The role of cellular antioxidant systems in immunity

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2 Summary

Metabolism and living in an oxygenate environment exposes organisms to reactive oxygen and nitrogen species (ROS/RNS), molecules such as hydrogen peroxide and hydroxyl radicals, capable of oxidizing and damaging various biomolecules. It was recognized early that these modifications are harmful. For example, it was suggested that damage by ROS could be responsible for the decline of functions with increasing age. However, it is clear now that ROS and RNS also have beneficial physiological roles. An example is the production of ROS and RNS by phagocytes of the immune system which is essential for the efficient elimination of invading pathogens. This is demonstrated in patients with defective phagocytic ROS production, who suffer from reoccurring, life-threatening infections (chronic granulomatous disease). Additionally, ROS and RNS, mainly hydrogen peroxide and nitric oxide, can also have signaling functions. For instance, in immune cells a role for ROS in signaling by the T and B cell receptor was found, and treating these cells with antioxidants inhibits signaling. These examples illustrate that oxidants clearly have beneficial and critical roles in life and cannot be just considered harmful byproducts. Indeed, cells have a multitude of enzymes, proteins and compounds to control and scavenge ROS and RNS, while still allowing for their beneficial functions. During my PhD I searched for a better understanding of the role and functions of ROS and cellular antioxidant systems in immune cells and responses by studying the role of the transcription factor Nrf2 (nuclear factor, erythroid derived 2, like 2) in inflammasome activation in dendritic cells (DC), and the functions of the antioxidant enzyme TrxR1 (thioredoxin reductase 1) in T lymphocytes.

Nrf2 activates transcription of genes by binding to the “antioxidant response element” (ARE) in the promoters of target genes. As this name indicates, many of the activated genes encode enzymes and proteins involved in cellular antioxidant systems. Stefan Freigang et al. in our lab showed the importance of Nrf2 for efficient inflammasome activation and interleukin (IL)-1β secretion by crystals. Inflammasomes are required for the activation of Caspase-1, which in turn cleaves and thereby activates pro-IL-1β. Processing of IL-1β is required for it to be bio-active. It then acts as a highly pro-inflammatory cytokine. In my PhD project focusing on Nrf2, we found that Nrf2 is mainly important for activation of the Nlrc3 inflammasome, which is activated by the
before mentioned crystals as well as other stimuli. In contrast, the Naip/Nlrc4 and Aim2 inflammasomes seemed to be less dependent on Nrf2. Nrf2 was already required upstream of IL-1β secretion for Caspase-1 processing and oligomerization of the inflammasome adaptor Asc (Apoptosis-associate speck-like protein containing a CARD domain). Trying to elucidate the role of Nrf2 in inflammasome activation, we found that transcriptional activity of Nrf2 is essential for efficient inflammasome activation and not some novel function independent of its capacity as a transcription factor. Despite its role in controlling antioxidant genes, we observed no change in steady-state ROS levels in the absence of Nrf2. Modulation of ROS was similarly not able to rescue inflammasome induction, suggesting a different mechanism than global changes in redox balance being responsible for the phenotype.

In a second project I investigated the role of TrxR1 in immune cells. TrxR1 is at the beginning of the cytosolic thioredoxin (Trx) system, which forms together with the glutathione (GSH) system the two main thiol based antioxidant systems in mammalian cells. Global deficiency of TrxR1 is embryonically lethal and we consequently used conditional knockouts of TrxR1. Cd4-Cre-mediated deletion of Txnrd1 (the gene encoding TrxR1) allowed the study of the role of TrxR1 in T cells. We found TrxR1 to be mainly redundant in steady state. In contrast, TrxR1-deficiency led to a massively reduced expansion upon T cell stimulation. This seems to be mainly due to a delayed entry in cell cycle and slower proliferation. A small decrease in cell survival possibly also contributed. Immediate activation and T cell receptor (TCR) signaling on the other hand was not impacted, indicating the defect is further downstream and not due to a block in TCR signaling. Trxs, downstream of TrxR1, are known to contribute to the biosynthesis of deoxy-nucleotides (dNTPs), the building blocks for new DNA. However, an insufficient supply of dNTPs was not the cause of the reduced proliferation in TrxR1-deficient T cells. Nevertheless, other biosynthesis deficiencies could be the cause of the reduced expansion, since, based on the treatment with antioxidants, there is no general problem with too much oxidation in T cells lacking TrxR1. Though, additional research is required to conclusively determine the precise role of TrxR1. Limited results for the function of TrxR1 in other immune cells suggest a differential requirement for TrxR1 in different cell types and possibly again a more important role upon cell activation.
These findings hint at the complexity of ROS/RNS and antioxidants interactions in cells with effects normally not mediated by gross changes in the cellular redox balance, but by more specific interactions.
3 Zusammenfassung


Nrf2 aktiviert die Transkription von Zielgenen nach Bindung an genetische „ARE“ Elemente. Nrf2 treibt die Expression von vielen Proteinen und Enzymen der zellulären Antioxidanssysteme. Stefan Freigang et al. in unserem Labor zeigten die Bedeutung von Nrf2 für die Inflammasomaktivierung und Sekretion von Interleukin (IL)-1β durch Kristalle und andere Stimulanzien. Inflammasome aktivieren Caspase-1, diese wiederum schneiden und dadurch aktivieren Pro-IL-1β, die inaktive Vorstufen des entzündungsfördernden Zytokins IL-1β. In dieser Arbeit
konnten wir zeigen, dass Nrf2 hauptsächlich wichtig für die Aktivierung von Nlrp3-Inflammasomen ist, während die Bedeutung von Nrf2 für Naip/Nlrc4- und Aim2-Inflammasome kleiner ist. Nrf2 wirkt schon vor der Sekretion von IL-1β auf die übergeordnete Prozessierung von Caspase-1 und die Oligomerisierung des Inflammasomadaptors Asc (Apoptosis-associate speck-like protein containing a CARD domain). Die Transkriptionsfaktoraktivität von Nrf2 ist essenziel für die effiziente Aktivierung von Nlrp3. Somit scheinen Nrf2-aktivierte Gene, und nicht etwa eine direkte Protein-Protein Interaktion von Nrf2 mit Inflammasomkomponenten für eine effiziente Aktivierung von Nlrp3 Inflammasomen wichtig zu sein. Wir konnten keinen Unterschied im Grundniveau von ROS zwischen Zellen mit oder ohne Nrf2 feststellen, trotz Nrf2s Rolle als transkriptioneller Aktivator von Antioxidansproteinien. Auch konnte die Manipulation von Zellen mit Antioxidantien die Inflammasomaktivierung in der Absenz von Nrf2 nicht retten. Diese Ergebnisse legen nahe, dass Nrf2 das Nlrp3 Inflammasom nicht über eine Veränderung des totalen zellulären ROS Niveaus beeinflusst.

Zusätzliche Forschung ist jedoch nötig um eine endgültige Antwort für die genaue Wirkung von TrxR1 in T Zellen zu finden. Erste Resultate für die Rolle von TrxR1 in anderen Immunzellen scheinen auf eine unterschiedliche Wichtigkeit von TrxR1 für verschiedenen Zelltypen hinzudeuten und wahrscheinlich wieder einer größeren Rolle in aktivierten Zellen.

Zusammengefasst zeigen diese Resultate die Komplexität der Interaktion von ROS und RNS mit zellulären Antioxidanssystem und dass Effekte nicht über globale Veränderung des Redoxgleichgewichtes verursacht werden, sondern durch spezifischere Interaktionen.
4 Introduction

For my PhD thesis I investigated the role of Nuclear factor, erythroid derived 2, like 2 (Nrf2) and Thioredoxin reductase 1 (TrxR1) in mouse immune cells and responses. Nrf2 and TrxR1 are important proteins for the cellular redox (reduction-oxidation) status and antioxidant response, i.e. to protect cells from excessive oxidants. Nrf2 is a master transcription factor driving expression of many proteins and enzymes involved in this process, whereas TrxR1 is the enzyme at the beginning of one of the major thiol-based antioxidant systems in the cytosol. The following chapters give an overview over the cellular redox system and the (mouse) immune system.
4.1 Cellular antioxidant systems

Reactive oxygen species (ROS) include the superoxide anion ($O_2^{-}$) and hydroxyl radicals ($OH^{•}$) as well as the non-radical hydrogen peroxide ($H_2O_2$) (Schieber 2014). Nitric oxide (NO) reacts with superoxide to form peroxynitrite (ONOO$^{-}$), a highly reactive nitrogen species (RNS) (Pacher 2007). These and other oxidants were originally thought to play mainly a detrimental role for cell function by damaging biomolecules, such as proteins and lipids. It has, however, become clear now that these molecules serve essential roles in, for example, cell signaling and immune defense (Harman 1956; Dupre-Crochet 2013; Holmstrom 2014; Schieber 2014). ROS/RNS can be generated from exogenous sources such as ionizing radiations or xenobiotics. However, there are also endogenous sources, mainly mitochondria and nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (Nox) complexes. Mitochondrial complex I and III are thought to be the main producers of superoxide in mitochondria in intact cells (Murphy 2009; Willems 2015). Nox was originally described in neutrophils, where a complex of membrane bound Nox2 (gp91$^\text{phox}$) and p22$^\text{phox}$ upon recruitment of cytosolic p40$^\text{phox}$, p47$^\text{phox}$, p67$^\text{phox}$ and RAC1 produces large amounts of superoxide after Neutrophil activation. This process was termed respiratory burst and is necessary for the killing of pathogens by neutrophils and other phagocytes. Nox1, 2, 3 and 5 produce superoxide, whereas Nox4 and the related Duox1 and 2 directly release hydrogen peroxide. Nox1 and Duox2 activation in colon epithelium was shown in response to commensal bacteria and to be important for tissue homeostasis. Endothelial cells express Nox1, Nox2, Nox4 and Nox5, which have roles in blood pressure regulations by counteracting NO signaling. Nox1 was also shown to promote, while Nox4 inhibited growth. Nox3 is highly expressed in the inner ear and important for a proper sense of balance (“head-tilt” mice have a mutated Nox3). In the thyroid Duox1 and Duox2 are involved in hormone biosynthesis (Bedard 2007; Dupre-Crochet 2013; Randall 2013; O’Neill 2015; Santillo 2015). Superoxide can dismutate to hydrogen peroxide and oxygen, a process greatly accelerated by abundantly expressed superoxide dismutases (Sod). Mammalian cells have a copper-zinc containing Sod ($Sod1$) mainly in their cytosol, a manganese-containing Sod in their mitochondria ($Sod2$) and can express an extracellular copper-zinc Sod ($Sod3$) (Fukai 2011; Bresciani 2015). Hydroxyl radicals are formed by Fenton reaction of hydrogen peroxide with iron ($Fe^{2+}$) or are derived from...
peroxynitrous acid (ONOOH). Hydroxyl radicals are highly reactive and react with most biomolecules such as lipids, nucleic acids and proteins. No specific enzymes are known to neutralize hydroxyl radicals (Finaud 2006; Halliwell 2013; Indo 2015). In contrast, several enzymes are able to neutralize hydrogen peroxide by converting it to water and oxygen. These are catalase, glutathione peroxidases (Gpx) and peroxiredoxins (Prx). The enzymatic antioxidant pathways, discussed in more detail in the next chapters, are complemented by dietary antioxidants such as vitamin C, vitamin E, carotenoids and flavenoids (Fig. 1a) (Pisoschi 2015).
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Fig. 1 Overview over cellular antioxidant systems and sources of reactive oxygen and nitrogen species (ROS and RNS). (a) Overview of ROS and RNS generation and degradation in mammalian cells. Cellular localizations of various antioxidant enzymes are indicated. For simplicity, the pathways of ROS/RNS generation and degradation are mainly shown in the cytoplasm, but they also happen in other compartments, where the enzymes are present. Adapted from (Lillig 2007; Nathan 2010; Hanschmann 2013) (b) The Thioredoxin (Trx) and Glutathione (GSH) system. Electrons for NADPH are used to reduce the mixed selenylsulfide of thioredoxin reductase (TrxR). TrxR either directly reduces targets or transfers electrons to Trx, which then can reduce protein disulfides. Additionally, NADPH is also the electron donor for Glutathione reductase (GR). GR reduces oxidized disulfide-linked glutathione (GSSG) to two reduced glutathiones (GSH). GSH is used to recycle glutaredoxin (Grx), which reduces protein disulfides and S-glutathionylated proteins. GSH is also used as the electron donor of other enzymes such as glutathione peroxidase (Gpx). NADPH is regenerated in the pentose phosphate pathway (PPP) by glucose-6-phosphate dehydrogenase (G6PD) and 6-phosphogluconate dehydrogenase (PGD) as well as by isocitrate dehydrogenase 1 (IDH1) and malic enzyme 1 (Me1). Adapted from (Brigelius-Flohe 2013; Hanschmann 2013; Hayes 2014). Additional abbreviations: ER oxidoreductin 1 (Ero1), nitric oxide synthases (Nos), NADPH oxidase (NOX), superoxide dismutase (Sod).
Antioxidant peroxidases: Catalase, glutathione peroxidases and peroxiredoxins

In mammals catalase is a homotetramer mainly localized in peroxisoms with high expression in liver, kidneys and erythrocytes. It requires heme to catalyze the dismutation of hydrogen peroxide to water and molecular oxygen without the need of an electron donor. However, catalase also has other activities and can even act as a pro-oxidant. For instance, a catalase-dependent increase in ROS was described in UV-treated keratinocytes (Kirkman 2007; Glorieux 2015). Expression of catalase seems to be non-essential in mice, since knockout mice are viable and fertile (Ho 2004). Another group of enzymes neutralizing peroxides are glutathione peroxidases (Gpxs). They belong to the thioredoxin protein family. Mouse Gpx1, Gpx2, Gpx3, and Gpx4 contain a selenocysteine (SeC, U), which is essential for full activity (Brigelius-Flohe 2013; Deponte 2013). SeC is encoded by the UGA stop codon and requires an additional 3’ untranslated region (3’-UTR) SeC insertion sequence in the messenger RNA for incorporation into the nascent protein. Selenocysteine is synthesized on its transfer RNA (tRNA^{Ser}{Sec}) from cysteine (Bulteau 2015). Gpx1 is found in cytosol and mitochondria of most cells and reduces hydrogen peroxide and other small organic hydroperoxides to water and alcohols, respectively. It uses two glutathiones (GSH; see next chapter) as electron donors. In contrast, Gpx4 also reduces larger hydroperoxides, such as phospholipid- and cholesterol hydroperoxides, found in membranes or lipoproteins. Consequently, Gpx4 is important for the redox balance of membranes. GSH is the main source of reducing equivalents for Gpx4, although protein thiols can substitute for GSH. The cytosolic splice form of Gpx4 is ubiquitously expressed, while a mitochondrial and sperm-nuclear form show more restricted expression. A global knockout of Gpx4 is lethal, whereas Gpx1, 2 or 3-deficient mice are viable. Gpx2 is mainly found in the intestine and Gpx3 extracellularly in plasma. Mouse Gpx5-8 (and most non-mammalian Gpxs) use a cysteine instead of a SeC in their active site (Brigelius-Flohe 2013; Deponte 2013). Peroxiredoxins (Prxs) are the third class of antioxidant peroxidases. They use a conserved cysteine, termed peroxiadic cysteine (C_p) for catalysis. C_p reacts as a thiolate (C_p-S’) with a peroxide (ROOH) forming sulfenic acid (C_p-SOH) and the corresponding alcohol (ROH or water for R=H). A second thiol can then attack the sulfenic acid forming a disulfide (C_p-S-S-R’). This thiol is from the resolving cysteine (C_R) of the other subunit of the Prx homodimer in 2-Cys Prxs (mammalian Prx1-4) or
of the same protein in atypical 2-Cys Prx (mammalian Prx5). The resulting disulfide is then generally reduced by disulfide exchange with thioredoxin (Trx), thus recycling the Prx by re-reduction. The third group of Prxs are 1-Cys Prxs (mammalian Prx6), which are regenerated by GSH. The different Prxs localize to various cellular compartments: Prx1 is found mainly in cytosol, nucleus, peroxisomes and extracellularly; Prx2 in cytosol, nucleus and at membranes; Prx3 in mitochondria; Prx4 in cytosol, endoplasmic reticulum (ER) and secreted; Prx5 in cytosol, mitochondria and peroxisomes; Prx6 in cytosol, lysosomes and vesicles (Rhee 2011; Hanschmann 2013; Karplus 2015). 2-Cys Prxs can be hyperoxidized, i.d. the sulfenic acid (C_P-SOH) is further oxidized to sulfinic acid (C_P-SO_2H) and possible to sulfonic acid (C_P-SO_3H). This inhibits the regeneration and recycling of the Prx, thus blocking peroxidase activity. Hyperoxidation of Prxs also seems to favor formation of decamers and bigger aggregates, which show molecular chaperone activity. The sulfinic acid can be reduced by ATP-dependent sulfiredoxins (Srx), thus restoring Prx peroxidase activity (Rhee 2011; Randall 2013). There are two main pathways in mammals providing reducing equivalents to antioxidant enzymes. These are the glutathione and the thioredoxin system discussed below.

The glutathione system

The tripeptide glutathione (GSH; γ-L-glutamyl-L-cysteinyl-glycine) forms the central part of the GSH system, one of the main thiol based antioxidant systems in mammalian cells (Fig. 1b). GSH and the oxidized form GSSG (two GSH linked by a disulfide) are also a major cellular redox buffer with concentrations reaching the mM range. GSH/GSSG are found in cytosol, nucleus, mitochondria, peroxisomes and endoplasmic reticulum (ER). With the exception of the ER, during homeostasis the ratio of GSH/GSSG is strongly in favor of the reduced GSH contributing to the reducing intracellular environment. De novo synthesis of GSH depends on glutamate cysteine ligase (GCL) and glutathione synthetase (GSS), which synthesize GSH from the individual amino acids. Additionally, oxidized GSSG can be reduced back to two GSH by glutathione reductase (GR, Gsr), which uses reduced NADPH as its electron donor. Glutathione-S-transferases (GST) catalyze the conjugation of GSH to substrates. This is an important step in the neutralization of many toxic molecules by decreasing their electrophilicity and increasing water solubility, thereby facilitating secretion. GSTs also glutathionylate proteins, thus forming a disulfide between GSH
and a protein cysteine. During oxidative stress the reversible S-glutathionylation might protect cysteines from over-oxidation to sulfinic/sulfonic acids (Kalinina 2014; Morris 2014). S-glutathionylation was also shown to be a post-translational modification regulating protein activity. Examples include IKKβ and p50 in the NF-κB (nuclear factor of kappa light polypeptide gene enhancer in B cells) pathway, which are inhibited by S-glutathionylation (Pineda-Molina 2001; Reynaert 2006). Glutathionylation can be reversed by glutaredoxins (Grx) via a thiol/disulfide exchange reaction. A nucleophilic attack of the N-terminal active site cysteine of Grx on GSH-mixed disulfide (GS-SR) leads to the formation of a Grx-glutathione disulfide and the release of the now reduced target (R-SH). A second GSH is then required to regenerate Grx releasing disulfide GSSG. This mechanism was termed the monothiol mechanism. Some Grx contain a second active site cysteine (CxxC motive) and can catalyze the reduction of protein disulfide bonds via a different mechanism (termed dithiol mechanism, though they still use the monothiol pathway for glutathionylated substrates). Here, a mixed disulfide between Grx and the target protein is formed. This is resolved by the second (C-terminal) cysteine, releasing the reduced target and forming an internal disulfide in Grx. Two GSH are then required to recycle the Grx in two steps via a mixed Grx-GSH disulfide. Alternatively, some Grxs are also re-reduced by thioredoxins (Trxs, described below). Grxs also serve together with Trxs as the electron donors of ribonucleotide reductase (RNR) in many organisms, including mammals, a function not related to its antioxidant role. RNR is the enzyme responsible for de novo synthesis of 2’-doxyribonucleotides, i.e the building blocks of DNA (Hawkins 2007; Lillig 2013).

