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The glutaredoxin/S-glutathionylation axis regulates interleukin 17A-induced pro-inflammatory responses in lung epithelial cells in association with S-glutathionylation of Nuclear Factor kappa B family proteins

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Abstract

Interleukin-17A (IL-17A) is a newly emerging player in the pathogenesis of chronic lung diseases that amplifies inflammatory responses and promotes tissue remodeling. Stimulation of lung epithelial cells with IL-17A leads to activation of the transcription factor, nuclear factor kappa B (NF- κ B), a key player in the orchestration of lung inflammation. We have previously demonstrated the importance of the redox-dependent post-translational modification, S-glutathionylation, in limiting activation of NF- κ B and downstream gene induction. Under physiological conditions, the enzyme glutaredoxin 1 (Grx1) acts to deglutathionylate NF- κ B proteins, which restores functional activity. In this study, we sought to determine the impacts of S-glutathionylation on IL-17A-induced NF- κ B activation and expression of pro-inflammatory mediators. C10 mouse lung alveolar epithelial cells, or primary mouse tracheal epithelial cells exposed to IL-17A show rapid activation of NF- κ B, and the induction of pro-inflammatory genes. Upon IL-17A exposure, sulfenic acid formation and S-glutathionylated proteins increased. Assessment of S-glutathionylation of NF- κ B pathway components revealed S-glutathionylation of RelA (RelA-SSG) and inhibitory kappa B kinase alpha (IKK α -SSG) after stimulation with IL-17A. SiRNA-mediated ablation of Grx1 increased both RelA-SSG and IKK α -SSG and acutely increased nuclear content of RelA, and tended to decrease nuclear RelB. SiRNA mediated ablation or genetic ablation of *Grx1* decreased the expression of NF- κ B regulated genes, KC and CCL20, in response to IL-17A, but conversely increased the expression of IL-6. Lastly, siRNA mediated ablation of IKK α attenuated nuclear RelA and RelB content and decreased expression of KC and CCL20 in response to IL-17A. Together, these data demonstrate a critical role for the S-

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glutathionylation/Grx1 redox axis in regulating IKK α and RelA S-glutathionylation and the responsiveness of epithelial cells to IL-17A.

Keywords

Interleukin-17A; glutaredoxin-1; protein S-glutathionylation; inhibitory kappa B kinase alpha; nuclear factor kappa B; redox

INTRODUCTION

The Interleukin-17 (IL-17) family is a newly emerging class of cytokines that plays a critical role in a variety of biological processes, including innate host defense and inflammation. This cytokine family consists of six members, IL-17A-F, with IL-17A the most extensively studied member to date. Upon binding of IL-17A to its heterodimeric receptors (IL-17RA/RC), various adaptor proteins containing unique interaction domains are recruited to the receptor complex, activating multiple pathways, which include mitogen activated protein kinases (MAPK) and nuclear factor kappa B (NF- κ B). Aberrant IL-17 signaling has been functionally linked to the pathogenesis of a variety of autoimmune and inflammatory diseases, including multiple sclerosis, systemic lupus erythematosus, psoriasis, rheumatoid arthritis and asthma [1–5]. Although T lymphocytes are the main source of IL-17A, the expression of IL-17Rs appears to be more ubiquitous [6], and the role of structural cells, including epithelial cells, as major responders to IL-17A has been well documented [7–9]. Airway epithelial cells have been shown to upregulate many pro-inflammatory mediators and genes involved in host-defense following stimulation with IL-17A. These include beta defensins, mucin genes and dendritic cell and neutrophil chemoattractants [7, 10, 11], in part due to the activation of the transcription factor NF- κ B.

Previous studies in our laboratory have demonstrated the importance of NF- κ B activation within lung epithelial cells in the pathogenesis of allergic airways disease in mouse models [12–14]. Two parallel NF- κ B activation pathways have been described to date: the classical and alternative pathways. In the classical pathway, inhibitor of kappa B kinase β (IKK β) becomes activated via phosphorylation, allowing it to then phosphorylate inhibitor of kappa B α (I κ B β), marking it for ubiquitination and subsequent proteasomal degradation. This event facilitates nuclear entry of the transcription factor heterodimer RelA/p50, and subsequent gene induction. In the alternative NF- κ B pathway, IKK α activation leads to the phosphorylation of p100 and its resultant processing to the active p52 form, and allows nuclear entry of the RelB/p52 heterodimer [15, 16]. The alternative pathway has typically been considered to function primarily in cells of the adaptive immune system and lymphoid organs, but more recent studies have demonstrated that both NF- κ B pathways can cooperate to control pro-inflammatory responses in lung epithelial cells [17].

