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Thioredoxin family proteins in a mouse model for allergic airway disease

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Summary

Thioredoxin (Trx) family proteins are omnipresent oxidoreductases with major functions in cellular redox signalling, catalysing post-translational redox modifications of cysteinyl thiol groups and regulating intracellular levels of the second messenger molecule hydrogen peroxide. Thioredoxins, glutaredoxins (Grxs) and peroxiredoxins (Prxs) are known to influence essential cellular pathways in inflammation, proliferation, and apoptosis which are determining factors in inflammatory diseases, such as asthma. To date primarily intracellular functions of Trx family proteins in health and disease have been described, however extracellular functions in redox signalling still need further investigation. Therefore, we aimed at examining distribution and functions of Trxs, Grxs and Prxs in immune response with a focus on extracellular distribution.

Allergic airway inflammation is characterized by chronic airway remodelling, bronchial obstruction and airway hyper-responsiveness. The accumulation of reactive oxygen species (ROS) in the epithelial lining fluid of the lung and the subsequent alterations of the redox state in the bronchial epithelial cells has been acknowledged in recent years. Several studies have suggested a beneficial effect for Trx1 as well as Grx1 by preventing a type-2 helper T-cell (Th2)-prone immune modulation followed by reduced airway remodelling. In this study, we investigated the expression levels of Trx family proteins in lung tissue and bronchoalveolar lavage (BAL) fluid in a mouse model of ovalbumin (OVA)-induced allergic airway inflammation. We discovered an increase of Grx2 and Prx4 in intracellular samples from mouse lungs upon airway inflammation. Exclusively under OVA-induced airway inflammation the presence of second isoform of Grx2, cytosolic Grx2c, was detected. Intraperitoneal injection of mice with recombinant Grx2WT during OVA-challenge had an anti-inflammatory effect, resulting in decreased asthmatic phenotype and significantly reduced total BAL cell count as well as eosinophilia. Administration of recombinant Grx2C40S mutant, lacking the capacity to catalyse the dithiol switch, did not display the same anti-inflammatory effect. An additional His-tag staining displayed an uptake of recombinant Grx2 in the epithelial cells and macrophages of the inflamed lung. Staining of lung sections for HIF1-α and pro-caspase3 increased after onset of allergic airway inflammation and decreased in mice treated with Grx2. The levels of Trx1, Grx1, Prx2 and Prx4 were increased in the extracellular fractions upon lung inflammation and Prx4 could only be detected in allergic airway inflammation, not in healthy mice. *In vitro* experiments stimulating macrophages elicited form Balb/c mice attempted to shed a light on functions of Trxs and Grxs in immune response. Grx2 and Trx1 have been identified

as potential activators of macrophages, inducing secretion of RANTES, IL-6, IL-10, and TNF-α, whereas cytokine levels of IL-4 and INF-γ have not been altered. Upon combined administration of redoxins with LPS/IFN-γ, mimicking acute inflammation, Trx1 reduced cytokine levels of TNF-α and INF-γ compared to LPS/IFN-γ alone. Treatment with Trx1/LPS/IFN-γ induced IL-6 and RANTES production to a level seen after LPS/IFN-γ stimulation only.

This thesis illuminates protein changes of Trxs, Grxs and Prxs in a murine model for allergic airway inflammation, with emphasis on extracellular distribution and functions. We indicate that alterations in protein levels are selectively regulated and contribute to a fine-tuned network of partners in redox signalling, providing potential for possible therapeutic approaches.

Zusammenfassung

Thioredoxine (Trxs) bilden eine Familie ubiquitärer Oxidoreduktasen, welche durch posttranslationale Redox-Modifikationen von Cysteinyl-Thiolgruppen sowie die Regulation der intrazellulären Wasserstoffperoxidgehaltes die zelluläre Redoxantwort steuern. Es ist bekannt, dass Thioredoxine, Glutaredoxine (Grxs) und Peroxiredoxine (Prxs) wesentliche Signalwege von Inflammation, Proliferation und Apoptose beeinflussen und somit in vielen Pathologien, insbesondere bei entzündlichen Erkrankungen wie dem allergischen Asthma, eine entscheidende Rolle spielen. Derzeit sind vorwiegend die intrazellulären Funktionen der Proteine der Trx-Familie in Gesundheit und Krankheit umfassend untersucht, doch die extrazellulären Funktionen bei der Redox-Signalübertragung bleiben bis zum heutigen Tage weitestgehend im Dunkeln. In dieser Arbeit haben wir uns daher zum Ziel gesetzt, Verteilung und Funktion von Proteinen der Trx-Familie in der Regulation der Immunantwort zu untersuchen, wobei der Schwerpunkt auf dem extrazellulären Vorkommen und den damit verbundenen Eigenschaften liegt.

Allergische Atemwegsentzündungen sind durch bronchiale Obstruktion und chronischen Umbau sowie Hyperreagibilität der Atemwege gekennzeichnet. Die Anhäufung reaktiver Sauerstoffspezies in der bronchialen Flüssigkeit der Lunge und die sich daraus ergebenden Veränderungen des Redoxzustands in den bronchialen Epithelzellen sind in den letzten Jahren in den Fokus der Asthmaforschung gerückt. Mehrere Studien beschrieben eine positive Wirkung von Trx1 und Grx1 in Atemwegsinfektionen, so würde eine Th2-typische Immunmodulation reduziert und verringere in der Folge den Umbau der Atemwegsstruktur – eine zentrale Pathologie des Asthmas.

In dieser Studie untersuchten wir die Expressionsniveaus von Proteinen der Trx-Familie in Lungengewebe und bronchoalveolärer Lavageflüssigkeit in einem Mausmodell der Ovalbumin (OVA) induzierten allergischen Atemwegsentzündung. Wir konnten eine Zunahme von Grx2 und Prx4 in intrazellulären Proben aus der Mäuselunge nach einsetzen der induzierten Atemwegsinfektion zeigen. Ausschließlich unter OVA-induzierter Inflammation wurde in diesen Proben eine zweite Isoform von Grx2, zytosolisches Grx2c, nachgewiesen. Die Behandlung von Mäusen mit intraperitoneal appliziertem, rekombinantem Grx2 parallel zur Induktion der Entzündung mit OVA, hatte eine entzündungshemmende Wirkung, die zu einer Verringerung des asthmatischen Phänotyps in der Immunhistochemie und einer signifikanten Reduktion der Gesamtzahl der Entzündungszellen, besonders der eosinophilen Granulozyten führte. Die Verabreichung der rekombinanten Grx2C40S-Mutante, der die Fähigkeit zur

Katalyse des Dithiol-Reaktionsmechanismus fehlt, hatte nicht die gleiche entzündungshemmende Wirkung. Eine zusätzlich durchgeführte His-Tag-Färbung zeigte eine Aufnahme von rekombinantem Grx2 in die Epithelzellen und Makrophagen der entzündeten Lunge. Die Färbung von Lungenabschnitten für HIF1-α und Pro-Caspase3 nahm nach Beginn der allergischen Atemwegsentzündung zu und ging bei den mit dem wildtyp Grx2 behandelten Mäusen deutlich zurück.

In den extrazellulären Fraktionen war die Konzentration von Trx1, Grx1, Prx2 und Prx4 unter Entzündungsbedingungen erhöht, zudem konnte Prx4 in dieser Studie erstmals nur in der Entzündung, nicht aber bei gesunden Mäusen nachgewiesen werden. In-vitro-Experimente, bei denen Makrophagen aus Balb/c-Mäusen stimuliert wurden, sollten Aufschluss über die Funktionen von Trxs und Grxs in der Immunantwort geben. Grx2 und Trx1 wurden als potenzielle Stimulatoren von Makrophagen identifiziert, die die Sekretion von RANTES, IL-6, IL-10 und TNF-α induzieren, während die Zytokinspiegel von IL-4 und INF-γ nicht verändert wurden. Bei kombinierter Verabreichung von Redoxinen mit LPS/IFN-γ, die einen Entzündungszustand nachahmt, wurde gezeigt, dass Trx1 die Zytokinspiegel von TNF-α und INF-γ im Vergleich zu LPS/IFN-γ allein senkt. Die Behandlung mit Trx1/LPS/IFN-γ induzierte die Produktion von IL-6 und RANTES auf ein Niveau vergleichbar mit der alleinigen Stimulation durch LPS/IFN-γ.

Diese Arbeit beleuchtet Proteinveränderungen von Thioredoxinen, Glutaredoxinen und Peroxiredoxinen in einem Mausmodell für allergische Atemwegsentzündungen mit besonderem Schwerpunkt auf der extrazellulären Verteilung und Funktion der Proteine. Wir zeigen, dass die Veränderungen der Proteinspiegel selektiv reguliert werden und zu einem fein abgestimmten Netzwerk von Partnern in der Redox-Signalgebung beitragen und somit Potenzial für mögliche Therapieansätze bieten.

Abbreviations

ASK 1	apoptosis signal-regulating kinase
BAL	bronchoalveolar lavage
caspase	cysteine- dependent aspartate-directed protease
Cys/C	cysteine
CBA	Cytometric Bead Array
COPD	chronic obstuctive pulmonary disease
DAN	2,3-diamino-naphthalene
DTT	dithiothreitol
DTNB	5,5-dithio-bis-(2-nitrobenzoic acid)
E.coli	Escherichia coli
ELISA	enzyme-linked immunosorbent assay
FAD	flavin adenine dinucleotide
FACS	fluorescence-activated cell sorting
GPx	glutathione peroxidases
GR	glutathione reductase
Grx	glutaredoxin
GSH	glutathione
GSNO	S-nitrosylated gluthathione
GSSG	glutathione disulfide
HIF	hypoxia-inducible factor
HIV	human immunodeficient virus
IFN-γ	interferon-γ
IHC	immunohistochemistry
IL	interleukin
IκB	Inhibitor of kappa B
IRP	iron regulatory proteins
JNK	C-Jun N-terminal kinase
LPS	lipopolysaccharide
MAP	mitogen-activated protein
MIF	macrophage inhibitory factor
NDP	nucleoside-diphosphate

NEM	N-ethylmaleimide
NFAT	nuclear factor of activated T cells
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NO	nitric oxide
NOS	nitric oxide synthase
Nrx	nucleoredoxin
OVA	ovalbumin
PBMCs	peripheral blood mononuclear cells
PBS	phosphate buffered saline
PDI	protein disulfide isomerase
PHD	prolyl hydroxylases
Prx	peroxiredoxin
RANTES	Regulated Upon Activation, Normally T-Expressed and Presumably Secreted
RNR	ribonucleotide reductase
RNS	reactive nitrogen species
ROS	reactive oxygen species
SOD	superoxide dismutases
TBP2	thioredoxin binding proteine 2
TCEP	Tris (2-carboxyethyl) phosphine hydrochloride
Th2	type-2 helper T-cell
Th1	type-1 helper T-cell
TLR	toll-like receptor
TNF	tumor necrosis factor
Tpx	thioredoxin peroxidase
TRAIL	TNF-related apoptosis-inducing ligand
TrxR	thioredoxin reductase
Trx	thioredoxin
Txnip	Trx interacting protein;
XO	xanthine oxidase

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Introduction

1.1 The concept of Redox signalling

Redox reactions – *i.e.* the transfer of electrons from a higher energetic level to a lower state of energy reducing oxygen to water – are a cornerstone of almost all known forms of life (Buchanan & Balmer, 2005). Redox reactions, such as the oxidative phosphorylation in the inner mitochondrial membrane, are also closely linked to the generation of reactive oxygen species (ROS). ROS are either radicals such as hydroxyl-, peroxyl radical or superoxide radical anion or non-radical small compounds *e.g.* hydrogen peroxide, singlet oxygen or peroxynitrite (Bae et al., 2011). However, ROS are not only produced during oxidative phosphorylation but are for instance also generated by cells of the innate immune system. Macrophages and neutrophile granulocytes produce superoxide radicals as a defence mechanism against microorganisms (Ischiropoulos et al., 1992). Despite their beneficial effect in the immune system, ROS have always been considered as the main facilitators of oxidative damage of lipids, nucleic acids or proteins and related to a large number of diseases such as neurodegenerative and cardiovascular disorders as well as cancer (Butterfield et al., 2006; Chinta & Andersen, 2008; Lillig & Holmgren, 2007). The common concept depicts ROS as oxidants opposed by a set of antioxidant compounds and enzymes *e.g.* ascorbic acid, carotenes, superoxide dismutase (SOD), and catalase which are in charge of scavenging ROS and preventing oxidative damage. An imbalance between those oxidants and antioxidants in favour of the oxidants has widely been defined as “oxidative stress” - leading to oxidative damage of cellular compounds and disruption of redox control (Sies & Cadenas, 1985). This concept of oxidative stress was maintained over a long period with little regards to the fact that redox reactions also lead to specific and directed redox modifications *e.g.* the oxidation of free thiol groups generating versatile compounds such as disulfides, sulfenic acids, persulfides or nitroso-thiols. The main target for those post-translational modifications are thiol groups of cysteinyl side chains of proteins and cofactors, but also methionyl and selenocysteinyl groups are subject to oxido-reduction. The reactivity of thiol groups thereby depends on the biochemical character of adjacent amino acids, for instance basic amino acids in close proximity to the thiol group will lower its pKa and at physiological pH enhance their affinity towards electrophilic targets.

In recent years increasing evidence has been established that redox modifications of proteins and in particular disulfide bond formation are physiological, specific and reversible

mechanisms of cellular signalling (Ghezzi et al., 2005; Jones, 2006). The concept of redox signalling not only pays attention to the mechanisms of oxidative damage by ROS and their role in several diseases, but also acknowledges that catalysed oxidative modifications of thiol groups have great influence on cellular signalling. Therefore, redox modifications - especially disulfide bond formation - are at the heart of redox signalling. In contrast to the former general opinion that the redox state is regulated by either the reducing or the oxidizing environment, it is now recognized that specific redox modifications can even occur in an overall oxidizing or reducing environment (Ghezzi, 2005). In particular, the common principle of a redox equilibrium between different subcellular compartments became obsolete and there is accumulating evidence that redox modifications are very differentially regulated in nuclei, mitochondria, the secretory pathway, and the extracellular space, pointing to varying effects of generation or depletion of ROS in each subcellular compartment (Go & Jones, 2008).

Central redox couples such as glutathione disulfide/glutathione (GSSG/GSH), cystine/cysteine, and $NADP^+$/NADPH are specifically regulated in distinct compartments within the cell, depending on a set of enzymes of the thioredoxin-fold family of proteins, most prominently thioredoxins (Trxs), glutaredoxins (Grxs) and peroxiredoxins (Prxs) (Hanschmann et al., 2013; Lillig & Holmgren, 2007). Trxs are ubiquitously expressed proteins in almost all organisms, cells and tissues. Today we have a broad knowledge about their cellular, nuclear and mitochondrial distribution, but the extracellular properties of those proteins are only beginning to emerge (Dammeyer & Arnér, 2011; Godoy, Funke, et al., 2011). Since the scope of protein redox modifications may be similar to that of phosphorylation or ubiquitinylation, the question how extracellular thiol/disulfide redox modifications affect physiological and pathological processes becomes increasingly relevant (Albrecht et al., 2011). The understanding of extracellular functions of Trx family proteins in these processes is currently expanding and therefore this thesis focusses on the extracellular functions of Trxs, Grxs and Prxs.

1.2 Thioredoxin family proteins

Thioredoxin – the founding member of an increasing family of versatile proteins – was originally characterized as an electron donor for the ribonucleotide reductase (RNR) of *E. coli* catalysing reactions important for DNA synthesis and repair (Laurent et al., 1964). In 1968, the amino acid sequence of Trx with the characteristic active site motive was determined followed by the identification of Grxs as GSH-dependent electron donors for the same RNR in absence of Trxs in 1976 (Holmgren, 1976). Besides Trxs and Grxs a large variety of proteins share the

common Trx fold but display very different functions, among them the disulfide oxidoreductase DsbA from *E.coli*, protein disulfide isomerases (PDI), glutathione S-transferase, glutathione-peroxidase as well as Prxs. The Trx fold is highly conserved and its most basic formation, represented in the *E.coli* Grx1, consists of a four stranded β-sheet surrounded by three α-helices. Trx family proteins in higher organisms have additional structural elements inserted, for instance human Trxs contain an additional N-terminal β-sheet and α-helix (Martin, 1995). Important features of the Trx fold are the C-X-Y-C active site motif and a cis-proline residue located in front of β-sheet strand three, which both have been found to determine the proteins reduction capacities (Lillig et al., 2008). Trx family proteins contain either one or two active site cysteine residues. The C-X-Y-C active site motif is located on a loop connecting β-sheet strand one and α-helix one and X and Y are usually hydrophobic amino acids, known to play an essential role for the biochemical properties of the protein. A mutational study demonstrated, that altering the active site motif of PDI from Cys-Gly-His-Cys to the Cys-Gly-Pro-Cys motif of Trx, changed the redox potential of the mutant with an increase in reducing activity (Horibe et al., 2004).

Trx family proteins – at least those with a mere negative redox potential – are oxidoreductases and catalyse 1) protein disulfide reduction, 2) protein de-/glutathionylation and protein de-/trans/S-nitrosylation and 3) reduction of hydrogen peroxide. The basis for these enzymatic reactions is the switch between the dithiol (reduced) and the disulfide (oxidized) active site of Trxs and Grxs, the so-called dithiol mechanism. The N-terminal active site Cys of Trxs has a low pKa value which allows it to initiate a nucleophilic attack at a target disulfide. Thus, a covalently bound mixed disulfide is generated which now can be reduced by the C-terminal Cys residue of the active site, leading to a reduced substrate and an oxidized disulfide containing Trx. The active site of the Trx can be reduced by Thioredoxin reductase (TrxR) receiving electrons from NAPDH, reviewed in 1995 by Holmgren (Holmgren, 1995).

In contrast to Trxs, the Grx system is more versatile considering the choice of its substrate. Redox active Grxs also catalyse de-/gluthathionylation via formation of a GSH-mixed disulfide by the monothiol mechanism. For this mechanism only the N-terminal active site Cys residue is required, forming a GSH-mixed disulfide intermediate, which is then subsequently reduced by a second molecule of GSH, releasing the oxidized GSSG and the reduced substrate protein. This reaction mechanism implies that oxidized Grxs are re-reduced differently from Trxs *i.e.* via GSH and glutathione reductase (GR) which also obtains electrons from NADPH. This reaction mechanism was first described by Holmgren in 1979 (Holmgren, 1979b).

Similar to the reaction mechanism of Trxs and Grxs, the reduction of hydrogen peroxide

catalysed by Prxs is a multistep reaction. In the first step the N-terminal Cys residue of the active site is oxidized by H_2O_2 which is reduced partially to water, yielding a sulfenic acid intermediate (Cys-SOH). In a second step the active site Cys residue is typically reduced by a resolving Cys residue outside the active site which forms a disulfide with the Cys-SOH in a nucleophilic replacement reaction yielding water as a product. Depending on the resolving Cys-residue they require for their catalytic activity, they are classified as 2-Cys, atypical 2-Cys and 1-Cys Prxs. In the case of 2-Cys Prxs (Prx1-4) the resolving Cys residue is located in the C-terminal region of the active site. Atypical 2-Cys Prxs (Prx 5) contain a second Cys-residue at the C-terminus of the protein, but not in the active site, whereas 1-Cys Prxs (Prx6) lack a second Cys-residue which is not required for their catalytic function (Rhee & Woo, 2011).

The reduction of protein-GSH-mixed disulfides, of intra- and intermolecular disulfide bonds as well as the reduction of hydrogen peroxide often leads to minimal but important conformational alterations of target proteins. Even the structure of Trxs and Grxs changes upon oxidation or reduction, forming intra- or intermolecular disulfides (Smeets et al., 2005). Since the list of interaction partners for Trx family proteins is still growing, as shown for Grx2 by Schütte and coworkers, those changes provide increasing evidence for the significance of Trxs, Grxs and Prxs in cellular redox signalling (Schütte et al., 2013). Not only folding and stability of target proteins are influenced (Sevier & Kaiser, 2002), but also their activity and localisation (Liu & Min, 2002). Therefore, one central question is which cysteinyl residues are important targets for oxidation or reduction in terms of redox signalling and which ones are simply sensitive to oxidative damage. This underlines the above-mentioned significance of the concept of redox signalling as a specific and organized process in which the proteins of the Trx family play an important role as catalysts of the reactions.

1.2.1 Thioredoxin systems

The mammalian Trx system comprising Trxs, TrxR as well as NADPH acting as electron donor, can be further characterized regarding the compartmentalisation of the system. Trx1 and the associated TrxR1 represent the predominantly cytosolic system whereas Trx2 and TrxR2 are mainly located in mitochondria. The 12 kDa protein Trx1 contains the highly conserved active site sequence Cys-Gly-Pro-Cys which is essential for the reduction of target proteins via the dithiol mechanism (Berndt et al., 2008). Furthermore, the mammalian Trx1 contains three other cysteinyl residues and even though they are located apart from the catalytic site they have an essential influence on the folding and stability of the protein and oxidation of those cysteines

leads to loss of enzymatic activity. In general, the active site and structural cysteines are targeted structures for inhibitors of Trx (Oblong et al., 1994).

Although Trx1 is referred to as a cytosolic protein, it can be translocated to the nucleus or even the extracellular space under certain conditions. Trx1 has been reported to be transferred to the nucleus upon various oxidative stimuli such as UV irradiation, while lacking a classical nuclear localisation sequence (NLS) (K. Hirota et al., 1999). Instead a basic region of the protein containing three lysyl residues was suspected to play a role in nuclear translocation (Spielberger et al., 2008). Nuclear Trx1 influences gene expression, *i.e.* reduction of a redox sensitive Cys-residue of NF-κB promotes DNA-binding by NF-κB and therefore induction of gene transcription (K. Hirota et al., 1999). Trx1 has also been detected in plasma under physiological and disease conditions (Gromer et al., 2004; Kasuno et al., 2003; H. Nakamura et al., 1996) and functions as a chemoattractant for several leukocyte populations (Bertini et al., 1999). Since Trx1 lacks an export signal, secretion of Trx1 was proposed to be independent from the Golgi-mediated secretory pathway (Rubartelli et al., 1992).

Various functions for Trx1 in the cytoplasm have been described. Primarily, it plays a crucial role in antioxidant defence, mainly through interaction with Prxs which reduce hydrogen peroxide in the cytosol and are then re-reduced by Trx1 (Chae et al., 1999). Secondly, cytosolic Trx1 binds to IκB and thus inhibits nuclear translocation of NF-κB and prevents NF-κB-dependent gene expression (K. Hirota et al., 1999). In contrast to NF-κB, activator protein-1 (AP-1) transcription activity is increased in transient Trx1-transfected cells pointing to an involvement of Trx1 in cell growth (Schenk et al., 1994). Third, growth promoting effects of Trx1 have been described for cancer cells as well as normal hepatocytes, monocytes and leukocytes (H. Nakamura et al., 2006; Sahaf & Rosén, 2000). Although the exact mechanism of growth regulation remained elusive, it is very likely that Trx1 acts via activation or regulation of transcription factors for instance NF-κB, AP-1 and p53 (Barone et al., 2012). Finally, Trx1 is involved in the regulation of apoptosis via Apoptosis signal regulating kinase 1 (ASK-1). Activation of ASK-1 leads to subsequent activation of downstream kinases such as c Jun N-terminal kinase (JNK) and p38 MAP (mitogen-activated protein) Kinase, which are key enzymes in pathways regulating proliferation and apoptosis (Saitoh et al., 1998). It is evident that ASK-1 is not only regulated via de-/phosphorylation but is also redox sensitive. Reduced Trx1 binds ASK-1 and by complexing inhibits its activity. Upon oxidizing conditions, the Trx1-ASK-1 complex dissociates and the kinase activates its downstream targets leading to apoptosis. Thus, the activation state of ASK-1 is adapted to the redox state of the cellular environment *e.g.* under oxidative challenges (Song & Lee, 2003).

Trx2 and the TrxR2 represent the mitochondrial Trx system. The 18 kDa Trx2 was first isolated from the rat heart and described as a protein containing the conserved Trx Cys-Gly-Pro-Cys active site sequence but lacking additional structural cysteines. Due to its 60 amino acid N-terminal mitochondrial translocation signal it is mainly localized in mitochondria, and cleavage of a mitochondrial translocation signal gives rise to a mature 12.2 kDa protein (Spyrou et al., 1997). Therefore, Trx2 has to be more resistant to oxidative conditions. Trx2 is proposed to play an important role in the mitochondrial antioxidant system since it inhibits TNFα-induced production of ROS, maintains cytosolic location of NF-κB and therefore blocks NF-κB activation and TNFα-induced apoptosis (Hansen et al., 2006).

TrxRs are 55-60kDa selenoproteins which form a homodimer with every subunit containing a flavin adenine nucleotide (FAD) domain as well as a NADPH-binding domain and an interface domain. The TrxR has two conserved active sites similar to those of the thioredoxins the N-terminal with a Gly-Cys-Sec-Gly sequence and the C-terminal active site with a Cys-Val-Asn-Val-Gly-Cys motif located close to the FAD domain. Due to the high activity and accessibility of selenocysteines, TrxR has a broad substrate specificity, with Trxs still being the main target (Rundlöf et al., 2004). In mammalians, three isoforms of TrxR were detected, a cytosolic (TrxR1), a mitochondrial (TrxR2) and a testis-specific isoform (TrxR3) (Turanov et al., 2006). Both the cytosolic (TrxR1) and the mitochondrial form (TrxR2) of the enzyme are essential for embryonic development since knockout of TrxR1 and 2 leads to early embryonic lethality. Although both isoforms exert similar functions it is noteworthy, that the TrxR2 is also essential for development of cardiac tissues and haematopoiesis, while TrxR1 knock-down is not directly affecting heart development (Jakupoglu et al., 2005).

In addition to Trxs, there is a large number of highly abundant proteins that share the common Trx fold, or at least one Trx fold domain. Among them nucleoredoxin (Nrx) is a versatile protein of 48 kDa present in the nucleus and the cytosol, with a higher expression in skin and testis (Godoy, Funke, et al., 2011). Nrx contains two N-terminal Trx-like domains with the active site motif Cys-Pro-Pro-Cys and a C-terminal domain similar to a PDI-domain lacking redox activity (Funato & Miki, 2007). Nrx was shown to have oxidoreductase activity in the insulin reduction assay and it is involved in regulation of transcription factors such as NF-κB and activator protein 1 (AP-1) (Kurooka et al., 1997). Overexpression of Nrx in HEK cells enhanced TNF-α induced NF-κB transcription, as well as transcription of AP-1 and CREB indicating a role for Nrx in inflammatory response (K. Hirota et al., 2000). Only recently, Prx1 was identified as an interaction partner of Nrx and in cells lacking Nrx, Prx1 was more oxidized compared to control, suggesting a role for Nrx in the transfer of oxidation equivalents from Prx1 to other

proteins as part of a redox signalling process (Urbainsky et al., 2018).

1.2.2 Glutaredoxin system

As already mentioned, the Grx system was originally detected in 1976 as an electron donor for RNR in *E.coli* mutants lacking Trx, but until today it became obvious that Grxs are not a simple Trx backup system, since they are involved in protein de-/glutathionylation, regulation of proliferation and apoptosis as well as coordination of iron sulfur clusters and iron homeostasis (Holmgren, 1976; Lillig & Berndt, 2013).

In the Grx system, electrons are transferred from NADPH to GSH through the glutathione reductase and then on to one of two redox active mammalian Grxs: Grx1 and Grx2 (Liedgens et al., 2020). Two additional Grxs, Grx3 and Grx5, lack oxidoreductase activity (Liedgens et al., 2020; Lillig et al., 2008; Trnka et al., 2020). Grxs are small proteins of around 12 kDa which share the common Trx-fold with Trxs and Prxs. Regarding their active site motifs Grxs can be categorized as class I (Cys-Pro-Tyr-Cys/Ser) and class II (Cys-Gly-Phe-Ser) Grxs, while class II Grxs can be further divided into single and multi-domain Grxs, according to their structure comprising either a single Grx domain or a Trx-like domain followed by several Grx-domains (Herrero & de la Torre-Ruiz, 2007; Liedgens et al., 2020; Trnka et al., 2020). Three dimensional structures of Grxs have been determined which revealed three structural features distinctive for Grxs: in addition to the conserved active site sequence, they show an accessible hydrophobic surface area for interaction with substrates and a GSH-binding site (Bushweller et al., 1992) reviewed in (Fernandes & Holmgren, 2004). Class I Grxs not only act in protein-disulfide reduction, but play an essential role for instance in the post-translational modification system catalysing protein de-/glutathionylation in the cytosol (Mieyal et al., 2008; Trnka et al., 2020). Due to the high affinity of Grx to the GSH moiety, Grx functions are closely linked to signal transduction via de-/glutathionylation of intracellular GSH-mixed disulfide targets (Lillig & Berndt, 2013). Class II Grxs do not show catalytic activity in the Grx-specific activity oxidoreductase assay (HED-assay), monothiol Grxs in *E.coli*, yeast and mammals have been proposed to play a key role in formation of iron-sulfur clusters and iron homeostasis (Mühlenhoff et al., 2010).