The thioredoxin system

The second thiol based redox system in mammals is the thioredoxin (Trx) system (Fig. 1b). NADPH is the original electron donor analogous to the GSH system and is used by thioredoxin reductases (TrxR) to re-reduce oxidized Trx. Trx was discovered as the electron of RNR (Laurent 1964), as was the case for Grx. In the context of antioxidant responses Trxs are used as the electron donor of many Prxs (as discussed above) and for methionine sulfoxide reductases (Msr), besides others. Similar to cysteine, methionine contains a sulfur atom susceptible to oxidation, which is reversed by Msrs (Boschi-Muller 2014; Achilli 2015). Trx contain a dithiol CxxC motive in their active site and reduce targets by disulfide exchange. In mammals Trx1
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is found in the cytosol, nucleus and extracellularly, whereas Trx2 is mitochondrial. Additional Trxs are found in testes (Miranda-Vizuete 2004; Mahmood 2013; Lu 2014b). The TrxRs have an analogous localization in mammals: TrxR1 is found in cytosol and nucleus, TrxR2 in mitochondria and a third form (TrxR3 or thioredoxin glutathione reductase) mainly in testes. Moreover, splice variants have been described but poorly characterized (Arner 2009). TrxR as well as GR belong to the same family of protein disulfide reductases and electrons from NADPH are transferred via flavin adenine dinucleotide (FAD) to a disulfide formed by two N-terminal cysteines (CVNVGC motif), which acts as the active site of GR. However, mammalian TrxRs contain a C-terminal extension, which acts as the actual main active site. This active site is formed by a cysteine and a selenocysteine (GCUG motif), which form a selenylsulfide upon reduction of target genes. TrxRs are head-to-tail homodimers and electrons from the N-terminal dithiol of one subunit are transferred to the C-terminal selenylsulfide of the other subunit to re-reduce the active site (Arner 2009; Lu 2014b). The higher reactivity of selenolate compared to thiolate and the relative open active site of mammalian TrxRs are thought to contribute to their wide substrate specificity. TrxRs also reduce several non-Trxs, substrates including Glutaredoxin 2 (Grx2), disulfide isomerase (PDI), dehydroascorbate (oxidized Vitamin C), selenites, lipoic acid and ubiquinone. Activity of Trxs can be inhibited by thioredoxin interacting protein (TXNIP), an endogenous inhibitor (Arner 2009; Mahmood 2013). Knockouts of both Txnr1 and Txnr2 (the genes encoding mouse TrxR1 and TrxR2) are embryonically lethal in mice at E8.5-E10.5 and at E13.5-E15.5, respectively. Embryos showed growth and developmental retardation in the case of TrxR1-deficient embryos and defective heart development and hematopoiesis in the absence of TrxR2. Trx1 and Trx2 are also indispensable for embryonic development. Trx1-deficient embryos died shortly after implantation and showed defective proliferation of the inner cell mass. Lack of Trx2 caused lethality between E10.5 to E12.5 with increased apoptosis and anterior neural tube defects (Matsui 1996; Nonn 2003; Conrad 2004; Jakupoglu 2005; Bondareva 2007).

Nrf2: A master regulator of antioxidant genes

Expression of many antioxidant genes is controlled by the transcriptional activator nuclear factor, erythroid derived 2, like 2 (Nrf2 or Nfe2l2). Nrf2 is a member of the cap’n’collar basic-region leucine zipper protein family. As a heterodimer with small
muculoaponeurotic fibrosarcoma (Maf) proteins (MafF, MafG, MafK) it binds to a DNA motive termed antioxidant response element (ARE, also called electrophile responsive element) and acts as a transcriptional activator. Several hundred Nrf2-target genes were identified including many phase I and II detoxifying enzymes, such as GSTs and multi-drug-resistance-associated protein transporters. Proteins from the GSH system (e.g. Gclc, Gclm, Gsr, xCT cystine importer, Grxs and Gpxs) as well as the Trx system (TrxRs, Trxs, Prxs) are other Nrf2-targets. Two other antioxidant genes upregulated by Nrf2 are heme oxygenase-1 (Hmox1) and NAD(P)H dehydrogenase, quinone 1 (Nqo1). Additionally, Nrf2 also promotes the pentose phosphate pathway, the major producer of reduced NADPH in mammalian cells, and other NADPH-generating enzymes with target genes including glucose-6-phosphate dehydrogenase (G6PD) and malic enzyme-1 (Me1) (Hayes 2014; Pall 2015). Nrf2 has a constitutive activity, but is mostly inhibit by Kelch-like ECH-associated protein 1 (Keap1) during homeostasis. Keap1 is the adaptor of a Cullin 3-based ubiquitin E3 ligase complex binding to Nrf2’s Neh2-ECH homology domain 2 (Neh2) promoting polyubiquitination and proteasomal degradation of Nrf2. Most Nrf2-inducers are oxidants or electrophiles which react with and modify cysteines of Keap1. This stops Nrf2 degradation allowing Nrf2 to enter the nucleus and to activate transcription of target genes. Alternatively, Nrf2 activators might also directly disrupt Keap1-Nrf2 interaction. The two double glycine repeat and C-terminal (DC) domains of a Keap1 homodimer bind one Nrf2 in its high-affinity ETGE and low affinity extended DLG (DLGex) motif. Oxidative modifications of Keap1 are thought to change the strength of the Keap1-DLGex interaction leading to stabilization of the bound Nrf2, thus blocking Keap1 from ubiquitinating further Nrf2 proteins. Nrf2 is also degraded by a Cul1-containing ubiquitin ligase complex containing the adaptor β-transducin repeat-containing protein (β-TrCP), which binds in the Neh6 domain of Nrf2 after phosphorylation by glycogen synthase kinase 3 (GSK3) (Hayes 2014; Suzuki 2015). An additional layer of control is provided by transcriptional regulation. Nrf2 transcription is enhanced by activation of NF-κB and aryl hydrocarbon receptor (Ahr) (Hayes 2014), as well as in a positive feedback loop by Nrf2 itself. Notably, phosphorylations by MAP kinases were shown to play only a minor role for Nrf2 activity (Sun 2009).
**Physiological functions of reactive oxygen species and cellular redox balance**

ROS production is associated with and is essential for optimal B and T cell activation. Similarly, ROS is an important mediator of platelet-derived growth factor (PDGF) or epidermal growth factor (EGF) induced proliferation and differentiation. Together this indicates that ROS plays an important role in cell signaling.

Indeed, EGF induces oxidation of a catalytic cysteine in protein tyrosine phosphatase 1B (PTP1B), which results in inactivation of this negative regulator of EGF receptor signaling. (Yang 2013; Schieber 2014). Signaling is thought to be mediated mainly by hydrogen peroxide. Hydroxyl radicals are too reactive to allow specificity and superoxide with is negative charge cannot diffuse easily through membranes and has low reactivity towards thiols. Hydrogen peroxide as a small polar molecule diffuses through membranes, a process enhanced by aquaporins, and readily reacts with thiols making it a suitable signaling molecule. As described above, (low-level) oxidations of thiols are reversible, thus allowing for the removal of the signal (Forman 2010). Peroxidases (catalase, Prxs, Gpxs) are rather abundant and have fast reaction rate constants, normally in the range of $10^5$-$10^7$ M$^{-1}$ s$^{-1}$ with hydrogen peroxide. Thus it is not completely clear, how hydrogen peroxide can react with target protein cysteines for signaling, as these reactions seem to be much slower. The floodgate hypothesis suggests close proximity of ROS production and target proteins with local inactivation of the antioxidant system as the mechanism allowing oxidation of target proteins. Indeed, hyperoxidation of Prxs can inhibit them and phosphorylation of Prx1 was shown to inhibit it in response to growth factor signaling (Woo 2010; Briehl 2015). Opponents of this theory argue this to be an unlikely mechanism due the free diffusion of hydrogen peroxide through membranes combined with the slow reaction rate with target cysteines leading to hydrogen peroxide diffusing out of the cell. Additionally, there are also many redundant peroxidases present. They suggest a direct role of the antioxidant system in signaling by acting as the first sensor for hydrogen peroxide. Oxidation might then be transferred from them to the target via disulfide exchange. Alternatively, direct protein-protein interaction which depend on the redox status of the antioxidant could mediate signaling (Forman 2010; Randall 2013). Indeed, hyperoxidation of some Prxs seems to promote their aggregation into higher order complexes (as discussed above), which could act as a signal. Another example is the control of apoptosis signal
regulating kinase 1 (Ask1) by Trx1/2 and Grx1. In their reduced state Trx/Grx bind and inhibit Ask1. Upon oxidation (or inhibition by TXNIP for Trxs) Ask1 gets activated. Ask1 is a MAP3K that promotes activation of the c-Jun N-terminal kinase (JNK) and p38 MAP kinase pathways leading to apoptosis and cell death in response to oxidative stress (Matsuzawa 2008; Hanschmann 2013). Oxidation of cysteines can inhibit transcription factors, e.g. NF-κB p50 is inhibited by cysteine oxidation in its DNA binding domain, which is reversed by nuclear Trx1 and Ref-1. Conversely, there are also reports of cytosolic ROS leading to NF-κB activation (Morgan 2011) hinting again at the complexity of ROS-mediated functions and the importance of the cellular localization.
4.2 The immune system

*Innate and adaptive immunity*

The immune system evolved to defend organisms against invading pathogens such as viruses, bacteria and parasites. Innate defense mechanisms have been described in every organism studied including bacteria, plants, and protozoa. Additional first lines of defense against invading pathogens in higher organisms include physical barriers in tissues with epithelial borders to the outside world (e.g. skin, mucosa) and secreted molecules with anti-microbial activity (e.g. acids, peptides) (Schroder 2006; Nakatsuji 2012). An adaptive immune system evolved in early jawed cartilaginous fish and in pre-jawed fish such as the lamprey, or even earlier. The literature often distinguishes the innate and adaptive immune systems, although the distinction can be blurred (Paul 2011; Schenten 2011). The innate immune system uses a limited amount of germ-line encoded pattern-recognition receptors (PRRs) to detect invariant pathogen-associated molecular patterns (PAMPs). PAMPs are not found in hosts, but are essential and ubiquitous for a group of pathogens, such as components of bacterial or fungal cell walls. Alternatively, unusual cellular localizations of molecules also found in hosts can be detected, such as cytosolic DNA. Additionally, activation can also occur in response to damage by recognition of danger-associated molecular patterns (DAMPs) or alarmins released by dying cells, e.g. extracellular adenosine triphosphate (ATP), high mobility group protein B1 (HMGB1) or Interleukin (IL)-33. Cells of the innate immune system include macrophages, dendritic cells (DCs), neutrophils, eosinophils, basophils, mast cells and innate lymphocytes (Takeuchi 2010; Ibrahim 2013; Blander 2014). Innate immunity enables a fast first response against pathogens and helps to initiate and shape the adaptive response, which is slower to form. Adaptive immunity relies on the clonal expansion of T and B cells specific for an antigens present on invading pathogens (Hodgkin 2007). Receptor diversity is generated by stochastic joining of gene segments dependent on recombination-activating genes (RAGs) during T and B lymphocyte development. Further diversity can be created by addition or removal of nucleotides at the joining segments. Consequently, functional T and B cell receptors (TCR and BCR) are not germ-line encoded like PRR and can detect nearly all possible antigens. Thus, there is a huge diversity of specificity but a very low frequency for a given specificity. Clonal expansion depends on each B or T cell expressing only receptors with one specificity, and rapid expansion of those few cells
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(clones) recognizing the antigens present. This selection and proliferation of cells with useful receptor specificity leads to the formation of a antigen-specific immune response. Adaptive immunity takes much longer than innate immunity to be effective, due to the originally low frequency of specific cells (Litman 2010; Hirano 2011). Another hallmark of adaptive immunity is memory, i.e. a faster response to a second exposure of an antigen (Sprent 2002; Schenten 2011).

**Pattern recognition receptors**

PRRs generally belong to five protein families. These are the Toll-like receptors (TLRs), RIG-I-like receptors (RLRs), C-type lectin receptors (CLRs), nucleotide-binding domain, leucine-rich repeat (LRR)-containing (or NOD-like) receptors (NLRs) and Aim2-like receptors (ALRs) (Brubaker 2015).

TLRs are transmembrane proteins with an extracellular LRR domain which mediates PAMP recognition, and an intracellular toll–interleukin 1 receptor (TIR) domain responsible for downstream signaling. TLRs localize to the plasma and endo-/lysosome membranes probing the extracellular compartment. TLR2 forms heterodimers with TLR1 or TLR6 which recognize components of bacterial and fungal cell walls such as triacyl lipoproteins, peptidoglycan (TLR1/2), lipoteichoic acid, diacyl lipoproteins and zymosan (TLR2/6). The flagellin sensing TLR5 is also found on the cell surface. Lipopolysaccharide (LPS), a major PAMP and endotoxin of gram-negative bacteria, is detected by TLR4 with the help of LPS-binding protein (LBP), CD14 and MD-2 on the cell surface and in endosomes. The other TLRs are found on endosomes and recognize nucleic acids: Double-stranded RNA is recognized by TLR3, single-stranded RNA by TLR7 and TLR8, unmethylated CpG DNA motifs by TLR9, while TLR13 binds bacterial 23S ribosomal RNA. The remaining known mouse TLRs are involved in the detection of *Toxoplasma gondii* (as a TLR11/12 heterodimer). Signaling by TLRs depends on myeloid differentiation primary response gene 88 (MyD88) or TIR-domain-containing adaptor inducing interferon-β (TRIF). TLR3 depends on TRIF, TLR4 uses both pathways and the remaining TLRs utilize MyD88. Further downstream TLR ligation leads to activation of the transcription factor NF-κB and in same cases interferon (IFN) regulatory factor (IRF) 3 and 7 (Kumar 2013; Sasai 2013; Lester 2014; Brubaker 2015).
Activation of the RLRs retinoic acid-inducible gene I (RIG-I), melanoma differentiation associated factor 5 (MDA5) and laboratory of genetics and physiology 2 (LGP2 or Dhx58) was shown mainly in response to viral infections and led to upregulation of antiviral genes and type I IFN production. RLRs recognize PAMPs associated with foreign RNA, such as 5’ triphosphorylated ends, double-stranded RNA and some viral RNA motifs. RIG-I and MDA5 activate the adaptor mitochondrial antiviral signaling protein (MAVS) via homotypic interaction of Caspase activation and recruitment domains (CARDs) on the RLRs and MAVS. Activated MAVS polymerizes and forms prion-like aggregates that mediate signaling ultimately leading to NF-κB, IRF3 and IRF7 activation (Loo 2011; Brubaker 2015).

Dectin-1 (Clec7a) and Dectin-2 (Clec4n) are examples for CLRs which activate NF-κB signaling in response to fungal β-glucans and α-mannans, respectively, besides other ligands. Many CLRs containing the defining C-type lectin-like domain however are soluble and opsonize pathogens without directly activating signaling (Brubaker 2015).

NLRs contain a C-terminal LRR domain, a central NACHT domain and varying N-terminal domains. The prototypical nucleotide-binding oligomerization domain containing 1 (Nod1) and Nod2 recruit RIPK2 via N-terminal CARD domains, leading to NF-κB and MAPK activation upon triggering. They recognize PAMPs associated with bacterial cell walls and membranes. Nod1 binds peptidoglycans containing meso-diaminopimelic acid, whereas Nod2 senses muramyl dipeptide. Many NLRs do not activate NF-κB signaling, but form pro-inflammatory protein complexes that have been termed inflammasomes, which are platforms for caspase-1 activation and IL-1β secretion (Fritz 2006). These are discussed in detail in the next chapter.

ALRs contain a pyrin and a DNA-binding HIN200 (PYHIN) domain and sense DNA. The ALR Interferon-γ-inducible protein 16 (IFI16, IFI204 in mice) was shown to detect cytosolic and nuclear DNA of invading pathogens, for example during herpes virus infections. IFI16 activation leads to an induction of type I IFNs via STING and it might be involved in inflammasome activation. The other known ALR, Absent in melanoma 2 (Aim2), forms an inflammasome upon binding to cytosolic double-stranded DNA and is discussed in the following chapter (Dempsey 2015; Diner 2015).
Inflammasomes

Inflammasomes are typically named after the PRR forming the sensor of the complex. Known inflammasome forming PRR are of the NLR and ALR family with the best studied proteins being the NLR family, pyrin domain containing 1b (Nlrp1b), Nlrp3, NLR family, CARD domain containing 4 (Nlrc4) and Aim2. Inflammasomes are large multimeric complexes which activate the zymogen pro-Caspase-1 (Casp1) by promoting autoproteolytic cleavage and generation of the active p10 and p20 fragments. Active Casp1 in turns cleaves the inactive pro-forms of the cytokines Interleukin (IL)-1β and 18, leading to their activation and secretion by a poorly understood unconventional secretion pathway. (Martinon 2002; Lamkanfi 2014).

DCs, macrophages and monocytes are the main producers of IL-1β and IL-18. Mature, cleaved IL-1β is a highly pro-inflammatory cytokine with local and systemic effects, such as promoting fever via the central nervous systems, release of acute phase proteins by the liver and upregulation of adhesion molecules promoting infiltration of immune cells into tissues. Effects on myeloid cells include activation and prolonging the life of neutrophils and macrophages. In lymphoid cells IL-1β is involved in promoting IL-17-producing T helper 17 (Th17), γδ T and group 3 innate lymphoid cells (ILCs) (Dinarello 2009; Garlanda 2013). IL-18 is a pro-inflammatory cytokine as well. It is associated with promoting IFN-γ production by Th1 and group 1 ILCs in synergy with IL-12. Other target cells activated by IL-18 include natural killer (NK) cells, macrophages, monocytes, DCs and neutrophils (Smith 2011; Garlanda 2013). The receptors for IL-1 and IL-18 are the IL-1R1/IL-1RacP and IL-18Rα/β dimers, respectively. Similar to TLRs they contain intracellular TIR domains and recruit MyD88 for signaling (Garlanda 2013).

The Pyrin domain (PYD) containing Nlrp3 and Aim2 depend on the adaptor Apoptosis-associate speck-like protein containing a CARD domain (Asc) to recruit Casp1 to the inflamasome upon activation. Asc contains a PYD and a CARD domain. Homotypic PYD-PYD interactions mediate binding between Nlrp3/Aim2 and Asc, which then uses its CARD to recruit Casp1 via the CARD of Casp1 (Lamkanfi 2014; Lu 2015). Nlrc4 contains itself a CARD instead of a PYD and can directly recruit Casp1 without the need for Asc. Nevertheless, Asc seems to still amplify Il-1β secretion by the Nlrc4 inflamasome by an unknown mechanism (Mariathasan 2004; Lamkanfi 2014).
Nlrc4 can also be seen as an adaptor, since the recognition of ligands for the Nlrc4 inflammasome is mediated by other NLRs: in mice cytosolic flagellin is recognized by NLR family, apoptosis inhibitory proteins (Naip) 5 and 6, whereas cytosolic bacterial type 3 secretion system (T3SS) needle and inner rod proteins are detected by Naip 1 and Naip2, respectively. The single human NAIP also recognizes T3SS needle protein. Naips contain N-terminal baculovirus inhibitor of apoptosis repeats (BIRs) instead of the PYD and CARD in Nlrps and Nlrcs, respectively. Nlrc4/Naip inflammasome activation was shown to be important in the immune response against bacterial infections, e.g. against Salmonella Typhimurium and Legionella pneumophila (Vance 2015).

Aim2 is another inflammasome-forming PRR, for which direct ligand recognition was shown. Aim2 recognizes cytosolic double-stranded DNA sequence-independently by binding it with its HIN200 domain (Jin 2012). Knockout mice showed the importance of Aim2 in the detection of the intracellular bacterial pathogens Francisella tularensis and partially Listeria monocytogenes, as well as in responses against DNA viruses such as mouse cytomegalovirus and vaccina virus (Jones 2010; Rathinam 2010).

Nlrp1b is activated by Bacillus anthracis lethal toxin. Though, the common C57B/6J laboratory mouse strain carries an inactive allele of Nlrp1b. In contrast, 129S1 and BALB/c mouse strains are susceptible (Cordoba-Rodriguez 2004; Boyden 2006).

Nlrp3 is probably the most and best-studied inflammasome. Gain-of-function mutations in human NLRP3 cause the spectrum of autoinflammatory diseases of cryopyrin-associated periodic syndromes (CAPS) with patients suffering from periodic outbreaks of inflammation accompanied by fever and rashes (Rigante 2014). The precise nature of the signal directly upstream of Nlrp3 activation is not known, despite a lot of research. Due to the broad range of stimuli activating Nlrp3, a direct interaction with the stimulants seems unlikely. Various bacterial, fungal and viral infections were shown to activate Nlrp3, including influenza virus, Candida albicans, L. monocytogenes or Staphylococcus aureus. Additionally, crystals and aggregates, such as monosodium ureate, aluminium salt, cholesterol crystals and β-amylloid aggregates, trigger Nlrp3. Extracellular ATP and pore-forming toxins, e.g. Nigericin, are other activators. Lysosomal damage and proteases seem to be involved in signaling by crystals and aggregates. Other suggested signal mechanisms are ROS, potassium efflux, calcium flux, mitochondrial damage (ROS, DNA, cardiolipin) or
cellular localization of inflammasome components (Latz 2013; Munoz-Planillo 2013; Lamkanfi 2014; Abais 2015).