Given the central role of NF- κ B in driving inflammatory responses, its activation is tightly regulated via protein-protein interactions as well as several post-translational modifications. Reversible oxidative modifications of cysteines, including sulfenic acid formation and protein S-glutathionylation (PSSG), have been shown to affect protein function [18–20]. PSSG represents the conjugation of the tripeptide antioxidant, glutathione (GSH), to

reactive, oxidizable cysteines within proteins. This modification has considerable implications for biological processes, as it can alter the function of various proteins, and both PSSG-induced activation and inactivation have been reported, depending on the protein target [21]. Work by our laboratory and others has demonstrated that the NF- κ B pathway, as well as upstream adaptor molecules such as TRAF6, are negatively regulated via PSSG [22–26]. Under physiological conditions, the thiol oxidoreductase glutaredoxin-1 (Grx1) can deglutathionylate proteins, thus restoring the native thiol form of the target cysteine residue [27]. Numerous studies initially focused on oxidant-induced PSSG, but more recent studies have demonstrated the role of this modification in the context of biologically relevant stimuli [22, 28–30].

Although IKK α acts as a positive regulator of IL-17A production in T-cells [31] and has also been shown to regulate IL-17A responses in synoviocytes, kidney cells and astrocytes [32], the role of epithelial IKK α in response to IL-17A is unclear. To date, it is also unknown whether IL-17A elicits redox perturbations in lung epithelial cells, and whether redox modifications alter pro-inflammatory responses to IL-17A. Because of the known regulation of the NF- κ B pathway through PSSG, the ability of Grx1 to reverse this modification, and the possible link between IKK α and IL-17A, we focused herein on the role of the Grx1/PSSG redox axis in regulating the IKK α -driven responsiveness of lung epithelial cells to IL-17A.

MATERIALS AND METHODS

Antibodies

The following antibodies were used in this study: rabbit anti-RelA, rabbit anti-RelB (Santa Cruz Biotechnology, Santa Cruz, CA), rabbit anti-Histone H3, rabbit anti-I κ B α (Cell Signaling Technology, Danvers, MA), mouse anti-GSH (Virogen, Watertown, MA), mouse anti-IKK α (Upstate, Millipore, Billerica, MA) Streptavidin conjugated-HRP (Jackson, West Grove, PA), goat anti-Grx1 (American Diagnostica, Stamford, CT) and mouse anti- β -actin (Sigma-Aldrich, Saint Louis, MO). The secondary HRP conjugated anti-rabbit and anti-mouse antibodies were from Amersham (Piscataway, NJ), anti-goat was from Jackson Laboratories (West Grove, PA). All fluorophore-conjugated antibodies were from Invitrogen (Carlsbad, CA).

Cell Culture

Primary mouse tracheal epithelial cells (MTECs) were isolated from WT mice or mice lacking the glutaredoxin-1 gene (referred herein as *Grx1* $-/-$), and cultured as previously described [33, 34]. A spontaneously transformed type II mouse lung alveolar epithelial cell line [35] (C10) was cultured as described previously [33]. Unless otherwise noted, 16 h prior to stimulation, C10 cells were switched to medium containing 0.5% FBS; MTECs were incubated for 16h in serum-free medium. Cells were stimulated with indicated concentrations of Interleukin-17A (IL-17A; R&D Systems, Minneapolis, MN) and harvested as previously described [36].

Transfection of siRNA

C10 cells were incubated with scrambled, non-targeting siRNA or Grx1 siRNA (Ambion, Carlsbad, CA) (all at 100 nM). C10 cells were incubated with SMARTpool scrambled nontargeting siRNA or SMARTpool IKK α siRNA (Dharmacon, Lafayette, CO) (all at 100nM). Cells were subsequently stimulated, harvested and analyzed as indicated.

Labeling of sulfenic acids using dimedone

Cells were switched to serum-free medium for 2 hours; in the final hour, cells were preincubated with 1 mM DCP-Bio1 (Kerafast, Boston, MA) in serum-free medium for 1h at 37° C. Medium containing DCP-Bio1 was removed, and fresh serum-free medium containing 100 ng/ml IL-17A was added to cells for indicated times. Cells were then fixed in 4% formalin and permeabilized with 0.2% Triton X100 in PBS for 10 min. The permeabilized cells were blocked in 5% BSA in PBS for 1h. The cells were then incubated with fluorescently labeled streptavidin (1:2000, SA alexa fluor 647) and the nucleus was counterstained with DAPI (1:4000). Cell images were acquired using a Zeiss LSM 510 META Confocal Laser Scanning Imaging System. All images were taken at 20 $\times$ magnification. The image files were converted to tiff format and brightness and contrast were adjusted equally in all images.