1.2.2.1 Glutaredoxins

Until today, four Grxs in mammals have been discovered: the class I Grx1 and Grx2 as well

as the class II Grx3 (also known as PICOT – protein kinase C interacting cousin of Trx) and Grx5. Grx1, the first described protein from the Grx family, is present in the cytosol in a lower concentration (1 μM) than Trx1(10 μM) but has a higher affinity for GSH as a substrate in respect to its low Km value for GSH and therefore reduces GSH-mixed-disulfides with greater efficiency than Trx (Holmgren, 1979a). Grx1 is not only involved in reduction of GSH-mixed disulfides and glutathionylated small compounds via the monothiol reaction mechanism, it also provides electrons for a number of other substrates such as ribonucleotide reductase (Lillig et al., 2008). Although mainly located in the cytosol, Grx1 has also been found to be an electron donor for human plasma glutathione peroxidase by Björnstedt *et al.* and later human Grx1 could be detected in blood plasma from healthy donors by a sensitive ELISA (Björnstedt et al., 1994; Lundberg et al., 2004). Additionally, Grx1 can be translocated to the nucleus and has been detected in the intermembrane space of mitochondria (Pai et al., 2007). Similar to the functions of Trx1, Grx1 is involved in regulation of gene transcription, mainly via glutathionylation of the p-50-subunit of NFκB, the c-Jun subunit of AP-1 and thereby inhibition of DNA-binding and gene transcription (K. Hirota et al., 2000; Pineda-Molina et al., 2001). As previously described for Trx1 (1.2.1) Grx1 is also involved in the regulation of TNFα induced apoptosis via ASK-1. Reduced Grx1 binds to C-terminal and N-terminal regions of ASK-1 and thus inhibits its activity. Upon oxidizing conditions, Grx1 as well as Trx1 release ASK-1 and therefore induce ASK-1 downstream pathways via cJun N-terminal kinase or p38 MAP kinase. In contrast to Trx1 it was found that Grx1 is not only sensitive to oxidation by ROS, but it also releases ASK-1 under conditions of high GSSG levels and thus also regulates apoptosis depending on the intracellular GSH/GSSG ratio (Song & Lee, 2003).

The second class I Grx, Grx2, shows some differences to Grx1, as a 17 kDa protein it is slightly larger and has a higher activity in the Grx-specific HED-assay using GSH as substrate (Johansson et al., 2004). In addition, Grx2 has a unique Cys-Ser-Tyr-Cys active site. Mutational studies showed that the single amino acid change in the Grx2 active site is essential for distinctive functions of the protein: Grx2 can receive electrons from Trx reductase and coordinates an iron-sulfur cluster [2Fe-2S] which bridges two Grx2 molecules forming a dimeric Grx2 complex that is enzymatically inactive (Lillig et al., 2005). For the dimeric holo Grx2 complex a function as redox sensor was suggested, since dissociation of the complex activates enzyme activity (Berndt et al., 2007).

Until today, three different isoforms of Grx2 have been described in human. The Grx2 gene is located on chromosome 1 and encodes five exons, that can be transcribed into three mRNA isoforms by alternative splicing (Lönn et al., 2008). The mitochondrial transcript variant Grx2a

was shown to be ubiquitously expressed and since it contains a N-terminal mitochondrial translocation signal, it plays an important role in regulation of the mitochondrial redox state and in modification of mitochondrial membrane proteins such as complex I via reversible protein de-/glutathionylation. The second isoform Grx2b was first characterised as a nuclear isoform, since nuclear staining of GFP-Grx2b fusion protein was seen in confocal microscopy (Lundberg et al., 2001). Later Lönn and coworkers described a more versatile distribution pattern of Grx2b, suggesting an exchange between nuclear and cytosolic localisation. Noteworthy, Grx2b transcripts could only be detected in testicular cells of healthy tissues and in transformed cell lines, *e.g.* HeLa, Jurkat and A549 cell line. In the following, the group detected a third isoform of Grx2, the Grx2c transcript variant which could also be detected exclusively in the testes and cancer cell lines. Due to the high susceptibility of growing spermatozoa towards oxidative conditions, Grx2c was proposed to have protective functions regarding spermatogenesis. The fact that Grx2c levels, detected by immunohistochemistry increased during spermatogenesis encouraged this hypothesis (Lönn et al., 2008). Gellert and co-workers linked Grx2c to cytoskeletal dynamics and hypothesized a correlation with cancer cell invasiveness, since it regulates the tetrameric collapsin response mediator protein 2 (CRMP2) through a dithiol-disulfide redox switch. Grx2c reduces CRMP2 and facilitates additional protein phosphorylation by various kinases and therefore induces activation of downstream pathways that control axonal outgrowth and guidance. Since Grx2c is also involved in alterations of phosphorylation of proteins like Vimentin and Stathmin, associated with various malignancies such as gastric cancer and renal cell carcinoma, an important role for Grx2c in tumor progression and cancer-specific cell survival is assumed (Gellert et al., 2020). Earlier studies, utilizing short-interfering-RNA (si-RNA) for silencing of Grx2 expression, revealed that Grx2a and Grx2c protected cells from cell death induced by chemotherapeutic agents such as Doxorubicin/Adriamycin. Grx2a reduced the ED_{50} for Doxorubicin almost 60-fold, thus underlining the general importance of Grx2a in the redox control of mitochondria, since a dose limiting factor of Doxorubicin treatment is thiol oxidation of mitochondrial proteins. In contrast to Grx2a, Grx2c was less efficient in preventing cell death induced by Doxorubicin (Enoksson et al., 2005). In conclusion Grx2 is a key protein in mitochondrial redox regulation which also has versatile functions regarding its testis and cancer-specific isoforms (Schütte et al., 2013). In contrast, Grx2 expression in mice is differentially regulated. There are six exons in the mouse Grx2 gene, comprising five transcript variants which then constitute three protein isoforms of Grx2, Grx2a, Grx2c, and Grx2d. Grx2a (17,3kDa) – similar to the human isoform – features a mitochondrial signalling peptide and is located mainly in

mitochondria, but transcribed in various tissues. Intriguingly, in contrast to the human Grx2c, mGrx2c (14kDa) is ubiquitously expressed in various tissues in mice in a cytosolic or nuclear location. The third isoform Grx2d is restricted to testis (Hudemann et al., 2009).

The 37 kDa class II Grx3 was originally detected as a protein kinase C interacting protein having a conserved C-terminal Trx domain. The multi domain Grx3 consists of two N-terminal Grx domains with the active site sequence Cys-Gly-Phe-Ser and the aforementioned C-terminal Thioredoxin domain with a Ala-Pro-Gln-Cys sequence (Haunhorst et al., 2010). The mainly cytosolic protein has also been found to be translocated to the nucleus under oxidative conditions *e.g.* treatment with hydrogen peroxide in human T-cells. As an interaction partner of protein kinase C (PKC), Grx3 can inhibit PKC-mediated activation of C-Jun N-terminal kinase as well as activation of transcription factors NF-κB and AP-1 and may therefore be involved in regulation of gene transcription (Witte et al., 2000). In addition, important functions for Grx3 in cardiomyocytes were described, among them increased contractility of Grx3 overexpressing cardiomyocytes due to enhanced calcium re-uptake by the sarcoplasmic reticulum and attenuation of cardiac hypertrophy via inhibition of calcineurin-NFAT signalling (Jeong et al., 2008). Eventually, Grx3 represents another Trx family member that has been characterized as an iron-sulfur cluster coordinating protein. Grx3 complexes two iron-sulfur clusters in a holo-dimeric conformation bound to the Grx domains of the protein. Haunhorst and co-workers demonstrated essential functions of Grx3 in cellular iron distribution and haemoglobin maturation in zebrafish and human cells (Haunhorst et al., 2013).

The second class II Grx, Grx5 possesses only one Grx domain and shares its active site sequence (Cys-Gly-Phe-Ser) with Grx3, but additionally contains a mitochondrial localisation sequence (MLS). Therefore, the 17 kDa Grx5 is primarily located in mitochondria and consistent with its single domain structure so far, no disulfide reductase activity for the protein has been described. Instead, the monothiol active site enables Grx5 to complex a [2Fe-2S] cluster in a dimeric conformation involving two molecules of GSH (Johansson et al., 2011). Therefore, Grx5 has long been suspected to play a role in iron homoeostasis and thus effect haematopoiesis, this hypothesis was supported by findings of Wingert and co-workers showing that Grx5 deficient zebra fish displayed a severe phenotype caused by hypochromic anaemia (Trnka et al., 2020; Wingert et al., 2005).

1.2.2.2 Glutathione

Glutahione, γ-L-glutamyl-cysteinyl-glycine (GSH), the major intracellular thiol compound

has been first described in the early 20th century by Hopkins (Hopkins, 1921). The ubiquitous tripeptide is synthesized in the cytosol in two ATP-dependent reaction steps. First glutamate and cysteine are linked by the glutamate cysteine synthetase (γGCS) in a rate limiting step, then glycine is bound to γ-L-glutamyl-cysteine by the enzyme glutathione synthetase. The major fraction of freshly synthesized GSH stays in the cytosol while minor fractions are transported to distinct cellular compartments such as mitochondria, the endoplasmic reticulum or the extracellular space, for instance the epithelial lining fluid of the lung (Comhair & Erzurum, 2005). GSH is present in the intracellular compartment primarily in the reduced (GSH) form. Oxidized GSSG can then be re-reduced by glutathione reductase (GR) utilizing electrons from NADPH. In the past, the redox state of proteins was directly linked to the ratio of GSH/GSSG e.g. the GSH/GSSG redox couple was suggested to determine the redox state in a certain compartment. The cytosol was thought to be a strictly reducing environment whereas the ER is rather oxidizing (Go & Jones, 2008). Today it is evident that the different GSH/GSSG levels e.g. in the ER are much rather the result of protein disulfide formation than its cause: the relatively high GSSG level may thus result from reduction of protein disulfides as part of a quality control mechanism for protein folding (Delaunay-Moisan et al., 2017).

In addition, various functions for GSH in cellular defence mechanisms were described. Not only is GSH involved in scavenging of ROS by direct interaction and in reduction of ROS acting as an electron donor for Grxs, but also it catalyses detoxification of xenobiotics in the liver via glutathione-S-transferase. Equally important is the enzymatic reduction of dehydroascorbate, where GSH functions as electron donor for either Grxs or protein disulfide isomerase (Forman et al., 2009). However, glutathione is also a key player in redox signalling and it regulates the oxidation state of protein cysteine residues either by direct glutathionylation or through enzymatic reaction with Grxs. Furthermore, it potentially protects cysteines from being over-oxidized by glutathionylation and a large number of proteins undergo post-translational glutathionylation with consequences for protein structures and functions (Lillig & Berndt, 2013).

1.2.3 Peroxiredoxins

Peroxidases – enzymes which catalyse the reduction of hydrogen peroxides – are represented in the mammalian cell by three different subfamilies, the catalases, the glutathione peroxidases and the Prxs. Among those, Prxs have a unique status, not only catalysing antioxidant reactions, but regulating cell signalling pathways, apoptosis and proliferation in interplay with ROS (Chae

et al., 1994; Forman et al., 2010). Mammalian Prxs comprise six proteins (Prx 1-6) which can be further categorized into three subfamilies: the 2-Cys Prxs (Prx 1-4) which contain both, the higly conserved N-terminal and C-terminal Cys residue, the atypical 2-Cys Prx (Prx5) that contains only the conserved N-terminal cysteine and requires a second Cys-residue for catalysis and the 1-Cys Prx (Prx6) which functions with only the N-terminal conserved Cys-residue (Chae et al., 1999). Intriguingly, 2-Cys Prxs form homodimers, which can be involved in formation of higher oligomeric structures. The homodimer is stabilized by the C-terminal Cys-residue of one subunit, interacting with the second subunit forming an intermolecular redox active disulfide. As the dimerization is favoured by oxidation of the active site, oligomerization is supported by reduction of the active site cysteines (Rhee et al., 1999). Indeed, the oligomeric structure of the Prxs was originally described for Torin, a protein discovered in the 1960 forming a decameric, "doughnut-shaped" structure consisting of a pentameric assembly of dimers, which was since confirmed to be equivalent to the mammalian Prx2 (Wood et al., 2002). The reduction of H_2O_2 is catalysed by Prxs in a two-step reaction. First the peroxidatic, N-terminal cysteinyl residue is oxidized to sulfenic acid while partially reducing H_2O_2 to water. In a second step the resolving cysteinyl residue forms a disulfide with the N-terminal thiol yielding a second molecule of water. This resolving cysteinyl residues is either located outside the active site on the same subunit, forming an intramolecular disulfide (for Prx5) or found on the adjacent subunit of the homo-dimeric protein, as shown for Prxs 1-4, forming an intermolecular disulfide. Both typical and atypical 2-Cys Prxs are re-reduced by Trxs (Rhee et al., 1999). Under increasing levels of cellular hydrogen peroxide, Prxs can be over-oxidized to a sulfinic and sulfonic form, initializing the stabilisation of decamers or even dodecamers of Prxs, which act as chaperones involved in protein folding (Jang et al., 2004; Wood et al., 2002).

Prxs are highly abundant proteins of 20-30 kDa with distinct subcellular localisation patterns. Prx1 is mainly localized in the cytosol but can also be found in the in various subcellular compartments such as mitochondria, peroxisomes, and the nucleus (Immenschuh et al., 2003). In addition, Prx1 has been detected extracellularly, for instance in sera from patients with several types of non-small-cell lung cancer (Chang et al., 2005). Functions in cellular signalling pathways have been confirmed for Prx1, since p38 MAPK activation state was altered in Prx1-knockout mice under cisplatin treatment, implying an involvement in the regulation of apoptosis (Ma et al., 2009). Similar to Prx1, Prx2 is mainly present in the cytosol or the nucleus and it was also described to have membrane-binding capacity (Woo et al., 2010). Prx 2 is considered to play a crucial role in inflammation through hydrogen peroxide/ROS mediated immune response in macrophages, since Prx2 deficient macrophages show an increased

inflammatory response under treatment with LPS (Salzano et al., 2014). Furthermore, mice deficient of Prx2 showed severe haemolytic anaemia (Low et al., 2008). With high reactivity towards hydrogen peroxide Prx2 interacts with STAT3, introducing disulfide bonds and therefore attenuating its transcriptional activity (Sobotta et al., 2015).

Although one of the least characterized Prxs, the mitochondrial Prx3 is proposed to be involved in protection from hydrogen peroxide produced by the mitochondrial respiratory chain, receiving electrons from the corresponding Trx in mitochondria, Trx2, and TrxR (Li et al., 2007). Later, Grx2 was discovered as a new electron donor for Prx3 in HeLa cells, since only silencing of both mitochondrial Trx2 and Grx2 inhibited the reduction of Prx3 (Hanschmann et al., 2010).

The 2-Cys Prx4 was found to be present in the cell as a 31 kDa precursor form and a 27 kDa form which lacks 36 N-terminal amino acids and was the only Prx detected in the endoplasmic reticulum. Previously the truncated form of Prx4 was is considered to be an active and secretable variant of the protein, shown to be released to the extracellular space, where it exerts heparin-binding functions under reducing conditions (Okado-Matsumoto et al., 2000). Later it was discovered that Prx4 is directed to the ER by interaction with ER-residing proteins and that binding to ERp44 is redox dependent, for instance Prx4 is released upon reduction by GSH (Yang et al., 2016). Prx4 functions in health and disease have since been further explored e.g. it reduces glutamate-induced stress in a neuronal cell line, reducing Ca^{2+} influx and “ROS” formation (Kang et al., 2020). Moreover, Prx4 was found to be elevated in serum of patients with sepsis (Schulte et al., 2011).

Prx 5, the only atypical 2-Cys Prx in humans, shows a very broad subcellular distribution, since it is detectable in the cytosol, nucleus, mitochondria and peroxisomes. Investigation of the crystal structure of Prx5 revealed that in contrast to the typical 2-Cys Prxs, there is no indication for dimerization of Prx5 (Knoops et al., 2011). Prx 5 is suggested to be an important player in regulation of cell signalling, for instance in inhibition of p53-induced apoptosis (Y. Zhou et al., 2000). Peroxiredoxin 6 is localized mainly in the cytosol and can also be transferred to lysosomes via a lysosomal targeting sequence (Sorokina et al., 2011). The 25 kDa protein contains only one active site Cys and no resolving Cys-residue for reduction of reaction intermediates (Cys-SOH) formed during interaction with H_2O_2 (Rhee & Woo, 2011). Although the nature of the reducing agent for the active site cysteine remains elusive, GSH has been proposed as a physiological electron donor (Lee et al., 2001).

Hydrogen peroxide and other ROS, which are by far the most important substrates of Prxs, have long been considered as oxidant agents damaging DNA, proteins and lipids. Today, there

is emerging evidence that Prxs are potent facilitators of redox signalling utilizing hydrogen peroxide as a messenger molecule to catalyse specific thiol oxidations, for instance formation of disulfide bonds or sulfenic acid derivatives or molecular chaperones and thus control various cell signalling pathways (Flohé, 2016; Forman et al., 2010).

1.3 Secretion of thioredoxin family proteins

Since the detection of Trxs as electron donors for RNR in the 1960s, intracellular functions of Trx family proteins haven been widely explored and importance of those proteins in redox regulation of cellular pathways. However, we are still scratching at the surface when it comes to extracellular functions of Trx family proteins. One explanation for this may thus be the limited knowledge on the mechanism of their secretion, as well as poor characterisation of extracellular interaction partners and catalysed reactions. Until today, five proteins of the Trx family have been detected in the extracellular environment, among them Trx1, TrxR, Grx1, Prx1, Prx2 and Prx4 and the current state of those investigations shall be summarized in this section.

Trx1 is the first Trx family protein that has been found to play an important role in the extracellular compartment. In 1992 two independent groups of researchers investigated the secretion of Trx1 and obtained consistent results. Trx1 was shown to be secreted from different cell lines, for instance fibroblasts and several neoplastic cell lines such as 9HTEO (airway epithelial cell line) and BM19 (lymphoblastoid cell line) under unstimulated conditions but not by resting T- and B-lymphocytes. In addition, activated T and B-cells also showed staining for Trx1 in the extracellular fraction (Rubartelli et al., 1992). These results cohere with findings by Ericson and co-workers, that activated normal and B-cells from chronic lymphatic leukaemia (B-CLL) show enhanced Trx1 expression and secretion (Ericson et al., 1992). Furthermore, known inhibitors of the ER-Golgi dependent secretory pathway such as Brefeldin A were utilized to show that Trx1 secretion is independent from conventional secretory routes (Rubartelli et al., 1992). The exact mechanism of this pathway of secretion still remains elusive – Trx1 secretion shares some features with the secretory route described for Interleukin 1β (IL-1 β) – yet there are distinct differences, since Trx1 could not be detected in intracellular vesicles and secretion was not decreased after treatment with inhibitors of cellular ABC transporters (Rubartelli et al., 1992). Plasma levels of Trx1 in healthy patients vary from 10-40 ng/ml with 25 ng/ml at average and in several diseases such as cancer, diabetes type 2 and HIV-infection those levels are increased (Kakisaka et al., 2002; H. Nakamura et al., 1996, 2006). In addition

to the reduction of ROS in direct association with Prxs, extracellular functions of Trx1 are closely related to functions of the immune system. Trx1 was described as a chemoattractant for polymorphonuclear cells and since no specific receptor for Trx1 was detected so far, the proposed mechanism for induction of chemotaxis is the alteration of surface structures of target molecules, e.g. thiol oxidation or isomerization, through the CGPC active site. Moreover, Bertini and co-workers proposed that the enhancement of inflammatory response by Trx1 may contribute to the pathologies of infectious diseases and furthermore Trx1 was suggested as sensor for oxidative stress related diseases, increasing the inflammatory response at the site of hypoxia (Bertini et al., 1999). A cleaved 10 kDa form of Trx1 was detected by Silberstein and co-workers in a myelomonocytic leukaemia cell line (U937 cells), firstly described as eosinophil cytotoxicity-enhancing factor (ECEF) (Silberstein et al., 1989). Later the polypeptide was established as Trx80, comprising the 80 N-terminal amino acids of Trx1 but lacking its disulfide reducing capacities and displaying higher chemotactic activity, especially for monocytes (Pekkari & Holmgren, 2004). Trx80 was detected in various cell lines e.g. monocytes, T-cells, transformed T-cell lines, activated platelets, but mostly secreted by activated monocytes, pointing to a regulation of secretion upon differentiation of monocytes to macrophages (Angelini et al., 2002; Rubartelli et al., 1992; Sahaf & Rosén, 2000). The mechanism of Trx1 truncation and how protein cleavage is controlled remains unclear, however as Trx80 is mainly localized at the plasma membrane facing extracellular surface, membranous protease cleavage was suggested. In addition, Trx80 has certain extracellular functions that could not be confirmed for Trx1, including strong chemokine activity on PMN's, autocrine induction of 'cluster of differentiation' (CD) surface proteins and cytokines (IL-12) and thereby a modulation of the Th1 T-cell response, reviewed by Pekkari and Holmgren (Pekkari & Holmgren, 2004).

Grx1 was already detected in plasma of healthy volunteers in 1998, however no elevation of plasma Grx1 levels was shown during open heart surgery (H. Nakamura et al., 1998). Subsequently, plasma levels of Grx1 were assessed by a sensitive ELISA in blood plasma of healthy donors with approximately 14 ng/ml mean (Lundberg et al., 2004). Recently, elevated plasma levels of Grx1 in patients with cardiovascular disease were found to correlate with the occurrence of adverse events such as myocardial infection and heart failure (Watanabe et al., 2021). Similar to Trx1 there was no secretory signal sequence detected for Grx1 and therefore secretion mechanism and its control is still unclear. Grx1 is expressed in cells of most tissues, but only several cells have been reported to secrete Grx1, among them peripheral blood mononuclear cells (PBMC's), a myelomonocytic leukaemia cell line (U937), and T-cell

leukaemia cell line Jurkat. Intriguingly Grx1 secretion in PBMC's was higher in unstimulated cells than in cells treated with 12-Otetradecanoylphorbol-13-acetate (TPA) were Grx1 secretion was strongly suppressed (Lundberg et al., 2004). In a study by Peltoniemi and co-workers Grx1 levels in alveolar macrophages of non-smokers, smokers and patients with chronic obstructive pulmonary disease (COPD) were compared and significantly higher Grx1 levels in macrophages of smokers were detected in comparison to non-smokers. In addition, Grx1 was found to be secreted into the sputum in all three groups and increased in patients with COPD, implicating an involvement of Grx1 in the regulation of extracellular GSH/GSSG ratio in oxidative stress related diseases (M. J. Peltoniemi et al., 2006). However, Grx1 is also an important electron donor for extracellular glutathione peroxidase 3 and therefore involved in reduction of hydroperoxides in the extracellular environment (Björnstedt et al., 1994). Grx1 was found to be secreted into the urine by distal convoluted tubule cells in a mouse model for ischemia-reperfusion injury, implicating a potential role as biomarker for the severity of kidney damage (Godoy, Oesteritz, et al., 2011). Grx2 was detected extracellular in sera from patients with allergic asthma and significantly decreased in comparison to healthy controls. (Hanschmann et al., 2020). In a study investigating various Trxs in pemphigus vulgaris Grx2 could be detected in blood serum, but no differences in comparison to healthy control patients was shown, however a significant decrease of Grx2 in PBMC’s of patients with pemphigus could be detected (Sliwiak et al., 2021).

Prx1, the cytosolic 2-Cys Prx, was detected in sera from patients with non-small cell lung cancer as well as in extracellular fractions from an adenocarcinoma cell line (A549) but not from a human non-cancer transformed epithelial cell line (BEAS 2B), indicating a potential function as biomarker for non-small cell lung cancer (Chang et al., 2005). Also striking similarities to the secretion of Trx1 were described for Prx1: the protein does not contain a secretory signal sequence and its secretion from A549 cells could not be inhibited by Brefeldin A (Chang et al., 2006). Prx1 as well as Prx2 was found in total cell extract of BAL fluid from controls and patients with sarcoidosis, the detected protein could either be secreted from immune cells or derived from intracellular fractions, however detection indicates involvement of the Prxs in pathologies of the lung (Kinnula et al., 2002). Elevated Prx2 levels in comparison with healthy controls were detected in plasma samples from patients with SARS (severe acute respiratory syndrome)-coronavirus infection and since Prx2 secretion from T-cells was suspected, an involvement in immune response of infected patients and possible role as biomarker for infectious diseases was suggested (Chen et al., 2004). Prx1 and Prx2 were also described as 'natural killer cell-enhancing factors A and B' by Sauri and co-workers, because in

a natural killer cell activity assay, especially NKEF A (Prx1) increased cytotoxicity of killers cells in its reduced form, a potential extracellular function of Prx1 and Prx2 (Sauri et al., 1996). Indeed NKEF A and B were elevated in plasma levels and CD8(+)T-cells of HIV-infected patients which had not yet received an antiviral therapy, pointing to an involvement of Prx1 and 2 in antiviral activity of CD8(+)T-lymphocytes (Geiben-Lynn et al., 2003). Recently it could be shown that Prx1 and Prx2 are exclusively present in serum as disulfide-linked homodimers, but that a nonclassical secretory pathway for Prx1 and Prx2 is depending on cysteine oxidation. Upon contact with inflammatory cytokine TNF-α or the oxidant menadione the catalytic cysteines of both Prx1 and Prx2 are oxidized and secreted as a dimer. Furthermore, Prx1 and Prx2 were found in extracellular supernatant and exosomes of HEK cells, suggesting an exosomal release mechanism directed by cysteine oxidation (Mullen et al., 2015). Moreover, it was demonstrated that Prx2 is released by macrophages in a glutathionylated form following stimulation by proinflammatory LPS. Intriguingly, Trx1 was shown to be released from macrophages along with Prx2, raising the question whether this is a possible secretion mechanism for Trx1 and part of an inflammatory circuit for Prx2, using Trx1 as an electron donor for reduction of H_2O_2 in the extracellular environment (Salzano et al., 2014).

For Prx4 there is strong evidence that the protein is secreted by an ER/Golgi-dependent pathway: first it possesses a leader peptide for secretion, which is cleaved shortly after synthesis and second it was shown to be localized in the ER and Golgi apparatus by co-labelling of calreticulin. In addition, extracellular heparin-binding capacities for the reduced form of Prx4 were identified, also binding to human umbilical vein endothelial cells was described, implying heparin-binding capacity as a possible mechanism for Prx4 to attach to a cell surface (Okado-Matsumoto et al., 2000). Although main extracellular functions of Prx4 were seen in reducing of hydrogen peroxide, which would be beneficial in redox-related diseases. Investigations in patients with sepsis showed elevated plasma levels of Prx4 correlating with severity of the disease and fatal outcome (Schulte et al., 2011). Recently Prx4 along with Prx1 and Prx2 was shown to induce NO production and IL-6 and TNF-α in a murine macrophage cell line and increased Toll-like-receptor4 (TLR4) expression, suggesting immune modulating properties of Prxs (Zhao et al., 2016). In summary, the secretion pathway of Prx4 is known as ER/Golgi related and Prx1 and Prx2 have been detected in lysosomal vesicles, but secretion mechanism of Trx1, Grx1 and Grx2 could not yet be identified.

1.4 Allergic airway inflammation

In the human body, the lungs are situated in a unique position due to the direct exposure of their mucosal epithelial surface to inhaled oxygen along with environmental pollutants such as cigarette smoke, diesel fuel particulates and other airborne pollutants. Therefore, the epithelial lining fluid contains a high amount of soluble ROS and RNS which not only have their source in airborne pollutants, but also come from oxidative phosphorylation and can be endogenously produced by immune cells, for instance macrophages and neutrophils (Comhair et al., 2005). The Trx system in interaction with GSH plays a key role in the regulation of ROS levels in the lung and the protection of bronchial epithelial cells as well as type I and II alveolar cells against oxidative damage. The importance of GSH has long been acknowledged, since GSH levels in the epithelial lining fluid of the lung were already found to be almost 100-fold higher than e.g. in the human plasma in the 1980's (Cantin et al., 1987). However, in the following years several studies focussed on the functions of Trx and TrxR in the developing lung, establishing that their expression is induced by exposure to oxygen in new-born primates (Das et al., 1999) and that Trx1 can be detected in bronchial epithelial cells and macrophages of the developing human lung (Kaarteenaho-Wiik & Kinnula, 2004). In contrast to the lungs of new-borns, Trx1, TrxR1 and Grx1 only show a weak staining in bronchial epithelial cells and macrophages of the adult lung, suggesting specific functions for Trx family proteins in development, regeneration and remodelling of the lung tissue and reduced activity in the healthy, matured lung (M. Peltoniemi et al., 2004).

Today in times of increasing prevalence of allergic diseases, especially asthma, with a prevalence of 4.3% doctor diagnosed asthma worldwide, the focus lies on a better understanding of the aetiology and pathology of allergic airway inflammation (To et al., 2012). Among the airway related disorders, such as chronic obstructive pulmonary disease (COPD) and pulmonary fibrosis, allergic asthma is already known to increase the oxidative burden through augmented production of ROS and RNS, thus the role of Trx family proteins became increasingly relevant in those disorders (Rahman et al., 1996, 2006).

