ensues. Our data suggest activation of ferroptotic pathways could be one of the prime drivers of intracellular (and extracellular) redox imbalance in T2-high asthma.

Previous reports from our laboratory showed that 15LO1 also contributes to downstream T2 immuno-inflammatory responses (6, 10, 14, 28). While multiple mechanisms may exist for these increases, it is widely appreciated that increased oxidative potential enhances receptor mediated downstream responses. Although ligand-receptor pathways are often thought of as linear paths from engagement to transcription/translation, many studies show that enhanced oxidative potential can augment or suppress signaling pathways, including those related to MUC5AC, while more recently,

314 oxidatively-driven epigenetic effects on gene transcription have also been reported (29-32).
315 However, given the lower intracellular GSH:GSSG after IL-13 stimulation, it is difficult to determine
316 the proportionate role that loss of GSH plays in downstream T2-pathway mRNA/protein expression.
317 This is especially difficult because under IL-13 conditions, upregulation of additional factors including
318 GPX4 and SLC7A11 (9), occur in an effort to maintain intracellular homeostasis. Similarly, increased
319 export of GSSG also occurred, potentially contributing to lower intracellular levels. Although the
320 mechanisms are unclear, multidrug resistance protein-1 (otherwise known as ABCC1) has been
321 reported to preferably export GSSG under conditions of high intracellular GSSG (33, 34).

322 Any immune or inflammatory settings in which amount or activity of any of these proteins
323 are lowered or do not increase appropriately (as we modelled with erastin) could dramatically perturb
324 homeostatic balance and enhance T2-inflammatory factor expression and oxidatively driven cell death.
325 In fact, worsening asthma control and exacerbations are consistently linked to lower lung GSH levels
326 and higher oxidative potential (35, 36). Exogenous factors, including various pollutants, could further
327 increase extracellular GSSG, perhaps limiting the export of GSSG against a concentration gradient.
328 Endogenously, interferon γ lowers SLC7A11 activity (37), suggesting that viruses, which activate
329 interferons of all types, could alter this fragile intracellular GSH homeostasis. This, in turn, would drive
330 oxidatively induced cell death, increase barrier dysfunction and T2-inflammation, potentially
331 identifying a mechanism by which viruses cause asthma exacerbations. Although not evaluated here,
332 lower intracellular GSH levels would also de-activate the GSH-dependent GPX4, further enhancing

ferroptotic cell death (8, 38). These factors could become self-perpetuating as more oxidative stress is generated by the inflammatory responses, promoting persistence of disease.

Our study has some limitations. GPX4 activity (levels of 15-HpETE-PE and 15-HETE-PE) was not directly measured in relation to inflammatory marker expression and cell death. However, we previously reported intracellular levels of these mediators in relation to GPX4 activity(7) that are likely similarly altered here. We chose to focus on SLC7A11 as playing a critical role in intracellular GSH levels, targeting SLC7A11 with erastin. Multiple other transporters and enzymes, including the availability of GSSG reductase, thioredoxins and activity of glutathione transferases are also involved in maintenance of the intracellular GSH-GSSG balance which could be evaluated in the future. Although our fresh epithelial brushing cell GSH data could have been influenced by non-epithelial (inflammatory) cell contributions, the percentages of these cell types in the brushings are typically less than 10% of total cells., and there was no correlation of BAL inflammatory cells and BAL GSH or GSSG. However, a more detailed evaluation of inflammatory cells and activity in tissues or epithelial brushings is needed before their contribution can be ruled out. Finally, further work is required to understand the mechanisms by which increased oxidative potential leads to increased T2-signature protein levels.

In conclusion, these findings strongly suggest that the altered airway redox imbalance consistently reported in asthma is primarily driven by high T2-associated 15LO1 activity in HAECs. This redox imbalance promotes enhanced expression of T2-associated proteins, leaving the cells

352 vulnerable to oxidatively induced cell death and, potentially, to asthma exacerbations. The 15LO1-

353 PEBP1 pathway should be investigated as a novel asthma therapeutic target.

354

Materials and Methods

Subjects

Subjects were enrolled in SARP or IMSA studies between February 2013 and January 2019 as previously described (39). All SARP participants were from the University of Pittsburgh site. Participants were never smokers, or had smoked less than five pack-years, with no smoking in the last year. HCs had no history of respiratory disease or recent respiratory tract infection and normal pulmonary function. All asthma patients met American Thoracic Society (ATS) criteria of asthma.(40) Patients were classified as severe asthma defined by 2014 European Respiratory Society/ATS guidelines (41). BAL fluid measurements from two different cohorts (SARP and IMSA) were combined to evaluate the GSH pathway.

Clinical and physiologic data

Prior to bronchoscopy, subjects underwent an extensive work up which included medical history, asthma control questionnaires, physical examination, FeNO concentration measurements, pulmonary function tests, and blood tests. Patients with exacerbation were identified from self-reported use of systemic corticosteroid courses lasting 3 or more days for asthma in the past 12 months on visit1. Questions regarding hospitalization for asthma were also reviewed. Asthma control was assessed with AQLQ. FeNO was measured before bronchodilator administration at a constant exhalation flow rate of

373 50 mL/s with a NIOX analyzer, according to ATS recommendations.(42) Baseline pulmonary function
374 tests were performed with standard medication withholds.

375

376 **Bronchoscopy**

377 Bronchoscopy with epithelial brushings and BAL were performed as previously described (6,
378 43, 44).

379

380 **ALI cell culture system and dicer siRNA**

381 After proliferation submerged culture condition, the first-passage HAECs were seeded in 12-
382 well transwell plates as previously described. When confluent, HAECs were shifted to ALI culture with
383 50µL apical media for 8 days to achieve full differentiation with/without IL-13 (10 ng/ml) stimulation
384 (R & D Systems, Minneapolis, MN, USA, #213-ILB) (10ng/ml).

385 DsiRNA KD of ALOX15 was performed in 12-well transwell plates as previously
386 described.(45) After transfection for 24 hours, IL-13 was added into lower chamber media every 48
387 hours for up to 7 days. ALOX15 DsiRNA was purchased from IDT (Coralville, IA, USA).

388 A selective enzymatic inhibitor of 15LO1, BLX2477 (gift from Dr. Hans-Erik Claesson,
389 Karolinska, Stockholm, Sweden) (15), was added at a final dilution of 2 mM for 24 hours. A selective
390 enzyme inhibitor of SLC7A11, erastin (Sigma-Aldrich, St. Louis, MO, #E7781),(19) was added at 10

μM into the medium for 24 hours before harvest, with Dimethyl sulfoxide or water added as vehicle control. Total protein from freshly brushed and cultured HAECs was collected in cell lysis buffer, homogenized by sonication and then centrifuged at 4,000 rpm for 10 minutes. The apical supernatant was used for GSH:GSSG and protein analysis.

RNA-sequencing data analysis of bronchoscopy brushings

RNA-sequencing data from two different cohorts [(SARP] and IMSA(GSE#158752)] were analyzed. The RNA-seq data for SARP are deposited to dbGaP at accessions phs001119.v1.p1 and phs001446, respectively. While awaiting data release via dbGaP, investigators may contact the senior author (Wenzel) of this study or go to the SARP website (46) to access to the data via the ancillary study mechanism for SARP. Count data for the SARP cohort was obtained from Dr. Stephanie Christianson/UCSF and as published recently (47).

To ensure harmonization with the SARP dataset, the count data for the IMSA cohort was generated using the same processing pipeline that was used to generate the SARP count data: reads were trimmed using TrimGalore (48) and aligned to Ensemble hg38 using STAR (49). Counts for known genes were then generated using featureCounts (50).

After obtaining count data for the IMSA cohort, the IMSA and SARP count data were processed together. The count data were filtered to remove lowly expressed genes, transformed using the variance stabilizing transformation from the DESeq2 package (51) , and then batch corrected using

ComBat (52). We found that processing the counts from the two cohorts together and correcting for batch effects with ComBat effectively harmonized the data from the two cohorts. This harmonized dataset was then used for the RNA-seq data analysis (Supplemental Table 5).