Alternatively, cells were harvested in the presence of dimedone to detect oxidized cysteines in cell lysates according to a previously described method [37]. In brief, cells were serum-deprived for 2h, and then stimulated with IL-17A and lysed in buffer containing 1mM dimedone. Excess dimedone was removed using Micro Bio-spin 6 columns (Bio-Rad, Hercules, CA). Samples were analyzed by SDS-PAGE, and extent of sulfenic acid labeling was assessed using anti-dimedone antibody (Millipore, Darmstadt, Germany).

Detection of S-glutathionylated proteins (PSSG)

Cells were exposed to IL-17A as indicated, and at the selected times lysates were prepared as described before [38]. In brief, samples were immunoprecipitated (IP) using GSH antibody (2 μ g/ml) and precipitated proteins were subjected to polyacrylamide gel electrophoresis and subsequent immunoblotting for IKK α [38]. As a reagent control, samples were treated with 50mM DTT prior to IP, to decompose PSSG. In selected experiments, cells were incubated with 250 μ M biotinylated glutathione ethyl ester (Bio-GEE) for 1h. Bio-GEE was prepared as previously described [39], and Bio-GEE-conjugated proteins were determined following nonreducing SDS-PAGE and Western blotting [39]. Western Blots were analyzed via densitometry, and

Total levels of PSSG in cell lysates were determined using the glutathione/glutathione reductase/NADPH/5,5'-dithiobis (2-nitrobenzoic acid) (DTNB) recycling assay, according to procedures described previously [40]. Briefly, lung tissue was homogenized in buffer containing 137 mM Tris-HCl, pH 8.0, 130 mM NaCl, and 1% NP-40. After determination of protein concentration, protein content was equalized and 500 μ g of protein was precipitated with acetone. Following centrifugation, the pellet was resuspended in buffer containing 0.1% Triton X-100 and 0.6% sulfasalicylic acid and freeze-thawed twice. Protein-associated glutathione was released with sodium borohydride, and total GSH levels were determined.

The sodium borohydride-sensitive fraction of GSH released from proteins was calculated and expressed as nanomoles GSH per milligram of protein.

mRNA analyses

Total RNA was isolated from cells and purified using the RNeasy kit (Qiagen, Valencia, CA) and reverse transcribed to cDNA for taqman gene analysis using SYBR green (Bio-Rad, Hercules, CA). Expression values were normalized to the housekeeping gene cyclophilin. Primers used were: KC; forward GCTGGATTACCTCAAGAA, reverse TGGGGACACCTTTTAGCATC, IL-6; forward CTGATGCTGGTGACAACCAC, reverse CAGAATTGCCATTGCACAAC, CCL20; forward AAGACAGATGGCCGATGAAG, reverse AGCCCTTTTCACCCAGTTCT, cyclophilin; forward TTCCTCCTTTCACAGAATTATTCCA, reverse CCAGTGCCATTATGG.

Enzyme-Linked Immunosorbent Assays (ELISA)

Cells were stimulated, and medium was assayed for KC, CCL-20 and IL-6 (R & D Systems, Minneapolis, MN) according to manufacturer's instructions.

Statistical analyses

Statistical analyses were performed using Graphpad Prism software (GraphPad, San Diego, CA) using one- or two-way ANOVA with Bonferroni correction for multiple comparisons. All experiments were conducted at least three times and data are presented as mean values plus the standard error of the mean.

RESULTS

IL-17A activates classical and alternative NF- κ B pathways

Recent work in our laboratory has provided insights into the interaction of the classical and alternative NF- κ B pathways in controlling inflammatory responses in airway epithelial cells [17]. IL-17A-induced classical NF- κ B activation in airway epithelial cells has been well documented [7, 10, 41, 42], but only recently was it demonstrated that IL-17A can also activate the alternative NF- κ B pathway [17]. Results in Figure 1A and B demonstrate activation of the classical NF- κ B pathway, as indicated by phosphorylated forms of RelA and I κ B α (Fig. 1A) as well as I κ B α degradation and increases in nuclear RelA content (Fig. 1B). Expression of I κ B α and A20, negative regulators of NF- κ B which are induced upon activation of NF- κ B, was enhanced following IL-17A exposure (Fig. 1C). In response to IL-17A, slight increases in nuclear RelB content occurred over time, indicative of alternative NF- κ B activation (Fig. 1B). These data demonstrate that IL-17A activates both classical and alternative NF- κ B in lung epithelial cells.

Cysteine oxidation and protein S-glutathionylation are increased following IL-17A exposure

Given that modifications of cysteines play an important role in protein regulation, we next investigated whether IL-17A stimulation leads to perturbations in the cellular redox environment through determination of cysteine redox modifications. We utilized dimedone

or a dimedone-based derivative (DCP-Bio1) to measure the extent of sulfenic acid formation following stimulation with IL-17A. Cells were either lysed in the presence of dimedone to trap sulfenic acid moieties or pre-incubated with DCP-Bio1 and then stimulated with IL-17A to visualize oxidation of cysteines via immunofluorescence. Results in Figure 2 demonstrate a rapid increase in sulfenic acid formation of numerous proteins based upon dimedone labeling and analysis via western blot (Fig. 2A) and immunofluorescence analysis (Fig. 2B). In cells stimulated with IL-17A, sulfenic acid residues were detectable predominantly in the periphery of the cell, suggestive of compartmentalization of sulfhydryl oxidations.