The pathology of allergic asthma can be summarized as an association of chronic inflammation, bronchial hyper-responsiveness and airway remodelling, resulting in intermittent airway obstruction with clinical symptoms such as shortness of breath, cough and chest pains (Maddox & Schwartz, 2002). The gross pathology of asthma is characterized by hyperplasia of goblet cells, mucus gland hyper-secretion, cell sloughing and mucus plugging, which are the underlying cause for obstruction of medium-sized bronchi and bronchioles. Obstruction and

airway hyper-responsiveness (AHR) are potentiated by hyperplasia and hypertrophy of smooth muscle cells, bronchial vessel dilation, mucosal oedema and collagen disposition resulting in a thickening of the airway wall. Microscopically the elicitation and migration of various inflammatory cells such as mast cells, eosinophils, neutrophils, lymphocytes and activated macrophages to the bronchial epithelium, the epithelial lining fluid and the mucus can be observed (Barrios et al., 2006).

The causal mechanisms of allergic asthma are closely linked to a hypersensitivity for environmental inhaled allergens which is suspected to be hereditary determined. So far, several genes have been identified that may be associated with allergic asthma, among them the genes that encode IgE, IL-4, IL-13 and TNF-α (Cookson, 2002). In addition to the sensitization to inhaled allergens such as house dust, cigarette smoke, fungi or pollen in the childhood, early infectious diseases have been associated with the development of allergic asthma (Maddox & Schwartz, 2002). During the onset of allergic asthma, the predominant immune response is coordinated by a subclass of $CD4^+$ lymphocytes, the Th2 cells, releasing for instance IL-4, IL-13 and IL-5 which induce an isotype switch from IgM to IgE and elicit eosinophils to the site of inflammation (Barnes, 2011; Fireman, 2003). Cross-linkage of IgE molecules by allergens cause the degranulation of mast cells, releasing inflammatory mediators such as histamine, leukotrienes as well as other mediators which now amplify the inflammatory response and secure the further recruitment and survival of inflammatory cells (Fireman, 2003). The chronic inflammatory environment may be the basis for irreversible long-term structural changes, e.g. increase in smooth muscle, epithelial metaplasia, and fibrosis which are summarized as airway remodelling (N. Hirota & Martin, 2013). Until today a large number of cytokines have been identified to play a key role in the pathophysiology of asthma, among them Th2-cytokines IL-4, -5, -13 as well as Th1-cytokines IL-1,-6 and TNF-α (Lambrecht et al., 2019). For instance IL-6 is a key player in systemic inflammation with elevated serum levels not only in septic patients but also in BAL fluid from patients with active severe asthma (Tillie-Leblond et al., 1999). IL-6 is known to play a key role in the transmission of the acute immune response to the directed immune response, inducing neutrophil apoptosis after early antigen detection and initiating transformation of monocytes to macrophages (Scheller et al., 2011). INF-γ is produced by $CD4^+$ Th1-cells and promotes activation and presentation of class I and class II MHC molecules on epithelial cells and macrophages, eliciting antigen presentation (Chung, 2001). Furthermore INF-γ induces differentiation of naïve lymphocytes to Th1-cells and inhibits Th2-cell differentiation. In 1997, Leonard and co-workers showed that INF-γ predisposes to the development of allergic disease and patients with asthma presented

significantly reduced INF-γ levels in response to allergen contact compared to control patients (Leonard et al., 1997). It also has been demonstrated that INF-γ inhibits the eotaxin induced recruitment of eosinophils to the lung which is an important pathologic factor in allergic airway inflammation (Fulkerson et al., 2004). The cytokine tumour necrosis factor-α (TNF-α) plays an important role in the induction of the cellular immune response. Produced by monocytes and macrophages it initiates cell differentiation of multiple target cells such as monocytes, endothelial cells, granulocytes and thrombocytes and therefore facilitates activation and survival of inflammatory cells, cell migration via endothelium and promotes tissue degeneration by induction of tissue destructive enzymes (Kalliolias & Ivashkiv, 2016).

The interplay of cellular apoptosis and tissue remodelling is a cornerstone of the pathophysiology of allergic asthma and caspases are important players in cellular response to inflammation and tissue homeostasis. Caspases comprise a family of cysteine proteases which are synthesized in the cell as inactive precursors consisting of four different domains, an amino-terminal domain of variable size (termed N-terminal polypeptide or pro-domain), a large subunit, a small subunit, and a linker region between the large and small domains flanked by asparagine residues, reviewed by (Nicholson & Thornberry, 1997). More than two decades ago crystal structure analysis of caspase-1 and caspase-3 bound to substrates have revealed that active caspases are composed of two heterodimers interacting via the small subunits to form a tetramer with two catalytic sites (Wilson et al., 1994). Caspases can be divided into initiator and effector caspases. Various stress stimuli and intracellular signalling pathways lead to oligomerisation and activation of initiator caspases-8, -9, and -10. These initiator caspases contain death effector domains (DED, caspase-8 and -10) or caspase-recruitment domains (CARD, caspase-2, -9, -1 and -11) and when two cleaved monomers of the effector caspase interact, they form an active dimer (Nuñez et al., 1998). In 1998, Stennicke and co-workers characterized caspase-8 as a strong initiator for pro-caspase 3 cleavage and activation (Stennicke & Salvesen, 1997). The apoptotic caspase, caspase 3 recognizes specific short peptide cleavage motives upon activation and cleaves cellular proteins where this motif is present and accessible which leads to protein degradation, to degradation of cell organelles and DNA fragmentation (Wall & McCormick, 2014). Once activated caspases need to be regulated and therefore different mechanisms of inactivation have been discussed. Intriguingly, S-nitrosylation plays an important role in this regulation process. In 1998, Rössig and co-workers revealed that NO could function as an inhibitor of caspase-3 via s-nitrosylation of an active site cysteine-residue (Cys 163) (Rössig et al., 1999). Recently, persulfidation of caspases was detected in apoptotically stimulated HeLa cells leading to attenuated caspase activity. These

effects could be antagonized by Trx and the Trx-reductase. Low intracellular levels of Trx/TrxR resulted in higher persulfidation of caspase-3 and 9 and decreased apoptosis. Even in resting HeLa cells a basal level of persulfidation of caspase could be detected, implicating a regulatory effect of de-/persulfidation for the induction of apoptosis (Braunstein et al., 2020).

Among the large variety of mediators, endogenous ROS and RNS such as superoxide anion ($O_2^{\cdot}$), its dismutation product H_2O_2, NO· and its by-products peroxynitrite ($ONOO^-$) and nitrite (NO_2) play an essential role in the amplification of the chronic inflammatory state. As mentioned before the lungs naturally display a high level of ROS and RNS in their epithelial lining fluid (Comhair et al., 2005). Intriguingly, these levels have shown to be increased in allergic asthma and studies investigating the main sources of oxidant molecules have discovered that not only migrating cells e.g. eosinophils and leukocytes but also resident cells such as alveolar type 2 cells, alveolar macrophages and endothelial cells are involved in production of RNS and ROS in asthma (Comhair & Erzurum, 2010; MacPherson et al., 2001). Furthermore, generation of ROS, first documented for the production of O_2^-, correlates to the localisation of immune cells at the site of inflammation (Sanders et al., 1995) and increases after bronchoprovocation with an inhaled antigen, as shown by Calhoun and co-workers (Calhoun et al., 1992). Due to their essential role in redox signalling and control of oxidative modifications Trxs, Grxs and Prxs stepped into the scope of asthma research in the past decade. Elevated levels of Trx1 had already been detected in various infectious diseases such as HIV and hepatitis C, influencing the immune response of monocytes and neutrophils (H. Nakamura et al., 1996). Therefore, it is to no surprise that serum Trx1 levels of patients with bronchial asthma were elevated during an asthma attack and could be correlated to the serum level of eosinophil cationic protein, while the forced expiratory volume (FEV_1) a main tool to measure the clinical condition of asthma patients, was inversely correlated to the Trx1 levels (Yamada et al., 2003). The effects of both, intravenous administration, and over-expression of Trx1 were investigated in a mouse model of OVA-induced allergic airway inflammation. Treatment of OVA-sensitized mice with recombinant human wild-type Trx1 but not the redox inactive mutant (C32S) was shown to decrease key features of asthma pathology for instance AHR and eosinophilic inflammation (Ichiki et al., 2005). Furthermore, Imaoka and coworkers discovered that administration of human recombinant Trx1 as well as over-expression of the protein in a chronic antigen exposure asthma model decreased airway remodelling and inhibited goblet cell hyperplasia, two major causes of bronchial obstruction in asthma (Imaoka et al., 2007). Also treatment of OVA-sensitized mice with Trx1, which reduces AHR and inflammation, leads to augmented mRNA levels of several cytokines such as IL-1α, IL-1β, IL-receptor antagonist and

IL-18 in comparison to untreated mice, suggesting that Trx1 reduces airway inflammation by up-regulation of Th_1-like cytokines (Ichiki et al., 2005).

In contrast, a recent study suggested that Trx1 suppresses airway inflammation independently from the systemic immune response by inhibiting production of macrophage migration inhibitory factor (MIF) which is an upstream modulator of airway inflammation. In Trx1 over-expressing mice (Trx-Tg) the levels of MIF as well as eotaxin and IL-13 were decreased but in vitro $CD4^+$-T-cell strains from wild-type and Trx-Tg displayed similar Th1 and Th2 responses (Torii et al., 2010). These ambiguous results indicate a multi-factorial role for Trx1 in allergic airway inflammation, especially since Trx1 itself was reported to reduce chemotaxis of eosinophils (Kobayashi et al., 2009). However, until today the role of Grx1 in the pathology of asthma remains unclear. Alterations of the GSH/GSSG ratio in favour of GSSG after antigen challenge have been discussed, but only recently changes in protein s-gluthathionylation (PSSG) were observed in allergic airway inflammation and suggested to contribute to the pathology of asthma (Comhair & Erzurum, 2005; Hoffman et al., 2012). Intriguingly, Reynaert and co-workers demonstrated that Grx1 - which is closely linked to the regulation of protein de-/glutathionylation - is up-regulated in a murine model of allergic airway inflammation. Furthermore, treatment of primary tracheal epithelial cells from OVA-sensitized mice with IFN-γ additionally increased Grx1 expression, resulting in attenuated PSSG, whereas TGF-β1 treatment decreased Grx1 expression, leading to enhanced PSSG (Reynaert et al., 2007). The importance of protein S-glutationylation was supported by recent data discovering that genetic ablation of Grx in a mouse model for airway inflammation increased overall PSSG in comparison to wild-type mice. A beneficial effect of enhanced protein S-glutathionylation was suggested, since Grx1 deficient mice showed resolution of airway hyper-responsiveness to control levels 7 days after antigen challenge and reduced mucus metaplasia (Hoffman et al., 2012). Although today no direct link between AHR-regulation and glutathionylation was provided, there is emerging evidence that protein S-glutathionylation is involved in the modulation of the inflammatory response for instance via regulation of the NF-κB pathway. NF-κB is a transcription factor which is crucial for the activation of various pro-inflammatory genes under oxidative stress conditions. Glutathionylation of the p50 subunit of NfκB and of the inhibitory kappa-β kinase (IKKβ) results in attenuation of pro-inflammatory gene transcription and thereby counteracts the activation of NF-κB by ROS sensitive signalling pathways that occurs in asthma (Comhair & Erzurum, 2010; Pineda-Molina et al., 2001). In addition to Trxs and Grxs, Prxs are involved in the inflammatory response in allergic airway inflammation. Inflammation was shown to increase Prx5 levels in OVA-sensitized mice but did

not change mRNA expression levels of Prx5 in tracheal epithelial cells, therefore Prx5 was determined in migrated leukocytes, indicating that inflammatory cells are rich in Prx5 and able to secrete the protein to the extracellular space (Krutilina et al., 2006). Furthermore, Prx1, which had already been shown to be secreted to the extracellular space, was implied in the protection against Th2-type airway inflammation and AHR (Chang et al., 2006). Prx1 knock-out and wild type mice were sensitized with OVA and additionally treated with the Th2-adjuvant aluminium potassium sulphate (Alum) or the Th1-stimulating Freund's adjuvant (Freund's). Ova/Alum-sensitized knock-out mice displayed a significantly increased leukocyte influx and cholinergic AHR as well as enhanced expression of IL-2 and decreased IFNγ levels in comparison to wild-type mice. In contrast Prx1 knock-out mice showed decreased leukocyte levels in BAL fluid during OVA/Freund's sensitization, thus an essential role for Prx1 in the regulation of the Th1/Th2 balance through suppression of IL-2 expression was suggested (Inoue et al., 2009).

In conclusion, these results suggest an important role for Trxs, Grxs and Prxs in allergic airway inflammation apart from their antioxidant capacities. Still, it remains elusive whether they function only in regulation of cellular redox signalling pathways or also contribute to inflammation as chemokine-like inflammatory mediators. However, it is intriguing that hypoxia itself could not yet be clearly linked to the pathology of asthma although hypoxic conditions are present in severe asthma exacerbations. A study revealed that a combination of OVA-sensitization and hypoxia exposure induced an increase in neutrophils and eosinophils, enhanced expression of HIF-1α (Hypoxia inducible factor 1α), eotaxin-1 and TGFβ, signs of airway inflammation. Furthermore, an increase of peri bronchial smooth muscle content and enhanced fibrosis were observed in comparison to normoxia (Baek et al., 2013). HIF-1α which is a key player in the cellular response to low oxygen levels, is induced under hypoxia and regulates transcription of various genes via activation of hypoxia response elements (Forsythe et al., 1996). Thus HIF-1α controls the expression of many hypoxia-regulated genes. The cellular adaptation to low oxygen and was recently implied in inflammatory processes contributing to the pathology of asthma. Studies investigating the effect of HIF knock-out found that partially HIF-1α deficient OVA-sensitized mice displayed increased IFNγ levels and reduced lung eosinophilia in comparison to wild-type mice (Guo et al., 2009) and HIF-1β knock-out mice showed decreased allergic airway inflammation and reduced production of Ovalbumin specific IgE and IgG (Baay-Guzman et al., 2012).

Finally allergic airway inflammation is induced and perpetuated by a complex interplay between allergen hypersensitivity, Th2-driven immune response that suggests a multifactorial involvement of Trxs, Grxs and Prxs, as outlined above.

1.5 Aims of the study

Trx family proteins are known to exert major functions in intracellular redox signalling and expression of these proteins in various tissues has been established in the past. In contrast extracellular functions of Trxs are much less understood – especially signalling functions in inflammation and immune response were addressed in recent publications. In this study, we aimed at investigating the expression pattern of Trx family proteins in a mouse model for allergic airway inflammation. Since distribution of Grxs, Trxs and Prxs has already been examined in lung histology of healthy mice, we wanted to explore protein changes under disease conditions closely linked to fundamental changes in redox state. Overall, we wanted to reach a better understanding of extracellular presence of Trx family proteins and get an insight into functional implications of those alterations *in vitro*. The central aims of this study were:

- to purify a sensitive Trx1 antibody for Western Blot analysis for further experiments.
- to examine the distribution pattern of Trx family proteins in a mouse model for allergic airway inflammation in the intracellular and the extracellular compartment.
- to get an insight into functional implications of Trx family proteins in allergic inflammation via investigation of HIF-1α and pro-caspase 3 levels.
- to further investigate Trx protein functions in OVA-induced airway inflammation upon treatment with recombinant Grx2 and the redox inactive Grx2C40S variant.
- to identify changes in cytokine secretion from murine macrophages after treatment with Trx1 and Grx2 as potential mechanisms for immune response.

2 Materials and methods

2.1 Materials

2.1.1 Instruments

Äkta Prime FPLC system	GE Healthcare (Chicago, USA)
Autoclave 5075 ELV	Tuttnauer (Breda, Netherlands)
Balances:	
Laboratory balance	Sartorius AG (Göttingen, Germany)
Precision balance BP 160P	Sartorius AG (Göttingen, Germany)
CASY TT cell counter	OMNI Life Sciences (Basel, Switzerland)
ChemiDoc XRS+ System	BioRad Laboratories Inc.(Hercules, USA)
Centrifuges:	
Refrigerated centrifuge 5402	Eppendorf SE (Hamburg, Germany)
Table centrifuge MR1822	Jouan™ (ThermoFisher Scientific, Waltham, USA)
Table centrifuge A14	Jouan™ (ThermoFisher Scientific, Waltham, USA)
Electrophoresis power supply unit	ThermoFisher Scientific (Waltham, USA)
FACSCanto II Flow cytometer	Becton Dickinson and Co. (Franklin Lakes, USA)
Fermenter	Bioengineering AG (Wald, Schweiz)
Heating block thermostat 5320	Eppendorf SE (Hamburg, Germany)
Magnetic stirrer R1000	Carl Roth GmbH (Karlsruhe, Germany)
Microscopes:	
Fluorescence microscope IX70	Olympus (Hamburg, Germany)
Light microscope Telaval	Carl Zeiss Jena GmbH (Jena, Germany)
Leica SP8 confocal microscope	Leica AG (Wetzlar, Germany)
pH-meter pH 538	WTW (Xylem analytics, Weilheim, Germany)
Plate reader MULTISKAN EX	ThermoFisher Scientific (Waltham, USA)
Plate reader Infinite M200	Tecan Group AG (Männedorf, Switzerland)
Rotating incubator WT17	Biometra (Göttingen, Germany)
Sterile benches	Nuaire (Plymouth, USA)
Thermocycler T3000	Biometra (Göttingen, Germany)
Ultra pure water system Mili-Q synthesis A10	Merck Milipore (Burlington, USA)
Vortex-Genie II	Scientific Industries (Bohemia, USA)
XCell II™ Blot Module	Invitrogen™ (ThermoFisher Scientific, Waltham, USA)
XCell SureLock® Mini Cell, PAGE-chamber	Invitrogen™ (ThermoFisher Scientific, Waltham, USA)

2.1.2 Chemicals and consumables

Chemicals used in this study were purchased in analytic grade from the following companies: Carl Roth GmbH (Karlsruhe, Germany), Merck Milipore (Burlington, USA), Merck KGaA (Darmstadt, Germany), AppliChem GmbH (Darmstadt, Germany), ThermoFisher Scientific (Waltham, USA) Roche Holding AG (Basel, Switzerland). Consumable supplies like pipette tips, Falcon tubes and cell culture bottles were obtained from Carl Roth GmbH (Karlsruhe, Germany), Greiner AG (Gremsmünster, Austria), Sarstedt (Nümbrecht, Germany). Various Kits used in this study were obtained from BioRad Laboratories Inc. (Hercules, USA), ThermoFisher Scientific, Waltham, USA) Promega (Madison, USA), Qiagen (Hilden, Germany), and Roche Diagnostics GmbH (Mannheim, Germany). Columns for protein and antibody purification and nitrocellulose membranes were purchased from GE Healthcare (Chicago, USA), ThermoFisher Scientific (Waltham, USA), Merck KGaA (Darmstadt, Germany) and Macherey-Nagel (Düren, Germany). SDS gradient gels from BioRad Laboratories Inc. (Hercules, USA). Compounds and substances such as antibiotics, buffer, cell media and supplements, enzymes, marker for DNA and proteins were purchased from Merck KGaA (Darmstadt, Germany), Carl Roth GmbH (Karlsruhe, Germany), New England Biolabs (Frankfurt, Germany) and PAA Laboratories (Cölbe, Germany).

2.1.3 Antibodies

Name	***Source***	***WB***	***IHC***	***Origin***
Actin	mouse	1:5000	1:1000	Santa Cruz (sc 47778)
GAPDH	rabbit	1:5000	-	Sigma Alldrich (G9545)
Grx1	rabbit	1:1000	1:200	Santa Cruz (sc 32943)
Grx2	rabbit	1:500	1:500	Lillig group
Grx3	rabbit	1:1000	1:250	Lillig group
GSH	mouse	1:1000		Virogen (101-A-250)
His-Tag	rabbit		1:1000	Invitrogen
Mouse-HRP	goat	1:5000	-	BioRad
Mouse-biotin	goat	1:1000	-	Invitrogen
Prx1	rabbit	1:1000	-	Lillig group
Prx2	rabbit	1:1000	1:200	Santa Cruz (sc 33572)
Prx4	rabbit	1:1000	1:200	Abcam (ab 59542)
Prx5	rabbit	1:1000	-	Lillig group
Rabbit-HRP	goat	1:5000	-	BioRad
Rabbit-biotin	goat	1:1000	-	Invitrogen
Alexa Fluor 568	goat	-	1:200	Invitrogen (A11011)

Alexa Fluor 647	rat		1:200	Invitrogen (A21247)
Hoechst 33342	-		1:10.000	Merck
hTrx1*	rabbit	1:1000	-	Lillig group
mTrx1*	rabbit	1.1000	-	Lillig group
Trx2	rabbit	1:1000	-	Lillig group

*Antibodies marked were purified in this study

2.1.4 Oligonucleotides

Name: target gene	***Endonucleases***	***Sequence***	***Target plasmid***
PREMH 39:hTrx1, Fw	NheI, BamHI	**gctagc**tacccatacgatgttccagatta cgct*atggtgaagcagatcgagagc*	pet15b
PREMH 40:hTrx1, Rw	NheI, BamHI	**ggatcc***ttagactaattcattaatggtgg cttc*	pet15b
PREMH 41:mTrx1, Fw	NheI, BamHI	**gctagc**tacccatacgatgttccagatta cgct*atggtgaagctgatcgagagc*	pet15b
PREMH 42:mTrx1, Rv	NheI, BamHI	**ggatcc***ttaggcatattcagtaatagag gcttc*	pet15b

2.1.5 Strains and plasmids

2.1.5.1 Bacteria

***E. coli* XL-1 blue mrf`**: Δ(mcrA)183 Δ(mcrCB-hsdSMR-mrr)173 *end*A1 *sup*E44 *thi*-1 recA1 *gyr*A1 *gyr*A96 *rel*A1 *lac* F`[*pro*AB*lac*I^{q}ZΔM15 Tn10(Tetr)]c Stratagene (tetracycline resistance)

***E. coli* BL21(DE3)pRIL:** *hasdS gal* (λI*ts*857 *ind* 1 *S*am7 *nin*5 lac UV5-T7 gene 1) Stratagene, (chloramphenicol resistance)

2.1.5.2 Cell lines

Primary macrophages were elicited from mice as immature exudate macrophages. These cells do not divide but do attach to the surface of the culturing plate. They were cultivated in 1640 RPMI medium and incubated at RT for 4 hours.

2.1.5.3 Plasmids

2.1.6 Animal model

All experiments were performed with ethical approval by the state of Hesse, Germany.

2.1.6.1 Mouse model for allergic airway inflammation

The Renz group (Department of laboratory medicine, Phillips University, Marburg) established a mouse model for induction of allergic airway inflammation by sensitization with Ovalbumin (OVA). Balb/c mice (Janvier laboratories, France) were housed under specific pathogen-free conditions in individual-ventilated cages (IVCs) in a 12-h light/dark cycle and were provided with a standard rodent diet and obtained food and water ad libitum. Balb/c mice (6-8 weeks) were sensitized with 10 mg OVA (Sigma, Merck, Darmstadt, Germany) in 200ml PBS by s.c. injection weekly for three weeks (day 0; 14 and 21) followed by exposure to 10 mg/ml OVA in PBS applied as aerosol for 20 min on three consecutive days and analysis after max. 48h. Control mice were sensitized and challenged with PBS.

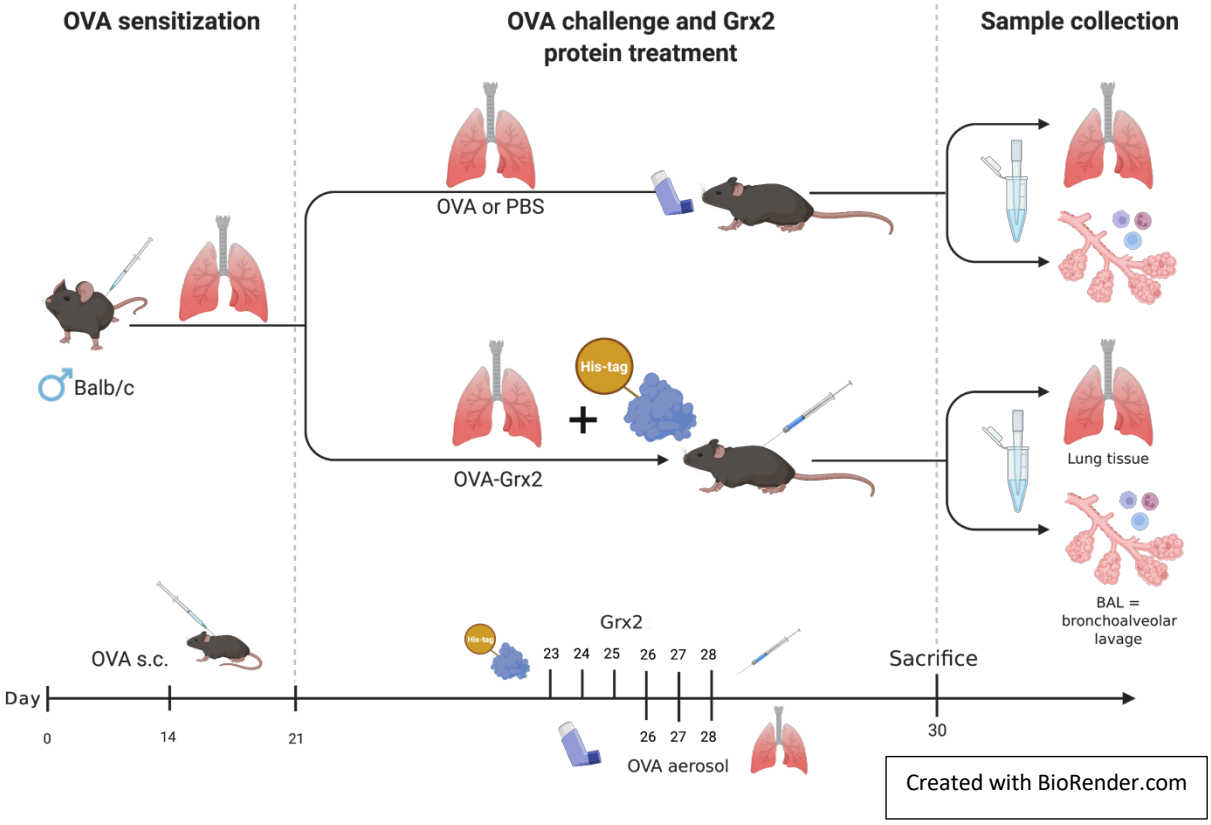

Figure 1 - Protocol for OVA-sensitization and challenge: *male Balb/c mice were sensitized with OVA injections i.p. day 0, day 14 and day 21 and challenged with OVA-aerosol daily from day 26-28. PBS*

was used as control. Mice were injected i.p. with 40 µg of recombinant his-tagged-Grx2, for 5 days respectively from day 23-27, beginning prior to OVA-challenge on day 26-2. 48h after OVA-challenge lung tissue and BAL samples were harvested.

To investigate the effect of several thioredoxin family proteins a second group of Balb/c mice were treated with i.p. injection of Grx2 parallel to induction of airway inflammation. Starting two days prior to challenge with OVA aerosol, they injected 40 µg daily of the corresponding Redoxin for five consecutive days followed by analysis. Mice were anaesthetized by i.p. injection of Ketamine (300mg/kg, Ketonarkon, Streuli pharma, Uznach, SA) and Xylazine (30 mg/kg, Rompun, Bayer, Leverkusen, Germany) and finally euthanized by cervical dislocation.

2.1.6.2 Isolation of lung tissue and bronchoalveolar lavage fluid from mice

NP40 buffer: 50 mM Tris, pH 7.4, 250 mM NaCl, 5 mM EDTA, 50 mM NaF, 1 mM Na3VO4, 1 % Nonidet P40 (NP40), 0,02 % NaN3

Animal experiments described in this study were solely performed by the Renz group according to ethical approval by the state of Hesse, Germany. Tissue obtained from those experiments was kindly provided for further investigation in the laboratory in Greifswald. Mice were anaesthetized as described above and bronchoalveolar lavage (BAL) was performed with a tracheal cannula containing 1ml PBS and BAL supernatant was stored at -20 °C. Then mice were sacrificed. Lung tissue was removed and lysed in NP40 buffer and vortexed until a homogenous solution was yielded and stored at -20 °C. Samples were tested for protein concentration using Bradford reagent.

2.1.6.3 Isolation of macrophages from mice

In collaboration with the Renz group (Department of laboratory medicine, Phillips University, Marburg) murine thiolglycolate-elicited exudate macrophages were isolated. For eliciting of immature macrophages Brewer thiolglycollate medium (3%) was injected to the peritoneal cavity of Balb/c mice, three days post treatment the mice were euthanized followed by isolation of macrophages. For harvesting of macrophages, the peritoneal cavity was rinsed with 5 ml PBS 2 times and the fluid containing elicited macrophages was aspirated and stored on ice. Cell suspension was then centrifuged at 13000 rpm at 4°C for 10 min and cell pellet was resuspended in 13 ml fresh 1640nRPMI medium (ThermoFisher Scientific, Waltham, USA). 4 fractions of 100 µl were stored on ice for FACS. Cell counting was performed using the

automated CASY TT cell counter (OMNI Life Sciences GmbH, Basel, Switzerland) according to protocol. Then 2 ml of cell suspension containing 4 Mio cells were seeded per cell culture flask supplemented with 4 ml of RPMI medium. After attachment of macrophages during 4h cells were washed in PBS and supernatant was discarded, again RPMI medium was added, and cells were mechanically harvested using a cell scraper, cell suspension was pooled in a 15 ml falcon and again cells were counted using CASY TT cell counter. 4x100µl were stored again for FACS.