Activation of inflammasomes and IL-1β secretion normally requires two signals in mouse cells. A first signal, usually via NF-κB, is needed for expression of pro-IL-1β and upregulation of expression of inflammasome components. In the case of Nlrrp3 this also leads to deubiquitination of the molecule that is required to allow oligomerization. This “primes” the cell for inflammasome activation by stimulus 2, in the case of Nlrrp3 the before mentioned crystals, ATP, etc. (Juliana 2012; Lopez-Castejon 2013; Py 2013; Lamkanfi 2014). Structure data of Asc-containing inflammasomes indicates that activated sensor proteins oligomerize, which in turn promotes the oligomerization of Asc into filaments formed by their PYD domains. Casp1 proteins are then recruited via CARD-CARD interactions and also form filaments, which emerge radially from the Asc-PYD fiber. Casp1 might then be activated due to the close proximity to each other (Cai 2014; Lu 2014a; Lu 2015). Upon activation nearly all Asc proteins in a cell are recruited to a huge, μm-sized, perinuclear “speck”. In DCs and macrophages inflammasome activation typically leads to a type of cell death termed pyroptosis, due to the simultaneous release of pro-inflammatory cytokines, such as IL-1β. Pyroptosis is characterized by a loss of membrane integrity and release of cytosolic components, followed by swelling, loss of mitochondrial membrane potential and morphological changes to the nucleus (Masumoto 1999; Fernandes-Alnemri 2007). Release of the Asc-speck after cell death was also suggested to promote inflammasome activation of neighboring cells by lysosomal destabilization and nucleation of further Asc and Casp1 polymerization (Baroja-Mazo 2014; Franklin 2014).

The gram-negative bacteria *Escherichia coli*, *Citrobacter rodentium* and *Vibrio cholerae* as well as cholera toxin B (CTB) induce IL-1β secretion and cell death. This was shown to be dependent on Casp11 (in mice; Casp4 and Casp5 are the human orthologues) and termed non-canonical inflammasome activation. Classical inflammasome activators, such as ATP, do not depend on Casp11. Release of IL-1β by non-canonical stimuli was still dependent on Nlrrp3, Asc and Casp1. Cell death however was completely independent of the classical inflammasome components (Kayagaki 2011). Sensing of these gram-negative bacteria seems to involve Lipid A, a
component of LPS, which activates Casp11 independently of TLR4 by an unknown mechanism (Kayagaki 2013).

**Mononuclear phagocytes**

Monocytes, macrophages and dendritic cells (DCs) comprise the mononuclear phagocyte system. The cells were traditionally distinguished by their morphology and phenotype. But their functions and characteristics are often overlapping making a clear-cut separation difficult at times. Macrophages are large cells efficient in phagocytosis of apoptotic cells, debris and invading pathogens, making them important for tissue homeostasis and host defense. DCs have a stellate morphology and their main role is as antigen-presenting cells (APCs), i.e. priming the T cell response, thereby linking innate and adaptive immunity (Hochreiter-Hufford 2013; Guilliams 2014; Mildner 2014). Blood monocytes were originally thought to continuously replenish tissue macrophage pools. But it is clear now that most tissue macrophages in homeostasis are actually embryonically derived and are maintained by local proliferation independent of adult hematopoiesis and influx of cells. Examples of embryonically derived tissue macrophages include microglia in the brain, alveolar macrophages in the lung and red pulp macrophages in the spleen. There are however also exceptions to this and macrophage populations, for example, in the skin and gut depend on a constant influx of cells from the blood (De Kleer 2014; Epelman 2014; Ginhoux 2014). In contrast, DCs and monocytes are dependent on adult hematopoiesis and are generated mainly form monocyte-macrophage-DC progenitors (MDPs). MDPs further differentiate into different DCs and common monocyte progenitors, which gives rise to monocytes. Monocytes are circulating cells in blood, spleen and bone marrow. In mice monocytes can be divided into classical (Ly-6C$^\text{hi}$ CCR2$^+$ CX3CR1$^{\text{int}}$) and non-classical (Ly-6C$^\text{lo}$ CCR2$^-$ CXCR1$^{\text{hi}}$) monocytes. Non-classical Ly-6C$^\text{lo}$ monocytes seem to differentiate from classical Ly-6C$^\text{hi}$ monocytes. They are found patrolling the luminal wall of blood vessel, where they are supposed to play are role in maintaining endothelial integrity. Inflammation and tissue injury are often accompanied by a huge influx of classical monocytes from the blood, which then differentiate and acquire functions and morphologies of macrophages or DCs. They are then sometimes referred to as monocyte-derived macrophages and DCs, respectively, to distinguish them from the tissue-resident macrophages and DCs present during homeostasis (Ginhoux 2014; Guilliams 2014).
DCs are important mediators linking innate with adaptive immunity by presenting antigens to and activating adaptive immune cells. Non-macrophage-derived DCs can be divided into classical DCs (cDCs) and plasmacytoid DCs (pDCs). pDCs lack the typical dendritic morphology and resemble plasma cells with a prominent secretory apparatus. They produce large amounts of type-I IFN in response to viral infection due to constitutive expression of IRF7. However, upon activation pDCs can also acquire a dendritic morphology and prime T cells. Similar to cDCs, pDC development depends on FMS-like tyrosine kinase 3 ligand (Flt3L). cDCs can be further divided into CD11b+ cDCs and CD8α+ (in lymphoid tissues) or CD103+ (in non-lymphoid tissues) cDCs. cDCs have a relative short half-life of 3-6 days and are constantly replenished by bone marrow hematopoiesis. New, immature DCs have endocytic activity. Activation by PAMPs or other inflammatory stimuli leads to DC maturation, which includes upregulation of antigen presentation by major histocompatibility complex (MHC) molecules and co-stimulatory molecules. This is accompanied by migration of the DC to the T cell zones of secondary lymphoid organs. Consequently, cDCs are typically the major population of APCs. A characteristic of CD103+CD8α+ cDCs is their high capacity for cross-presenting endogenous antigens to CD8+ T cells (Satpathy 2012; De Kleer 2014; Mildner 2014). DCs promote T cell activation by presenting antigen-MHC complexes to cognate T cell receptors (signal 1) and providing co-stimulation (signal 2), e.g. by CD80/86 to CD28 on the T cell. Additionally, DCs might secrete cytokines to further activate T cells and promote polarization (signal 3) (den Haan 2014). Interestingly, another way of DCs to control T cell proliferation is the modulation of the extracellular redox balance and providing reduced cysteines to T cells. Naïve T cells lack the cystine transporter x_c system and depend on the DCs to reduce cystine to cysteine, which the T cells are able to utilize (Angelini 2002; Yan 2010; Buck 2015).

**T cells**

T cells are one of the major arms of the adaptive immunity. T cell receptors (TCRs) contain dimers of α- and β-chains or γ- and δ-chains, expressed by αβ T cells and γδ T cells, respectively. Mature TCR chains are not germ-line encoded, but are the result of somatic rearrangement mediated by recombination-activating genes (RAG1/2). This generates TCR-specificities against nearly all possible antigens. T cells develop in the thymus and are dependent on adult bone marrow hematopoiesis (Nikolich-Zugich
αβ T cells development can be broadly divided by the expression of the co-receptors CD4 and CD8. TCRβ rearrangement happens in CD4⁺ CD8⁻ double negative (DN) thymocytes in the cortex and subcapsular zone. Cells with functional TCRβ rearrangement and surface expression of a pre-TCR complex, formed by TCRβ, invariant pre-TCR α-chain and CD3 signaling molecules, start migrating back into the thymic cortex. This is accompanied by expression of CD4 and CD8 and rearrangement of the TCRα-chain. CD4⁺ CD8⁺ double-positive (DP) cells with a successfully rearranged TCRα-chain expressing a mature αβ-TCR are selected for useful avidities. αβ T cells recognize peptides presented by major histocompatibility complexes (MHC) and not whole native proteins. Intermediate avidity to self peptide-MHC is required for positive selection, thus restricting T cells for recognition of host MHCs, while T cells with no/low affinity to endogenous MHCs die. Negative selection on the other hand leads to depletion of cells with too high avidity for self, thus limiting the chances for autoreactivity. Down-regulation of either of the co-stimulatory CD4 and CD8 molecules leads to single-positive (SP) cells, which undergo further negative selection in the thymic medulla before leaving the thymus. CD4⁺ SP (CD4SP or just CD4 T cell in the periphery) T cells are restricted to peptides presented in MHC class II, whereas CD8⁺ SP T cells recognize peptides in the context of MHC class I (Koch 2011; Shah 2014).

CD4 T cells depend on antigen presentation by MHC class II, which is only present on professional APCs (mainly DCs, Macrophages, B cells). MHC class II are loaded with peptides in the phago-endosomal compartment, thus allowing the presentation of peptides derived from extracellular pathogens phagocytosed by the APC. Alternatively, also cellular proteins are processed via autophagy (Neefjes 2011; Roche 2015). CD4T cells are central in orchestrating the immune response by other cells and are hence also called T helper (Th) cells. (Activated) CD4 T cell can be divided into subsets based on their function and cytokine production.

Th1 cells are characterized by expression of the transcription factor T-bet and production of IFN-γ. The Th1 phenotype is promoted by IL-12p70 and IFN-γ via STAT4 and STAT1-dependent signaling, respectively. A Th1 response is typically associated with cell-mediated immunity against viruses and other intracellular pathogens. Functions of IFN-γ include promoting class switching of B cells to IgG2a and activating macrophages (Schroder 2004; Zhu 2008; Luckheeram 2012).
Th2 cells require the transcription factor GATA3, which is induced by STAT6 in response to IL-4. Additionally STAT5 downstream of IL-2 is required for full Th2 commitment. Effector cytokines of Th2 cells include IL-4, 5 and 13. IL-4 promotes B cell class switching to IgE and IgG1. Physiological roles of Th2 responses are the defenses against extracellular parasites such as helminths. Overshooting Th2 responses are associated with asthma and other allergies (Zhu 2008; Luckheeram 2012; Kolls 2013).

Th17 cells are important for the control of extracellular bacterial and fungal infections. Over-activation is often observed in autoimmune diseases. Induction of the Th17-defining transcription factor RAR-related orphan receptor γt (RORγt) depends on transforming growth factor β (TGFβ) in the presence of IL-6 or IL-21. Later IL-23 is required for expansion and survival of Th17 cells. IL-6, 21 and 23 signal via STAT3. As the name indicates, Th17 cells produce large quantities of IL-17A and F, additionally also IL-21 and IL-22 are produced (Zhu 2008; Luckheeram 2012).

T follicular helper (Tfh) cells are found in the follicular/B cell regions of secondary lymphoid organs and are important for long-term humoral responses by B cells. The transcription factor Bcl6 was identified as the master regulator of Tfh development (Crotty 2011).

T regulatory (Treg) cells are negative regulators of immunity, suppressing the activity of other cells. Tregs are important to maintain peripheral tolerance and suppress autoimmunity. They express the transcription factor forkhead box P3 (FOXP3) and high levels of CD25 (IL2Rα), the high affinity chain of the IL-2 receptor. Tregs can be directly formed in the thymus from precursors with relative high avidities for self-antigens/MHCs or are induced in the periphery. TGFβ is critical for the induction of Tregs. Effector cytokines of Treg include the mostly immuno-suppressive IL-10 and TGFβ (Josefowicz 2012; Luckheeram 2012). Interestingly, inhibition of effector T (Teff) cells by Treg seems to also involve inhibition of the redox modeling by APCs by interfering with GSH synthesis and taking up reduced Cysteine from the extracellular space (Yan 2010).

CD8 T cells or cytotoxic T lymphocytes (CTLs) recognize peptides presented in MHC class I complexes. In contrast to MHC class II, class I molecules are expressed on all nucleated cells. Peptides presented by MHC class I molecules are derived from
proteasomal degradation of cellular proteins and thus reflect proteins expressed inside the presenting cell. This allows the detection of antigens from intracellular pathogens, such as viruses, which would otherwise be invisible to immune cells. Alternatively, peptides from phagocytosed proteins can also be loaded onto MHC class I by cross-presentation, a process mainly reserved for some DCs. This is important to allow the original priming and activation of antigen-specific naïve CD8 T cells (Neefjes 2011; Joffre 2012). Activated CTLs induce cell death of target cells upon detection of cognate antigen-MHC class I by their TCR. Activation leads to degranulation and release of pore forming and lytic proteins, such as perforin and granzymes. These mediate induction of apoptosis in the target cells. Alternative pathways include the expression of Fas ligand (FasL) and tumor necrosis factor, which upon binding on target cell receptors also initiate apoptosis and cell death (Chavez-Galan 2009).

Effector T cells rapidly expand upon exposure to their cognate antigen. Upon clearance of antigen, T cell numbers normally contract by programmed cell death. However a small pool of memory cells remain which confer a fast and strong response to a second encounter of the same antigen. Memory T cells can be broadly divided into central memory (T_{CM}), effector memory (T_{EM}) and tissue-resident memory (T_{RM}) T cells. T_{CM} are mainly found in secondary lymphoid organs and have high proliferative potential, but low immediate effector capacity. T_{EM} are in addition found patrolling non-lymphoid organs and have low proliferation potential, but higher effector activity. Finally, T_{RM} seem to be mainly found at the site of the previous infection and do not circulate (Kaech 2012; Mueller 2013).

Two other T cell populations, γδ T and invariant natural killer T (iNKT) cells, are often described as innate-like cells, due to restricted use of only certain TCR chains in many populations and fast cytokine production and activation. iNKTs in mice use a Vα14-Jα18 α-chain combined with a restricted set of β-chains. iNKTs are restricted to CD1d, a MHC-like molecule, and seem to recognize (glyco-)lipids presented on CD1d. iNKT develop from DP thymocytes, but unlike normal αβ T cells undergo further proliferation and activation already in the thymus and then resembling more activated or memory T cells than naïve cells. They express for example the memory/activation marker CD44 and do not require activation by an APC for cytokine production (Salio 2014; McEwen-Smith 2015). γδ T cells derive from DN cells, but fetal γδ T cells already seed some tissues. The different γδ populations are
more diverse than iNKTs with some more resembling innate cells and other more adaptive cells. Less is known about the antigens recognized by the TCRs of γδ T cells (Kisielow 2013; Vantourout 2013).

**B cells**

B cells are the second type of adaptive immune cells. They are mediators of the humoral immunity by secretion of their B cell receptors (BCR) in a soluble form as antibodies. Similar to T cells they rely on RAG-mediated recombination to generate a huge repertoire of BCRs with different antigen-specificities. Unlike T cell, BCR/antibodies recognize the whole 3D structure of the intact antigens and do not depend on antigen processing and presentation of peptides on MHC molecules. B cell develop in the bone marrow. In a process analogous to T cells, B cells first rearrange the V\textsubscript{H}-D\textsubscript{H}-J\textsubscript{H} gene segments of their BCR heavy chain followed by expression of a pre-BCR with a surrogate light chain. This is followed by the rearrangement of the V\textsubscript{L}-J\textsubscript{L} segments of the light chain. Functional recombination leads to expression of IgM as the BCR on the surface and egression of the immature B cell from the bone marrow. Immature B cells undergo final maturation in the spleen (Pieper 2013). Mature B cells can be divided into B1, marginal zone (MZ) and follicular B cells. B1 and MZ rapidly respond to antigen in a T cell independent fashion, thus can also be called innate-like. In contrast, the majority of follicular B cell responses depend on CD4T help. Upon BCR-dependent recognition of antigen an extrafollicular response quickly produces a short-lived population of antibody producing cells. Alternatively, B cells form germinal centers (GC) in the follicular zone of secondary lymphoid organs with the help of Tfh cells. B cells rapidly proliferate in GC and further mutate their BCRs in a process termed somatic hypermutation. BCR are selected for high affinities and form long-lived antibody-producing plasma cells or memory B cells. This generates antibodies with much higher affinity than the quicker extrafollicular response (Kurosaki 2015; Nutt 2015).
4.3 Aim of this thesis

A general importance of ROS and RNS in immune cells has been recognized. However, the detailed functions and requirements of antioxidant enzymes and systems in individual types of immune cells are poorly understood. To help unraveling the functions of antioxidant system in immune cells and responses I have been using global and conditional knockouts of the transcription factor Nrf2 and the antioxidant enzyme Txnrd1 (TrxR1) respectively, in mice for my PhD projects. Nrf2 is a transcriptional activator of a plethora of genes with antioxidant functions. While the global knockout of Nrf2 shows little overt phenotypes, previous findings from our lab showed a defect in Nlrp3 inflammasome activation in Nrf2-deficient macrophages and DCs. In one of my PhD projects I focused on further characterization of this finding by investigating activation of other inflammasomes in Nrf2-deficient cells and by trying to understand the mechanism, by which Nrf2 influences inflammasome activation. Global knockouts of Txnrd1 are non-viable. Consequently, we used Cre-mediated deletion to investigate the role of TrxR1 specifically in certain immune cells. Data from Mai Matsushita in our lab (Matsushita 2015) demonstrated the importance of Gpx4, another selenocysteine-containing antioxidant enzyme, in T cell survival and expansion following activation. In my second project, I also mainly focused on the role of TrxR1 in T cells and studied the effect of TrxR1-deficiency in T cell homeostasis and during immune responses.
4.4 References


binding and activation mechanisms of the AIM2 inflammasome and IFI16 receptor. Immunity 36, 561-571.


5 Results

5.1 Nrf2 transcriptional activity controls Nlrp3 inflammasome activation independent of reactive oxygen species levels

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Nuclear factor, erythroid derived 2, like 2 (Nrf2) is the master transcriptional activator of many cellular antioxidant enzymes. Here we demonstrate an important role of Nrf2 in activation of inflammasomes containing Aim2, Nlrc4 and Nlrp3 in particular. Lack of Nrf2 caused a defect in Asc oligomerization into Specks, caspase-1 cleavage and consequently IL-1β secretion. Absence of Nrf2 resulted in delayed detoxification of cellular reactive oxygen species (ROS), which was, however, not responsible for the deficient inflammasome activation. Expression of Nrf2 mutants with defective or altered transcriptional activation capacity demonstrated the importance of Nrf2 in its function as a transcription factor, suggesting that Nrf2 target gene(s) are required for efficient inflammasome activation rather than direct interaction of Nrf2 with inflammasome components.
Introduction

Secretion of the pro-inflammatory cytokine Interleukin-1β (IL-1β) is tightly regulated. Apart from control of the expression of its inactive pro-form, it needs proteolytic cleavage by Caspase-1 (Casp1) for release and activity (Sims 2010; Netea 2015). Casp1, in turn, is present as an inactive zymogen (pro-Casp1) and needs autoproteolytic processing to become an active protease capable of cleaving pro-IL-1β (Cerretti 1992; Thornberry 1992). Caspase-1 is activated in large multimeric complexes termed inflammasomes (Martinon 2002). They are typically comprised of a sensor protein, the adaptor Asc (Apoptosis-associated speck-like protein, caspase recruitment domain [CARD] containing) encoded by the Pycard gene and Casp1. Most inflammasomes contain a sensor protein from the NLR (Nod-like receptor) family including the well studied Nlrp1 (NLR family, pyrin domain containing 1), Nlrp3 and Nlrc4 (NLR family, CARD domain containing 4)/Naips (NLR family, apoptosis inhibitory protein) inflammasomes. In other inflammasomes the sensor comes from the PYHIN (pyrin and HIN200 domain–containing) protein family, where Aim2 (absent in melanoma 2) is the most investigated member (Lamkanfi 2014; Lu 2015).

Casp1 activation and secretion of mature IL-1β requires at least two stimuli. Activation of NF-kB (signal 1) upregulates the expression of IL-1β and inflammasome components (i.e. Nalp3). Subsequently, an inflammasome activator (signal 2) is needed to trigger inflammasome assembly and consequently IL-1β processing (Bauernfeind 2009; Netea 2009). The importance of this tight regulation is highlighted in patients with gain-of-function mutations in Nlrp3 which abrogate the need for stimulus 2. These patients suffer from a spectrum of autoinflammatory diseases called cryopyrin-associated periodic syndromes (CAPS) characterized by episodes of local inflammation and fever (Brydges 2009; Meng 2009; Rigante 2014).

Signal 2 is provided by pathogen-associated molecular patterns (PAMPs) and danger-associated molecular patterns (DAMPs) caused by tissue damage or cellular stress. Aim2 directly recognizes cytosolic double stranded DNA, for example during Francisella tularensis and vaccinia virus infection (Burckstummer 2009; Fernandes-Alnemri 2009; Hornung 2009; Roberts 2009; Jones 2010; Rathinam 2010; Jin 2012). Mouse Nlrc4 uses different Naips as receptors to sense cytosolic bacterial flagellin or type 3 secretion system components (Amer 2006; Franchi 2006; Miao 2006; Miao
2010; Kofoed 2011; Zhao 2011; Rayamajhi 2013; Yang 2013; Vance 2015). Nlrp1b recognizes *Bacillus anthracis* lethal toxin in susceptible mouse strains such as 129S1 and BALB/c leading to inflammasome activation, while resistant mouse strains, e.g. C57BL/6J, carry a mutated Nlrp1b allele (Cordoba-Rodriguez 2004; Boyden 2006). The precise mechanism(s) leading to the activation of the Nlrp3 inflammasome remains still unclear. Various bacterial (e.g. *Neisseria gonorrhoeae*) (Duncan 2009), fungal (e.g. *Candida albicans*) (Gross 2009; Joly 2009), viral (e.g. influenza virus) (Allen 2009; Thomas 2009), and parasitic infections (e.g. *Schistosoma mansoni*) (Ritter 2010) can activate Nlrp3. In addition, crystals and aggregates, such as monosodium urate (Martinon 2006), cholesterol (Dewell 2010; Freigang 2011) and aluminum salt crystals (Eisenbarth 2008; Hornung 2008) as well as β-amyloid aggregates (Halle 2008) trigger the Nlrp3 inflammasome. Bacterial pore forming toxin (e.g. Nigericin) and extracellular ATP (Mariathasan 2006) are other Nlrp3 inflammasome activators. Due to the broad range of triggers it is unlikely that Nlrp3 detects these stimuli directly. Instead it is thought that Nlrp3 senses cell damage or changes in cellular homeostasis, though the exact nature of this common upstream signal remains unknown (Latz 2013; Lamkanfi 2014). Intracellular reactive oxygen species (ROS) (Cruz 2007; Nakahira 2011; Zhou 2011), mitochondrial (Nakahira 2011; Zhou 2011; Shimada 2012; Iyer 2013) or lysosomal damage (Halle 2008; Hornung 2008), cellular localization of Nlrp3 and Asc (Misawa 2013; Park 2013; Subramanian 2013) and changes in potassium (Munoz-Planillo 2013) and calcium (Murakami 2012) levels have been implied in Nlrp3 activation.