GSH and GSSG measurement

GSH and GSSG levels were measured, as previously described (21). GSH and GSSG levels were determined as the difference of low molecular weight thiols. BAL fluid, cell lysates or apical culture supernatants were incubated with glutathione peroxidase (Sigma-Aldrich, #G6137), cumene hydroperoxide (Sigma-Aldrich, #247502), glutathione reductase (Sigma-Aldrich, #G9297), and NADPH (Sigma-Aldrich, # N1630) for 30 minutes at 25°C. 10mM thiol Fluorescent Probe IV (Sigma-Aldrich, #1173888-41-9) was then used to identify the low molecular weight thiols and fluorescence measured at 400 nm excitation and 465 nm emission. A standard curve was established by the addition of GSH to phosphate buffer solution. Intracellular GSH and GSSG levels were corrected for intracellular protein, determined by Pierce BCA Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA, #23225) using albumin as a standard. The lower limit of detection for this assay was 0.016 μ M or 0.13 nmol/mg protein.

High-performance liquid chromatography analysis of low molecular mass thiols and disulfides

Quantification of GSH in BAL fluid was performed as reported by Steele *et al* (53). Briefly, GSH was derivatized with 4-fluoro-7-aminosulfonylbenzofurazan (ABD-F) to the fluorescent GS-ABD analyte. After separation on a C18 reverse phase high-performance liquid chromatography column (Beckman, 4.6 x 150 mm; particle size, 5 μ), GS-ABD was detected with a Waters 474 scanning fluorescence detector ($\lambda(\text{ex}) = 390 \text{ nm}$; $\lambda(\text{em}) = 530 \text{ nm}$). Authentic GSH was used as an external standard. GSSG in the samples was reduced with tris(2-carboxyethyl)phosphine (TCP) to GSH, and then derivatized with ABD-F to obtain the total concentration of thiols ($[\text{GSSG}] = [\text{GSH}]_{\text{total}} - [\text{GSH}]_{\text{prior to reduction with TCP}}$).

LC-MS/MS analysis of oxygenated phospholipids

Lipids were extracted using the Folch procedure, and phosphorus was determined by a micro-method as described previously (7, 21). Phospholipids and their hydroxy- and hydroperoxy-derivatives, including 15-HETE-PE and 15-HpETE-PE, were analyzed by LC/MS using a Dionex Ultimate 3000 HPLC system coupled on-line to a Orbitrap Fusion Lumos mass spectrometer (Thermo Fisher Scientific) using a normal phase column (Luna 3 μm Silica (2) 100 Å, 150 x 2.0 mm, (Phenomenex)). The column was maintained at 35 °C. The analysis was performed using gradient solvents (A and B) containing 10 mM ammonium formate at a flow rate of 0.2 ml/min. Solvent A contained isopropanol/hexane/water (285:215:5, v/v/v), and solvent B contained isopropanol/hexane/water (285:215:40, v/v/v). All solvents were LC/MS-grade. The column was eluted for 0-23 min with a linear

gradient from 10% to 32% B; 23-32 min with a linear gradient of 32%–65% B; 32-35 min with a linear gradient of 65%–100% B; 35-62 min held at 100% B; 62-64 min with a linear gradient from 100% to 10% B; followed by an equilibration from 64-80 min at 10% B. Analysis was performed in negative ion mode at a resolution of 140,000 for the full MS scan in a data-dependent mode. The scan range for MS analysis was m/z 400-1800 with a maximum injection time of 128 ms using 1 microscan. An isolation window of 1.0 Da was set for the MS scans. Capillary spray voltage was set at 3.5 kV, and capillary temperature was 320 °C. The S-lens RF level was set to 60. Ion source conditions were set as follows: spray voltage = 4 kV, sheath gas = 20 (arbitrary unit), auxiliary gas = 4 (arbitrary unit), sweep gas = 0 (arbitrary unit), transfer tube temperature = 300 °C, RF-Lens level = 50%. Analysis of raw LC/MS data was performed using software package Compound Discoverer™ 2.0 (Thermo Fisher Scientific) with an in-house generated analysis workflow and oxidized PL database. Briefly, peaks with S/N ratio of more than 3 were identified and searched against oxidized PL database. Lipids were further filtered by retention time and confirmed by a fragmentation mass spectrum. Deuterated PLs (Avanti Polar Lipids) were used as internal standards. Values for m/z were matched within 5 ppm to identify the lipid species.

Western Blot

Cell lysates and basilar supernatants were run on 4-12% sodium dodecyl sulphate-polyacrylamide (SDS-PAGE) gels (Invitrogen, Carlsbad, CA, USA) and transferred on polyvinylidene

466 difluoride membrane (Invitrogen). For supernatant studies, 40 µl of culture media was loaded as
467 previously described (14).

468 Primary antibodies against SLC7A11 (Cell Signaling Technology, #12691, 1:500 dilution),
469 iNOS (BD Biosciences, San Jose, CA, USA, #610329, 1:500 dilution), 15LO1 (Abnova, Taipei, Taiwan,
470 #H00000246-D01P, 1:1000 dilution), GPX4 (Abcom, Cambridge, MA, USA, #125066, 1:1000
471 dilution), POSTN (Santa Cruz Biotechnology, Dallas, TX, #67233, 1:500), and CCL26 (R&D Systems,
472 #AF653, 1:500 dilution) were used. GAPDH (Novus Biologicals, #NB300-320, 1:1000 dilution) or β-
473 actin (Sigma-Aldrich, #A5441, 1:2000 dilution) was measured as control. Membranes were developed
474 using Amersham imager 600 (GE Healthcare Life Sciences) with SuperSignal West Femto Maximum
475 Sensitivity Substrate (Thermo Fisher Scientific, #34096). Densitometry analysis was performed using
476 Image J Software.

478 **MUC5AC and LDH measurements**

479 MUC5AC protein expression was quantified by ELISA (Novus Biologicals, #NBP2-76703).
480 LDH by the Lactate Dehydrogenase Assay Kit (Abnova, #KA0878) according to the manufactures'
481 instructions. LDH was measured in both the culture medium and cell lysates, and LDH release (%) was
482 expressed as (LDH levels in culture medium)/[(LDH levels in culture medium) + (LDH levels in cell
483 lysis)] ×100.

Statistical Analysis

All statistical analyses were performed with JMP Pro software version 14 (SAS Institute Inc., Cary, NC, USA). Data were visualized with GraphPad Prism software version 8 (San Diego, CA) software. For GSH pathway measurements, each colored symbol represents an individual patient (circles=HC, triangles=M/MA, squares=SA) or individual human cell line which can be followed through multiple figures. Most values were log-transformed because as they were linearly distributed; AQLQ scores were subjected to quantile normalization. Intergroup comparisons were performed by parametric t-test and ANOVA methods, or χ^2 tests. Cells from specific donors under two conditions were compared using matched-pair analysis. The Pearson's correlation was employed to analyse relationships among the continuous data. Bonferroni correction was applied to analyses correlating BAL fluid or intracellular GSH, GSSG, and GSH:GSSG to up to 5 gene expression variables or clinical parameters to adjust for multiple comparisons. A p value of <0.05 was considered statistically significant for univariate/two-group analyses. Any p-value passing this significance (and correction) threshold is in bold text.

Study approval

The studies were approved by the University of Pittsburgh Institutional Review Board (IMSA, PRO15050456; local SARP, PRO12070359) and all subjects provided written informed consent prior to participation in the study and were identified by different numbers, not by names.

