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The Crosstalk between Nrf2 and Inflammasomes

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Zusammenfassung

Nrf2 ist ein Transkriptionsfaktor, der die Expression von vielen cytoprotektiven Genen reguliert. Nrf2 ist imstande, auf eine Reihe von exogenen und endogenen Arten von Stress zu reagieren, indem es die Expression von Zielgenen induziert, deren Genprodukte den schädlichen Auswirkungen von zellulärem Stress entgegenwirken. Somit übt Nrf2 einen Schutzmechanismus aus, der das Überleben der Zelle unter Stressbedingungen sicherstellen kann.

Nrf2 ist besonders in der Chemoprevention von Krebs und Arzneimittel-verursachter Toxizität von Bedeutung. Nrf2 wurde ein schützender Effekt in verschiedenen Krankheitsmodellen zugeschrieben und man geht davon aus, dass die Aktivierung des Transkriptionsfaktors mittels Nrf2-aktivierender Substanzen einen therapeutischen Effekt ausübt. Nrf2 hat jedoch auch eine "dunkle Seite". Diese hat sich in Mutationen von Genen offenbart, die für Proteine im KEAP1-NRF2 Signalweg kodieren und so zu einer Überaktivierung von Nrf2 in unterschiedlichen Arten von Krebs führen. Interessanterweise wurde beschrieben, dass Nrf2 für die Aktivierung von Inflammasomen nötig ist, jedoch ist diese Rolle von Nrf2 bei Entzündungen umstritten.

Inflammasome sind Komplexe des angeborenen Immunsystems, die sich nach Detektion einer Reihe unterschiedlicher exogenener und endogenener Stressfaktoren – sogenannter PAMPs und DAMPs – zusammenfügen. Dies führt zur Aktivierung der Protease Caspase-1, die wiederum die entzündungsauslösenden Zytokine pro-IL-1β und pro-IL-18 in deren reife Form überführt und für deren Ausschleusung aus der Zelle sorgt. Somit resultiert Inflammasomaktivierung in einer Entzündung im Organismus. Darüber hinaus bewirkt Inflammasomaktivierung Pyroptose, eine lytische Form von Zelltod, die die Entzündungsreaktion fördert.

Es wurde berichtet, dass oxidativer Stress und reaktive Sauerstoffspezies (ROS), die PAMPs und DAMPs im Signaltransduktionsweg nachgeschaltet sind, eine Rolle in der Aktivierung vom NLRP3-Inflammasom spielen. Daher ist es überraschend, dass der Transkriptionsfaktor Nrf2, der für ROS-detoxifizierende Enzyme kodiert, für die Inflammasomaktivierung benötigt wird. Der zugrunde liegende Mechanismus war bisher unbekannt. Ausserdem wurde kürzlich berichtet, dass bestimmte Nrf2-aktivierende Substanzen, die die Expression von Nrf2-Zielgenen induzieren, die Inflammasomaktivierung blockieren.

Im Zuge meiner Doktorarbeit habe ich das Zusammenspiel von Nrf2 einerseits und von Nrf2-Aktivatoren andererseits mit dem Inflammasom studiert. Wir untersuchten die Rolle von Nrf2 bei der Inflammasomaktivierung und konnten mithilfe von Zellen der Maus und des Menschen bestätigen, dass Nrf2 für die Inflammasomaktivierung benötigt wird. Da Nrf2 ein Transkriptionsfaktor ist, wurde spekuliert, dass die Expression von Nrf2-Zielgenen für die Inflammasomaktivierung nötig ist. Unsere Resultate zeigen dagegen, dass Nrf2-Zielgene keine

Rolle in der NLRP3-Inflammasomaktivierung spielen. Weder in peritonealen Makrophagen von Mäusen, die eine konstitutiv aktive (ca) Version von Nrf2 exprimieren, noch in menschlichen Keratinozyten mit überexprimiertem caNrf2 war die Inflammasomaktivierung verstärkt, obwohl Nrf2-Zielgene verstärkt exprimiert wurden. Die Analyse der Sekretion von reifem IL-1β nach Inflammasomaktivierung in Keratinozyten mit überexprimiertem Nrf2, einer Version von Nrf2 ohne Zellkernlokalizierungssequenz (NLS) oder dominant negativem (dn) Nrf2 hat gezeigt, dass die Aktivierung des NLRP3-Inflammasomes nicht mit der Expression von Nrf2-Zielgenen korreliert ist, sondern eher mit der Verfügbarkeit von Nrf2 im Zytoplasma. Dies führte zu der Hypothese, dass eine direkte oder indirekte physische Interaktion zwischen dem Nrf2/Keap1-Komplex und dem Inflammasom dem Bedarf von Nrf2 für die Inflammasomaktivierung zugrunde liegt. Tatsächlich konnten wir eine Interaktion von überexprimierter Caspase-1 und allen Komponenten des Nrf2/Keap1/Cul3/Rbx1-Komplexes nachweisen.

Wir haben auch die Rolle von Nrf2-aktivierenden Substanzen bei der Aktivierung vom Inflammasom untersucht. Verschiedene dieser Substanzen blockierten das Inflammasom in Keratinozyten, THP-1-Zellen und menschlichen peripheren mononuclearen Blutzellen. In der Maus, also im lebenden Organismus, konnten wir zeigen, dass eine orale Zufuhr der Nrf2-aktivierenden Substanzen Sulforaphan (SFN) und Dimethylfumarat (DMF) die Inflammasom-abhängige Entzündung unterbinden kann. Das ist besonders interessant, da DMF als Medikament für die Behandlung von Psoriasis und Multipler Sklerose (MS) verwendet wird. Bei beiden Krankheiten wird ein Mitwirken des Inflammasoms im Krankheitsverlauf diskutiert. Der molekulare therapeutische Mechanismus von DMF in Patienten, die an Psoriasis oder MS leiden, ist weitgehend unbekannt. Unsere Resultate deuten darauf hin, dass die anti-inflammatorische Wirkung von DMF – zumindest teilweise – auf eine Inhibierung des Inflammasoms zurückzuführen ist.

Zusätzlich haben wir untersucht, ob der Effekt von Nrf2-Aktivatoren und von Nrf2 auf das Inflammasom von den Zielgenen des Transkriptionsfaktors abhängig ist. Die Unterbindung der Proteinsynthese mittels Cycloheximid hat die SFN-vermittelte Hemmung des Inflammasoms in Keratinozyten und THP-1-Zellen nicht verhindert. Ausserdem hat SFN die Inflammasomaktivierung in dendritischen Mauszellen komplett gestoppt - sowohl normalen als auch Zellen ohne Nrf2. Diese Resultate zeigen, dass SFN das Inflammasom unabhängig von Nrf2 inhibiert.

Wir haben auch die Konsequenzen der NLRP3-Inflammasomaktivierung für die Expression und Aktivität von Nrf2 untersucht. Hier hat sich gezeigt, dass Nrf2-Protein rasch mittels eines zum Teil von Keap1-unabhängigen Mechanismus abgebaut wird. Diese Daten deuten darauf hin, dass der das Überleben von Zellen unterstützende Nrf2-Signalweg und der Zelltod-induzierende

Inflammasom-Signalweg in ein und derselben Zelle nicht zum selben Zeitpunkt aktiv sein können.

Zusammenfassend lässt sich sagen, dass die Resultate dieser Arbeit eine wichtige Rolle von Nrf2 und insbesondere von Nrf2-aktivierenden Substanden bei der Aktivierung des NLRP3-Inflammasoms belegen und eine neue Funktion von Nrf2 im Prozess der Entzündung zeigen, die unabhängig von der Expression von Nrf2-Zielgenen ist.

Summary

Nrf2 is a transcription factor that regulates the expression of many cytoprotective genes. In response to different exogenous and endogenous kinds of stress, Nrf2 upregulates its target genes, whose gene products help to combat harmful cellular stressors. Thereby Nrf2 represents a protective pathway, which allows cell survival under stress conditions.

Nrf2 is particularly important for chemoprevention in cancer development and protective in drug-induced toxicity. In many disease models protective effects have been attributed to Nrf2 and Nrf2 induction by Nrf2 activating compounds was shown to be beneficial. However, Nrf2 has also a "dark side". Many mutations in the genes encoding proteins of the KEAP1-NRF2 pathway causing Nrf2 hyperactivity in different cancer types have been found. Interestingly, it was reported that expression of Nrf2 is required for inflammasome activation; however, this role of Nrf2 in inflammation is controversially discussed.

Inflammasomes are innate immune complexes, which assemble upon sensing of a wide range of different exogenous or endogenous stimuli, so called pathogen-associated molecular patterns (PAMPs) and danger-associated molecular patterns (DAMPs). This leads to activation of the protease caspase-1, which in turn processes the pro-inflammatory cytokines pro-IL-1β and pro-IL-18 and regulates their secretion. Thus, inflammasome activation results in inflammation *in vivo*. Furthermore, inflammasome activation induces pyroptosis, a lytic form of cell death, which supports inflammation.

Oxidative stress and reactive oxygen species (ROS) downstream of PAMPs and DAMPs have been implicated in NLRP3 inflammasome activation. Therefore, it is surprising that the transcription factor Nrf2, which induces the expression of ROS-detoxifying enzymes, has a role in inflammasome activation. So far, the underlying molecular mechanism was unknown. In addition, it was recently reported that certain Nrf2 activating compounds, which induce the expression of Nrf2 target genes, block inflammasome activation.

During my thesis, I studied the crosstalk between Nrf2 and Nrf2 activating compounds with inflammasomes. We investigated the role of Nrf2 in inflammasome activation and by using human and murine cells, we could confirm a requirement of Nrf2 for inflammasome activation. Since Nrf2 is a transcription factor, it has been speculated that Nrf2 target gene expression is required for inflammasome activation. In contrast, our results show that Nrf2 target genes are not involved in NLRP3 inflammasome activation. Neither in peritoneal macrophages derived from mice expressing constitutively active (ca) Nrf2, nor in human primary keratinocytes (HPKs) overexpressing caNrf2, inflammasome activation was increased, although target gene expression was induced. Analysis of the secretion of mature IL-1β upon inflammasome

activation in HPKs overexpressing wild-type Nrf2, a nuclear localization sequence (NLS)-deficient mutant of Nrf2, or dominant negative (dn) Nrf2, demonstrated that NLRP3 inflammasome activation is not correlated with the expression of Nrf2 target genes, but rather with the abundance of Nrf2 in the cytoplasm. Therefore, we hypothesized that a direct or indirect physical interaction between the Nrf2/Keap1 complex and inflammasomes might underlie the requirement of Nrf2 for inflammasome activation. Indeed, we detected an interaction between overexpressed caspase-1 and all components of the Nrf2/Keap1/Cul3/Rbx1 complex.

We also analysed the effect of Nrf2 activating compounds on inflammasome activation. Several of these compounds blocked the inflammasome in HPKs, THP-1 cells and in human peripheral blood mononuclear cells (PBMCs). *In vivo* in mice, we could show that oral application of the Nrf2 activating compounds sulforaphane (SFN) and dimethyl fumarate (DMF) was also able to dampen inflammasome-dependent inflammation. This is particularly interesting, since DMF is used as a drug for the treatment of patients suffering from psoriasis and multiple sclerosis (MS). In both diseases an involvement of inflammasomes is discussed. The mechanisms of action of DMF in psoriasis or MS patients are only poorly characterised. Our results suggest that the anti-inflammatory activity of DMF – at least in part – is attributed to inflammasome inhibition.

In addition, we examined whether the effect of Nrf2 activators and of Nrf2 on the NLRP3 inflammasome is dependent on Nrf2 target gene expression. Blockade of protein synthesis by cycloheximide did not prevent SFN-mediated inflammasome inhibition in HPKs and THP-1 cells. Furthermore, SFN completely abolished inflammasome activation in bone marrow-derived dendritic cells (BMDCs) from both, wild-type and Nrf2 KO mice. These results demonstrate that SFN inhibits the inflammasome independently of Nrf2.

Finally, we studied the consequence of NLRP3 inflammasome activation on Nrf2 expression and activity. We found Nrf2 to be rapidly degraded by a mechanism that is in part independent of Keap1. These data suggest that the pro-survival Nrf2 pathway and cell death-inducing inflammasome activation, cannot be active in one cell at the same time.

Taken together, the findings described in this thesis prove an important role of Nrf2 and particularly of Nrf2 activating compounds in NLRP3 inflammasome activation and point to a novel function of Nrf2 in inflammation, independently of Nrf2-induced gene expression.

Chapter 1 – Introduction

1.1 Inflammation and the innate immune system

Inflammation is an acute response to infection and tissue damage to limit harm to the body. It is initiated upon the sensing of signs of acute danger or disturbances of the steady state [1]. The immune system evolved to sustain this homeostatic state, which is achieved by a coordinated response to environmental threats or internal danger signals that involve complex crosstalk among distinct immune cell types [2].

The immune system of vertebrates consists of two complementary arms: the innate and the adaptive immune system. The evolutionary more ancient innate immune system is the first to be activated by pathogens or danger signals and is usually sufficient to clear the infection. However, when the innate immune system is overwhelmed, it triggers and directs the adaptive arm, thus activating specific B and T cells for pathogen clearance [3].

Once activated, the innate immune system initiates the inflammatory response by secreting pro-inflammatory cytokines and chemokines, inducing the expression of adhesion and co-stimulatory molecules in order to recruit immune cells to the site of infection, and to trigger the adaptive immune response [3].

The innate immune system relies on its capability to detect invading microbes, tissue damage, or stress via germline-encoded pattern-recognition receptors (PRRs). These receptors recognize a limited number of well-conserved microbial structures termed pathogen-associated molecular patterns (PAMPs) and host-derived danger signals (danger-associated molecular patterns, DAMPs) [4]. PRRs are expressed by many cell types, including macrophages, monocytes, dendritic cells, neutrophils and epithelial cells [3].

There are five major families of PRRs with different localization in the cell. The transmembrane toll-like receptors (TLRs) and C-type lectin receptors (CLRs) and the intracellular NOD-like receptors (NLRs), RIG-I-like receptors (RLRs) and absent in melanoma 2 (AIM2)-like receptors (ALRs). While most receptors evolved to modulate the transcriptional response of cells after activation, members of the NLR and ALR protein family can form multi-molecular protein complexes termed inflammasomes.

1.2 Inflammasomes, caspase-1 and interleukin-1

Inflammasomes are innate immune complexes, which assemble after sensing of PAMPs and DAMPs, leading to proteolytic maturation and release of the pro-inflammatory cytokines interleukin-1β (IL-1β) and IL-18. These multi-molecular protein complexes consist of a cytosolic (or nuclear in the case of IFI16) pattern-recognition receptor, the adaptor molecule apoptosis-associated speck-like protein containing a caspase activation and recruitment domain (ASC) and the effector enzyme caspase-1. The receptor is either a member of the NLR or ALR protein

family; the latter being also called pyrin and HIN domain-containing (PYHIN) protein family. Structurally, the NLRs contain a carboxy-terminal leucine-rich repeat (LRR) domain, a conserved central NACHT domain, which is essential for nucleotide binding and protein oligomerization, and a variable amino-terminal domain that defines several NLR subfamilies (Figure 1). Members of the NLRP subfamily carry an amino-terminal pyrin domain (PYD).

Following activation and oligomerization, NLRPs recruit ASC, which is composed of a PYD and an C-terminal caspase activation and recruitment domain (CARD). ASC acts as an adaptor between the PYD of the respective NLRP and the CARD of pro-caspase-1. However, NLRC4 is able to interact directly with pro-caspase-1 through its CARDs.

The PYHIN-containing inflammasomes comprise AIM2 and interferon-inducible protein 16 (IFI16). AIM2 and IFI16 both contain a conserved C-terminal DNA-binding hematopoietic IFN-inducible nuclear protein with 200-amino acids domain (HIN-200) and an N-terminal PYD domain, which mediates interaction with ASC and, subsequently, pro-caspase-1 [5, 6].

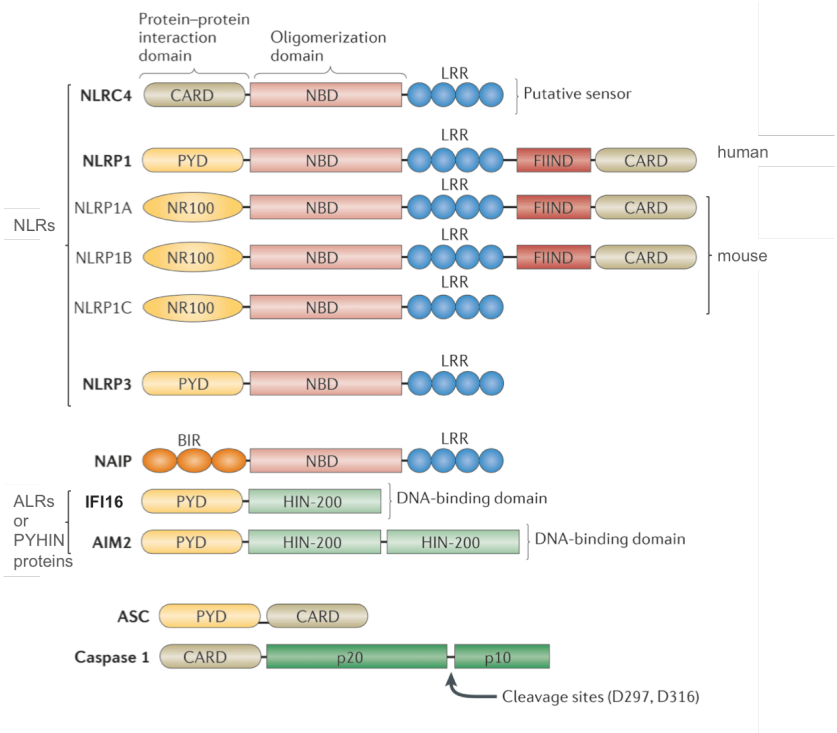

Figure 1: (modified from [6]): Inflammasome components and domain structure. The activation and formation of inflammasome complexes is mediated through several protein domains. In NOD-like receptors (NLRs), the sensory component is formed by the leucine-rich repeat (LRR). Oligomerization of NLRs is mediated by the nucleotide-binding domain (NBD). The pyrin domain (PYD) mediates protein-protein interactions between the inflammasome sensor and ASC, which also contains a PYD. The CARD of ASC mediates protein-protein interactions with the CARD of pro-caspase-1. NLRP1 contains a unique function-to-find domain (FIIND). In the murine proteins NLRP1a, NLRP1b and NLRP1c, the amino-terminal PYD is replaced by an NR100 domain (amino-terminal domain of rodent NLRP1 of about 100 amino acids), which has no known homologue in humans. NLR-family apoptosis-inhibiting proteins (NAIPs) are a subfamily of the NLRs and contain a bacculovirus inhibitor of apoptosis repeat (BIR) domain. In AIM2 and IFI16, the HIN-200 DNA-binding domain is the sensory component; the PYD mediates interaction with ASC [6].

Caspases are cysteine proteases that initiate or execute cellular programs, leading to inflammation or cell death. They are produced in cells as catalytically inactive zymogens and usually undergo proteolytic processing during activation. Caspases are categorized as either

pro-apoptotic or pro-inflammatory. The pro-inflammatory caspases are comprised of caspase-1, -11 and -12 in mouse and caspase-1, -4, -5 and -12 in humans [7]. Inflammasomes function to convert inactive pro-caspase-1 to active caspase-1, which then in turn mediates proteolytic processing of the inactive precursor cytokines pro-IL-1β and pro-IL-18, generating the biologically active cytokines IL-1β and IL-18, respectively. Active caspase-1 is also known to induce pyroptosis, a lytic form of cell death, which supports inflammation [8].

The cytokine IL-1 is an important pro-inflammatory mediator, which is produced at sites of injury or immunological challenge. It coordinates mechanisms such as cellular recruitment to a site of infection or injury, and the regulation of sleep, appetite, and body temperature [9]. IL-1 exerts its biological functions through binding to the IL-1 receptor type I (IL-1RI), which is expressed by almost all cell types. Agonistic ligands of IL-1RI are IL-1α and IL-1β, which are encoded by separate genes. Both cytokines are expressed as pro-proteins; however, only pro-IL-1β requires proteolytic processing for activation. IL-1β is translated as an inactive 31-kDa pro-IL-1β precursor and maturation requires cleavage by caspase-1 before its active 17-kDa form is able to bind and activate IL-1RI. Receptor binding activates NF-κB and MAP kinase signalling pathways. IL-1 activity is regulated by the IL-1R antagonist, which binds IL-1RI, but lacks signalling activity [10]. Since IL-1β is such a critical and potent cytokine, its activity is rigorously controlled by expression, maturation, and secretion. Pro-inflammatory stimuli induce expression of inactive pro-IL-1β, but maturation and release are controlled by inflammasomes. There are several types of inflammasomes that become activated upon encounter with distinct DAMPs and/or PAMPs (Figure 2).

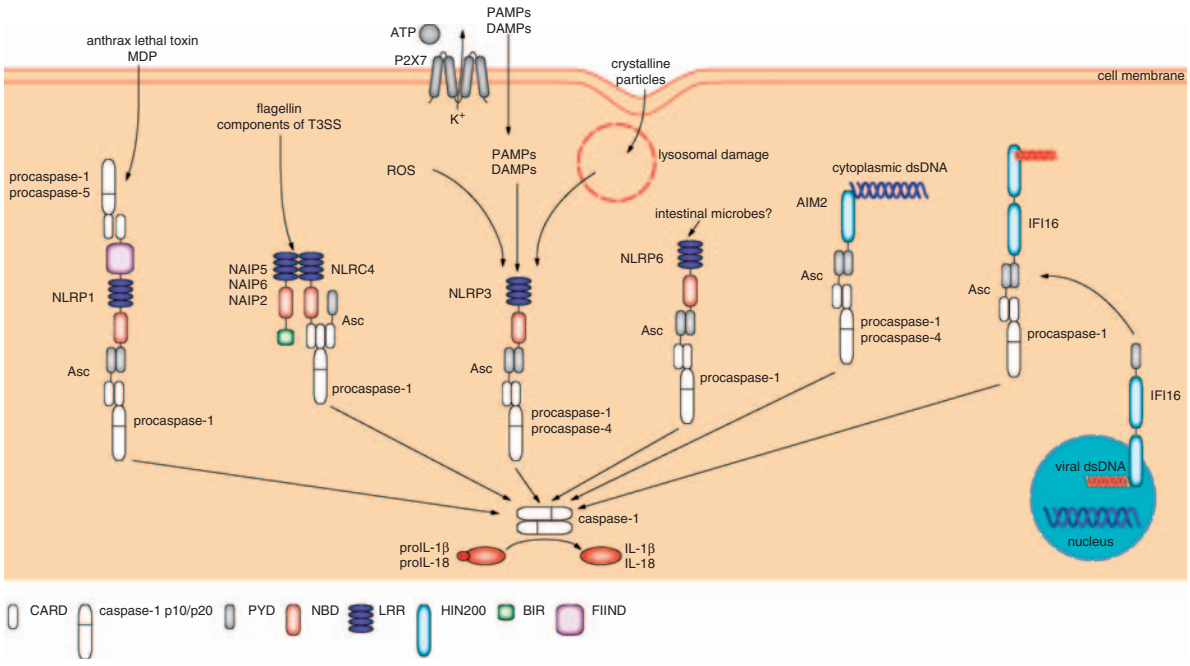

Figure 2 [11]: The different types of inflammasomes. The NLRs (NLRP1, NLRC4, NLRP3 and NLRP6) and the ALRs (AIM2 and IFI16) build up inflammasomes upon sensing of different PAMPs and/or DAMPs (see text below for

details). Upon activation, all inflammasomes recruit ASC and caspases-1, which induces activation of the protease. This leads to activation of pro-IL-1β and pro-IL-18, and subsequent secretion of the active cytokines.

NLRP1 inflammasome

NLRP1 was the first member of the NLR family to be identified to form an inflammasome complex [12]. In humans there is a single gene encoding NLRP1, whereas mice have a cluster of three homologous genes (*Nlrp1a*, *b* and *c*) (Figure 1). Human NLRP1 was shown to directly bind and be activated by muramyl dipeptide (MDP), a peptidoglycan fragment from both gram-positive and gram-negative bacteria. Interestingly, NLRP1 ligands are species-specific, since murine NLRP1b is not activated by MDP, but by lethal toxin from *Bacillus anthracis* [13, 14] (Figure 2). It was shown that MDP-mediated NLRP1 inflammasome activation is enhanced, but not strictly dependent on the adaptor protein ASC. Apart from caspase-1, caspase-5 has also been implicated in binding NLRP1 [12, 15]. NLRP1 is highly expressed in the skin and is required for UVB-induced caspase-1 activation in human primary keratinocytes (HPKs) [16] (see below).

NLRP3 inflammasome

The NLRP3 inflammasome is the most extensively studied type of inflammasome, since it is activated by many different PAMPs and DAMPs (Figure 2) and plays a prominent role in a variety of inflammatory diseases (see below). In most cell types, NLRP3 is only expressed at low levels and requires priming, also referred to as 'signal one', which is an NF-κB-activating stimulus. This signal is usually provided by Toll-like receptor agonists, such as LPS, and by inflammatory cytokines, such as tumour necrosis factor (TNF)-α, and leads to increased expression of NLRP3 and pro-IL-1β.

Subsequently, NLRP3 inflammasome activation requires a second, distinct stimulus ('signal two'), which consists of a wide range of infection- and stress-associated signals [17]. NLRP3 activators include PAMPs and toxins from bacterial, viral, fungal and protozoan origin [15], as well as host-derived DAMPs such as ATP, hyaluronan, amyloid-β fibrils, and uric acid crystals [18-21]. Additionally, the NLRP3 inflammasome drives inflammation in response to a number of environmental irritants, including silica, asbestos [22-24] and UVB irradiation [16].

Due to the diverse nature of the known NLRP3 activators it is unlikely that all of these different stimuli are sensed by NLRP3 directly. Instead, it is generally believed that a common cellular downstream event triggered by these stimuli activates the NLRP3 inflammasome. Several models for NLRP3 inflammasome activation have been proposed so far. The three most prominent are the ion flux model, the lysosome rupture model, and the reactive oxygen species (ROS) model [15, 25]. According to the ion flux model NLRP3 activation is dependent on changes in cytosolic levels of K^+, Ca^{2+}, and H^+. It was shown for several NLRP3 activators to induce potent

ion fluxes [15]. According to the lysosome rupture model inefficient clearance and phagocytosis of crystalline or large particles lead to phagosomal destabilisation and lysosome rupture. The ensuing release of cathepsins into the cytosol triggers inflammasome activation through an uncharacterised pathway [25]. In a third model ROS is a crucial factor. Oxidative stress in the form of enhanced ROS levels has been widely implicated in NLRP3 activation and many NLRP3-activating stimuli trigger the generation of ROS [25]. It was suggested that NLRP3 senses increased levels of ROS by a complex of thioredoxin and thioredoxin-interacting protein (TXNIP) [26] or by oxidized mitochondrial DNA that is released from dysfunctional mitochondria [27, 28]. However, the precise role of ROS in NLRP3 inflammasome activation is still debated.

Apart from mitochondrial DNA and the generation of mitochondrial ROS [29], the translocation of cardiolipin from the inner to the outer mitochondrial membrane has been suggested to induce NLRP3 inflammasome activation [30]. Finally, a more recent model includes relocalization of NLRP3 to mitochondria [31]. Although mitochondria are crucially involved in NLRP3 inflammasome activation, their precise function in this process has yet to be fully clarified.

The realization that the caspase-1-deficient mice used by most researchers lack in addition also caspase-11 expression led to the discovery of an alternative or non-canonical NLRP3 inflammasome pathway [32]. Non-canonical inflammasome activation involves the activation of caspase-11 by cytosolic LPS originating from gram-negative bacteria by a mechanism that is independent of the traditional LPS receptor TLR4 [33, 34]. However, unlike caspase-1, caspase-11 does not directly cleave pro-IL-1β and pro-IL-18 [32].

NLRC4 inflammasome

In contrast to the diverse stimuli that activate NLRP3 inflammasomes, the NLR family CARD domain-containing protein 4 (NLRC4) inflammasome (also known as IPAF) responds to a more limited set of stimuli. NLRC4 is activated in response to flagellin or structural components of the type III secretion system [35-38] expressed by different bacterial pathogens, including *Legionella pneumophila*, *Pseudomonas aeruginosa*, *Salmonella typhimurium*, and *Shigella flexneri* [39-42].

These bacterial proteins are directly bound by NLR-family apoptosis-inhibiting proteins (NAIPs), which interact with NLRC4 to trigger inflammasome assembly (Figure 2). In mice, Naip1 and Naip2 recognize bacterial needle and inner rod proteins of the type III secretion system, respectively, whereas both Naip5 and Naip6 bind cytosolic flagellin. In contrast, in humans only one NAIP has been characterised and was shown to bind the type III secretion system needle protein [43, 44].

NLRC4 contains a CARD that can directly interact with the CARD of caspase-1 in the absence of ASC. This interaction may explain why NLRC4 is able to induce pyroptosis independently of ASC. Nevertheless, ASC is required for NLRC4-mediated IL-1β and IL-18 release [35, 45].

NLRP6 inflammasome

NLRP6 is highly expressed in epithelial cells and in goblet cells in the intestine and was shown to have a crucial role in maintaining intestinal health and homeostasis [46-49]. Deficiency in NLRP6 in mice results in alterations in the composition of colonic microbiota, leading to an expansion of colitogenic bacteria. Because these alterations in the microbiota were also found in IL-18-deficient mice, it was suggested that NLRP6 activation might trigger IL-18 release [47]. However, a more recent study demonstrated that NLRP6 contributes to mucus secretion by goblet cells through the regulation of autophagy in an IL-1β- and IL-18-independent manner [49]. The mechanisms by which these events lead to an altered microbiota in NLRP6-deficient mice are still not clear. Also the signals that lead to NLRP6 activation await identification.

AIM2 and IFI16 inflammasomes

AIM2 and IFI16 belong to the family of AIM2-like receptors (ALRs) or PYHIN proteins. They directly bind to their ligand, double-stranded DNA (dsDNA), via the HIN-200 domain, whereas the PYD domain recruits ASC for caspase-1 activation [50] (Figure 2). Structural analysis demonstrated that binding of DNA to the positively charged HIN domain of AIM2 results in a conformational change, AIM2 oligomerization and subsequent inflammasome activation. Interestingly, AIM2 does not appear to recognize a specific sequence or structure of dsDNA, but a minimum sequence length of 80 base pairs is required for effective AIM2 activation [51].

AIM2 typically senses cytosolic dsDNA [52-55] from DNA viruses such as mouse cytomegalovirus (CMV) and vaccinia virus [56], as well as from bacteria such as *Francisella tularensis* [56-58], *Listeria monocytogenes* [59-63], *Streptococcus pneumonia* [64], and *Mycobacterium tuberculosis* [65]. By contrast, IFI16, which is located in the nucleus, forms an inflammasome in response to Kaposi sarcoma-associated herpes virus infection [66].