Structural analysis of *in vitro* assembled Asc-containing inflammasomes showed that the sensor proteins nucleate the formation of short fibrils composed of the pyrin domains (PYD) of Asc upon triggering. From these central Asc fibrils Casp1 CARD filaments emancipate radially probably recruited by the CARDs of Asc (Cai 2014; Lu 2014; Sahillioglu 2014). Upon stimulation, almost every Asc molecule aggregates in a µm-sized detergent-resistant perinuclear ‘Speck’. Speck formation in macrophages and dendritic cells is accompanied by cell death through pyroptosis characterized by cell swelling, plasma membrane integrity loss and rupture, resulting in the release of cytosolic content into the extracellular space (Masumoto 1999; Fernandes-Alnemri 2007).
The transcription factor Nrf2 (nuclear factor, erythroid derived 2, like 2; Nfe2l2) (Moi 1994) is the transcriptional activator responsible for expression of phase II detoxifying and antioxidant enzymes (Venugopal 1996; Itoh 1997). Nrf2 is a member of the cap’n’collar basic-region leucine zipper transcription factor family, which recognizes and binds to the antioxidant response element (ARE, also called electrophile responsive element) as a heterodimer together with a small Maf protein (i.e. MafF, MafG, MafK) (Itoh 1997; Dhakshinamoorthy 2000; Gong 2001; Motohashi 2004). Nrf2 drives transcription of genes under basal conditions and upon induction (Hayes 2000; McMahon 2001; Chanas 2002). Nrf2 activity is thought to be mainly regulated by its inhibitor Keap1 (Kelch-Like ECH-Associated Protein 1), which targets Nrf2 for proteasomal degradation by promoting its ubiquitination by a Cullin 3-containing E3 ubiquitin ligase complex (Itoh 1999; Dhakshinamoorthy 2001; Kobayashi 2004). Many Nrf2 inducers are soft electrophiles, which oxidize cysteines in Keap1, thereby preventing Keap1 from targeting freshly translated Nrf2 for degradation (Levonen 2004; Wakabayashi 2004; Eggler 2005; Tong 2006; Takaya 2012). Additionally, Nrf2 activity is modulated by other mechanisms (Hayes 2014) such as phosphorylation (Huang 2002; Sun 2009) and transcriptional regulation (Kwak 2002; Miao 2005). Antioxidant enzymes upregulated by activation and nuclear translocation of Nrf2 include Heme oxygenase-1 (Hmox1), NAD(P)H dehydrogenase (quinone 1) (Nqo1) and Peroxiredoxins (Prdx). Other Nrf2-dependent cytoprotective genes include many enzymes involved in the glutathione redox system (e.g. glutathione S-transferases and glutaredoxin 1), glutathione peroxidases and multi-drug-resistance-associated protein (MRP) transporters. Additional Nrf2-dependent genes are involved in carbohydrate and lipid metabolism (Wakabayashi 2010; Hayes 2014; Pall 2015).

In the present study, we further investigate the mechanism of Nrf2-mediated Nlrp3 inflammasome activation.
Results

*Nrf2 is required for efficient IL-1β secretion by BMDCs and BMDMs.*

We previously reported that Nrf2-deficiency in hematopoietic cells protects mice from atherosclerosis due to decreased IL-1 secretion by myeloid cells in response to cholesterol crystals (Freigang 2011; Freigang 2013). Similarly, bone marrow derived dendritic cells (BMDC) and macrophages (BMDM) secreted strikingly reduced amounts of IL-1β upon stimulation of the Nlrp3 inflammasome by the DAMP ATP, the bacterial toxin Nigericin and Al(OH)₃ crystals independent of the TLR or NLR ligand used to trigger signal 1 ([Fig. 2a-c, e]). Inflammasome activation is typically accompanied by pyroptotic cell death. Consistently, NLRP3 activation induced cell death in wild-type BMDC, which was strongly decreased in the absence of Nrf2 as measured by Lactate dehydrogenase (LDH) release and by staining of dead and dying cells with 7-AAD and Annexin-V ([Fig. 2d, f]). In parallel to decreased IL-1β secretion, Nlrp3-triggered BMDCs also secreted less IL-1α ([Fig. 2g]). In contrast, Nrf2⁺/⁻ BMDCs had no defect in secretion of IL-12/23p40 after stimulation with various TLR and NLR ligands ([Fig. 2h]), while IL-6 production was reduced ([Fig. 2i]).

To study the role of Nrf2 on other inflammasomes, we activated the Aim2 and Nlrc4 inflammasomes by cytoplasmic delivery of double-stranded DNA (p(dA:dT)) and flagellin (FliC), respectively. Nrf2-deficient BMDCs secreted less IL-1β compared to wild-type BMDC ([Fig. 2j]), However, the defect in Nrf2-deficient BMDC appeared to be more pronounced for the Nlrp3 compared to the Aim2 and Nlrc4 inflammasomes ([Fig. 2k]). Consequently, we focused on Nlrp3 to further address the mechanism of Nrf2-mediated inflammasome activation.
Fig. 2 Nrf2 is required for efficient IL-1β secretion by BMDCs and BMDMs. (a) Wild-type and Nrf2-deficient BMDCs were primed with R837 (5μg/ml) before addition of ATP, Nigericin or Al(OH)₃ at concentration indicated for 40min or 4h, respectively. Total stimulation time for all samples was 7h. IL-1β secretion was measured in supernatants by ELISA. (b) Bone marrow derived macrophages (BMDM) were stimulated with LPS (30 ng/ml) or R837 (5 μg/ml) before addition of ATP (2mM) or Al(OH)₃ (200μg/ml) for 1h or 5h, respectively. IL-1β levels in supernatants were assessed by ELISA. (c) IL-1β levels in supernatants of BMDCs
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primed with CpG (100 nM), LPS (400 ng/ml), LTA (5 µg/ml), MDP (10 µg/ml), poly(I:C) (50 µg/ml), R837 (5 µg/ml), Zymosan A (10 µg/ml) or left untreated followed by addition of 2 mM ATP for 1h (left) or 200 µg/ml Al(OH)₃ for 5h (right) was measured by ELISA. (d, e) BMDCs were primed with R837 and then stimulated by addition of 200 µg/ml Al(OH)₃, 2 mM ATP or 15 µM Nigericin for indicated times. (d) Cell death (necrosis) was assessed by lactate dehydrogenase (LDH) release. Values show percentages of maximal LDH release after Triton-X lysis normalized to release after stimulation with R837 only. (e) IL-1β levels in supernatants measured by ELISA. (f) BMDCs were stained with 7-AAD and Annexin-V after treatment with R837/ATP, R837/Nigericin for 80 min or R837/Al(OH)₃ for 4h, as described above in (d, e). Samples were analyzed by flow cytometry. A representative sample is shown with numbers in each quadrant indicating average percentage ± standard deviation of the triplicates analyzed. (g) IL-1α levels in supernatants of BMDC cultures treated as described in (a). (h, i) IL-12/23p40 and IL-6 levels in supernatants of BMDC stimulated with TLR and NLR ligands as described in (c). (j) R837-primed BMDCs were transfected with 0.25 µg p(dA:dT) or 50 ng FliC for 4h to stimulate the NLRC4 and Aim2 inflammasome, respectively, or treated with the transfection reagents Lipofectamin 2000 and Profect P1 only as unstimulated controls (transf ctr). IL-1β in supernatants was measured. (k) Values show ratio of IL-1β in supernatants of Nrf2-deficient to wild-type BMDCs. Dots represent average values from individual experiments. R837-primed BMDCs were stimulated with ATP, Al(OH)₃ or transfected with p(dA:dT) and FliC. Bars show mean ± standard deviation. Dots in (k) represent ratios of individual experiment. Two-Tailed Student t-test was used to compare wild-type and Nrf2⁻⁻ groups, except for (k) were one-sample t-test with theoretical mean of 1 was used.
**Results**

**IL-1β and Caspase-1 processing, as well as ASC assembly is defective in Nrf2-deficient cell upon inflammasome triggering**

Secretion of mature IL-1β requires cleavage of its pro-form by Casp1, which in turn has to be proteolytically processed to become active. Consistent with reduced amounts of IL-1β measured by ELISA, we detected less secreted mature IL-1βp17 by western blot in supernatants of Nrf2−/− compared to wild-type DC stimulated with ATP, Nigericin or Al(OH)₃. Additionally, Casp1 processing and secretion is reduced in Nrf2-deficient BMDCs as well (Fig. 3a). No increase in processed mature IL-1βp17 and Casp1p10 was found in total lysates of Nrf2−/− cells, indicating defective IL-1β processing rather than secretion. Having established that both Casp1 and IL-1β processing is defective, we looked at Asc oligomerization, i.e. Asc Speck formation, an upstream event of Casp1 activation. Flow cytometry can be used to detect Speck formation by measuring the decrease in fluorescence signal width associated with Asc aggregation to a single Speck (Sester 2015). We observed reduced Speck formation in Nrf2-deficient BMDCs stimulated with the Nlrp3 activators ATP, Nigericin and Al(OH)₃ both by staining endogenous Asc and using cells expressing an Asc-GFP fusion protein enabling acquisition of live, unfixed cells (Fig. 3b-e). Similar results were obtained when we analyzed Speck formation by immunocytochemistry (data not shown). Notably, overexpression of Asc-GFP in BMDCs did not restore IL-1β secretion in Nrf2-deficient cells (Fig. 3f).

mRNA levels of Nlrp3, Nlrc4, and Il1b were about 50% reduced in Nrf2-deficient BMDCs after stimulation with R837 indicating a slightly reduced signal 1, although protein levels appeared comparable as assessed by Western blot (Fig. 3a, g). These findings suggest that (i) Nrf2 is required upstream of Speck formation, i.e. for Asc oligomerization, (ii) recruitment of Asc to the inflammasome, or (iii) activation and oligomerization of Nlrp3 itself. Additionally, Nrf2 appears to promote optimal signal 1 stimulation.
Fig. 3 Nrf2 is essential for ASC assembly and processing of IL-1β and Caspase-1

(a) Maturation of Caspase-1 and IL-1β was assessed by Western blot in cell lysates and supernatants of R837-primed BMDCs stimulated with ATP (2mM, 40min), Nigericin (5µM, 40min) and Al(OH)₃ (200 µg/ml, 4h) or p(dA:dT) (3 ng, 4h) and FliC (200 ng, 4h) transfected with Lipofectamine 2000 and Profect P1, respectively.

(b-e) Asc Speck formation in Nrf2−/− and wild-type BMDC. R837-primed BMDCs were stimulated with 5mM ATP, 10µM Nigericin and 200µg/ml Al(OH)₃. (b, d) Cells were stained with anti-ASC mAb followed by PE-labeled secondary antibody, or (c, e) expressed retrovirus-driven Asc-GFP fusion protein. Speck formation was measured by flow cytometry. Cells with an Asc speck have a changed signal pulse shape during acquisition on a flow cytometer due to the different distribution of fluorophore inside the cell compared to cells without a speck. This results in a smaller pulse width for cells with a speck. (b, e) Shown are representative dot plots. Cells in gated area formed Specks. (d, e) Quantification of flow cytometry data. Values show percent Speck+ cells (f) An Asc-GFP fusion protein or just GFP as a control was overexpressed by retroviral transduction in wt and Nrf2-deficient BMDCs. IL-1β
levels in the supernatants of GFP+ R837-primed cells stimulated with ATP were measured by ELISA. (g) Relative expression of the indicated genes in Nrf2−/− BMDCs compared to wt. Bar and lines show mean ± standard deviation (a-h). Dots represents results from independent experiments in (g) with error bars indicating standard deviation. Two-Tailed Student t-test was used to compare wild-type and Nrf2−/− groups, except for (g) were one-sample t-test with theoretical mean of 1 was used.

Nrf2 mediation of inflammasome activation is independent of changes in the intracellular redox balance

Many of Nrf2’s targets include genes involved in the cellular antioxidant response. Therefore, we investigated if changes in intracellular ROS and antioxidant capacities might be responsible for the defective inflammasome activation in Nrf2-deficient cells. Dyes based on reduced fluorescein, i.e. CM-H2DCFCA (5-(and-6)-chloromethyl-2’ , 7’-dichlorodihydrofluorescein diacetate, acetyl ester) that turn fluorescent upon removal of acetate groups by cellular esterases and oxidation by ROS can be used to assess total cellular ROS levels. Addition of this dye to BMDC in the absence of any stimulation lead to a spontaneous increase in fluorescence, indicating generation of ROS, probably caused by the dye itself (Bonini 2006). The peak response in fluorescence-induced oxidation by ROS was comparable in wt and Nrf2−/− BMDCs indicating that ROS production was unaffected in the absence of Nrf2. However, ROS detoxification was significantly delayed in Nrf2−/− cells (Fig. 4a, b) When cells were stimulated with ATP after ROS levels returned to baseline subsequent to CM-H2DCFCA staining, wild-type cells actually had more ROS than Nrf2−/− BMDCs despite the presence of Nrf2. No induction of ROS was observable upon stimulation with the crystalline Nltp3 activator MSU (Fig. 4e). We used a variety of modulators of ROS and cellular redox balance to further characterize the role of ROS in the defective inflammasome activation in Nrf2−/− cells. Addition of the antioxidants ascorbic acid (aa) and α-Tocopherol (α-Toc) to cells stimulated with R387 and ATP had no effect on IL-1β secretion. N-acetyl-cysteine (NAC) inhibited inflammasome activation at high concentration (Cruz 2007; Petrilli 2007; Nakahira 2011), but failed to decrease the difference between wt and Nrf2−/− BMDCs. Interestingly, addition of cell-
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permeable Catalase (coupled to polyethylene glycol) boosted IL-1β secretion by both wt and Nrf2\(^{-/-}\) BMDCs. Similarly culture of BMDCs in medium supplemented with β-Mercaptoethanol did not rescue inflammasome activation (data not shown). Decreasing the cellular antioxidant potential by inhibition of the glutathione system with L-Buthionine-sulfoximine (BSO), an inhibitor of glutamate-cysteine ligase, or by inhibiting cystine import by the \(x_{\text{c}}\)- transporter system with L-Glutamic acid (L-Glu) or sulfasalazine (SAS) (Gout 2001; Conrad 2012) did not abolish the IL-1β secretion defect or inhibited both wt and Nrf2\(^{-/-}\) cells to a similar extent. Treatment of cells with Diphenyleneiodonium chloride (DPI) and (2R,4R)-4-aminopyrrolidine-2,4-dicarboxylate (APDC) were used to inhibit Nlrp3 inflammasome activation with conflicting results (Cruz 2007; Dostert 2008; Bauernfeind 2011; Munoz-Planillo 2013). We were not able to reproduce an effect of APDC with concentrations described in literature. The Nrf2 inducers Diethylmaleate (DEM) and \(\text{tert-Butylhydroquinone (tBHQ)}\) did not stimulate IL-1β secretion in wild-type and Nrf2\(^{-/-}\) BMDCs indicating that Nrf2 activation alone is insufficient to trigger or enhance inflammasome activation (Fig. 4d). We next overexpressed several Nrf2-target genes involved in ROS detoxification such as Malic enzyme 1 (Me-1), Peroxiredoxin 1 (Prdx1), NAD(P)H dehydrogenase, quinone 1 (Nqo1) as well as Thioredoxin-1 (Txn1) and combinations of Txn1 together with Thioredoxin reductase-1 (Txnrd1) or Prdx1 in Nrf2-deficient BMDC. Expression of these genes failed to restore IL-1β secretion in Nrf2-deficient cells, in contrast to complementation with retrovirus driven wild-type Nrf2 (Fig. 4e). A role of cysteine oxidation has been suggested in regulating Caspase-1 activity (Meissner 2008). We precipitated proteins with reversible oxidative modifications (Purification of Reversibly Oxidized Proteins: PROP) (Templeton 2010) and probed for Caspase-1 and Asc. We observed no increase in Caspase-1 oxidation in Nrf2\(^{-/-}\) BMDCs. Without exogenous oxidation by \(H_2O_2\) only very little oxidized Asc was detectable and no difference was observed (Fig. 4f). Taken together these findings indicate that reduced inflammasome activation in Nrf2-deficient cells is not due to generally elevated oxidative stress.
Fig. 4 Nrf2 mediation of inflammasome activation is independent of changes in the intracellular redox balance. (a-c) ROS levels were measured with the CM-H2DCFDA dye by flow cytometry. Oxidation of the dye leads to an increase in fluorescence signal. (a) Kinetics of the oxidation and following reduction of the dye in wt and Nrf2-deficient BMDCs are shown for the times after dye addition indicated on top. (b) Comparison of ROS levels at indicated times after dye addition. (c) Changes in ROS levels were determined after 15 and 25 min ATP (5mM) and MSU (300µg/ml) stimulation in wt and Nrf2−/− cells 210 min after CM-H2DCFDA addition. (d) IL-1β levels in supernatants of R837/ATP stimulated BMDCs in the absence or presence of titrating concentrations of α-Tocopherol (αToc; 33, 100, 333µM), Catalase-PEG (Cat; 333, 1000, 3333U/ml), DL-Dithiothreitol (DTT; 1, 3, 10mM), N-acetyl-L-cysteine (NAC; 1, 3, 10mM), L-Buthionine-sulfoximine (BSO; 100, 300, 1000µM), L-Glutamic acid (L-Glu; 1, 3mM) and Sulfasalazine (SAS; 100, 300µM); ascorbic acid (30, 300, 3000µM); 2R,4R-APDC (APDC; 10, 30, 100µM), Diphenyleneiodonium chloride (DPI; 10, 20, 30, 40µM), Diethylmaleate (DEM; 10, 30, 100µM) and tert-Butylhydroquinone (tBHQ; 10, 30, 100µM). The same untreated control is shown in the left and right graphs for easier comparison of treated samples to controls. (e) Several candidate Nrf2 target genes encoding for antioxidant enzymes (i.e. Malic enzyme-1 (Me-1), Thioredoxin-1 (Txn1), Peroxiredoxin 1 (Prdx1), NAD(P)H dehydrogenase, quinone 1 (Nqo1) were retrovirally expressed in Nrf2-deficient BMDCs and IL-1β secretion was measured in supernatants after R837 and ATP stimulation. Nrf2-deficient BMDC transduced with retrovirus expressing wild-type Nrf2 or GFP only were used as positive and negative controls. (f) Oxidation of cysteines in Casp1 and Asc was assessed by precipitation of oxidized proteins (PROP) in BMDCs primed with 5ug/ml R837 or 400ng/ml LPS and stimulation as
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described in legend to Fig. 3a or for 10min with 500µM H₂O₂ as positive control. Input (total) and elution of PROP were probed for Casp1 and Asc by Western blot. Black triangles mark the Casp1 band in PROP. Bars indicate mean ± standard deviations. Two-Tailed Student t-test was used to compare wild-type and Nrf2−/− groups in (d). 1-way ANOVA and Dunnetts multiple comparison test was used to compare groups overexpressing proteins to the GFP only expressing control.

Transcriptional activity of Nrf2 is necessary for efficient inflammasome activation

To investigate whether or not the Nrf2 transcriptional activity is required for Nlrp3 inflammasome activation, we over-expressed wild-type and several Nrf2 mutants with different properties (Fig. 5a) in Nrf2-deficient BMDCs, and compared their ability to restore IL-1β secretion. Expression of Nrf2 containing a deletion in the DNA binding domain including one of the nuclear localization signals (ΔNLS) (Jain 2005) and 3 Nrf2 mutants carrying individual mutations in the 3 putative nuclear localization signals (NLSm1-m3) (Theodore 2008). Furthermore, a mutant Nrf2 was constructed lacking the capacity to interact with the chromo-ATPase/helicase CHD6 due to a F584A mutation in the Neh3 domain (Nioi 2005). Nrf2−/− BMDCs were infected with the corresponding retroviruses, GFP expressing cells were sorted, stimulated with R837 and ATP and analyzed for IL-1β production. The capacity to reconstitute IL-1β secretion showed a strong correlation with Nrf2 transcriptional activity, i.e. the ability to induce expression of Nrf2 targets genes including Nqo1, Prdx1 and Hmox1 (Fig. 5b, c).