2.1.7 Data analysis

2.1.7.1 Western Blot analysis

The computer-based programme ImageJ (NIH) was utilized to analyse and quantify protein bands from Western blots. Statistical significance of Western Blot results was determined using the Student's unpaired t-test for two group comparison. Statistical significance was indicated as *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

2.1.7.2 Figures

Figures and Diagrams were created using ImageJ, GNU Image Manipulation Program, Inkscape and Microsoft Excel.

2.2 Methods

2.2.1 Molecular biological methods

2.2.1.1 Polymerase Chain Reaction

Standard techniques such as Agarose gel electrophoresis, ligation, restriction of DNA with Endonucleases and DNA extraction were performed according to standard protocols. Ligation of DNA into pGEM-T was performed according to manufacturer`s instructions (ThermoFisher Scientific, Waltham, USA) For DNA extraction and gel elution NucleoSpin Gel and PCR clean-up Kit (Macherey-Nagel, Düren, Germany) were used. The Polymerase chain reaction (PCR) is a well-established method for amplification of specific DNA sequences. PCR was performed in a thermocycler with a preparation of 2-20ng DNA-template, 25mM dNTP`s, 10 pmoles/µl

specific primer and reaction buffer containing 1,5mM $MgCl_2$. First the DNA was denatured at 92°C for 2 min and reaction paused at 80°C followed by the addition of 2U DyNAzyme EXT Polymerase (ThermoFisher Scientific, Walsham, USA). Then 29 cycles followed with denaturing DNA at 90°C for 1:45 min, annealing primers for 1:30 min at a primer specific temperature and elongation of DNA at 72°C for 1 min. subsequently. After a final elongation time of 10 min. at 72°C the PCR product was loaded on a 1% Agarose gel for control

2.2.1.2 DNA-sequencing

All plasmids generated in this study were sent to Seqlab (Göttingen, Germany) for sequencing in concentrations of 100-300 ng/µl DNA. Results were analysed with Ncbi blast and ExPASy

2.2.1.3 Transformation and cultivation of bacteria

LB-Medium: 1% (w/v) NaCl, 0,5% (w/v) yeast extract, 1 % (w/v) tryptone, pH 7.4;

Competent bacteria *E.coli* XL1blue were generated chemically based on rubidiumchloride method and were already available. Aliqouts of 200µl of competent bacteria were thawed and supplemented with 25ng plasmid DNA or ligation product and incubated for 20 min. on ice. Heat shock was performed for 90 seconds at 42°C on a heating block, followed by incubation on ice for 5 min. and addition of 800µl LB-medium. After an additional incubation time of 45 min at 37°C, the bacteria were spun down for 2 min at 10000 rpm in a centrifuge (Eppendorf SE, Hamburg, Germany), the pellet was resuspended in 50µl LB-Medium and transformed bacteria were transferred to an Agar-plate, containing antibiotics (Ampicilline, Chloramphenicol, Kanamycin or Tetracycline) for selective resistance testing and incubated over night at 37°C.

2.2.1.4 Protein expression

LB-Medium: 1% (w/v) NaCl, 0,5% (w/v) yeast extract, 1 % (w/v) tryptone, pH 7.4; Washing buffer: 300mM NaCl, 25mM NaH_2PO_4, 25mM Na_2HPO_4, pH 8.0;

Bacteria for protein expression were incubated in a 5l Fermenter to yield high amounts of recombinant protein. 5l of LB-medium containing selective antibiotics (Ampicilline, Chloramphenicol, Kanamycin or Tetracycline) were supplemented with 1% of *E.coli* BL21

(DE3) transformed with pet15b plasmid under controlled conditions at 37°C. Cells were incubated until they had reached the exponential growth phase with an OD_{600} of 0.6, at that time point temperature was reduced to 22°C and expression of the recombinant protein was induced by adding 0.5M IPTG. Cells were harvested after 20h by centrifugation at 5000x g for 10min at 4°C. The resulting pellet was resuspended in 20ml Washing buffer, centrifuged and stored at -20°C.

2.2.2 Cell biological methods

2.2.2.1 Cell cultivation

All cell lines and primary cells used in this study were cultivated at 37°C in a humidified atmosphere containing 5% CO2 and 20% O2. Cell lines and the appropriate culturing medium are listed in 2.1.5.2. To prevent fast growing cells from stress, medium was generally changed every three days, therefore medium was discarded, cells were washed in PBS and new medium was added. When cells were confluent, they were splitted. Subsequently medium was removed, cells were gently washed in PBS and incubated for a short time at 37°C in trypsin to detach cells. After adding fresh prewarmed medium the reaction was stopped, the cells could be splitted and cells were centrifuged for 5 min at 700 rpm to remove the trypsin, before seeding them out in a ratio of 1:2 or 1:3. Since primary macrophages do not divide, they were not splitted and medium was not changed, because they were already harvested after three days.

2.2.2.2 Cell counting

Cells were counted using a Neubauer counting chamber according to manufacturer's recommendations. Subsequently 20 µl of cell suspension was added to the counting chamber and cells were counted manually using a light microscope. Primary macrophages were counted using the CASY TT cell counting system according to manufacturer's recommendations.

2.2.2.3 Cell lysis

Primary macrophages were mechanically harvested using a cell scraper, cell suspension was pooled and cell number determined using CASY TT cell counting system.

2.2.2.4 Macrophage stimulation

200.000 cells per well were seeded on a 96-well microtiter plate. Redoxins e.g. mTrx1 and hGrx2c (AG Lillig) were reduced using 10mM DTT and incubated for 30 mins. For DTT removal protein solution was washed using a NAP5 column (Thermo Fisher Scientific, Waltham, USA) and separated with 3 ml PBS. Protein concentration was determined with NanoDrop. Freshly reduced protein was then added to the microtiter plate in a concentration of 40µl and pre-incubated for 1h. Then 20 ng/ml LPS (Sigma Aldrich, Merck KGaA, Darmstadt, Germany) and/or 10 ng/ml INF-γ (Sigma Aldrich, Merck KGaA, Darmstadt, Germany) were added to each well. After macrophage stimulation for 4-72h expression of cytokines were measured using CBA.

2.2.2.5 Fluorescence-activated cell sorting

MACS buffer: PBS, 0.5% BSA, 2mM EDTA, MACS BSA Stock solution and 1:20 MACS Rinsing Solution (Miltenyi Biotec);

Fluorescence-activated cell sorting (FACS) allows the separation of subpopulations of white blood cells via fluorescence-labelled antibody detection in a flow cytometer. For cell surface marker analysis cell suspension was elicited as described in 2.1.6.3. Cell samples were centrifuged for 10 min at 10000 rpm at 4°C and pellet was resuspended in FC block antibody to block Fc-receptors of macrophages and 100µl were pipetted on a 96-well microtiter plate. FITC-coupled antibody CD 45 was added in a concentration of 1:100 and F4/80 was added in concentration 1:20. Samples were incubated on 96-well plate for 30 mins in the dark at RT. Cells were subsequently washed in 100 µl ice cold MACS buffer and centrifuged on the 96-well plate for 7 mins and supernatant was discarded. Finally, each sample was resuspended in 100 µl MACS buffer and added to a FACS tube. Analysis was performed using FACSCanto II Flow cytometer (BD, Franklin lakes, USA).

2.2.2.6 Cytometric Bead Array

For quantification of cytokine levels in macrophage supernatant a cytometric bead array system was used, since it allows for analysis of multiple parameters in one sample and the application of a minimal sample volume for simultaneous measurement. 100µl of cell free supernatant from each macrophage sample were analysed using mouse cytometric bead 23-plex assay (M60-009RDPD, BioRad, Hercules, USA) according to manufacturer's recommendations. Standards were prepared and serially diluted in a 96-well plate and all

samples were evaluated by specific antibody-coated 6.5 µm capture beads and phycoerythrin-conjugated secondary antibodies for detection. Cytokine measurements were performed using the Bio-Plex-200 Suspension Array System (BioRad, Hercules, USA). Analysis of standard curves and cytokine levels was performed in BioPlex Manager software (BioRad).

2.2.3 Biochemical methods

2.2.3.1 Protein purification

Washing buffer: 300mM NaCl, 25mM NaH_2PO_4, 25mM Na_2HPO_4, pH 8.0; Equlibration buffer: 30 mM imidazole in Washing buffer, pH 8.0; Elution buffer: 250mM imidazole in Washing buffer, pH 8.0;

Recombinant Proteins which were cloned containing a His-tag were purified according to the IMAC (immobilized metal affinity chromatography) principle using the Äkta Prime FPLC system (GE Healthcare, Chicago, USA). Cell pellets were defrosted and lysed in 20ml Washing buffer containing 5 mg Dnase I and 20mg lysozyme (ThermoFisher Scientific, Waltham, USA) and incubated for 30 min at RT. Cells were further homogenized with an ultrasound applying 85% amplitude, in cycles of 0.5 seconds for 2:30 min on ice, followed by centrifugation at 10000 x g for 30 min at 4°C. Supernatant containing the recombinant protein was loaded on a equilibrated HisTrap column (Merck KGaA, Darmstadt, Germany), unspecific binding was avoided by an additional washing step with Washing buffer. Finally, His-tagged proteins which had bound to the HisTrap column were eluted in fractions of 1 ml with Elution buffer and stored at 4°C.

2.2.3.2 Affinity purification of antibodies

Coupling buffer: 0,1 M $NaHCO_3$, 0,5 M NaCl, pH 8.3; Elution buffer:100mM glycine pH 2.5.

For specific detection of protein levels, polyclonal antibodies from rabbit serum were used. Serum of immunized rabbits containing specific antibodies was already available. To gain high specificity of antibodies for the required protein, the antibodies from the serum were affinity purified using antigen coupled to cyanogen bromide (CnBr) activated Sepharose (Merck KGaA, Darmstadt, Germany). 5mg of CnBr-activated-Sepharose was diluted in 1,5 ml cold 1mM HCL, the solution was added to a PD10 desalting column (ThermoFisher Scientific,

Waltham, USA) and washed with additional 150 ml of 1mM HCL and equilibrated with 2 ml Coupling buffer. 5-10 mg protein diluted in Coupling buffer were added to the column and incubated for 90 min at RT on a rocking table for antigen coupling. Free reactive groups were blocked through incubation with 6 ml 1M ethanolamine, pH 8.0, overnight at 4°C. After washing the column with 0.1 M acidic acid containing 0.5 M NaCl, pH 4,0 and 0,1 M Tris containing 0.5 M NaCl, pH 8.0, subsequently in three repeated steps. The column was equilibrated with 10 ml 10 mM Tris pH 7.5 and 10 ml 10mM Tris pH 8.8. Finally, 1 ml of serum was added and unspecific binding compounds were washed from the column with 10 ml 10 mM Tris pH 7.5, 10 ml 10 mM Tris pH 7.5 containing 0.5M NaCl and with another 3 ml 10 mM Tris pH 7.5. Specific binding antibodies were eluted with 500µl Elution buffer in Eppendorf tubes already supplemented with 100 µl 1M Tris pH 8.8 for neutralisation of the solution. Antibody containing fractions were tested using Bradford reagent, pooled and rebuffered in 1x PBS via a NAP 5 column according to manufacturer's recommendations. Protein concentrations were determined using NanoDrop and aliquots were stored at -20°C

2.2.3.3 Biotinylation of purified antibodies

Since the antibody concentration yielded from affinity purification can often be very low, the antibody solution was concentrated by centrifugation in an AmiconUltra centrifugation tube (cut off 10 kDa) at 4000x g for 10 min at RT. For biotinylation of antibodies 200 µg of NAS-LC-biotin (Thermo fisher Scientific, Waltham, USA) were added to 2 mg of antibody in 1x PBS and incubated for at least 2 hours on ice. The antibody was loaded on a PD 10 column and eluted with 1x PBS, aliqouts were frozen and stored at -20°C.

2.2.3.4 Determination of protein concentrations

To determine the total protein concentration of a sample, such as cell or tissue lysate, colorimetric measurement with Bradford reagent from the BioRad Protein Assay Kit was performed according to manufacturer's recommendations. The assay was performed on a 96-well plate with both, the BSA standards (0,0 - 0,8 mg protein) and the samples applied as duplicates. The protein amount was measured spectrophotometrically at 280nm on a plate reader Plate reader (Multiscan Ascent ThermoFisher Scientific, Waltham, USA) and total protein concentration was calculated using the extinction coefficient for each protein gathered from ProtParam (www.expasy.org).

2.2.3.5 SDS-polyacrylamide gel electrophoresis

Loading dye: 0.3 M Tris/HCl, pH 6.8, 50 % (v/v) glycerol, 5 % (w/v) SDS, 10 mM EDTA, 0.1 % bromophenol blue; Running buffer: 250 mM Tris, 1.92 M glycine, 1% (w/v) SDS;

The SDS polyacrylamide gel electrophoresis (SDS-PAGE) is a frequently used technique for separation of proteins by their size. Since the anionic detergent SDS leads to unfolding of the protein and binds to its hydrophobic and hydrophilic groups applying a negative charge to the SDS-protein complex, proteins can be distinguished by size when moved through a polyacrylamide gel matrix. For sample preparation 5-75µg protein were diluted in 1x Loading dye and protein disulfide groups were reduced by incubating with 100mM DTT combined with neutralised 50mM TCEP for 30 min. Then, proteins were denatured for 10 min at 94°C and 5-30µl of the samples were loaded on a gel along with pre-stained or unstained page ruler (Fermentas, ThermoFisher Scientific, Waltham, USA) for determination of molecular weight of the separated proteins. For the non-reducing SDS-PAGE either 4-20% Mini-Protean® or 18% Criterion® TGX Stain Free Precast gels (BioRad, Hercules, USA) were run for 30-45 min at 200 V according to manufacturer's recommendations. Since the Stain Free Precast gels include trihalo compounds that interact with tryptophane residues of proteins, they could be visualized by UV light after running the SDS-PAGE in a StainFree Imager (BioRad, Hercules, USA) and no protein staining was needed. Before imaging, gels were washed 2 times in aqua bidest.

2.2.3.6 Western Blot

Transfer buffer: 20 mM Tris, 0.02 % (w/v) SDS, 150 mM glycine, 20 % (v/v) methanol; TBS: 25 mM Tris, 150 mM NaCl, 2.7 mM KCl, pH 7,4; TBST: TBS, 0.05% (v/v) Tween 20, pH 7.4; Blocking buffer: 5% (w/v) milk powder and 1% (w/v) BSA in TBST; Stripping buffer: 0.1M NaOH, 1%(w/v) SDS, 100mM DTT

Western Blot is a method depending on antibody based specific detection of proteins which are separated by SDS-PAGE and transferred to a PVDF membrane by electrophoretic transfer. In this study wet-blot technique was used as well as a Semi-Dry blotting method. For preparation PVDF membranes (Macherey-Nagel, Düren, Germany) were equilibrated in methanol, ECL nitrocellulose membranes (Merck KGaA, Darmstadt, Germany) in aqua bidest. The wet-blot technique was performed in a blotting chamber (BioRad, Hercules, USA) for 120 min at 40 V

on ice. Semi-Dry Blot was performed in a Semi-Dry electrophoretic Transfer Cell (BioRad, Hercules, USA) at 25 V for 60 min. at RT. The membranes were washed 3 times with TBS and incubated for 1h in Blocking buffer at RT to avoid unspecific binding, followed by incubation with primary antibody diluted in Blocking buffer as described in (table 2.1.3) at 4°C overnight on a rocking table. Before adding the secondary horseradish-peroxidase coupled antibody (HRP-antibody, 1:5000) diluted in blocking buffer without BSA, the membranes were washed 5 times in TBST for approximately 5 min. each. After 1h of incubation with the secondary antibody at RT, the membrane was again washed 5 times with TBST. If augmentation of the antibody signal was needed, instead of HRP-coupled-antibody a biotin-labelled secondary antibody was added, followed by incubation for 1h at RT. Membrane was again washed 5 times in TBST, then HRP-coupled Streptavidin in TBS was added, incubated for another 1h at RT and membrane was finally washed 5 times in TBST. For protein detection by chemiluminescence, which was performed using the ChemiDoc system (BioRad, Hercules, USA), SuperSignal West Femto and Pico solutions (ThermoFisher Scientific, Waltham, USA) were added to the membrane in a dilution of 9:1according to manufacturer's recommendations. For re-staining of the PVDF-membrane, membranes were washed 2 times for 5 min in TBS, 2 times for 5 min in Stripping buffer and again 2 times in TBS, all steps performed on a moving incubator at RT.

2.2.3.7 Immunohistochemistry

Washing buffer: 0.25% Triton X-100 in PBS (PBST); Blocking buffer: 10% goat serum, 10% Triton X-100 in 240mM PB, 4M NaCl.

Lung tissue was fixed in 4% paraformaldehyde directly after harvesting and processed for paraffin embedding. Sections of 16µm were cut on a microtome and placed on slides coated with poly-l-lysine. Paraffin embedded slides of PBS-control lung and OVA-induced asthma lung tissue were kindly provided by the Renz group (Marburg). Staining was performed using a streptavidin-biotin-peroxidase detection system and visualized with aminoethyl carbazole as chromogen. Before staining, sections were deparaffinized and to inhibit endogenous peroxidases slides were incubated in 3% hydrogen peroxide for 10 min. Sections were washed for three times in PBS and non-specific antibody binding was blocked using 10% goat serum in PBS for 1h. Subsequently sections were incubated overnight at 4°C with the appropriate concentration of primary antibody in blocking buffer. In each case, negative controls comprising matched immunoglobulins in buffer alone were used. The following day the slides

were washed three times in PBS and then incubated with a biotinylated anti-mouse or anti-rabbit secondary antibody for 60 min at RT. For antigen staining a streptavidin-HRP system was utilized according to manufacturer's recommendations (Jackson, Cambridgeshire, UK). After incubation with the matching substrate aminoethyl carbazole (Invitrogen, ThermoFisher Scientific, Waltham, USA) for 5 min at RT sections were counterstained with Mayer's haematoxylin and covered with a drop of Mowiol mounting medium (Merck KGaA, Darmstadt, Germany) Finally, slides were evaluated by microscopy with a light microscope Telaval (Carl Zeiss Jena, Germany). Picture white balance was optimized using ImageJ (NIH, USA).

2.2.3.8 Immunofluorescence

Citrate buffer: 10 mM Trisodium citrate dihydrate, pH 6.0. Washing buffer: 0.25% Triton X-100 in PBS (PBST); Blocking buffer: 10% goat serum, 10% Triton X-100, 240mM PB, 4M NaCl.

For immunofluorescence analysis lung sections were deparaffinized using a descendent alcohol series (99,9%, 96%, 70%), washed in Aqua dest. and transferred to a glass cuvette filled with citrate buffer. Slides were heated in a microwave (600 W) for 3x3 mins. After cooling down for 20 mins. slides were washed in PBS 2x5 min. and non-specific antibody binding was blocked using blocking buffer for 1h at RT. Subsequently sections were incubated overnight at 4°C with the appropriate concentration of primary antibody in blocking buffer. The following day slides were washed 2 times in washing buffer and incubated with fluorescence-coupled secondary antibody 1:200 in Blocking buffer for 1h at RT in the dark. Subsequently slides were again washed 2x in washing buffer and nuclear staining was performed adding 1:10.000 Hoechst 33342 (Merck KGaA, Darmstadt) in PB for 10 min. Finally, after a last washing step, slides were mounted with Mowiol. Images were acquired with Leica SP8 confocal microscope using *Leica software* (Leica AG, Wetzlar, Germany). Images were analysed in ImageJ and assembled in Inkscape.

3 Results

3.1 Generation and evaluation of a sensitive Trx1 antibody

Western blot analysis is considered a relevant tool in detection of protein levels in different types of samples. For the aims of this study the comparison of protein levels of Trx family proteins in different compartments was crucial, therefore we aimed to generate a sensitive antibody against human as well as mouse Trx1. For antibody generation, serum of immunised rabbits (human and mouse Trx1) was already available. To obtain highly specific antibodies we used affinity purification as described in 2.2.3.2. Antibodies were biotinylated with NHS-LC-biotin according to protocol described in 2.2.3.3. Simultaneously bacteria were transfected with hTrx1-pet15b/mTrx1 and cultivated expressing Trx1 His-tag-coupled protein using a 5l Fermenter (2.2.1.4). The freshly purified mTrx1 as well as hTrx1 antibodies were then tested using the antigens yielded from protein purification in the ÄKTA FLPC prime system (2.2.3.1), data not shown.

3.2 Intracellular levels of selected Trx family proteins in allergic airway inflammation

At the Department for Laboratory Medicine at the University of Marburg, Renz and co-workers, namely Christoph Hudemann, PhD, established a mouse model for induction of allergic airway inflammation by sensitization with OVA (2.1.6.1) to investigate expression levels of selected Trx family proteins in a murine model for allergic airway inflammation. OVA sensitization was performed by subcutaneous injections whereas induction of inflammation by OVA-challenge was achieved by OVA aerosol inhalation. Control mice were sensitized and challenged with PBS. In a second group mice with OVA-induced asthma were treated with i.p. injection of 40µg of Grx2 (2.1.6.1) to display changes in asthmatic phenotype and bronchial expression of the investigated proteins.

In this study we evaluated the expression patterns of Trx family proteins (Trx1, Trx2, Grx1, Grx2, Prx2, Prx4) in the samples provided by the Marburg laboratory utilizing Western blot technique as well as immunohistochemistry. In the following lung tissue samples were considered to represent the intracellular compartment whereas BAL-samples reflect the extracellular compartment. Lung tissue and BAL-samples of PBS-control and OVA-mice as

well as Grx2-treated mice were generated (described in 2.1.6.2 and performed by Christoph Hudemann) and transported from Marburg to the laboratory in Greifswald under close control of temperature and constant cooling of the samples. Samples were lysed in NP40 buffer and vortexed until a homogenous solution was reached and stored at -21° degrees. Total protein concentration of cell or tissue lysate was measured using colorimetric measurement with Bradford reagent from the BioRad Protein Assay Kit according to manufacturer's recommendations as explained in 2.2.3.4. 40 µg of cell lysate were reduced with 100 mM of DTT for 20 min at room temperature and another 10 min at 94° Celsius. Subsequently, 50 mM TCEP was added to reach complete disulfide reduction. The prepared samples were then analysed by Western blot as described in 2.2.3.6. All antibodies used in this study are listed in table 2.1.3. As loading control, the housekeeping enzyme glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) was utilized.

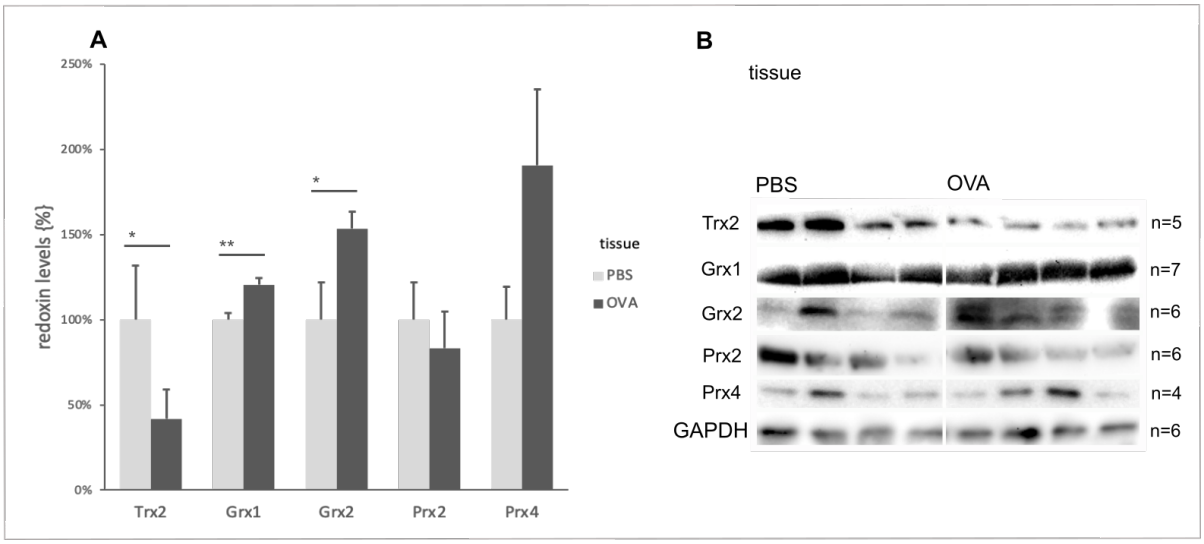

Figure 2 - intracellular expression pattern of Trx family proteins in lung tissue samples: *protein levels of 5 redoxins were detected by Western Blot in PBS and OVA lung, control GAPDH (B), quantification of protein levels using imageJ, regarding PBS control as 100%, shown are means, error bars indicate SD, * p<0.05, ** p<0.01, *** p<0.00, n = 4-7, as indicated above, Student's T-test.*

Protein levels of Trx2, Grx1, Grx2, Prx2 and Prx4 in the control and OVA-lung tissue samples were investigated. For Prx2, no significant changes in protein levels were detected in PBS- and OVA-lungs. In contrast, Trx2 levels decreased in OVA-lung tissue samples in comparison to control-lung (41.8%, p value = 0.047, unpaired t-test analysis). Protein levels of Grx1(120.6%, p= 0.003) and Grx2 (153.5%, p= 0.036) were significantly augmented upon lung inflammation. Although Prx4 levels seemed to be increased in OVA-lung tissue, but this effect was not considered significant due to low statistical power of the results (190%, p value = 0.1, unpaired

t-test). Grx2 specific bands were detected at expected protein size level with an affinity purified sensitive serum antibody (1:500). Intriguingly, Grx2 showed staining for a second protein band above the band detected in the PBS lung (3.2.1).

3.2.1 Grx2 levels are augmented in allergic airway inflammation

Western blot analysis indicated a significant increase of intracellular Grx2 levels in OVA-lung compared to the Grx2 levels detected in the control-lung (334%, p= 0.0001). Intriguingly, in a total number of 6 mice suffering from OVA-induced allergic airway inflammation a second Grx2 band could be detected. This specific staining above the expected 17.3 kDa band for Grx2a matched the Grx2c isoform. However, this could not be detected in the PBS-treated control mice. Western blot results suggested the presence of Grx2a accompanied by the Grx2c isoform as a unique feature of the inflamed lung tissue.

Furthermore, total Grx2 levels were also augmented in lung tissue of OVA-mice treated with 40 µg of recombinant Grx2 and the increase could be attributed to the additional Grx2c staining in Western blot. To evaluate the accuracy of the second isoform stained in the previous experiments, we aimed at blocking Grx2 antibody with antigen prior to staining. Grx2 affinity purified serum antibody (1:500) was blocked with 100 µg of mGrx2c and incubated for 2h at RT. Subsequently Western blotting of blocked Grx2 showed the disappearance of the second isoform staining. This underlines the specificity of serum Grx2 antibody and supports the detection of a second isoform in intracellular samples from OVA-induced allergic airway inflammation.

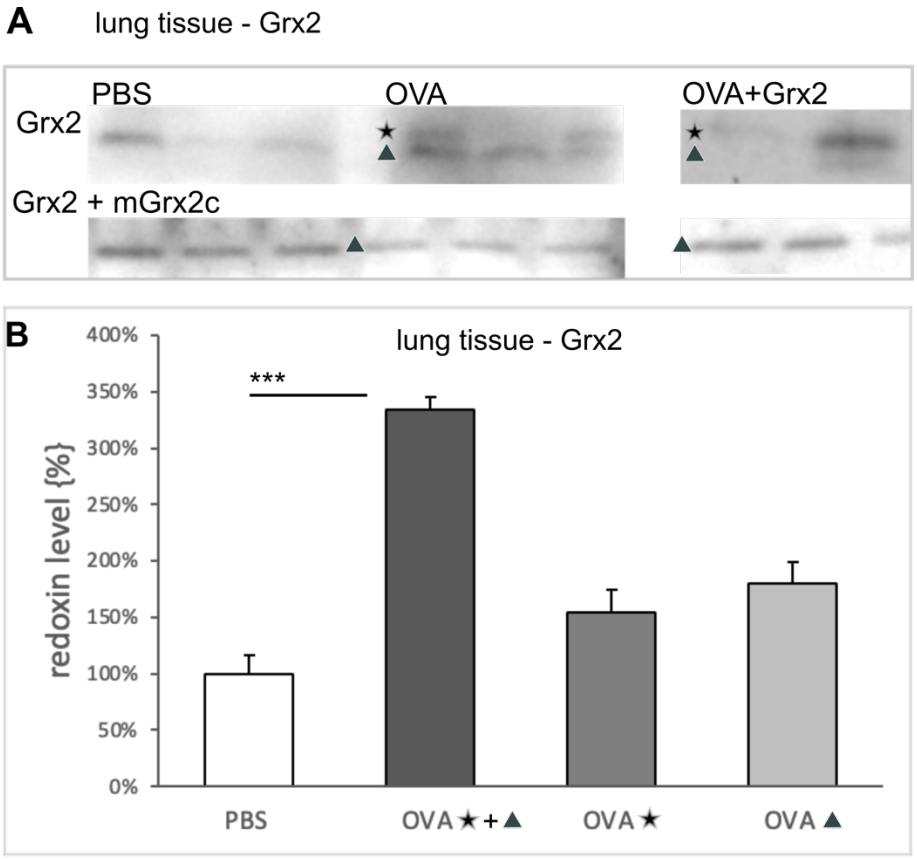

Figure 3 – Grx2 level in allergic airway inflammation: ***A*** *Western Blot analysis of lung tissue samples from PBS mice, OVA challenged mice and OVA mice treated systemically with Grx2 using Grx2 affinity purified antibody*

★marks the staining for Grx2c isoform (upper band), ▲marks suspected Grx2a isoform (lower band); lower series of Western Blots: blocking Grx2 antibody with mGrx2c antigen removes the second isoform; ***B*** *Quantification of Grx2 levels from Western blot, regarding PBS as 100% comparing with OVA Grx2 total and each isoform respectively, n = 6, error bars indicate mean ± SD, significant differences between PBS control and OVA group are indicated, *** p<0.001, Student's T-test.*

3.3 Extracellular levels of selected Trx family proteins in allergic airway inflammation

As described in chapter 1.3., intracellular functions of Trx family proteins have been widely explored in the past, but regarding extracellular functions of Trxs, Grxs and Prxs we are just scratching at the surface. Therefore, investigating extracellular samples in a murine model of OVA-induced allergic airway inflammation is a cornerstone of this study. To detect protein

levels in extracellular compartments, BAL samples were harvested by rinsing of trachea and bronchial tubes with PBS (2.1.6.3). Total protein concentrations of BAL samples were measured using colorimetric measurement with Bradford reagent from the BioRad Protein Assay Kit, according to manufacturer's recommendations as explained in 2.2.3.4. 20 µg of sample was reduced with 100 mM of DTT for 20 min at room temperature and another 10 min at 94° C. Subsequently freshly NaOH neutralised 50 mM TCEP was added to reach further disulfide reduction. The prepared samples were then analysed for protein levels of Trx1, Grx1, Prx1 and Prx4 by Western blotting. 18% Criterion Stain-free precast gels (BioRad, Hercules, CA, USA) were used, since they enable application of 45 µl of sample and for the BAL fluid samples we sought to apply the highest possible sample quantity to the gel.