1.3 Keratinocytes as sensors of danger

The skin is our largest organ and provides the first line defence of the human body against injury and infection. The ability of the skin to carry out multiple roles is closely related to its structure, which is composed of an outer epidermis overlying an inner dermis, separated by a basement membrane [67] (Figure 3). The epidermis is a stratified epithelium, which is mainly composed of keratinocytes. These cells differentiate and migrate in an ordered manner starting from the basal layer upwards to form a layered structure. Other cell types of the human epidermis include

melanocytes, which produce the pigment melanin, specialized dendritic cells, the Langerhans cells, and tissue-resident $CD8^+$ T cells [68].

Human keratinocytes can be regarded as immune sentinels, since they can recognize PAMPs and DAMPs through TLRs and the inflammasome machinery. Keratinocytes express TLRs on the cell surface (TLR1, TLR2, TLR4, TLR5 and TLR6) and at endosomes (TLR3 and TLR9). TLR engagement leads to activation of host cell signalling pathways and subsequent innate and adaptive immune responses with antimicrobial peptide, cytokine and chemokine production. Furthermore, human keratinocytes express inflammasome proteins and can therefore secrete active IL-1β [68]. In contrast to myeloid cells, human keratinocytes constitutively express inflammasome proteins as well as pro-IL-1β. Irradiation with a physiological dose of UVB activates caspase-1 and induces the secretion of mature IL-1β, which was described to involve both NLRP1 and NLRP3 inflammasomes [16]. In addition, human primary keratinocytes also feature a functional AIM2 inflammasome, which is activated upon transfection of cells with dsDNA [69, 70]. Furthermore, interferon (IFN)-γ-primed keratinocytes are able to sense infection by herpes simplex virus (HSV) and modified vaccinia virus Ankara (MVA) via different inflammasomes [71].

Inflammatory factors produced and secreted by keratinocytes include – among others - the cytokines IL-1α and –β, IL-6, IL-10, IL-18, TNF-α, and the chemokines CC-chemokine ligand 20 (CCL20) and a diverse set of CXC-chemokine ligands (CXCLs), which attract immune cells into the skin [68]. Thereby, keratinocytes are able to modulate innate and adaptive immune responses.

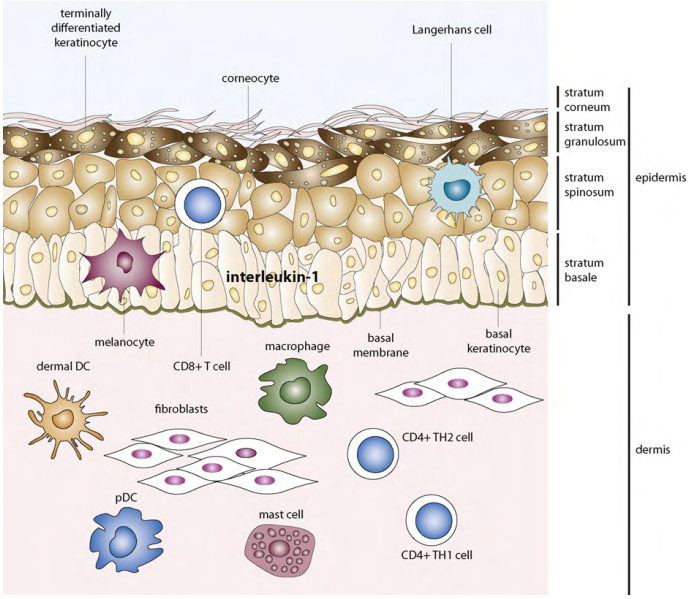

Figure 3 [72]: The cellular composition of human skin. Through its architecture and cellular composition the skin provides protection from injury and infection. Besides professional immune cells such as macrophages, neutrophils, dendritic cells and lymphocytes, keratinocytes have been demonstrated to play an important regulatory role in cutaneous inflammatory and immune responses by producing various types of cytokines. Keratinocytes are a major source of IL-1. When activated and secreted, the cytokine induces an inflammatory response through activation and recruitment of immune cells [72].

1.4 Inflammasome-mediated disorders

As described above, inflammation is a physiological reaction to infection and injury. In this context inflammation is thought to be beneficial because inflammatory reactions are required for successful elimination of pathogens and induction of tissue repair processes. A controlled inflammatory response subsides after clearance of the initial insult. However, failure to eliminate the danger signal may result in prolonged inflammation or persistent damage to local tissue that can promote the development of chronic inflammatory diseases [73]. Chronic inflammation is a sign of several pathologies.

The following part will give an overview about some major human diseases associated with abnormalities in inflammasomes and so-called sterile inflammation. These include auto-inflammatory diseases, which are caused by genetic mutations in inflammasome-related genes, chronic inflammatory metabolic diseases and neurological disorders, and inflammasome-associated autoimmune diseases.

Auto-inflammatory Syndromes

Auto-inflammatory diseases result primarily from aberrant innate immune signalling and are mediated mainly by cytokines of the innate immune system, most notably IL-1β, in the absence of antigen-specific humoral responses. In that regard they differ from autoimmune disorders, in which adaptive immune components such as autoreactive T cells or immunoglobulins to self-antigens play a role [3, 15].

Hereditary fever disorders

Genetic studies in the human population revealed that mutations in inflammasome-related genes are associated with the development of auto-inflammatory conditions, namely hereditary periodic fevers (HPFs), which include a number of rare inherited disorders associated with systemic and tissue inflammation.

CAPS

NLRP3 was previously named cryopyrin, since it was found to be associated with cold-induced fevers in patients suffering from cryopyrin-associated periodic syndromes (CAPS) [74]. These conditions are caused by mutations in the *NLRP3* gene and include familial cold auto-inflammatory syndrome (FCAS), Muckle-Wells syndrome (MWS) and neonatal-onset multisystem inflammatory disease (NOMID). FCAS is the mildest form of the disease and presents with cold-induced fevers and skin rashes. MWS patients may, in addition, develop amyloidosis together with deafness and arthritis. NOMID patients have the most severe symptoms that may lead to very serious neurological impairment caused by chronic

polymorphonuclear meningitis [15]. The disease-causing mutations in CAPS are believed to confer gain-of-function to NLRP3, leading to a hyperactive inflammasome and IL-1β overproduction; however, the mechanisms of action of these mutant proteins are still unclear. Anti-IL-1 therapy is highly effective in the treatment of CAPS, illustrating the crucial role of IL-1 in disease progression [75-77]. However, a recent study also supports a role for IL-18 and cytokine-independent effects of the inflammasome, such as pyroptosis, in CAPS pathology [78].

FMF

Familial Mediterranean fever (FMF) is an auto-inflammatory disease associated with mutations in the Pyrin-encoding *MEFV* gene and is characterised by recurrent episodes of fever with inflammation of serosal surfaces [79]. There is significant neutrophil infiltration into the affected tissues, which is consistent with the IL-1-driven inflammation seen in inflammasome-dependent syndromes. Most of the FMF-causing pyrin mutations are found in the C-terminal PRY-SPRY domain, however, the molecular mechanism, by which these mutations lead to exaggerated pyrin inflammasome activation, remain unclear. Possible therapies for FMF patients include treatment with Anakinra, an IL-1 receptor antagonist, and/or colchicine to dampen or prevent inflammatory FMF attacks in patients [2, 73].

NLRC4-associated inflammatory disease

Missense mutations in the NACHT domain of the *NLRC4* gene have been associated with macrophage activation syndrome (MAS)-like illness or severe enterocolitis [80, 81]. The syndromes are characterised by spontaneous activation of the NLRC4 inflammasome. However, in contrast to CAPS, NLRC4-MAS patients benefited only partly from IL-1β neutralization during Anakinra therapy, suggesting that other inflammasome effector mechanisms are involved in the pathology. Indeed, patients present with extremely high levels of circulating IL-18 and partly increased macrophage cell death, making IL-18 neutralization and pyroptosis inhibition promising candidates for possible future therapy [2, 80, 81].

Chronic auto-inflammatory metabolic diseases and neurological disorders

Other auto-inflammatory diseases are also associated with aberrant inflammasome activation, but in contrast to the syndromes described above they are caused by chronic exposure to inflammasome activators. These so called inflammasomopathies are metabolic diseases and neurological disorders, neither of which were traditionally considered to be inflammatory diseases. However, it is increasingly recognized that the diseases have an inflammatory component that contributes significantly to the disease process [82].

It is now well established that immunity and metabolism are interconnected and that a metabolic disease can be associated with a chronic inflammatory state. Recent studies have implicated inflammasomes (especially the NLRP3 inflammasome) as an important factor in obesity and metabolic diseases [10]. Obesity is the most common underlying condition for many diseases in the Western world, and obesity-associated inflammation can give rise to multiple metabolic disorders, such as type 2 diabetes (T2D) and atherosclerosis.

Type 2 Diabetes

The development of insulin resistance, a defining feature of T2D, highly correlates with obesity and chronic inflammation. Initially, insulin resistance can be successfully compensated by increased insulin secretion; however, as the disease progresses, this compensation fails and results in elevated blood glucose levels and diabetes. This development is often the result of pancreatic decline and β-cell failure [10]. Inflammation in the pancreas, by IL-1β and other inflammatory pathways, has been strongly linked to apoptosis and reduced function of β-cells [83].

Although the molecular pathogenesis is not fully understood, the activation of the NLRP3 inflammasome has been implicated in the development of insulin resistance and the pathogenesis of T2D. In line with this, a number of studies have shown that mice deficient in NLRP3, caspase-1, or ASC show improved glucose tolerance and insulin sensitivity when exposed to a high-fat diet (HFD) [26, 84-87]. Multiple potential mechanisms, by which the NLRP3 inflammasomes may become activated during T2D, have been proposed.

The first direct implication of the inflammasome in insulin resistance came from the finding that chronic hyperglycaemia triggers IL-1β secretion in pancreatic β-cells through inflammasome activation mediated by thioredoxin-interacting protein (TXNIP) [26, 88]. It was shown earlier that hyperglycaemia upregulates TXNIP expression in pancreatic islet cells [89] and the subsequent identification of TXNIP as a direct ligand of NLRP3 has provided an elegant explanation for how elevated glucose levels might activate the inflammasome. Increased levels of ROS were shown to cause a conformational change in TXNIP, which leads to dissociation from thioredoxin and, in turn, association with NLRP3 and inflammasome activation [26]. However, it is important to mention that, although TXNIP is reported to be a ligand for NLRP3, an involvement of TXNIP in NLRP3 inflammasome activation could not be reproduced by others [90].

A second potential mechanism is mediated by islet amyloid polypeptide (IAPP), a 37-amino-acid peptide hormone that is secreted from β-cells along with insulin and whose deposition in human pancreatic islets has been strongly linked to the development of T2D [91]. *In vitro*, IAPP is capable of activating the NLRP3 inflammasome in macrophages and dendritic cells via IAPP

phagocytosis and lysosome destabilisation [90], and transgenic mice expressing human IAPP have elevated pancreatic IL-1β [92].

More recently, in a rat model of T2D, endocannabinoids were described to activate the NLRP3 inflammasome in pancreas infiltrating macrophages in a cannabinoid receptor (CB_1R)-dependent manner. Intriguingly, blockade of CB_1R by an inhibitor delayed the progress of T2D in this animal model, which implicates CB_1R as a potential therapeutic target in T2D [93].

Finally, palmitate, a saturated fatty acid, and ceramide were shown to accumulate in serum and adipose tissue, respectively, in response to HFD and to induce NLRP3 inflammasome activation [86, 87].

Regarding a therapeutic strategy for treating T2D, clinical trials have suggested that IL-1 blockade may significantly ameliorate T2D [94, 95]. Another therapy for T2D is the use of the anti-diabetic drug glibenclamide, also known as glyburide, which, interestingly, was also identified as a NLRP3 inflammasome inhibitor [96].

Atherosclerosis

Obesity and metabolic abnormalities predispose individuals to complications such as cardiovascular disease. The cause of cardiovascular disease is most commonly attributed to atherosclerosis, a chronic condition, which is characterised by thickening of the artery walls due to the accumulation of fatty metabolites and cholesterol. The subsequent invasion of macrophages elicits a chronic inflammatory response to the cholesterol crystals and - since they cannot be cleared - the unstable atherosclerotic plaques can rupture and cause thrombosis [52].

The innate immune system has a central role in the inflammatory processes implicated in atherosclerotic progression through TLRs and the NLRP3 inflammasome [73]. Macrophages can recognize oxidized low-density lipoproteins (ox-LDL) and cholesterol crystals at the cell surface via TLRs in combination with CD36 [97]. The uptake of ox-LDL results on the one hand in priming of macrophages and induction of pro-IL-1β expression, and on the other hand in intracellular conversion of ox-LDL to cholesterol crystals within macrophages [97, 98]. These cholesterol crystals have been shown to activate the NLRP3 inflammasome *in vitro* in both mouse [98] and human cells [99] through phagosome destabilisation.

Cholesterol-crystal deposition in atherosclerotic vessel walls has long been known to occur, but has been thought to be a late sign of atherosclerotic lesions; however, investigations have shown that these crystals are present already at very early stages of disease [98].

A potential role of the inflammasome in atherosclerosis was corroborated *in vivo* through the observation that chimeras of LDL receptor-deficient mice, which are prone to developing atherosclerotic plaques, reconstituted with NLRP3-, ASC-, and IL-1α/β-deficient bone marrow cells had reduced serum IL-18 concentrations and were protected from atherosclerosis [10, 98].

Using a second genetic model of atherosclerosis, apolipoprotein E (ApoE)-deficient mice, subsequent studies have also demonstrated that caspase-1 ablation protects against atherosclerosis [100, 101].

In addition, it has been shown that IL-1 plays a role in atherosclerosis. Both IL-1β deficiency and IL-1 blockade can ameliorate atherosclerosis in mice [102-104]. However, another study has suggested a more prominent role for IL-1α, rather than IL-1β, in the pathogenesis of atherosclerosis [105]. Clinical trials testing the effect of IL-1 blockade on atherosclerosis in humans are ongoing [15, 73].

Gout

Gout is an auto-inflammatory disease characterised by severe and painful joint inflammation. This form of inflammatory arthritis is associated with metabolic disturbances leading to increased levels of uric acid in the blood (hyperuricemia) and the deposition of monosodium urate (MSU) crystals within joints [17].

The prevalence for gout has significantly increased during the past century concomitant with increased rates of obesity, diabetes, and metabolic syndrome. Western diets rich in purines, of which uric acid is a metabolite, often lead to the hyperuricemia observed in gout. If the concentration of uric acid in the blood exceeds a certain threshold, MSU crystals are formed [15]. Although MSU crystals were identified as having a causal role in gout more than 100 years ago, the molecular mechanisms underlying the resultant joint inflammation have only become clear during the past decade. In 2006, it was discovered that MSU crystals induce a sterile IL-1β-mediated inflammatory response via activation of the NLRP3 inflammasome [21]. Accordingly, IL-1 blockade was subsequently found to be a highly effective treatment for gout [106, 107].

Neurodegenerative disorders

There is increasing evidence suggesting that inflammasomes also contribute to the pathogenesis of neurodegenerative disorders. These diseases are associated with the accumulation of protein aggregates in the central nervous system (CNS) and with neuroinflammation that promotes disease development and progression [15, 108].

Alzheimer's disease

Alzheimer's disease (AD) is a chronic neurodegenerative disease characterised by cognitive dysfunction and progressive memory decline (dementia). Disease severity correlates with the deposition of amyloid-β protein in so-called senile plaques [6, 73]. Amyloid-β was the first molecule associated with a neurodegenerative disorder to be shown to activate the inflammasome. Phagocytosis of fibrillar amyloid-β was found to trigger activation of the NLRP3 inflammasome in microglia, the main innate immune cells within the CNS, and caused both

endosomal rupture and cathepsin B release [20]. A direct link between the NLRP3 inflammasome and AD has been shown in a murine model, in which transgenic mice develop chronic deposition of amyloid-β; however, animals that are lacking NLRP3 or caspase-1 are mostly protected from amyloid pathology [109]. Similarly, the observation that active caspase-1 can be found in the brain tissue of patients with minimal cognitive impairment and AD indicates that inflammasome activation may be an important early step in disease pathogenesis [73, 109].

Amyotrophic lateral sclerosis

Amyotrophic lateral sclerosis (ALS) is a neurodegenerative disease that results from the progressive death of motor neurons, which eventually leads to loss of control of voluntary muscles and, finally, paralysis and death [15]. Similarly to AD discussed above, ALS is associated with the accumulation of protein aggregates. Mutations in the superoxide dismutase 1 (*SOD1*) gene that result in the formation of toxic misfolded protein aggregates are thought to be a major contributor to pathogenesis [6]. Mutant SOD1 was found to trigger inflammasome activation in microglia in a model of ALS, and caspase-1 or pro-IL-1β deficiency significantly ameliorated neurodegenerative disease and improved survival in mice expressing the mutant form of SOD1 [110].

Parkinson's disease

Parkinson's disease (PD) is characterised by the loss of dopamine-generating neurons in the substantia nigra within the mid-brain and the presence of intraneuronal aggregates composed mainly of α-synuclein [108]. Similarly to aggregated amyloid-β, aggregated α-synuclein was shown *in vitro* to induce the production of IL-1β in a process dependent on cathepsin B and the NLRP3 inflammasome [111]. In a PD mouse model, in which the disease is induced by loss of dopaminergic neurons caused by treatment with a neurotoxin, mice deficient in NLRP3 are protected [112]. This result provides *in vivo* evidence for a link between the NLRP3 inflammasome and PD.

Inflammasome-associated autoimmune diseases

As described above, several auto-inflammatory diseases are strongly linked with aberrant inflammasome activation. By contrast, the understanding of the role of inflammasomes in autoimmunity is less clear. The following section lists some selected autoimmune disorders, where a role of the inflammasome was implicated.

Essentially, autoimmune diseases are defined by hyperactivation of the adaptive immune system against self-antigens. However, the innate immune system was shown to influence the development of autoimmune responses [2, 113]. IL-1β and IL-18 are critical for the initiation and control of the adaptive immune response. Among other effects on T and B cells, IL-1β and

IL-18 play a crucial role in driving the differentiation and amplification of Th17 and Th1 cells, respectively [114], and Th17 responses are implicated in many autoimmune disorders [115].
Genome-wide association studies revealed that genetic variations in *NLRP1* are linked to vitiligo and vitiligo-associated Addison's disease [116], systemic lupus erythematosus (SLE) [117], Addison's disease and type 1 diabetes [118], celiac disease [119], rheumatoid arthritis [120], autoimmune thyroid disease [121] and systemic sclerosis [122]. The mechanisms, by which *NLRP1* single nucleotide polymorphisms (SNPs) influence autoimmunity, remains largely unclear; however, one study describes that autoimmunity-associated NLRP1 variants may lead to increased IL-1β processing in response to inflammatory stimuli, such as TLR ligands [123].
SNPs in the NLRP3 locus have also been associated with a wide range of disorders, including type 1 diabetes [124], celiac disease [124] and psoriasis [125].

Multiple sclerosis

Multiple sclerosis (MS) is an autoimmune inflammatory demyelinating disease of the CNS mediated by myelin-specific autoreactive T cells. Investigations into a role for inflammasomes in MS have focused on the peripheral immune response, in which activated T cells and macrophages infiltrate the CNS during MS relapses. These events can be modelled in mice by experimental autoimmune encephalitis (EAE) [6]. Studies using NLRP3 or ASC knockout mice have shown that the induction of EAE is dependent on the NLRP3 inflammasome [126, 127]. NLRP3 deficiency and subsequent loss of IL-1β and IL-18 signalling dramatically delayed the course of disease and alleviated disease severity by reducing T cell priming and subsequent T cell trafficking into the CNS [126-128]. Inflammasome activation was also implicated in a chemically induced demyelination disease model (cuprizone model) [129]. Clinically, caspase-1 expression is elevated in MS plaques and also in peripheral blood mononuclear cells (PBMCs) from MS patients compared to healthy controls [114].
However, a role for NLRP3 and ASC in EAE has not been detected in all studies and seems to be dependent on the intensity of immunization in the disease model [130]. Aggressive immunization of mice, for example with heat-killed mycobacteria (Mtb), was able to induce EAE even in the absence of NLRP3 or ASC, whereas low dose Mtb immunization required NLRP3 and ASC for EAE induction [82, 131]. Furthermore, it was demonstrated that interferon (IFN)-β, which is used as a treatment for MS patients, is only effective, when EAE is NLRP3-dependent. This suggests that IFN-β may therapeutically inhibit the NLRP3-IL-1β-IL-18 axis in MS [82, 131, 132].

AIM2 inflammasome and autoimmunity

As AIM2 has been shown to recognize self-DNA in the cytosol, this inflammasome has also been linked to numerous diseases, including systemic lupus erythematosus (SLE) and psoriasis. The

level of AIM2 expression has been associated with severity of disease in SLE patients, and AIM2 inflammasome blockade was found to ameliorate development of autoimmunity in a model of lupus that is induced via immunization with DNA from apoptotic cells [133]. AIM2 has also been linked to the chronic skin disorder psoriasis: keratinocytes of psoriatic lesions have increased levels of both dsDNA and AIM2, and AIM2 can form an inflammasome and release IL-1β in response to cytosolic DNA in cultured keratinocytes [69, 74]. This suggests that AIM2 may respond to self-DNA released during cell damage to drive chronic inflammatory disease.

1.5 Nrf2, a master regulator of cytoprotection

Cells are continuously exposed to ROS produced during internal metabolism and in response to environmental toxic agents. Controlled production of ROS in the cell in response to physiological cues serves useful purposes, because they act as signalling molecules to regulate pathways involved in cell division, inflammation, immune function, autophagy, and stress response [134]. However, uncontrolled production of high levels of ROS and reactive nitrogen species (RNS) results in oxidative stress. Both, oxidative and electrophilic stress, caused by toxic insults, impair cell function, can damage cellular macromolecules, resulting in severe cell damage, and contribute to the development of cancer and other pathophysiological states [135].

Cells can adapt to electrophilic and oxidative stress via the activation of the transcription factor nuclear factor erythroid derived 2, like 2 (Nrf2), which upregulates the expression of numerous cell defence genes. Nrf2 regulates directly approximately 250 genes that are involved in cell protection and homeostasis, including those for antioxidant proteins, detoxification enzymes, drug transporters and numerous other cytoprotective genes, and it also influences energy metabolism, inflammation and cell growth [136].

Nrf2 is a member of the cap 'n' collar (CNC) subfamily of basic region leucine zipper (bZip) transcription factors, which also includes nuclear factor erythroid 2 p45 (NF-E2 p45), Nrf1 and Nrf3 [137]. Nrf2 contains six functional domains termed Nrf2-ECH homology (Neh) domains 1 to 6 [138] (Figure 4A). In the C-terminal region, within Neh1, Nrf2 has a characteristic CNC bZip domain: the basic region contributes to DNA binding and the leucine zipper to heterodimerization with its transcriptional partners, the small Maf (musculoaponeurotic fibrosarcoma homolog) proteins [139, 140]. Transcription activation is conferred by three regions, the so-called transactivation domains: Neh4 and Neh5 in the N-terminal part, as well as Neh3 at the C-terminus [135]. Neh4 and Neh5 also act as translocation domains. The Neh6 domain contains a so-called degron motif involved in Nrf2 turnover and Keap1-independent degradation (see below). The N-terminal Neh2 domain mediates binding to kelch-like ECH-associated protein 1 (Keap1), a negative regulator of Nrf2 activity [138].

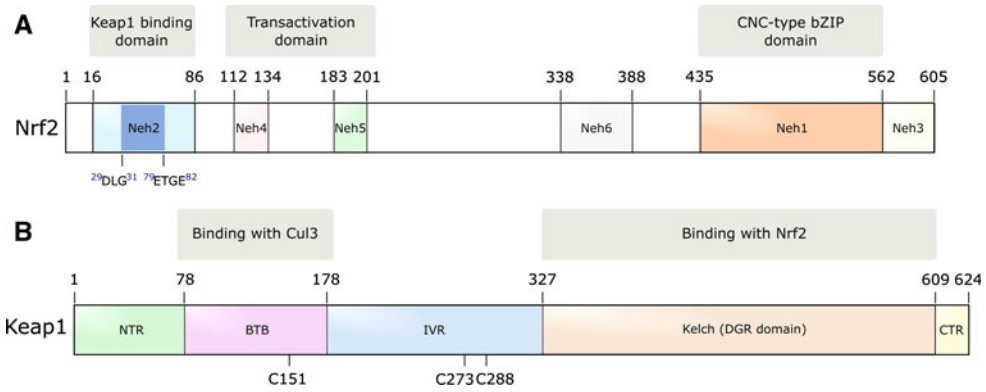

Figure 4 [141]: Domain structures of (A) Nrf2 and (B) Keap1. (**A**) For Nrf2, the Neh2, Neh4, Neh5, Neh6, Neh1 and Neh3 domains are indicated. Furthermore, the location of the 'DLG' and 'ETGE' motifs within the Neh2 domain, through which Nrf2 binds to Keap1, are depicted. Neh1 contains the bZip DNA binding and heterodimerization domain, through which Nrf2 interacts with small Maf proteins and binds to DNA as a heterodimer. The Neh4 and Neh5 domains act synergistically to bind the transcriptional co-activator CBP. The Keap1-independent negative regulation of Nrf2 is controlled by the Neh6 domain. (**B**) For Keap1, the positions of the N-terminal region (NTR), the BTB domain, the intervening region (IVR), the Kelch (DGR) domain and the C-terminal region (CTR), and the location of C151, C273 and C288 are shown.

Nrf2 is an ubiquitously expressed protein with particularly high levels being present in epithelial and myeloid cells [142, 143]. However, Nrf2 is constantly degraded with a half-life of less than 20 min [144]. This rapid turnover maintains cellular Nrf2 at a low level. As mentioned above, the transcription factor Nrf2 plays a central role in the inducible expression of many cytoprotective genes in response to oxidative and electrophilic stress. A cardinal feature of Nrf2 is that its activity and hence expression of its target genes are maintained at low basal levels under normal homeostatic conditions, but increase rapidly in response to a wide spectrum of oxidants and electrophiles [145]. Nrf2 target genes contain in their promoter region a regulatory element, the so-called antioxidant response element (ARE) or electrophile-response element (EpRE). To date, approximately 250 genes that contain ARE/EpRE sequences have been reported in mice and humans [145]. Target genes of Nrf2 are central to the phase II antioxidant/detoxifying response and involved in glutathione synthesis, elimination of ROS, detoxification of xenobiotics and drug transport [146]. Examples of important Nrf2 target genes are listed in Table 1. Thus, the up-regulation of Nrf2 target genes helps the cell to combat harmful stressors such as ROS and electrophilic xenobiotics, effectively providing a cellular survival mechanism [147].

Category	Gene	Gene symbol
Antioxidant proteins	Glutamate cysteine ligase, catalytic subunit	*GCLC*
	Glutamate cysteine ligase, modifier subunit	*GCLM*
	Sulfiredoxin 1	*SRXN1*
	Thioredoxin reductase 1	*TXNRD1*
	Peroxiredoxin 1	*PRDX1*
Drug metabolizing enzymes and transporters	Aldo-keto reductases	*AKRs*
	Glutathione S-transferases	*GSTs*
	Multidrug resistance-associated proteins	*MRPs*
	NAD(P)H: quinone oxidoreductase 1	*NQO1*
	UDP-glucuronosyltransferases	*UGTs*
NADPH synthesis	Glucose-6-phosphate dehydrogenase	*G6PD*
	Malic enzyme 1	*ME1*
Stress-response and metal-binding proteins	Ferritin	*FTL*
	Heat shock proteins	*HSPs*
	Haeme-oxygenase 1	*HMOX1*
	Metallothionein	*MT1*

Table 1 (adapted from [148]): Examples of key Nrf2 target genes

The importance of Nrf2 in the antioxidant defence *in vivo* has been extensively studied using Nrf2 knockout (KO) mice. The animals have a normal embryonic development, are fertile and at least young mice do not show obvious abnormalities under normal housing conditions [149]. However, since they exhibit lowered basal and induced levels of ARE-regulated genes in multiple tissues, Nrf2 KO mice are more susceptible to drug-induced toxicity, and cancer development is increased upon exposure to various carcinogens [143, 150, 151].

Keap1 negatively regulates Nrf2

Due to the broad effects and important functions of Nrf2, its activity is tightly controlled. The best characterised mechanism of Nrf2 regulation is mediated by interaction with Keap1. Keap1 tightly regulates Nrf2 at the protein level. It is mainly present in the cytoplasm where it is tethered to the actin cytoskeleton [152] and acts as a repressor by retaining Nrf2 in the cytoplasm and mediating its degradation via the ubiquitin-proteasome pathway.

Keap1 contains two known protein-interacting domains: the BTB (bric-a-bric, tramtrack, broad complex) domain, which mediates homodimerization and binding of Keap1 to the protein Cullin 3 (Cul 3), and the Kelch repeats or double glycine repeat (DGR) domain mediating binding to Nrf2 [135] (Figure 4B).

Under normal homeostatic conditions, Nrf2 is highly unstable due to its interaction with Keap1, which is a substrate adaptor of the Cul3-RING box protein (Rbx1) E3 ubiquitin ligase complex

for Nrf2 ubiquitination. According to the two-site binding model, Nrf2 is recognized by dimeric Keap1 through two key motifs in the Nrf2 Neh2 domain (Figure 5A). The Kelch domain of each Keap1 protein binds to the 'DLG' or 'ETGE' motifs within Neh2, recognized as low affinity- and high affinity-binding sites, respectively. Nrf2 is subsequently polyubiquitinated at seven lysines within the Neh2 domain and thereby marked for proteasomal degradation [153-156].

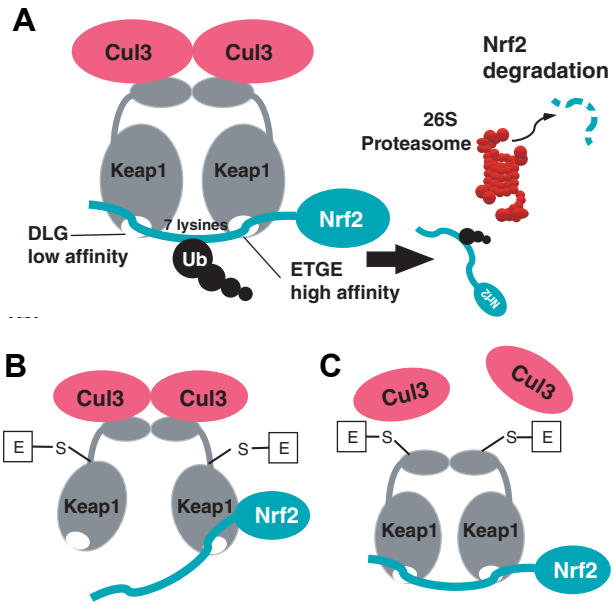

Figure 5 [146]: Structure of the Keap1-Nrf2 complex. (**A**) Two-site binding model of Keap1 and Nrf2. A Keap1 homodimer binds to a single Nrf2 molecule at two sites, the 'DLG' and 'ETGE' motifs. This conformation is believed to stimulate Keap1-mediated ubiquitination of Nrf2 through presenting seven target lysine residues to the ubiquitin ligase complex. (**B** and **C**) Proposed mechanisms of the Keap1-Nrf2 system in response to electrophiles. (**B**) In a model referred to as hinge-and-latch, electrophiles directly modify reactive cysteine residues of Keap1 and alter the conformation of the Keap1 homodimer. This leads to a dissociation of the low-affinity 'DLG' motif from Keap1 (latch), while the high-affinity 'ETGE' motif remains associated with the other Keap1 molecule (hinge). (**C**) In an alternative model the interaction between Keap1 and Cul3 is disrupted upon modification of Keap1.