670 **Data and materials availability**

671 All data for this manuscript are included in the paper and raw data are available for review on request.

506

507 **Author contributions**

508 TN and SEW designed the study. JBT performed HAECs culture. TN, YD, SS, YN, JZ and VK
509 performed research studies. TN, AJS and SEW performed statistical analyses. MG, SC, SG, and JF
510 performed RNA-seq analysis. TN, VK and SEW interpreted the data. TN, AJS, and SEW wrote the
511 manuscript. All authors were involved in the preparation and review of the manuscript and approved
512 the final submitted version.

513

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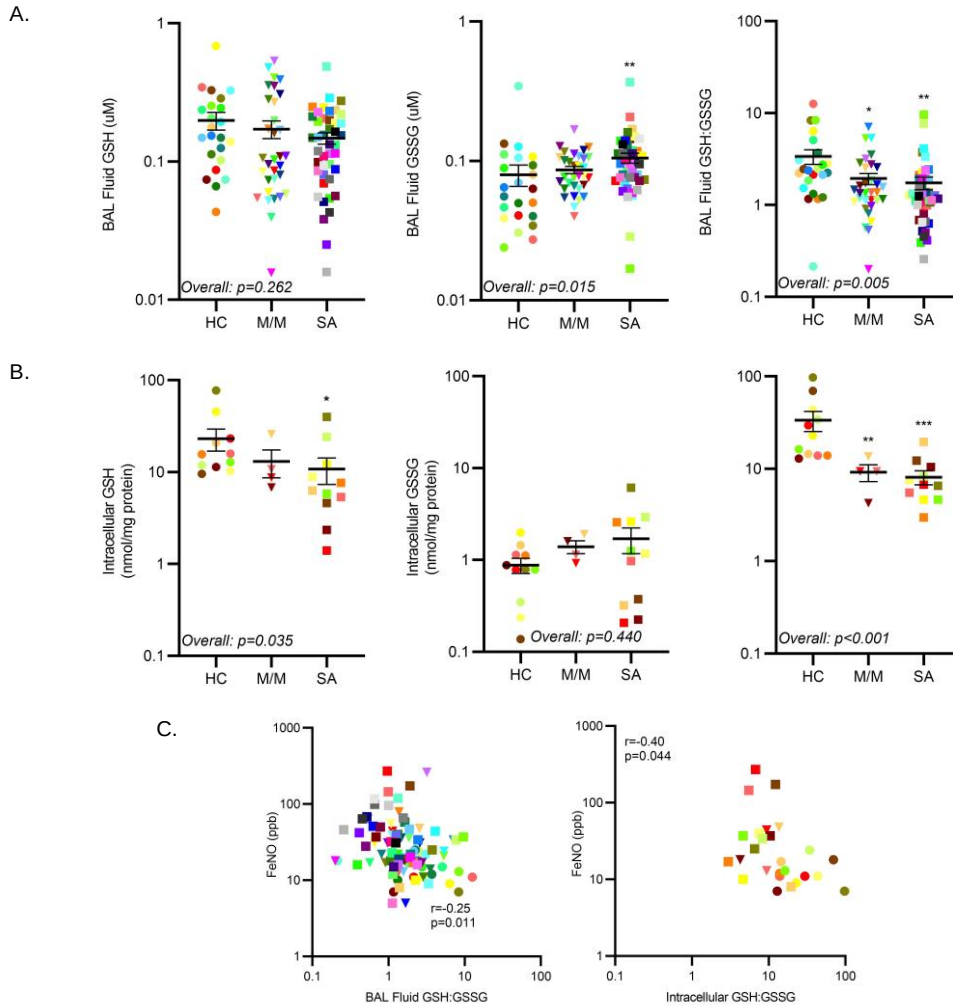


Fig. 1. Abnormal redox balance, as measured by higher BAL fluid oxidized glutathione (GSSG) and lower intraepithelial cell GSH is observed in asthmatic bronchoalveolar lavage (BAL) fluid, as compared to healthy controls. GSH, GSSG, and GSH:GSSG were measured by enzymatic assay in BAL fluid and in fresh epithelial cells from healthy controls (HCs), mild/moderate and severe asthma patients. **(A)** Higher levels of GSSG and lower GSH:GSSG were observed in asthmatic BAL fluid. **(B)** Intracellular GSH levels and GSH:GSSG were lower in fresh asthmatic epithelial cells compared to HCs. **(C)** BAL fluid ($n=98$) and intracellular GSH:GSSG ($n=26$) positively correlated with FeNO. ANOVA with intergroup comparisons by t-test were used for subject group comparisons. Pearson's correlations were used for GSH:GSSG vs FeNO. Bonferroni corrected significant p-values for 3 groups was set at 0.0166. All data were log-transformed prior to analysis and data converted back to the linear scale for presentation. Bars represent mean and error bars represent SEM (* $p<0.01$, ** $p<0.001$).

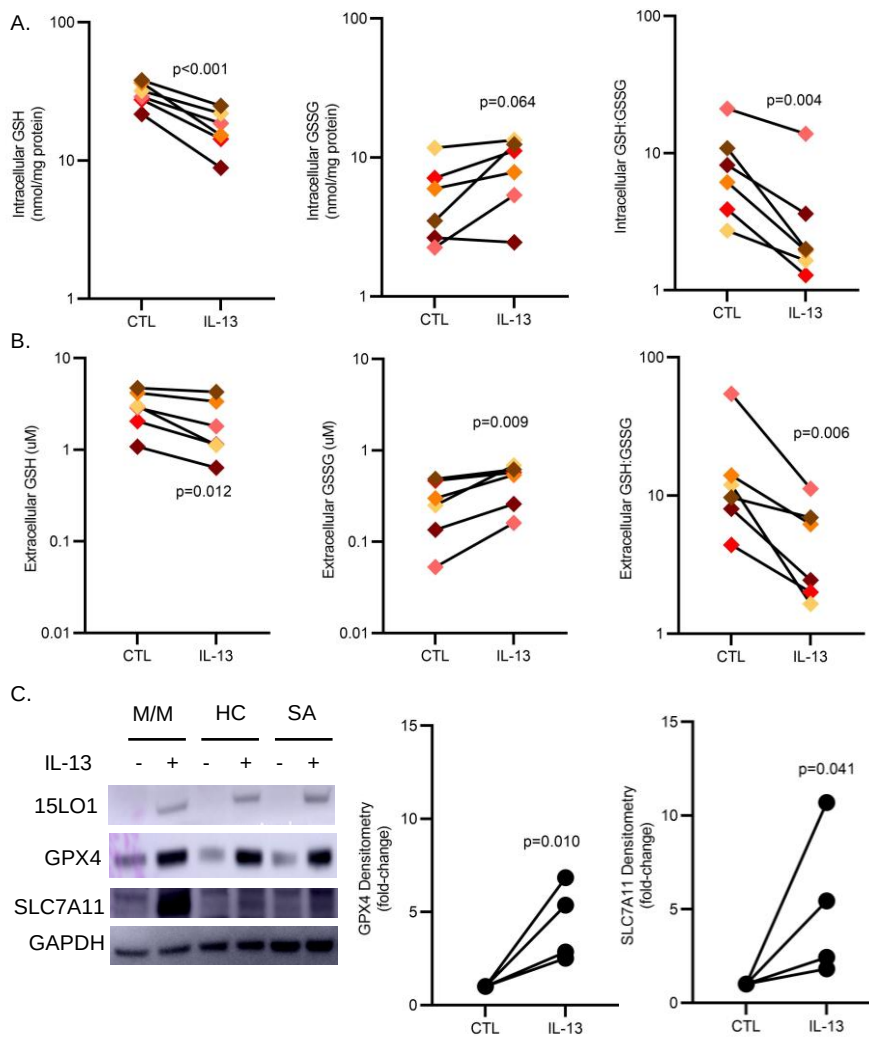
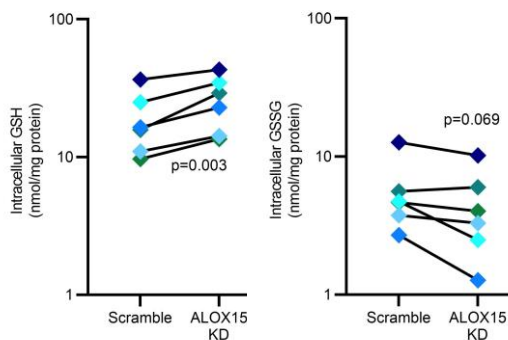


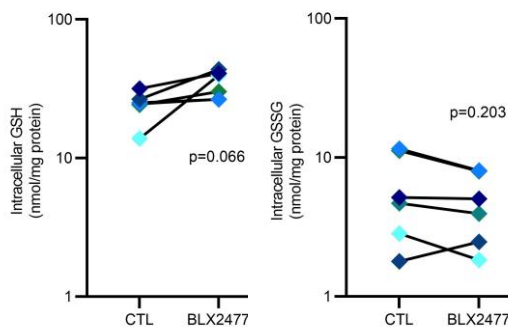
Fig. 2. IL-13 lowers alters the intra- and extracellular oxidative potential by decreasing intracellular GSH and GSH:GSSG and increasing extracellular GSSG.