Since we cannot completely rule out the possibility that the described mutations cause misfolding of the protein, we also expressed the A502Y mutant of Nrf2. This mutant still acts as a transcriptional activator, but activates a largely different set of genes (Kimura 2007) demonstrating that it is a properly folded and active protein. Reconstitution of wild-type Nrf2, but not Nrf2A502Y mutant, restored expression of Nqo1, while both efficiently enhanced the expression of HO-1. Notably, the Nrf2A502Y mutant failed to rescue defective IL-1β secretion in Nrf2−/− BMDC demonstrating that Nrf2 transcriptional activity is required for inflammasome activation (Fig. 5d, e). Since we established the need for Nrf2 transcriptional activity, we checked if proteins known to be necessary for inflammasome activation are downregulated in stimulated
Nrf2-deficient BMDCs. The chaperones HSP90 and SGT1 (encoded by Sugt1 gene) are described to be needed for Nlrp3 activation (Mayor 2007). However, we did not see a difference in their expression in Nrf2−/− BMDCs making them unlikely to mediate the reduced inflammasome activation (Fig. 5f). The anti-apoptotic proteins Bcl-2, Bcl-XL (Bcl2l1) and A1/Bfl-1 (Bcl2a1a), as well as plasminogen activator inhibitor 2 (PAI-2; Serpinb2) were suggested to suppress IL-1β secretion (Bruey 2007; Greten 2007; Zhou 2011; Shimada 2012). Nrf2-deficient BMDCs showed a small increase in Bcl2l1 levels fitting to the lower levels of IL-1β secreted (Fig. 5g). However treating BMDCs with ABT-737 an inhibitor of Bcl-2 and Bcl-XL/Bcl2l1 at higher concentrations (Rooswinkel 2012) did not lead to an increase of IL-1β secretion, making it unlikely for the small difference in Bcl2l1 levels to be responsible for the decreased inflammasome activation (Fig. 5i). Another mechanism of Nlrp3 regulation is the deubquitination of Nlrp3 by BRCC3 (Juliana 2012; Lopez-Castejon 2013; Py 2013). Though, no difference on the mRNA levels was detected indicating this process is not affected in Nrf2−/− BMDCs (Fig. 5i). Autophagy is another process described to inhibit inflammasome activity (Shi 2012), and additionally autophagy-deficiency led to increased inflammasome activation (Nakahira 2011; Zhou 2011). However, Nrf2-deficient BMDCs had a decrease in autophagy as assessed by levels of LC3. This is in accordance with literature identifying the ubiquitin-LC3 adapter p62/Sequestosome1 (Sqstm1) as an Nrf2-target (Jain 2010), suggesting a decrease in autophagy turn-over in Nrf2-deficient cells. Incidentally, p62 is also a negative regulator of Keap1, thus creating a positive feedback loop with Nrf2 (Komatsu 2010; Lau 2010) (Fig. 5j).

Together these findings show that one or several target genes of Nrf2 are required for efficient inflammasome activation and not a direct interaction of Nrf2 with another protein. We were, however, not able to identify a specific Nrf2-dependent target or mechanism necessary for efficient inflammasome activation.
**Fig. 5 Transcriptional activity of Nrf2 is necessary for efficient inflammasome activation.** (a) Schema depicting Nrf2 protein organization in several domains and location of introduced mutations. Various putative nuclear localization signals (NLS) were mutated (NLSm1, NLSm2, NLSm3) or deleted (dNLS). The A502Y mutation shifts Nrf2 binding specificity, whereas the F584A mutation disrupts the binding of Nrf2 to the helicase CHD6. (b) IL-1β production by Nrf2-deficient BMDCs with retroviral expression of various Nrf2 mutants (i.e. NLSm1, NLSm2, NLSm3, and Nrf2<sup>F584A</sup>), and wild-type Nrf2 stimulated with R837 and ATP. (c) The expression of the Nrf2 targets Nqo1, Prdx1 and Hmox1 was determined by RT-qPCR after 5h tBH4 (50μM) stimulation. (d) IL-1β production and (e) Nqo1, Prdx1 and Hmox1 mRNA expression by Nrf2-deficient BMDCs reconstituted with wild-type Nrf2, the Nrf2<sup>A502Y</sup> mutant or GFP control vector after stimulation with (d) R837/ATP or (e) tBH4. (f-h) Relative expression of indicated genes in R837-primed Nrf2-deficient BMDCs compared to wt cells. Dots indicate results from independent experiments. (i) R837-primed BMDCs were treated with indicated concentrations of the BH3 mimetic...
ABT-737 one hour before stimulation with ATP. IL-1β secretion was measured by ELISA. (j) LC3-I (upper band) and LC3-II were detected by Western blot in lysates from BMDCs stimulated with Everolimus (Evr, 20µM; 2h), R837 + ATP (R+A; 5µg/ml, 4h + 2mM, 1h), R837 only (5µg/ml, 5h), LPS (1µg/ml, 16h) or left untreated (left panel). Expression of Sqstm1 (encoding p62) in wt and Nrf2-deficient BMDCs stimulated for 7h with R837 or left untreated (right panel). Error bars show standard deviation. Bars indicate means (a-e, i, j) and dots (f-h) results from individual experiments. 1-way ANOVA and Dunnetts multiple comparison test was used to compare samples expressing Nrf2 or Nrf2 mutants to the GFP only expressing control (b-e). One-sample t-test with theoretical mean of 1 was used in (f-h). Two-Tailed Student t-test was used to compare wild-type and Nrf2−/− groups in (i, j).
Discussion

Nrf2-deficient BMDCs show reduced Asc Speck formation and consequently reduced processing of pro-Casp1 and pro-IL-1β as well as reduced secretion of mature IL-1β. This indicates a defect in Nlrp3 oligomerization, recruitment of Asc or Asc oligomerization. Based on the finding that the ability of Nrf2 mutants to rescue IL-1β secretion correlates with their ability to drive expression of Nrf2 targets we show here that this phenotype is caused by a lack of Nrf2 transcriptional activity and not by some novel non-transcriptional activity of Nrf2. Similarly Nrf2A502Y, which drives expression of a largely different set of genes compared to wild-type Nrf2 (Kimura 2007), fails to rescue the reduced inflammasome activation. A single mutation in its DNA-binding domain is unlikely to affect protein-protein interaction of Nrf2 with other proteins, thus strongly suggesting that Nrf2 transcriptional activity, and not some novel interactions with other proteins is required for efficient inflammasome activation. In addition to up-regulating gene expression upon oxidative/electrophilic stress, Nrf2 has a basal activity and drives expression of genes even during non-induced conditions (Hayes 2000; McMahon 2001; Chanas 2002). We did not see an induction of Nrf2 target genes in stimulated cells, indicating no Nrf2 activation. Similarly treatment of cell with Nrf2 inducers failed to increase IL-1β secretion in wt cells. Together these findings suggest that expression of genes by basal and not induced Nrf2 activity is required for efficient Nlrp3 inflammasome activation.

Nlrp3 inflammasome activation and consequently IL-1β secretion needs two signals. Signal 1 by NLR/TLR ligands drives transcription of IL-1β mRNA and up-regulates expression of inflammasome components, whereas signal 2 by DAMPs induces oligomerization of Nlrp3, followed by Asc and Casp1 recruitment. In contrast to our reports of a pro-inflammatory role of Nrf2 in atherosclerosis (Freigang 2011), Nrf2 has often been described to be anti-inflammatory in other inflammation models (Kim 2010). For example Nrf2-deficient mice have been shown to have exacerbated colitis (Khor 2006; Osburn 2007) and experimental autoimmune encephalomyelitis (Johnson 2010) with an increased in pro-inflammatory cytokines such as TNF-α, IL-1β, IL-6 and IL-12p40. In contrast, we found in addition to the reduced IL-1β secretion by Nrf2−/− BMDCs reduced levels of IL-6. This suggests a pro-inflammatory role of Nrf2 in this context. However, levels of IL-12/23p40 were unchanged showing that Nrf2-deficiency does not cause a general down-regulation of cytokine secretion. Further
investigating the effect of Nrf2 on signal 1 we found some decrease in IL-1β mRNA in Nrf2-deficient BMDCs, although no consistent change in protein levels by Western blot was detected. Similarly inflammasome components seem to be present in comparable amounts. Together this indicates a minor role of Nrf2 in transcriptional priming of inflammasome components and IL-1β. In addition to regulation via transcription, TLR/NLR signaling was suggested to regulate inflammasome activation by post-translational modifications such as deubiquitination of Nlrp3 (Juliana 2012; Lopez-Castejon 2013). BRCC3 is the major deubiquitinating enzyme responsible for this process (Py 2013). We detected comparable levels of BRCC3 in Nrf2-deficient BMDCs, suggesting no role of Nrf2 in deubiquitination of Nlrp3. Nevertheless, we cannot exclude that Nrf2 might play a role for post-translational modifications of inflammasome components by other (so far unknown) mechanisms. Autophagy, which is also involved in inflammasome regulation, similarly seems to not be the cause of the decreased activation in Nrf2-deficient cells.

Nrf2 is the major transcriptional activator of antioxidant genes, and ROS have been implied to play a role in signal 2 of Nlrp3 inflammasome activation. Consequently, we investigated how modulating ROS would change or rescue IL-1β secretion by Nrf2-deficient BMDCs. Interestingly, Nrf2-deficient BMDCs have ROS levels comparable to wt cells during steady-state and only have a defect in degrading ROS upon an oxidative insult. This suggests that Nrf2 is dispensable during homeostatic control of ROS levels and only needed for efficient degradation of ROS during oxidative stress. This is in line with untreated Nrf2 knockout mice having no overt phenotype (Chan 1996; Itoh 1997). Treatment of Nrf2-deficient BMDCs with antioxidants did not rescue the reduced IL-1β secretion. High concentrations of NAC inhibited inflammasome activation as reported (Cruz 2007; Nakahira 2011), although to a similar extent in wt and Nrf2−/− BMDCs indicating that they do not target the pathway defective in Nrf2-deficient cells. Similarly, retroviral expression of Nrf2-driven antioxidant proteins did not rescue the impaired inflammasome activation. Together this data shows that globally elevated ROS levels are not responsible for the reduced Nlrp3 inflammasome activation in Nrf2−/− cells. It is still possible that localized changes in ROS might be responsible for the phenotype, since ROS mediated signaling seems to be rather local and specific (Forman 2010; Randall 2013; Levonen 2014).
Together this data shows that one or several target genes of Nrf2 are required for efficient Nlrp3 inflammasome activation by interacting with the Nlrp3 activating signaling cascade and possibly to some extent by influencing priming. However, further investigations are still needed to unravel how Nrf2 precisely contributes to inflammasome activation. This is complicated by the fact that the signaling pathway leading to Nlrp3 activation is still not completely understood.
**Materials & Methods**

**Mice & Cells**

C57BL/6J (“wild-type”) animals were bought from The Jackson Laboratory (Bar Harbor, Maine, USA). Nrf2-deficient mice (Nfe2l2tm1Mym) (Itoh 1997) backcrossed to C57BL/6 for more than eight generations, were a gift from M. Yamamoto at RIKEN BioResource Center, Japan. Mice were housed in individually ventilated cages under specific pathogen free conditions at ETH Phenomics Center (EPIC) (Zurich, Switzerland). All animal experiments were approved by the local animal ethics committee (Kantonales Veterinärsamt Zürich, licenses 167/2011 and ZH270/2014), and performed according to local guidelines (TschV, Zurich) and the Swiss animal protection law (TschG).

**Cell culture**

Bone-marrow cells from femur and tibur of sex-matched 6-12 week old mice were differentiated into BMDCs in RPMI-1640 medium (Gibco) supplemented with GM-CSF (supernatant from X63-GMCSF cell line (Zal 1994)), 2mM L-glutamine (GE Healthcare), 10mM HEPES (Lonza), 100 U/mL Penicillin, 100 µg/mL Streptomycin (Gibco), 10% FCS (Gibco). Fresh medium was added on d3 and d6 of culture. Non-adherent cells were harvested and used in experiments on d7 to d9 of culture. BMDM were cultured analogously, but with medium supplemented with supernatant from L929 instead of X63-GMCSF cells. Adherent BMDM detached by washing plates with cold PBS + 2mM EDTA were used in experiments. Cells were generally primed with 5µg/ml R837 (Tocris Bioscience) unless indicated otherwise. CpG (Microsynth), Ultra-pure LPS from *Escherichia. coli* O111:B4 (InvivoGen), Lipoteichoic acid from *Staphylococcus aureus* (InvivoGen), Muramyl dipeptide (InvivoGen), Polyinosinic-polycytidylic acid HMW (InvivoGen) and Zymosan A (Sigma-Aldrich) were used at indicated concentrations. Cells were primed for 3h before stimulation for 4h with 200µg/ml Al(OH)₃ (SERVA Electrophoresis) or transfection of poly(deoxyadenylic-thymidylic) acid sodium salt (p(dA:dT); Sigma-Aldrich) with Lipofectamin 2000 (Life Technologies) at a 1:2 ratio or transfection with high-purity flagellin (FliC) from *Salmonella* typhimurium (Adipogen) with Profect P1 (Targeting Systems) at a 1:5 ratio. Alternatively cells were primed for 4 to 6h before stimulation with 2mM Adenosine 5’-triphosphate disodium salt (ATP;
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Sigma-Aldrich), 5µM Nigericin sodium salt (Sigma-Aldrich) or as indicated. Other compounds used were ABT-737 (Adipogen), L-ascorbic acid (Sigma-Aldrich), (2R,4R)-4-aminopyrrolidine-2,4-dicarboxylate (APDC; Sigma-Aldrich), L-Buthionine-sulfoximine (BSO; Sigma-Aldrich), Catalase-polyethylene glycol (Catalase-PEG; Sigma-Aldrich), Diethylmaleate (DEM; Sigma-Aldrich) and tert-Butylhydroquinone (tBHQ; Sigma-Aldrich), Diphenyleneiodonium chloride (DPI; Sigma-Aldrich), DL-Dithiothreitol (DTT; Sigma-Aldrich), L-Glutamic acid (L-Glu; Sigma-Aldrich), N-Acetyl-L-Cysteine (NAC; Sigma-Aldrich), Sulfasalazine (Sigma-Aldrich) and (±)-α-Tocopherol (α-Toc; Sigma-Aldrich).

ELISA and Cytotoxicity

IL-1β, IL-12/23p40 and IL-6 in supernatants were quantified by sandwich ELISA using the following antibody pairs: B122&13-7112-85, C15.6&C17.8 and MP5-20F3&MP5-32C11 (eBioscience). Cell death was assessed by measuring the release of Lactate dehydrogenase with the Pierce LDH Cytotoxicity Assay Kit (Life Technologies). R837-primed cells lysed with the provided lysis buffer were used to determine 100% LDH release, while cells treated only with R837 and no stimulus 2 were set as 0% release.

Immunoblot analysis

3 Mio. BMDCs were seeded in 6 well-plates and stimulated in serum-free OptiMEM (Gibco). Cells were lysed and supernatants were precipitated with chloroform/methanol as described elsewhere (Jakobs 2013). PVDF membranes (GE Healthcare) were probed with antibodies against Caspase-1p10 (SantaCruz sc-514), Asc (Adipogen AG-25B-0006-C100), Nlrp3 (Adipogen AG-20B-0014-C100), IL-1β (R&D Systems AF-401-NA), HMGB-1 (R&D Systems MAB1690) and β-Actin (Sigma-Aldrich A3854) and corresponding secondary horseradish peroxidase-coupled antibodies: bovine anti-goat IgG (SantaCruz sc-2352), goat anti-mouse IgG (Southern Biotech 1030-05) and goat anti-rabbit IgG (SouthernBiotech 4050-05). Proteins with oxidized cysteines were precipitated as described (PROP) (Templeton 2010).

Retroviral expression of genes

For expression of genes Nrf2, Me-1, Txn1, Txnrd1, Prdx1 and Nqo1 were PCR amplified from mouse tissue cDNA. QuikChange II Site-Directed Mutagenesis Kit
(Agilent Technologies) was used to introduce site-directed mutations. Genes were then cloned upstream of the IRES into the retroviral vector pMYs-IRES-GFP. The Asc-GFP cassette from the pTJ-hsASC-StrepII-TagGFP2-8H vector (a kind gift of V. Hornung) was cloned into pMYs-IRES-puro vector to enable expression of an Asc-GFP fusion protein to assess speck formation. Retroviruses were produced in the Phoenix-eco packaging cell line and concentrated by centrifugation at 10’000g for 1h. BMDCs were spin-infected twice on d2-d4 and GFP+ single cell were sorted 48h-72h after the last infection with a FACSaria III (BD Bioscience).

**Flow cytometry**

Cells were acquired on a FACS Canto II or FACS Calibur (BD Bioscience) and data was analyzed in FlowJo software (Tree Star).

Asc Speck formation was assessed by analyzing the change of signal pulse width to area upon aggregation of Asc into specks as described elsewhere (Sester 2015). Briefly, cells expressing Asc-GFP were directly analyzed by flow cytometry. For staining of endogenous Asc, cells were fixed by addition of 4 volumes ethanol and stained with anti-Asc antibody (Adipogen AG-25B-0006-C100) and goat anti-rabbit Ig-PE (SouthernBiotech) after blocking with 3% BSA, 2% goat serum and Fc block (clone 2.4G2, 1µg/ml) in PBS. During acquisition on a flow cytometer cells with Asc specks produce a GFP or PE signal, respectively, with a smaller signal pulse width compared to cells without a speck due to the differential distribution of the fluorophores. To analyze cell death cells were stained with Annexin-V-APC (BD Bioscience) and 7-AAD (eBioscience) in Annexin-V binding buffer. Total cellular ROS was quantified by adding 15ng/ml CM-H2DCFDA (Life Technologies) to cells and observing fluorescein signal at indicated times by flow cytometry.
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5.1.1 Thioredoxin reductase 1 is required for fast T cell cycling and anti-viral responses

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T lymphocytes are an important arm of the adaptive immunity. Upon recognition of its cognate antigen, T cell receptor (TCR) signaling leads to cell activation and rapid proliferation. This signaling requires reactive oxygen species (ROS). However, excessive amounts of ROS are harmful and cells require antioxidant systems to protect them from oxidative stress and, proliferating cells in particular, for biosynthesis of new biomolecules by providing reducing equivalents. Given the role of ROS in T cells and their fast proliferation, we investigated the role of thioredoxin reductase 1 (TrxR1) in T cells and other leukocytes. TrxR1 is the enzyme at the beginning of the thioredoxin (Trx) system in the cytosol which forms together with the glutathione (GSH) system the two main mammalian antioxidant pathways. Lack of TrxR1 had only a minor effect on peripheral T cells in homeostasis in Txnrd1<sup>fl/fl</sup>; Cd4-Cre mice. However, during infection with lymphocytic choriomeningitis virus (LCMV) TrxR1-deficient T cells failed to expand and control virus infection. In vitro stimulation revealed delayed cell cycle entry and longer cycling time as the main culprit for the reduced expansion of TrxR1-deficient T cells. Analysis of other immune cells revealed a role of TrxR1 in dendritic cells and some macrophage populations in homeostasis. After stimulation the before unaffected B cells and neutrophils also showed a defect. Together this suggests a role of TrxR1 mainly in rapidly proliferating immune cells and sufficient compensation for the lack of TrxR1 in most cells during homeostasis.
Introduction

Reactive oxygen and nitrogen species (ROS and RNS), e.g. hydrogen peroxide, superoxide and nitric oxide, can react with various biomolecules, such as proteins, lipids and nucleic acids, damaging and inactivating them. Consequently, cells developed various mechanisms to cope with oxidative stress helping them neutralize ROS/RNS and repair damaged caused. The main cellular sources of ROS are the mitochondrial respiratory chain and NADPH oxidases (NOX complexes). While ROS/RNS can be harmful, it has become clear that they also have physiological functions (Holmstrom 2014; Schieber 2014). Examples include the production of ROS/RNS by phagocytes to help them kill pathogens (respiratory burst) (Dupre-Crochet 2013) and the role of ROS/RNS in signal pathways. ROS is required for efficient T cell activation (Yang 2013), and changes in the redox status have also been suggested to skew T cell differentiation into subsets. T regulatory (Treg) cells were shown to have a stronger antioxidant capacity compared to T effector (Teff) cells (Gostner 2013; Kesarwani 2013; Weinberg 2015). Hence ROS levels have to be tightly balanced to protect cells from damage and to allow proper function. The two main antioxidant systems in mammals are the glutathione (GSH) and the thioredoxin (Trx) system. Nicotinamide adenine dinucleotide phosphate (NADPH) is used as the electron donor by glutathione reductase (GR) to recycle oxidized glutathione (GSH). GSH is then used amongst other to reduce glutaredoxins (Grx). Electrons (from NADPH) are also transferred via thioredoxin reductases (TrxR) to thioredoxins (Trx) and are then mainly used to reduce protein disulfides. Together with thioredoxin-interacting protein (TXNIP), an endogenous Trx inhibitor, this forms the Trx system. Mammalian TrxRs are selenocysteine-containing flavoenzymes with broad substrate specificities allowing reduction of many different proteins apart from Trx and some non-disulfide substrates such as selenite and dehydroascorbic acids in addition. Trx is used as the electron donor for the antioxidant enzymes (2-Cysteine) peroxireductases (Prx) and methionine sulfoxide reductases (Msr), reducing peroxides and methionine sulfoxides, respectively (Mahmood 2013; Lu 2014). TrxR1 (gene name: Txnrd1) and Trx1 (Txn1) are cytosolic and nuclear proteins, whereas TrxR2 (Txnrd2) and Trx2 (Txn2) are mainly localized in mitochondria. The third mammalian TrxR, Thioredoxin glutathione reductase (TGR; gene Txnrd3), is together with some specific Trxs expressed in testes (Miranda-Vizuete 2004). However, several less
characterized alternative splice forms of TrxRs have been described with potentially different localization (Arner 2009). Apart from their role as antioxidants, both Trx and Grx also serve as electron donors for ribonucleotide reductase (RNR), required for the synthesis of new 2’-deoxyribonucleotides (dNTPs) for DNA synthesis (Laurent 1964; Holmgren 1976; Zahedi Avval 2009). Trxs regulate NFκB and AP-1 signaling (Hayashi 1993; Schenk 1994; Hirota 1997) and have been involved in regulation of apoptosis. Trxs in their reduced state bind and inhibit apoptosis signal-regulating kinase 1 (ASK-1) upstream of c-Jun N-terminal kinases (JNK) and p38 MAP kinases pathway thereby coupling redox status with apoptosis (Saitoh 1998).