Keap1 is a cysteine-rich protein: Murine Keap1 and the human homologue contain 25 and 27 cysteine residues, respectively, some of which act as sensors of oxidative stress. Electrophilic agents or ROS can modify reactive cysteine residues in a domain of Keap1 called the intervening region (IVR region) (Figure 4B). Modification of these cysteine residues triggers a conformational change in Keap1, which leads to stabilisation and activation of Nrf2.

According to the hinge-and-latch model (Figure 5B), it is believed that the conformational change in the Keap1 homodimer disrupts the low affinity interaction between the Kelch domain and the 'DLG' of Nrf2, which impairs ubiquitination and blocks proteasome-mediated degradation of Nrf2. As a consequence, Nrf2 levels are increasing; because Keap1 is saturated with Nrf2 that is not ubiquitinated and degraded, *de novo* synthesised Nrf2 is able to bypass Keap1 and to accumulate in the nucleus. An alternative mechanism proposes that modification of Keap1 by electrophiles impairs the integrity of the Keap1-Cul3 E3 ligase complex, because Cul3 dissociates from Keap1 upon exposure to Nrf2 activating compounds [157] (Figure 5C).

Loss of the Cul3-Keap1 interaction results in a decline in Keap1-Cul3 E3 ligase activity and consequently less ubiquitination of Nrf2.

According to both models, Nrf2 is stabilised upon modification of Keap1 and can accumulate in the nucleus, where it forms heterodimers with small Maf proteins. These Nrf2-Maf heterodimers recognize and bind AREs in the regulatory regions of Nrf2 target genes. This allows the transcription factor to upregulate the expression of these genes (Figure 6).

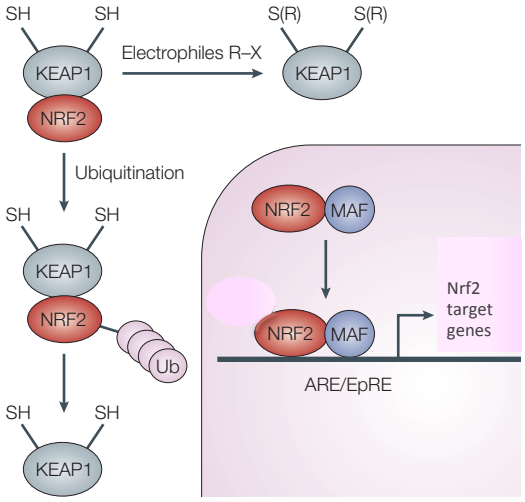

Figure 6 (modified from [158]): The Keap1/Nrf2/ARE pathway. Keap1 binds Nrf2 and retains it in the cytosol, where it has a short half-life and undergoes ubiquitination. Electrophiles modify reactive cysteine residues of Keap1, which enables Nrf2 to translocate to the nucleus, where it interacts with small Maf proteins and binds to ARE/EpRE to cause enhanced transcription of Nrf2 target genes.

Other mechanisms of Nrf2 regulation and crosstalk of Nrf2 with other signalling pathways

Nrf2 activity is tightly controlled at the protein level by proteasomal degradation. Although Keap1 is the most important regulator of Nrf2, recent results also demonstrated Keap1-independent mechanisms of Nrf2 degradation, and it became clear that other E3 ubiquitin ligase complexes contribute to the regulation of Nrf2 (Figure 7).

Phosphorylation of the Neh6 domain of Nrf2 by glycogen synthase kinase 3β (GSK-3β) creates a modification that enhances the ubiquitination of Nrf2 by the β-TrCP (β-transducin repeat-containing protein)-Skp1 (S-phase kinase-associated protein 1)-Cul1 (Cullin1)-Rbx1 E3 ubiquitin ligase complex [159, 160]. It was proposed that GSK-3β is regulated by growth factors. In turn, these growth factors activate Nrf2 through stimulation of upstream protein kinases and subsequent inhibitory phosphorylation of GSK-3β [136, 145]. Furthermore, GSK-3β has been also reported to induce export of Nrf2 from the nucleus because of its influence on the subcellular location of the Src family kinase Fyn. In particular, it has been reported that GSK-3β controls the nuclear abundance of Fyn, which in turn phosphorylates Nrf2 to stimulate nuclear export of Nrf2 [161, 162].

In addition, the E3 ubiquitin ligase HMG (high mobility group)-coA reductase degradation 1 (Hrd1), which is involved in endoplasmic reticulum-associated protein degradation (ERAD), also controls Nrf2. Hrd1 targets the Neh4-5 domain of Nrf2 for ubiquitination and degradation by the proteasome [163].

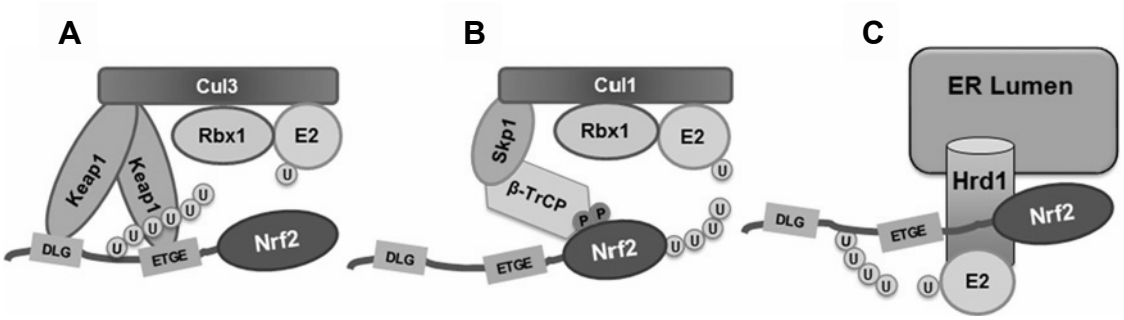

Figure 7 [147]: The three E3 ubiquitin ligases for Nrf2. (**A**) Keap1-Cul3-Rbx1 E3 ubiquitin ligase, (**B**) *β*-TrCP-Skp1-Cul1-Rbx1 E3 ubiquitin ligase, (**C**) Hrd1 E3 ubiquitin ligase.

Apart from degradation through ubiquitin-proteasome systems, Nrf2 is subjected to a positive regulation by other proteins through disruption of the Nrf2-Keap1 interaction. Several proteins with motifs identical or similar to the 'ETGE' motif of Nrf2 were identified, which can compete with Nrf2 for Keap1 binding, thus stabilising Nrf2 [147, 164]. One example is the autophagy adaptor protein p62, which mediates the formation of protein aggregates destined for autophagic turnover. The expression of p62 is elevated due to autophagy-deficiency, leading to increased degradation of Keap1 [165]. Via its 'STGE' motif, p62 directly interacts with Keap1, which allows p62 to sequester Keap1 into the autophagosomes. This impairs the ubiquitination of Nrf2, leading to increased Nrf2 activity [166]. Further examples of proteins, which disrupt the Keap1-Nrf2 binding and thereby activate Nrf2 are cyclin-dependent kinase inhibitor p21 [167], dipeptidyl peptidase 3 (DPP3) [164], partner and localizer of BRCA2 (PALB2) [168], and phosphoglycerate mutase 5 (PGAM5) [169].

Protein kinases have also been proposed to play a role in the regulation of Nrf2 activity. Phosphorylation of serine residue 40 (Ser^{40}) of Nrf2 by protein kinase C (PKC) has been suggested to promote the release of Nrf2 from Keap1 [170, 171]. The mitogen-activated protein kinase (MAPK) signalling cascade is activated by oxidative stress and also has been implicated in Nrf2 control. It was shown that a number of Nrf2 inducers modulate MAPK activity. Studies with kinase inhibitors suggest that extracellular signal-regulated protein kinase (ERK) and c-Jun N-terminal kinase (JNK) positively regulate Nrf2 activity, whereas $p38^{MAPK}$ has been reported to both positively and negatively regulate Nrf2 activity [145, 172-174]. The effect of MAPK activity on ARE-dependent gene expression might be due to either the direct phosphorylation of Keap1 and/or Nrf2, or through an indirect, less characterised mechanism. Mutation of putative MAPK phosphorylation sites in Nrf2 has only little impact on the activity of the transcription factor, suggesting that MAPK plays only a limited role in the activation of Nrf2 [175].

Nrf2 is also regulated at the transcriptional level. The expression of Nrf2 can be upregulated by some oncogenes (see below). In addition, Nrf2 was shown to be regulated post-transcriptionally by microRNAs (miRNAs) [143].

Crosstalk between the Nrf2 and NF-κB pathways

The nuclear factor-κB (NF-κB) complex is a key transcription factor that mediates immune responses to bacterial and viral infections, inflammation, aspects of development, cell proliferation and protection against UV radiation [176]. Pro-inflammatory cytokines and bacterial lipopolysaccharide (LPS) are potent NF-κB activators, acting on extracellular receptors and initiating intracellular phosphorylation events, which coordinate signalling and conditional cell responses [177]. Phosphorylation of IκBα, the negative regulator of NF-κB, leads to an interaction with the *β*-TrCP-Skp1-Cul1 complex driving IκBα ubiquitination and proteasomal degradation, releasing NF-κB subunits, which then translocate to the nucleus [178]. There, NF-κB homo- and hetero-dimers associate with κB regulatory DNA sequences upstream of NF-κB target genes and initiate the transcription of these genes.

NF-κB was implicated in the modulation of Nrf2 transcription and activity, having both positive and negative effects on Nrf2 target gene expression. On the other hand, the absence of Nrf2 can exacerbate NF-κB activity, leading to increased cytokine production. The interplay between the Nrf2 and NF-κB pathways occurs through a range of complex molecular interactions and is often cell type- and tissue context-dependent. Although convincing evidence for important functional interactions between the two pathways exists, many aspects of the conditional and dynamic nature of the crosstalk are still unknown [177].

The best established mechanism of inhibition of Nrf2 by NF-κB is the competition of Nrf2 and the NF-κB subunit p65 for the transcriptional co-activator CBP (CREB-binding protein)-p300 complex. CBP-p300 has an intrinsic histone acetyl transferase activity, which leads to local acetylation of histones and subsequent loosening of the chromatin structure. Furthermore, it also acetylates non-histone proteins like Nrf2 and p65, which is thought to augment assembly of the transcriptional machinery and to enhance gene transcription [179, 180]. Therefore, overexpression of p65 is thought to limit the availability of CBP for Nrf2 complex formation, prioritizing transcription of NF-κB-driven genes [177]. In addition, p65 can promote the association of histone deacetylase (HDAC) 3 with MafK, thus preventing binding of Nrf2 to MafK and, therefore, decreasing expression of ARE-regulated genes [180].

Another protein linking the NF-κB and Nrf2 pathways is *β*-TrCP, a component of the *β*-TrCP-Skp1-Cul1 E3 ligase complex. It recognizes and binds phosphorylated substrates and marks them for proteasomal degradation. As mentioned earlier in this chapter, *β*-TrCP-Skp1-Cul1 is involved in limiting nuclear Nrf2 levels; however, the canonical role of *β*-TrCP, as described

above, is the regulation of IκBα degradation in response to cytokines. Therefore, *β*-TrCP function can lead to augmentation of NF-κB activity as well as to inhibition of Nrf2-ARE-mediated transcription [177].

As stated previously, NF-κB is currently thought to have a dual role in the regulation of Nrf2 activity. Certain cell types show induction of Nrf2 protein and increased target gene expression regulated by NF-κB in response to TNF-α. It was demonstrated that Nrf2 contains several κB sites in its proximal promoter, which are subject to binding and transcription initiation by p65 [181]. This underlies high basal Nrf2 activity in acute myeloid leukaemia (AML) cells and is believed to be the prime cause of chemoresistance of AML cells [177, 181].

The modulation of Nrf2 in response to NF-κB activation can act as a protective mechanism against the consequences of inflammation [177]. For instance, activation of the small GTPase Ras-related C3 botulinum toxin substrate 1 (RAC1) by LPS can induce Nrf2-mediated heme oxygenase-1 (HO-1) expression, which in turn dampens the pro-inflammatory activity of NF-κB [182, 183]. There is a general consensus that Nrf2 is protective against systemic infections, via mechanisms targeting NF-κB and modulating pro-inflammatory gene expression in macrophages [184-186]. Nrf2 is protective against endotoxic shock and severe sepsis triggered by microbial infection [184]. In the same study, Nrf2-deficient mouse embryonic fibroblasts (MEFs) revealed enhanced activity of IκB kinase-β (IKKβ), the activator kinase of NF-κB. Also others showed an augmentation of NF-κB-dependent cytokine production in the absence of Nrf2, which is thought to be the underlying cause for the neurodegenerative phenotype in Nrf2 KO mice [177] (see below).

The role of Nrf2 in oncogenesis

Nrf2 regulates the expression of numerous cytoprotective genes. Owing to its role in protecting the cell from cytotoxic compounds, Nrf2 is especially important for chemoprevention of cancer development. The role of Nrf2 in this field has been widely studied [187]. As a central player in cytoprotection, Nrf2 has been traditionally considered as a transcription factor that prevents cancer development and is therefore a tumour suppressor [188]. It was shown in multiple studies that Nrf2 KO mice are more sensitive to carcinogenesis [151, 189-191], and Nrf2 ablation has been related to enhanced metastasis [192, 193]. Accordingly, there are multiple reports describing the beneficial effects of Nrf2 signalling in cancer chemoprevention [194], and different Nrf2 activating compounds have been used in clinical trials for cancer prevention [188].

As Nrf2 promotes cell survival under stress, cells heavily rely on Nrf2 activation to prevent cell death. Hence, it is reasonable to assume that increased Nrf2 activity could be also protective for cancer cells and, therefore, support tumour growth. Indeed, some years ago it was reported for

the first time that as a result of somatic Keap1 mutations, rendering the protein inactive, Nrf2 constitutively localizes into the nucleus. Such mutations were found in biopsies from lung cancer patients and also in lung cancer cell lines [195-197]. Thereafter, several papers have reported elevated activity of Nrf2 via multiple mechanisms in a wide number of solid cancers and leukaemias [187]. Furthermore, mounting evidence has indicated that Nrf2 hyperactivation is associated with resistance to chemotherapeutic agents and a poor prognosis in many cancer types [198-201].

In addition to protecting against cell death, many Nrf2 target genes are involved in metabolic pathways, like glucose metabolism, purine biogenesis and fatty acid oxidation, which are associated with rapid growth and proliferation of cancer cells. It was proposed that enhanced Nrf2 activity can lead to metabolic reprogramming, which in turn can alter cellular behaviour and lead to cancer progression [147, 201-204].

The wide-ranging observations regarding the role of Nrf2 in cancer suggest that Nrf2 plays distinct roles in the initiation and promotion of cancer [205]. There is no direct evidence that Nrf2 activating chemopreventive drugs themselves are carcinogenic; in fact, many are potent and safe agents for the suppression of carcinogenesis in mouse models of cancer [206]. However, if enhancement of oxidative stress represents an important therapeutic approach to cancer, then there is good reason to suggest that one should consider blocking Nrf2 activity in fully malignant cells and thereby increase oxidative stress. In summary, it was suggested that Nrf2 has a dual role in cancer development. Nrf2 activity is desirable for the host organism in early stages of tumorigenesis, when the host is seeking to control premalignant carcinogenesis, but is undesirable at later stages, when it makes fully malignant cancer cells resistant to chemotherapy [206]. However, this has also been challenged, since Nrf2 activation had a pro-tumorigenic effect in the early phase of skin cancer development [204]. Thus, the protective effect may be restricted to patients, which do not yet have oncogenic mutations and where NRF2 activation could protect cells from acquisition of such mutations through enhanced oxidative stress.

Mechanisms of NRF2 hyperactivation in cancer

Cancer cells utilize several mechanisms to enhance Nrf2 activity. Among the most frequent mechanisms leading to Nrf2 hyperactivity in cancer are somatic mutations in the key components of the KEAP1-NRF2 pathway. Loss-of-function mutations in the human *KEAP1* gene have been found in carcinomas of lung [196, 207-210], gallbladder [211], ovary [212], breast [213, 214], liver [210] and stomach [210]; these mutations result in constitutive Nrf2 activity. In the *NRF2* gene, gain-of-function mutations have been observed in squamous cell carcinomas (SCC) of the oesophagus, skin, lung and larynx [207, 215, 216]. These mutations are found

exclusively within the 'ETGE' and 'DLG' motifs of NRF2, leading to decreased Keap1-binding affinity and inhibition of NRF2 degradation [197, 206]. Furthermore, somatic mutations leading to NRF2 hyperactivation have also been found in the coding regions of the *Cul3* [217, 218] and *Rbx1* gene [219].

In addition to somatic mutations in the key components of the Keap1-Nrf2 pathway, several other mechanisms have been found to cause constitutive Nrf2 activation in cancer (Figure 8). For instance, epigenetic modifications, such as promoter methylation of the *Keap1* gene, suppress expression of the gene and result in Nrf2 activation [220]. Hypermethylation of the *KEAP1* promoter has been shown in lung, colorectal and prostate cancer [221-223]. Another epigenetic mechanism includes miRNAs. For example, the Keap1-targeting miR-200a was shown to decrease Keap1 expression in human breast cancer cells and, thereby, enhance Nrf2 activity [224]. In contrast, in oesophageal SCC, decreased expression of Nrf2-targeting miRNAs was shown to enhance Nrf2 activity [225].

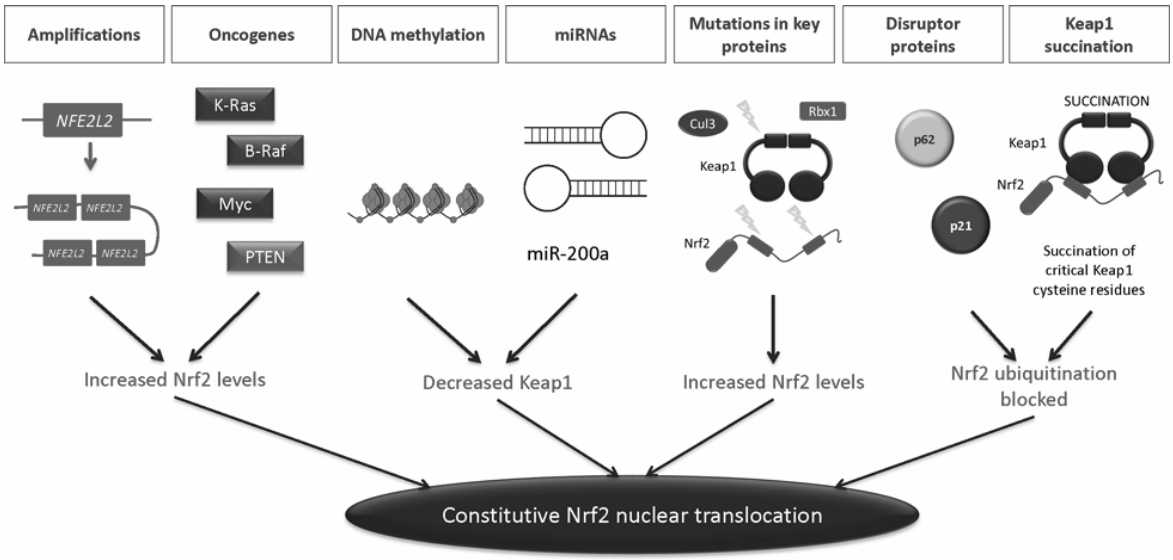

Figure 8 [197]: Mechanisms that lead to increased Nrf2 activity in cancer. Mutations in the genes encoding key components of the Keap1-Nrf2 pathway (Keap1, Nrf2, Cul3 or Rbx1) are the most common mechanisms that either disrupt the binding between Nrf2 and Keap1 or prevent ubiquitination and degradation of Nrf2. The *Nrf2* gene can also be amplified. Other mechanisms include DNA methylation of the *Keap1* promoter, regulation by miRNAs, proteins that disrupt the Keap1-Nrf2 binding, Keap1 succination and transcriptional activation by oncogenes and hormones. See text for details.

Among non-genetic mechanisms, multiple proteins that disrupt the binding between Nrf2 and Keap1, so-called disruptor proteins, have been identified. This mechanism of Nrf2 regulation has already been mentioned in the chapter above (see page 30). It seems that by interfering with the normal regulation of the Keap1-Nrf2 pathway, some proteins are associated with carcinogenesis and/or malignant transformation. One example is p62, an autophagy-related protein that is able to target Keap1 for autophagosomal degradation [166]. The protein is commonly upregulated in various human cancers [226]. Importantly, it was shown that persistent activation of Nrf2 due to

Keap1-p62 aggregate formation contributes to the growth of human hepatocellular carcinomas [227]. It was suggested that because the p62-dependent mechanism of Nrf2 activation takes place upon compromised autophagy, it takes much longer to attenuate, since protein aggregates have to be resolved and new Keap1 protein has to be translated before the functional Keap1-Nrf2 axis can be restored again. Consequently, p62-dependent prolonged Nrf2 signalling leads to increased cell survival and potential cellular transformation [147, 228]. Another example of a protein that disrupts the Keap1-Nrf2 interaction is p21, which competes with Keap1 for Nrf2 binding [167]. A recent study uncovered a link between p21-dependent Nrf2 activation and TGF-β-induced drug resistance in SCC. It was shown that in TGF-β-responding SCC stem cells, TGF-β transcriptionally activates p21, which stabilises Nrf2, thereby markedly enhancing glutathione metabolism and diminishing effectiveness of anti-cancer therapy [201].

The Keap1-Nrf2 pathway may also be subjected to succinylation, a posttranslational modification, in cancer. Fumarate hydratase inactivation in renal carcinomas leads to accumulation of fumarate, which forms 2-succinyl adducts with specific Keap1 cysteine residues, resulting in Nrf2 activation [229, 230].

Oncogenes also contribute to the enhancement of Nrf2 activity in cancer. It has been demonstrated that the oncogene products K-Ras, B-Raf, and c-Myc can stimulate transcriptional activation of Nrf2 via the MEK-ERK-Jun pathway [231]. Some years later, K-Ras was shown to facilitate activation of Nrf2 through a TPA (12-*O*-tetradecanoylphorbol-13-acetate)-responsive element (TRE) in the regulatory region of Nrf2 [232].

Finally, Nrf2 may also be upregulated by gonadotropins and sex steroid hormones, such as follicle-stimulating hormone (FSH), luteinizing hormone (LH) and *β*-oestradiol (E2). This indicates that hormones also regulate Nrf2 especially in cancers, in which they play a fundamental role, such as breast, ovarian, endometrial and prostate cancer [197, 233].

The role of Nrf2 in other diseases

Protective role of Nrf2 in disease

As mentioned above, consistent with the role of Nrf2 as a central regulator of the adaptive response to oxidative stress, Nrf2 KO mice are sensitive to diverse oxidative insults and their susceptibility to carcinogenesis is increased. Oxidative stress is linked not only to cancer, but also to various non-malignant diseases [137]. Primarily on the basis of studies with Nrf2 KO mice and cells derived from them, the protective role of Nrf2 in various diseases has been established. For example, Nrf2 KO mice are sensitive to neurodegenerative disorders, as shown in mouse models for Parkinson's disease [234], Alzheimer's disease [235], amyotrophic lateral sclerosis [236], and multiple sclerosis [237]; inflammatory disorders, such as inflammatory

bowel disease [238]; pulmonary diseases, such as asthma [239] and pulmonary fibrosis [240]; models of liver toxicity [241, 242]; and insulin resistance [243]. Furthermore, activation of the Nrf2 defence response in mice has been shown to protect against neurodegenerative diseases, cardiovascular diseases, acute lung injury, chronic obstructive pulmonary diseases, diabetes, autoimmune disease, inflammation, and cancer [244, 245]. These studies show the importance of Nrf2 as a protective and disease-preventing factor.

Nrf2 in atherosclerosis

Nrf2 is involved in the pathogenesis of cardiovascular diseases, including atherosclerosis, a chronic inflammatory disease of the vascular arterial walls (see also 'Atherosclerosis' chapter on page 22). During atherosclerosis, Nrf2 signalling modulates many physiological and pathophysiological processes, such as lipid homeostasis, foam cell formation, macrophage polarization, redox regulation and inflammation [246]. In contrast to many other inflammatory disorders alleviated by Nrf2 (see above), atherosclerosis can be exacerbated by Nrf2. However, the molecular basis for this phenomenon is a matter of debate. In apolipoprotein E (ApoE)-deficient atherosclerotic mice, multiple investigators have revealed that Nrf2 deficiency reduces atherosclerotic lesions [247-250]. The transplantation of Nrf2-deficient bone marrow cells reduced atherosclerotic lesions in ApoE-deficient recipient mice, indicating that Nrf2 in bone marrow-derived cells positively affects progression of atherosclerosis in ApoE KO mice [250]. However, opposite results were obtained by using a different genetic model of atherosclerosis, LDL receptor (LDLR) KO mice. LDLR and Nrf2 double-deficient mice have exacerbated atherosclerotic phenotypes compared to LDLR single-KO mice. The bone marrow transplantation of Nrf2-deficient cells into LDLR-deficient mice exacerbates atherosclerosis compared with the transplantation of wild-type cells, suggesting that Nrf2 in bone marrow-derived cells negatively affects progression of atherosclerosis in LDLR KO mice [251, 252]. These results indicate that Nrf2 exhibits both pro- and anti-atherogenic effects in mice in a genetic background-dependent manner [246]. A mechanistic understanding of how Nrf2 affects atherogenic processes remains elusive. Nonetheless, currently available data strongly suggest a role of Nrf2 in the control of atherosclerosis [253].

Nrf2 activating compounds

A variety of compounds that increase Nrf2 activity have been identified. They can be components of food, dietary supplements, metabolites, or synthetic agents [254]. These so-called ARE or Nrf2 inducers (also termed Nrf2 activators) are structurally diverse and have few common properties, except that they are electrophiles or pro-electrophiles, which need to be metabolically activated to become electrophilic. These compounds are capable of reacting with

nucleophilic thiols, including cysteine sulfhydryl groups on target proteins and this thiol reactivity has been correlated with their ability to enhance the activity of Nrf2.

Chemical classes of ARE-inducing electrophiles include flavonoids, phenolic compounds, isothiocyanates, Michael acceptors, 1,2-dithiol-3-thiones, dimercaptans and heavy metals [254]. The most thoroughly studied Nrf2 inducers are sulforaphane (SFN), diethyl maleate (DEM), *tert*-butylhydroquinone (tBHQ), dimethyl fumarate (DMF), 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 (15d-PGJ_2), and 2-cyano-3,12 dioxooleana-1,9 dien-28-oic acid (CDDO) triterpenoids.

The underlying mode of action of these compounds involves oxidation or conjugation of cysteine residues on Keap1, especially $Cys^{151, 273, 288}$ [255-257] (Figure 9). As described in the chapter above, the modification of Keap1 at cysteine residues results in a conformational rearrangement of Keap1, which in turn leads to an inhibition of the ubiquitin ligase activity of Keap1, allowing Nrf2 to induce target gene expression.

The functional significance of the Keap1 cysteine residues, which are targeted by oxidative modification, has been examined in several experiments using site-directed mutagenesis of Keap1. Cys^{273} and Cys^{288} are crucial for maintaining the structural integrity required for Keap1 to associate with Nrf2. The substitution of one or both of these cysteine residues renders Keap1 unable to direct ubiquitination of Nrf2 [256]. Modification of Cys^{151} by ARE inducers including tBHQ, SFN and DMF probably inhibits the Keap1-Cul3 interaction and thereby prevents the ubiquitination and degradation of Nrf2 [157, 256, 257]. Thus, Cys^{151} is a critical residue for a subset of Nrf2 activators (Figure 9). By contrast, 15d-PGJ_2, nitro fatty acids, heavy metals such as $CdCl_2$ and some arsenic species are Cys^{151}-independent Nrf2 inducers [258-261]. It was suggested that the different chemicals that trigger the Keap1-Nrf2 system are associated with distinct patterns of Keap1 cysteine modification. The unique utilization of sensor cysteine residues has resulted in the development of the concept of a 'cysteine code', whereby the biological responses are similar even when structurally different inducers react with distinct cysteine residues of Keap1 [257, 262].

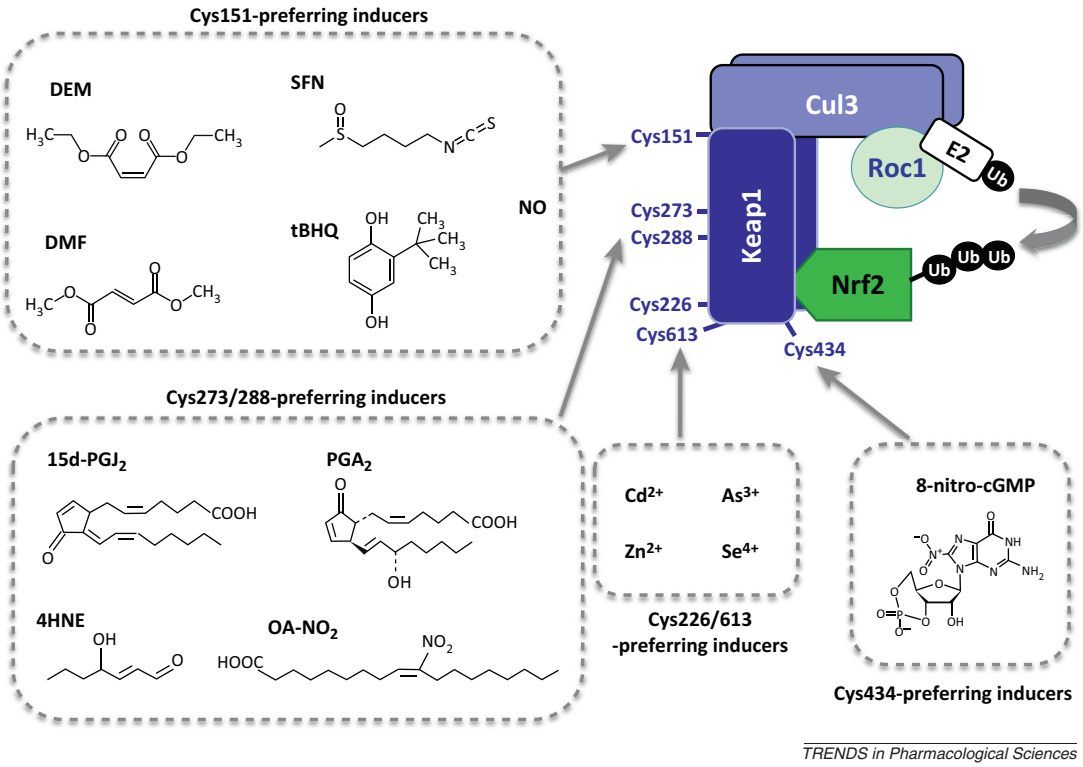

Figure 9 [262]: Distinct utilization of Keap1 cysteine residues by different chemicals that trigger Nrf2 activation. Electrophilic inducers and heavy metals modify cysteine residues in Keap1 and thereby impair Keap1 function, subsequently leading to Nrf2 derepression and activation. See text for details.