(A) IL-13 decreases intracellular GSH levels and lowers GSH:GSSG ($n=6$). **(B)** IL-13 also decreases extracellular GSH levels but increases extracellular GSSG, lowering GSH:GSSG ($n=6$). **(C)** IL-13 (10 ng/ml) increases expression of 15LO1, GPX4 and SLC7A11 by western blot, with GPX4 and SLC7A11 increases quantified by densitometry ($n=4-12$). Matched-pair analysis of log-transformed data was used to compare conditions.

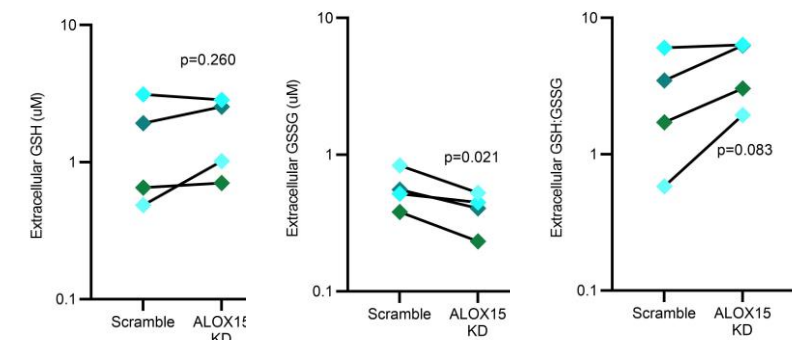
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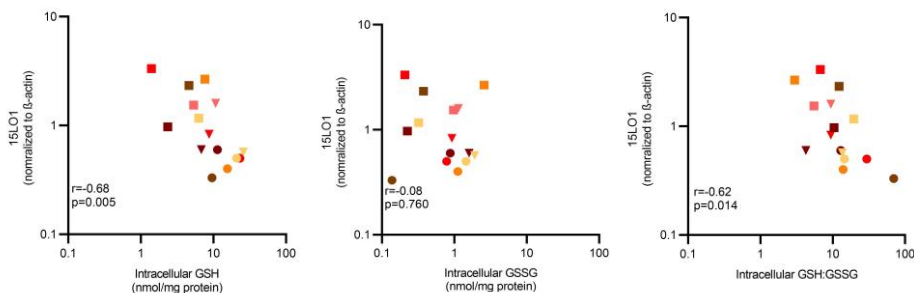
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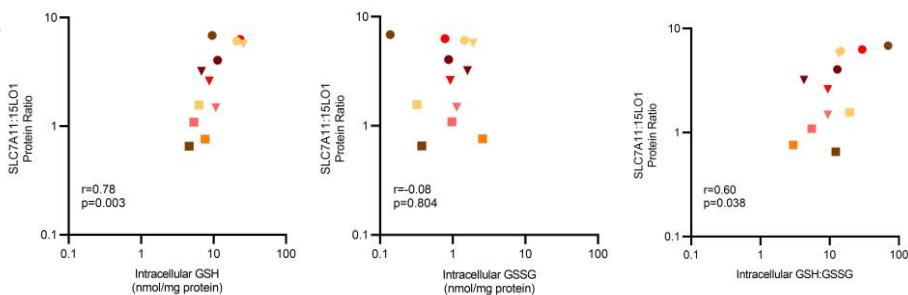
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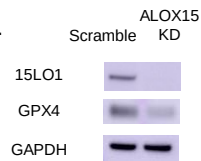
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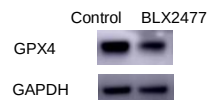


Fig. 3. 15LO1 expression and activity regulate intracellular redox balance by lowering GSH and GSH:GSSG. (A) Under IL-13 conditions, ALOX15 (15LO1) knockdown (KD) increases intracellular GSH and GSH:GSSG towards control conditions (n=6). (B) Under IL-13 conditions, inhibition of 15LO1 by the specific 15LO1 inhibitor BLX2477 also restores intracellular GSH and GSH:GSSG (n=6). (C) ALOX15 KD lowers extracellular GSSG and restores a normal GSH:GSSG (n=6). (D) Representative western blots demonstrating ALOX15 KD also decreases GPX4 protein expression (n=4). (E) Representative western blots show decreased expression of GPX4 under conditions of 15LO1 inhibition (n=4). (F) *Ex vivo* 15LO1 protein expression correlates with intraepithelial cell GSH and GSH:GSSG (n=15). (G) Indexing SLC7A11, as a critical regulator of intracellular GSH levels, to 15LO1 improves the relationship of 15LO1 to intracellular GSH (n=12). Matched-pair analysis (A, B, and C) of log transformed data was used to compare conditions, while Pearson's correlation was used for F and G.