Because sulfenic acid formation is believed to be a gateway for further cysteine modifications, we next asked if S-glutathionylation (PSSG) was also increased in response to stimulation of cells with IL-17A. Glutathione-conjugated proteins from cell lysates were assessed by non-reducing SDS-PAGE analysis. Incubation with an anti-GSH antibody revealed a time-dependent increase in overall glutathione-adducted proteins in response to IL-17A (Fig. 3A). Overall PSSG content measured following release of GSH from precipitated proteins also showed trends towards increases in PSSG in epithelial cells stimulated with IL-17A at early time points (Fig. 3B). Extensive data exists to support a functional role for PSSG of NF- κ B components in controlling the activity of this pathway [22–25], including work in our laboratory demonstrating the importance of IKK β S-glutathionylation (IKK β -SSG) [23, 43]. Therefore, we next examined the glutathionylation status of specific proteins in the NF- κ B pathway after IL-17A stimulation. Although we were unable to consistently detect IKK β -SSG in response to IL-17A (data not shown), we did detect increases in RelA-SSG and IKK α -SSG (Fig. 3C), which occurred in a biphasic manner. The bi-phasic changes in RelA-SSG and IKK α -SSG could reflect alterations in expression of Grx1. However, assessment of Grx1 protein content revealed no differences in expression (Fig. 3C) suggesting that time-dependent alterations of PSSG in response to IL-17A are not a mere reflection of differences in Grx1 content. Overall, these data indicate that IL-17A stimulation leads to a rapid increase in sulfenic acid and PSSG in lung epithelial cells, and that RelA and IKK α are targets for PSSG.

Glutaredoxin 1 plays an important role in controlling epithelial cell responsiveness to IL-17A

Under physiological conditions, the oxidoreductase Grx1 can catalyze the deglutathionylation of proteins, restoring their native structure and function, and IKK β is known to be a substrate for this enzyme [23, 43]. Therefore, we hypothesized that Grx1 plays a role in IL-17A signaling by deglutathionylating NF- κ B proteins, namely RelA and IKK α . First, we evaluated the effects of siRNA-mediated ablation of Grx1 on NF- κ B activation by assessing nuclear content of RelA and RelB in response to IL-17A. RelA nuclear content was increased acutely after ablation of Grx1, compared to siRNA control cells exposed to IL-17A (Fig. 4A). In contrast, while nuclear content of RelB increased in response to IL-17A in siRNA control transfected cells, nuclear content of RelB in cells subjected to Grx1 ablation did not appear to change (Fig. 4A), indicating differential regulation of both NF- κ B pathways by Grx1. The kinetics of nuclear RelA and RelB accumulation in response to IL-17A were considerably different as compared to Fig. 1B,

likely the result of transfection of the cells. Interestingly, ablation of Grx1 also led to a baseline increase in mRNA expression of A20, which was further increased in response to IL-17A, compared to siRNA controls (Fig. 4B), indicating that certain facets of the NF- κ B signaling pathway may be intrinsically dampened in the absence of Grx1.

We next analyzed the impact of siRNA-mediated ablation of Grx1 on the expression of pro-inflammatory cytokines induced by IL-17A. IL-17A-induced mRNA expression of KC and CCL20 was substantially decreased following ablation of Grx1 (Fig. 5A), and similar decreases in content of these cytokines were apparent subsequent to Grx1 ablation (Fig. 5B), pointing to a requirement of Grx1 in augmenting the production of these cytokines. Intriguingly, the ability of IL-17A to increase expression of IL-6 mRNA and protein was markedly increased in C10s following siRNA mediated ablation of Grx1, in contrast to siRNA control transfected cells which demonstrated only small increases in IL-6 upon stimulation with IL-17A (Fig. 5A & B). Similar patterns of expression of KC, CCL20 and IL-6 were seen in primary mouse tracheal epithelial cells (MTECs) lacking the glutaredoxin-1 gene (*Grx1*) following stimulation with IL-17A (Fig. 5C), further solidifying the importance of Grx1 in controlling epithelial cell responses to IL-17A. These data suggest an important functional role for Grx1 in controlling IL-17A-induced proinflammatory signaling. These findings also illuminate the unique impact of Grx1 in regulating pro-inflammatory responses to IL-17A in lung epithelial cells, which are highly dependent on the target gene.