Because many studies have demonstrated that Nrf2 plays important roles in the protection against various diseases and cancer (see above), there is substantial interest in identifying and developing Nrf2 activators for therapeutic use [244, 262]. Electrophilic Nrf2 inducers have been the subject of widespread research over the last 10-15 years [263]. Many inducers have been shown to protect against chronic degenerative diseases in various animal models of carcinogenesis, cardiovascular disease and neurodegeneration [141].

Additionally, Nrf2 inducers have anti-inflammatory effects, which are in general less characterised and only partially Nrf2 dependent. DMF for example, is known to target various cellular pathways and is involved in inhibition of pro-inflammatory cytokine signalling, inhibition of NF-κB nuclear translocation, inhibition of dendritic cell maturation, and suppression of lymphocyte and endothelial cell adhesion molecule expression [264-269]. In one study, SFN alleviated LPS-induced inflammation in mouse peritoneal macrophages, which has been attributed to Nrf2 activation, as Nrf2-deficient macrophages did not exhibit this anti-inflammatory capacity [270]. However, to date it is not clear, whether and how the anti-inflammatory effect of Nrf2 activators is related to Nrf2 target gene expression.

One of the most potent naturally occurring Nrf2 activators is the isothiocyanate SFN, which is abundant in cruciferous vegetables such as broccoli [271]. SFN was shown to inhibit carcinogenesis in different tissues [206], to have neuroprotective effects [141, 254] and was in

general suggested to be a promising drug for the treatment of various pathologies that have both oxidative stress and inflammatory components [141].

DMF is an Nrf2 inducer, which has been successfully used for the treatment of the inflammatory skin disease psoriasis [272, 273]. An oral formulation of fumaric acid esters, with DMF being the main component, was registered by the German drug administration under the name Fumaderm and has become a standard drug for the systemic therapy of psoriasis in Germany [267]. In studies involving patients with relapsing-remitting multiple sclerosis, orally administered DMF or BG-12, a new oral formulation of DMF, reduced the appearance of new inflammatory lesions in the CNS [274, 275]. In accordance with these data, DMF was shown to have significant neuroprotective effects in experimental autoimmune encephalomyelitis (EAE), a mouse model of multiple sclerosis [276], as well as in transgenic mouse models of Huntington's disease [277]. Moreover, DMF has cardioprotective effects [278] and was also described to reduce melanoma growth and metastasis in animal models [279]. Recently, DMF has been approved under the name Tecfidera for the treatment of relapsing multiple sclerosis by the U.S. Food and Drug Administration (FDA) [205]. Whereas the exact mechanisms, by which DMF exerts its clinical efficacy, remain to be clarified, the effects are believed to be at least in part mediated through Nrf2 activation [205].

One of the most promising chemical Nrf2 inducers is CDDO, derived from oleanolic acid, which has antioxidant and anticancer properties. The methyl ester derivate (CDDO-Me) is a potent inducer of Nrf2 already at low nanomolecular concentrations [280]. CDDO-Me has been studied in clinical trials, under the generic name bardoxolone methyl, to assess its potential for the treatment of a variety of disorders. CDDO-Me entered phase III clinical trials for the treatment of diabetes-associated chronic kidney disease, although this trial was stopped due to toxicity concerns [262, 281].

The electrophilic inducers described above are all cysteine reactive compounds, therefore off-target effects may be anticipated, because the agents can target cysteine residues in multiple proteins. For example, SFN was shown to target NF-κB [282] as well as both Jun and Fos of the AP1 complex [283]. Proteomic and other analyses revealed that synthetic CDDOs target a range of proteins containing reactive cysteine residues [206, 284]. However, the effects on Nrf2 induction by these compounds are often observed at a lower concentration range, suggesting that Keap1 is a more accessible, reactive or sensitive target to these agents [263].

In addition to electrophilic inducers, small molecules, that can directly disrupt the Keap1-Nrf2 protein-protein interaction, have been emerging as attractive new inducers of Nrf2 activity [205]. Whereas these non-electrophilic compounds are expected to become safe chemical Nrf2 inducers upon optimization, they are still in early development and further improvements are required [205, 263].

1.6 Crosstalk between Nrf2 and Nrf2 activators with inflammasomes

Nrf2 and Inflammasomes

As mentioned above, atherosclerosis is a complex, multi-factorial disease and the molecular mechanisms that initiate und sustain the underlying inflammatory process are incompletely understood. It is known that IL-1 conveys many atherogenic effects and IL-1 signalling severely enhances atherosclerosis and vascular inflammation [102, 103]. Another atherogenic factor is oxidative stress and it was shown that oxidized lipids contribute to disease progression [285]. Accordingly, activation of the Nrf2 pathway was considered to protect from atherosclerosis by inducing the expression of antioxidant proteins [286-288]. Paradoxically, studies using Nrf2 KO mice, reported that Nrf2 has pro-atherogenic effects in ApoE KO mice [247-250]. In one of the latter studies it was suggested that the pro-atherogenic function of Nrf2 was due to cholesterol crystal-induced production of the pro-inflammatory cytokines IL-β and –α, which is dependent on Nrf2 expression [249]. This study describes for the first time a function of Nrf2 as a positive regulator of inflammasome activation. The authors demonstrated that Nrf2 expression is required for cholesterol crystal-induced NLRP3 inflammasome activation [249]. Three years later, it was reported that Nrf2 expression is required for NLRP3 and AIM2, but not NLRC4 inflammasome activation in mice and murine cells [289]. The pro-inflammatory effect of Nrf2 is an unexpected finding; on the one hand, Nrf2 positively regulates the expression of antioxidant proteins, such as ROS detoxifying enzymes, and on the other hand, oxidative stress, particularly mitochondrial ROS, is required for NLRP3 inflammasome activation. The molecular mechanisms underlying the crosstalk between inflammasomes and Nrf2 are not known. However, according to the function of Nrf2 as a transcription factor, a role for Nrf2 target genes in inflammasome activation is discussed [249, 289].

Nrf2 activators and inflammasomes

As already described above, Nrf2 activating compounds are considered to have Nrf2-independent anti-inflammatory effects, which are less well characterised. Several publications demonstrate an anti-inflammatory role for Nrf2 activators by inhibition of inflammasome activation. For example, it has been reported that fumaric acid esters prevent pyroptosis in the human monocytic cell line THP-1 [290]. Curcumin, a phenolic natural compound derived from the plant *Curcuma longa*, is an established Nrf2 activator and blocks NLRP3 inflammasome activation and septic shock [291]. Also arsenic species were shown to inhibit several inflammasomes [292]. In addition, treatment of macrophages with the Nrf2 activator 15d-PGJ_2

inhibits inflammasome activation very efficiently [293]. The same research group identified in a more recent study SFN as an inhibitor of multiple inflammasomes independently of Nrf2 [294]. However, the molecular mechanisms underlying inflammasome inhibition by Nrf2 activators are completely unknown.

1.7 Aims of this PhD thesis

Although inflammasomes have been the subject of intensive research for more than a decade, the exact mechanisms of inflammasome activation are still poorly understood. We and others have identified that expression of the transcription factor Nrf2 supports inflammasome activation; however, the underlying molecular events are unknown.

The aim of this thesis was to study the crosstalk between Nrf2 and Nrf2 activating compounds with inflammasomes. The first task was to investigate the role of Nrf2 in inflammasome activation in human cells, and in murine cells in vitro and in vivo. Since Nrf2 is a transcription factor, a possible involvement of Nrf2-induced gene expression in NLRP3 inflammasome regulation should be examined.

The second goal of this thesis was to analyse the effect of Nrf2 activating compounds on inflammasome activation in different cell types and in various mouse models, and the role of Nrf2 target genes.

Finally, the question, which molecular mechanisms underlie the crosstalk between Nrf2 and inflammasomes, should be addressed.

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Chapter 2 – Materials and Methods

General remarks

Material and methods are adapted from the dissertation of Gerhard Strittmatter (ETH Diss.-No 22564). Standard methods are described only briefly, but modifications of commonly used protocols are stated. Deviations from protocols from single experiments can be found in the respective figure legends.

2.1 Materials

2.1.1 Chemicals and other materials

Acetic acid	FLUKA CHEMIE, *Buchs, Switzerland*
Acetone	MERCK, *Darmstadt, Germany*
Acrylamide/bisacrylamide (30:0.8)	ROT, *Karlsruhe, Germany*
Adenosine triphosphate	SIGMA, *Munich, Germany*
AEBSF	SIGMA, *Munich, Germany*
Agar	DIFCO, *Detroit, US-MI*
Agarose	CAMBREX, *East Rutherford, US-NJ*
Ammonium persulfate (APS)	SIGMA, *Munich, Germany*
Ammonium pyrrolidinedithiocarbamate (PDTC)	SIGMA, *Munich, Germany*
Ampicillin	SIGMA, *Munich, Germany*
ANTI-FLAG®M2 Affinity Gel	SIGMA, *Munich, Germany*
Aprotinin	SIGMA, *Munich, Germany*
BAPTA-AM	SIGMA, *Munich, Germany*
BBL™Trypticase™ Soy Broth	BD, *Franklin Lakes, US-NJ*
Bovine serum albumin (BSA)	SIGMA, *Munich, Germany*
Bromophenol blue	SIGMA, *Munich, Germany*
BSA Fraction V	PAA, *Pasching, Austria*
Calcium chloride ($CaCl_2$)	FLUKA CHEMIE, *Buchs, Switzerland*
Chloroform	FLUKA CHEMIE, *Buchs, Switzerland*
Complete proteinase inhibitor cocktail	ROCHE, *Rotkreuz, Switzerland*
Cycloheximide	SIGMA, *Munich, Germany*
Deoxynucleotide triphosphates (dNTPs)	ROCHE, *Rotkreuz, Switzerland*
Dimethyl fumarate (DMF)	SIGMA, *Munich, Germany*
Dimethylsulfoxide (DMSO)	MERCK, *Darmstadt, Switzerland*
Dinitrofluorobenzene (DNFB)	SIGMA, *Munich, Germany*
Dithiothreitol (DTT)	SIGMA, *Munich, Germany*

Ethanol (EtOH)	SIGMA, *Munich, Germany*
Ethylenediaminetetraacetic acid (EDTA)	SIGMA, *Munich, Germany*
FastStart Universal SYBR Green Master	ROCHE, *Rotkreuz, Switzerland*
Ficoll-Paque PLUS	GE HEALTHCARE, *Little Chalfont, UK*
Flasks and (multiwell) dishes for cell culture	NUNC, *Roskilde, Denmark*
GelGreen	BIOTIUM, *Hayward, US-CA*
Glycerol	MERCK, Darmstadt, Germany
Glycine	ROT, Karlsruhe, Germany
Hydrogen chloride (HCl)	FLUKA CHEMIE, *Buchs, Switzerland*
Isopropanol	SIGMA, *Munich, Germany*
Ketamine	VETERINARIA, Zürich, Switzerland
Magnesium chloride ($MgCl_2$)	FLUKA CHEMIE, *Buchs, Switzerland*
Methanol	FLUKA CHEMIE, *Buchs, Switzerland*
Methyl cellulose (Methocel)	SIGMA, *Munich, Germany*
MG132	SIGMA, *Munich, Germany*
Microfilter units (0.22/0.45 µm)	MILLIPORE, *Billerica, US-MA*
Milk powder (low fat)	MIGROS, *Zurich, Switzerland*
Nigericin	ENZO LIFE SCIENCES, *New York, US-NY*
Nitrocellulose membrane (Protran 0.2 µm)	GE HEALTHCARE, *Little Chalfont, UK*
Nonidet P-40	SIGMA, *Munich, Germany*
Olive oil	MIGROS, *Zurich, Switzerland*
Paraformaldehyde (PFA)	SIGMA, *Munich, Germany*
Phorbol 12-myristate 13-acetate (PMA)	SIGMA, *Munich, Germany*
Poly(dA:dT)	SIGMA, *Munich, Germany*
Potassium chloride (KCl)	FLUKA CHEMIE, *Buchs, Switzerland*
Potassium dihydrogen phosphate (KH_2PO_4)	FLUKA CHEMIE, *Buchs, Switzerland*
Propidium iodide	ROCHE, *Rotkreuz, Switzerland*
Protein A sepharose (PAS)	GE HEALTHCARE, *Little Chalfont, UK*
Saponin	SIGMA, *Munich, Germany*
Sodium chloride (NaCl)	FLUKA CHEMIE, *Buchs, Switzerland*
Sodium dodecyl sulphate (SDS)	SIGMA, *Munich, Germany*
Sodium hydrogen phosphate (Na_2HPO_4)	FLUKA CHEMIE, *Buchs, Switzerland*
Sodium hydroxide (NaOH)	FLUKA CHEMIE, *Buchs, Switzerland*
Sulforaphane (SFN)	SIGMA, *Munich, Germany*
tert-Butylhydroquinone (tBHQ)	SIGMA, *Munich, Germany*
Tetramethylethylendiamine (TEMED)	SIGMA, *Munich, Germany*

TMB ELISA Substrate Solution	eBIOSCIENCES, *San Diego, USA-CA*
Triton-X 100	SIGMA, *Munich, Germany*
TRIZMA Base (TRIS)	SIGMA, *Munich, Germany*
TRIzol®	THERMO FISHER SCIENTIFIC, *Waltham, US-MA*
Tryptone	DIFCO, *Detroit, US-MI*
Tween 20	ROT, *Karlsruhe, Germany*
Ultrapure LPS (upLPS)	INVIVOGEN, *Toulouse, France*
Uric acid	SIGMA, *Munich, Germany*
Whatman 3MM paper	WHATMAN, *Maidstone, England*
Xylazine	VETERINARIA, *Zürich, Switzerland*
Yeast extract	DIFCO, *Detroit, US-MI*
Zymosan-A from *Saccharomyces cerevisiae*	SIGMA, *Munich, Germany*
β-mercaptoethanol	SIGMA, *Munich, Germany*
ε-amino caproic acid	SIGMA, *Munich, Germany*
15-deoxy-Δ-^{12,14}-prostaglandin J_2 (15d-PGJ_2)	SIGMA, *Munich, Germany*

Preparation of MSU crystals

Monosodium urate (MSU) crystals (provided by G. Fenini, *University Hospital Zurich)* were prepared by crystallization of a supersaturated solution of uric acid under mildly basic conditions. Briefly, uric acid (250 mg) was added to a solution of NaOH (33 mM, 45 ml), the solution was boiled until the uric acid was dissolved and passed through a filter. NaCl solution (5 M, 1 ml) was added and crystallization was performed at 4° C. Crystals were filtered, then dried using a speedvac, weighted and autoclaved.

2.1.2 Protein and DNA size standards

Prestained protein molecular weight marker	THERMO FISHER SCIENTIFIC, *Waltham, US-MA*
GeneRuler DNA ladder mix	THERMO FISHER SCIENTIFIC, *Waltham, US-MA*
GeneRuler 100 bp DNA ladder	THERMO FISHER SCIENTIFIC, *Waltham, US-MA*

2.1.3 Cell culture media, additives and reagents

Anti-anti antibiotic-antimycotic (A/A)	GIBCO BRL, *Paisley, Scotland*
Blasticidin	INVIVOGEN, *Toulouse, France*
DMEM (order no. 41966-029)	GIBCO BRL, *Paisley, Scotland*
Doxycycline	SIGMA, *Munich, Germany*
Fetal bovine serum (FBS), heat inactivated	GIBCO BRL, *Paisley, Scotland*
GlutaMAX	GIBCO BRL, *Paisley, Scotland*

Keratinocyte-SFM, EGF, BPE (order no. 17005)	GIBCO BRL, *Paisley, Scotland*
OptiMEM (order no. 11058-021)	GIBCO BRL, *Paisley, Scotland*
Puromycin	SIGMA, *Munich, Germany*
Red Blood Cell Lysis Buffer (order no. R7757)	SIGMA, *Munich, Germany*
RPMI 1640 (order no. 61870)	GIBCO BRL, *Paisley, Scotland*
Sodium pyruvate 100 mM	GIBCO BRL, *Paisley, Scotland*

2.1.4 Transfection reagents

INTERFERin	POLYPLUS, *Illkirch, France*
Lipofectamine 2000	INVITROGEN, *Basel, Switzerland*
TransIT-X2	MIRUS BIO LLC, *Madison, US-WI*

2.1.5 Kits

CytoTox 96 LDH assay kit	PROMEGA, *Madison, US-WC*
ECL plus western blotting detection system kit	AMERSHAM, *Uppsala, Sweden*
ELISA development kits for human and mouse IL-1β	R&D SYSTEMS, *Minneapolis, US-MN*
Gel extraction/PCR purification kit	MACHEREY-NAGEL, *Düren, Germany*
NBT/BCIP substrate kit	PROMEGA, *Madison, US-WC*
Plasmid Midi/Maxi kit	QIAGEN, *Düsseldorf, Germany*
RevertAid First Strand cDNA Synthesis Kit	THERMO FISHER SCIENTIFIC, *Waltham, US-MA*
RNeasy Mini kit	QIAGEN, *Düsseldorf, Germany*

2.1.6 Enzymes

Accutase	PAA, *Parsching, Austria*
Calf intestinal phosphatase (CIP)	NEB, *Ipswich, US-MA*
DNA polymerase I (Klenow)	NEB, *Ipswich, US-MA*
DNA-restriction enzyme buffers	NEB, *Ipswich, US-MA*
DNA-restriction enzymes	NEB, *Ipswich, US-MA*
Gateway LR Clonase II Enzyme mix	LIFE TECHNOLOGIES, *Carlsbad, US-CA*
Mung Bean Nuclease	NEB, *Ipswich, US-MA*
Pfu DNA polymerase	THERMO FISHER SCIENTIFIC, *Waltham, US-MA*
Proteinase K	ROCHE, *Rotkreuz, Switzerland*
T4 DNA ligase	NEB, *Ipswich, US-MA*
T4 Polynucleotide Kinase (PNK)	NEB, *Ipswich, US-MA*
Taq DNA polymerase	THERMO FISHER SCIENTIFIC, *Waltham, US-MA*

Taq DNA polymerase	ROCHE, *Rotkreuz, Switzerland*
Trypsin	GIBCO BRL, *Paisley, Scotland*

2.1.7 Primary Antibodies

α-Asc (rabbit, polyclonal, AL177)	ADIPOGEN, *Liestal, Switzerland*
α-Caspase-1 (CARD, rabbit, polyclonal, sc-622; used for detection of procaspase-1 and CARD)	SANTA CRUZ, *Santa Cruz, US-CA*
α-Caspase-1 (p10, rabbit, polyclonal, sc-514)	SANTA CRUZ, *Santa Cruz, US-CA*
α-Cullin 3 (rabbit, polyclonal)	Prof. Dr. M. Peter, *ETH Zurich, Switzerland*
α-Flag M2 (mouse, monoclonal, F1804)	SIGMA, *Munich, Germany*
α-HA (rabbit, polyclonal, sc-805)	SANTA CRUZ, *Santa Cruz, US-CA*
α-IL-18 (rabbit, polyclonal, PM014)	MBL, *Woburn, US-MA*
α-IL-1β (human) (mouse, monoclonal, MAB201)	R&D SYSTEMS, *Minneapolis, US-MN*
α-IL-1β (mouse) (goat, polyclonal, AF-401-NA)	R&D SYSTEMS, *Minneapolis, US-MN*
α-Keap1 (goat, polyclonal, sc-15246)	SANTA CRUZ, *Santa Cruz, US-CA*
α-Lamin A/C (goat, polyclonal, sc-6215)	SANTA CRUZ, *Santa Cruz, US-CA*
α-Ly6G/Ly6C (Gr1) (mouse) (rat, monoclonal, APC-conjugated, 108412)	BIOLEGEND, *San Diego, USA-CA*
α-Myc (mouse, monoclonal, 631206)	CLONTECH LABORATORIES, *Mountain View, US-CA*
α-Neutrophil [7/4] (mouse) (rat, monoclonal, FITC-conjugated, ab53453)	ABCAM, *Cambridge, UK*
α-NLRP3 (mouse, monoclonal, Cryo-2)	ADIPOGEN, *Liestal, Switzerland*
α-NQO1 (mouse, monoclonal, ab28947)	ABCAM, *Cambridge, UK*
α-Nrf2 (rabbit, polyclonal, sc-13032)	SANTA CRUZ, *Santa Cruz, US-CA*
α-Nrf2 (rabbit, polyclonal, sc-722)	SANTA CRUZ, *Santa Cruz, US-CA*
α-Rbx1 (ROC1) (rabbit, monoclonal, ab133565)	ABCAM, *Cambridge, UK*
α-α-Tubulin (mouse, monoclonal, CP06)	CALBIOCHEM, *Darmstadt, Germany*
α-β-Actin (mouse, monoclonal, AC-15)	SIGMA, *Munich, Germany*

2.1.8 Secondary Antibodies

Manufacturers' order number is indicated in parentheses.

α-Goat IgG (AP-conjugated, V1151)	PROMEGA, *Madison, US-WC*
α-Goat IgG (HRP-conjugated, 401515)	CALBIOCHEM, *Darmstadt, Germany*
α-Mouse IgG (AP-conjugated, S372B)	PROMEGA, *Madison, US-WC*
α-Rabbit IgG (AP-conjugated, S373B)	PROMEGA, *Madison, US-WC*

2.1.9 siRNAs

All siRNA duplexes were purchased from Sigma *(Munich, Germany)* or Microsynth *(Balgach, Switzerland).*

Caspase-1 (1)

Sense	5'-r(GGC AGA GAU UUA UCC AAU A)dTdT-3'
Antisense	5'-r(UAU UGG AUA AAU CUC UGC C)dGdA-3'

Caspase-1 (2)

Sense	5'-r(AAG AGA UCC UUC UGU AAA GGU)dTdT-3'
Antisense	5'-r(ACC UUU ACA GAA GGA UCU CUU)dTdT-3'

Cullin 3 (1)

Sense	5'-r(CAA CAC UUG GCA AGG AGA C)dTdT-3'
Antisense	5'-r(GUC UCC UUG CCA AGU GUU G)dTdT-3'

Cullin 3 (2)

Sense	5'-r(AAC AAC UUU CUU CAA ACG CUA)dTdT-3'
Antisense	5'-r(UAG CGU UUG AAG AAA GUU GUU)dTdT-3'

Keap1 (1)

Sense	5'-r(GUG UUA CGA CCC AGA UAC A)dTdT-3'
Antisense	5'-r(UGU AUC UGG GUC GUA ACA C)dTdT-3'

Keap1 (2)

Sense	5'-r(CCU UAA UUC AGC UGA GUG U)dTdT-3'
Antisense	5'-r(ACA CUC AGC UGA AUU AAG G)dTdT-3'

Nrf2 (1)

Sense	5'-r(GAG AAA GAA UUG CCU GUA A)dTdT-3'
Antisense	5'-r(UUA CAG GCA AUU CUU UCU C)dTdT-3'

Nrf2 (2)

Sense	5'-r(CCU UAU UCU CCU AGU GAA U)dTdT-3'
Antisense	5'-r(AUU CAG UAG GAG AAU AAG G)dTdT-3'

Rbx1 (1)

Sense	5’-r(AAG AAG CGC UUU GAA GUG AAA)dTdT-3’
Antisense	5’-r(UUU CAC UUC AAA GUG CUU CUU)dTdT-3’

Rbx1 (2)

Sense	5’-r(CUG CUG UUA CCU AAU UAC AAA)dTdT-3’
Antisense	5’-r(UUU GUA AUU AGG UAA CAG CAG)dTdT-3’

Scrambled (ctrl.)

Sense	5’-r(UUC UCC GAA CGU GUC ACG U)dTdT-3’
Antisense	5’-r(ACG UGA CAC GUU CGG AGA A)dTdT-3’

VEGF

Sense	5’-r(CUG AUG AGA UCG AGU ACA U)dTdT-3’
Antisense	5’-r(AUG UAC UCG AUC UCA UCA G)dTdT-3’

2.1.10 Primers

Primers for mouse genotyping

Nrf2 KO

Forward	5’-GCC TGA GAG CTG TAG GCC-3’
Reverse wild-type	5’-GGA ATG GAA AAT AGC TCC TGC C-3’
Reverse KO	5’-GGG TTT TCC CAG TCA CGA C-3’

Primers for real-time PCR

Human primers

GAPDH

Forward	5’-AAG GTC GGA GTC AAC GGA TT-3’
Reverse	5’-CTC CTG GAA GAT GGT GAT GG-3’

GCLC

Forward	5’-GGA AGG AAG GTG TGT TTC CTG G-3’
Reverse	5’-ACT CCC TCA TCC ATC TGG CAA-3’

GCLM

Forward	5’-CCA GAT GTC TTG GAA TGC ACT G-3’
Reverse	5’-AGG ACT GAA CAG GCC ATG TCA-3’

NQO1

Forward	5’-GTG ATA TTC CAG TTC CCC CTG C-3’
Reverse	5’-AAG CAC TGC CTT CTT ACT CCG G-3’

NRF2

Forward	5’-CCA GGT TGC CCA CAT TC-3’
Reverse	5’-TCC CAA ACT TGG TCA ATG TCC-3’

RPL27 (1)

Forward	5’-TCA CCT AAT GCC CAC AAG GTA-3’
Reverse	5’-CCA CTT GTT CTT GCC TGT CTT-3’

RPL27 (2)

Forward	5’-ATC GCC AAG AGA TCA AAG ATA A-3’
Reverse	5’-TCT GAA GAC ATC CTT ATT GAC G-3’

SRXN1

Forward	5’-CAA GGT GCA GAG CCT CGT-3’
Reverse	5’-GAT GGT CTC TCG CTG CAG TT-3’

Murine primers

Aim2

Forward	5’-GTC ACC AGT TCC TCA GTT GTG-3’
Reverse	5’-CAC CTC CAT TGT CCC TGT TTT AT-3’

Asc

Forward	5’-CTT GTC AGG GGA TGA ACT CAA AA-3’
Reverse	5’-GCC ATA CGA CTC CAG ATA GTA GC-3’

Caspase-1	
Forward	5’-ACA AGG CAC GGG ACC TAT G-3’
Reverse	5’-TCC CAG TCA GTC CTG GAA ATG-3’

Gclm	
Forward	5’-TCC CAT GCA GTG GAG AAG AT-3’
Reverse	5’-AGC TGT GCA ACT CCA AGG AC-3’

Gstp1	
Forward	5’-CCT CTG TCT ACG CAG CAC TGA ATC C-3’
Reverse	5’-TTC CAG CTC TGG CCC TGG TCA G-3’

Nlrc4	
Forward	5’-TTG AAG GCG AGT CTG GCA AAG-3’
Reverse	5’- GGC GCT TCT CAG GTG GAT G-3’

Nlrp3	
Forward	5’-ATT ACC CGC CCG AGA AAG G-3’
Reverse	5’-TCG CAG CAA AGA TCC ACA CAG-3’

pro-IL-1β	
Forward	5’-TGG ACC TTC CAG GAT GAG GAC A-3’
Reverse	5’-GTT CAT CTC GGA GCC TGT AGT G-3’

RPS29	
Forward	5’-GGT CAC CAG CAG CTC TAC TG-3’
Reverse	5’-GTC CAA CTT AAT GAA GCC TAT GTC C-3’

Srxn1	
Forward	5’-CGG TGC ACA ACG TAC CAA T-3’
Reverse	5’-TTG ATC CAG AGG ACG TCG AT-3’

2.1.11 Plasmids

Vectors

pBluescript II KS/SK (+)	STRATAGENE, *La Jolla, US-CA*
pcDNA3	INVITROGEN, *Basel, Switzerland*
pCG	described by Beer *et al.* [1]
pCMV	CLONTECH, *Palo Alto, US-CA*
pMD2.G	Prof. Dr. J. Tschopp, *University of Lausanne, Switzerland*
psPAX2	Prof. Dr. J. Tschopp, *University of Lausanne, Switzerland*
pLenti CMVtight Puro DEST (w768-1) (Addgene: 26430)	described by Campeau *et al.* [2]
pLenti CMV rtTA3 Blast (w756-1) (Addgene: 26429)	described by Campeau *et al.* [2]
pENTR1A no ccDB (w48-1) (Addgene: 17398)	described by Campeau *et al.* [2]
pLenti CMVtight eGFP Puro (w771-1) (Addgene: 26431)	described by Campeau *et al.* [2]

Expression constructs

To generate plasmids for protein expression, open reading frames were subcloned into the vector plasmid and sequenced. Nrf2_NLS (Δ nuclear localization sequence) was generated using a protocol for deletion mutagenesis by overlap extension PCR. Keap1 C151W was generated by site-directed mutagenesis. Both methods are described in chapter 2.2.4. Lentiviral vectors were generated by subcloning into pENTR1A no ccDB (w48-1) and cloned into the destination vector pLenti CMVtight Puro DEST (w768-1) by recombination using the LR clonase II.

pIRESneo Nrf2	Prof. Dr. S. Werner, *ETH Zurich, Switzerland*
pIRESneo caNrf2	Prof. Dr. S. Werner, *ETH Zurich, Switzerland;* described by Schafer *et al.* [3]
pBluescript KS (+) dnNrf2	Prof. Dr. S. Werner, *ETH Zurich, Switzerland;* described by Alam *et al.* [4]
pCMV6-XL5 Keap1	ORIGENE, *Rockville, US-MD*
pcDNA3 Cullin3	Prof. Dr. M. Peter, *ETH Zurich, Switzerland*
pcDNA3 Rbx1	Prof. Dr. M. Peter, *ETH Zurich, Switzerland*
pCMV Nrf2_NLS (Δ nuclear localization sequence)	described by Jain *et al.* [5]

pCMV Keap1 C151W described by Zhang *et al.* [6]

Insert (GeneBank accession no.)	**Vector**	**Tag**	
Nrf2 (wt) (NM_001145412)	pCMV	5'-HA	
Nrf2 (wt)	pLenti CMVtight Puro DEST (w768-1)	-	
dnNrf2	pLenti CMVtight Puro DEST (w768-1)	-	
caNrf2	pLenti CMVtight Puro DEST (w768-1)	-	
Nrf2_NLS	pLenti CMVtight Puro DEST (w768-1)	-	nt 1472-1526 (Δ 54 nt)
Keap1 (wt) (NM_012289)	pLenti CMVtight Puro DEST (w768-1)	-	
Keap1 C151W	pLenti CMVtight Puro DEST (w768-1)	-	nt 451-453 (tgt→tgg)
pro-caspase 1 (wt) (NM_033292, nt 18-1232)	pLenti CMVtight Puro DEST (w768-1)	3'-FLAG	described by Sollberger *et al.* [7]

2.1.12 Bacterial strains

E. coli XL1-Blue	STRATAGENE, *La Jolla, US-CA*
Stbl3 bacterial strain	THERMO FISHER SCIENTIFIC, *Waltham, US-MA*
Listeria monocytogenes	PD Dr. Pal Johansen, *University Hospital Zurich, Switzerland*

2.1.13 Eukaryotic cell lines

COS-1	ATCC, *Manassas, US-VA*
THP-1	ATCC, *Manassas, US-VA*
HEK293T	ATCC, *Manassas, US-VA*

2.1.14 Standard buffers

Co-IP buffer

NaCl	100 mM
EDTA	15 mM
Triton-X100	0.1 % (w/v)
TRIS/HCl (pH 9.5)	50 mM

PBS

NaCl	140 mM
KCl	30 mM
Na_2HPO_4	6.5 mM
KH_2PO_4	1.5 mM

Adjusted to pH 7.4

PBST

PBS	
Tween	0.1 %(v/v)

2.2 Methods

2.2.1 Cell biological methods

Cultivation and storage of eukaryotic cells

Cells were grown in petri dishes or flasks as adherence or suspension cultures. They were incubated in a CO_2 incubator (37 °C, 95 % relative humidity, 5 % CO_2), cultivated in growth medium and propagated as specified in the following table:

Cell type	Growth medium		Propagation	Passages
	Basal medium	Supplements		
COS-1	DMEM	10 % FBS, 1 % A/A	Detaching and splitting 1/10 when confluency was reached	~P10-P35
HEK293T	RPMI 1640	10 % FBS, 1 % A/A, sodium pyruvate (1 mM), GlutaMAX (1:100)	Splitting 1/10-1/25 when confluency was reached	~P10-P35
THP-1 macrophages	RPMI 1640	10 % FBS, 1 % A/A	Splitting 1/10-1/25 at a density of about 10^6 cells/ml with a medium change every third day	~P10-P35
Human primary keratinocytes	Keratinocyte-SFM	EGF, BPE (aliquots provided by manufacturer)	Detaching and splitting 1/3-1/5 at a density of about 90 %, medium change every second day	Up to P5
Murine primary macrophages	RPMI 1640	10 % FBS, 1 % A/A	No propagation	
Murine bone marrow-derived dendritic cells	RPMI 1640	10 % FBS, 1 % A/A, GlutaMAX (1:100), mGM-CSF (1000 U/ml), 50 µM β-mercaptoethanol	Differentiation for 10 days, subsequent detaching and seeding without mGM-CSF, no further propagation	

To passage adherent cells, they were washed three times with PBS. To detach from the plastic surface, they were incubated 4-5 min with a suitable amount of trypsin (0.05 % [w/v] in PBS) at 37 °C in the incubator. The trypsinization was stopped by resuspending the cells in fresh medium containing at least 10 % FBS and subsequent seeding. For human primary keratinocytes trypsinization was stopped by resuspending the cells in fresh medium and addition of a volume of FBS corresponding to the amount of trypsin. The cells were then

centrifuged (200 x g, 3 min, room temperature) and resuspended in fresh medium with subsequent seeding.