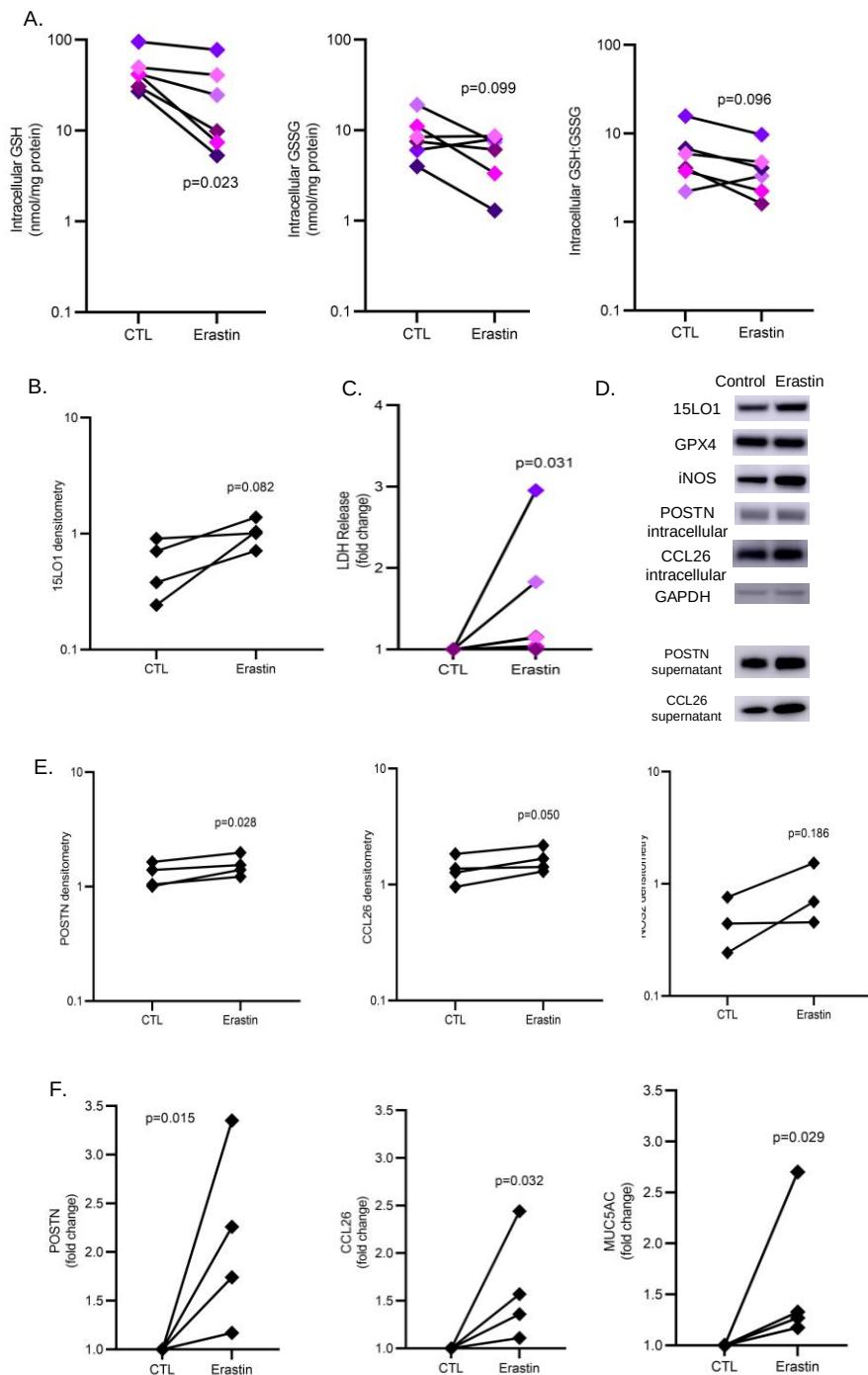


Fig. 4. Activity of the cystine-glutamate antiporter SLC7A11 modulates intracellular redox, cell death and Type-2 signature protein expression. (A) Inhibition of SLC7A11 activity by erastin lowers intracellular GSH and GSH:GSSG (n=6). **(B)** This decrease in GSH:GSSG is accompanied by increasing expression of 15LO1 (n=4). **(C)** The decreased GSH:GSSG raises LDH release, consistent with activation of oxidatively-driven cell death (n=6). **(D)** Representative western blot of intracellular and secreted Type-2 signature markers following SLC7A11 chemical inhibition with erastin for 24 hrs (n=3-4). **(E)** Densitometry results showing increases in intracellular periostin, CCL26 and iNOS following treatment with erastin (n=3-4). **(F)** Densitometry results showing parallel fold-change increases in basilar secretion of periostin (POSTN) and CCL26, with increased apical secretion of MUC5AC protein following treatment with erastin (n=4). Matched-pair analysis following log-transformation was used to compare the conditions.

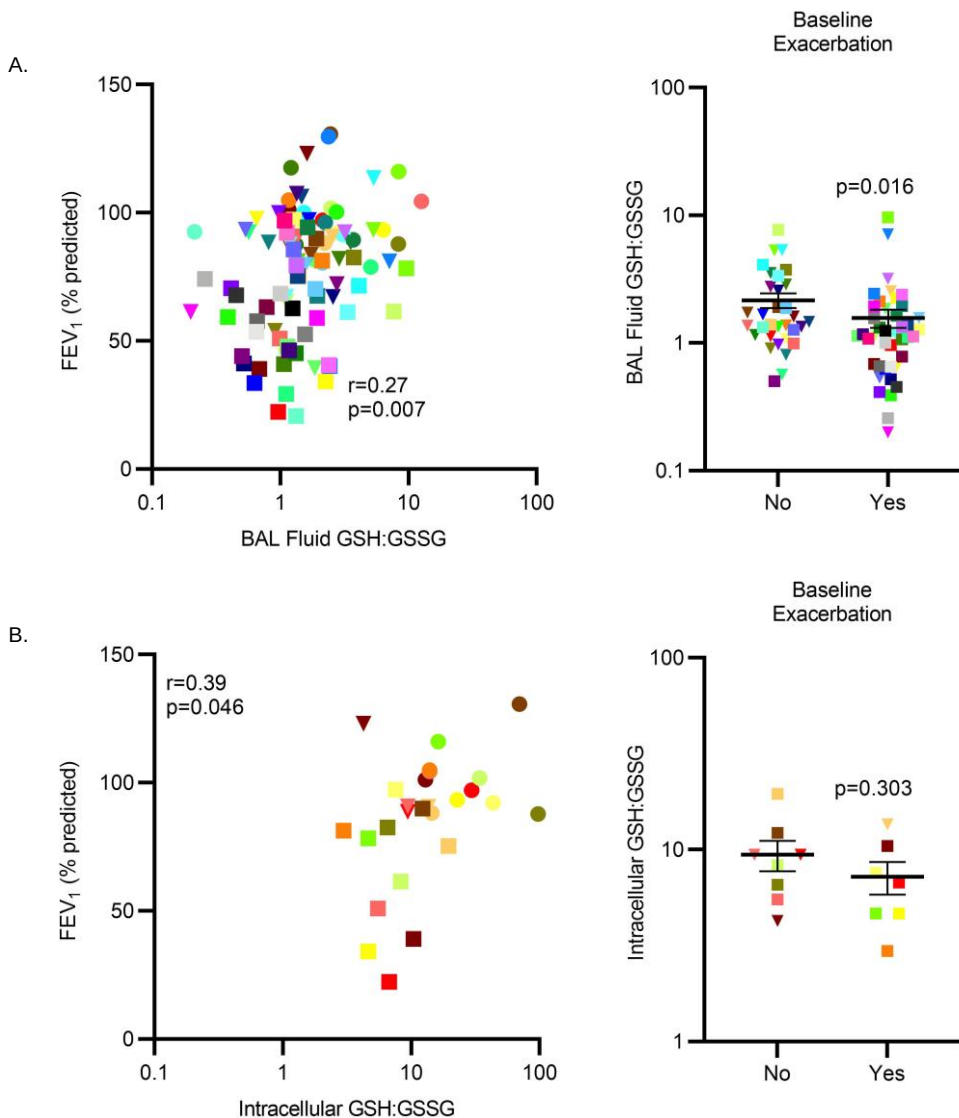


Fig. 5. *Ex vivo* extra- and intracellular redox balance is associated with clinical outcomes relevant to asthma. (A) Extracellular BAL fluid GSH:GSSG correlates with lung function ($n=98$) as measured by FEV₁% predicted ($n=75$) and associates with asthma exacerbations. **(B)** Intracellular GSH:GSSG correlates with FEV₁% predicted ($n=26$), while in small numbers the association with baseline exacerbations ($n=15$) is not significant. Pearson's correlation testing was performed for comparisons of FEV₁% predicted with GSH:GSSG, while T-tests were used for group comparisons. Data were log transformed except for FEV₁% predicted, which was normally distributed. Bars represent mean and error bars represent SEM.

727 **Tables**

728 **Table 1. Characteristics of all patients combined**

	HC (n = 23)	M/M (n = 32)	SA (n = 43)	P value
Age (years)	41 ± 12	39 ± 14	47 ± 10	0.008
Female/male	15/8	25/7	27/16	0.468
BMI (kg/m²)	27 ± 5	29 ± 6	33 ± 7	0.007
History of exacerbation*, yes/no	NA	11/21	32/11	<0.001
Inhaled corticosteroids, yes/no	NA	18/14	43/0	<0.001
ACQ	NA	0.9 (0.4-1.5)	2.3 (1.4-3.5)	<0.001
AQLQ	NA	6.0 (5.5-6.5)	4.2 (3.3-5.1)	<0.001
FeNO (ppb)	15 (11-18)	22 (17-37)	37 (18-58)	<0.001
FEV₁ (% predicted)	98 ± 14	85 ± 19	61 ± 20	<0.001

729 Data as means ± SDs

730 p values by ANOVA, Mann-Whitney, Kruskal-Wallis, or χ^2 tests

731 Exacerbation*, self-reported use of systemic corticosteroid ≥ 3 days in past 12 months

Table 2. Relationships of clinical, physiologic and inflammatory factors to extracellular BAL fluid and intracellular GSH and GSSG

	<u>FeNO (ppb)</u>	<u>FEV1% predicted</u>	<u>AQLQ total score</u>
<u>BAL Fluid</u>	n=98	n=98	n=75
GSH (μM)	r=-0.11 p=0.298	r=0.09 p=0.364	r=0.20 p=0.081
GSSG (μM)	r=0.29 p=0.004	r=-0.29 p=0.003	r=-0.17 p=0.155
<u>Intracellular</u>	n=26	n=26	n=15
GSH (nmol/mg protein)	r=-0.56 p=0.003	r=0.37 p=0.062	r=0.66 p=0.008
GSSG (nmol/mg protein)	r=-0.14 p=0.48	r=-0.04 p=0.852	r=0.52 p=0.049

Bold p-values pass Bonferonni Correction.