Apart from its intracellular role, Trx1 is also found extracellularly, where, for example, its secretion by antigen-presenting dendritic cells (DC) is required for efficient proliferation of T cells after activation by the DCs (Angelini 2002; Castellani 2008). A critical role of the Trx system for mouse development became evident in global knock-out of Txnrd1, which were embryonically lethal between E8.5-10.5 with growth and developmental retardation (Jakupoglu 2005; Bondareva 2007). Despite being one of the major antioxidant enzymes, the specific role of TrxR1 in the immune system remains poorly described. We have been characterizing its role in lymphocytes using conditional knockouts. We generated Txnrd1^{fl/fl}; Cd4-Cre mice lacking Txnrd1 mainly in T cells and invariant natural killer (iNKT) cells. Moreover, we generated Txnrd1^{fl/fl}; Ert2-Cre mice allowing inducible deletion of Txnrd1 by Tamoxifen treatment. Our results demonstrate that TrxR1 is essential for development of iNKT cells, but is largely dispensable for homeostasis of peripheral αβ T cells. However, T cell expansion depends critically on TrxR1 in vitro and in vivo.
Results

**Txnrd1 is mostly dispensable for T cell homeostasis**

We recently demonstrated the importance of the selenocysteine enzyme Glutathione peroxidase 4 for peripheral T cell homeostasis and responses against pathogens (Matsushita 2015). Thioredoxin reductase 1 (TrxR1; gene Txnrd1) is another selenoenzyme involved in the cellular redox balance and antioxidant response. To continue expanding our understanding of the role of the cellular antioxidant system in T cells, we generated Txnrd1<sup>fl/fl</sup>; Cd4-Cre mice. In these mice, Cre recombinase leads to a selective deletion of Txnrd1 in cells expressing or having expressed CD4. These are mainly CD4 and CD8 αβ T as well as invariant natural killer T (iNKT) cells which all derive from CD4<sup>+</sup>CD8<sup>+</sup> double positive thymocytes (Stritesky 2012). Thymic CD4 and CD8 T cell development was unaffected in Txnrd1<sup>fl/fl</sup>; Cd4-Cre mice (Fig. 6a). TrxR1-deficiency had no effect on peripheral αβT cells in lymph nodes, while numbers of CD4<sup>+</sup> and CD8<sup>+</sup> T cells were consistently reduced in spleen of Txnrd1<sup>fl/fl</sup>; Cd4-Cre mice (Fig. 6b-e). While the large majority of peripheral T cells in naïve mice are in a resting state characterized as CD62L<sup>hi</sup>CD44<sup>lo</sup>, there are a few (central) memory T cells present as CD62L<sup>lo</sup>CD44<sup>hi</sup> or CD62L<sup>hi</sup>CD44<sup>hi</sup> cells. In the absence of TrxR1, the population of CD8<sup>+</sup> CD62L<sup>hi</sup>CD44<sup>hi</sup> cells was reduced by approximately 50% (Fig. 6f-i). To assess the ability of Txnrd1-deficient T cells in direct competition with wild-type counterparts, we generated mixed bone marrow chimeras (50:50 WT:KO). In this situation, peripheral CD4 and CD8 T cell lacking TrxR1 showed a clear disadvantage compared to wild-type cells (Fig. 7a) in spleen and lymph nodes and the disadvantage was even more pronounced for activated/memory T cells (Fig. 7b). Interestingly, TrxR1-knockouts were almost completely devoid of iNKT cells in the thymus (Fig. 6a) and peripheral lymphoid and non-lymphoid organs (i.e. liver) (Fig. 6b-e) indicating a crucial role of TrxR1 in iNKT cells development or survival.

These data show the requirement of TrxR1 for the thymic generation of iNKTs. In contrast, thymic selection of αβT cell in the DP stage is not dependent on TrxR1. Expansion of iNKT cells in the thymus occurs from a subset of CD4<sup>−</sup>CD8<sup>+</sup> double positive (DP) thymocytes. In contrast, αβT cell expansion during development only occurs in the CD4<sup>−</sup>CD8<sup>−</sup> double negative 2 (DN2) and CD4<sup>+</sup>CD8<sup>+</sup> DP stage before
positive/negative selection. To explain abrogated iNKT cell but normal αβ T cell development in Txnrd1^fl/fl; Cd4-Cre mice, we speculated that TrxR1 might be required for αβ T cell expansion before deletion of Txnrd1 gene and absence of the protein. To study this possibility we generated Txnrd1^fl/fl; Ert2-Cre mice allowing conditional gene depletion upon injection of TAM. These mice have visibly smaller thymi already one week after deletion (not shown). Analysis showed a massive reduction in total cellularity and numbers of all analyzed thymocyte populations including DN and DP precursors, mature single positive (SP) αβ T cells and γδ T cells, as well as iNKT cells. Due to the massive loss of the DP, which account for 80% of all thymocytes, the frequency of all other cell populations was increased (Fig. 7c). However, the reduced thymic generation of new T cells had no major effect on peripheral populations two weeks after deletion, with only a small decrease of αβ T cells in spleens (Fig. 7d).

Taken together, these results demonstrate that TrxR1 is crucial for the expansion of all T cell subset precursors but not T cell selection in the thymus. While T cell homeostasis remains largely intact in the periphery, except for a reduction of splenic and activated/memory T cells in naïve mice, a crucial role of TrxR1 for peripheral T cell homeostasis becomes evident by their disadvantage in reconstitution of lethally irradiated mice compared with wild-type cells in a competitive situation.
Fig. 6 Txnrd1-deficient T cells have a minor defect in the periphery and secondary lymphoid organs. (a) Thymocytes of Txnrd1fl/fl, Cd4-Cre mice and Cre-
negative *Txnrd1*fl/fl litters were stained with anti-CD4, CD8, TCRβ, TCRδ, NK1.1 and CD1d-PBS57-tetramer (to stain invariant NKT cells). Cells were pre-gated as FSC/SSC lymphocytes and singlets. Example FACS plots and gating are shown on top with frequency of lymphocyte gate indicated. Number of cells per thymus are plotted below with the following gating: double-negative cells (DN; CD4− CD8−), double-positive thymocytes (DP; CD4+ CD8+), single positive CD4 and CD8 T cells (CD4T and CD8T; TCRβ+ CD1d-PBS57-tetramer+ plus CD4+CD8− and CD8+CD4−, respectively), iNKT cells (TCRβint CD1d-PBS57-tetramer+) and γδ T cells (TCRδ+ TCRβ−). (b–e) Spleen, liver, mesenteric and inguinal lymph node cells of *Txnrd1*fl/fl and *Txnrd1*fl/fl; *Cd4-Cre* animals were stained for CD4 T, CD8 T, iNKT, γδ T and NK cells and NK cells (NK1.1+ TCRβ− TCRδ+) as in (a). (f–i) Activation/memory status of CD4 and CD8 T cells in spleen, liver, mesenteric and inguinal lymph nodes was assessed by staining for CD62L and CD44. Dot blots (top panels) gated on CD4 and CD8 T cells, respectively and graphs (bottom panels) with quantification for all mice are shown. Numbers and bars show mean±standard deviation. Circles in graphs represent individual mice. Two-Tailed Student t-test was used to compare Cre-negative with *Cd4-Cre* groups.
**Fig. 7 Trx1-deficient cells are at a disadvantage in competitive situations and TrxR1 is required for expansion of T cell precursors (a, b)** Lethally irradiated wild-type mice were reconstituted with a mixture of wild-type and *Txnrd1^fl/fl*, *Cd4-Cre* bone marrow expressing CD45.1 and CD45.2, respectively. After reconstitution thymic, splenic, liver and inguinal LN cell populations were stained as in **Fig. 6**. Ratios of *Txnrd1^fl/fl*, *Cd4-Cre* to wild-type cells for the indicated populations normalized to B cells (gated as CD19^+^ lymphocytes) are plotted. (c, d) *Txnrd1* was deleted by treating *Txnrd1^fl/fl*, *Ert2-Cre* inducible knock-out and control Cre-negative
mice with Tamoxifen (TAM). Animals were analyzed 2 weeks after deletion. Thymi (c) were stained as described for Fig. 6a. Representative dot blots with gating and frequencies plus graphs with frequencies and cell numbers are shown. (d) CD4, CD8, γδ T and iNKT cells in spleen, liver and iLN were analyzed as described for Fig. 6b-e. Numbers and bars show mean±standard deviation. Circles in graphs represent individual mice. Two-Tailed Student t-test was used to compare Cre-negative with Ert2-Cre groups.
Results

T cells require TrxR1 for efficient expansion in vivo.

Given the stronger effect of TrxR1-deficiency in activated/memory compared to naïve αβ T cells, we next investigated T cells in an immune response. Mice were infected with LCMV WE. We monitored proliferating cells in blood and spleen during infection by measuring incorporation of the thymidine analog EdU by flow cytometry. EdU was administered 16h before analysis. During all time points analyzed CD4 and CD8 T cells from Txnrd1^{fl/fl}; Cd4-Cre mice showed strikingly reduced proliferation. Consequently at day 7 post infection there was a massive defect in the total number of proliferating T cells when they lacked TrxR1 (Fig. 8a, b). In fact, virus-specific T cells were undetectable by gp33-41 tetramer staining at day 7 p.i. (Fig. 8c, d). Consequently, Txnrd1^{fl/fl}; Cd4-Cre mice failed to clear LCMV WE infection (Fig. 8g). Staining of 7-AAD and Annexin-V during infection showed comparable frequencies of dead/dying CD4+ and CD8+ cells suggesting reduced T cell expansion results from defects in cell proliferation rather than cell survival (Fig. 8e, f). To investigate T cell proliferation in a sterile environment, where differences in viral titers cannot confound results, we treated Txnrd1^{fl/fl}; Cd4-Cre and wt controls with the superantigen Staphylococcal enterotoxin B (SEB). SEB binds to and polyclonal activates all T cells with a TCRβ carrying the Vβ8.1 or Vβ8.2 chain. Consistent with viral infection, we observed a strikingly reduced expansion of SEB specific T cells (Fig. 8h). Deficiency of the antioxidant enzyme Gpx4 in T cells has been reported to blunt primary but not recall T cell expansion following viral infection (Matsushita 2015). To assess the role of TrxR1 in memory/recall responses, we have generated Txnrd1^{fl/fl}; Ert2-Cre mice. These mice ubiquitously express Ert2-Cre, but only delete floxed genes upon administration of Tamoxifen (TAM). We infected these mice with LCMV WE allowing clearance of LCMV and generation of a memory in the presence of TrxR1. Thereafter the Txnrd1 gene was deleted by TAM injection, and mice subsequent challenged with Listeria monocytogenes expressing the LCMV epitope gp33-41 (Listeria-gp33). Gp33-41-specific memory T cells lacking TrxR1 failed to expand upon secondary infection (Fig. 8i). However, the remaining gp33-41-specific memory cells present before reinfection were able to protect mice from an otherwise lethal dose of Listeria (Fig. 8j, k). Together these data show a critical role of TrxR1 for expansion of activated T cells.
Results (cont.)
Fig. 8 T cells require TrxR1 for efficient expansion in vivo. (a-f) Txnrd1fl/fl and Txnrd1fl/fl; Cd4-Cre mice infected with 200 pfu LCMV WE i.v. and analyzed after 3, 4 and 7 days of infection (d7 mice were infected separately) (a, b) Proliferation of CD4 and CD8 cells was assessed by EdU incorporation in blood and spleen. Mice were injected with 0.5mg EdU i.p 16h before analysis. Dot plots (left) show representative results from d7 with numbers indicating percentage of EdU⁺ of CD4⁺ and CD8⁺ cells, respectively. Graphs show percentage (middle) and total number of EdU⁺ CD4⁺ and CD8⁺ T cells per µl blood or per spleen (right) (c, d) On day 7 of infection activation of CD4 and CD8 T cells was assessed by down-regulation of CD62L. Gp33.41 virus-specific CD8 cells were stained with tetramers. Dot plots with gating and frequencies are shown (left panels) as well as quantification of data (right panels). Additionally, total numbers of CD4 and CD8 cells per µl blood and per spleen are shown. (e, f) Cell death in LCMV-infected mice was assessed by staining dead and dying cells with Annexin-V and 7-AAD. Percentage of alive CD4 and CD8 for blood and spleen are shown. (g) LCMV titers over time were determined by plaque assay of blood from mice infected with 500 pfu LCMV WE at indicated times after infection. (h) Sterile expansion of T cell was induced by injecting mice with
100 µg of the superantigen Staphylococcal enterotoxin B (SEB). Differences in expansion between \( Txnrd1^{fl/fl} \) and \( Txnrd1^{fl/fl} \); \( Cd4-Cre \) mice were assessed by staining CD4 and CD8 T cells carrying the affected TCR\( \beta \) chains V\( \beta \)8.1 or V\( \beta \)8.2. Kinetics in blood plus spleens at d3 were analyzed. (i-k) \( Txnrd1^{fl/fl} \) and \( Txnrd1^{fl/fl} \); \( Ert2-Cre \) mice were infected with LCMV WE. Following Tamoxifen (TAM) induced deletion of \( Txnrd1 \), memory T cell responses were assessed by infecting mice with \( Listeria monocytogenes \) expressing the LCMV epitope gp33-41. (i) Frequencies of total CD8 and antigen-specific gp33-41 tetramer\(^+\) CD8 cells at the indicated days of infection are shown in blood for the primary LCMV and the secondary Listeria infection. Additionally splenic T cells were analyzed at day 5 of Listeria infection when mice were sacrificed. (j) Mice without primary LCMV infection (grey), consequently lacking a gp33-41-specific memory response, were infected in parallel to the mice with primary LCMV infection (black lines). Weight changes were monitored over the course of the infection for both groups. Mice lacking primary LCMV infection were all moribund at day 4 of Listeria infection and were euthanized. (k) Listeria titers in liver and spleen were determined at d4 and d5 for animals without and with primary LCMV infection, respectively. Means±standard deviations are shown. Circles in graphs represent individual mice. Two-Tailed Student t-test was used to compare Cre-negative with Cre-positive groups.
TrxR1 is required for efficient T cell proliferation and survival in vitro independently of antioxidants, ribonucleotide reductase and cell death pathways.

We next studied activation, proliferation, and differentiation of T cells lacking TrxR1 in vitro to define the underlying molecular mechanisms. T cells from Txnrd1<sup>fl/fl</sup>; Cd4-Cre and Txnrd1<sup>fl/fl</sup> mice were sorted and labeled with CFSE prior to stimulation with plate-bound anti-CD3 and anti-CD28 (Fig. 9a). Txnrd1-deficient T cells showed impaired proliferation (i.e. slower CFSE dilution) and consequently reduced numbers of cells after two to four days of stimulation (Fig. 9b). We used the precursor cohort method (Hawkins 2007) to analyze time series data of CFSE-labeled TrxR1-sufficient and deficient T cells. This allowed calculation of division times and time to first division. For both CD4<sup>+</sup> and CD8<sup>+</sup> cells, we found a longer mean division time as well as time needed to start dividing (Fig. 9c). Furthermore, Txnrd1-deficient T cells showed delayed up-regulation of Ki-67 upon anti-CD3/anti-CD28 stimulation (Fig. 9d, e). Ki-67 is absent in quiescent cells (G<sub>0</sub>), but present in cycling cells (G<sub>1</sub>/S/G<sub>2</sub>/M) (Scholzen 2000). These data indicate a delay in cell cycle entry in the absence of TrxR1. Consistently, analyzing the DNA content by PI staining, we observed fewer Txnrd1-deficient cells in S and G<sub>2</sub> at d1.5 However, at later time points the distribution of cells in the different stages of the cell cycle were comparable, indicating no specific block at a certain cell cycle checkpoint (Fig. 9f). TrxR1 is a major player in the cellular antioxidant response and we speculated, whether treatment with exogenous antioxidants might be able to rescue the reduced proliferation of TrxR1-deficient T cells. Addition of the antioxidants ascorbic acid (aa), DL-Dithiothreitol (DTT), N-acetyl-L-cysteine (NAC), cell-membrane permeable catalase or the flavin enzyme inhibitor diphenyleneiodonium chloride (DPI) however failed to restore proliferation of TrxR1-deficient T cells (Fig. 10a). For cells to proliferate they have to duplicate their genome and therefore require deoxyribonucleotide building blocks. The enzyme ribonucleotide reductase (RNR) reduces ribonucleotides to 2'-deoxy-ribonucleotides (dNTPs) and catalysis the rate-limiting step for de novo dNTP synthesis (Holmgren 2010). Reduced Trx1, which is recycled by TrxR1, is an electron donor for RNR (Zahedi Avval 2009). We supplemented T cell cultures with deoxy-nucleosides (dNs) to investigate, if the reduced proliferation in TrxR1-deficient cells is caused by a lack of dNTPs. However, dNs addition did not rescue defective proliferation (Fig. 10b). Further, TrxR1-deficient
Results

Cells had no increase in DNA breaks as measured by γ-H2AX (Fig. 10c). DNA breaks accumulate in cells with a reduced dNTP pool as shown in the positive control of RNR-inhibitor hydroxyurea treated cells (Saintigny 2001) (Fig. 10c). These data strongly suggest that dNTP synthesis due to impaired RNR activity is not the cause for defective T cell proliferation in the absence of TrxR1. We observed a small decrease in viability of Txnrd1-deficient T cells after anti-CD3/anti-CD28 stimulation in vitro (Fig. 10d) indicating increased cell death contributes to impaired expansion of T cells lacking TrxR1. Viability was not rescued by treatment with antioxidants (i.e. ascorbic acid and α-Tocopherol) or IL-2 and IL-7 that are known to promote T cell survival and proliferation. Inhibition of cell death pathways did also not increase survival (Fig. 10e, f). Ferroptosis was inhibited with the iron chelators ciclopirox olamine (CPX) and Deferoxamine (DFO) or the inhibitor Ferrostatin-1 (Fer-1), necroptosis with Necrostatin-1 (Nec-1) and apoptosis with the pan-caspase inhibitor z-VAD-fmk. A defect in TCR signaling and hence T cell activation could explain the reduced expansion of TrxR1-deficient T cells. However, anti-CD3/anti-CD28 mediated upregulation of the activation markers CD25, CD44 and CD69, which occurs prior cell division, was comparable in TrxR1-deficient and wild-type T cells (Fig. 10g). These findings show the importance of TrxR1 for T cell proliferation by regulation of cell cycle entry as well as cell cycle time and to a smaller degree for increasing survival of cells in vitro.
Fig. 9 TrxR1 is required for efficient T cells proliferation (a) Magnetically-sorted T cells from Txnrd1<sup>fl/fl</sup> (black line) and Txnrd1<sup>fl/fl</sup>; Cd4-Cre (grey line) mice were CFSE labeled and stimulated in anti-CD3/anti-CD28 coated plates in vitro. CFSE dilution was measured by flow cytometry at indicated times of stimulation. (b) Total CD8<sup>+</sup> T cells per well were counted over time during anti-CD3/anti-CD28 stimulation. (c) Proliferation parameters of CFSE-labeled Txnrd1-deficient and wild-type CD4 and CD8 T cells were determined with the precursor cohort method (Hawkins 2007). The reciprocal of the slope is the mean division time and the intersection with mean division number y=1 indicates the time to first division. (d, e) T cells leaving G<sub>0</sub> and entering cell cycle were detected by Ki-67 staining and analysis by flow cytometry during anti-CD3/anti-CD28 stimulation of magnetically sorted T cells. Histograms of Ki-67 staining (d) with gating for CD4 (left) and CD8 (right).
(right) cells and quantification (e, CD4 top, CD8 bottom) at indicated times of stimulation are depicted. (f) DNA content of in vitro stimulated CD4 cells was determined with propidium iodide (PI) from d1 to d3 of stimulation. Means±standard deviations are shown. Two-Tailed Student t-test was used to compare Cre-negative with Cd4-Cre groups except for (e), where 1-way ANOVA with Bonferroni’s multiple comparison test for each time point was used (planned comparison).
Results