For long-term storage, cells were suspended, centrifuged (200 x g, 4 min, room temperature) and resuspended in growth medium containing 10 % DMSO. They were slowly frozen at ~ 1 °C/min in cryotubes and stored in liquid nitrogen.

Establishment of primary eukaryotic cell cultures

Primary human keratinocytes

Keratinocytes were isolated from foreskin as described [8]. Biopsies were from different donors at an age of 8-11 years (provided by the Tagesklinik für Kinderchirurgie, *Fällanden, Switzerland*).

Primary mouse macrophages

Adult mice were sacrificed by CO_2 exposure and disinfected with 70 % ethanol. Subsequently, 5 ml of PBS was injected into the peritoneum. The mice were slightly rolled and massaged to bring the cells in suspension. After 5 min, a cut was made in the abdominal skin with a scissor and the peritoneum was punctured carefully. The PBS containing residual peritoneal macrophages was aspirated. The cells were collected, washed once by centrifugation (200 x g) and seeded in RPMI 1640 medium (10 % FBS, 1 % A/A) at required densities for experiments without further propagation.

Primary mouse bone marrow-derived dendritic cells (BMDCs)

Adult mice at an age of 8-12 weeks were sacrificed by CO_2 exposure and disinfected with 70 % ethanol. Femur and tibia were cut with a scissor and flushed with PBS or RPMI 1640 medium. The bone marrow cells were collected, homogenized with a syringe and passed through a cell strainer (70 µm Nylon, BD, *Franklin Lakes, US-NJ*). Cells were washed once by centrifugation (200 x g, 5 min, room temperature), treated with red blood cell lysis buffer (SIGMA, *Munich, Germany*), followed by washing and counting. Subsequently, the cells were seeded in 10-cm dishes at a density of 5 x 10^6 cells per dish and differentiated for 10 days in RPMI 1640 medium (10 % FBS, 1 % A/A, GlutaMAX (1:100), mGM-CSF (1000 U/ml), 50 µM β-mercaptoethanol). Fresh medium was added at day 3, day 6 and day 8. After differentiation, the cells were detached from the plastic surface by scratching, washed once by centrifugation, counted and seeded in RPMI 1640 medium (10 % FBS, 1 % A/A, GlutaMAX [1:100]) at required densities for experiments.

Isolation of peripheral blood mononuclear cells (PBMCs) from human blood

Blood was diluted with 2 volumes of PBS, slowly layered onto Ficoll-Paque PLUS (GE HEALTHCARE, *Little Chalfont, UK*) and centrifuged (200 x g, 20 min, room temperature) at slow acceleration and breaking rate. PBMCs were collected from inter-phase, washed twice with PBS and treated with red blood cell lysis buffer (SIGMA, *Munich, Germany*). Subsequently, PBMCs were again washed, resuspended in RPMI 1640 medium, counted and seeded at required densities.

Transient transfection of cells with plasmids

Eukaryotic cells were transfected using the TransIT-X2 reagent (MIRUS BIO LLC, *Madison, US-WI*) or Lipofectamine 2000 reagent (INVITROGEN, *Basel, Switzerland*) according to the manufacturers' instructions. For HEK 293T and COS-1 cells 0.01-8 μg plasmid DNA per well of a 6-well plate was diluted in OptiMEM and cells were grown in OptiMEM medium after transfection. For human primary keratinocytes 0.1-2 μg plasmid DNA was diluted in pure keratinocyte-SFM. The cells were transfected in keratinocyte-SFM containing EGF and BPE and the medium was changed 5 h after transfection.

siRNA transfection

Cells were transfected with siRNAs using INTERFERin (POLYPLUS, *Illkirch, France*) according to the manufacturers' instructions. Concentration of siRNA was adjusted to 10 nM and INTERFERin was adjusted to 1 μl/ml. Final culture volume was 1.2 ml per well of a 12-well plate.

Transfection of cells with poly(dA:dT)

Cells were transfected with poly(dA:dT) (SIGMA, *Munich, Germany*) using Lipofectamine 2000 (INVITROGEN, *Basel, Switzerland*) according to the manufacturers' instructions. Concentration of poly(dA:dT) was adjusted to 0.5-2 μg/ml and Lipofectamine to 2 μl/ml. Final culture volume was 1 ml per well of a 12-well plate.

Inducible overexpression of proteins from primary keratinocytes using a lentiviral system

To generate keratinocytes, which overexpress a gene of interest in an inducible manner, we used the lentiviral system described by Campeau *et al.* [2]. DNA was cloned with the appropriate restriction enzymes from expression vectors into the pENTR1A no ccDB (w48-1) vector that had been linearized by restriction enzymes. For this purpose, the pENTR1A vector and the flanking restriction sites of the gene of interest were digested. The cleaved pENTR1A vector was treated with calf intestinal phosphatase (CIP; described in 2.2.4) followed by purification on a 1 %

agarose gel using the MACHEREY-NAGEL gel extraction kit. DNA insert and vector were ligated by *T4* DNA ligase. Competent bacteria were transformed, single colonies picked, and DNA isolated by miniprep. After sequencing, the gene of interest was subcloned into the lentiviral pLenti CMVtight Puro DEST (w768-1) using the Gateway LR Clonase II enzyme mix (LIFE TECHNOLOGIES, *Carlsbad, US-CA*) according to the manufacturers' instructions. Subsequently, lentivirus was produced, cells infected, and expression induced. See below for a detailed protocol.

Lentivirus production

Lentivirus was produced by transfection of HEK 293T cells with a mix of either the pLenti CMVtight Puro DEST (w768-1) vector encoding the desired gene of interest or pLenti CMV rtTA3 Blast (w756-1) encoding a reverse tetracycline-controlled transactivator 3 (rtTA3) and the two packaging vectors psPAX2 and pMD2.G. The cells were plated in 6-cm dishes at a density of 1.5 x 10^6 cells per dish in DMEM (10 % FBS, no A/A) and transfected the day after seeding. For transfection, the medium was changed to 2.5 ml DMEM (10 % FBS, no A/A). Plasmids were mixed gently in a ratio of 1:3:4 to a total of 8 µg (1 µg psPAX2, 3 µg pMD2.G, and 4 µg pLENTI vector) in 0.6 ml OptiMEM. 18 µl of TransIT-X2 transfection reagent (MIRUS BIO LLC, *Madison, US-WI*) were added and the mix was incubated for 15 min at room temperature.

The transfection mix was added drop wise onto the HEK 293T cells. The day after, 1 ml of DMEM (10 % FBS, no A/A) was added. 44-48 h post transfection, the supernatant of the HEK 293T cells was collected and centrifuged at 200 x g for 4 min at 4 °C before filtration through a 0,45 µm filter. The supernatants of this centrifugation step were distributed to Eppendorf tubes and centrifuged at 17'000 x g for 4-6 h. The resulting supernatant was carefully removed without disturbing the virus pellet and leaving 100-200 µl of medium in the tube. The virus pellet was resuspended in K-SFM with additives avoiding the formation of bubbles and directly added to keratinocytes.

Transduction of keratinocytes and induction of gene expression

HPKs were thawed and seeded in 6-cm dishes in 4 ml of K-SFM with additives. Subsequently, lentivirus-containing supernatants collected from HEK 293T cells (described above) were added: 500 µl of lentivirus-containing supernatants with pLenti CMVtight Puro and the gene of interest and 500 µl of lentivirus-containing supernatants with pLenti CMV rtTA3 Blast.

24 h after transduction, the medium was replaced with 5 ml of K-SFM and the cells were left for another 24 h before applying selection (blasticidin 1 µg/ml and puromycin 0.5 µg/ml). After 24-48 h, transduced HPKs were seeded in 12-well plates. Expression of the gene of interest was induced by adding doxycycline (1 µg/ml) for 20 h the day after. After replacing the medium with 1 ml of K-SFM, the cells treated with the desired stimulus.

CRISPR/Cas9-mediated genome editing in THP-1 cells

This method was established by Dr. G. Strittmatter and G. Fenini (both *University Hospital Zurich*).

CRISPR/Cas9 cloning

gRNAs were designed using an online tool on the web platform Benchling (https://benchling.com). Single-stranded forward and reverse DNA oligos were ordered from Microsynth *(Balgach, Switzerland)* and dissolved in ddH_2O to 100 µM.
The following reaction mix was prepared to phosphorylate and anneal the oligos:

Forward oligo	1.0 µl
Reverse oligo	1.0 µl
10x *T4* NEBuffer	1.0 µl
T4 PNK	1.0 µl
ddH_2O	6.5 µl

The reaction was carried out in a Mastercycler gradient (EPPENDORF, *Hamburg, Germany*) PCR machine using the following program:

30 min	37 °C
5 min	95 °C

Followed by cooling down from 95 °C to 25 °C with ΔT=5 °C/min.
LentiCRISPR v2 vector (Addgene Plasmid #52961) described by Sanjana *et al.* [9] was digested using the restriction enzyme BsmBI according to the following settings:

DNA	5.0 µg
BsmBI	2.5 µl
10x NEBuffer 3.1	5.0 µl
ddH_2O	add. 50 µl

60 min	37 °C
20 min	80 °C (heat inactivation)

Afterwards, the digested vector was treated with calf intestinal phosphatase (CIP) by adding 2 µl of CIP to 50 µl of digestion reaction and incubation for 60 min at 37 °C. The digested vector was purified on a 0.8 % agarose gel using the MACHEREY-NAGEL gel extraction kit. The annealed

phosphorylated oligos were diluted 1:200 in ddH_2O and ligated into BsmBI-digested and CIP-dephosphorylated LentiCRISPR v2 according to the following protocol:

LentiCRISPR v2 (BsmBI-digested and CIP-treated)	50 ng
Phosphorylated oligos (1:200 dilution)	1.0 µl
10x *T4* ligase buffer	2.0 µl
T4 ligase	1.0 µl
ddH_2O	add. 19 µl

16 h (over night)	16 °C
10 min	65 °C
∞	4 °C

Stbl3 bacteria were transformed with 5-10 µl of ligated lentiCRISP v2 and plated on LB/Ampicillin agar plates. Colonies were picked followed by small scale plasmid preparation (described in 2.2.4).

Lentivirus production

Lentivirus was produced by transfection of HEK 293T cells with a mix of ligated lentiCRISP v2 and the two packaging vectors psPAX2 and pMD2.G. Cells (7.6×10^4 cells/cm^2) were plated in 6-well plates in RPMI 1640 medium (10 % FBS, sodium pyruvate (1 mM), GlutaMAX [1:100]) without antibiotics (RPMI complete [no A/A]) and transfected the following day.

For transfection, the medium was changed to 2.5 ml RPMI complete (no A/A). Plasmids were mixed gently in a molar ratio of 1:1.65:3.5 to a total of 2.96 µg (0.25 µg psPAX2, 0.75 µg pMD2.G, and 1.96 µg pLENTI vector) in 0.25 ml of OptiMEM. 7.5 µl of TransIT-X2 transfection reagent (MIRUS BIO LLC, *Madison, US-WI*) were added and the mix was incubated for 15 min at room temperature.

The transfection mix was added dropwise onto the HEK 293T cells. The day after, fresh medium was added. 44-48 h post transfection, the supernatant of the HEK 293T cells was collected and centrifuged at 200 x g for 4 min at 4 °C before filtration through a 0,45 µm filter. One virus pellet per 6-well plate was added to 10^6 THP-1 cells in RPMI complete (no A/A). 24 h after transduction, medium was changed to RPMI 1640 (10 % FBS, sodium pyruvate (1 mM), GlutaMAX (1:100), 1 % A/A). After additional 24 h, puromycin was added to a final concentration of 5 µg/ml for selection.

Irradiation of cells with UV light

60-30 min after medium change, cells were irradiated with the desired dose of UVB. For this purpose, the cover of the culture dish was removed and the cells were exposed to a UV source (UV802L, WALDMANN, *Villingen-Schwenningen, Germany*). For a dose of 86.4 mJ/cm^2 the distance to the irradiation source was 7 cm and the irradiation time was 7 min 13 sec (UV802L). Control cells were covered with aluminium foil and placed under the UV lamp.

Stimulation of protein secretion from cultured cells

Murine BMDCs or murine macrophages (2-5 x 10^5 cells/cm^2) were grown in 24-well plates (ELISA) or 6-well plates (Western blot), primed with 0.1 μg/ml upLPS for 16 h and subsequently stimulated with nigericin (20 μM), zymosan (20 μg/ml), MSU (150 μg/ml), or were transfected with poly(dA:dT) (1 mg/ml) for 6 h. For stimulation with ATP (5 mM), medium was changed after 30 min and conditioned medium was harvested after 6 h.

Human keratinocytes were transfected with siRNA and 48-72 h after transfection, the medium was changed if not otherwise indicated and cells were irradiated with UVB at a dose of 86.4 mJ/cm^2 or stimulated with nigericin (5 μM). For treatment with Nrf2-activating compounds, the medium was changed before addition of the activator; cells were irradiated or stimulated with nigericin (5 μM) 15-45 min later. Secreted proteins were collected after 5 h.

THP-1 cells (3 x 10^4 cells/cm^2) were grown in 24-well plates (ELISA) or 6-well plates (Western blot) and differentiated with PMA (27 nM) for 3 days. After a medium change, differentiated cells were primed with 0.1 μg/ml upLPS for 16 h and subsequently stimulated with nigericin (5 μM) or MSU (150 μg/ml). Conditioned medium was harvested after 6 h.

Peripheral blood mononuclear cells (3 x 10^5 cells/cm^2) isolated from human blood were grown in flat-bottom 96-well plates, primed with 0.1 μg/ml upLPS for 16 h and subsequently stimulated with nigericin (5 μM).

Measurement of protein release from stimulated cells

After stimulation of cells, the supernatant was removed and centrifuged at 1'500 x g for 3 min at 4 °C. Adherent cells were lysed in culture medium containing 2 or 10 % Triton-X100 for 10 min. For Western blotting proteins in the supernatant were precipitated with acetone (2.5 volumes of acetone). For ELISA and LDH measurements supernatants and lysates were diluted as desired. Each experiment was performed with triplicate dishes. ELISA and LDH measurements were performed in 96-well plates. Cytokine levels were determined by ELISA kits against human and murine IL-1β (R&D SYSTEMS, *Minneapolis, US-MN*) and TMB ELISA Substrate Solution (eBIOSCIENCES, *San Diego, USA-CA*). LDH activity was measured by the CytoTox 96 assay (PROMEGA, *Madison, US-WC*). All assays were performed according to the manufacturers'

instructions. Cytokine concentration and optical densities after stop of the chromogenic reactions were measured by the Spectra Max190 plate reader (MOLECULAR DEVICES, *Sunnyvale, CA*) and using the Softmax software (version 4.3.1, provided by the device manufacturer) with the appropriate templates.

The percent of release was individually calculated for each well according to the formula

$$\% \text{ lysis} = \frac{SN}{SN+Lys}$$

(SN is supernatant and Lys is lysate)

For the calculation of cytokine release, absolute concentrations were used. For the calculation of LDH release, optical densities were used.

2.2.2 Animal experiments

Animals

Animals were housed and fed according to federal guidelines. All experiments involving mice were approved by the local veterinary authorities.

Nrf2 knockout mice [10] were kindly provided by Dr. Yuet-Wai Kan (*University of California, San Francisco*); caNrf2 mice in C57Bl/6 background were kindly provided by Prof. Dr. G. Rogler (*University Hospital Zurich*). Mice expressing caNrf2 in myeloid cells were generated by mating of transgenic mice expressing Cre under control of the *LysM* gene promoter [11] with transgenic mice expressing caNrf2 under control of a β-actin promoter and CMV enhancer. To avoid expression of the transgene in all cells, the caNrf2 cDNA is flanked by loxP site, allowing expression of the caNrf2 transgene in the presence of Cre recombinase [12].

Gavage feeding

Mice were fed with Nrf2-activating compounds dissolved in vehicle as indicated. A volume of 0.2 ml was gavaged using a reusable feeding needle (20 Gauge, 30 mm long) (FINE SCIENCE TOOLS, *Heidelberg, Germany*).

MSU-induced peritonitis

The *in vivo* mouse peritonitis model was described by Martinon *et al.* [13] and Chen *et al.* [14]. Mice were injected intraperitoneally (i.p.) with 2 mg MSU crystals in 0.5 ml sterile PBS. After 6 h, mice were sacrificed by CO_2 exposure and peritoneal cavities were washed with 5 ml of PBS. The lavage fluids were centrifuged at 200 x g for 5 min and total numbers of infiltrating cells were counted by a hematocytometer. Cells were analysed by FACS and total numbers of neutrophils ($Ly6G^+$ $7/4^+$ cells) were determined.

Analysis of contact hypersensitivity (CHS)

The mouse contact hypersensitivity (CHS) model was described by Watanabe *et al.* [15]. Mice were sensitized by external application of 50 µl of 0.5 % DNFB in acetone:olive oil (3:1, v/v) on the left ear at day 0. At day 5, sensitized mice were elicited on the right ear by topical application of 50 µl of 0.5 % DNFB in acetone:olive oil. Ear thickness of the left ear was measured before and 24 h after elicitation with a digital gauge (MITSUTOYO, *Kanagawa, Japan*). For ear thickness measurements mice were anesthetized by intraperitoneal injection of ketamine (0.06 mg per g body weight) and xylazine (0.003 mg per g body weight) dissolved in PBS.

Listeria monocytogenes infection and determination of colony-forming units

Listeria monocytogenes infection in mice was performed according to the protocol described by Johansen *et al.* [16]. *Listeria monocytogenes* was grown in tryptic soy broth (BD, *Franklin Lakes, US-NJ*) and 2 x 10^4 colony-forming units (CFUs) in a volume of 0.2 ml were injected intraperitoneally into mice. Livers were collected after 2 days for bacterial counts. The organs were homogenized and passed through a cell strainer (70 µm Nylon, BD, *Franklin Lakes, US-NJ*). Cells were lysed in 0.5 % saponin (SIGMA, *Munich, Germany*), serial lysate dilutions were made in PBS and plated on tryptic soy agar. After 16 h incubation at 37 °C, organ titers were determined.

2.2.3 Microbiological methods

Cultivation and storage of E. coli strains

E. coli cells were suspended in an appropriate amount of LB-medium and grown at 37 °C on a shaker (180-230 rpm) until the required OD_{600} was reached.

LB-Medium

Tryptone	1 % (w/v)
Yeast extract	0.5 % (w/v)
NaCl	1 % (w/v)

If required, 100 µg/ml Ampicillin was added to the medium. To cultivate *E. coli* on agar plates, they were spread over the plate and incubated at 37 °C. Agar plates were produced by adding 1.5 % (w/v) agar to the LB-medium before autoclaving. This medium was poured into petri dishes. If required, an appropriate amount of antibiotic was added to the medium after cooling down to ~55 °C. Plates with *E. coli* were sealed with parafilm and stored at 4 °C.

Preparation of transformation-competent E. coli

A fresh overnight culture of transformation-competent *E. coli* (XL-1 Blue) was diluted 1:100 with LB-medium and shaken at 37 °C until the optical density at 600 nm was between 0.4 and 0.5. After centrifugation (600 x g, 10 min, 4 °C), the bacterial pellet was carefully resuspended in cold, sterile 100 mM $MgCl_2$ solution (1/4 of the original volume). The mixture was incubated on ice for 30 min, centrifuged as described above and the pellet was resuspended in cold, sterile 100 mM $CaCl_2$ solution (1/50 of the original volume). The mixture was incubated 3-4 h on ice. Cold, sterile glycerol was added to a final concentration of 30 %, the mixture was aliquoted and stored at -80 °C.

2.2.4 Molecular biological methods

Transformation of competent E. coli with plasmid DNA

100 µl transformation-competent *E. coli* cells were mixed with 10 µl plasmid or ligation mix (~100 ng DNA) and incubated on ice for 30-60 min. The mixture was subjected to a heat-shock (2 min at 42 °C) and put on ice. 600 µl LB-medium was added to the mix and shaken for 30-60 min at 37 °C. Subsequently, the mix was plated on LB plates, containing the corresponding selection marker, and incubated overnight at 37 °C.

Preparation of plasmid DNA from E. coli

Small scale plasmid preparation with the Plasmid Midi Kit

In order to quickly get small amounts of plasmid DNA, solutions from the Plasmid Midi Kit (QIAGEN, *Düsseldorf, Germany*) were used. This method is based on an alkaline lysis of the cells with subsequent precipitation of proteins, lipids and genomic DNA followed by centrifugation. The plasmid DNA in the supernatant was precipitated by isopropanol. The obtained purity and amount of plasmid DNA is sufficient for a subsequent analysis by restriction enzyme digest or sequencing.

4 ml of 5 ml overnight bacterial LB culture with the appropriate antibiotic was transferred to a microcentrifuge tube and centrifuged (1'500 x g, 6 min, 4 °C). The pellet was resuspended in 250 µl Resuspension buffer I by pipetting up and down. 250 µl of Lysis buffer II were added and incubated for 5 min at room temperature while inverting the tubes. To stop lysis and to precipitate proteins and lipids, 350 µl of Neutralization buffer III were added. The tubes were inverted several times and centrifuged (17'000 x g, 15 min). The supernatant was transferred to a new tube with 600 µl isopropanol and mixed. This step precipitates plasmid DNA. After incubating the tubes 15 min on ice, they were centrifuged (17'000 x g, 5 min). The pellets were

washed with 1 ml 70 % ethanol and centrifuged again (17'000 x g, 5 min). The supernatant was removed and the plasmid pellet was dried at room temperature. The DNA containing pellet was resuspended in 20-30 µl dH_2O and the nucleic acid concentration was determined. Plasmid preparations were stored at -20 °C.

Resuspension buffer I

EDTA	10 mM
RNAse A	100 µl/ml
TRIS/HCl (pH 8.0)	50 mM

Stored at 4 °C after addition of RNAse A

Lysis buffer II

SDS	1 % (w/v)
NaOH	200 mM

Neutralization buffer III

Potassium acetate (3 M, pH 5.5)

Middle scale plasmid extraction with the Plasmid Midi/Maxi Kit

The Plasmid Midi/Maxi Kit (QIAGEN, *Düsseldorf, Germany*) was used and plasmid isolation was performed according to the manufacturers' instructions. Plasmid DNA was stored at -20 °C.

Preparation of genomic DNA from mouse ears for genotyping

DNA from mouse ear biopsies was isolated according to the HotSHOT genomic DNA preparation method [17]. Mouse ears were clipped and 100 µl of alkaline lysis buffer were added to the biopsies. The samples were incubated in 1.5 ml Eppendorf tubes for 30 min at 95 °C. Afterwards, the tubes were cooled down to 4 °C and 100 µl of neutralization buffer were added, before the samples were centrifuged (17'000 x g, 10 min, 4 °C). The supernatants were used for genotyping PCR.

Alkaline lysis reagent

NaOH	25 mM
EDTA	0.2 mM

Neutralization reagent

TRIS/HCl	40 mM

Determination of the nucleic acid concentration

In order to determine the concentration of DNA or RNA solutions, the absorption at 260 nm was measured. This was done using an UVS-99 Micro-Volume Spectrophotometer (ACTGENE, *Piscataway, US-NJ*) according to the manufacturers' instructions. ddH_2O or the elution solution from the appropriate isolation kit was used as a blank. Because proteins in solution absorb light at 280 nm, the ratio between OD_{260} and OD_{280} was taken as an indicator for the purity of the nucleic acid. The optimal value is 1.8, indicating a pure nucleic acid solution.

Agarose gel electrophoresis of DNA

DNA molecules were separated by agarose gel electrophoresis for analytical and preparative purpose. Gels with an agarose content of 0.8-2 % (w/v) in TAE buffer were used. Agarose concentration was dependent on the fragment length. Electrophoresis was performed at 100-150 V. To visualize DNA bands, GelGreen (1 x) (BIOTIUM, *Hayward, US-CA*) was added to the gel. The DNA samples were mixed with 6 x loading buffer (THERMO FISHER SCIENTIFIC, *Waltham, US-MA*) before loading them into the slots.

1 x TAE	
TRIS/HCl	40 mM
Acetic acid	40 mM
EDTA	1 mM
pH 8.0	

The bands were visualized under UV light and photographed. As size standard 100 bp or 1 kb DNA ladder were used, depending on the analysed fragment length. For cloning of fragments, the bands of interest were excised with a scalpel under UV light. The UV irradiation was kept as short as possible because UV damages DNA. The DNA was purified from the gel with the Gel Extraction Kit (MACHEREY-NAGEL, *Düren, Germany*) according to the manufacturers' instructions.

Polymerase chain reaction (PCR)

PCR allows fast amplification of DNA fragments from a DNA template.

Preparative PCR reactions

Two primers flanking the coding sequence and containing introduced restriction sites complementary to those in the target expression vector were used. The following reaction mix was prepared in a PCR tube on ice:

PCR reaction mix

Template DNA	~5-100 ng
5'-Primer (50-100 μM)	1 μl
3'-Primer (50-100 μM)	1 μl
dNTPs (2.5 mM each)	1 μl
Pfu DNA polymerase	0.5 μl
10 x PCR buffer	5 μl
ddH_2O	add. 50 μl

The PCR reaction was carried out in a Mastercycler gradient (EPPENDORF, *Hamburg, Germany*) PCR machine. The temperature program was adjusted to the desired parameters - depending on fragment length, primer annealing temperature and specificity of the amplification.

PCR program

First denaturing step	5-8 min	95 °C	
Denaturing step	1 min	94 °C	25-35 cycles
Primer annealing	30-60 sec	45-55 °C	
Primer extension	1-2 min	72 °C	
Last primer extension	10 min	72 °C	
Storage until analysis		4 °C	

Specificity and efficiency of the reaction were tested by running 5 μl of the reaction mix on an agarose gel. The reaction product was purified by preparative agarose gel electrophoresis with subsequent gel extraction.

Analytic PCR reactions for mouse genotyping

Genotyping of the Nrf2 KO mice was performed by PCR. Mouse ear DNA was used as template and amplified with 3 primers annealing to a chromosomal region outside the Nrf2 KO construct

and inside the KO construct or the corresponding wild-type region. The wild-type allele results in a 300 bp fragment and the KO in a 220 bp fragment, which can be separated on a 2 % agarose gel. The following reaction mix was prepared in a PCR tube on ice:

PCR reaction mix

Template DNA	2 µl
5'-Primer (10 µM)	0.2 µl
3'-Primer 1 (10 µM)	0.2 µl
3'-Primer 2 (10 µM)	0.2 µl
$MgCl_2$ (100 mM)	0.6 µl
dTNPs (10 mM)	0.2 µl
Taq DNA polymerase	0.2 µl
10 x PCR buffer (with KCl)	2 µl
ddH_2O	add. 20 µl

The PCR reaction was carried out in a Mastercycler gradient (EPPENDORF, *Hamburg, Germany*) PCR machine.

PCR program

First denaturing step	5 min	95 °C	
Denaturing step	30 sec	95 °C	35 cycles
Primer annealing	30 sec	56 °C	
Primer extension	1 min	72 °C	
Last primer extension	5 min	72 °C	
Storage until analysis		4 °C	

One-step site-directed mutagenesis

Site-directed mutagenesis was used to generate the expression plasmid encoding Keap1 with a mutated cysteine (C151W). The approach is based on the QuickChange™ site-directed mutagenesis system developed by STRATAGENE (*La Jolla, US-CA*) and was described by Liu *et al.* [18]. A 38-mer primer pair was designed with a single nucleotide modification at nt 451-453 (tgt→tgg), which results in an amino acid change from cysteine to tryptophan at position 151. The primer-extension reaction was performed as described for preparative PCR. Template DNA was digested by using the restriction enzyme DpnI and remaining circular nicked DNA was transformed into competent *E. coli*.