Lower “n” for AQLQ is secondary to exclusion of healthy controls for whom the score doesn’t apply

737 **Table 3. Relationships of HAEC gene expression with BAL fluid (n=84) and intracellular (n=23)**

738 **GSH pathways**

<u>BAL Fluid</u>	ALOX15	CCL26	MUC5AC	NOS2	POSTN
GSH (μM)	r=-0.04	r=-0.02	r=0.13	r=0.05	r=0.10
	p=0.726	p=0.866	p=0.228	p=0.662	p=0.359
GSSG (μM)	r=0.30	r=0.19	r=0.21	r=0.23	r=0.25
	p=0.005	p=0.082	p=0.052	p=0.030	p=0.020
GSH:GSSG	r=-0.23	r=-0.14	r=-0.03	r=-0.12	r=-0.08
	p=0.032	p=0.183	p=0.795	p=0.276	p=0.454
<u>Intracellular</u>					
GSH (nmol/mg protein)	r=-0.52	r=-0.59	r=-0.30	r=-0.61	r=-0.64
	p=0.006	p=0.002	p=0.139	p=<0.001	p=<0.001
GSSG (nmol/mg protein)	r=-0.29	r=-0.35	r=0.07	r=-0.23	r=-0.32
	p=0.153	p=0.081	p = 0.745	p=0.252	p=0.106
GSH:GSSG	r=-0.22	r=-0.23	r=-0.38	r=-0.37	r=-0.31
	p=0.273	p=0.268	p=0.058	p=0.060	p=0.129

739 **Bold p-values pass Bonferonni Correction.**

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カタログ

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Supplemental Figure 1

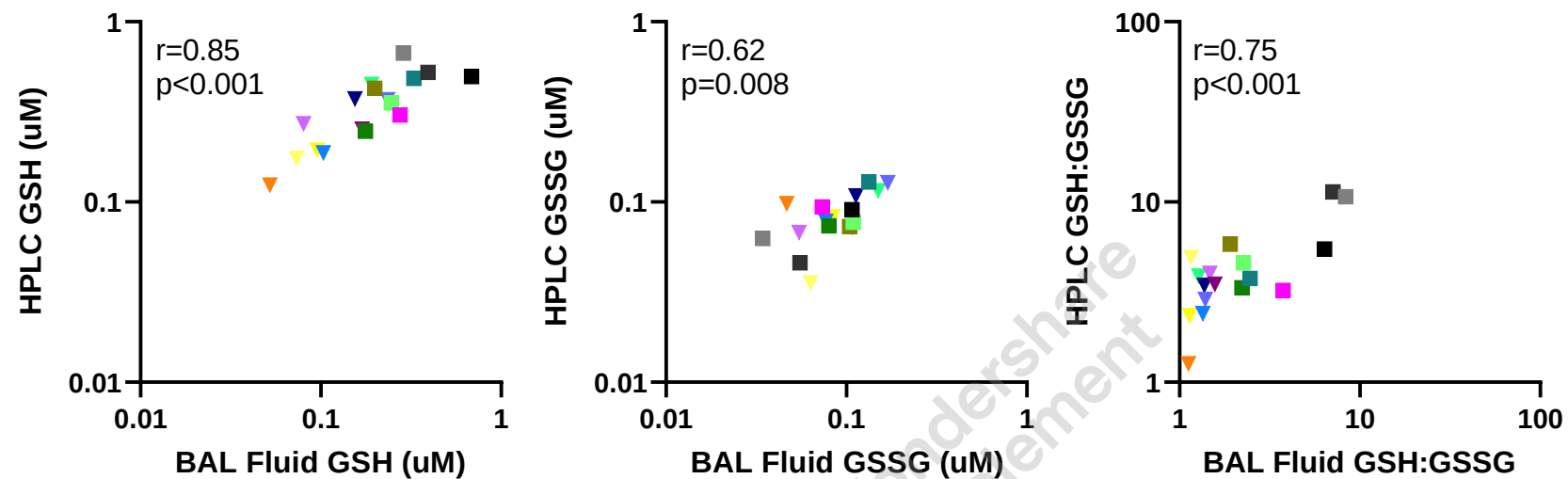


Fig. 1. GSH and GSSG levels and GSH:GSSG measured by enzymatic method correlated strongly with high-performance liquid chromatography (HPLC) measurements (n=17).

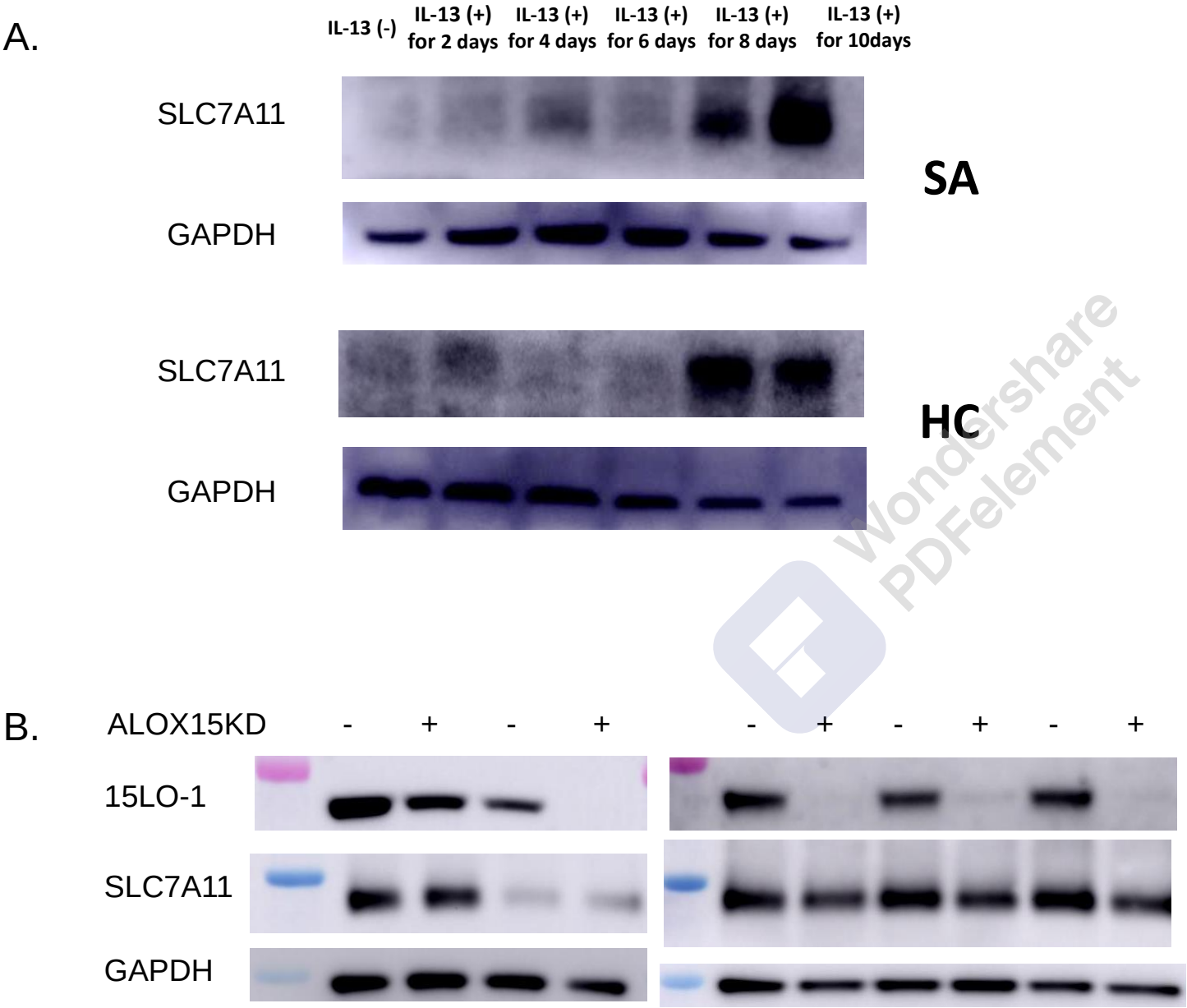
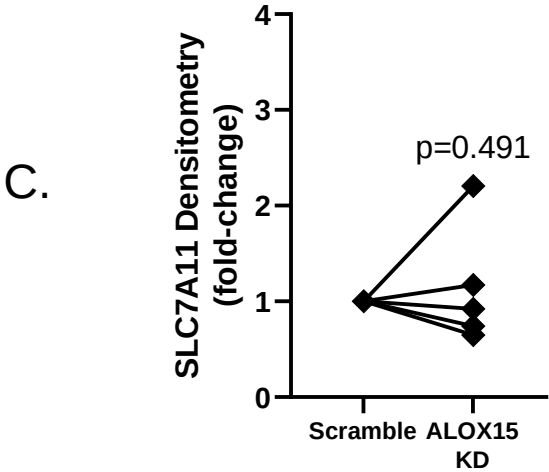