S-glutathionylation of RelA and IKK α is controlled by glutaredoxin 1

In order to further understand the mechanism by which Grx1 controls IL-17A-induced pro-inflammatory responses, we first evaluated the overall patterns of PSSG following ablation of Grx1. Cells were incubated with a biotinylated form of GSH (Bio-GEE) 1h prior to stimulation with IL-17A, and harvested at various time points. Results in Fig. 6A demonstrate a time-dependent increase in numerous Bio-GEE-conjugated proteins in response to IL-17A, some of which are further increased following Grx1 ablation, even in unstimulated control cells. We next determined whether RelA-SSG and IKK α -SSG are potential substrates for Grx1-mediated deglutathionylation, which may act to control IL-17A-induced pro-inflammatory signaling. Immunoprecipitation of S-glutathionylated proteins revealed a biphasic pattern of both RelA-SSG and IKK α -SSG in siRNA control transfected cells stimulated with IL-17A (Fig. 6B). The kinetics of increases in RelA-SSG and IKK α -SSG are somewhat different to earlier observations in Fig. 3C, likely due to the transfection protocol used. Nonetheless, IL-17A-mediated increases in RelA-SSG and IKK α -SSG were more pronounced subsequent to Grx1 ablation (Fig. 6B). Taken together, these data suggest a direct role for Grx1 in controlling the extent of both RelA-SSG and IKK α -SSG.

Loss of IKK α results in dampened IL-17A responses in airway epithelial cells

The relationship between IKK α and IL-17A has been established in various cell types, but the role of epithelial IKK α in controlling IL-17A responses is unknown. Therefore, we investigated the functional link between IKK α and IL-17A signaling in airway epithelial cells. Ablation of IKK α using siRNA resulted in small reductions in nuclear content of both

RelA and RelB in response to IL-17A stimulation (Fig. 7A), in conjunction with diminished expression of KC and CCL20 (Fig. 7B). Ablation of IKK α using siRNA did not alter IL-6 expression in response to IL-17A (data not shown). These data collectively indicate a role for both Grx1 and IKK α in controlling airway epithelial cell responses to IL-17A stimulation, and that IKK α -SSG correlates with the magnitude and pattern of IL-17A-induced pro-inflammatory responses.

DISCUSSION

The Interleukin-17 (IL-17) family of cytokines has garnered much recent attention. For example, a protective role of IL-17A has been demonstrated in host defense against bacterial infections [44–47]. Conversely, a pathogenic role of IL-17A has emerged in settings of autoimmune diseases, neutrophilic inflammation, cancer, and fibrotic tissue remodeling [48–51]. Therefore, an understanding of the molecular mechanisms whereby IL-17 family members elicit biological responses is of critical importance towards the development of therapeutics aimed at targeting this family of cytokines [6, 52, 53]. IL-17A is the most well characterized member, and proximal signaling events induced at the IL-17RA/C complex have been well characterized [6]. Thiol/redox perturbations are emerging as cardinal regulators of many biological pathways, yet it remains unclear whether IL-17A elicits any redox changes and whether these events affect the cellular responses to IL-17A. In the present study we demonstrate that exposure to IL-17A induced rapid protein thiol oxidations, evidenced by increases in sulfenic acid formation and S-glutathionylated proteins. We also demonstrated that RelA and IKK α are both targets for IL-17A-induced PSSG, and that IKK α contributes to pro-inflammatory signaling in lung epithelial cells. Furthermore, Glutaredoxin-1 (Grx1) controlled both RelA-SSG and IKK α -SSG, and regulated the profile and magnitude of pro-inflammatory responses. Overall these findings illuminate a new dimension of IL-17A-induced biological responses that involve a thiol/redox control mechanism.

Studies herein have focused on the response of airway epithelial cells to IL-17A. The airway epithelium is the first line of defense against inhaled insults and stimuli, and proper functioning of airway epithelial cells is necessary to maintain proper homeostasis in the lung [54, 55]. The airway epithelium also plays a vital role in responding to both external and internal signals to control the extent of inflammatory responses. Thus, airway epithelial cells are a major target of IL-17A, and have previously been shown to produce various host defense and pro-inflammatory mediators upon IL-17A exposure [7, 8, 10, 11]. IKK α plays a role in both classical and alternative NF- κ B pathways, but also has NF- κ B independent functions [56]. IKK α was originally demonstrated to play a role in adaptive immune responses and lymphoid organ development [57]. More recently, a functional role for IKK α in regulating polarization of IL-17-producing T helper 17 lymphocytes has been demonstrated [31] and IKK α has also been shown to regulate the responsiveness of synoviocytes, kidney cells and astrocytes to IL-17A [32]. The role of IKK α in regulating the responsiveness of epithelial cells to IL-17A had not been previously demonstrated. Results from the present study not only show the functional importance of IKK α in regulating the responsiveness of epithelial cells to IL-17A, but also show that the outcome of IL-17A signaling in epithelium is controlled by the Grx1/PSSG axis, in association with IKK α -SSG.