Deletion mutagenesis by overlap extension PCR

To generate the expression plasmid encoding Nrf2 with a deletion of the nuclear localization sequence (Nrf2_NLS; nt 1472-1526 [Δ 54 nt]) a standard protocol for overlap extension PCR mutagenesis was applied. The deletion of the 54 nucleotides was introduced by two successive PCR steps as described [19]. In the first step, two pairs of primers are used to generate DNA fragments with overlapping ends containing the deletion. The resulting two PCR fragments are gel purified and used as a template for the second step, where the two PCR products, owing to terminal complementarity, recombine and become amplified.

Digestion of DNA by restriction enzymes

For an analytical digest 1-5 μg of plasmid DNA were digested with 0.5-1 μl restriction endonuclease (most 10 U/μl) in a volume of 10 μl. This mix was doubled for a preparative digest. The appropriate buffer was used according to the recommendations of the manufacturer. For double digestion each enzyme was used at the amount described above. The reaction tubes were incubated for 2-5 h at the recommended temperature. The exact amount of enzyme was adjusted by considering the desired reaction time and suitability of buffer conditions. 10 μl of the digestion mix were used for gel electrophoresis. The complete digestion mix was used for fragment purification.

Generation of blunt ends with DNA polymerase I, Large (Klenow) fragment or Mung Bean nuclease

To prepare PCR products or digestion products with blunt ends for ligation, either the DNA polymerase I, Large (Klenow) fragment, or the Mung Bean Nuclease (both NEB, *Ipswich, US-MA*) were used. Klenow polymerase removes 3'-overhangs and fills in 3' recessed (5'-overhang) ends, whereas Mung Bean nuclease removes 3' and 5' extensions from DNA ends. The sample DNA was mixed with 0.5 μl enzyme in a final volume of 50 μl and incubated for 45 min at 37 °C for the Klenow polymerase or at 30 °C for the Mung Bean Nuclease.

Dephosphorylation of DNA ends

Alkaline phosphatise removes phosphate residues from DNA ends. Two ends without phosphate cannot be linked by *T4* DNA ligase. This can be used to avoid intra- and intermolecular ligation of vector DNA. Dephosphorylation was done mainly with vectors, which had been digested with a single restriction enzyme or to avoid self-ligation after incomplete digestion with two enzymes. The reaction was carried out in a final volume of 50 μl with 0.5 μl calf intestinal phosphatise (CIP; 30 U/μl). Incubation time was 1 h at 37 °C. After the reaction, the enzyme was removed by DNA purification using the PCR purification kit (MACHEREY-NAGEL, *Düren, Germany*).

DNA ligation

DNA insert and vector were ligated by *T4* DNA ligase. The optimal stoichiometric ratio of vector to insert was assumed to be ~1:4 for sticky end ligations and ~1:10 for blunt end ligations. The following reaction mix was incubated for 18 h at 4 °C:

DNA ligation mix	
10 x Ligation buffer	2 µl
Vector	1 µl
Insert	4 µl/10 µl
T4 ligase (U/µl)	1 µl
ATP (100 M)	1 µl
ddH_2O	add. 20 ml

Isolation of total cellular RNA from eukaryotic cells

RNA isolation was performed from one well of a 6-well plate using the RNeasy Mini kit (QIAGEN, *Düsseldorf, Germany*) according to the manufacturers' instructions.

Isolation of total RNA from tissue samples using TRIzol®

Nucleic acids can be separated from proteins and lipids by phenol extraction with subsequent ethanol precipitation. TRIzol® reagent (THERMO FISHER SCIENTIFIC, *Waltham, US-MA*) is a solution of phenol and other components and was used for RNA isolation from liver, spleen or colon according to the manufacturers' instructions.

Tissue was homogenized in TRIzol® reagent using an Ultra Turrax (KINEMATICA AG, *Luzern, Switzerland*). 0.2 ml of chloroform per 1 ml of TRIzol® reagent was added and shacken vigorously for 15 seconds. To separate the aqueous phase from the phenol phase, the solution was centrifuged (12'000 x g, 15 min, 4 °C). The nucleic acids of the resulting aqueous phase were precipitated with 0.5 ml of isopropanol per 1 ml of TRIzol® used for homogenizazion. This solution was incubated for 10 min at room temperature and subsequently centrifuged (12'000 x g, 10 min, 4 °C) to pellet the RNA. The RNA pellet was washed with 75 % ethanol, dried for 10 min and resuspended in RNase-free water. RNA was stored at -80 °C.

Preparation of cDNA by reverse transcription

Synthesis of cDNA from total cellular RNA was performed using the RevertAid First Strand cDNA Synthesis Kit (THERMO FISHER SCIENTIFIC, *Waltham, US-MA*) according to the manufacturers' instructions using oligo dT primers.

Quantitative Real-time PCR (qRT-PCR)

Relative quantification of gene expression at the RNA level was performed using the LightCycler 480 SYBR Green Master or the FastStart Universal SYBR Green Master (both ROCHE, *Rotkreuz, Switzerland*). The real-time PCR was performed with cDNA from total cellular RNA or from tissue RNA and a primer pair was designed to a fragment of ~150 bp in length flanking an intron-exon border of the desired gene. To quantify the relative expression level of a certain gene, two reaction mixes were prepared. One contained the primer pair targeting the gene of interest, the other contained a primer pair targeting an internal reference gene (RPL27, GAPDH or RPS29). The following mixture was prepared on ice in ThermoFast or LightCycler 480 Multiwell detection plates:

qPCR reaction mix

Template DNA	~5-100 ng
5'-Primer (100 μM)	0.5 μl
3'-Primer (100 μM)	0.5 μl
2 x SYBR Green PCR Master Mix	5 μl
ddH_2O	add. 10 μl

The PCR reaction and detection were performed in the LightCycler 480 96-well version (ROCHE, *Rotkreuz, Switzerland*) or the ViiA 7 Real-Time PCR System (LIFE TECHNOLOGIES, *Carlsbad, US-CA*) according to the manufacturers' instructions. The following temperature program was applied:

Real-time PCR program

Initiation step	2 min	50 °C	
First denaturing step	10 min	95 °C	
Denaturing step	10 sec	95 °C	40 cycles
Primer annealing and extension	30 sec	58 °C	
	15 sec	95 °C	dissociation curve
	20 sec	60 °C	
	15 sec	95 °C	

Specificity of the reaction was ensured by surveying the dissociation curve of a given primer pair. Data processing was performed using the LightCycler 480 software provided by the

manufacturers according to the guidelines. Data evaluation and statistical analysis followed the rules of the ΔCT method described by the system manufacturer.

2.2.5 General protein methods

Acetone precipitation

2.5 volumes of acetone were mixed with the sample. After overnight incubation at -20 °C the mixture was centrifuged (17'000 x g, 60 min, 4 °C) and acetone was removed. The pellet was resuspended in 1 x sample loading buffer before complete drying.

SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

To separate proteins according to their size by SDS-PAGE, the buffer system of Laemmli [20] was used. The electrophoresis was carried out in vertical direction using the SE 250 mini gel system (HOEFER, *Holliston, US-MA*) in 1 mm thick polyacrylamide gels of appropriate acrylamide concentration depending on the protein size. The gel contains a 1 cm long 5 % stacking gel and a 6 cm long 8-20 % separating gel.

Before loading the samples, they were mixed with an equal volume of 2 x sample loading buffer, sonicated and incubated for 5 min at 95 °C. Electrophoresis was performed at 75 V until the loading dye reached the separating gel and then at 100-120 V until the loading dye reached the bottom of the gel. Prestained protein molecular weight marker (THERMO FISHER SCIENTIFIC, *Waltham, US-MA*) was used in all gels.

Separating gel (4 mini gels or one large gel)

Acrylamide concentration	8 %	10 %	12.5 %	15 %	18 %	20 %
Acrylamide/bisacrylamide (30:0.8)	11.4 ml	13.3 ml	16.8 ml	20.0 ml	23 ml	24.2 ml
TRIS/HCl (1 M, pH 8.8)	15.0 ml	15.0 ml	15.0 ml	15.0 ml	15.0 ml	15.0 ml
ddH_2O	13.4 ml	11.5 ml	7.4 ml	4.2 ml	1.2 ml	-
SDS (10 % [w/v])	400 µl	400 µl	400 µl	400 µl	400 µl	400 µl
APS (20 % [w/v])	170 µl	170 µl	200 µl	200 µl	200 µl	200 µl
TEMED	16 µl	16 µl	16 µl	16 µl	16 µl	16 µl

Stacking gel (4 mini gels)

Acrylamide concentration	5 %
Acrylamide/bisacrylamide (30:0.8)	2.7 ml
TRIS/HCl (1 M, pH 6.8)	2.0 ml
ddH_2O	10.8 ml
SDS (10 % [w/v])	160 µl
APS (20 % [w/v])	80 µl
TEMED	16 µl

SDS-PAGE running buffer

TRIS/HCl (pH 8.0)	25 mM
Glycine	192 mM
SDS	0.1 % (w/v)

2 x sample loading buffer

TRIS/HCl (pH 8.0)	100 mM
Glycerol	20 % (v/v)
SDS	10 % (v/v)
Bromophenol blue	0.01 % (w/v)
DTT (1M)	20 % (v/v)

Western blot

For the detection of specific proteins by Western blotting, electrophoretically separated proteins were transferred onto a nitrocellulose membrane. After blocking of unspecific binding sites, the membrane was incubated with the primary and the corresponding secondary antibody. Secondary antibodies are coupled to an enzyme that catalyzes the detection reaction.

Protein transfer by semi-dry blotting

Three sheets of Whatman 3MM paper were soaked in anode buffer I and placed on the anode side of the blotting apparatus. Two sheets of Whatman paper were soaked in anode buffer II and placed on top of the first sheets. The nitrocellulose membrane was equilibrated in anode buffer II and subsequently placed onto the Whatman papers, followed by the gel, which was carefully pressed onto the membrane to avoid air bubbles in between. Finally, three sheets of Whatman paper soaked in cathode buffer were place on top of the gel. Blotting was performed in a semi-dry blotter (Z340502, SIGMA, *Munich, Germany*) at 75 mA per gel for 30 min.

Anode buffer I

Methanol	20 % (v/v)
TRIS/HCl	300 mM

Anode buffer II

Methanol	20 % (v/v)
TRIS/HCl	25 mM

Cathode buffer

Methanol	20 % (v/v)
TRIS/HCl	25 mM
ε-amino caproic acid	40 mM

Incubation with antibodies and visualization of protein bands

Unspecific binding sites were blocked with 3 % milk powder (MIGROS, *Zurich, Switzerland*) in PBST (blocking solution) for at least 30 min. The membranes were then incubated with the primary antibody in blocking solution overnight at 4 °C. After washing three times in PBST for 5 min, the membranes were incubated with the corresponding secondary antibody in blocking solution for 30-60 min at room temperature. Secondary antibodies were coupled to alkaline phosphatise (AP) or horseradish peroxidase (HRP). After incubation, the membranes were washed three times for 5 min.

AP detection was performed using the NBT/BCIP substrate kit (PROMEGA, *Madison, US-WC*). The membrane was washed once with AP buffer and incubated in AP buffer containing 16.5 µl BCIP and 33 µl NBT per 5 ml of buffer. Reaction was stopped with H_2O when desired band intensities were obtained.

AP buffer

TRIS/HCl (pH 9.5)	100 mM
NaCl	100 mM
MgCl2	5 mM

HRP detection was performed using the enhanced luminescence principle [21]. Substrate solution (ECL plus western blotting detection system; AMERSHAM, *Uppsala, Sweden*) was prepared according to the manufacturers' instructions. ~1 ml ECL solution was evenly distributed on the membrane and covered with a clear plastic foil. After 5 min incubation, residual ECL solution was removed and the blot was exposed to an X-ray film. After the desired

incubation time (usually 5 sec-30 min), the X-ray film was developed with a developing apparatus (FPM-100A, FUJFILM, *Tokio, Japan*).

Imaging of blots and stained gels

Blots, films and stained gels were scanned using the CanonScan 9950F device (CANON, *Tokio, Japan*). Images were acquired with 300 dpi resolution without filters or other altered acquisition settings. Digital image processing was performed using the Photoshop software (ADOBE SYSTEMS SOFTWARE, *San Jose, CA*). Processing was restricted to cutting. When the observed bands in the original blots were too weak for meaningful digital and print reproduction, brightness and contrast adjustments were applied to the whole image until the scientific result reflected the one seen on the original blot.

2.2.6 Co-immunoprecipitation (Co-IP)

Co-IP of endogenous proteins in human primary keratinocytes

Protocol for co-IP in HPKs was described by Sollberger *et al.* [22]. Human primary keratinocytes were grown to 80 % confluency in 10 cm dishes and, if desired, transfected with siRNA. One dish was harvested in 150 µl co-IP buffer with complete protease inhibitor cocktail (ROCHE, *Rotkreuz, Switzerland*) according to the manufacturer's instructions. Lysates of 4 dishes were pooled and homogenized using a dounce tissue grinder pestle (SIGMA, *Munich, Germany*) on ice, followed by centrifugation (17'100 x g, 20 min, 4 °C). The supernatant was used for co-IP: Protein solution was mixed with 1 volume of co-IP buffer containing Aprotinin (1:100) and AEBSF (1:400) or complete protease inhibitor cocktail (ROCHE, *Rotkreuz, Switzerland*) and incubated with 20 µg of antibody (caspase-1 [sc-622]) or with a corresponding amount of an isotype control (HA) for 3 h at 4 °C on a rocker. After centrifugation (17'000 x g, 10 min, 4 °C), the supernatants were mixed with 150 ml of protein A sepharose (50 mg/ml) (GE HEALTHCARE, *Little Chalfont, UK*) and incubated for 90 min at 4 °C on a rocker. The protein A sepharose beads were washed 4 times with 1 ml of co-IP buffer and resuspended in 70-100 ml of 2 x sample loading buffer.

Alternatively, co-IP was performed using an ANTI-FLAG® M2 Affinity Gel (SIGMA, *Munich, Germany*). HPKs were grown in 10-cm dishes and transduced with lentiviral constructs encoding FLAG-tagged caspase-1 or GFP (described in 2.2.1). After antibiotic selection for 3 days, expression of the gene of interest was induced by the addition of doxycycline. After 24 h, cells were collected in lysis buffer, centrifuged and resulting supernatants were subjected to immunoprecipitation following the manufacturer's protocol.

2.2.7 Cell fractionation

The protocol for producing nuclear and cytoplasmic cell fractions was described by Suzuki *et al.* [23]. HPKs were grown in 10-cm dishes to 80-90 % confluency. Cell fractionation was performed on ice. Cells were washed twice with ice-cold PBS, detached from the plastic surface by scratching and collected in 1 ml of PBS per 10-cm dish. After a short spin in the centrifuge for 10 sec, the supernatant was discarded and cells were resuspended in 0.1 % NP-40/PBS by pipetting up and down 5 times using a p1000 micropipette cut about 3 mm off at the end to enlarge the opening. An aliquot of 300 µl was removed, mixed with 100 µl of 4 x Laemmli buffer and kept on ice until sonication; this fraction is the "whole cell extract". The remaining solution was again spinned down shortly (10 sec). The supernatant was transferred to a new tube, mixed with 1/3 volume of 4 x Laemmli buffer and kept on ice; this is the "cytoplasmic fraction". The pellet was resuspended in 0.1 % NP-40/PBS by pipetting up and down once. The resulting solution was spinned down for 10 sec, the supernatant was discarded and the nuclear pellet was resuspended in 200 µl of 1 x Laemmli buffer. The resulting nuclear fraction was kept on ice until sonication. All fractions were incubated for 5 min at 95 °C before loading on a SDS-polyacrylamide gel.

4 x Laemmli sample buffer

TRIS/HCl (pH 6.8)	250 mM
β-mercaptoethanol	20 % (v/v)
SDS	8 % (w/v)
Glycerol	20 % (v/v)
Bromophenol blue	0.008 % (w/v)

2.2.8 Flow cytometry

Cell surface staining

Cells were washed once with PBS and transferred to 96-well U-bottom plates. After fixation with 2 % PFA for 15 min, the cells were washed 3 times with FACS buffer (2 % FCS in PBS), followed by incubation with the 1° antibodies (fluorochrome-conjugated) in a dilution of 1:300 in FACS buffer for 25 min at 4 °C. After 3 washes with FACS buffer, cells were taken up in 200 µl FACS buffer and analysed directly using a FACSCanto (BD, *Franklin Lakes, US-NJ*) with FACS DIVA software (BD, *Franklin Lakes, US-NJ*).

2.2.9 Statistical analysis, data acquisition and evaluation

Statistical analysis was performed using the Prism Software (GraphPad Software, *San Diego, CA*). When multiple groups were compared to one control group, one-way ANOVA with posterior

Dunnett's correction was performed. Different treatments were compared to control treatment either by Student's *t*-test or by Mann-Whitney test, when the populations did not follow a Gaussian distribution. Unpaired *t*-test with Welch's correction was used when the two population variances were not assumed to be equal.

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Chapter 3 – Results

3.1 Opposing effects of Nrf2 and Nrf2-activating compounds on the NLRP3 inflammasome independent of Nrf2-mediated gene expression

Martha Garstkiewicz, Gerhard E. Strittmatter, Serena Grossi, Jennifer Sand, Sabine Werner, Lars E. French, Hans-Dietmar Beer

Nrf2 is a transcription factor with a central role in cytoprotection by regulating the expression of genes, which code for antioxidant proteins and detoxifying enzymes. Furthermore, a role of Nrf2 in inflammasome activation has recently been demonstrated, but the underlying molecular mechanisms remain known. Inflammasomes are innate immune complexes, which assemble upon sensing of a wide range of PAMPs and DAMPs. Their activation leads to the secretion of pro-inflammatory cytokines, which culminates in an inflammatory response. In this study we addressed the role of Nrf2 in inflammasome activation. We show that Nrf2 expression supports NLRP3 inflammasome activation and demonstrate that the requirement is independent of Nrf2 target genes. Conversely, Nrf2 activators were found to inhibit inflammasome activation.

The manuscript will be submitted for publication in the near future and I will be first author.
My contributions to the study were:

- Generation of BMDCs, isolation of peritoneal macrophages and performing experiments with these cells (as shown in Figures 1, 2 and 6)
- ELISA measurements of IL-1β secretion from HPKs (as shown in Figures 1 and 3)
- qRT-PCR analysis (as shown in Figures 1, 2, 4, 5 and S1)
- Lentiviral transduction of HPKs and analysis of IL-1β secretion by Western blot (as shown in Figure 2)
- Treatment of THP-1 cells and PBMCs with Nrf2 activators and analysis of IL-1β secretion (as shown in Figure 3)
- Performing mouse experiments (as shown in Figure 4)
- Analysis of Nrf2 protein expression and IL-1β secretion in HPKs and THP-1 cells by Western blot (as shown in Figures 3 and 5)
- siRNA transfection of HPKs with subsequent analysis of Nrf2 protein expression by Western blot (as shown in Figures 5 and S1)
- Cellular fractionation of HPKs (as shown in Figure S1)
- Design of experiments, statistical analysis of experiments and involvement in the writing of the manuscript

Abstract

The transcription factor Nrf2 regulates expression of genes required for protection from xenobiotic and oxidative stress. Under normal conditions Nrf2 is constantly degraded upon ubiquitination mediated by the Nrf2 inhibitor Keap1. Inflammasomes represent stress-induced immune complexes. They are critically involved in acute and chronic inflammation through caspase-1-mediated activation of pro-inflammatory cytokines. Here we identified Nrf2 as a positive regulator of the NLRP3 inflammasome. In contrast, Nrf2 activating compounds, including the anti-inflammatory drug dimethyl fumarate (DMF), inhibit inflammasome activation. Both effects are independent of the transcriptional activity of Nrf2 and, at least in part, not interdependent. On the other hand, NLRP3 inflammasome activation induces a rapid and partly caspase-1- and Keap1-independent degradation of Nrf2. These experiments argue against a simultaneous activation of both stress-related pathways. Finally, we provide evidence that the cross-regulation of both pathways is controlled by a physical interaction between the Nrf2/Keap1 and NLRP3 complexes.

Introduction

The transcription factor nuclear factor erythroid derived 2, like 2 (Nrf2) is a major regulator of cytoprotection [1]. In response to endogenous and exogenous stresses caused by reactive oxygen species (ROS) or electrophiles Nrf2 induces expression of target genes involved in antioxidant defence and compound detoxification [2]. Therefore, Nrf2 plays a key role in protection of the body against drug toxicity and stress-induced (inflammatory) diseases [3, 4]. However, elevated expression of Nrf2 target genes confers also advantages for cancer cells by increasing cancer chemoresistance and enhancing tumour cell growth and survival [2, 5]. In several types of tumour cells activating mutations in the *NRF2* gene or inactivating mutations in the gene encoding the Nrf2-binding protein Kelch-like ECH-associated protein 1 (*KEAP1*) have been identified, resulting in enhanced activity of the transcription factor. This results in enhanced target gene expression, subsequent ROS protection and metabolic changes of cancer cells positively affecting their proliferation, as well as chemo- and radioresistance [2, 5].

The adaptor protein Keap1 is a repressor of Nrf2, mediating its constant degradation under homeostatic conditions [5]. Keap1 links Nrf2 by physical interaction to the E3 ubiquitin ligase Cullin 3 (Cul3) and the RING-box protein 1 (Rbx1), resulting in ubiquitination of the transcription factor and its subsequent degradation by the proteasome pathway. Small amounts of Nrf2 can escape from Keap1/Cul3/Rbx1-mediated degradation, translocate to the nucleus and are responsible for a basal level of target gene expression. In contrast, electrophiles inactivate Keap1 upon chemical modification of some of its cysteine residues (in humans 27). This induces

a conformational change of the Nrf2/Keap1/Cul3/Rbx1 complex and inhibition of Nrf2 degradation. Newly synthesised Nrf2 bypasses Keap1 and translocates to the nucleus, where it accumulates and increases target gene expression [5].

Several Nrf2 activating compounds such as sulforaphane (SFN), an isothiocyanate found in broccoli sprouts, have been identified. They represent electrophilic agents reacting with cysteine residues of Keap1 [6]. SFN is widely acknowledged as an agent providing chemopreventive benefits in humans and mice. Naturally occurring as well as synthetic Nrf2 activating compounds are currently in preclinical studies and clinical trials for different applications, such as prevention of neurodegenerative diseases or cancer [7-9]. Interestingly, it has been recently demonstrated that dimethyl fumarate (DMF), an efficient drug for the treatment of patients suffering from psoriasis or multiple sclerosis (MS) [6, 10, 11], is cardioprotective via activation of Nrf2 [12]. Psoriasis and MS are common inflammatory diseases affecting mainly the skin or the central nervous system, respectively. The molecular mechanisms underlying the therapeutic effects of DMF in both conditions are only poorly understood; however, a role of inflammasomes in both diseases is being discussed [13, 14].

Inflammasomes comprise a group of innate immune complexes, which induce an inflammatory response upon sensing of several different exogenous and endogenous stress factors [15]. They play an important role in immunity, and particularly the NLRP3 (NACHT, LRR and PYD domains-containing protein 3) inflammasome is involved in the pathogenesis of common (auto)inflammatory diseases, such as atherosclerosis, type 2 diabetes mellitus, or gout [15]. Inflammasomes consist of a central sensor and scaffold protein such as NLRP3, the adaptor protein ASC (apoptosis-associated speck-like protein containing a CARD) and the protease caspase-1. Upon stress factor detection inflammasomes are assembled, caspase-1 is activated and in turn processes and thereby activates the pro-inflammatory cytokines pro-interleukin (IL)-1β and -18, which induce inflammation upon their proteolytic processing and secretion [16]. Inflammasome activation is associated with a lytic form of cell death termed pyroptosis [15]. Recently, it has been reported that Nrf2 expression is required for inflammasome activation in murine cells *in vitro* and *in vivo* [17, 18] but this requirement is controversially discussed [19, 20]. The underlying mechanisms are unknown, but an involvement of Nrf2 target genes in inflammasome activation has been suggested [17, 18]. In addition, there is evidence that high concentrations of certain Nrf2 activators inhibit inflammasome activation. Surprisingly, it has been suggested that this occurs independently of Nrf2 [19, 20].

Here, we demonstrate that expression of Nrf2 supports inflammasome activation in murine and human cells. However, Nrf2 activating compounds block activation of caspase-1 in different cell

types and they dampen inflammasome-dependent inflammation *in vivo*. Both effects are not caused by changes in Nrf2 target gene expression. Mechanistically, we identified an interaction of components of the Nrf2/Keap1/Cul3/Rbx1 complex with caspase-1, demonstrating a physical link to inflammasomes. As Nrf2 is quickly degraded upon inflammasome activation, cells can activate either Nrf2, which induces cytoprotection and supports survival, or caspase-1, which results in inflammation and cell death.

Results

Nrf2 expression is required for efficient inflammasome activation

To determine if the basal activity of Nrf2 is required for inflammasome activation, we generated bone marrow-derived dendritic cells (BMDCs) from Nrf2-deficient mice and wild-type littermates, activated the NLRP3 as well as the AIM2 inflammasomes by several potent inducers, and analysed the secretion of mature IL-1β as a readout for caspase-1 activation. Consistent with published data [17, 18], secretion of IL-1β by Nrf2-deficient BMDCs was severely impaired as demonstrated by Western blot and ELISA (Figure 1A and B). As a control we analysed expression of pro-IL-1β and of several inflammasome proteins at the mRNA and protein level. However, expression of these genes was not significantly affected by the loss of Nrf2 (Figure 1C and D).

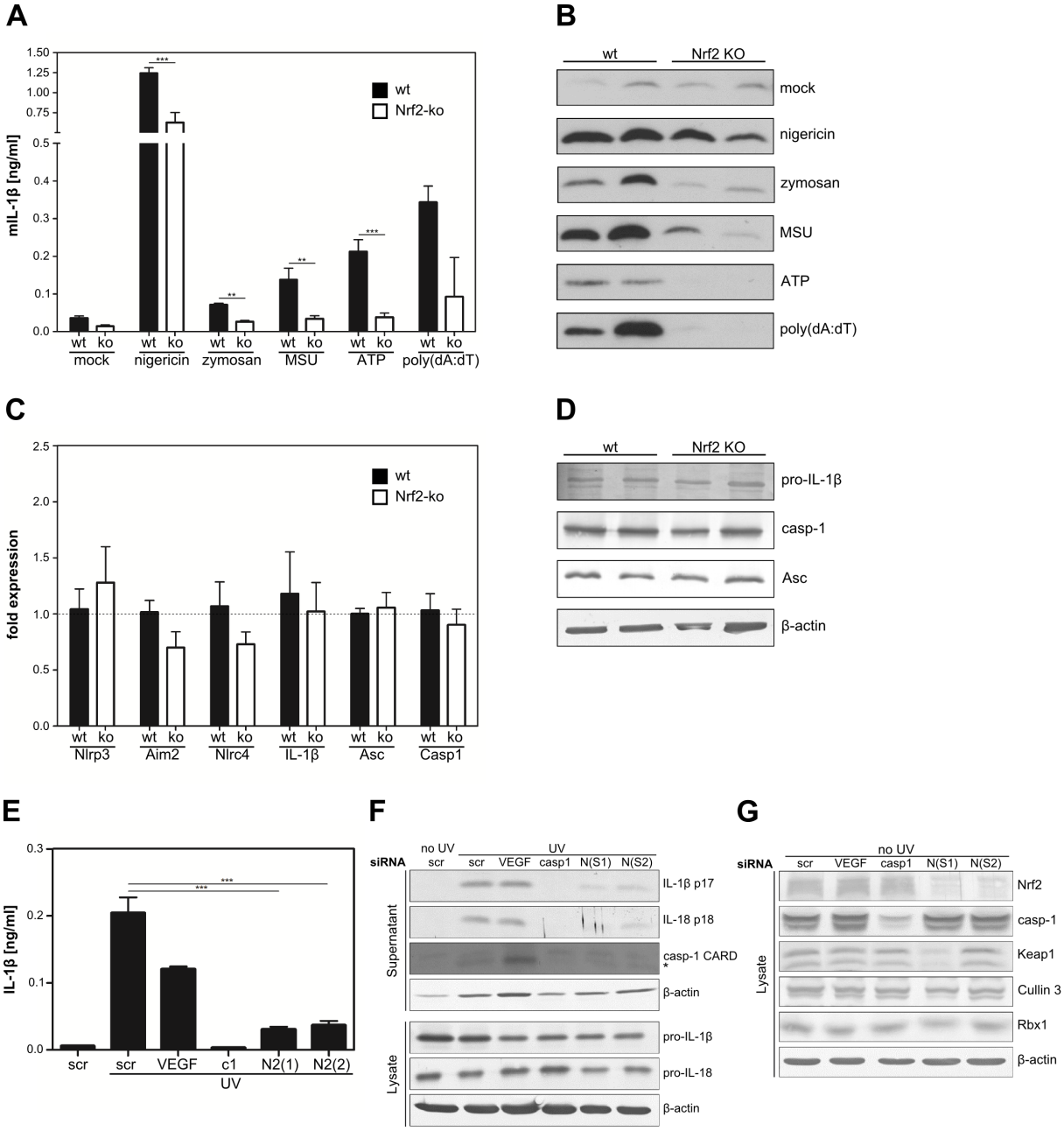
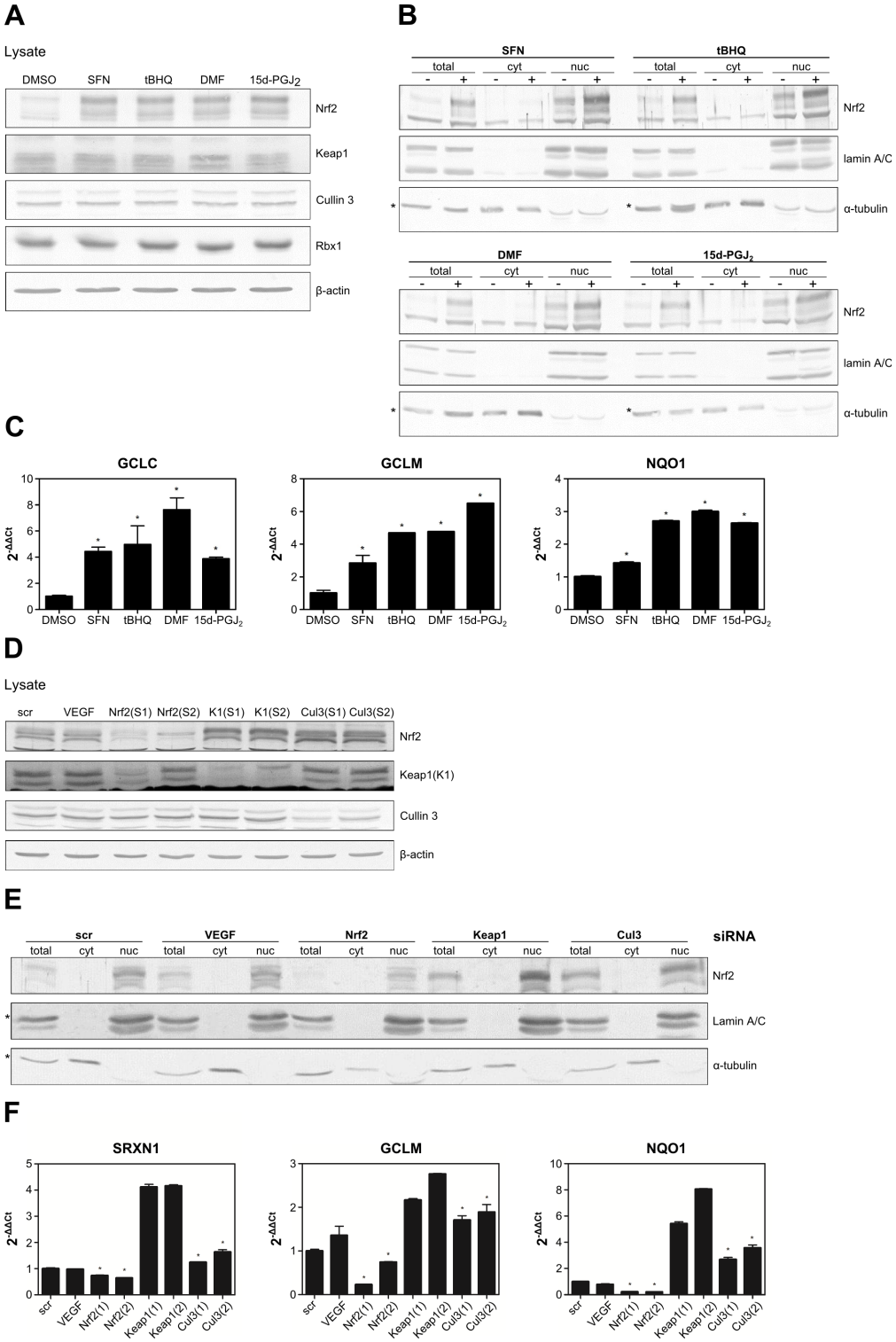


Figure 1: Nrf2 expression is required for full inflammasome activation. (**A**-**D**) Bone marrow (BM) cells were isolated from Nrf2-deficient mice and wt littermates and differentiated into dendritic cells (DCs). (**A**, **B**) After priming with upLPS overnight, BMDCs were treated with the NLRP3 inflammasome activators nigericin (20 µM), zymosan (20 µg/ml), MSU (150 µg/ml), ATP (5 mM) or transfected with poly(dA:dT) (1 µg/ml) for activation of the AIM2 inflammasome. After 6 h, supernatants were analysed for secretion of IL-1β by (**A**) ELISA or (**B**) Western blot. Mock-treated primed BMDCs were analysed for expression of inflammasome proteins and pro-IL-1β at the (**C**) mRNA level by qRT-PCR or at the (**D**) protein level by Western blot. (**B**, **D**) Samples from two mice per genotype are shown. (**E**-**G**) Human primary keratinocytes (HPKs) were transfected with specific siRNAs as indicated (scr: scrambled, VEGF: vascular endothelial growth factor (additional control), c1: caspase-1, N2 or N: Nrf2), 3 d later (**E**, **F**) irradiated with UVB or mock treated and harvested after 5 h (**G**). Inflammasome activation was analysed by (**E**) ELISA measurement of IL-1β in the supernatant or by (**F**) western blotting as indicated. Specific bands are marked with an asterisk. (**G**) Western blot for analysis of expression of Nrf2/Keap1 complex proteins and caspase-1 of mock-treated HPKs after transfection with caspase1, Nrf2 or control siRNAs. Statistics: (**A**) Error bars represent the mean ± SD of a representative experiment performed with three mice per genotype. Mann-Whitney test was performed. (**E**) One-way ANOVA.