Fig. 2. (A) Representative western blots showing time course of SLC7A11 protein expression in response IL-13 stimulation *in vitro*. **(B)** Representative western blots showing the inconsistent impact of ALOX15 siRNA knockdown on SLC7A11. **(C)** Densitometric analysis of the fold changes.



Supplemental Figure 3.

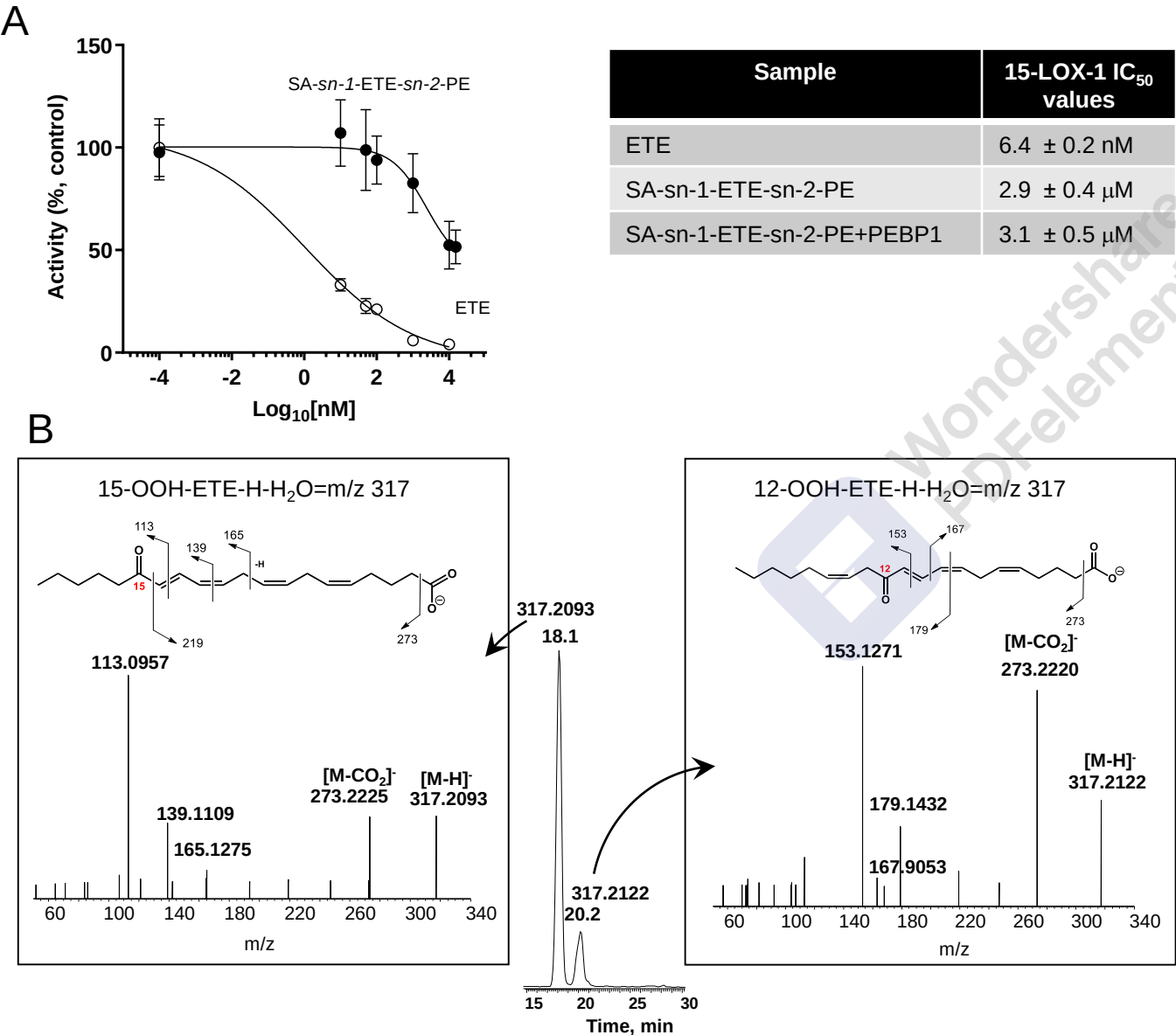


Fig 3. Effect of BLX2477 on oxidation of free eicosanotetraenoic acid (ETE) and stearyl-sn-1-eicosatetraenoyl-sn-1-phosphatidylethanolamine (SA-sn-1-ETE-sn-2-PE) by 15-LOX-1 in the absence and in the presence of PEBP1.

(A) Concentration dependent inhibition of 15LO1 activity by BLX2477 in the presence of ETE (open circles) or in the presence of SA-sn-2-ETE-sn-2-PE (closed circles) (left panel). The data are presented as % of control. 100% of 15LO1 activity in the absence of BLX2477 was used as the control (n=5-7). IC₅₀ values of 15LO1 for BLX2477. IC₅₀ values were calculated using GraphPad Prism (GraphPad Software, Inc.)

(B) 15LO1 oxidation of ETE results in the formation of two oxidized products 15-OOH-ETE and 12-OOH-ETE. Base peak profile of oxygenated ETE formed during 15LO1-driven reaction (middle panel). MS² spectrum and structural formulas showing the fragments formed during the analysis of molecular ions with m/z 317.2 corresponding to 15-OOH-ETE without water (left panel) and 12-OOH-ETE without water (right panel).

Experimental conditions: ETE or SA-sn-1-ETE-sn-2-PE were integrated into OA-sn-1-OA-sn-2-PC (100 mM, at ratio of 1:1) liposomes and incubated with 15LO1 (0.4mM) for 2.5 and 5 min at 37°C. Reaction was started by the addition of 3 μM 13 HpODE. At the end of incubation lipids were extracted and analyzed by LC/MS.

Supplemental Figure 4.