These observations have broad implications not only for IL-17A-induced pro-inflammatory responses, but also for other stimuli that signal through IKK α , as these may also be regulated by Grx1-mediated PSSG. However, additional studies are needed to map the reactive cysteine within IKK α that is the target for PSSG induced by IL-17A, and to determine its functional importance.

The functional impact of RelA-SSG had been established previously, as studies have shown both inhibition of RelA nuclear entry [22, 58] and DNA binding [59] following RelA-SSG. Paradoxically, we demonstrate here that IL-17A-induced RelA-SSG corresponded to increases in nuclear RelA, which were further enhanced following ablation of Grx1. These results indicate that, in response to IL-17A, nuclear translocation of RelA may not be affected by its S-glutathionylation, but that RelA-SSG could interfere with DNA binding, as has been shown previously following oxidant-triggered PSSG *in vitro* [59]. Further investigation is needed to determine the functional role of RelA-SSG in response to IL-17A, which was beyond the scope of the current study.

It is plausible that other components of the IL-17 pathway, in addition to RelA and IKK α , may be targets for thiol/redox control. In this regard, TRAF6 was recently described as a target for Grx1-mediated deglutathionylation in the context of TLR/IL1R signaling [26]. Since the IL-17R complex also utilizes this adaptor molecule for the activation of NF- κ B [6], it is plausible that TRAF6 is also a target for PSSG in cells stimulated with IL-17A. A recent study demonstrated the importance of A20 in negative feedback regulation of IL-17A-induced cellular responses [60]. A20 is one of several de-ubiquitinases that has been shown to be a target for reversible oxidations which regulate its activity [61]. The active site cysteine of A20 in particular was demonstrated as a target of hydrogen peroxide-induced oxidation, resulting in inhibition of its deubiquitinase activity [61]. It is plausible that PSSG also inhibits de-ubiquitinase activity of A20, thereby regulating the responsiveness of epithelial cells to IL-17A, scenarios that will require further investigation. It is worthy to mention that Grx1 status regulates mRNA expression of A20, and that absence of Grx1 led to a two fold increase of A20 mRNA. Despite this apparent link between A20 and Grx1, the specific mechanisms whereby Grx1 regulates A20 remain to be further elucidated.

The opposing outcome of IL-17A-induced KC and CCL20 expression compared to IL-6 expression following ablation of Grx1 is intriguing. While ablation of Grx1 attenuated IL-17A-induced expression of KC and CCL20, ablation of Grx1 augmented the ability of IL-17A to induce IL-6, and similar effects were observed in two distinct epithelial cell cultures. These results clearly demonstrate that ablation of Grx1 does not dampen the overall pro-inflammatory response, but rather that ablation of Grx1 modifies the nature of the pro-inflammatory response of epithelial cells to IL-17A. It remains unclear whether the altered cellular responses to IL-17A following ablation of Grx1 are indeed due to altered function of NF- κ B, and whether this involves RelA/p50 or RelB/p52 heterodimers, the latter of which are known to bind to a broader spectrum of kappaB sites [62]. Following ablation of Grx1, the overall nuclear content of RelA was acutely increased, whereas nuclear RelB content tended to decrease in response to IL-17A, as compared to respective siRNA controls. Thus, an altered balance of RelA/p50 or RelB/p52 dimers could account for the observed changes of pro-inflammatory responses to IL-17A. In this regard, prior studies have indicated that

active RelB dimers can repress transcription of *IL1B* and *IL12B* genes [63], and that in association with decreased RelB, IL-6 levels increased [64]. C-Rel is important in the transcriptional regulation of *IL6* [65], which raises the possibility that Grx1 may alter *IL6* gene expression via c-Rel. Alternatively, it is possible that other transcription factors modulate pro-inflammatory gene expression following modulation of Grx1 which could account for the differential responses of IL-6 expression as compared to KC and CCL20. Future studies mapping transcription factor occupancy at the promoters of these cytokine genes will be required to fully understand these scenarios.

Results from the present study demonstrate increases in sulfenic acid formation and PSSG in response to IL-17A. Numerous studies have previously demonstrated these oxidative events under conditions of overt stress, or in response to direct exposure to an oxidant. The subtle patterns of oxidations demonstrated herein are reflective of a biological stimulus that elicits a cellular response without creating overt cellular stress, nor inducing cell death. The source of oxidants following IL-17A stimulation remains unknown. Increased sulfenic acid formation was observed as early as five minutes following IL-17A stimulation, and appeared to be localized primarily near the cell periphery (Fig. 2B). In the context of TLR/IL-1R signaling, previous studies have observed an oxidant-dependent recruitment of TRAF6 to the receptor complex, via non-phagocytic NADPH oxidase 2 (Nox2), which is necessary for signal propagation [66]. The pattern of sulfenic acid formation observed herein could potentially reflect a similar NADPH oxidase-induced signaling event that is localized to the IL-17R complex. Activation of Nox2 following engagement of the IL17R may potentially be responsible for the production of oxidants, which in turn lead to sulfenic-acid containing proteins. Indeed, further analyses will be required to fully determine the source of oxidants and the specific subcellular localization of these oxidative events.