Fig. 10 Trx1 is required for proliferation and survival in vitro independently of antioxidants, ribonucleotide reductase and cell death pathways. (a) MACS-sorted anti-CD3/anti-CD28 stimulated T cells were treated with the antioxidants ascorbic acid (aa; 0.8, 4, 20μM), Catalase-polyethylene glycol (catalase; 278, 1136, 4545 U/ml), Diphenyleneiodonium chloride (DPI; 62.5, 250, 1000nM), DL-Dithiothreitol (DTT; 111, 333, 1000 μM) and N-acetyl-L-cysteine (NAC; 2, 8, 32 mM) during stimulation and counted after 3 days. (b) Cultures of anti-CD3/anti-CD28 stimulated T cells were supplemented with deoxy-nucleosides (dN) at day 0 and day 2 of culture. CD4+ and CD8+ cells were counted after 3 days. Deoxy(d)-Adenosine, dCytidine,
dGuanosine and Thymidin were added to final concentrations of 62.5, 125 and 417µM for each nucleoside. (c) γ-H2AX and actin were probed in western blots of 50µg cell lysate from Txnrd1\textsuperscript{fl/fl} and Txnrd1\textsuperscript{fl/−}; Cd4-Cre T cells stimulated for indicated days with anti-CD3/anti-CD28. As a positive control for DNA damage cell treated with 2mM of the ribonucleotide reductase inhibitor hydroxyurea (HU) were used. (d) Cell death during the first 24 hours of anti-CD3/anti-CD28 was assessed by staining cells with Annexin-V and 7-AAD. FACS plot of cells stimulated for 1 day and gating (left), as well as quantification of alive (Annexin-V - 7-AAD) cells before stimulation and after 24 hours of incubation with and without stimulation (right) is shown. (e, f) Analysis of cell survival of CD4\textsuperscript{+} (e) and CD8\textsuperscript{+} (f) T cell treated with antioxidants, cytokines and cell death inhibitors after 1 day of anti-CD3/anti-CD28 stimulation. Survival of cell with the added antioxidants ascorbic acid (aa; 1, 6, 30 µM) and α-Tocopherol (αToc; 3, 16, 80 µM) as well as the cytokines Interleukin-2 (IL-2; 1:500, 1:100, 1:50) and IL-7 (1:100, 1:50, 1:10) are shown in the left graphs. Results for treatment with the ferroptosis inhibitors ciclopirox olamine (CPX; 8, 63, 500 nM), Deferoxamine (DFO; 1, 10, 50 µM) and Ferrostatin-1 (Fer-1; 0.5, 2, 8 µM); the Necroptosis inhibitor Necrostatin-1 (Nec-1; 1, 4, 20 µM) and the apoptosis inhibitor z-VAD-fmk (zVAD; 2, 12, 60 µM) are to the right. (g) Up-regulation of the activation markers CD25, CD44 and CD69 was measured by flow cytometry during the first 8 hours of anti-CD3/anti-CD28 T cell stimulation. Histograms for the 3 markers for CD4\textsuperscript{+} (top) and CD8\textsuperscript{+} (bottom) cells after 0 (light grey), 2 (dark grey) and 8 hours (black) of stimulation are shown on the left. Txnrd1\textsuperscript{fl/fl} cells are plotted as solid lines and Txnrd1\textsuperscript{fl/−}; Cd4-Cre cells as dashed lines. Median fluorescence intensities (mfi) for indicated times are shown in the graphs to the right. Means±standard deviations are shown. Two-Tailed Student t-test was used to compare Cre-negative with Cd4-Cre groups.
B cells require Txnrd1 for mounting an efficient antibody response, but Txnrd1 is dispensable for B cell homeostasis.

B cells are the other major lymphocyte type besides T cells. To study the role of TrxR1 in B cells we analyzed Txnrd1\(^{\text{fl/fl}}\); Ert2-Cre and mixed wild-type : Txnrd1\(^{\text{fl/fl}}\); Ert2-Cre bone marrow chimeras. We found no decrease in B cell precursors in bone marrow, including cycling cells (Fig. 11a). Similarly, different B cell populations in the spleen were unchanged. We found no difference in CD23\(^+\) (mainly follicular B cells), CD21/35\(^+\) marginal zone B cells and double-negative CD23\(^-\)CD21/35\(^-\) cells (B1 and transitional-1 B cells) (Fig. 11b). Peripheral B cell in blood, liver and inguinal lymph node were as well not affected by TrxR1-deficiency (Fig. 11c). Unlike T cells, TrxR1-deficient B cells seem to not have a disadvantage compared to wild-type cells in a competitive situation in wild-type : Txnrd1\(^{\text{fl/fl}}\); Ert2-Cre mixed bone marrow chimeras upon TAM-induced deletion (Fig. 11d). We generated mixed Igh-\(J^{-}\) (B cell knock-out) : Txnrd1\(^{\text{fl/fl}}\) \pm \ Ert2-Cre (4:1 ratio) bone marrow to investigate the importance of TrxR1 in B cells during an immune response without affecting T cell help. In these mice most T cells (and other non-B leukocytes) are derived from Igh-\(J^{-}\) cells, hence are wild-type for the Txnrd1 locus. In contrast, all B cell are Txnrd1\(^{\text{fl/fl}}\) \pm \ Ert2-Cre. After Tamoxifen-induced deletion of Txnrd1 in B cells, mice were immunized with Q\(\beta\)/ssRNA virus-like particles (VLPs). VLPs promote a strong antibody response in the absence of an infectious agent (Jennings 2008). We measured serum titers of Q\(\beta\)-specific antibodies as a read-out for B cell function. Mice with TrxR1-deficient B cells had a decreased IgM response after one week and after class switching a reduced IgG2b/c response after two weeks. Interestingly, antibody titers in most mice with Txnrd1\(^{\text{fl/fl}}\); Ert2-Cre B cells did increase to levels of Cre-negative mice at later time points (Fig. 11e). These findings show that TrxR1 is dispensable for B cells during steady-state, but required for an efficient antibody response. This is similar to T cells, where TrxR1 also seems to be mainly required upon activation of cells and not for peripheral homeostasis.
**Results**

Fig. 11 B cells require Txnrd1 for mounting an efficient antibody response, but Txnrd1 is dispensable for B cell homeostasis. (a-c) B cell were analyzed in Tamoxifen (TAM) treated Txnrd1fl/fl and Txnrd1+/−, Ert2-Cre mice (same mice as used in Fig. 7e, d). (a) Gating strategy for B220+ bone marrow (BM) cells is shown with numbers indicating frequencies (top) and total cell numbers for individual mice shown below. (b) Splenic B cells (CD19+ B220+) were separated for expression of CD23 and CD21/35. Gating and frequencies shown in dots plots on top with number of cells per spleen below. (c) Cell numbers and frequency of B cells (gated as CD19+ lymphocytes) in inguinal LN, liver and blood. (d) B cell populations were analyzed in mixed wild-type (CD45.1+): Txnrd1fl/fl; Ert2-Cre (CD45.2−) bone marrow chimeras after treatment with Tamoxifen. The relative change in frequencies of Txnrd1fl/fl; Ert2-Cre cells compared to frequencies of blood CD19+ cells before TAM treatment are plotted with gating done as in (a-c). (e) Lethally irradiated mice were reconstituted
with 4 parts *Igh-J* knockout and 1 part *Txnrd1<sup>fl/fl</sup> or *Txnrd1<sup>fl/fl</sup>; Ert2-Cre* bone marrow. Consequently all B cells are *Txnrd1<sup>fl/fl</sup> and *Txnrd1<sup>fl/fl</sup>; Ert2-Cre*, respectively. In contrast, most T and other non-B cells are wild-type for the *Txnrd1* alleles. Mice were immunized with Qβ virus-like particles after Tamoxifen induced deletion of *Txnrd1*. Serum anti-Qβ IgM, IgG2b and IgG2c titers were measured by ELISA at indicated time points after immunization. Means±standard deviations are shown. Two-Tailed Student t-test was used to compare *Cre*-negative with *Ert2-Cre* groups.

**Dendritic cells have a defect upon deletion of *Txnrd1***

We used *Txnrd1<sup>fl/fl</sup>; Ert2-Cre* mice and mixed wild-type : *Txnrd1<sup>fl/fl</sup>; Ert2-Cre* bone marrow chimeras to investigate the importance of TrxR1 in myeloid cells during steady state. Two weeks after *Txnrd1*-deletion we detected reduced numbers of dendritic cells (CD11c<sup>+</sup> MHC class II<sup>+</sup>) in spleen and inguinal LNs (Fig. 12a, b). In the competitive situation of chimeras also Ly-6C<sup>−</sup> CD11b<sup>+</sup> F4/80<sup>+</sup> monocytes/macrophages seem to have a defect in the absence of TrxR1 in spleen, blood and lungs (Fig. 12c). In contrast, eosinophils, neutrophils and classic (Ly-6C<sup>+</sup>) monocytes do not require TrxR1 during homeostasis (Fig. 12b, c). Since we mainly saw an effect of TrxR1-deficiency on activated and proliferating T cells, we speculated if neutrophils might be more affected during emergency hematopoiesis with its much-increased proliferation of neutrophil precursors (Manz 2014). Tamoxifen-treated *Txnrd1<sup>fl/fl</sup>; Ert2-Cre* mice were injected with non-lethal doses of LPS and blood neutrophils as well as bone marrow neutrophils and precursor were analyzed. While there was only a tendency for a decrease in blood neutrophilia, a significant drop in bone marrow precursors was found (Fig. 12d, e). Together this suggests a differential requirement of TrxR1 for various myeloid cells and possible analogous to T cells a more important role after stimulation at least for neutrophils.
Results

**Fig. 12** Dendritic cell have a defect upon deletion of *Txnrd1*. (a, b) *Txnrd1*<sup>fl/fl</sup> and *Txnrd1*<sup>−/−</sup>; Ert2-Cre mice were treated with Tamoxifen (TAM) to delete *Txnrd1*. After 2 weeks myeloid populations in spleen, inguinal lymphnodes (iLN), lung and blood were analyzed with the gating strategy shown in (a). Total cell counts per spleen, 2 iLN, lung and μl blood for indicated populations are shown in (b) (same mice were used for Fig. 11a-e). (c) Lethally irradiated mice were reconstituted with a mixture of wild-type (CD45.1<sup>+</sup>) and *Txnrd1*<sup>−/−</sup>; Ert2-Cre (CD45.2<sup>+</sup>) bone marrow (same mice were analyzed for Fig. 11d) and following Tamoxifen treatment myeloid
Results

cells were analyzed as in (a), though gating in lung and blood included F4/80 and MHC class II to separate DCs and Monocytes/Macrophages analogous to spleen/iLN gating. For the different population the relative change in frequencies of CD45.2⁺ Txnr1fl/fl; Ert2-Cre cells compared to untreated blood CD11b⁺ CD45.2⁺ cells are shown, i.e. values below 1 indicate a decrease in frequency upon deletion of Txnr1.

(d, e) Emergency myelopoiesis was induced by injecting mice twice with 35 µg LPS in a two day interval and analyzing bone-marrow (BM) and blood one day after the last injection. FACS blots were pre-gated on alive CD11c⁻ CD11b⁺ and alive CD11c⁻ cells for BM and blood, respectively. Neutrophils and precursors in the BM were gated as shown (d). Results from 4 independent experiments are summarized in (e) with circles representing means from experiments and lines connecting corresponding means from Cre-negative with Ert2-Cre mice. Means±standard deviations are shown. Two-Tailed Student t-test (paired for e, others unpaired) was used to compare Cre-negative with Ert2-Cre groups.
Discussion

Oxidants were originally thought to be only harmful for life by damaging biomolecules as for example suggested in the free radical theory of ageing proposed more than half a century ago (Harman 1956). However, it is clear now that the role of ROS/RNS is more complex and they are also required for cellular functions. Together with growth factor signaling, TCR signaling was one of the first processes, where ROS were shown to have a beneficial function (Holmstrom 2014; Schieber 2014). Consequently, cells have to balance cytoprotective antioxidant capacity while allowing ROS/RNS to mediate signaling. An antioxidant system is also required to ensure reversibility of signaling by removing oxidative modifications. The Trx system is one of the main antioxidant pathways in mammalian cells. TrxR1 is at the beginning of the pathway in the cytosol and its importance is highlighted by the embryonic lethality of the global TrxR1 knockout (Jakupoglu 2005; Bondareva 2007). Considering the known requirement of ROS in T cell signaling and the role of different redox status for different T cell subsets, we analyzed the effect of TrxR1-deficiency in T cells. Txnrd1fl/fl, Cd4-Cre mice showed normal T cell development in the thymus. Peripheral CD4 and CD8 αβ T cells were unchanged in the organs analyzed with the exception of a small reduction in spleens. A reduction in activated (CD62Llo and/or CD44+) T cells was also observable in several organs. Supporting a role of TrxR1 in activated T cell was the drastic reduction of invariant NKT cells already in the thymus. Unlike T cells, iNKTs get pre-activated during their development in the thymus and undergo further expansion after the DP stage resembling more memory than naïve T cells (Das 2010). In the competitive situation of mixed bone-marrow chimeras, the contribution of TrxR1-deficient cells to peripheral T cell populations in all analyzed organs was smaller compared to the one from wild-type cells. This suggests an intrinsic defect in the absence of TrxR1. This defect is however small enough to be mostly compensated in the absence of competition. iNKTs need presentation of endogenous ligands by CD1d on thymic DP cells for their development (Stritesky 2012; Salio 2014). The lack of a rescue of iNKT cells in the presence of wild-type DP cells in the mixed bone marrow chimeras argues against TrxR1 being required for CD1d-mediated presentation of ligands, but suggests an intrinsic role of TrxR1 in iNKT development. Activating T cells by either an infection with LCMV or in a sterile environment by the superantigen SEB revealed a
severely defective expansion of TrxR1-deficient T cells. Together with the reduction in activated T cells and iNKT cells in naive animals this suggest a role for TrxR1 in the expansion of the cells, but less importance for homeostasis. When Txnrd1 is deleted in the context of a memory response, while being intact during the primary response, TrxR1-deficient T cells failed to expand, but still mounted a protective immune response. This shows again the importance of TrxR1 for expansion, but suggest it is dispensable for T cell function. The complete collapse of thymic T cell population in Tamoxifen-treated Txnrd1fl/fl; Ert2-Cre mice, i.e. deletion of Txnrd1 already in bone-marrow and thymic DN T cell precursors, also fits with a requirement of TrxR1 for T cell expansion, since expansion during T cell development happens before the DP stage (Kawamoto 2003), i.e. before Txnrd1-deletion in Txnrd1fl/fl; Cd4-Cre mice. The abolition of thymic output had no big effect on peripheral T cell population, which do not depend on TrxR1, as shown with the Txnrd1fl/fl; Cd4-Cre mice. Additionally, peripheral T cell pools are relatively independent of thymic output for several weeks in the absence of a thymus in adult mice (Miller 1965; Doenhoff 1979). Together this suggest an important contribution of TrxR1 for expansion of T cells, therefore TrxR1 could be required for activating signaling, survival after activation or proliferation. To better understand the precise role of TrxR1 for T cell expansion, we used in vitro anti-CD3/anti-CD28 stimulation. Cells also had a defective expansion in vitro, which could not be rescued by antioxidant treatment, suggesting no general lack of antioxidants causing the defect. It is however important to remember that even wild-type T cells require a source of reducing equivalents for growth in vitro, consequently medium is already supplemented with the reducing agent β-mercaptoethanol (Cerottini 1974; Chang 1982). TrxR1 was not required for TCR and co-stimulatory signaling, since activation marker up-regulation was comparable in TrxR1-deficient and wild-type T cells. Proliferation of T cells, however, was affected by deletion of Txnrd1 since TrxR1-deficient T cells had a delayed cell cycle entry and an increased cell cycle time. Proliferating cells need new dNTPs for DNA replication, which are provided by RNR (Holmgren 2010). The Trx system is a known electron donor of RNR, so we speculated a lack of dNTPs could lead to the slower proliferation of TrxR1-deficient cells. However, supplementation with dNs failed to rescue proliferation and Txnrd1fl/fl; Cd4-Cre T cell had no signs of increased DNA damage, expected to occur in situation of inadequate dNTP supplies. This data thus indicates a different function of TrxR1 for proliferation.
differences in the distribution of cells in G1, S or G2 phase by staining cellular DNA content. This argues against a defect at a specific cell cycle checkpoint. In addition to the decreased cell division, TrxR1-deficient T cells also had a minor increase in cell death, which was not rescued by cytokine supplementation or inhibition of cell death pathways by various inhibitors. Investigating other immune cells using inducible deletion of TrxR1 in adult Txnrd1^{fl/fl}; Ert2-Cre mice, we found no defect in B cell development and peripheral cell numbers. However, upon stimulation by VLPs mice with TrxR1-deficient B cell had a defective antibody response, mimicking the situation in T cells, which also show a much stronger phenotype upon activation. The cause of the reduced serum titers still needs to be further characterized. It is not clear yet, if it is due to a reduced expansion of B cells, differentiation defects or reduced antibody secretion. Interestingly, titers of mice with TrxR1-deficient B cells seem to approach wild-type levels later after immunization. Investigating myeloid cells in naïve Txnrd1^{fl/fl}; Ert2-Cre, we found a reduction of DCs in spleen, LNs and lungs and F4/80+ Ly-6C+ macrophages in spleen, lung and blood. Neutrophils and Ly-6C+ monocytes/macrophages were unchanged despite their short lifespan (Rankin 2010; Yona 2013). Similar to the stronger phenotype in T and B cells upon activation, increasing proliferation of neutrophil precursors during emergency hematopoiesis, seems to lead to a reduction in cells in the absence of TrxR1. Together this data highlights the importance of TrxR1 for fast proliferating cells. The relatively mild phenotype in non- and slow proliferating cells suggests a compensatory mechanism. A likely candidate for this is the GSH system. Indeed, the inhibition of GSH synthesis by BSO leads to cell death of TrxR1-deficient T cells (data not shown) and compensation for the lack of TrxR1 by the glutathione system was shown for mouse embryonic fibroblasts and HeLa cells (Mandal 2010; Du 2012), whereas wild-type cells are not affected by BSO. Differences in reliance on the Trx versus the GSH system in various cell types might also explain their different susceptibility to TrxR1 deletion, a point warranting further investigation. Mechanistically, the role of TrxR1 is also not clear yet and needs further research. Even tough dNTP supplies seems to be sufficient in TrxR1-deficient cells, TrxR1 might be needed for other anabolic pathways during fast proliferation. Future research is also needed to confirm that the differences in B and myeloid cells are indeed caused by defective proliferation similar to T cells and not by another mechanism, such as cell death.
**Materials & Methods**

**Mice**

Txnrd1\(^{fl/fl}\) mice (Jakupoglu 2005) were a kind gift of M. Conrad (Helmholtz Zentrum, Munich, Germany). Cd4-Cre and Ert2-Cre mice are described elsewhere (Lee 2001; Hameyer 2007). C57BL/6J and B6 Ptprc\(^{a}\) (CD45.1) animals were bought from The Jackson Laboratory (Bar Harbor, Maine, USA). For bone-marrow chimeras recipients were irradiated twice with 4.75 Gy with a 4 hour break in a RS 2000, Rad Source Technologies Inc., Alpharetta, USA. Mice were reconstituted with bone-marrow from hind legs of donor mice the next day and used for experiments after 8 or more weeks. For Tamoxifen induced deletion Txnrd1\(^{fl/fl}\); Ert2-Cre and control Txnrd1\(^{fl/fl}\) Cre-negative litter mates were injected with 2mg Tamoxifen i.p. on two consecutive days and mice used in experiments the earliest after one week. Tamoxifen (Sigma-Aldrich) was dissolved in 1 part ethanol (50mg/ml) and mixed by vortexing with 1 part Cremophor EL (BASF), followed by addition of 8 parts sterile PBS (Gibco). Mice were housed in individually ventilated cages under specific pathogen free conditions at ETH Phenomics Center (EPIC) (Zurich, Switzerland). All animal experiments were approved by the local animal ethics committee (Kantonales Veterinärsamt Zürich, licenses 167/2011, ZH270/2014, 25/2014, 113/2012, 127/2012), and performed according to local guidelines (TschV, Zurich) and the Swiss animal protection law (TschG).