Human primary keratinocytes (HPKs) constitutively express pro-IL-1β and inflammasome proteins, and UVB irradiation induces secretion of mature IL-1β and -18 in an inflammasome-dependent manner [21]. Treatment of HPKs with the Nrf2 activating compounds SFN, DMF, *tert*-butylhydroquinone (tBHQ) or 15-deoxy-Δ-^{12,14}-prostaglandin J_2 (15d-PGJ_2) resulted in fast and robust stabilisation and nuclear accumulation of Nrf2 protein, whereas expression of the other Nrf2 complex proteins Keap1, Cul3, and Rbx1 was not affected (Figure S1A and B). Nrf2 stabilisation and nuclear accumulation was accompanied by induction of classical Nrf2 target genes (Figure S1C). In addition, knock-down of Keap1 or Cul3 expression induced stabilisation of Nrf2, its nuclear accumulation, and enhanced target gene expression (Figure S1D-F). These experiments demonstrate that the Nrf2 pathway is functional in HPKs [22].

Therefore, we knocked down Nrf2 expression in HPKs using siRNA and analysed inflammasome activation upon UVB irradiation (Figure 1E-G). In Nrf2 knock-down HPKs caspase-1 activation was inhibited, and secretion of IL-1β and -18 was reduced, demonstrating that Nrf2 expression is also required for efficient inflammasome activation in human keratinocytes.



Supplementary Figure 1: Nrf2 activation in human primary keratinocytes (HPKs). (**A**-**C**) HPKs were treated with the Nrf2 activators SFN (10 µM), tBHQ (10 µM), DMF (50 µM) and 15d-PGJ2 (10 µM). After 1 h, the cells were harvested and analysed for expression of the indicated proteins using total cell lysates (**A**) or cytoplasmic and nuclear lysates (**B**). Western blots for the nuclear protein lamin A/C and the cytoplasmic protein α-tubulin served as controls. Specific bands are marked with an asterisk. (**C**) Total RNA was isolated after 8 h and qRT-PCR was performed for quantification of expression of the Nrf2 target genes glutamate-cysteine ligase, catalytic subunit (GCLC), glutamate-cysteine ligase, modifier subunit (GCLM), and NAD(P)H dehydrogenase, quinone 1 (NQO1). (**D**-**F**) HPKs were transfected with siRNAs for 3 d as indicated. Scrambled (scr) siRNA and siRNA targeting the unrelated vascular endothelial growth factor (VEGF) served as controls. Western blots of (**D**) total lysates or (**E**) nuclear and cytoplasmic lysates and (**F**) qRT-PCR for expression of target gene expression. Statistics: (**C**, **F**) Mann-Whitney test, (**F**) related to scr control.

Nrf2-induced gene expression is not involved in inflammasome regulation

Since Nrf2 is a transcription factor, it is likely that a reduction of Nrf2 target gene expression underlies the inhibition of the NLRP3 inflammasome upon ablation of Nrf2 expression. To determine if activation of Nrf2-mediated gene expression has the opposite effect and results in enhanced maturation of pro-IL-1β, we characterised peritoneal macrophages isolated from transgenic mice expressing a constitutively active (ca) mutant of Nrf2 [23] in myeloid cells. This mutant lacks the domain, which mediates binding to Keap1. However, secretion of mature IL-1β and consequently NLRP3 inflammasome activation was not changed upon caNrf2 expression (Figure 2A and B), although expression of Nrf2 target genes was induced (Figure 2C).

To further address the possibility that Nrf2 target genes regulate NLRP3 inflammasome activation, we performed experiments in HPKs. We transduced these cells with lentiviral constructs encoding wild-type Nrf2 or Keap1, or mutant proteins. After induction of expression the cells were irradiated with UVB, resulting in inflammasome activation as reflected by secretion of mature IL-1β (Figure 2D). As a control, mRNA levels of Nrf2 target genes were determined (Figure 2E). Overexpression of wild-type Nrf2 indeed increased secretion of IL-1β (Figure 2D). However, and consistent with the results obtained with macrophages from caNrf2-transgenic mice, overexpression of the caNrf2 mutant increased target gene expression to a similar extent, but did not enhance pro-IL-1β maturation. An Nrf2 mutant lacking the nuclear localization sequence (Nrf2_NLS) slightly increased target gene expression, but strongly increased IL-1β production (Figure 2D and E). Wild-type Keap1 as well as a mutant, which cannot mediate Nrf2 degradation, increased IL-1β in the supernatant of HPKs, although the proteins influenced Nrf2 target gene expression in an opposite manner. These results demonstrate that NLRP3 inflammasome activation is not correlated with the expression of Nrf2 target genes, but rather with the availability of Nrf2 in the cytoplasm.

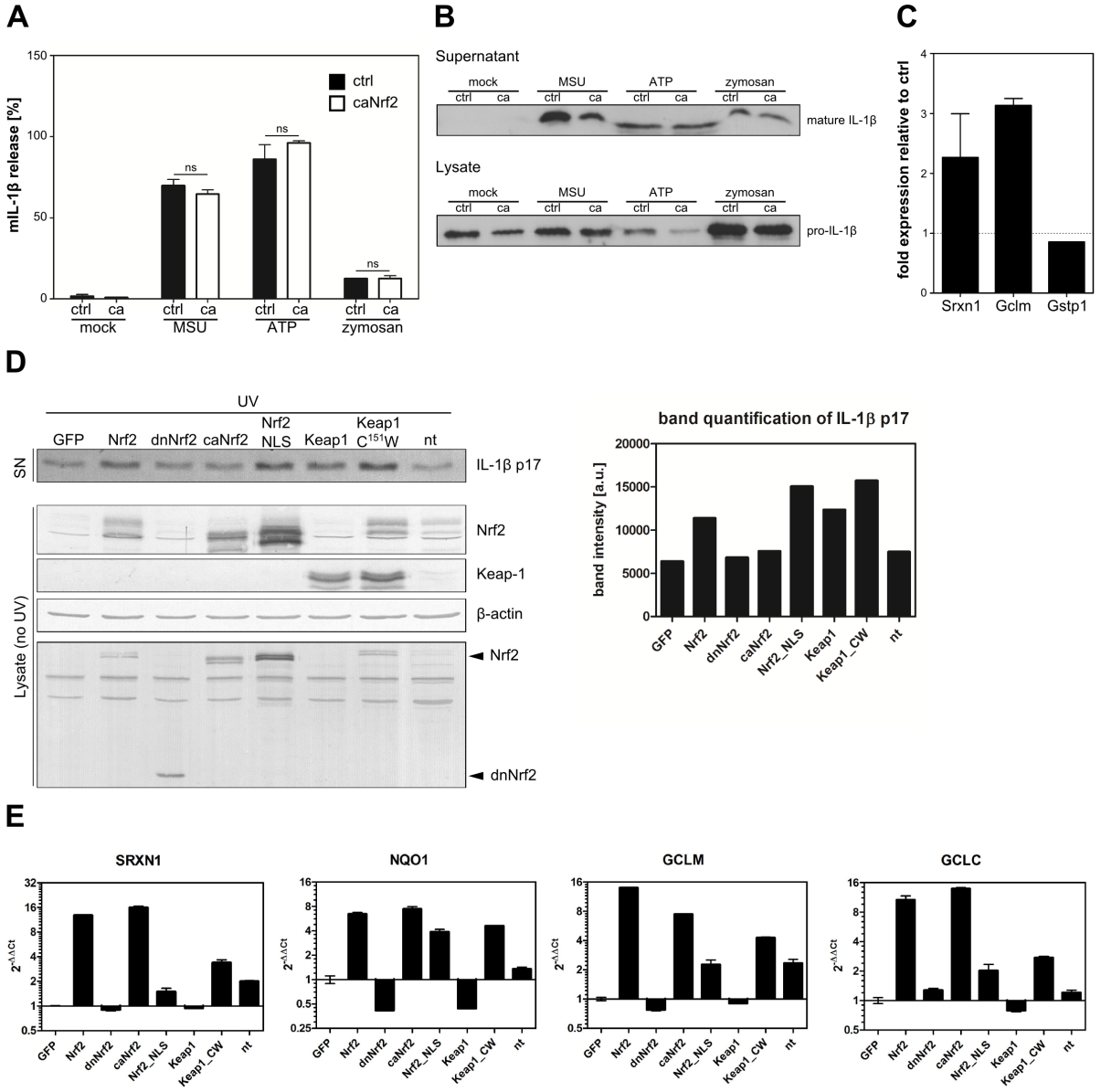


Figure 2: Nrf2 target genes are not involved in NLRP3 inflammasome regulation. (**A**-**C**) Peritoneal macrophages were isolated from mice, which overexpress a constitutively active (ca) mutant of Nrf2 in myeloid cells and from control mice. Cells were treated as described (Figure 1A) and analysed for NLRP3 inflammasome activation by IL-1β measurement in supernatants by (**A**) ELISA or (**B**) Western blot. (**C**) Expression of the Nrf2 target genes sulfiredoxin 1 (SRXN1), NAD(P)H dehydrogenase, quinone 1 (NQO1), glutamate-cysteine ligase, modifier subunit (GCLM), and glutamate-cysteine ligase, catalytic subunit (GCLC) was determined by qRT-PCR. (**D**, **E**) HPKs were transduced with lentiviral constructs encoding the indicated proteins (GFP: green fluorescent protein, dnNrf2: dominant negative Nrf2, caNrf2: constitutively active Nrf2, Nrf2_NLS: Nrf2 lacking nuclear localization domain, nt: not transduced). Transduced cells were selected by cultivation in antibiotic-containing medium for 1 d. Expression was induced with doxycycline 3 d later. (**D**) Cells were irradiated with UVB and 5 h later lysates and supernatants were harvested and analysed for the expression and activation of the indicated proteins by Western blot. (**E**) HPKs were harvested, and expression of the indicated Nrf2 target genes was determined by qRT-PCR. Statistics: (**A**) Error bars represent the mean ± SD of a representative experiment performed with three mice per genotype. Mann-Whitney test was performed.

Nrf2 activators inhibit NLRP3 inflammasome activation

To determine the effects of Nrf2 activating compounds on inflammasome activation, we treated keratinocytes with different doses of SFN, tBHQ, DMF or 15d-PGJ_2 and irradiated the cells with UVB. These compounds inhibited inflammasome activation in a dose-dependent manner as reflected by detection of reduced amounts of processed caspase-1 and mature IL-1β and -18 in the supernatant (Figure 3A-D). SFN and 15d-PGJ_2 were much more efficient than tBHQ and DMF. The anti-inflammatory effect of Nrf2 activators is not restricted to human keratinocytes, since they also inhibited IL-1β secretion in the human monocytic cell line THP-1 (Figure 3E) and in human peripheral blood mononuclear cells (PBMCs) (Figure 3F). As the Nrf2 activators were added to the cells only 15 to 30 min prior to inflammasome activation, it is unlikely that Nrf2 target genes are involved in inflammasome inhibition. To further test this possibility, we treated HPKs (Figure 3G) or THP-1 cells (Figure 3H) with cycloheximide, which blocks protein synthesis (Supplementary Figure 2B). If added just before treatment of cells with SFN, cycloheximide did not prevent inflammasome inhibition by the Nrf2 activator. These experiments provide strong evidence that induction of Nrf2 target genes does not underlie inflammasome inhibition by SFN.

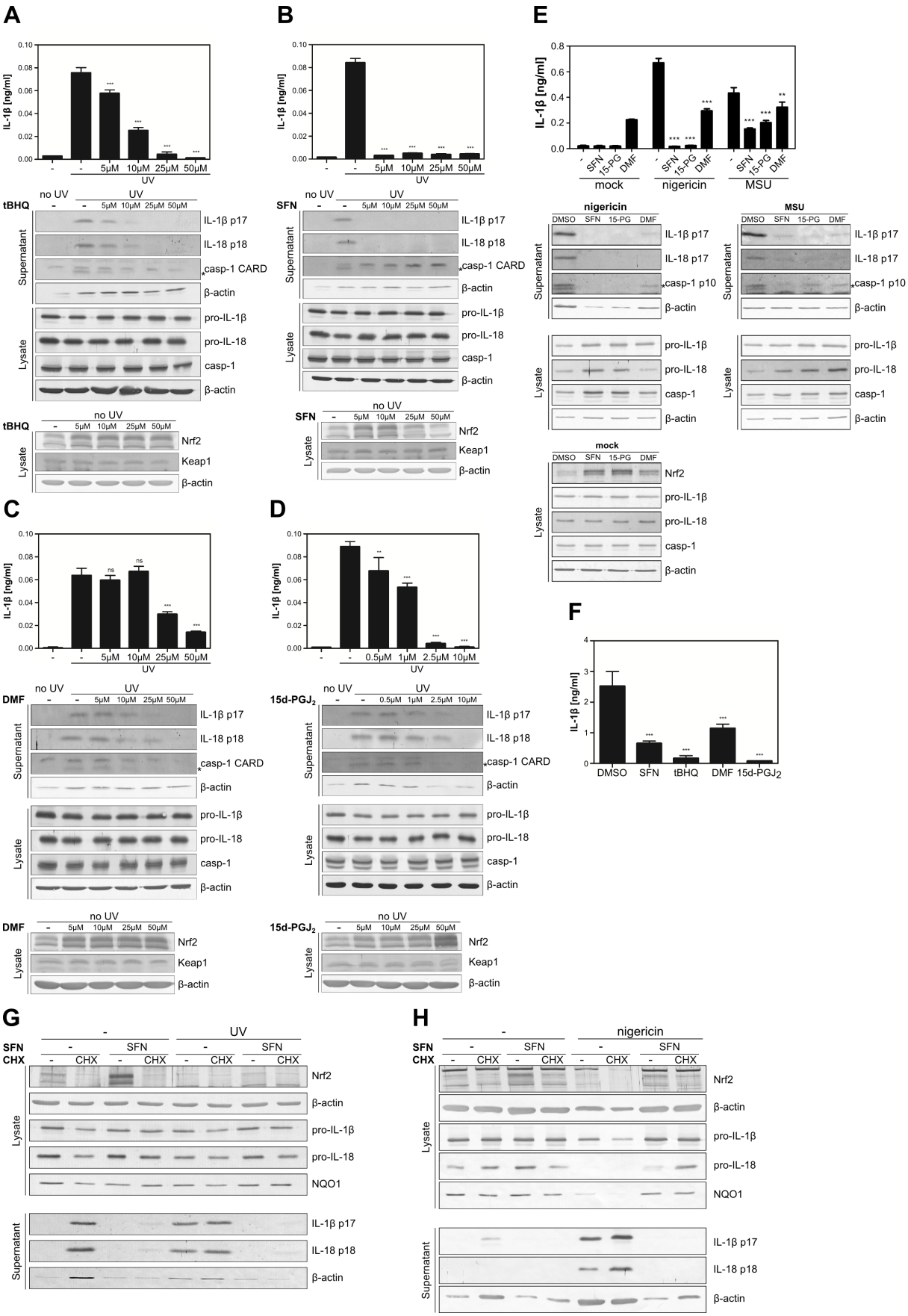
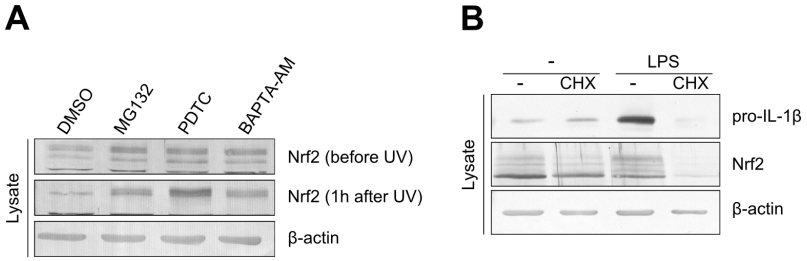


Figure 3: Nrf2 activation blocks inflammasome activation. HPKs were treated with the indicated concentrations of the Nrf2 activating compounds (**A**) tBHQ, (**B**) SFN, (**C**) DMF or (**D**) 15d-PGJ2, irradiated with UVB 30 min later and harvested after 5 h. ELISA measurements were performed for quantification of IL-1β secretion and Western blots for

analysis of expression and activation of the indicated proteins. Specific bands are marked with an asterisk. (**E**) THP-1 cells were differentiated with PMA (27 nM) for 3 d, primed with upLPS (100 ng/ml) overnight, and 1 h before inflammasome activation (5 μM nigericin, 150 μg/ml MSU) treated with SFN (10 μM), 15-PGJ$_2$ (10 μM) or DMF (50 μM) (15-PG: 15-PGJ$_2$). Cells and supernatants were harvested after 5 h and analysed for inflammasome activation by ELISA measurement of IL-1β and Western blots as indicated. (**F**) Freshly isolated PBMCs from human blood were primed overnight with upLPS (100 ng/ml) and treated with SFN (10 μM), tBHQ (10 μM), DMF (50 μM) or 15-PGJ$_2$ (10 μM) 1 h before inflammasome activation by nigericin (5 μM). ELISA measurement for secretion of IL-1β as readout for inflammasome activation was performed after 5 h. (**G**) HPKs or (**H**) differentiated and primed THP-1 cells were pretreated with cycloheximide (CHX, 30 μg/ml) for 1 h before SFN (10 μM) was added to the cells and after an additional hour the inflammasome was activated by (**G**) irradiation with UVB or (**H**) treatment with nigericin (5 μM). Cells and supernatants were harvested after (**G**) 6 h or (**H**) 3.5 h and analysed for inflammasome activation by Western blot as indicated. Statistics: One-way ANOVA.



Supplementary Figure 2: (**A**) HPKs were treated with MG132 (1 μM), PDTC (500 μM) or BAPTA-AM (12.5 μM) for 10 min and harvested (before UV) or irradiated with UVB and harvested after 1 h. Western blots showing expression of Nrf2. (**B**) Differentiated and primed THP-1 cells were pretreated with cycloheximide (CHX, 30 μg/ml) for 1 h before priming cells with upLPS overnight. Western blots show expression of the indicated proteins.

DMF dampens inflammasome-dependent inflammation

An important open question is whether Nrf2 activating compounds are able to block inflammasome-dependent inflammation *in vivo*. Although DMF is used as a drug for the treatment of the inflammatory diseases psoriasis and MS, its mode of action is poorly characterised. However, in both diseases an involvement of inflammasomes is discussed [13, 14]. Monosodium urate (MSU) crystal-induced peritonitis is a mouse model of inflammation, which is dependent on IL-1, IL-1R1, MyD88 and the NLRP3 inflammasome [24, 25]. Recently, it has been shown that Nrf2 expression is required for this type of inflammation [17]. Most importantly, high concentrations of the Nrf2 activators 15d-PGJ$_2$ and SFN, when injected into the peritoneum, blocked inflammasome activation and reduced MSU-induced peritonitis [19, 20]. We chose a different way of administration and supplied mice with SFN or DMF by gavage to determine a potential anti-inflammatory activity of the Nrf2 activators *in vivo*. Since DMF was less potent in inflammasome inhibition than SFN at the same concentrations (Figure 3), we treated mice with this drug for six instead of two days before induction of peritonitis (Figure 4A and D). We analysed the cellular infiltrate in the peritoneum 6 h post injection of MSU crystals. The number of neutrophils was significantly reduced in SFN- and DMF-treated compared to control mice (Figure 4B and E). As a control for the SFN and DMF treatment we determined expression of Nrf2 target genes in the liver and found increased mRNA expression (Figure 4C and F).

These results demonstrate that SFN and DMF, when orally administered, inhibit inflammation in an NLRP3 inflammasome-dependent mouse model.

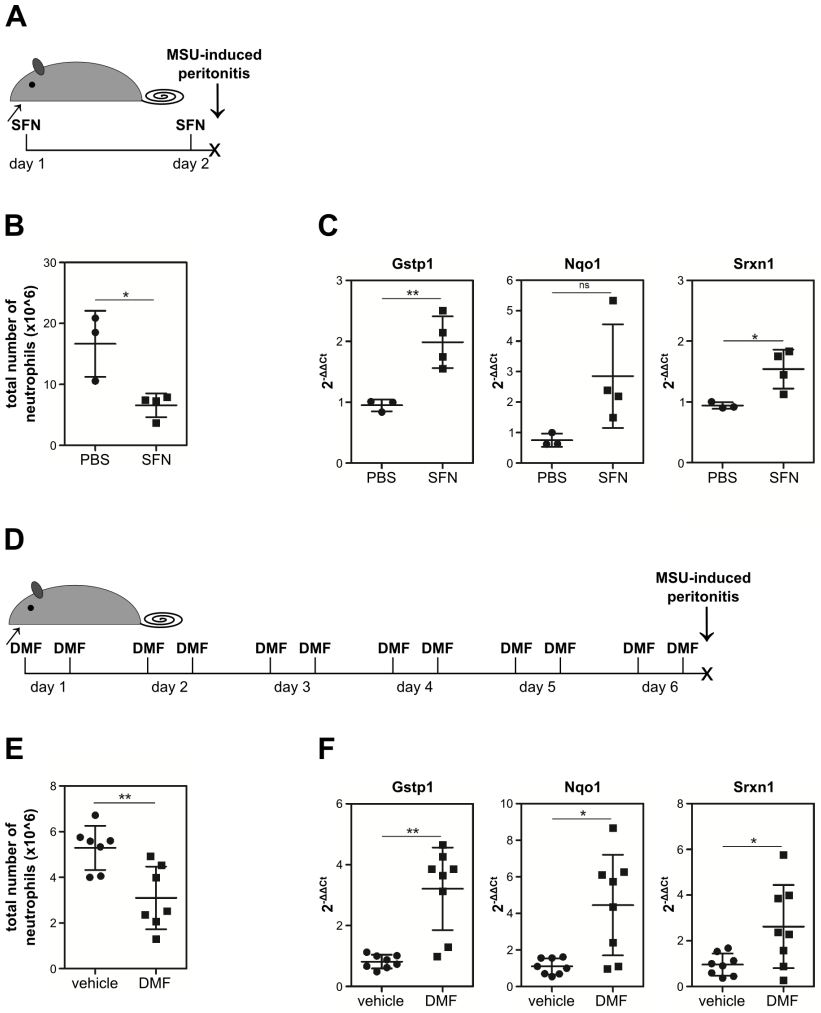
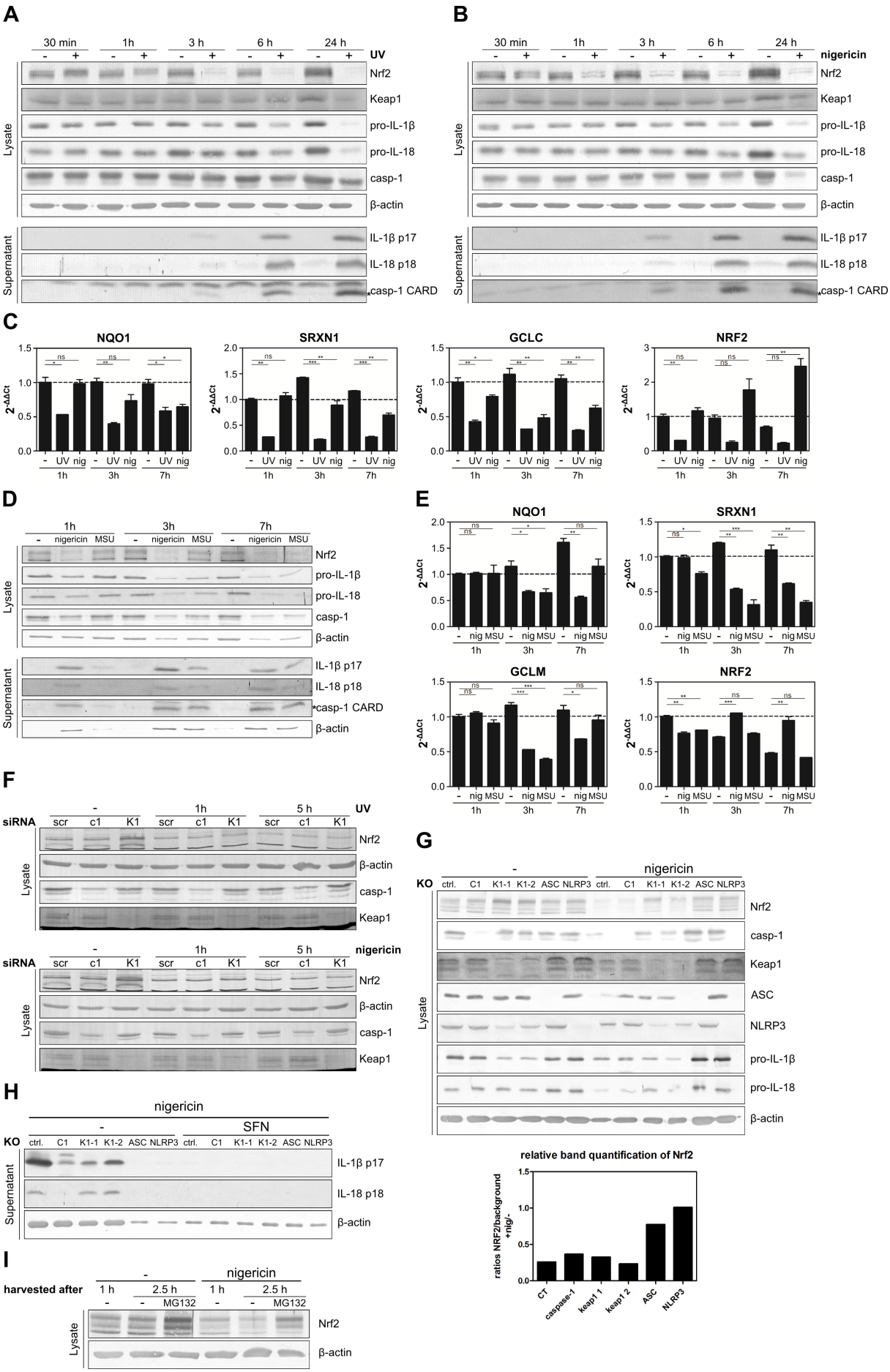


Figure 4: Nrf2 activators dampen peritonitis. Mice treated by gavage with (**A**, **B**, **C**) SFN (25 mg/kg) in PBS or (**D**, **E**, **F**) DMF (20 mg/kg) in H_2O containing 0.08 % methocel and 10 % DMSO, vehicle-treated mice served as control. Regime for (**A**) SFN or (**D**) DMF treatment. Peritonitis was induced by peritoneal injection of 2 mg MSU crystals. (**B**, **E**) After 6 h the number of neutrophils of the peritoneal lavage was determined by flow cytometry. (**C**, **F**) At the same time, the liver was isolated and analysed for the expression of the Nrf2 target genes Gstp1, Nqo1 and Srxn1 by qRT-PCR. Statistics: Student's *t*-test.

NLRP3 inflammasome activation downregulates Nrf2 expression

Next, we investigated the activity of Nrf2 upon activation of the NLRP3 inflammasome, since inflammasome activation is ROS-dependent [26]. Interestingly, UVB irradiation of HPKs induced a strong and fast downregulation of Nrf2 protein levels, while caspase-1 activity was induced (Figure 5A). This is surprising, since UVB irradiation is a strong inducer of ROS production and it can be anticipated that the cells would benefit from Nrf2 activation. UVB-induced IL-1β secretion by HPKs requires expression of NLRP1 and NLRP3 [21]. Both nigericin and MSU crystals are considered as "true" NLRP3 activators [15], but HPKs cannot phagocytose MSU crystals. Therefore, we treated HPKs with nigericin only and THP-1 cells with either of these NLRP3 activators. These treatments also resulted in a fast downregulation of Nrf2 protein levels (Figure 5B and D), while only UVB irradiation strongly downregulated Nrf2 mRNA expression

(Figure 5C and E). Therefore, NLRP3 inflammasome activation most likely induces Nrf2 protein degradation. Interestingly, this effect does not require caspase-1 expression and activity and is partially independent of Keap1 as determined by siRNA-mediated knock-down of these proteins in HPKs or knockouts in THP-1 cells. However, inflammasome activation-induced Nrf2 degradation was blocked upon ablation of ASC or NLRP3 expression (Figure 5G) and upon treatment of cells with the ROS blocker PDTC or the Ca^{2+} chelator BAPTA-AM (Supplementary Figure 2A). Although Keap1 expression is partially dispensable for Nrf2 degradation by inflammasome activation, the transcription factor is directed to the proteasome under these conditions, since Nrf2 degradation was inhibited by the proteasome inhibitor MG132 (Figure 5I and Supplementary Figure 2A).