The biochemical links between sulfenic acid intermediates and PSSG in biological settings remain poorly understood. Sulfenic acid intermediates can be unstable and give rise to further oxidations [67]. PSSG of sulfenic acid intermediates is believed to be important in the protection of cysteines against overoxidation [68]; therefore, it is plausible that sulfenic acid intermediates observed in response to IL-17A are the precursor to PSSG. The mechanisms that govern PSSG in response to IL-17A also remain unknown. PSSG can occur spontaneously, notably under conditions of oxidative stress when an abundance of oxidized glutathione (GSSG) is present [19]. Enzymatic catalysis of PSSG by glutathione S-transferase Pi (GSTP) has been demonstrated under conditions of oxidative stress [69], or in response to the death inducing ligand FasL [70]. It remains unknown at this time whether GSTP, a protein that is highly expressed in lung epithelial cells [71], plays a role in the catalysis of IKK α -SSG in response to IL-17A.

In summary, results from the present study demonstrate that the pro-inflammatory response elicited by IL-17A is controlled by the S-glutathionylation/Grx1 redox axis in association with RelA-SSG and IKK α -SSG. Therefore, avenues that target the protein thiol redox status can be exploited to harness the cellular response to IL-17A and have the potential to impact host defense and diseases associated with aberrant IL-17 signaling.

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Highlights

- Thiol redox changes in response to IL-17A are unknown to date
- IL-17A increases cysteine oxidation, S-glutathionylation, RelA-SSG and IKK α -SSG
- IL-17A-induced RelA-SSG and IKK α -SSG is controlled by glutaredoxin-1 (Grx1)
- IL-17A-induced pro-inflammatory gene expression is controlled by Grx1
- Thiol redox-based regulatory mechanisms may control IL-17A- induced inflammation

Figure 1. Activation of classical and alternative NF- κ B pathways in lung epithelial cells in response to IL-17A

(A) Mouse alveolar type II cells (C10) were stimulated with IL-17A (100ng/ml) and NF- κ B was assessed in whole cell lysates by measuring phosphorylated forms of RelA (pRelA) and I κ B α (pI κ B α). β -actin; loading control. Right panels: densitometric evaluation of pRelA and pI κ B α . Data reflect arbitrary units, following normalization to loading control, β -actin. (B) Assessment of nuclear RelA and RelB content and degradation of cytosolic I κ B α following stimulation with IL-17A (50ng/ml) in C10 cells. β -actin; loading control for cytosolic lysates, (labeled as C); Histone H3 (H3) was used as a loading control for nuclear lysates, (labeled as N). Right and bottom panels: densitometric evaluation of nuclear RelA, nuclear RelB and I κ B α . Data reflect arbitrary units, following normalization to respective loading controls. (C) Evaluation of mRNA expression of NF- κ B-dependent negative regulators A20 and I κ B α via real-time PCR in C10 cells stimulated with IL-17A (50ng/ml). Results were normalized to the housekeeping gene cyclophilin and are presented as relative expression. *p < 0.05 (ANOVA), compared to untreated controls (Sh).

Figure 2. IL-17A increases cysteine oxidation in lung epithelial cells

(A) C10 cells were stimulated with IL-17A (50ng/ml) for indicated times and subsequently harvested in the presence of dimedone (1mM) to trap the sulfenic acid form (SOH) of cysteines. Samples were analyzed via reducing SDS-PAGE, and overall sulfenic acid formation was determined using an anti-dimedone antibody (1:1000). -Dim, minus dimedone control. (B) Assessment of SOH formation (red) via immunofluorescence in C10 cells pre-incubated with DCP-Bio1 (1mM) for 1h and stimulated with 100ng/ml IL-17A for indicated times. An anti-biotin Alexafluor-647 secondary antibody was used to visualize DCP-Bio1- bound proteins. Nuclei are counterstained with DAPI (blue). No DCP-Bio1; omission of DCP-Bio1 label.