Western blot results show an increase of several Trxs in OVA samples compared to PBS control. Protein levels of Trx1 (290%, p= 0.012), Grx1 (354%, p= 0.0012), Prx1 (136%, p=0.017), Prx2 (266%, p= 0.019) and Prx4 (for details on Prx4 section 3.3.1) significantly increased in OVA samples. As already experienced for Prx2 analysis in lung tissue samples, Prx2 antibody showed a repeated and even staining at approx. 70 kDa not fitting the expected protein mass of 21 kDa. However, since two independent Western Blot experiments showed specific staining for Prx2 at 70 kDa, detection of Prx2 in disulfide-linked homodimeric form was assumed.

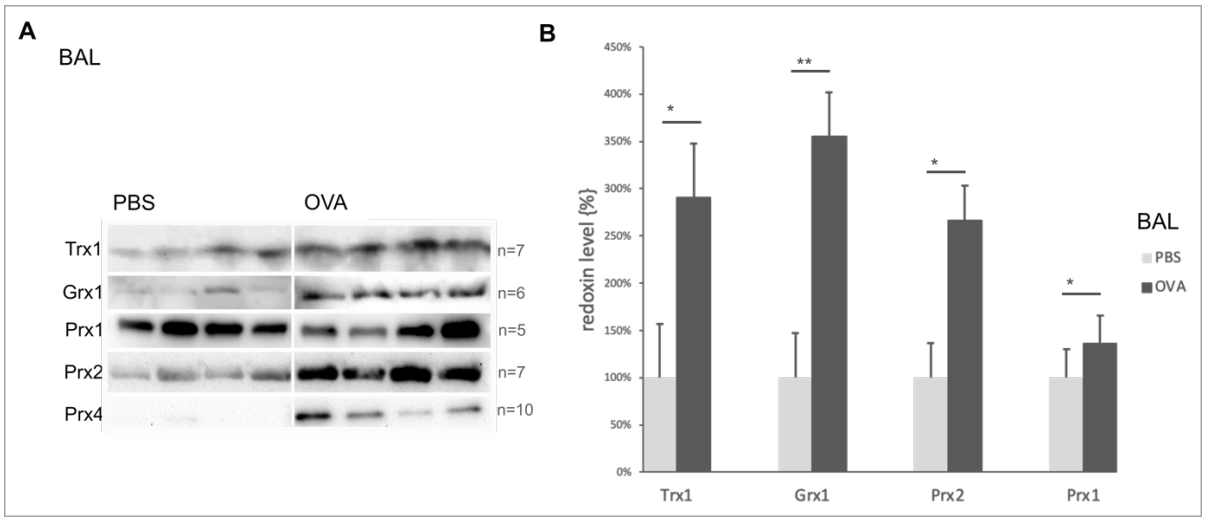

Figure 4 - extracellular redoxin levels in BAL samples: ***A*** *samples from bronchoalveolar lavage from PBS control and OVA-challenged mice (n=6) were pre-treated with DTT/TCEP to reduce disulfides and analysed by Western Blot;* ***B*** *Quantification of Western Blots was performed using ImageJ, data are presented in % regarding PBS mice as 100%, n = 5-10 as indicated above, shown are means, error bars indicate ±SD, significant differences between PBS control and OVA group are indicated *p<0.05,*

*** p<0.01, ***p<0.001 (Student's T-test). For quantification of Prx4 levels see fig.5.*

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3.3.1 Prx4 is detectable in the extracellular compartment in allergic airway inflammation but not in healthy mice

For further understanding of different expression patterns of thioredoxin family proteins we subsequently analysed the presence of Prx4 in the extracellular compartment of the lung and Western blot technique revealed unexpected results: Prx4 – which has already been described to be secreted to the extracellular compartment – could be identified in BAL samples upon airway inflammation, however it could not be detected in the healthy control-lung. Results were validated by two independent Western blot experiments with n = 5 and n = 5 different mouse samples respectively.

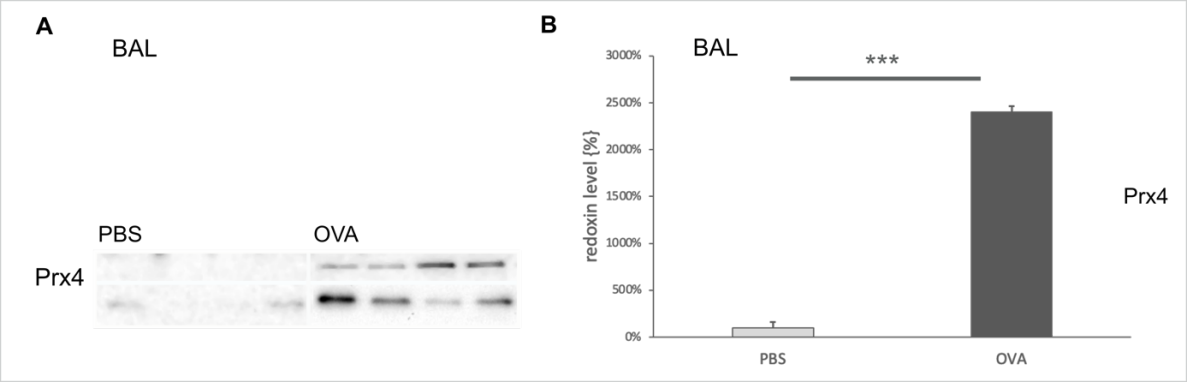


***Figure 5 - extracellular Prx4 levels in BAL samples:** **A** BAL samples from PBS control and OVA-challenged mice were pre-treated with DTT/TCEP to reduce disulfides and analysed by Western Blot, two independent WB experiments shown; **B** Quantification of Western Blots was performed using ImageJ, data are presented in % regarding PBS mice as 100%, error bars indicate mean ± SD, significant differences between PBS control and OVA group are indicated, n =5 in two independent Western blot experiments, ***p<0.001, Student's T-test.*

To further investigate the phenotype displayed in the murine model of OVA-induced allergic airway inflammation we performed immunohistochemistry. The slides containing lung tissue of PBS-control mice and OVA-mice were kindly provided by the Renz group, namely Christoph Hudemann, PhD in Marburg and were further processed and stained in the laboratory in Greifswald (see also 2.2.2.7). Representative HE-staining gives a first indication of the

histological changes observed upon allergic inflammation. The images illustrate the induction of airway thickening, mucus production and inflammatory cell migration after onset of OVA-induced airway inflammation (figure 9). Subsequently, Histology indicates an increase of Prx4 in OVA-lung in comparison to control. In addition, IHC showed an increase and altered protein localisation for Prx4. For instance, alveolar-macrophages and cells of the pseudostratified epithelium of bronchioles displayed a higher staining for Prx4 in comparison to the control lung tissue (figure 6). These findings strongly support Western blot results and indicate a different distribution pattern of Prx4 in the epithelium and alveolar immune cells upon OVA-induced allergic inflammation.

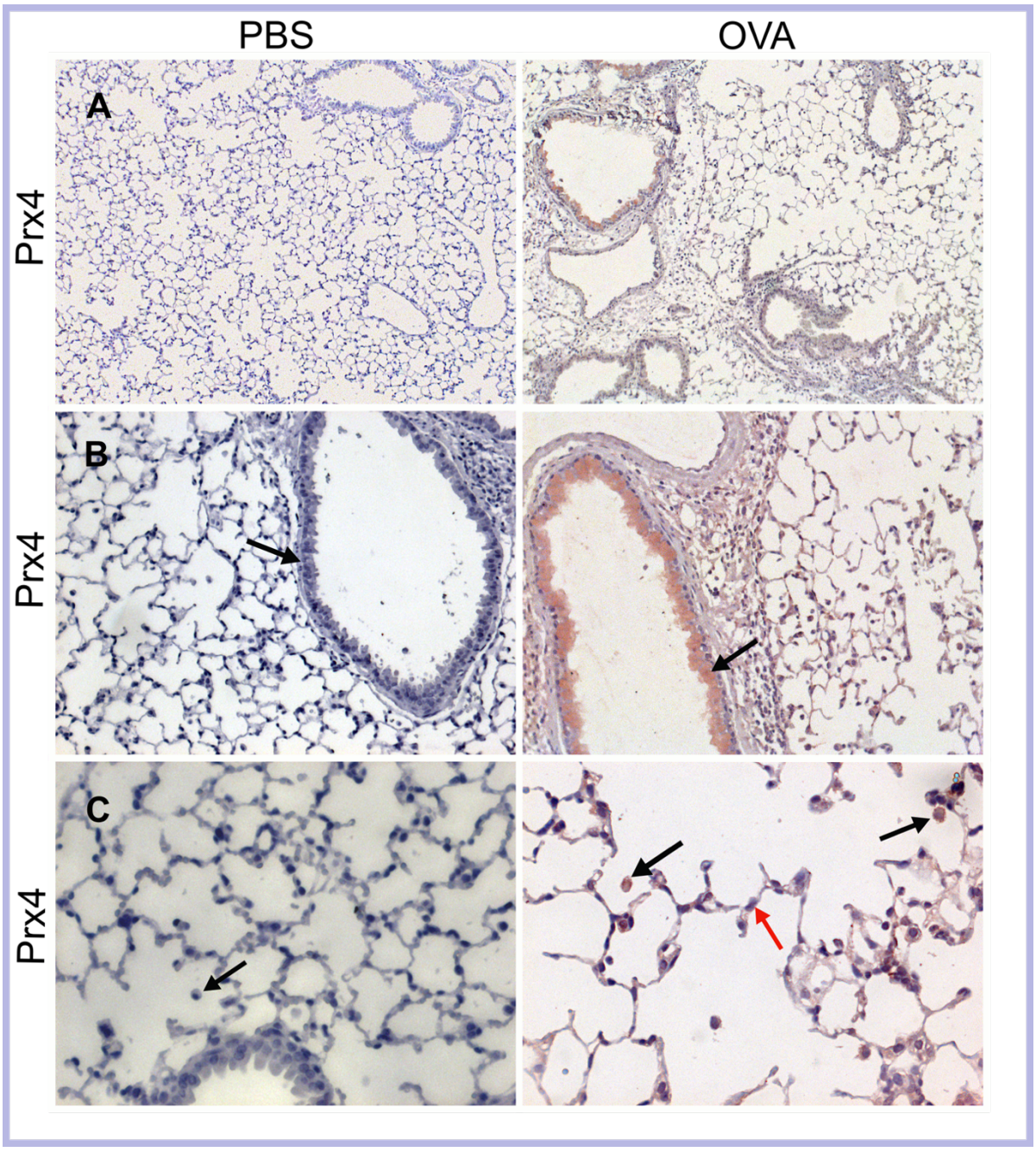
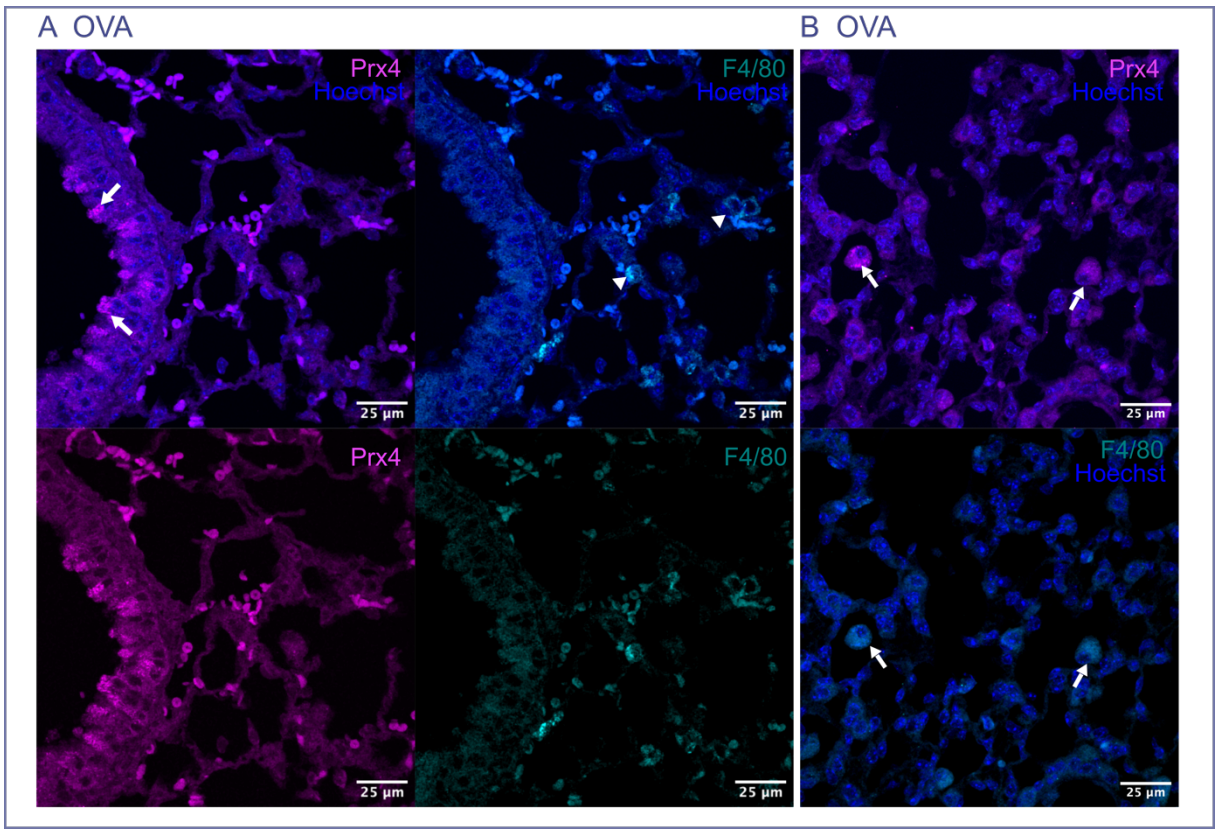


Figure 6 – Immunohistochemistry of mice lungs: *lung sections from PBS control and OVA-challenged mice were analysed by immunohistochemistry for Prx4;* ***A*** *100x magnification;* ***B*** *black arrows mark pseudostratified columnar epithelium of the primary bronchioles, Prx4 staining of bronchiolar epithelium increases after OVA-challenge, 200x magnification;* ***C*** *black arrows mark alveolar macrophages, showing increased Prx4 staining in OVA-lung, red arrow: type I pneumocyte, 400x magnification.*

To corroborate our findings from IHC images, we performed IF-staining against Prx4 in OVA-lung and control mice accompanied by co-staining for F4/80 – a valuable marker for activated macrophages. IF images verified that infiltrating macrophages detected by F4/80 were positive for Prx4 upon inflammation, whereas Prx4 positive macrophages could not be detected in control lung sections. Interestingly, we could also demonstrate that Prx4 staining was increased in epithelial cells of larger and smaller bronchioles indicating a redistribution of the protein to secretory sites of the cells (figure 7). The IHC and IF-staining presented was performed for lung sections from at least n = 6 different animals for each condition (OVA/PBS) and representative images are displayed.



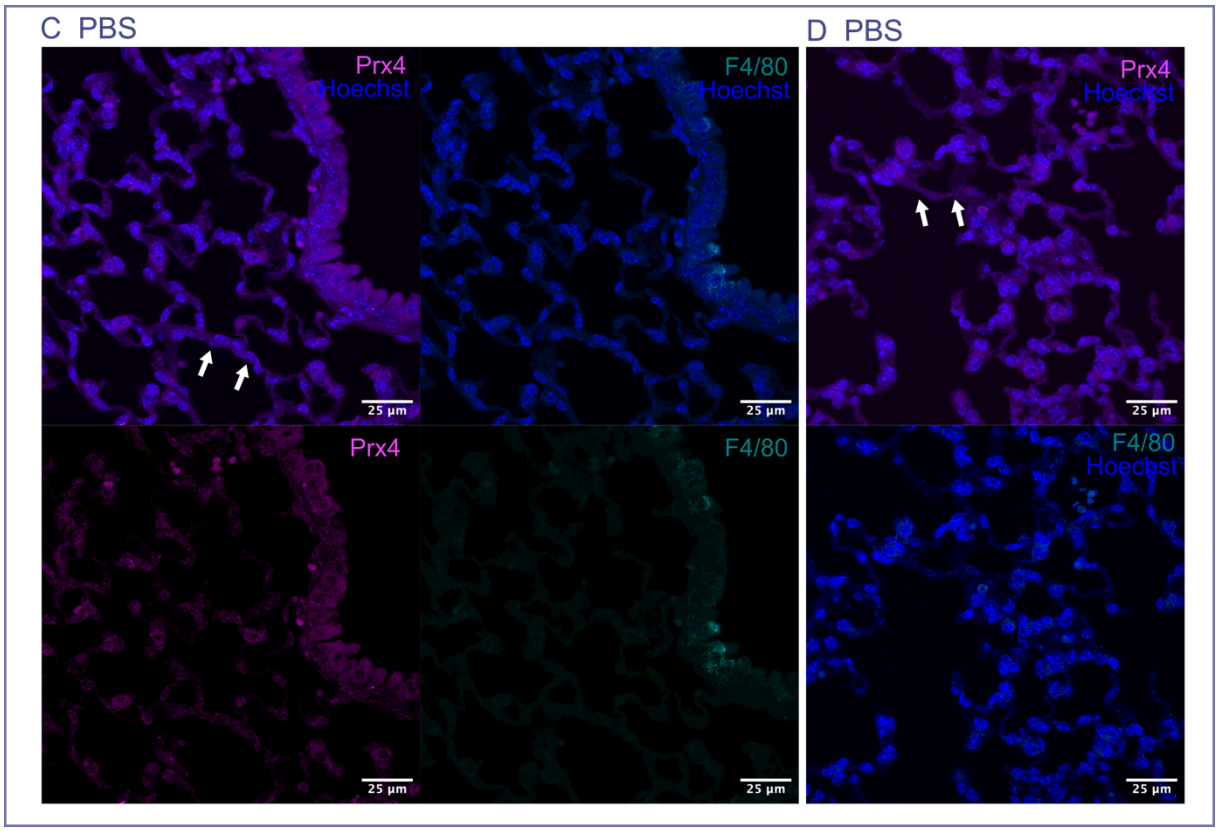


Figure 7 – Immunofluorescence of mice lungs: *inflamed OVA-exposed lungs (OVA) and control lungs (PBS) were stained for Prx4 and the macrophage-marker F4/80 using immunofluorescence, nucleic acid stain was performed utilizing Hoechst 33342.* ***A:*** *OVA lung with enhanced mucus production, arrows indicate Prx4 in upper layer of pseudostratified epithelium of bronchioles, arrowheads highlight macrophage staining for F4/80.* ***B:*** *OVA lung with thickening of bronchiolar epithelium and infiltrating immune cells, arrows indicate corresponding staining of macrophages for F4/80 and Prx4.* ***C:*** *PBS lung bronchus with a representative section of healthy alveolar structure (arrows).* ***D:*** *no signs of infiltrating immune in cells F4/80 staining.*

To investigate, whether the increase of Prx4 is accompanied by alterations of other Trxs involved in inflammatory response we stained the corresponding lung sections with sensitive antibodies against Nrx and Prx2. The results suggest a constant expression of Nrx and Prx2 in control lung and upon allergic airway inflammation.

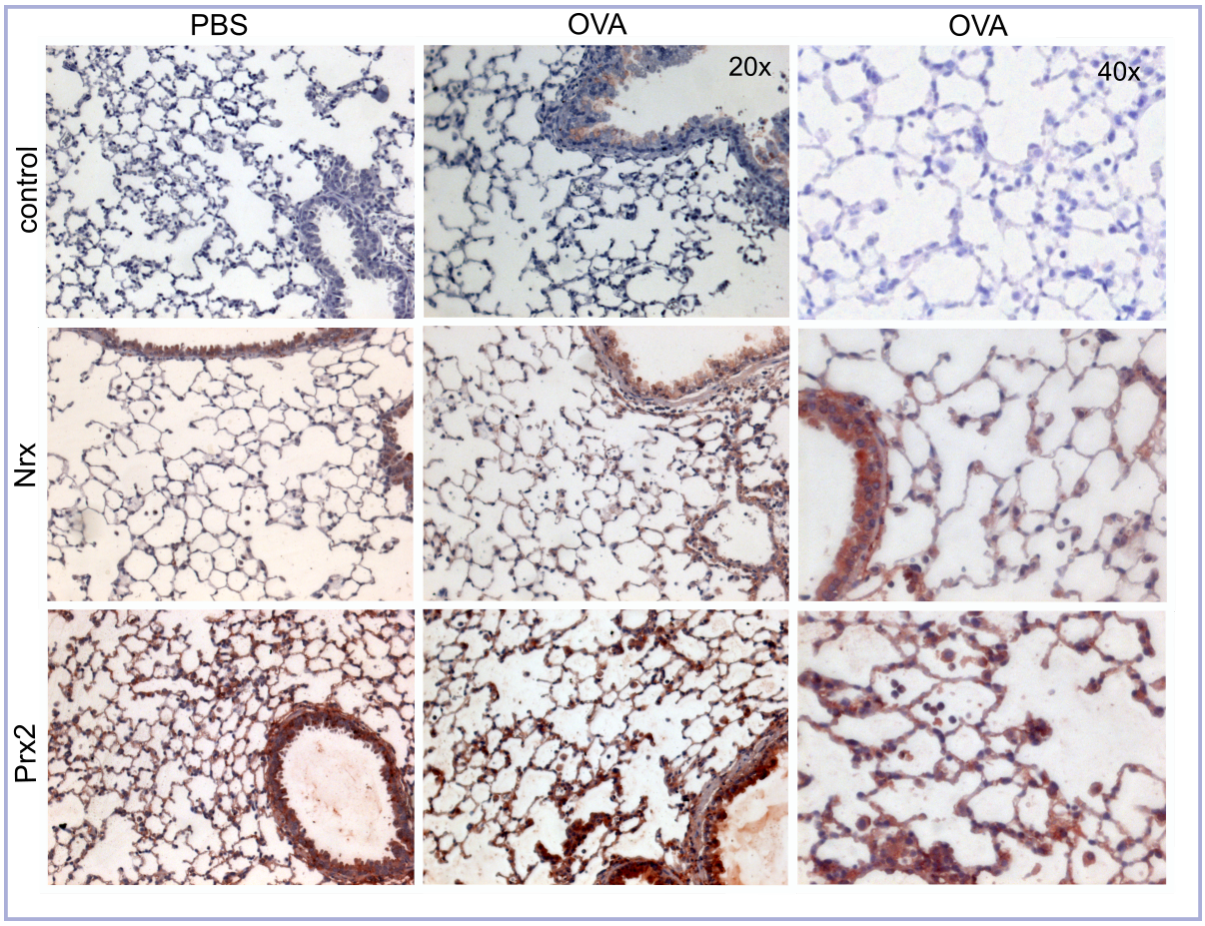


Figure 8 – Immunohistochemistry of mice lungs: *lung sections from PBS control and OVA-challenged mice were stained with Prx2 and Nrx compared to control staining; magnifications 200x and 400x are displayed.*

3.4 Regulatory role of selected redoxins in treatment of allergic airway inflammation

Experiments from various groups have already established the crucial role of Trx1 in allergic airway inflammation (Ito et al., 2011). So far, function of Grxs have not been investigated in a model of lung inflammation, therefore, in collaboration with Christoph Hudemann in the Marburg laboratory, we wanted to investigate Grx2 function in acute airway inflammation. After onset of OVA-induced allergic airway inflammation one branch of Balb/c mice were treated with 40 µg of recombinant redoxin for 5 days respectively on day 23-27.

In this study, we investigated the alterations in Trx family protein levels in PBS control lung, OVA-lung and OVA+Grx2 lung. Samples were prepared as described above (2.2.3.6). Protein levels were subsequently assessed using Western Blot technique.

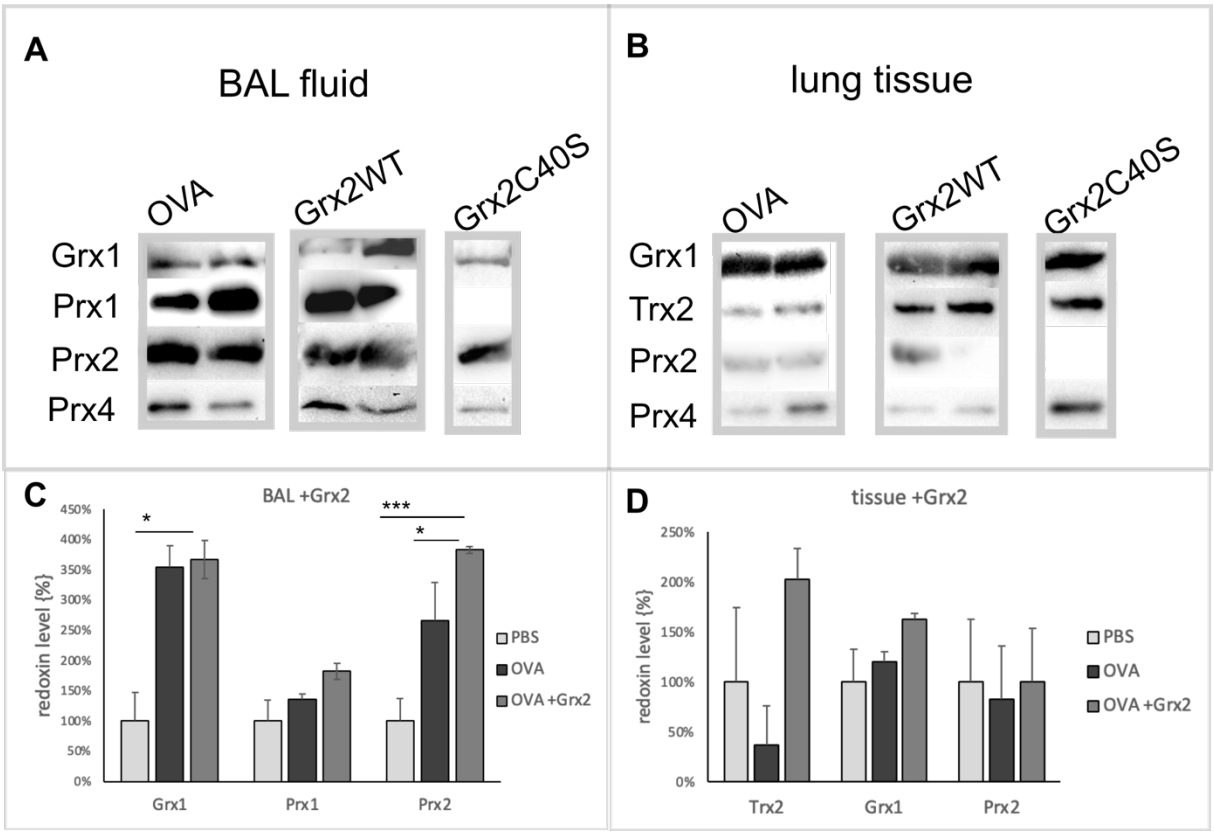


Figure 9 - Western Blot analysis of BAL fluid and lung tissue after treatment with different Grxs, A - B: *Western Blot analysis of BAL (**A**) and tissue samples (**B**) from OVA mice treated with Grx2WT (n = 4) or Grx2C40S (n = 2) respectively, in comparison to OVA without treatment (n = 6);* ***C-D:*** *quantification of BAL (**C**) and tissue samples **(D)**, samples of OVA+Grx2WT lung in comparison to PBS and OVA without treatment, data are presented in % regarding PBS mice as 100%, error bars indicate mean ± SD, significant differences between PBS control and OVA+Grx2 group are indicated *p<0.05, **p<0.01, ***p<0.001 (Student's T-test);*

Protein level of Grx1, Prx1, Prx2 and Prx4 displayed no significant changes upon treatment with Grx2WT compared to OVA conditions in BAL fluid. However, in comparison to PBS control Grx1 and Prx2 levels significantly increased in OVA+Grx2WT lung. Moreover, Prx2 increased significantly after treatment with Grx2 in comparison to OVA treatment alone (figure 8). Grx1 appears to be reduced after treatment with Grx2C40S mutant. Due to the small sample number of animals treated with the Grx2C40S mutant (n = 2), quantification of Western blot results was not performed. Evaluation of protein levels in lung tissue samples of OVA+Grx2WT

mice indicated an increase in Trx2 compared to OVA samples, but changes were not significant. Protein levels of Grx1 and Prx2 were not altered under different treatments Grx2WT or Grx2C40S. Intriguingly, Prx4 levels in BAL samples increased significantly upon treatment with Grx2 compared to OVA samples. Not only the onset of inflammation but also the administration of recombinant Grx2 augmented Prx4 levels in extracellular samples. Compared to Grx2WT the treatment with Grx2C40S mutant displayed a decrease in extracellular Prx4, although this was not statistically significant (figure 10).