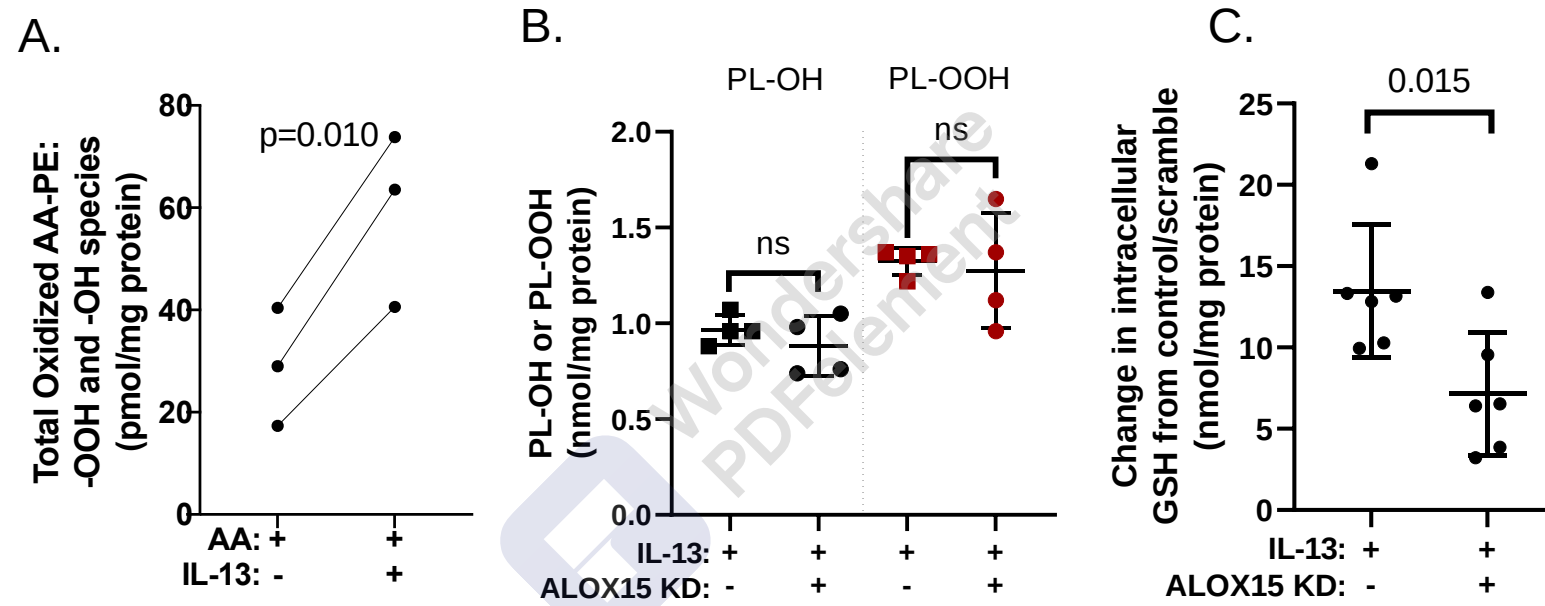


Fig 4. (A) Changes in hydroperoxy- and hydroxy-arachidonic acid (AA) species, including 15-HpETE and 15-HETE, following IL-13 with AA supplementation in HAECS. (B) Changes in total hydroperoxy- (PL-OOH) and hydroxy-phospholipids (PL-OH) following IL-13 and ALOX15/15LO1 KD compared to control/scramble in HAECS. (C) Changes in intracellular GSH.

Supplemental Figures.

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(B) 15LO1 oxidation of ETE results in the formation of two oxidized products 15-OOH-ETE and 12-OOH-ETE. Base peak profile of oxygenated ETE formed during 15LO1-driven reaction (middle panel). MS^2 spectrum and structural formulas showing the fragments formed

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32 **Supplemental Table 1. Characteristics of fresh epithelial cell subset**

	HC (n = 11)	M/M (n = 4)	SA (n = 11)	P value
Age (years)	44 ± 14	35 ± 18	52 ± 11	0.189
Female/male	8/3	2/2	7/4	0.706
BMI (kg/m²)	27 ± 4	28 ± 2	30 ± 7	0.478
History of NA exacerbation*, yes/no	NA	1/3	6/5	0.310
Inhaled corticosteroids, yes/no	NA	1/3	11/0	0.001
ACQ	NA	0.6 (0.2-1.2)	1.7 (1.3-3.0)	0.023
AQLQ	NA	6.3 (5.9-6.7)	5.2 (3.9-5.8)	0.013
FeNO (ppb)	11 (9-17)	31 (14-47)	37 (17-45)	0.011
FEV₁ (% predicted)	102 ± 13	98 ± 17	65 ± 25	<0.001

33 Data as means ± SDs

- 34 p values by ANOVA, Mann-Whitney, Kruskal-Wallis, or χ^2 tests
- 35 Exacerbation*, self-reported use of systemic corticosteroid ≥ 3 days in past 12 months





Supplemental Table 2. Relationships between extracellular (BAL fluid) and intracellular

GSH pathways in asthma and HCs

	<u>BAL fluid</u>		
	GSH (μ M)	GSSG (μ M)	GSH:GSSG
<u>Intracellular</u>			
GSH (nmol/mg protein)	$r=0.41$ $p=0.037$	$r=-0.29$ $p=0.156$	$r=0.58$ $p=0.002$
GSSG (nmol/mg protein)	$r=0.26$ $p=0.191$	$r=-0.09$ $p=0.645$	$r=0.33$ $p=0.104$
GSH:GSSG	$r=0.13$ $p=0.517$	$r=-0.19$ $p=0.352$	$r=0.24$ $p=0.234$

Supplemental Table 3. Non-parametric correlations for BAL eosinophils (absolute, % BAL cells) and GSH, GSSG and GSH:GSSG

Eosinophils

	Absolute eosinophil numbers	% BAL Cells
<i>BAL Fluid:</i>		
GSH	rho=0.02 p=0.877	rho=-0.08 p=0.417
GSSG	rho=-0.01 p=0.938	rho=0.05 p=0.644
GSH:GSSG	rho=-0.05 p=0.610	rho=-0.18 p=0.078
<i>Intra-epithelial:</i>		
GSH	rho=-0.25 p=0.227	rho=-0.33 p=0.102
GSSG	rho=-0.26 p=0.199	rho=-0.29 p=0.148

GSH:GSSG

rho=-0.07

rho=-0.14

p=0.730

p=0.482

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58 **Supplemental Table 4. Non-parametric correlations of inflammatory cells (% BAL cells)**59 **versus BAL fluid and intracellular/intra-epithelial GSH, GSSG, and GSH:GSSG.**

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61 **Other Inflammatory Cells**

	Neutrophils	Macrophages	Lymphocytes
	(% BAL cells)	(% BAL cells)	(% BAL cells)
<i>BAL Fluid:</i>			
GSH	rho=-0.06	rho=0.01	rho=-0.01
	p=0.586	p=0.989	p=0.914
GSSG	rho=0.16	rho=-0.15	rho=0.07
	p=0.147	p=0.156	p=0.542
GSH:GSSG	rho=-0.19	rho=0.14	rho=-0.09
	p=0.089	p=0.206	p=0.431

Intra-epithelial:

GSH	rho=-0.07	rho=0.01	rho=0.13
	p=0.725	p=0.985	p=0.527
GSSG	rho=0.15	rho=0.17	rho=-0.12
	p=0.481	p=0.403	p=0.557
GSH:GSSG	rho=-0.32	rho=0.01	rho=0.07
	p=0.117	p=0.956	p=0.744

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64 **Supplemental Table 5. Correlation of T2 signature genes and ALOX15 expression.**

	ALOX15 expression
CCL26 expression	r=0.58
	p<0.001
MUC5AC expression	r=0.41
	p<0.001
NOS2 expression	r=0.67
	p<0.001
POSTN expression	r=0.56
	p<0.001

65 **Bold p-values pass Bonferonni Correction**

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