Figure 3. Increases in protein S-glutathionylation in C10 cells stimulated with IL-17A
 (A) C10 cells were stimulated with 50ng/ml IL-17A, and whole cell lysates were harvested at indicated times. SDS-PAGE analysis was performed under non-reducing conditions and GSH-conjugated proteins were assessed using an anti-GSH antibody (1:1000). A separate sample was incubated with (+DTT) to decompose S-glutathionylated proteins. (B) Following stimulation with 50ng/ml IL-17A, C10 cells were harvested at indicated times and overall S-glutathionylation was evaluated using the DTNB recycling assay. (C) C10 cells were stimulated with 50ng/ml IL-17A and harvested as indicated. S-glutathionylated proteins (200μg protein/sample) were immunoprecipitated (IP) using an anti-GSH antibody (2μg/ml) overnight at 4o C. Following IP, samples were subjected to SDS-PAGE and Western Blot analysis using RelA and IKKα antibodies. WCL: Whole cell lysates to confirm equal content of RelA and IKKα, or Grx1. +DTT, control sample incubated with DTT to decompose S-glutathionylated proteins. β-actin: loading control.

Bottom panel: densitometric evaluation of IKK α -SSG and RelA-SSG. Data reflect arbitrary units, following normalization to total IKK α or RelA.

Figure 4. Ablation of Grx1 alters NF-κB activation and mRNA expression of A20

(A) Assessment of NF-κB activation following siRNA-mediated ablation of Grx1 or control siRNA and stimulation with IL-17A (50ng/ml). Nuclear (N) and cytosolic (C) lysates were harvested from C10 cells and nuclear content of RelA and RelB (top panels) was assessed. Histone H3 (H3); loading control. Bottom panels: Evaluation of Grx1 in cytosolic lysates to confirm siRNA-mediated ablation. β-actin; loading control. Graphs represent densitometric evaluation of nuclear RelA and RelB. Data reflect arbitrary units, following normalization to the loading Histone H3. (B) C10 cells were subjected to siRNA-mediated ablation of Grx1 or control siRNA, and stimulated with IL-17A for indicated times, and A20 mRNA expression was evaluated. Results were normalized to the housekeeping gene cyclophilin and are presented as relative expression.

Figure 5. Grx1 siRNA or genetic ablation of Glrx1 alters pro-inflammatory responses in lung epithelial cells

C10 cells stimulated with IL-17A (50ng/ml) for indicated times were analyzed for mRNA expression (A) and protein levels (B) of KC, CCL20 and IL-6 following siRNA-mediated ablation of Grx1 or control siRNA. (C) Expression of KC, CCL20 and IL-6 in WT or *Glrx1*^{-/-} primary mouse tracheal epithelial cells (MTECs) following stimulation with IL-17A (20ng/ml). Expression values were normalized to the housekeeping gene cyclophilin, and data are presented as relative expression. * p 0.05 (ANOVA) compared to untreated controls (Sh); † p 0.05 (ANOVA) compared to scr siRNA groups at the same time point.

Figure 6. Grx1 ablation promotes S-glutathionylation of RelA and IKKα

C10 cells were subjected to siRNA-mediated ablation of Grx1 or control siRNA. (A) Cells were pre-incubated with Bio-GEE (250μM) for 1h prior to stimulation with IL-17A (50ng/ml), and subsequently harvested at indicated times. SDS-PAGE performed under non-reducing conditions, followed by Western Blot analysis of Bio-GEE-conjugated proteins assessed via streptavidin-HRP. -BG; control wherein the Bio-GEE label was omitted. ns; non-specific bands. (B and C) Following siRNA-mediated ablation of Grx1, C10 cells were stimulated with IL-17A (50ng/ml) and harvested at indicated times. S-glutathionylated proteins were immunoprecipitated with GSH antibody (2μg/ml) and resolved via SDS-

PAGE and western blotting for IKK α (B) or RelA (C). Bottom panel: western blot showing confirmation of Grx1 ablation. Right panels: densitometric evaluation of IKK α -SSG and RelA-SSG. Data reflect arbitrary units, following normalization to total IKK α or RelA.

Figure 7. SiRNA-mediated ablation of IKKα modulates expression of pro-inflammatory mediators in lung epithelial cells

(A) Assessment of nuclear RelA and RelB following siRNA-mediated ablation of IKKα or control siRNA and stimulation with IL-17A (50ng/ml). Nuclear (N) and cytosolic (C) lysates were harvested from C10 cells and nuclear content of RelA and RelB was assessed. Histone H3 (H3) was used as a loading control. Bottom panels: Assessment of IKKα in cytosolic lysates to confirm siRNA-mediated ablation. β-actin; loading control. Graphs represent densitometric evaluation of nuclear RelA and RelB. Data reflect arbitrary units, following normalization to the loading Histone H3. (B) Assessment of KC and CCL20 mRNA expression following siRNA-mediated ablation of IKKα and stimulation with IL-17A (50ng/ml) at indicated times. Values were normalized to the housekeeping gene cyclophilin, and data presented and relative expression. * p 0.05 (ANOVA) compared to untreated controls (Sh); † p 0.05 (ANOVA) compared to scr siRNA groups at the same time point.