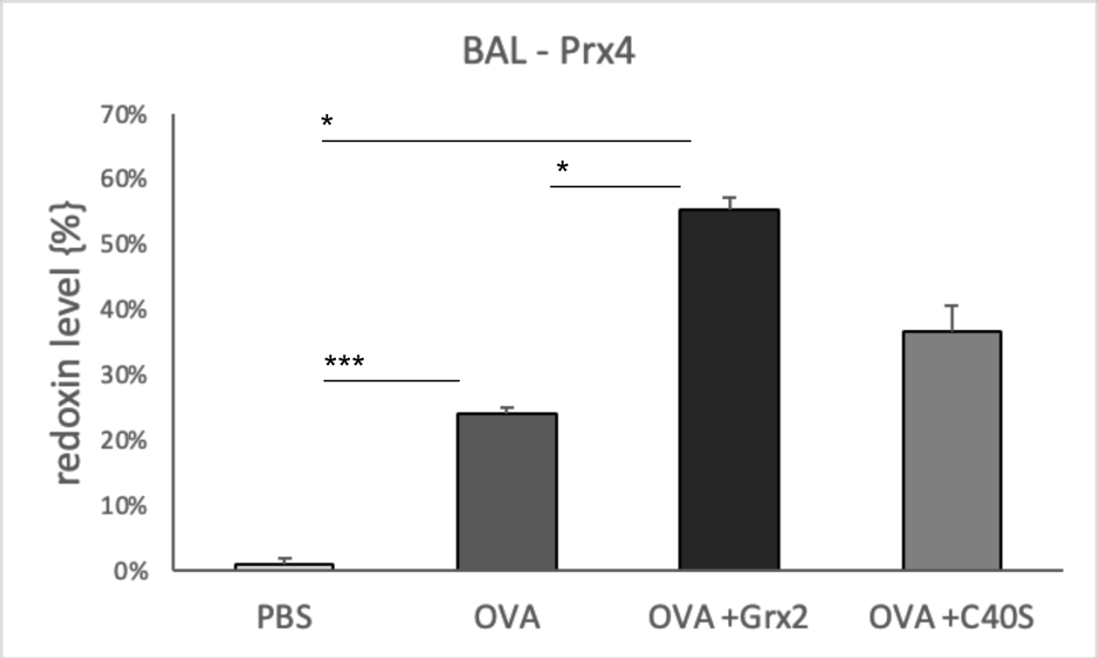


Figure 10 – Quantification of Prx4 levels in BAL upon different conditions: *Prx4 levels detected in BAL fluid by Western Blot with a sensitive antibody were quantified using ImageJ software, PBS was regarded as 1% to simplify data presentation, Prx4 increased in OVA lung (24%, p=0.0001) and after treatment with Grx2WT (55%, p= 0.04) compared to PBS control. Prx4 increased in Grx2 treated mice compared to OVA (p= 0.04); Prx4 decreased after treatment with Grx2C40S mutant compared to treatment with Grx2WT (low statistical power, p=0.08); OVA mice treated with Grx2WT (n = 4) or Grx2C40S (n = 2),OVA without treatment and PBS (n = 6); error bars indicate mean ± SD, significant differences between groups are indicated * p<0.05, ** p<0.01, ***p<0.001, Student's T-test;*

However, we did not detect substantial changes in all Trx levels after Grx2 treatment and this observation raised the question whether Grx2 administration influences the set of immune cells present in BAL fraction in comparison to PBS, OVA and the Grx2C40S-mutant, lacking the capacity to catalyse the dithiol switch. Therefore, analysis of total BAL cell count, eosinophils, macrophages and lymphocytes was performed. With a flexible tracheal cannula, BAL samples

were yielded from balb/c mice in 1ml PBS. Total cell count and individual cell count was determined using FACS. Total BAL cell counts significantly increased in OVA compared to PBS lung. Grx2 treatment significantly decreased BAL cell numbers whereas the redox inactive Grx2-C40S mutant did not show the capacity to reduce total BAL cell count. Significant induction of eosinophils and lymphocytes was also shown in OVA lung compared to PBS control. Eosinophil numbers were reduced under Grx2WT treatment and increased again under Grx2C40S administration. Intriguingly cell counts of macrophages did not change significantly between all analysed conditions.

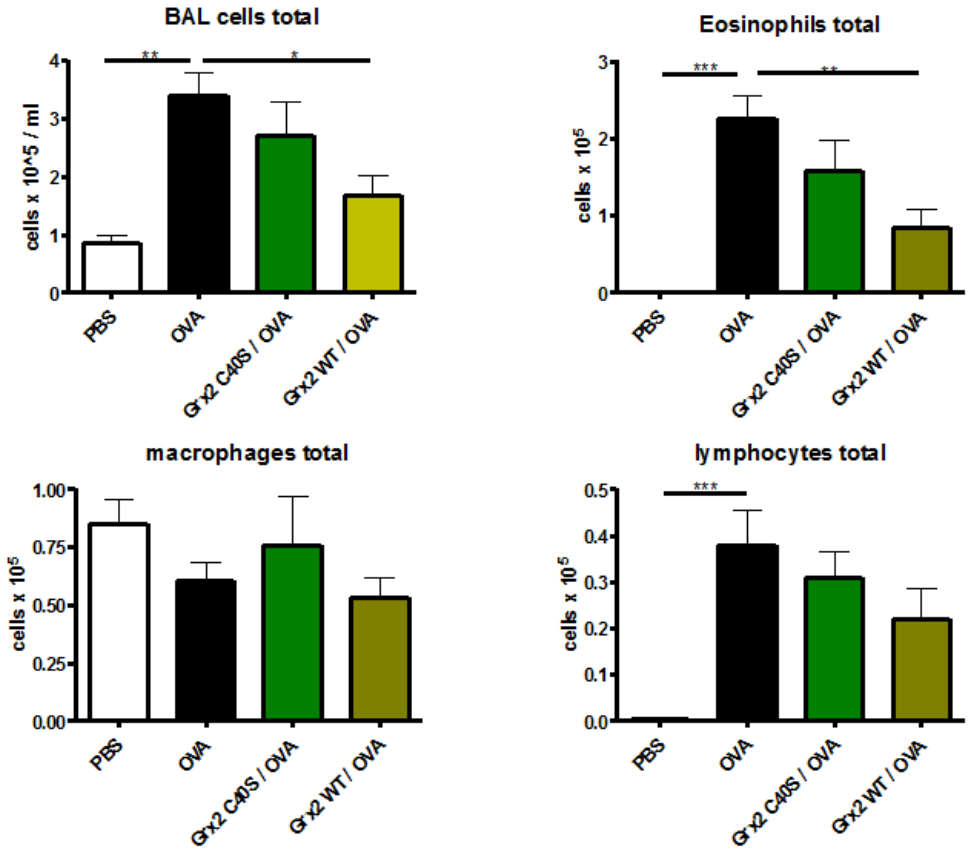


Figure 11 – Grx2 treatment reduces total and differential immune cell count in BAL samples*: cell count of total BAL cells, eosinophils, macrophages and lymphocytes in PBS control lung compared to lung after OVA-sensitization and treatment with Grx2WT or Grx2C40S, data from n=5 mice are shown as means ± SD, significant changes are marked between PBS and OVA (**p<0,01, ***p< 0,001) and OVA and protein treatment (*p<0,05), total BAL cells and eosinophils reduced under Grx2WT treatment, macrophage cell count did not change significantly under different conditions.*

For further understanding of changes in allergic phenotype Immunohistochemistry was performed with slides of lung tissue samples kindly provided by Renz group at the Marburg laboratory. Representative HE-staining of PBS, OVA and OVA+Grx2WT lung tissue displayed

a decrease in histological key features of allergic airway inflammation such as hyperplasia of goblet cells, mucus gland hyper-secretion, cell sloughing and migration of immune cells under Grx2 treatment.

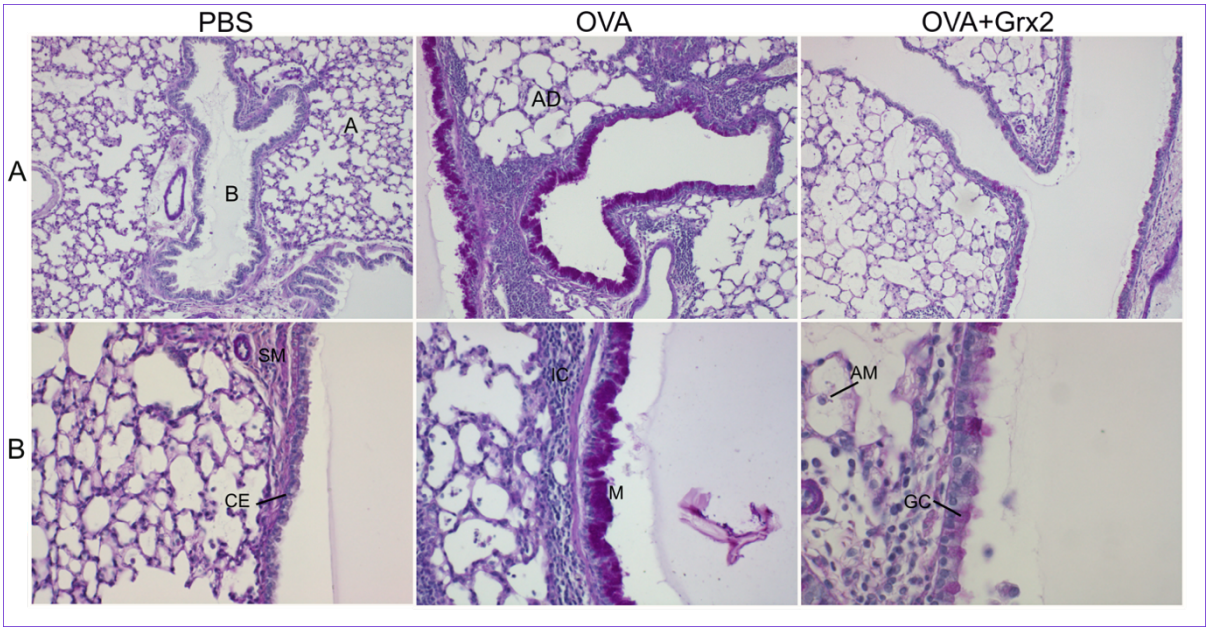


Figure 12 - Representative Haematoxylin and Eosin (HE) Staining *of histological changes after OVA-challenge and onset of allergic airway inflammation in comparison to PBS control lung and decrease of allergic key features after Grx2 treatment.* ***A*** *100x magnification, B=primary bronchioles, A= alveoli, AD = alveolar duct containing smooth muscle cells,* ***B*** *200x magnification, CE=pseudostratified columnar epithelium of the primary bronchioles, SM = smooth muscle cells in the Tunica muscularis, IC = immune cells in the Lamina propria, M = Mucus secreted from goblet cells, GC = goblet cells, AM = alveolar macrophages within alveoli.*

To gain better understanding of functional associations between Grx2 treatment and changes in histological appearance, IHC for HIF-1α and pro-caspase-3 were performed. Activation of Hif-1α itself is known to be redox regulated and interaction with Trxs has been established (J. Zhou et al., 2007). Histological evaluation of HIF-1α presence showed a decrease in OVA+Grx2 lung, comparable to staining for HIF-1α in PBS-lung, suggesting redox regulation of HIF-1α through Grx2. However, pro-caspase-3 levels were found to be increased in OVA-lung and decreased in OVA+Grx2 lung. Results indicate reduced apoptotic activity in the OVA+Grx2 lung. Caspases are important players in initiation and exertion of programmed cell death. Therefore, pro-caspase-3 has become a valuable marker for programmed cell death induced by various stimuli and in this study, we detected pro-caspase-3 presence in lung tissue samples to get an insight into cellular apoptotic activity. Immunostaining for pro-caspase-3 revealed decreased protease levels in OVA+Grx2-lung in comparison to OVA-lung, suggesting

reduced apoptotic activity after application of recombinant Grx2 intraperitoneally.

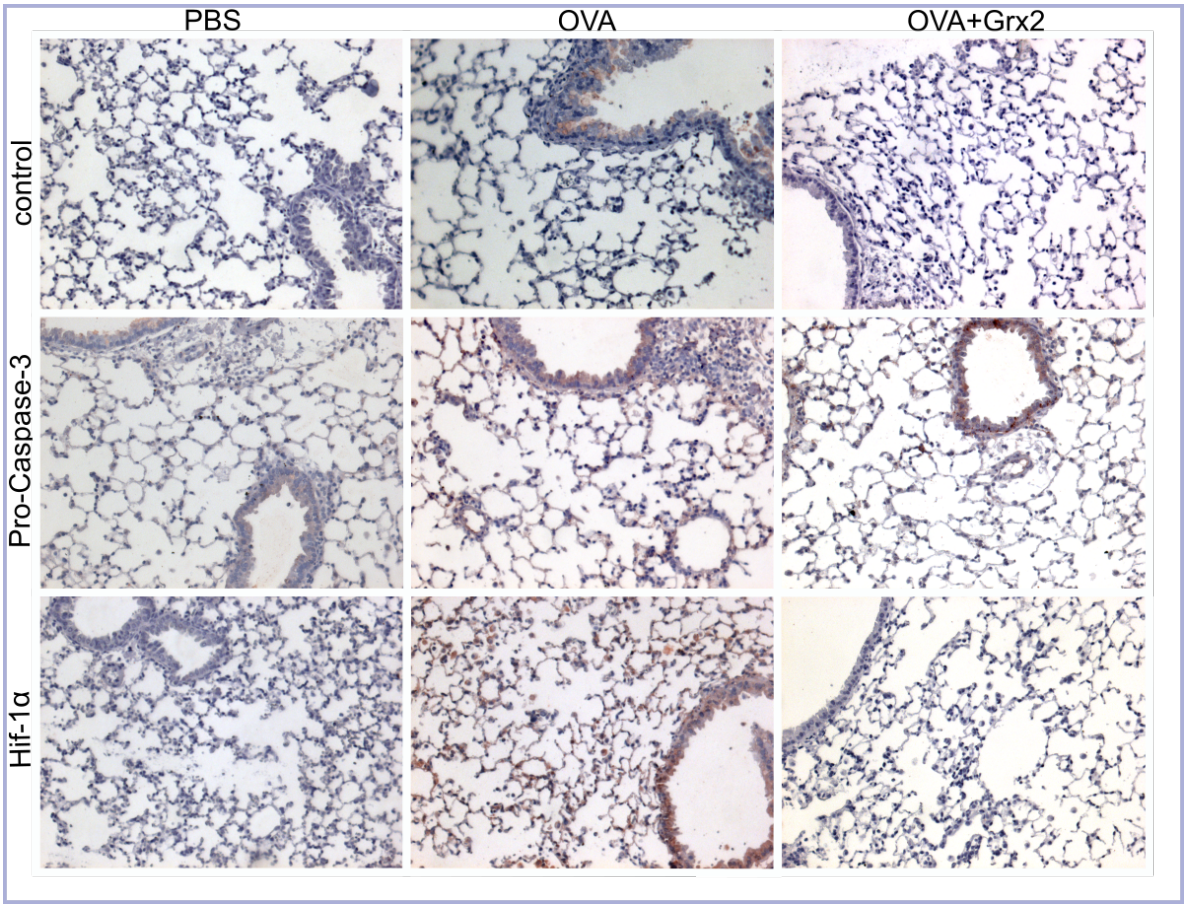


Figure 13 – Grxs modulate protein expression in allergic airway inflammation*: lung sections of mice stained for Hif-1α, which is known to be redox regulated, shows a decrease during Grx2 treatment, comparable to control-lung. Pro-Caspase3 levels are increased in OVA-lung and decreased in OVA+Grx2-lung, indicating reduced apoptotic activity in the OVA+Grx2 lung, magnification 200x.*

Pulmonal uptake of recombinant Grx2 was investigated by immunostaining with a biotinylated His-tag-antibody (1:500 anti-rabbit AB, 2.1.3.). Only the recombinant Grx2 applicated via intraperitoneal injection contains a His-tag and can thus be differentiated from autologous Grx2. Protein level of his-tagged Grx2 was augmented in OVA+Grx2 lung, underlining potential uptake of Grx2 in allergic airway inflammation. Corroboratively, IHC displayed a clear staining for His-tag antibody in bronchial epithelial cells as well as alveolar cells after treatment with recombinant Grx2WT.

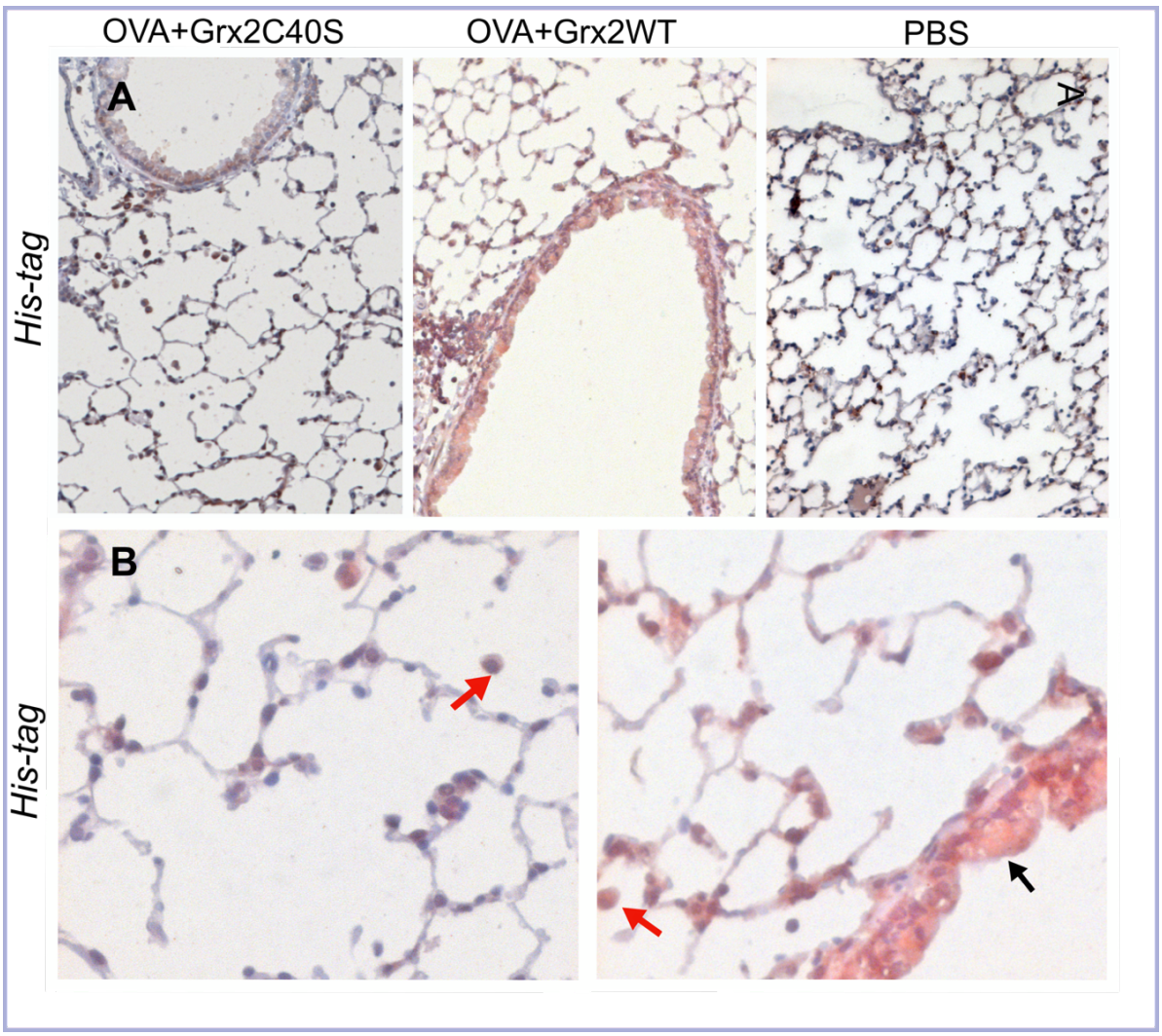


Figure 14 - Protein level of intraperitoneal applicated recombinant Grx2: *pulmonal Grx2 uptake was assessed by immunohistochemistry using a specific His-tag-antibody,* ***A:*** *comparison of Grx2C40S and Grx2WT in lung sections, staining intensity of Grx2C40S resembles PBS, Grx2WT lung shows a stronger His-tag staining, indicating an increased Grx2 uptake, 20x magnification;* ***B:*** *direct comparison of Grx2WT and Grx2C40S staining, alveolar macrophages (red arrows), pneumocytes type I and the pseudostratified bronchiolar epithelium (black arrow) show a strong His-tag staining in Grx2WT sections but not in Grx2C40S, magnification 400x.*

3.5 Cytokine expression in macrophages from allergic airway inflammation after treatment with Trx1 and Grx2

To further explore the influence of extracellular Trxs as possible cytokine-like proteins in the inflamed lung, we then investigated *in vitro* stimulation of primary macrophages with

recombinant Trx1 and Grx2 individually and under inflammation conditions with simultaneous addition of LPS and INF-γ. All experiments regarding macrophage function and stimulation were performed in the Marburg laboratory at the Renz group together with Christoph Hudemann, PhD, as a guest scientist and under ethical approval of the state of Hesse, Germany. First, balb/c mice (n=5) were injected i.p with 1ml Brewer thioglycolate medium to elicit macrophages to the peritoneal cavity. After 72h mice were sacrificed and peritoneal cavity was rinsed with PBS followed by RPMI medium. Cell counting was performed using CasyTT cell counter (OLS OMNI Life Science GmbH&Co KG, Bremen, Germany). F4/80 and CD45 antibodies were utilized for cell separation via FACS (2.2.2.5).

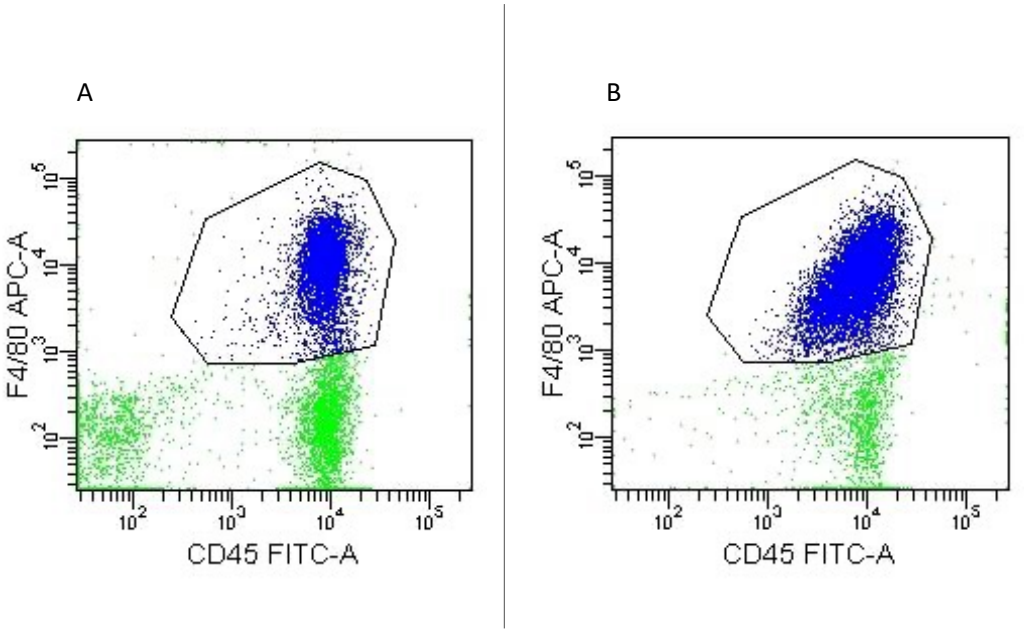


Figure 15 – Macrophage cell count: *macrophages elicited from Balb/c mice were counted using FACS with CD45 and F4/80 antibody,* ***A*** *before and* ***B*** *after seeding.*

After cell cultivation and attachment in RPMI medium for 4h at 37°C, attached cells were harvested and counted. 200.000 cells per well were seeded on a 96-well microtiter plate and prepared for macrophage stimulation. After pre-treatment with 10mM DTT, 40 µg of mTrx1 and hGrx2c, respectively, were added and pre-incubated for 1h. Subsequently, 20 ng/ml LPS (lipopolysaccharide) and/or 10 ng/ml INF-γ were added to induce macrophage simulation as positive control, medium was used as negative control. After 4h and 72h of incubation the levels of RANTES, IL-4, IL-6, IL-10, TNF-α and INF-γ were determined using a mouse cytometric bead array kit according to manufacturer´s recommendations and measured with BioRad-Flex-

200 (BioRad, Hercules, CA, USA). The changes in cytokine expression were directly compared to stimulation with LPS and INF-γ, which are potent inductors of cytokine expression targeting macrophages. Macrophage stimulation by LPS and INF-γ is comparable to the cytokine stimulation seen under conditions of asthmatic airway inflammation and will be referred to as an OVA-like state in the following.

Upon 4hrs and 72h stimulation with hGrx2c as well as mTrx1 increased the overall cytokine levels of RANTES, IL-6, IL-10 as well as TNF-α and INF-γ compared to PBS control. Cytokine levels of INF-γ were augmented by sole stimulation with LPS and INF-γ, whereas INF-γ levels decreased under Grx2 and Trx1 stimulation alone and increased again under combination of each redoxin with LPS and INF-γ. Suggesting a Th1-immune response to be reduced under treatment with both redoxins. Comparable to LPS and INF-γ treatment, TNF-α expression was also reduced under Grx2 treatment. In contrast, TNF-α was induced significantly under Trx1 alone and Trx1 combined with LPS and INF-γ. Furthermore, induction of IL-6 was lower after stimulation with Grx2 then under LPS and INF-γ treatment or LPS and INF-γ and Grx2 combined.