**Infections and in vivo stimulation**

Lymphocytic choriomeningitis virus (LCMV) WE was originally from Rolf Zinkernagel (University of Zurich, Zurich, Switzerland) and propagated in L929 cells with virus aliquots stored at -80°C. Mice were infected with 200 or 500 pfu LCMV WE i.v. Blood diluted 1:5 in MEM medium (Life Technologies) + 2% FCS (Gibco) was stored at -80 °C until LCMV titers were determined in MC57 cells (Battegay 1991). For memory experiments mice were injected i.v. with 50’000 cfu *Listeria monocytogenes* expressing the LCMV gp\(_{33-41}\) epitope in PBS. 10-fold serial dilutions of organ homogenates on BHI plates were used for determination of titers. 10µg Qβ virus like-particles loaded with ssRNA (kind gift of Martin Bachmann, University of Zürich, Zurich, Switzerland) were injected i.p. Blood seum was collected in Microtainer tubes (BD Bioscience) and stored at -20 °C after separation.
Nunc Immuno MaxiSorb 96 well plates (Sigma-Aldrich) were coated with 50μl 1μg/ml Qβ-VLP over night at 4 °C, followed by washing and blocking with PBS + 2% FCS + 0.05 % Tween 20. Plates were incubated with 1:3 serial dilutions of 1:50 pre-diluted serum, followed by anti-IgM, IgG2b (Bioreba), IgG2c (Southern Biotech) alkaline phosphatase conjugated antibodies and detection. 35μg LPS (Ultrapure, *E. coli* 0111:B4; InvivoGen) was injected i.p. at day 0 and day 2 for analysis of emergency haematopoiesis at day 3. 100μg Staphylococcal enterotoxin B (Sigma-Aldrich) was injected i.p. per mouse and blood/organs analyzed at indicated days.

*Staining and flow cytometry*

Organs were digested with collagenase type IV (600 U/ml; Sigma-Aldrich) and DNase I (20 μg/ml; Boehringer) for analysis of lung and myeloid cells in spleen and lymph nodes. Erythrocytes were removed by incubating cells with ACK buffer (155mM NH₄Cl, 10mM KHCO₃, 0.1mM EDTA (Sigma-Aldrich) in H₂O, pH 7.4) for 5 min. For preparation of liver cells, bulk hepatocytes were removed by centrifugation at 20 g for 5 min, followed by underlaying supernatants with Lympholyte-M (Cedarlane) or 30 % Percoll (Sigma-Aldrich) in PBS for analysis of lymphocytes and myeloid cells, respectively. After 20 min centrifugation at 800 g interphase cells and pellet, respectively, were used. Bone-marrow cells were extracted from femur and tibur by cutting both ends of the bone and flushing out the marrow with a 25G needle and PBS + 2 % FCS. Cells were stained in 96 well V-bottom plates in PBS + 2 % FCS unless indicated otherwise. H-2D(b)-LCMV gp₃₃₋₄₁ tetramer (KAVYNFATM) and CD1d-PBS57 tetramer was kindly provided by the National Institute of Health, USA. Fixable viability dye eFluor® 780 (eBioscience) and LIVE/DEAD® fixable yellow dead cell stain (life technologies) were stained in pure PBS before antibody staining. For EdU labeling mice were injected with 0.5mg EdU (life technologies) 16 hours before sacrificing mice. Cells were labeled before staining with antibodies with the Click-iT Plus EdU Alexa Fluor® 488 Flow Cytometry Assay Kit (life technologies) according to manufacture’s instructions adapted to staining of 2-3 million cells in 96 well plates: Washing steps were done twice with 200μl volume, fixing in 50μl and staining by adding 150μl reaction cocktail to cell resuspended in 30μl permeabilization buffer. The following antibodies were used: Fc block (anti-CD16/CD32; clone 2.4G2, home-made); CD4-APC (GK1.5), IgM-bio (R6-60.2), TCRgd-bio (GL3), streptavidin-BV711, CD21/35-FITC (7G6), NK1.1-FITC
experiments cells were fixed with 4% Formaldehyde (all Aldrich) or 37°C for PI staining and Propidium iodide (Sigma-Aldrich) staining cells were fixed with ethanol after washing in PBS by resuspending cells in 20µl PBS and addition of 180µl ethanol, followed by freezing at -20°C over night. Fixed cells were resuspended in 38mM citrate buffer pH 7.5 (Fluka) supplemented with 40µg/ml RNase A (invitrogen) and 20µg/ml PI for 30min at 37°C for PI staining and then directly acquired. Cells were acquired on a FACSCanto II, LSRFortessa or FACSCalibur (BD Bioscience) and data was analyzed in FlowJo software (Tree Star).

Magnetic cell sorting and in vitro stimulation

Spleen or lymph node (inguinal, axillary, brachial) cells were sorted with CD4 (L3T4), CD8α (Ly-2) or CD90.2 microbeads (MACS, Miltenyi Biotec) according to manufacturer’s instructions. Sorted T cells in IMDM + GlutaMAX, 10 % FCS, 100 U/mL Penicillin, 100 µg/mL Streptomycin, 50 µM β-Mercaptoethanol (all Gibco) were then stimulated in plates coated with 50 µl of 4 µg/ml anti-CD3 (145-2C11; home-made) and 2 µg/ml anti-CD28 (37.51; home-made) in PBS. For time-course experiments cells were fixed with 4 % Formaldehyde in PBS (Sigma-Aldrich) or

CD3, CD4-PE (GK1.5), CD44-PE (IM7), CD62L-PE (MEL-14), Siglec-F-PE (E50-2440), TCRb-PE-Cy7 (H57-597) (BD Bioscience); CD127-APC (SB/199), CD62L-APC (MEL-14), Ly-6C-APC (HK1.4), TCRb-APC (H57-597), c-kit/CD117-APC-Cy7 (2B8), CD19-APC-Cy7 (6D5), CD4-APC-Cy7 (GK1.5), CD8-APC-Cy7 (53-6.7), Ly-6C-APC-Cy7 (HK1.4), Streptavidin-APC-Cy7, CD44-bio (IM7), F4/80-bio (BM8), CD127-BV421 (A7R34), F4/80-BV421 (BM8), I-A/I-E-BV510 (M5/114.15.2), CD69-FITC (H1.2F3), Ly-6C-FITC (HK1.4), Ly-6G-FITC (1A8), CD43-PE (S11), CD8-PE (53-7.3), Ki-67-PE (16A8), CD11c-PE-Cy7 (N418), CD4-PE-Cy7 (GK1.5), CD45.2-PE-Cy7 (104), CD8-PE-Cy7 (53-6.7), Streptavidin-PE-Cy7, B220-PerCP (RA3-6B2), CD11b-PerCP (M1/70), CD8-PerCP (53-6.7), streptavidin-PerCP, CD4-PerCP-Cy5.5 (RM4-5) (Biolegend); CD11c-APC (N418), CD19-APC (MB19-1), CD45.2-APC (104), CD8-APC (53-6.7), streptavidin-APC, CD103-bio (2E7), CD24/HSA-FITC (M1/69), CD4-FITC (GK1.5), CD45.2-FITC (104), CD62L-FITC (MEL-14), CD8-FITC (53-6.7), Vb8.1/8.2-FITC (KJ16-133), CD115-PE (AFS98), CD19-PE (eBio1D3), CD23-PE (B3B4), CD25-PE (PC61), CD45.2-PE (104), I-A/I-E-PE (M5/114.15.2), NK1.1-PE (PK136), CD44-PerCP-Cy5.5 (IM7), KLRG1-PerCP-eFluor710 (2F1) (eBioscience). To analyze cell death cells were stained with Annexin-V-APC (BD Bioscience) and 7-AAD (eBioscience) in Annexin-V binding buffer. For Ki-67 and Propidium iodide (PI, Sigma-Aldrich)
ethanol as described above in the case of PI or Ki-67 staining. For CFSE-labeling 10⁷/ml PBS-washed cells were incubated for 10 min at 37 °C with 5 μM CFSE (5- (and-6)-Carboxyfluorescein Diacetate, Succinimidyl Ester; life technologies) in PBS + 0.1% BSA (bovine serum albumin; Sigma-Aldrich), followed by quenching with FCS-containing medium or PBS. Analysis of proliferation kinetics of CFSE-labeled cells was done as described elsewhere (precursor cohort method) (Hawkins 2007). Inhibitors and compounds were added at the beginning of culture if not indicated otherwise. The following substances were used (from Sigma-Aldrich if no other company indicated): L-ascorbic acid, Catalase-polyethylene glycol, Ciclopirox olamine, 2'-Deoxyadenosine monohydrate, 2'-Deoxycytidine hydrochloride, 2'-Deoxyguanosine monohydrate, Deferoxamine, diphenylenciodonium chloride, DL-Dithiothreitol, Ferrostatin-1 (ChemBridge Corporation), N-Acetyl-L-Cysteine, Necrostatin-1 (inactive) (Merck), (±)-α-Tocopherol, Thymidine and Z-Val-Ala-Asp(OMe)-CH2F (zVAD-FMK; Peptitde Institute).

**Western blot**

PBS-washed cells were lysed in Lämmli buffer (4% SDS, 20% glycerol, 120mM Tris, pH 6.8) by boiling and sonication. Protein concentrations were measured with BSA protein assay kit (Thermo Scientific) in 1:10 diluted samples. 50μg total protein per lane were separated by SDS-PAGE and blotted on PVDF membranes (Amersham Hybond P 0.45 PVDF, Sigma-Aldrich). Membranes were probed with anti-pγ-H2AX (Ser139; JBV301; Milipore) and anti-β-Actin (A3854; Sigma-Aldrich) and chemiluminescence detected with a ChemiDoc MP (BioRad).

**Statistical analysis**

Statistical analysis was done with Prism 5 (GraphPad) using unpaired and paired two-tailed T test with confidence interval set to 95 % and 1-way ANOVA with planned comparison with Bonferroni post test with alpha set to 0.05. Abbreviations used in the graphs are: ns: P > 0.05; *: P ≤ 0.05; **: P ≤ 0.01; ***: P ≤ 0.001; ****: P ≤ 0.0001
Addendum

Fig. 13 Supplementary data TrxR1: Ova-induced allergic Asthma and Tregs in Txnrd1<sup>fl/fl</sup>; Cd4-Cre mice. (a, b) Allergic asthma was induced by sensitizing Txnrd1<sup>fl/fl</sup> and Txnrd1<sup>fl/fl</sup>; Cd4-Cre mice with 50µg OVA/Alum i.p. at day 0 and 6 followed by challenge with 20µg OVA challenge i.t. on day 10, 11 and 12. Bronchoalveolar lavage (BAL; (a)) and draining LNs (dLN; (b)) were analyzed on day 15 by flow cytometry. Cell numbers and frequencies are plotted for eosinophils (Eo; CD11b<sup>+</sup>CD11c<sup>-</sup>SiglecF<sup>+</sup>), neutrophils (Neutro; CD11b<sup>+</sup>CD11c<sup>-</sup>SiglecF<sup>-</sup>Gr-1<sup>+</sup>) and CD4 and CD8 T cells (CD4<sup>+</sup> or CD8<sup>+</sup> plus TCR<sup>β</sup><sup>-</sup>). (c) OVA-specific IgG1 levels were determined by ELISA of BAL and serum from the same animals as in (a, b). (d) Proliferation of regulatory T cells (Treg) was boosted by injecting mice with IL-2/anti-IL-2 (JES601A12) immuno-complex (IL-2ic). Expansion of Treg cells was analyzed after 5 days in spleen, LN and blood of IL-2ic or PBS only treated Txnrd1<sup>fl/fl</sup> and Txnrd1<sup>fl/fl</sup>; Cd4-Cre mice by staining FoxP3 in CD4<sup>+</sup> cells. Means±standard deviations are shown. Two-Tailed Student t-test was used to compare Cre-negative with Cd4-Cre groups in (a, b). 1-way ANOVA with Bonferroni’s multiple comparison test was used in (d).
In contrast to the drastic phenotype during LCMV infection with a near absence of T cell expansion in Txnrd1[^1]f/f, Cd4-Cre, there was a nearly normal response during a model of ovalbumin (OVA) induced allergic asthma with comparable eosinophilia and antibody response (Fig. 13a-c). This suggests a sufficient T helper response, despite a slight decrease of CD4 T cells in BAL. The difference to the striking phenotype in LCMV could be due to lower T cell proliferation in the OVA-Asthma model and as demonstrated by the memory experiment T cell function seems to be functional in TrxR1-deficient cells.

Preliminary data indicates a trend for decreased frequency of CD4^+ FoxP3^+ Treg cells already in naïve Txnrd1[^1]f/f; Cd4-Cre mice. Upon stimulation with IL-2/anti-IL-2 immuno-complex (IL-2ic) Treg cells were however able to expand, although to a lower level then Cre-negative cells (Fig. 13d). Tregs were shown to proliferate during homeostasis (Hori 2002), which could explain the difference in untreated animals, but this still needs further investigation.

Preliminary data for metabolism acquired using a Seahorse Bioscience XF24-3 to analyze oxygen consumption rate (OCR; a measure of mitochondrial respiration) and extracellular acidification rate (EAR; a measure for glycolysis) of ex vivo and in vitro stimulated (2 days anti-CD3/anti-CD28) T cells was mostly inconclusive due to large variations (data not shown). A trend for a decreased OCR in stimulated TrxR1-deficient T cells was however present in both experiment, but requires further study to confirm.
References


6 Concluding Remarks

In my PhD thesis, I looked at the role of antioxidant systems in immune cells. One of my projects shows the importance of transcriptional activity of Nrf2 for efficient Nlrp3 inflammasome activation in BMDCs and BMDMs, whereas other inflammasomes were less affected by the absence of Nrf2. Reconstitution of mutated Nrf2 in Nrf2-deficient cells demonstrated the need for transcriptional activity and not a transcription factor independent role of Nrf2 for inflammasome activation. This strongly suggests Nrf2-target gene(s) to be required for Nlrp3 activation. Many Nrf2 target genes are involved in the cellular antioxidant defense. Nevertheless, baseline ROS levels seem to be comparable in Nrf2-deficient cells compared to wt cells, and Nrf2 seems to be mainly required for the removal of oxidative stress upon an insult. This probably reflects the lack of major phenotypes in Nrf2-deficient mice in untreated/unstressed situations and suggests the presence of compensatory mechanisms. Similarly, treatment with antioxidant had no big effect, again suggesting no global problem with ROS in the absence of Nrf2. Understanding the role of Nrf2 in Nlrp3 inflammasome activation is complicated by the fact that the mechanisms leading to Nlrp3 activation are still not understood. While NOX-derived ROS as well as mitochondrial-derived ROS were implied in Nlrp3 inflammasome activation, the above findings argue against a change in ROS as the cause of the phenotype in Nrf2-deficient BMDCs. In addition, in the absence of Nrf2 more ROS would be expected, actually promoting inflammasome activation. Activation of inflammasomes can lead to pyroptosis in minutes, which is accompanied by mitochondrial damage. Consequently, ROS production could be a secondary effect of the cell death and not directly involved in inflammasome activation. Together, this data indicates that the defective inflammasome activation in the absence of Nrf2 is not due to changes in total redox status, but due to a more specific interaction. Similar to the small role of Nrf2 in steady-state, TrxR1 seems to be dispensable in resting cells or slow growing cells. In contrast, activated T cells fail to properly proliferate in the absence of TrxR1. Blocking the GSH system, the second cellular thiol-redox system, completely abolished the residual proliferation of TrxR1-deficient T cells in vitro. This demonstrates partial redundancy and compensation by this second redox system. However, fast proliferation seems to specifically require TrxR1. Similar to the defective inflammasome activation in the absence of Nrf2, antioxidants were not able
to compensate for the lack of TrxR1. This suggests that TrxR1 has specific functions beyond just keeping the total cellular redox balance in control. While the apparent function of supplying the RNR with reducing equivalents seems to be redundant, TrxR1 could still be needed for other metabolic functions rather than antioxidant functions. More research is, however, required to understand the unique non-redundant role of TrxR1. Further studies could also continue to look at the role of TrxR1 in other cell types than T cells. It would be interesting to see if other cell types also show a proliferation defect, and if there are differences in their dependency on TrxR1, possible caused by differential expression of compensatory pathways such as the GSH system. Overall, this work demonstrates the importance of antioxidant systems for immune cells and highlights the complexity of redox regulation in cells. Biological effects of oxidants and antioxidants seem to rarely change the total cellular redox status, but act more specifically.
7 Appendix

7.1 Author contributions

This Thesis was written by me with correction by Manfred Kopf. I performed the experiments with the exception of Fig. 4a-c and Fig. 5b,c, which were done by Esther Rosenwald and Jacqueline Weber, respectively, together with Jan Kisielow. Experiments in the Thioredoxin reductase-1 project (Fig. 6-Fig. 13) were partially done together with Jonathan Muri, at the time a student ("Semester work" and Master thesis), who I supervised.

7.2 Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tr>
<td>3'-UTR</td>
<td>3'-untranslated region</td>
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<tr>
<td>aa</td>
<td>ascorbic acid</td>
</tr>
<tr>
<td>AhR</td>
<td>aryl hydrocarbon receptor</td>
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<td>Aim2</td>
<td>Absent in melanoma 2</td>
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<td>APC</td>
<td>antigen-presenting cells</td>
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<td>APDC</td>
<td>(2R, 4R)-4-aminopyrrolidine-2, 4-dicarboxylate</td>
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<td>ARE</td>
<td>antioxidant response element</td>
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<td>Asc</td>
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<td>aToc</td>
<td>(+)-α-Tocopherol</td>
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<tr>
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<td>Nos</td>
<td>nitric oxide synthase</td>
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<td>pDC</td>
<td>plasmacytoid dendritic cell</td>
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<tr>
<td>PDGF</td>
<td>platelet-derived growth factor</td>
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<td>PDI</td>
<td>protein disulfide isomerase</td>
</tr>
<tr>
<td>pfu</td>
<td>plaque-forming unit</td>
</tr>
<tr>
<td>PGD</td>
<td>6-phosphogluconate dehydrogenase</td>
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<tr>
<td>PI</td>
<td>propidium iodide</td>
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<td>PPP</td>
<td>pentose phosphate pathway</td>
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<td>Prdx1</td>
<td>peroxiredoxin 1 (gene)</td>
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<td>PRR</td>
<td>pattern-recognition receptors</td>
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<tr>
<td>Prx</td>
<td>peroxiredoxin</td>
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<td>PTP1B</td>
<td>protein tyrosine phosphatase 1B</td>
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<tr>
<td>PYD</td>
<td>pyrin domain</td>
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<td>PYHIN</td>
<td>pyrin and HIN200 domain–containing</td>
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<td>p.i.</td>
<td>post infection</td>
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<td>RAG</td>
<td>recombination-activating genes</td>
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<td>retinoic acid-inducible gene I</td>
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<td>ribonucleotide reductase</td>
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<td>RNS</td>
<td>reactive nitrogen species</td>
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<tr>
<td>RORγt</td>
<td>RAR-related orphan receptor γt</td>
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<tr>
<td>ROS</td>
<td>reactive oxygen species</td>
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<td>Sulfasalazine</td>
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<td>Selenocysteine</td>
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<td>Sod</td>
<td>superoxide dismutases</td>
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<tr>
<td>SP</td>
<td>single-positive</td>
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<td>Sqstm1</td>
<td>p62 or sequestosome 1</td>
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<tr>
<td>Srx</td>
<td>sulfiredoxin</td>
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<tr>
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<td>Tamoxifen</td>
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<td>tBHQ</td>
<td>tert-Butylhydroquinone</td>
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<td>central memory T (cell)</td>
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<tr>
<td>TCR</td>
<td>T cell receptor</td>
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<td>Teff</td>
<td>effector T (cells)</td>
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<td>Toll-like receptor</td>
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<td>Treg</td>
<td>regulatory T (cell)</td>
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<td>TRIF</td>
<td>TIR-domain-containing adaptor inducing interferon-β</td>
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<td>T&lt;sub&gt;RM&lt;/sub&gt;</td>
<td>tissue-resident memory T (cell)</td>
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<tr>
<td>Trx</td>
<td>thioredoxin</td>
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<td>TrxR</td>
<td>thioredoxin reductases</td>
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<td>thioredoxin-interacting protein</td>
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<td>Txnrd1</td>
<td>thioredoxin reductase 1 (gene)</td>
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<td>VLP</td>
<td>virus-like particle</td>
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<td>wild-type</td>
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<tr>
<td>zVAD</td>
<td>N-Benzylxocarbonyl-Val-Ala-Asp (O-Me) fluoromethyl ketone</td>
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<tr>
<td>β-TrCP</td>
<td>β-transducin repeat-containing protei</td>
</tr>
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</table>
7.3 Acknowledgment

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