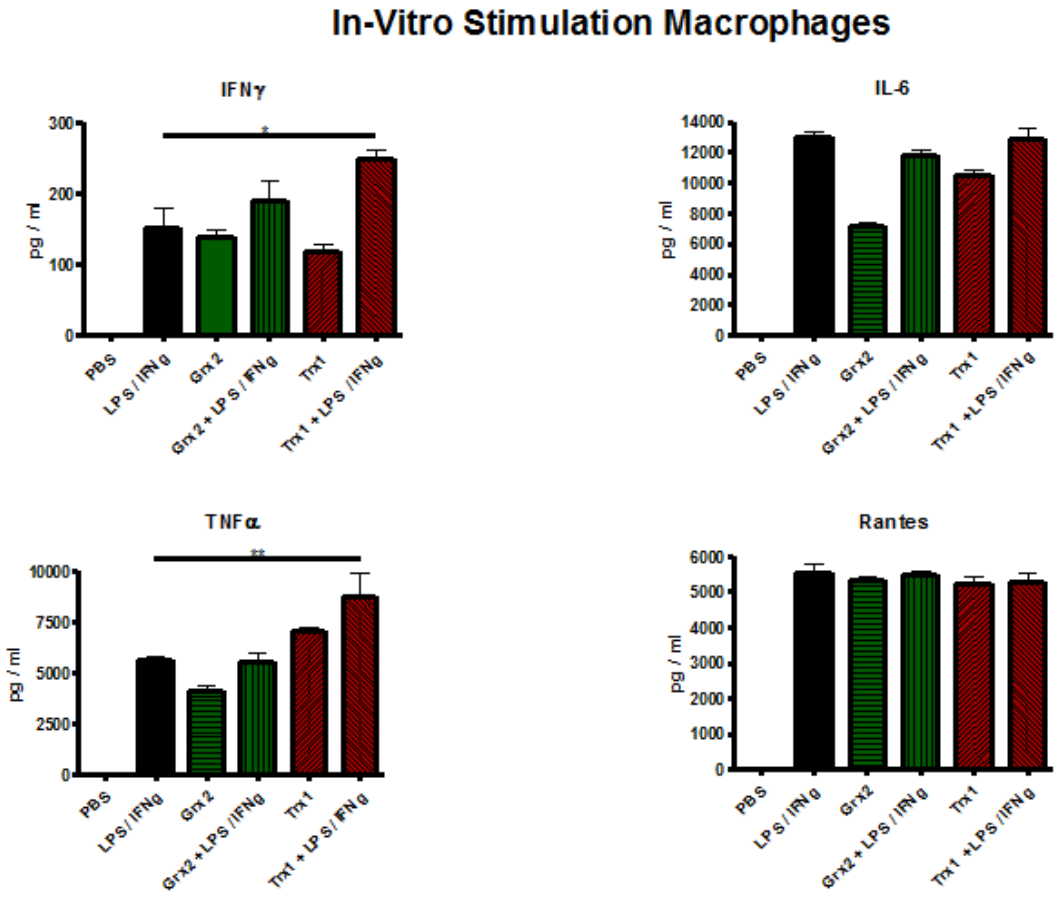


Figure 16 – cytokine levels after macrophage stimulation: *macrophages were stimulated with Grx2, Trx1 and/or LPS+IFN-γ in RPMI medium, after 4 and 72hrs cytokine levels were measured with a*

*cytometric bead array, data after 72hrs of stimulation are shown, n= 5 per group, shown are means ± SD, *= p<0.05, ** =p<0.01;*

In contrast, IL-6 levels were equally increased after Trx1 and Trx1+ LPS and INF-γ stimulation. Finally, RANTES levels, measured after 4 and 72hrs of macrophage stimulation, were induced in comparison to PBS control, but showed no significant alterations between the five different stimulation conditions. Overall Grx2 and Trx1 stimulation induced cytokine expression in macrophages and upon activation with LPS and INF-γ stimulation of macrophages with mTrx1 additionally changes cytokine expression of IL-6, IL-10, TNF-α and INF-γ. These results indicate a very distinct regulatory effect on macrophage stimulation.

4 Discussion

4.1 Trx family proteins in allergic airway inflammation – presence and functions

Intracellular expression and distribution of thioredoxin family proteins has been widely explored in the past decade. Mapping the physiological occurrence of those proteins was the aim of publications such as “The Redox Atlas of the Mouse“ assembled in 2010 by Godoy and the Lillig group and “Redox atlas of the rat brain“ published as “Thioredoxin and glutaredoxin system proteins - immunolocalization in the rat central nervous system“ by Aon-Bertolino et al. 2011 both in Biochimica et Biophysica Acta (Aon-Bertolino et al., 2011; Godoy, Funke, et al., 2011). Moreover, in 2011 Dammeyer and coworkers compiled the “Human Redox Atlas“ from data of the human protein atlas and indicated a large variety of expression patterns of Trxs, Grxs and Prxs in different human tissues (Dammeyer & Arnér, 2011). Since then, changes of *intracellular* protein levels and expression patterns have been implicated in hypoxia-related disorders, for instance in renal ischaemia and reperfusion injury in ischaemia and reperfusion injury of cardiac tissue e.g. myocardial infarction in diabetes mellitus and most importantly in lung inflammation (Godoy, Oesteritz, et al., 2011; Hanschmann et al., 2013). Physiological and pathological conditions and signals for the translocation of Trx family proteins to the *extracellular* compartment are to date poorly understood, inviting us to search for a disease model to investigate processes of redox signalling inside and outside the cell.

The unique position of the pulmonal mucosa at the interface of inhaled air - not only containing oxygen but also airborne pollutants - and the bloodstream leads to a high abundance of ROS and RNS in the epithelial lining fluid (Comhair et al., 2005; Fitzpatrick et al., 2009). Due to this extraordinary exposure of pulmonal epithelial cells, the role of the GSH and Trx systems in pathological conditions of the lung emerged in Redox research (T. Nakamura et al., 2005). Therefore, a model for the understanding of inflammatory processes of asthma had to be established and until today murine models, challenging the lung with external antigens, are frequently used for research. In the 1990s a mouse model for OVA-induced allergic airway inflammation was established and remained a valuable tool for the investigation of asthma-like pathologies until today (Blyth et al., 1996). Since OVA is the most conveniently available and well tested antigen, this study relied on the model for OVA-induced airway inflammation established by our collaboration partners in the Marburg laboratory. Limitations occurring in a murine model, for instance the difference in airway behaviour, the influence of the strain and

age of the animals on immune response, the route and timing of antigen exposure must be taken into consideration when comparing findings in the mouse model to the conditions of asthma in the human body (Bates et al., 2009; Kips et al., 2003). These differences between the murine model and the human disease are additionally underlined by the fact that mice do not naturally develop a condition comparable to human asthma (Bates et al., 2009). Therefore, murine models of asthma are generally to be considered artificial and only a cautious and considerate interpretation and transfer of the results to the human pathology is possible.

In this study, we used a well-established murine model for allergic airway inflammation to examine protein levels of 9 members of the Trx family with a special interest in extracellular distribution patterns. The collected data from Western blot of intracellular lung tissue showed the presence of Grx1, Grx2, Trx2, Prx2 and Prx4 in control and inflamed lung. Protein levels of Grx1 and Prx2 remained constant in PBS and OVA-lung. Prx2 detection by IHC in lung sections also displayed a constant staining in bronchial epithelial cells and alveolar cells, confirming results from the Western blots. Trx2 was significantly reduced in OVA-samples. Grx2 and Prx4 levels significantly increased in OVA in comparison to PBS control. Surprisingly, Grx2 levels not only increased after OVA-challenge in the lung tissue samples but showed a specific staining for a second Grx2 isoform in Western blot analysis. Staining pattern for Trx1 in the lung tissue samples appeared blurred and uneven. mTrx1 is a 11,6 kDa protein, staining at a 70 kDa range is not specific for Trx1. Multiple attempts to improve staining for Trx1 in lung tissue samples were unsuccessful, thus Western blot results for Trx1 were excluded from further interpretation.

In extracellular BAL fluid samples, Grx1, Trx1, Prx1 and Prx2 could be detected both in control and OVA samples. Intriguingly Grx1, Trx1 and Prx2 levels increased in OVA lung samples whereas Prx4 could only be detected after OVA challenge and not in control mice samples. Prx1 levels stayed even in PBS and OVA samples. In comparison to intracellular samples, Trx1 could be detected with specific bands at expected protein sizes in BAL samples. All data are summarized in table 1.

Redoxin	*lung tissue samples*		*lung BAL samples*		*lung tissue*
	PBS	OVA	PBS	OVA	Redox Atlas
Grx1	+	+	+	++	+
Grx2	+	++	/	/	+
Trx1	/	/	+	++	+
Trx2	++	+	+	+	++
Prx1	+	+	+	+	+
Prx2	+	+	+	++	++
Prx4	+	+	-	++	+

Table 4: Distribution of Trx family proteins in OVA-induced allergic airway inflammation: *presence of redoxins in lung tissue and BAL samples comparing PBS control and OVA conditions, analysis by WB, redoxin present = +; redoxin increased = ++; redoxin not detected =-; no data = /; in comparison to the redoxin levels depicted in the Redox Atlas of the Mouse (Godoy, Funke, et al., 2011)*

Experiments from various groups have already established the crucial role of Trx family proteins in allergic airway inflammation. Trx1 levels were found to be increased in serum from patients with asthma exacerbation (Ito et al., 2011; Yamada et al., 2003) and in a murine model for airway inflammation Trx1 administration prior to OVA challenge reduced eosinophils and histological key features in the lung (Ichiki et al., 2005). These findings are supported by our experiments, establishing an increase in Trx1 in BAL-samples of the asthmatic lung. Grx1 levels were found to be increased in an OVA-induced murine model of allergic airway disease, in addition Grx1 and Grx2 mRNA increased in lung epithelial cells (Reynaert et al., 2007). A role for Grxs in antioxidant defence was suggested, but the underlying mechanism remained unclear. Today we can confirm an increase in Grx1 levels in BAL samples and an increase in Grx2 in lung tissue upon lung inflammation.

Prx2 knockout mice were reported to show a more severe asthmatic phenotype in OVA-induced airway inflammation and decreased expression of TLR4 (Moon et al., 2008). In contrast, Prx1 was suggested to have a pro-inflammatory effect, in Prx1 knockout mice eosinophil count in BAL significantly increased after OVA and Alum induced onset of allergic airway inflammation compared to WT mice (Inoue et al., 2009). In a proteomic analysis of lung tissue and BAL samples from OVA-induced airway inflammation Prx1 levels were shown to be increased in inflammation (Fajardo et al., 2004). In contrast, enhanced pulmonary fibrosis was found in Prx1 knockout mice treated with cytostatic drug bleomycin, now suggesting a protective role for Prx1 in oxidative stress and lung injury (Kikuchi et al., 2011). In this study,

we did not observe significant changes in Prx1 levels and did not further investigate functional aspects of Prx1 due to the inconclusive results found in literature.

Nrx so far has not been reported to be directly involved in allergic airway inflammation, however we found strong and constant staining for Nrx in control and OVA induced lung inflammation. Nrx was also detected in lung tissue sections by Godoy and co-workers. in the Redox Atlas of the Mouse (Godoy, Funke, et al., 2011). Several functions of Nrx in immune response have been reported, for instance stabilization of the interaction of flightless homolog 1 and myeloid differentiation primary response protein MyD88, suppressing LPS-induced NF-κB activity (Stottmeier et al. 2016). In addition, potential regulatory functions of gene expression were suggested. Nrx over-expression in HEK293 cells led to an increased NF-κB activation, following stimulation by TNF-α (K. Hirota et al., 2000).

A cornerstone of this study is the analysis of protein levels by Western Blot – a method with certain limitations concerning reproducibility and specificity of staining. Frequent reasons for these disturbances include unspecific antibodies and incorrect tissue processing. To minimize disturbances, specific antibodies were used which were extensively tested in the past by the Lillig group (Godoy, Funke, et al., 2011) and a large number of antibodies were affinity purified in the working group, for instance affinity purification of mTrx1 and hTrx1 antibody is also a part of this study (see 2.2.2.2). Furthermore, IHC of mouse lung sections was used to evaluate changes in lung phenotype and distribution of Trx family proteins after OVA-challenge. Similar problems such as unspecific or false-negative staining may occur due to unspecific antibodies, incorrect tissue processing, embedding, fixation or staining. Again, we tried to minimize errors by using tested and affinity purified antibodies and performing control staining. Still, there might be uncertainties about IHC results since this technique precludes the use for quantitative analysis and displays only qualitative alterations of protein staining. However, the combination of both methods, WB and IHC, allows for reasonably reliable statements on changes in protein levels and protein distribution.

4.2 Prx4 secretion in allergic airway inflammation – involvement in immune response?

This thesis provides further insight into extracellular secretion of Trxs and their involvement in immune response. In this study we showed for the first time the extracellular occurrence of Prx4 in BAL samples in a mouse model for OVA-induced lung injury by Western blot analysis. In BAL samples from PBS control lung, Prx4 could not be detected. In addition, IHC indicated

an increase as well as an altered subcellular distribution of Prx4 in OVA mice compared to control lung. For instance, alveolar-macrophages and epithelial cells of bronchioles exhibited a higher staining for Prx4 in comparison to the control lung tissue, highlighting the redistribution of Prx4 towards the secretory compartment. Nevertheless, one could argue that IHC staining of the untreated control lung tissue (figure 6, 3.3.1.) is too weak in contrast to the OVA lung tissue, indicating a faulty staining procedure. For IHC and IF staining we used a sensitive Prx4 antibody which was extensively validated previously by our group (Godoy, Funke, et al., 2011) reducing the probability of non-specific staining. Therefore, we assumed that the enhanced Prx4 staining in OVA mice is caused by an increase in protein levels – an assumption which is also supported by the results of an IF staining performed in OVA and PBS control lung. IF showed an increase in Prx4 in activated macrophages co-stained with F4/80 antibody and in the pseudostratified epithelium of bronchioles. These findings establish up-regulation of Prx4 levels as well as redistribution of Prx4 to epithelial and inflammatory cells such as alveolar macrophages, in OVA-induced allergic inflammation, indicating a modulating role in immune response.

When the summarising the literature outside the “redox-biology”-community, scavenging of hydrogen peroxide and other ROS is presumed to be the main extracellular function of Prxs and the proteins are often mentioned as “antioxidants” alongside other components such as GSH. Indeed, Prxs are highly effective in scavenging hydrogen peroxide not only due to their high intracellular abundance but also due to low K_M values for hydrogen peroxide (Low et al., 2007). But recent experiments suggested that Prxs are destined for more. As already established in the introduction (see also 1.2.3) Prxs facilitate the neutralization of peroxynitrite and regulate cell signalling pathways by oxidation of cysteine residues and formation of high molecular weight aggregates that act as cellular chaperones (Jang et al., 2004; Manta et al., 2009). As an essential prerequisite for the formation of higher oligomeric structures Prxs contain a conservative peroxidate cysteine residue located in the N-terminal part of the protein that is crucial for catalysis and can selectively be oxidized to a sulfenic acid. 2-Cys-Prxs such as Prx4, share an additional cysteine referred to as a resolving cysteine. It interacts with the cysteine sulfenic acid of another, oxidized Prx4 molecule leading to the formation of a homodimer via an intermolecular disulfide bond. As the dimerization is favoured by oxidation of the active site, formation of a monomer is supported by reduction of the active site cysteines (Wood et al., 2002). Intracellular excess of hydrogen peroxide may lead to an additional hyperoxidation of the N-terminal cysteine residue to sulfinic acid leading to peroxidase inactivation. This sulfinic acid can only be re-reduced by Sulfiredoxins and p-53 inducible sestrins (Biteau et al., 2003;

Budanov et al., 2004). Hyperoxidized Prx2 was shown to form high molecular weight (HMW) decamers assembling intracellular in large filamentous constructs inducing a cell cycle arrest in mitogenesis (Phalen et al., 2006). In the HMW state, Prxs exert chaperone functions interacting for instance with ubiquitin C-terminal hydrolase-L1 (UCH-L1), preventing this protein from either thermal or oxidant-dependent inactivation (Lee et al., 2018). The switch in Prx activity between function as a hydrogen peroxidase and formation of decamers by hyperoxidation is suggested to operate as a "floodgate" for hydrogen peroxide dependent cell signalling - under regular hydrogen peroxide levels, Prxs function as peroxidases maintaining a constant level of intracellular ROS and inhibiting redox signalling. In contrast an intracellular rise in peroxide levels leads to inactivation of 2-Cys-Prxs favouring hydrogen peroxide accumulation leading to peroxidation of susceptible target proteins (Wood et al., 2003). Finally, in a chaperone state, some peroxiredoxins may be exported from the cell possibly acting as biomarkers of oxidative damage (Mullen et al., 2015) see also chapter 1.3.

Among the 2-Cys-Prxs, Prx4 is the only protein located in the ER, which is known to be an overall more oxidizing environment. Prx4 is primarily located in pancreas, spleen, liver, lung and testis. First it was hypothesized that a second 27 kDa Prx4 isoform lacking 36 N-terminal amino acids is an actively tailored and secretable variant of the protein (Okado-Matsumoto et al., 2000). Instead, Tavender and co-workers revealed that Prx4 is rather contained in the ER and that intracellular levels of the short Prx4 lacking the N-terminal sequence are not significant (Tavender et al., 2008). Subsequently it was demonstrated, that Prx4 localisation in the ER is favoured by interactions with endoplasmatic reticulum resident protein ERp44. Prx4 is oxidized, scavenging excess hydrogen peroxide produced in the ER, and as such is recognized by ERp44 forming an intermolecular disulfide. This interaction prevents Prx4 secretion and provides oxidative agents that facilitate protein folding, via protein disulfide isomerases (PDI) or other ER oxidoreductases (Yang et al., 2016). Prx4 was postulated to have a proof-reading capacity for disulfide bonds in the ER in interplay with protein disulfide isomerases ERp46 or P5 and to contribute to control of protein folding in the ER (Sato et al., 2013). In 2019 Lipinski and co-workers detected Prx4 in extracellular vesicles of activated macrophages together with inflammasomes and caspase-1 - known to regulate the activity of IL-1β (Lipinski et al., 2019). The inflammasome NLRP3 (NOD-like receptor pyrin domain containing 3) oligomerizes and forms a molecular platform by recruiting the adapter protein ASC (apoptosis-associated speck-like protein containing a CARD) and pro-caspase-1 subsequently leading to cleavage of pro-IL-1β to yield active IL-1β (Schroder & Tschopp, 2010). In its HMW state Prx4 was shown to form a redox sensitive complex with caspase 1. A Prx4 decamer, consisting of five disulfide

linked Prx4 dimers, interacts with cysteine 397 of caspase-1 forming a disulfide and integrating caspase-1 into the HMW complex which is then transferred to extracellular vesicles. In Prx4 deficient mice the release of IL-1β is augmented and mechanistic experiments revealed that the redox active complex of HMW-Prx4 and caspase-1 is essential for caspase inactivation and attenuation of IL-1β function. Moreover, the presence of Prx4 in vesicles together with inflammasome proteins and caspase-1 led to the conclusion that extracellular function of caspase-1 in activating immune response of surrounding cells can be attenuated by Prx4 (Lipinski et al., 2019).

In a nutshell, Prx4 is characterized as an ER-residing protein involved in ER redox homeostasis, introducing disulfide bonds to regulate protein folding and upon secretion exerting extracellular functions in redox regulation of immune response. Indeed, differentially regulated extracellular levels of Prx4 were reported in various pathologic conditions such as diabetes, neurodegenerative disease, cancer, sepsis and inflammation (Hanschmann et al., 2013). Prx4 levels were found to be elevated in serum of patients with type 2 diabetes and negatively correlated with triglyceride levels (El Eter & Al-Masri, 2015). In an INS-1E pancreatic beta-cell model, Prx4 overexpression resulted in a significantly increased intracellular insulin levels and glucose-induced insulin secretion, suggesting a regulating role of Prx4 in ER folding of pro-insulin (Mehmeti et al., 2014). In 1997, Prx4 was described to negatively regulate NF-κB activation in HeLa cells (Jin et al., 1997). Since then, increased protein levels of Prx4 and Prx1 have been detected in human small cell lung carcinoma and lung adenocarcinoma (Jiang et al., 2014). In a neurodegenerative model for Alzheimer's disease β-amyloid-oligomer induced cell toxicity was reduced by Prx4 via attenuation of the formation of H_2O_2 and ER stress (Kam et al., 2019). In patients with septic shock high serum concentrations of Prx4 were associated with increased disease severity (Schulte et al., 2011). Intriguingly, LPS injection in mice *in vivo* led to elevated Prx4 levels, whereas the selective caspase-1 inhibitor YVAD significantly reduced levels of extracellular Prx4 after LPS stimulation, thus indicating that Prx4 secretion is linked to caspase-1 activation (Lipinski et al., 2019). Treatment of macrophages from the murine macrophage cells line RAW 246.7 with Prx1, Prx2 and Prx4 significantly induced the production of proinflammatory mediators such as TNF-α and IL-6 via nuclear translocation of NFκB (Zhao et al., 2016). These findings highlight the involvement of Prx4 in regulatory pathways inside and outside the cell facilitating an increased adaptability to environmental influences.

As already introduced in chapter 1.4., the interplay of cellular apoptosis and tissue remodelling is a cornerstone of the pathophysiology of allergic asthma and caspases are

important players in cellular response to inflammation and tissue homeostasis (Datta et al., 2013). In the inflamed lung, apoptosis can be initiated as a result of excess NO and H_2O2 production, ER stress, bacterial toxins, and DNA damage. Caspases regulate downstream processes of apoptosis such as protein and cell organelle degradation and DNA fragmentation. Caspase-3 is a main effector caspase for apoptosis whereas caspase-1, -4, and -5 primarily regulate inflammatory response, reviewed by Shalini (Shalini et al., 2015). Apoptosis of bronchial cells was hypothesized to be involved in airway injury and increased airway permeability resulting in tissue remodelling (Barnes, 2017). In both well-established models for allergic asthma, OVA-induced and house dust mite induced asthma, apoptosis of bronchial epithelial cells was demonstrated (Hoffman et al., 2012; Truong-Tran et al., 2003). In 2003 administration of a pan-caspase inhibitor in a mouse model for OVA-induced allergic airway inflammation revealed decreased lung inflammation (Iwata et al., 2003) administration of the same inhibitor in a mouse model for acute septic lung injury showed similar reduction of severe lung injury (Messer et al., 2013).

In 2017, Rao and co-workers analysed the influence of Prx4 overexpression on chondrocyte apoptosis in a rat model for osteoarthritis. Lentivirus dependent overexpression of Prx4 decreased IL-β induced apoptosis via attenuation of caspase 3 and 9 activity and attenuated ROS and NO production in chondrocytes. Localisation of Prx4 in ER and Golgi apparatus led to the conclusion that regulatory effects of Prx4 on apoptosis were attributed to phosphatidylinositol 3-kinase/AKT signalling (Rao et al., 2017). These findings gave reason to further investigate the influence of Prx4 on apoptosis in allergic lung inflammation and the mechanistic relationship between Prx4 and the precursor pro-caspase-3. In this study we utilized pro-caspase-3 as a valuable marker for programmed cell death and Immunohistochemistry results show an increase of pro-caspase 3 in OVA lung in comparison to PBS-control and displayed a decrease of pro-caspase 3 in Grx2-OVA lung, indicating a decreased apoptotic activity in the Grx2 treated OVA lung. These data lead to the conclusion that Grx2 treatment also has an inhibitory effect on pulmonary apoptosis. Intriguingly, staining of pro-caspase-3 was highest in epithelial cells of bronchioles and alveoli, corresponding to the sites in which Prx4 expression was increased in OVA lung. These results allow different assumptions. Primarily increased pro-caspase-3 levels may indicate a decreased activation of pro-caspase-3 to caspase-3 by formation of an active tetramer resulting in reduced apoptotic activity. Persulfidation of pro-caspase-3 at an active site thiol occurs on a basal level under non-apoptotic conditions and inhibits activation of the enzyme (Braunstein et al., 2020). A precisely regulated equilibrium for the persulfidation of the caspase precursor could be an explanation

for the different pro-caspase-3 levels under inflammation conditions. If constant intracellular Prx4 levels contribute to a ground level of persulfidation of the catalytic site cysteine (163-Cys) of pro-caspase leading to further protein stabilisation, Prx4 would then be inhibiting apoptosis under physiologic conditions. After OVA-challenge and induction of airway inflammation, Prx4 was found to be secreted to the extracellular compartment. We hypothesize, that the shift of Prx4 to the extracellular space facilitates depersulfidation of pro-caspase-3 and its subsequent cleavage to active caspase-3 inducing downstream pro-apoptotic pathways. The Trx/TrxR system is involved in depersulfidation, since TrxR inhibition displayed an attenuating effect on caspase depersulfidation and activation (Braunstein et al., 2020). This possible mechanism of Prx4 involvement in apoptotic signalling obviously needs to be further investigated. Research should be focussed on the effect of overexpression of Prx4 on pro-caspase persulfidation *in vitro* for example via a biotin switch assay and compared to changes of hydrogen sulfide modifications after inhibition of Prx4.

Finally, in the group of OVA-mice with additional Grx2 treatment during OVA challenge, Prx4 levels remained constant in the intracellular samples and increased in the extracellular compartment whereas pro-caspase-3 staining decreased under these conditions.

Braunstein and coworkers noted that the Trx/TrxR system is involved in de-/persulfidation of caspases, since TrxR inhibition displayed an attenuating effect on caspase de-/persulfidation and activation (Braunstein et al., 2020). A possible influence of Grx2 on the equilibrium between persulfidation and depersulfidation may be exerted via interaction with TrxR, since Grx2 is an alternative substrate for TrxR2 (Johansson et al., 2004).

Extracellular detection of Prx4 in OVA and OVA-Grx2 mice is one of the most intriguing findings of this thesis, yet we can only begin to understand the mechanisms of Prx4 secretion. Prx4 was described forming HMW decamers under excess of hydrogen peroxide in the ER and subsequently being secreted from the cell in vesicles. Therefore, we assume, that formation of H_2O_2 under conditions of inflammation in OVA mice induces Prx4 accumulation in vesicles, which are subsequently secreted from the ER to the extracellular space with messaging functions. Certainly, this assumption needs further confirmation from mechanistic experiments. For instance, the stimulation of Prx4 secretion *in vitro* and the subsequent administration of Brefeldin A – a potent inhibitor of vesicular transport from Golgi-apparatus – could determine if Prx4 is secreted by an ER/Golgi dependent pathway.

IHC confirmed the presence of Prx4 in viable epithelial cells, still one might argue that the extracellular presence of Prx4 could also be the result of cell death and undirected release of the protein from apoptotic cells. Further experiments need to rule out this possibility, for

instance changes in mRNA levels of Prx4 could be investigated in OVA lung samples. Similar experiments were performed by Krutilina and co-workers and inflammation was shown to increase Prx5 levels in OVA-sensitized mice but did not change mRNA expression levels of Prx5 in tracheal epithelial cells, therefore Prx5 was determined in migrated leukocytes, indicating that inflammatory cells are rich in Prx5 and able to secrete the protein to the extracellular space (Krutilina et al., 2006).

Finally, the mechanisms of extracellular Prx4 influence on macrophages and immune response still undefined and should be further investigated. Since Grx2 and Trx1 addition to macrophage cultures showed alterations in cytokine levels in this study (3.5) *in vitro* treatment of macrophages with Prx4 and subsequent analysis of cytokine levels in comparison with LPS and INF-γ stimulation should be attempted.

4.3 Grx2 treatment prevents severe allergic phenotype, presumably via cellular uptake and redox-dependent inactivation of Hif-1α

Grx2 is equipped with a unique dithiol Cys-Ser-Tyr-Cys active site that enables formation of a dimeric complex coordinated by an iron-sulfur (2Fe-2S) cluster bridging two Grx2 molecules. Grx2 can thus switch between an inactive dimeric and an active monomeric state and functions as a redox sensor have been described by Lillig and co-workers in 2005. This enzymatically active state signifies a crucial linkage between the Trx and the Grx system, since Grx2 receives electrons either from TrxR2 or GSH. In most cells Grx2 is present in the dimeric (holo) Grx2 form (Lillig et al., 2005). Moreover, Grx2 constitutes 3 isoforms in mice, mitochondrial Grx2a, Grx2c with cytosolic localization, and testis specific Grx2d isoform which is unique for mice (Hudemann et al., 2009). Mouse Grx2c is known to form the above-mentioned iron-sulfur cluster and reduce substrates such as low molecular weight proteins with disulfide active sites, first detected in human Grx2 (Berndt et al., 2007). Recently, detoxification of NO by Grx2 has been described as a protective factor in neuroinflammation, stating that Grx2 protection is not linked to its enzymatic activity as oxidoreductase but to the capacity to coordinate an iron-sulfur cluster. Disassembly of the dimeric Grx2 using GSH as a ligand forming S-nitroso glutathione (GSNO) protects oligodendroglial cells from nitration reducing neuro-inflammation *in vitro* (Lepka et al., 2017). With these key features in mind, the results of this study suggest a similar protective function of Grx2 in acute airway inflammation *in vivo.* Western blot analysis showed low Grx2 levels in control lung of Balb/c mice and detected an increase in Grx2 in the OVA-sensitized lung. Moreover, a second isoform of Grx2 could be detected in OVA lung, supposedly matching the 14 kDa Grx2c. Specificity of Grx2 antibody was tested by blocking with antigen (mGrx2c) prior to repeated western blot analysis and staining for the second Grx2 isoform vanished. Specific sandwich-ELISA quantification of Grx2 protein levels in lung tissue confirmed a 1,3-fold increase of Grx2 in acute airway inflammation (figure 14).

To further investigate these findings, a Grx2-specific sandwich ELISA was performed for quantification of Grx2 levels in control- and OVA-lung tissue samples. ELISA protocol was established and performed by Eva-Maria Hanschmann, PhD, at the Lillig group. Cell lysate of OVA- and control-lung diluted in incubation buffer and protein standards from 0-32µg/ml were added to a 1:500 dilution of primary antibody, colorimetric reaction was measured using a Tecan 96-well plate Reader

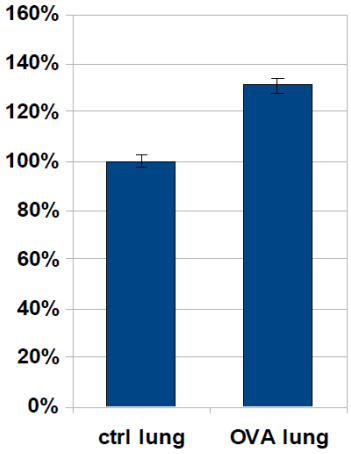


Figure 17 - Grx2 protein levels are augmented upon airway inflammation: *Quantification of Grx2 protein levels in control- and asthmatic lung through Grx2 specific sandwich ELISA, values are presented in %, regarding control lung as 100%, error bars indicate mean ± SD, n = 6;*

IHC of lung cross sections from the same mouse model for acute allergic airway inflammation published by Hanschmann and co-workers in 2020 support our findings, showing strong staining for Grx2 especially in epithelial cells of bronchioles after OVA-challenge in comparison to PBS-control (Hanschmann et al., 2020). The increase of Grx2 upon inflammation, which until today could be confirmed by three independent methods, does however not provide any conclusions about protein function. To elucidate protein function of Grx2 in acute airway inflammation Balb/c mice were injected with 40μg Grx2 recombinant protein or the Grx2 C40S mutant on day 23-27 after OVA sensitization. OVA+Grx2WT lungs display a decrease in histological key features of asthma under Grx2 treatment accompanied by a decrease in staining for HIF 1α and pro-caspase-3 comparable to PBS control. Intriguingly, cellular uptake of recombinant Grx2 after treatment with the redoxin could be established by staining with a specific His-Tag-antibody, which was strongly positive in the animals treated with Grx2WT compared to control. However, His-tag staining appeared to be lower after treatment with recombinant Grx2C40S mutant, indicating that dithiol mechanism of Grx2 is crucial for Grx2 function and uptake upon lung inflammation. Overall, these data imply a differential role for Grx2 in inflammation, which is facilitated by an increase in intrinsic Grx2 levels but also by an uptake of extracellular Grx2.

The Grx2C40S mutant utilized in this study was first applied by Johansson and co-workers in 2004, stating that mutation of the C-terminal active site motive from cysteine to serine led to a decreased affinity for formation of GSH-mixed disulfides but conserved the capacity to perform de-/glutathionylation (Johansson et al., 2004). Since then, Grx2 C40S mutant serves as a model for a monothiol mechanism in Grx2, unable to perform direct de-/nitrosylation in the absence of GSH (Ren et al., 2019). In our study we utilized Grx2C40S mutant to investigate whether the dithiol mechanism is crucial for Grx2 effects on lung inflammation. We observed decreased Prx2 and Prx4 levels in WB in comparison to treatment with Grx2WT. Nevertheless, numbers of probed animals (n = 2) in case of Grx2C40S mutant are too small to be significant. However, also the uptake of Grx2C40S into lung tissue was reduced compared to Grx2WT concluding from histology of lung cross sections. IHC of lung sections with pro-caspase-3

antibody showed a decrease in Grx2C40S samples compared to OVA, but in comparison to treatment with Grx2WT pro-caspase-3 levels appeared to be still higher. Clearly, this could not be quantified in IHC, therefore a confirmation by WB should be performed. In PAS-stained lung sections from OVA-induced airway inflammation, Hanschmann and co-workers observed significantly less goblet cell formation after Grx2C40S treatment similar to Grx2WT treatment and in comparison to untreated OVA mice. The total number of BAL cells from Grx2 treated mice was similar in Grx2WT and Grx2C40S, however the number of eosinophils significantly increased under Grx2C40S administration compared to wildtype, indicating a loss of anti-inflammatory properties in the redox inactive mutant (Hanschmann et al., 2020).

Since hypoxia is a key player in pathogenesis of asthma it is worthwhile to look at the role of HIF-1α in inflammation. HIF-1 is a heterodimeric transcription factor which consists of two subunits, HIF-1α and HIF-1β. Under hypoxic conditions, oxygen-dependent inactivation by ubiquitination and proteosomal degradation is paused and cytosolic HIF-1α dimerizes with nuclear HIF-1β and leads to transcriptional activation of vascular endothelial growth factor (VEGF) and other target genes involved in response to hypoxia (Forsythe et al., 1996; Semenza, 2000). HIF-1α hydroxylation at proline 402 and 564 in the oxygen-dependent degradation (ODD) domain is performed by prolyl-hydroxylases (PHD). Thus HIF-1α controls the expression of many hypoxia-regulated genes and the cellular adaptation to low oxygen and was implied in inflammatory processes contributing to the pathology of asthma (Baek et al., 2013; Tsapournioti et al., 2013). Confirming the results of our study, HIF-1α and HIF-1β were found to be increased in bronchial epithelial cells as well as in macrophages and lymphocytes in a model for OVA-induced allergic airway inflammation (Huerta-Yepez et al., 2011). Studies investigating the effect of HIF knock-out found that partially HIF-1α deficient OVA-sensitized mice displayed increased IFNγ levels and reduced lung eosinophilia in comparison to wild-type mice. HIF-1β knock-out mice showed decreased allergic airway inflammation and reduced production of ovalbumin specific IgE and IgG (Guo et al., 2009). HIF knock-out in T-lymphocytes reduced hypoxia dependent differentiation of CD4+ T-cells in various inflammatory mouse models, suggesting a role in regulation of the immune response depending upon different external stimuli (McGettrick & O'Neill, 2020). A key regulatory mechanism of HIF-1α function – in addition to the hydroxylation at Pro402/564 – is the S-nitrosylation of a cystine residue (Cys533 in mice) located in the ODD-domain leading to protein stabilization (Li et al., 2007) which is independent from prolyl-hydroxylase degradation (Jaakkola et al., 2001). The s-nitrosylated HIF-1α is reported to form structurally more stable GSH adducts leading to conformational stabilization and activation of the protein (Watanabe et al., 2016).

HIF-1α activation subsequently induces transcription of various target genes such as glucose transporters and enzymes of glycolysis, the chemokine receptor CXC4R and the expression of VEGF (Dehne & Brüne, 2009; Staller et al., 2003). These target genes exert different functions in cancer, hypoxia related disorders and inflammation. NO mediated S-nitrosylation of HIF-1α was reported in cancer cells under radiation which led to formation of reactive oxygen species such as NO (Li et al., 2007). In context with s-nitrosylation and formation of S-nitroso-glutathione (GSNO), Grx was first reported to have denitrosylation capacity of S-nitrosocysteines as well as S-nitrosylated proteins and GSNO by Ren and coworkers in 2019. In S-nitrosylated proteins from HEK-cells Grxs exerted denitrosylase activity, even when lacking the N-terminal active site cysteine residue (C40S mutant) suggesting, that denitrosylation can either be performed via dithiol mechanism like Trx1 or monothiol mechanism comparable to Grx-mediated glutathionylation (Ren et al., 2019).

Starting from there, the role of Grx in HIF-1α regulation was investigated *in vivo* in a mouse model for hind limb ischaemia. In Grx knockout mice stable HIF-1α GSH-adducts increased, presumably stabilizing HIF-1α and leading to VEGF mediated angiogenesis and revascularization of the limb (Watanabe et al., 2016). Grx-overexpression impairs revascularization also via HIF (Murdoch et al., 2014). However, the induction of HIF-1α target genes such as VEGF promoting angiogenesis, endothelial cell growth and vascular permeability seems to be beneficial in the ischemic tissue, yet the exact same mechanisms appear to be fatal in allergic airway inflammation, leading to airway hyperresponsiveness and airway remodeling. This raises the question whether there is a mechanistic switch providing the possibility to turn on and off HIF-1α activity and translation of its target genes whenever needed.

In our study we detected increased levels of Grx2 in lung tissue of OVA treated mice and decreased asthmatic features in mice after treatment with Grx2WT starting 5 days prior to OVA-challenge. Evaluation for HIF-1α in IHC displayed a strong increase for HIF staining in OVA-induced airway inflammation but after Grx2WT treatment staining was strongly reduced. Even after treatment with Grx2C40S, we discovered a decrease in HIF, but not to the same extend as experienced with Grx2WT.

Supporting our results, Hanschmann and co-workers reported that NO production was increased in immortalized murine macrophage cell line RAW264.7 and this effect was attenuated by wildtype Grx2. Intriguingly the Grx2 mutant C40S - lacking the capacity to form a dithiol - could not decrease the NO levels compared to control, implicating that in this inflammation model the dithiol mechanism plays an important role (Hanschmann et al., 2020). Therefore, we hypothesize that a possible mechanism of HIF-1α regulation in airway

inflammation may be the Grx2 mediated denitrosylation of s-nitrosylated HIF, leading to destabilization and subsequent degradation of the protein. Protein denitrosylation represents a possible mechanistic switch for HIF-1α activity and function. Crucial roles of HIF-1α in allergic asthma, such as induction of monocyte chemotactic protein 1 (MCP-1/CCL2) contributing to airway hyperresponsiveness, promotion of enhanced glycolysis and induction of VEGF-mediated airway remodelling may thus be reduced by interaction of Grx2 with HIF-1α *in vivo* as shown in this study. Verification of the hypothesized interaction of Grx2 with HIF-1α could be assessed in the future by investigating the influence of Grx2 admission to intracellular levels of S-nitroyslated HIF-1α *in vitro*.

4.4 Treatment with Trx1 and Grx2 alterates immune response and reduces allergic phenotype in a mouse model of allergic airway inflammation

Considering the pathology of allergic asthma, the condition can be summarized as an association of chronic inflammation, bronchial hyper-responsiveness and airway remodeling, resulting in intermittent airway obstruction with clinical symptoms such as shortness of breath, cough and chest pains (Maddox & Schwartz, 2002). The chronic inflammatory process in the asthmatic lung is amplified and perpetuated by a complex network of structural changes, recruitment of various cell types and secretion of different mediator proteins, thus it is no surprise that not every aspect of the etiology and pathology of allergic asthma has yet been understood. The causal mechanisms of allergic asthma are closely linked to the activity of Th2-cells. Th2-cells stimulate effector cells of the allergic cascade such as eosinophils, mast cells and dendritic cells through a set of mediators and cytokines: IgE, IL-3, -4,-5,-9, -13 and TNF-α (Cookson, 2002). Due to those findings, asthma was depicted as a Th2-cell prone disease linked to atopy since the 1990s. In the following, research on asthma treatment focussed on inhibition of Th2-cells and Th2-associated mediators i.e. IL-antibodies without reaching a clinical trial state, reviewed by Holgate (Holgate et al., 2009). In view of failing therapeutic alternatives the concept of asthma as a strictly Th2-regulated disease had to be revisited. Subsequently, alterations in the lung epithelium caused by environmental pollutants such as cigarette smoke, fuel particulates, viruses and other airborne pollutants were propagated to initiate signals that transfer from the epithelial layer to the underlying mesenchyme and induce inflammatory and remodelling processes in this compartment (Holgate, 2011). Alongside these

observations, the susceptibility of the lung epithelium to oxidants was eventually acknowledged. Furthermore, it was reported, that the lung epithelial cells lack the capability of producing INF-γ and INF-β in response to infection by rhinoviruses (Wark et al., 2005). Since both factors – susceptibility to oxidants and viruses lead to cellular apoptosis, the mechanisms of epithelial dysfunction have entered the focus of interest. In this study we already revealed distributional changes of Trx family proteins in lung inflammation *in vivo*. Considering the functions of Grxs and Trxs in protecting the epithelial barrier and exerting redox signalling functions in lung inflammation – we aimed at investigating the influence of Trxs on immune cells in vitro. Cell count of immune cells from BAL samples of inflamed mice showed an increase in total immune cells, as well as eosinophils, lymphocytes and macrophages. Whereas treatment of the OVA-challenged mice with Grx2 could reduce the number of total immune cells and eosinophils significantly, administration of the redox inactive Grx2-C40S mutant failed to reduce the immune cell count. These results confirm findings published by Hanschmann and co-workers in 2020 that treatment with Grx2 and Trx1 reduced the BAL cell count in a mouse model of OVA-induced asthma. Moreover, they also corroborate a significant suppression of eosinophils after s.c. administration of Trx1 and Grx2, whereas Grx1 and Prx2 treatment did not alter the immune cell count in BAL fluid (Hanschmann et al., 2020).

Since Grx2 treatment reduced the cell count of all analysed immune cells other than macrophages in vivo, we hypothesized that oxidoreductases exert their influence on macrophages by changing the cytokine expression. We analysed cytokine levels of RANTES, IL-4, IL-6, IL-10, TNF-α and INF-γ secreted by macrophages after treatment with different recombinant redoxins Trx1 and Grx2, and under conditions similar to allergic asthma with co-stimulation by LPS and INF-γ. In general, it could be shown that mTrx1 and hGrx2c alone have cytokine-like effects on macrophages, inducing secretion of RANTES, IL-6, IL-10, INF-γ and TNF-α. Cytokine production of macrophages treated with Grx2 alone showed a decrease in IL-6, INF-γ and TNF-α in comparison to OVA-conditions. In combination with LPS/INF-γ, Grx2 administration again increased levels of IL-6, INF-γ and TNF-α whereas RANTES levels remained similar to treatment with LPS/INF-γ or Trx1. Especially IL-6 production decreased after addition of Grx2, suggesting immune modulating function for Grx2 in airway inflammation. IL-6 facilitates T-lymphocyte differentiation towards Th2-cells via STAT3 activation and induction of IL-4 production (Scheller et al., 2011). However, the effect of Grx2 on macrophage cytokine production could not be seen under OVA conditions to the same extent.

Intriguingly, cytokine levels changed significantly after treatment with Trx1 under OVA-conditions (+LPS/INF-γ) compared to separate administration of LPS/INF-γ. The additional

increase of INF-γ and TNF-α in macrophages treated with Trx1+LPS/INF-γ suggests a modulation of Trx1 towards a Th1-prone immune response. INF-γ is produced by CD4 + TH1-cells and promotes activation and presentation of class I and class II MHC molecules on epithelial cells and macrophages therefore eliciting antigen presentation (Chung, 2001). Overall INF-γ facilitates a Th1-prone immune response and counteracts several mechanisms postulated in the pathology of asthma. In contrast, TNF-α plays an important role in apoptosis and the induction of the cellular immune response by facilitating activation and survival of inflammatory cells, cell migration via endothelium and promoting tissue degeneration by induction of tissue destructive enzymes (Kalliolias & Ivashkiv, 2016).

Trx1+LPS/INF-γ treatment increased the levels of IL-10, supporting the theory of overall anti-inflammatory properties of Trx1. The anti-inflammatory cytokine IL-10, secreted by various cell types i.e. macrophages, dendritic cells and lymphocytes, supresses excessive immune response by inducing T-regulatory cells promoting allergen-specific tolerance and furthermore by complex mechanisms including inhibition of INF-γ, TNF-α and IL-6 production(Schülke, 2018). In conclusion, IL-10 increase counteracts some of the pathological mechanisms postulated in asthma, for instance the increased allergen sensitivity and airway hyper responsiveness. Moreover, reduced airway hyper responsiveness in mice treated with Trx1 and Grx2 measuring the methacholine induced bronchial reactivity by head-out body plethysmography has been demonstrated (Hanschmann et al., 2020).

Another player in allergic airway inflammation is a chemokine found in the literature under the name RANTES (regulated on activation, normal T cell expressed and secreted) or C-C Chemokine ligand 5 (CCL5 – due to its C-C-structural motif). In 1996 it came to the attention of asthma researchers, as it was detected in the airways of asthma patients and enhanced eosinophil recruitment through the CCR3 receptor (Powell et al., 1996). Findings on the influence of CCL5 on airway inflammation have been contradictory, since several studies reported an increase in CCL5 levels and a subsequent decrease in airway hyperresponsiveness after allergen challenge (Koya et al., 2006). However, it seems to be established that CCL5 plays a key role in virus infections for instance it induces cytotoxic T-lymphocyte activation in response to RSV infection potentiating lung inflammation after allergen contact (Catalfamo et al., 2004). Therefore, we also investigated the CCL5/RANTES levels after macrophage stimulation and detected a clear increase after stimulation in comparison with medium control but there were no significant changes in CCL5 levels comparing the different stimulation approaches.

Finally, IL-4 is a major cytokine in Th2-prone immune response in asthma, not only inducing

allergen specific IgE production in B-cells but also facilitates enhanced production of glycoproteins by goblet cells and cell migration of eosinophils via IL-4 induced VCAM-1 expression (Lambrecht et al., 2019; Steinke & Borish, 2001). Alterations in IL-4 levels therefore lay in the focus of asthma research and therapeutic strategies involve the IL-4 receptor antagonist Dupilumab, which significantly reduced exacerbation rates in asthma patients and has a beneficial effect in patients with corticosteroid refractory nasal polyposis (Castro et al., 2018; Jonstam et al., 2019).

Separate treatment with Grx2 and Trx1 led to an increase in IL-4 cytokine secretion, however, treatment with Trx1+LPS/INF-γ did not yield significant changes in IL-4 levels in comparison to LPS/INF-γ alone. These results allow two conclusions to be drawn: either there is no significant effect of Trxs on IL-4 levels or the effect on IL-4 is exerted differently and could not be detected by our experimental setup. The protein structure of IL-4 contains three active thiols which are crucial for binding of IL-4R-α receptor and in a reduced state prevent IL-4 binding to its receptor. In 2009 Curbo and co-workers showed a possible reduction of IL-4 intramolecular disulfides by Trx1 and PDI, detecting 10-fold increase of NEM-biotin labelled thiols in IL-4 in the presence of Trx1 (Curbo et al., 2009). This reversible redox modification could be a potential mechanism of Trx1 influence on IL-4 activity, not by regulating the IL-4 secretion but rather modulating the capacity of IL-4 to bind to the IL-4R-α receptor. Encouraged by the findings of Curbo and co-workers we performed a NEM-biotin switch assay, to determine changes in free thiols groups after treatment with Grx2, Trx1 and LPS/IFN-γ. Unfortunately, results indicated no significant alterations in free thiol groups between the 5 different composites and the negative control. Among several reasons for the insufficient outcome of this experiment, rapid autooxidation during sample extraction and preparation could obscure changes in redox state of proteins. The administration of 1 M TCEP neutralized with NaOH was utilized to reduce reversible redox modifications including disulfide bonds, s-nitrosylation and sulfenic acid formations. Additionally, samples were frozen immediately after sample extraction, however further oxidation of free thiols cannot be excluded. Moreover, incomplete alkylation of thiol groups by NEM is a possible reason for incorrect detection of free thiol groups (Rogers et al., 2006). Finally, independent repetition of the experiment would be necessary to gain reliable information and therefore further insights into the changes in redox state of cytokines after treatment with Trxs could be a promising approach to Trx functions in immune response.

Reviewing the results from our macrophage *in vitro* stimulation, the protein preparation of mGrx2 and hTrx1 must be taken into close consideration. Recombinant proteins were reduced

with DTT and excess DTT was removed using a NAP 5 column, however endotoxin removal was not conducted in these experiments. Endotoxins, consisting of lipopolysaccharides from bacterial cell membranes, are potent activators of macrophages and monocytes and therefore dramatically interfere with the experimental setup used in this study. In 2020, Hanschmann and co-workers showed no significant changes in INF-γ levels after stimulation with either Grx1, Grx2, Trx1 or Prx4, therefore the results with INF-γ increase shown in this study could be influenced by endotoxins added with the recombinant protein. In future experiments, endotoxin removal should be performed e.g. using standardized endotoxin removal columns to avoid unspecific macrophage stimulation.

4.5 Extracellular functions of thioredoxin family proteins in allergic airway inflammation – relevance and future perspectives

This study has provided novel insight into spacial and - to some extend - functional changes of Trx family proteins in a murine model for allergic airway inflammation. The characterization of important Trxs, Grxs and Prxs in healthy mice and after OVA-induced onset of lung inflammation has shown distinct alterations in the redoxin levels in the inflamed lung. In intracellular lung samples, Grx2 and Prx4 levels were found to be increased upon inflammation. We have identified a second Grx2 isoform, likely Grx2c, present upon lung inflammation. Therefore, we have further investigated the influence of treatment with either Grx2WT or the redox inactive Grx2C40S mutant in a mouse model for OVA-induced allergic airway inflammation. Histological features of asthma in mice lungs have been reduced after treatment with Grx2 WT. His-tag staining of lung sections has shown an uptake of the recombinant protein into lung epithelial cells and therefore indicated a targeted function for Grx2 in airway inflammation. Levels of Trx family proteins after treatment with recombinant Grx2 have remained inconclusive, the assessment of changes in Trxs after treatment with Grx2WT or Grx2C40S need further investigations, for instance sample number would have to be increased for more valid statements. However, HIF und caspase results have shown an increase after OVA-induced onset of inflammation in IHC and have indicated a decrease under treatment with Grx2. We have suggested HIF-1α as a potential target for Grx2 signalling, since HIF-1α decreased under treatment with Grx2 in OVA lung. Denitrosylation of a critical active site cysteine is known to induce destabilisation and degradation of HIF-1α with subsequent structural changes in bronchial airways such as remodelling and chemotaxis of inflammatory

cells. We hypothesized that upon lung inflammation upregulated Grx2 leads to denitrosylation and deactivation of HIF1α. This hypothesized interaction of Grx2 with HIF-1α clearly needs further investigation, for instance by testing the denitrosylation capacity of Grx2 in GSNO pre-incubated HIF-nitroso thiols in a fluorimetric diaminonaphthalene-(DAN) assay (Misko et al., 1993).

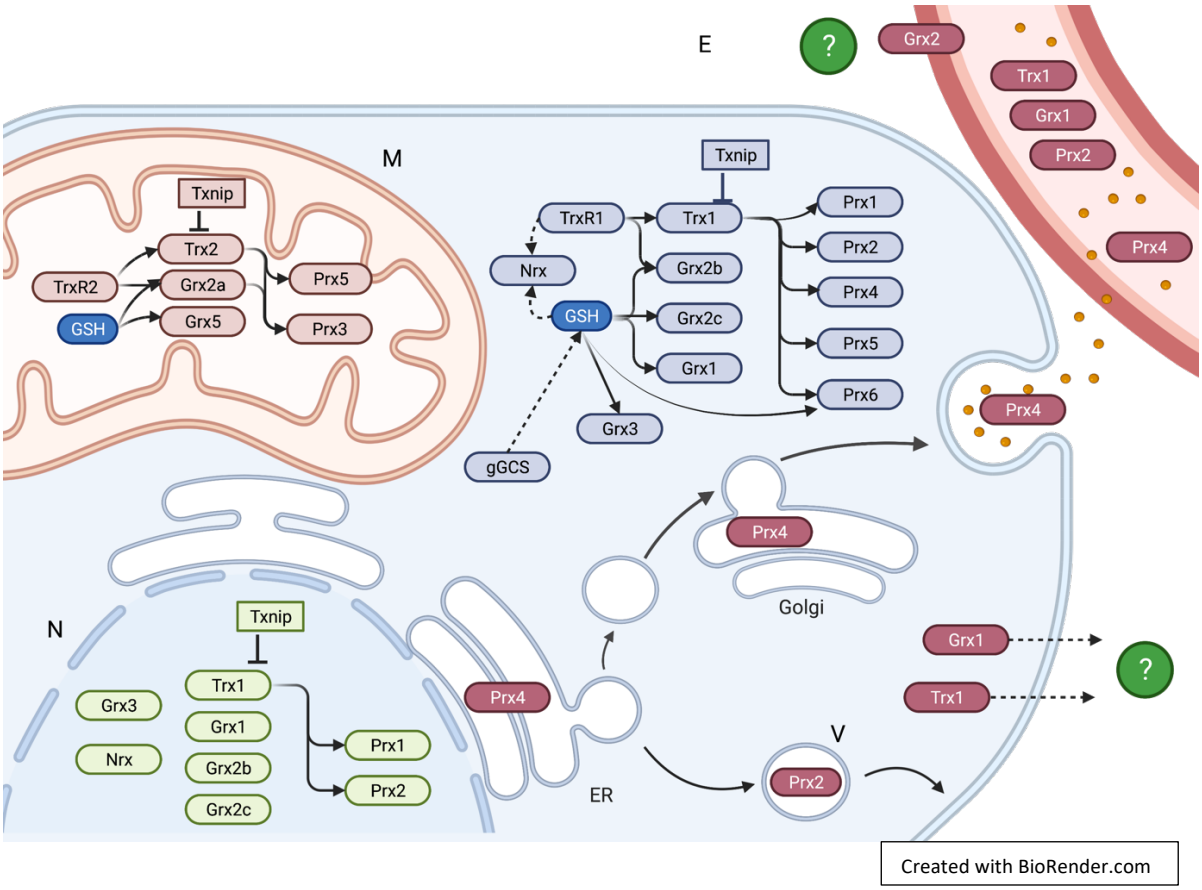


Figure 18 – Compartmentalisation of Trxs, Grxs and Prxs in the human cell and extracellular space and confirmed interactions in redox signalling: *Thioredoxin localisation is shown in different intracellular and extracellular compartments, green in the nucleus (N), brown in mitochondria (M), blue in the cytosol and red in the extracellular space (E). The secretory compartments are depicted, endoplasmatic reticulum (ER), Golgi apparatus (golgi), lysosomal vesicles (V), To date secretion of Prx4 via ER/golgi pathway is known, for Prx2 and Prx1 a lysosomal secretion upon cysteine oxidation is assumed, for Trx1 and Grx1 an alternative secretion pathway is considered but not confirmed, secretion of Grx2 is still under investigation.*

Eventually, the focus of our research shifted towards the extracellular compartment of the lung, represented by samples from the bronchoalveolar lavage fluid obtained through rinsing of the trachea and bronchial tubes. Why did we take such a keen interest in BAL fluid samples? The lung epithelial lining fluid scavenging environmental pollutants, particulates and airborne

viruses plays a key role in the onset of asthma by initiating inflammatory and remodeling signals in the underlying tissue. The structural changes in the inflamed lung such as hyperplasia of goblet and smooth muscle cells, mucus hypersecretion, vessel dilation and activation of inflammatory cells are enabled by this process. In the epithelial lining fluid ROS and RNS are ubiquitously present also in the healthy lung, therefore the cells are well equipped with redox active enzymes such as superoxide dismutase, catalase, glutathione peroxidase and particularly Trxs, Grxs, Prxs (Comhair & Erzurum, 2010). Not only environmental air pollutants but alveolar cells and most importantly inflammatory cells such as macrophages, neutrophils and eosinophils produce excess ROS upon inflammation leading to disturbances of the redox equilibrium (Kinnula, 2005). In this study, we have discovered increased levels of Trx1, Grx1, Prx2 and Prx4 in extracellular samples of inflamed mouse lungs. Intriguingly, Prx4 has been detected only upon airway inflammation in BAL fluid and Prx4 levels were shown to be constant under Grx2 treatment. IHC experiments have confirmed the presence of Prx4 in the epithelial cells of the inflamed lung and IHC has demonstrated reduced asthmatic phenotype after OVA+Grx2 treatment in HE-staining.

Moreover, we have identified an increase in total BAL inflammatory cells after OVA-challenge and an increase in eosinophils, which were reduced again after treatment with Grx2WT. *In vitro* treatment of macrophages elicited from Balb/c mice with recombinant Grx2 and Trx1 was an attempt to shed light on functional mechanisms in inflammation. Grx2 and Trx1 have been identified as potential activators of macrophages, inducing secretion of RANTES, IL-6, IL-10, and TNF-α, whereas cytokine levels of IL-4 and INF-γ have not been altered. Upon combined administration of redoxins with LPS/IFN-γ, mimicking an inflammatory state, Trx1 was shown to reduce cytokine levels of TNF-α and INF-γ compared to LPS/IFN-γ alone. Treatment with Trx1/LPS/IFN-γ induced IL-6 and RANTES production similar to a level seen after LPS/IFN-γ stimulation.

Macrophage activation must be reviewed critically since endotoxin removal was not performed during preparation of the recombinant proteins. Intriguingly, similar experiments stimulating macrophages from PBS and OVA mice with Trx1, Grx1, Grx2 and Prx2 after endotoxin removal also indicated no effect on IFN-γ levels and confirmed our results regarding the influence of Trx1 and Grx2 on IFN-γ (Hanschmann et al., 2020).

In addition, our findings of extracellular Prx4 in lung inflammation would suggest experiments on macrophage stimulation by Prx4, those experiments are currently performed by Hudemann and co-workers and will enlighten Prx4-macrophage interaction. In conclusion, our findings have demonstrated a specific regulation of Trx family proteins in allergic airway

inflammation in the intracellular and extracellular compartment. Clearly Trxs, Grxs and Prxs not only reduce oxidative damage by scavenging H_2O_2, but they interact in differential regulatory pathways most importantly stimulating and regulating inflammatory cells such as macrophages. Since anti-inflammatory capacity of Grx2 in treatment of allergic airway inflammation has been established in a mouse model, it is tempting to suggest a serious clinical value for Grx2 and its interaction partners from our study. However, the results obtained from the animal model cannot be directly transferred to a clinical setting. Since the murine model for OVA-induced allergic airway inflammation only mimics an asthma-like condition, it cannot capture the full complexity of inflammatory processes in human asthma and further investigations with asthmatic volunteers are required.

The first attempt to confirm increased Grx2 levels in human was performed by Hanschmann and co-workers and they have detected reduced serum levels of Grx2 in patients with acute asthma. Reduced serum levels of Grx2 could also indicate the need for external administration of a redox active Grx as a therapeutic approach. In a recent review the importance of protein S-glutathionylation in asthma and other chronic lung diseases was highlighted and the need to search for a “suitable” Grx for asthma therapy has been emphasised (Janssen-Heininger et al., 2020).

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6 Appendix

Eidesstattliche Erklärung

Hiermit erkläre ich, dass ich die vorliegende Dissertation selbständig verfasst und keine anderen, als die angegebenen Hilfsmittel benutzt habe.

Die Dissertation ist bisher keiner anderen Fakultät, keiner anderen wissenschaftlichen Einrichtung vorgelegt worden.

Ich erkläre, dass ich bisher kein Promotionsverfahren erfolglos beendet habe und dass eine Aberkennung eines bereits erworbenen Doktorgrades nicht vorliegt.

Greifswald, den 17.04.2024 ……