A
30 min
1h
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Keap1
pro-IL-1β
pro-IL-18
casp-1
β-actin
Lysate
IL-1β p17
IL-18 p18
casp-1 CARD
Supernatant
B
nigericin
C
NQO1
SRXN1
GCLC
NRF2
2-ΔΔCt
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MSU
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siRNA
scr
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K1
5 h
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KO
ctrl.
C1
K1-1
K1-2
ASC
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ASC
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I
harvested after
2.5 h
MG132
relative band quantification of Nrf2
ratios NRF2/background +nig/-
CT
caspase-1
keap1 1
keap1 2

Figure 5: Nrf2 is degraded upon NLRP3 inflammasome activation. (**A**-**C**) HPKs were irradiated with (**A**) UVB or treated with (**B**) nigericin (5 μM) and cells and supernatants were harvested at different time points as indicated. Western blots for expression and activation of the indicated proteins. Specific bands are marked with an asterisk. (**C**) Expression of Nrf2 and Nrf2 target genes was determined by qRT-PCR. (**D**, **E**) THP-1 cells were differentiated with PMA (27 nM) for 3 d, primed with upLPS (100 ng/ml) overnight and treated with nigericin (5 μM) or MSU (150 μg/ml). Cells and supernatants were harvested at different time points as indicated and analysed for (**D**) expression and activation of the proteins as indicated by Western blot and (**E**) expression of Nrf2 and Nrf2 target genes by qRT-PCR. (**F**) HPKs were transfected with specific siRNAs as indicated (scr: scrambled, c1: caspase-1, K1: Keap1), 2 d later irradiated with UVB or treated with nigericin (5 μM). Cells were harvested after 1 h or 5 h, and lysates were analysed for the expression of the indicated proteins by Western blot. (**G**, **H**) Differentiated and primed THP-1 cells with knockout of the indicated genes were treated with (**G**) nigericin (5 μM) or (**H**) additionally pretreated with SFN (10 μM). 3.5 h later lysates and supernatants were harvested and analysed for the expression and activation of the indicated proteins by Western blot. (**I**) Differentiated and primed THP-1 cells were treated with nigericin (5 μM). After 1 h, cells were harvested directly (1 h) or treated with MG132 (1 μM) or mock-treated. After 2.5 h, cells were harvested and analysed for the expression of the indicated proteins by Western blot. Statistics: One-way ANOVA.

The Nrf2/Keap1/Cul3/Rbx1 complex physically interacts with caspase-1

Our experiments demonstrate that Nrf2 target genes are most likely not involved in the cross-talk between Nrf2 and the NLRP3 inflammasome, pointing to a novel mechanism, by which the transcription factor is linked to inflammation. Since overexpression experiments in HPKs (Figure 2D and E) revealed a correlation between the amount of cytoplasmic Nrf2 and inflammasome activation, it seems possible that Nrf2 supports NLRP3 inflammasome activation by a direct or indirect physical interaction with the immune complex. To address this point, we performed co-immunoprecipitation (co-IP) experiments with an antibody for caspase-1 and lysates of HPKs. However, we were not able to detect an interaction between caspase-1 and Nrf2 (results not shown). Interestingly, however, interaction of caspase-1 with Rbx1 was found. The specificity of the band was verified by knock-down of Rbx1 expression (Figure 6A). In addition, we overexpressed a FLAG-tagged version of caspase-1 in HPKs and precipitated the protease with an ANTI-FLAG ® M2 Affinity Gel (Figure 6B). In this precipitate endogenous Nrf2, Keap1, Cul3, and Rbx1 were detected, demonstrating that overexpressed caspase-1 interacts with these proteins. However, treatment of HPKs with SFN did not prevent, but rather enhanced the interaction between caspase-1 and Rbx1 (Figure 6C). To address the question whether Nrf2 complex proteins interact with inflammasome proteins directly, we performed co-IP experiments with lysates of transfected COS-1 or HEK293T cells. However, interactions of Nrf2, Keap1 and Rbx1 with caspase-1, pro-IL-1β and NLRP3 could not be detected in a reproducible manner (results not shown).

These experiments demonstrate a physical crosstalk between the Nrf2 and NLRP3 complexes, which may explain the requirement of Nrf2 expression for NLRP3 inflammasome activation. Most likely, this interaction is not direct, but mediated by unknown proteins. The fact that Rbx1 bound to caspase-1 also upon SFN treatment of HPKs raises the possibility that Nrf2 activators inhibit inflammasome activation through a different molecular mechanism. To address this possibility, we treated BMDCs from wild-type and Nrf2 knockout mice with SFN or vehicle (Figure 6 D and E). Whereas Nrf2 ablation reduced IL-1β maturation and, therefore,

inflammasome activation, SFN completely abolished secretion of the cytokine independently of Nrf2 expression. In addition, inflammasome inhibition by SFN is also Keap1 independent (Figure 5H). These experiments demonstrate that Nrf2 ablation and SFN inhibit NLRP3 inflammasome activation by different molecular mechanisms.

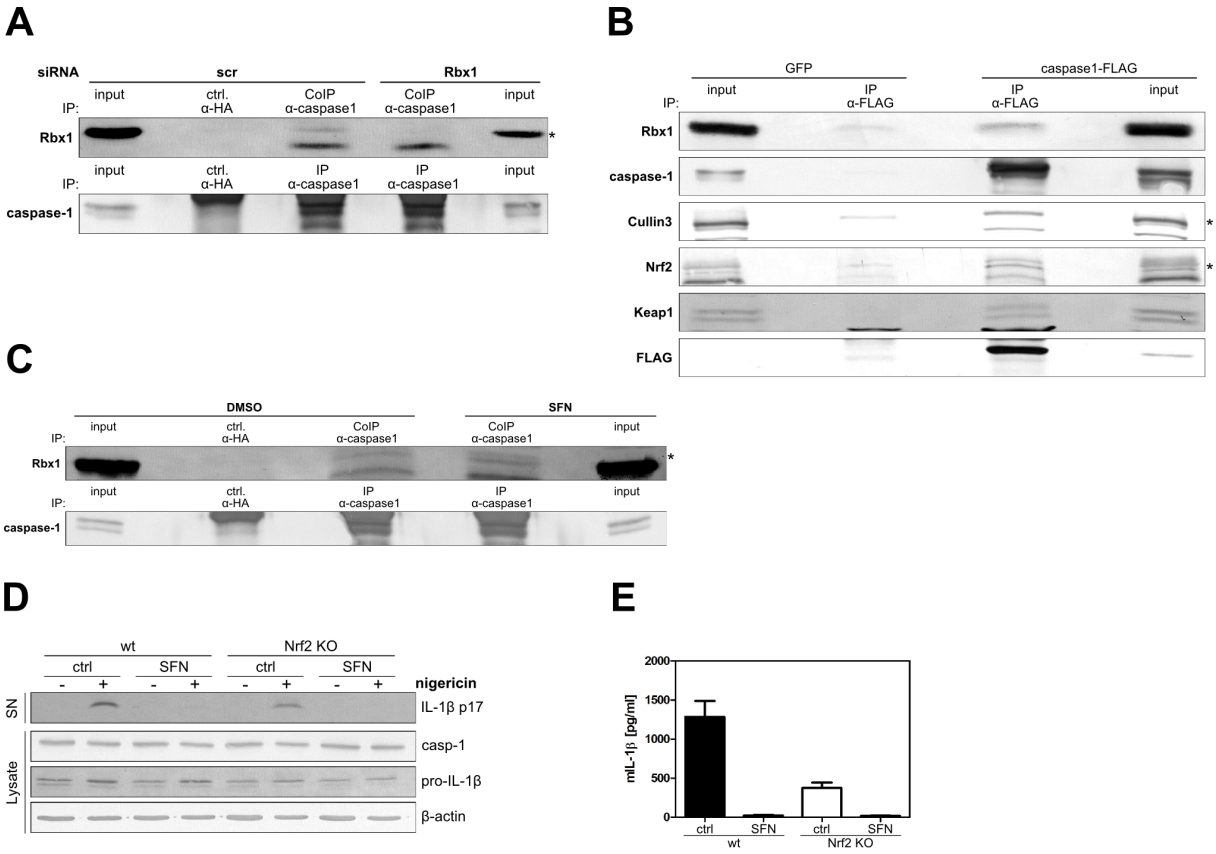

Figure 6: Nrf2 and SFN influence the NLRP3 inflammasome by different mechanisms. (**A**) HPKs were transfected with scrambled siRNA for control or with Rbx1-specific siRNA. 3 d later cells were harvested and IPs were performed with a caspase-1-specific or with an HA antibody, the latter served as isotype control. Western blots for caspase-1 and Rbx1. (**B**) HPKs were transduced with lentiviral constructs encoding FLAG-tagged caspase-1 or GFP under the control of a Tet-On inducible promoter. After selection for 3 d expression was induced by the addition of doxycycline (1 µg/ml). Cells were harvested after 24 h and IP was performed with an ANTI-FLAG® M2 Affinity Gel (Sigma). Western blots showing expression and interactions of the indicated proteins. (**C**) HPKs were treated with SFN (50 µM) or the solvent DMSO and 2 h later harvested. IPs were performed with a caspase-1-specific antibody. Western blots for caspase-1 and Rbx1. (**D**, **E**) DCs were differentiated from the bone marrow of wt and Nrf2 knockout mice (n=4), primed overnight with upLPS and treated with the solvent DMSO (ctrl) or SFN (10 µM). After 1 h, BMDCs were treated with 5 µM nigericin and harvested 4.5 h later. (**D**) Western blots for expression and activation of the indicated proteins and (**E**) ELISA for quantification of secretion of IL-1β. (**A**-**C**) Specific bands are marked with an asterisk.

Discussion

Both, inflammasomes and the Nrf2 transcription factor are activated by stress factors. Whereas numerous pathogen- and danger-associated molecular patterns (PAMPs and DAMPs) induce inflammasome activation and subsequently inflammation, Nrf2 is activated by ROS and/or electrophiles. Interestingly, ROS have been discussed as inducers of NLRP3 inflammasome activation downstream of PAMPs and DAMPs [15]. Nrf2 activation induces expression of ROS-detoxifying enzymes and represents a survival pathway [5]. In contrast, inflammasome activation causes cell death, termed pyroptosis [15]. Recent evidence suggests that Nrf2 is a

positive regulator of the NLRP3 type of inflammasomes [17, 18]. However, this is challenged by results from other studies [19, 20]. High concentrations of certain Nrf2 activators are able to block inflammasome activation [19, 20]. Nrf2 activators induce expression of Nrf2 target genes, whereas ablation of Nrf2 expression has the opposite effect. Therefore, it is unexpected that both – Nrf2 activation and Nrf2 ablation – have the same consequence, namely inhibition of the NLRP3 inflammasome. The anti-inflammatory effects of Nrf2 activators are consistent with previous data where SFN ameliorated the development of experimental autoimmune encephalomyelitis (EAE) [27] and blocked IL-1β production by peritoneal macrophages [28]. DMF is successfully used for the treatment of psoriasis and MS [6, 29], two inflammatory diseases with a suggested involvement of inflammasomes [13, 14]. However, the known target genes of Nrf2 are not obvious candidates for mediating a direct anti-inflammatory activity. This raises the possibility that Nrf2 activators exert their anti-inflammatory activity independently of Nrf2, which has also been previously suggested [19, 20].

Here, we present evidence that Nrf2 ablation as well as Nrf2 activating compounds block activation of the NLRP3 inflammasome. Most importantly, our experiments show that both effects are not mediated by Nrf2 target genes. Whereas Nrf2 ablation only dampens inflammasome activation, Nrf2 activators are able – dependent on the dose – to completely block caspase-1 activation. This suggests that two different molecular mechanisms are underlying NLRP3 inflammasome inhibition by Nrf2 ablation and Nrf2 activating compounds. First, a physical (most likely indirect) interaction of the Nrf2 complex with caspase-1 might contribute to the requirement of Nrf2 expression for inflammasome activation, since cytoplasmic Nrf2 supports NLRP3-dependent IL-1β production. Second, how SFN, DMF and other Nrf2 activators block inflammasome activation is as yet unknown and has to be determined in the future. It has been speculated that SFN might covalently modify not only Keap1, but also other proteins. However, inflammasome proteins and, in particular, caspase-1 do not seem to be a direct target [20].

The mechanisms of action of DMF in patients suffering from psoriasis or MS are only poorly characterised. Our results suggest that DMF exerts its anti-inflammatory activity - at least in part - via inflammasome inhibition. This raises the possibility that DMF may also be useful for the treatment of other inflammatory diseases with an involvement of inflammasomes. In addition, SFN might be even more efficient in these patients, as our results demonstrate inflammasome inhibition at lower SFN concentrations *in vitro* and *in vivo* compared to DMF. However, since Nrf2 activating compounds inhibit inflammasome activation independently of Nrf2 target genes, not all of them may block inflammasomes.

Surprisingly, NLRP3 inflammasome activation induces rapid Nrf2 degradation, which is only partially inhibited upon knock-down/knockout of caspase-1 and Keap1. Cell death induced by inflammasome activation can be essential for immunity due to killing of intracellular pathogens. In addition, this cell death contributes to the limitation of the inflammasome-dependent inflammatory response. Nrf2 activation would be detrimental in this context, because it supports survival of cells.

Several publications demonstrate a protective role of Nrf2 using Nrf2 knockout mice and cells [3, 22]. In these experiments Nrf2 activators lost their activity in the absence of Nrf2. Our results raise the possibility that the protective effects of Nrf2 in wild-type mice in comparison to Nrf2 knockout mice might be also mediated by inflammasomes and not solely by induction of Nrf2 target genes.

Taken together, we present evidence for an important and complex crosstalk between the Nrf2 and inflammasome pathways, which is most likely mediated by different mechanisms independent of Nrf2 target genes. However, more effort is required for the characterisation of the underlying molecular events. This is also important, since Nrf2 activators are being used and tested for the treatment of inflammatory and other types of diseases.

Acknowledgements

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3.2 Further results

This chapter contains additional experiments and results concerning the data presented in the chapter above (3.1). The following results will not be included in the manuscript, which will be prepared for publication. Yet, in the context of this thesis, they are relevant. On the one hand, these data point to experimental limitations, and on the other hand, they offer the possibility for a broader discussion of the subject.

My contributions to this part of the study were:

- Design and performing of mouse experiments (as shown in Figures 7, 8, 12-15)
- ELISA measurements of IL-1β secretion from HPKs (as shown in Figures 9 and 10)
- Inflammasome reconstitution by transient transfection of HEK 293T cells (as shown in Figure 11)
- Breeding of mice and performing the experiment (as shown in Figure 16)
- Analysis of Nrf2 protein expression by Western blot (as shown in Figure 17)
- Design and statistical analysis of experiments

Nrf2 ablation and inflammasome-dependent inflammation in vivo

It was reported that Nrf2 expression is required for NLRP3 and AIM2 inflammasome activation in murine cells *in vitro* and *in vivo* [1, 2]. Using BMDCs isolated from Nrf2 KO mice, we could confirm a requirement of Nrf2 expression for inflammasome activation *in vitro* in murine cells (Figure 1A-D). To assess, whether Nrf2 is also required for inflammasome activation *in vivo*, we induced peritonitis in Nrf2 KO mice with MSU crystals. We analysed the cellular infiltrate in the peritoneal cavity 6 h post injection of MSU crystals. The number of infiltrating neutrophils was reduced in Nrf2 KO mice compared to wild-type control animals; however, the reduction was not significant. This experiment was repeated twice and Nrf2 KO mice always displayed reduced influx of neutrophils, but the reduction was not statistically significant in either case (Figure 7).

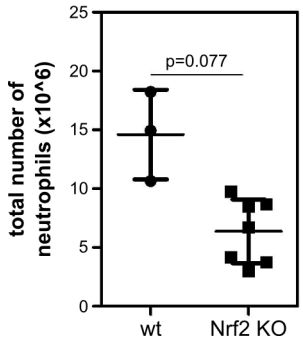

Figure 7: MSU crystal-induced peritonitis in Nrf2 KO and control wild-type mice. Peritonitis was induced in Nrf2 KO mice and wt littermates by peritoneal injection of 2 mg MSU crystals in PBS. After 6 h, the cellular infiltrate in the peritoneal cavity was analysed and the number of neutrophils was determined by flow cytometry. Figure shows combined results of two representative experiments. Number of mice in both experiments: first experiment: n=2 for wild-type and n=3 for Nrf2 KO; second experiment: n=1 for wild-type and n=4 for Nrf2 KO. Statistics: Unpaired *t*-test with Welch's correction.

In a second step we wanted to test Nrf2 KO mice in a different mouse model of inflammation, which is inflammasome-dependent. We chose contact hypersensitivity (CHS), also known as mouse ear-swelling model, which is an established model for skin inflammation. CHS is a delayed-type hypersensitivity reaction induced by sensitizing chemicals that penetrate the skin surface. In CHS, priming of the adaptive immune system depends on the concomitant activation of the innate immune system and it was described that inflammasome activation contributes to CHS [3]. We sensitized mice by topical application of dinitrofluorobenzene (DNFB) to the skin of one ear and after five days, mice were elicited by application of DNFB to the other ear (Figure 8A). Swelling of the second ear, which is dependent on inflammasome activation, was measured before and 24 h after DNFB challenge. Ear thickness was slightly reduced in Nrf2 KO mice treated with DNFB compared to wild-type mice, although not to a significant extent (Figure 8B).

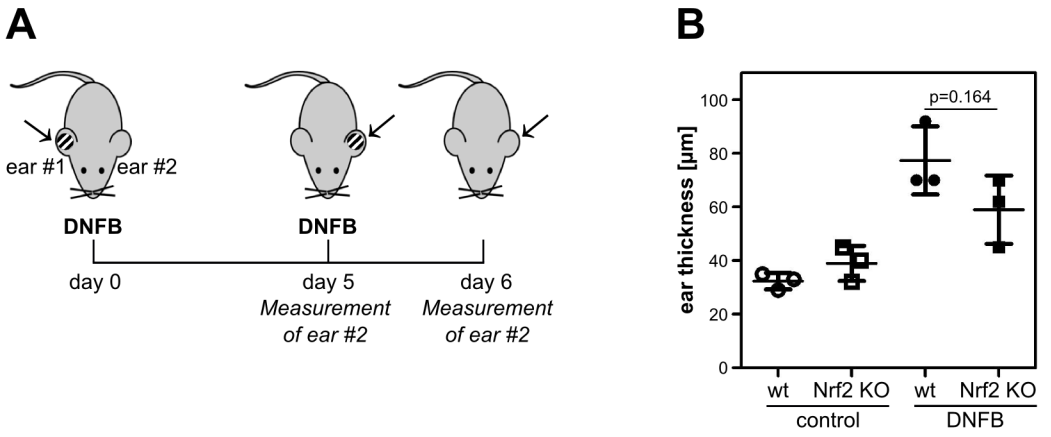

Figure 8: CHS or ear-swelling mouse model with Nrf2 KO and control wild-type mice. 50 µl of 0.5 % DNFB in acetone:olive oil (3:1, v/v) was applied to one ear of each mouse (ear 1). After 5 d, the thickness of the other ear (ear 2) was measured with a digital thickness gauge before mice were elicited by painting 'ear 2' with 50 µl of the DNFB solution. Ear thickness of 'ear 2' was measured 24 h later. Control mice were painted with vehicle. Statistics: Student's *t*-test.

Knock-down of Cul3 and Rbx1 does not interfere with inflammasome activation in HPKs

Our experiments demonstrate that Nrf2 expression is required for efficient inflammasome activation not only in murine, but also in human cells. Knock-down of Nrf2 expression in HPKs using siRNA inhibited caspase-1 activation and reduced the secretion of IL-1β and -18 upon UVB irradiation (Figure 1E-G). Under homeostatic conditions, Nrf2 is bound in the cytoplasm to Keap1, which mediates the interaction to the E3 ubiquitin ligase Cul3 and Rbx1. To determine whether any of the other components of the Nrf2/Keap1/Cul3/Rbx1 complex contributes to efficient inflammasome activation in HPKs, we performed siRNA transfection to knock-down expression of Keap1, Cul3, or Rbx1, respectively. Knock-down of Keap1 expression using two different (out of four) siRNA sequences strongly impaired inflammasome activation as reflected by reduced secretion of IL-1β and -18 (Figure 9). However, according to the morphology of the cells, the knock-down of Keap1 using these two siRNA sequences had toxic effects, which is also indicated by the increased level of β-actin in the culture supernatant. Knock-down of Keap1 expression with two additional, different siRNA sequences did not impair inflammasome activation (results not shown). Moreover, knockout of Keap1 expression in THP-1 cells did not impair secretion of IL-18 (Figure 5G and H). It seems that knock-down and particularly knockout of Keap1 expression results in reduced expression of pro-IL-1β, which in turn causes lower secretion levels of IL-1β; however, pro-IL-18 expression does not seem to be dependent of Keap1 expression. In conclusion, further experiments are required to elucidate the specific functions of Keap1 in inflammasome activation.

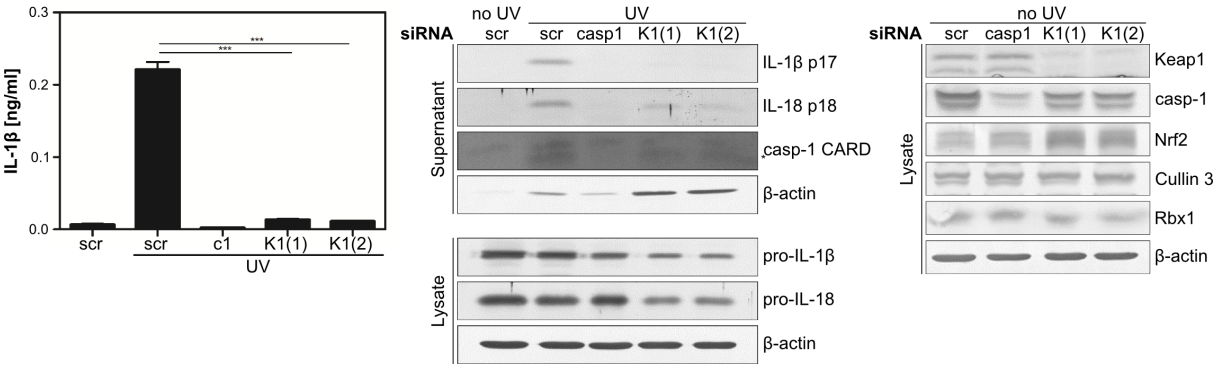

Figure 9: Knock-down of Keap1 expression in HPKs. HPKs were transfected with specific siRNAs as indicated (scr: scrambled, c1: caspase-1, K1: Keap1), irradiated with UVB 3 d later and harvested after 5 h or mock-treated ('no UV'). Inflammasome activation was analysed by ELISA measurement of IL-1β in the supernatant or by Western blots as indicated. Specific bands are marked with an asterisk. Statistics: One-way ANOVA.

Knock-down of Cul3 expression using two different siRNA sequences did not interfere with inflammasome activation (Figure 10A). In contrast, siRNA-mediated knock-down of Rbx1 expression indeed resulted in reduced secretion of IL-1β as measured by ELISA (Figure 10B). However, we observed that the knock-down of Rbx1 expression inhibited proliferation compared to cells transfected with scr siRNA (results not shown). This suggests that the reduced amount of secreted IL-1β upon knock-down of Rbx1 expression is not mediated by a direct effect on inflammasome activation. Rather, Rbx1 is an important protein, whose reduction inhibits cell proliferation and causes toxicity for HPKs, which in turn impairs inflammasome activation. In summary, our experiments suggest that Cul3 and Rbx1 are dispensable for inflammasome activation.

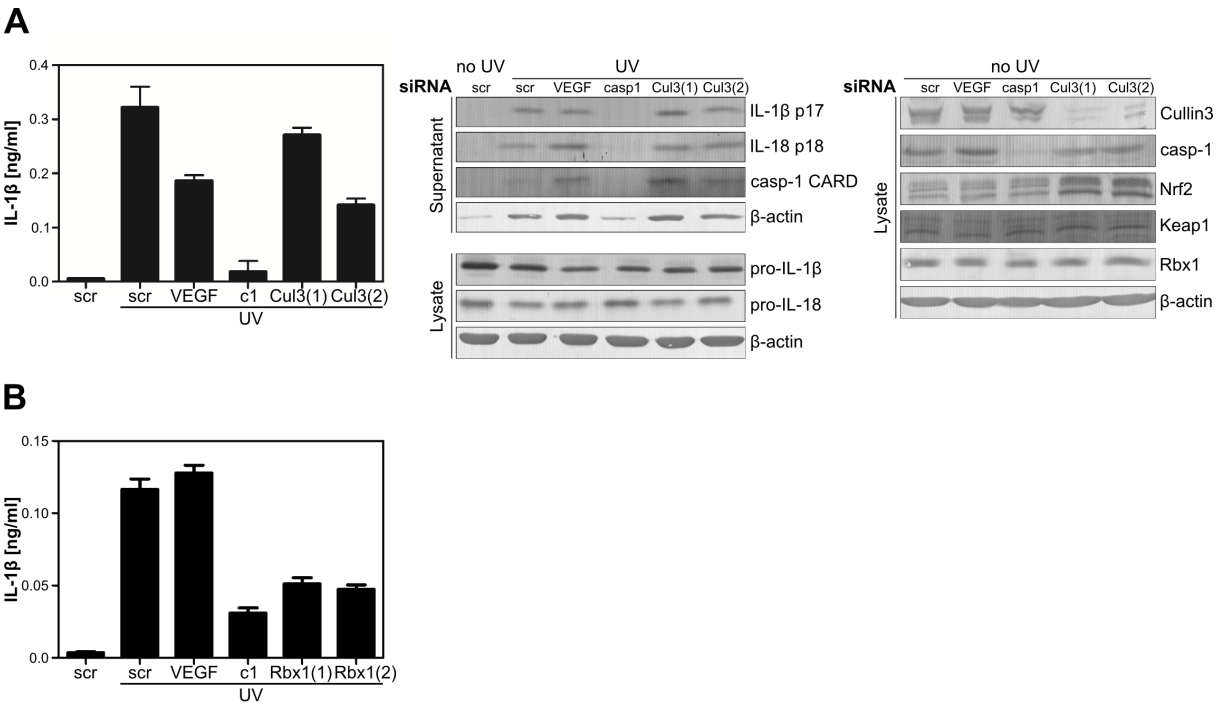

Figure 10: Knock-down of (A) Cul3 and (B) Rbx1 in HPKs. HPKs were transfected with siRNA for (**A**) 3 d or (**B**) 2 d as indicated. Scrambled (scr) siRNA, siRNA targeting the unrelated vascular endothelial growth factor (VEGF) and

siRNA targeting caspase-1 (c1) served as controls. Cells were irradiated with UVB and harvested after 5 h or mock-treated ('no UV'). Inflammasome activation was analysed by (**A**, **B**) ELISA measurement of IL-1β in the supernatant or (**A**) by Western blots as indicated.

Nrf2 increases IL-1β production in HEK 293T cells with a reconstituted NLRP3 inflammasome

Our results indicate that the amount of Nrf2 protein in the cytoplasm positively correlates with inflammasome activation. On the one hand, a knock-down of Nrf2 expression reduced caspase-1 activation (Figure 1E-G); on the other hand, overexpression of Nrf2 using lentiviral constructs increased secretion of IL-1β in HFKs (Figure 2D). We wanted to test whether Nrf2 would also influence pro-IL-1β processing by a reconstituted inflammasome. A functional NLRP3 inflammasome can be reconstituted in HEK 293T cells through transfection of plasmids encoding pro-IL-1β, NLRP3, pro-caspase-1, and ASC [4]. We co-transfected HEK 293T cells with these plasmids, which resulted in processing of pro-IL-1β (Figure 11, lane 2 and 3) and in secretion of mature IL-1β into the culture supernatant (not shown). In a second step, we additionally transfected cells with a plasmid encoding Nrf2, which led to a mild increase in pro-IL-1β processing by the reconstituted NLRP3 inflammasome (Figure 11, lane 4-7). This result strengthens our hypothesis that NLRP3 inflammasome activation is correlated with the availability of Nrf2 in the cytoplasm.

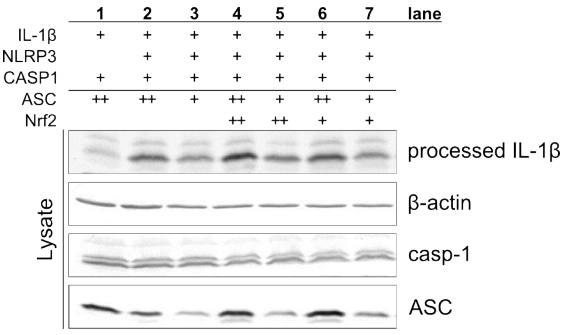

Figure 11: Nrf2 supports processing of pro-IL-1β in HEK 293T cells with a reconstituted NLRP3 inflammasome. The NLRP3 inflammasome was reconstituted in HEK 293T cells by transfection of cells with plasmids encoding pro-IL-1β, NLRP3, caspase-1, and ASC, in addition to a plasmid encoding Nrf2. (+) and (++) indicate that a low and a high concentration of DNA was transfected, respectively. Cells were harvested 48 h after transfection and analysed by Western blot as indicated.

Nrf2 activators and inflammasome-dependent inflammation in vivo

As mentioned above, two studies reported that the Nrf2 activators 15d-PGJ_2 and SFN, respectively, block inflammasome activation and reduce MSU crystal-induced peritonitis [5, 6]. In both studies the Nrf2 activator was injected intraperitoneally (i.p.) before injection of MSU crystals to induce peritonitis. We were wondering, whether DMF is also able to dampen inflammasome-dependent inflammation *in vivo* using the same approach. To this end, we injected DMF i.p. into mice, followed by injection of MSU crystals. After 4 h, mice were again

treated with DMF and at 6 h, peritoneal lavage was performed and cell recruitment to the peritoneum was measured (Figure 12A). We found that unlike after injection of 15d-PGJ_2 or SFN, the number of neutrophils was not changed after injection of DMF (Figure 12B). As a control for the DMF treatment we determined expression of Nrf2 target genes in the infiltrating cells. However, an increase in Nrf2 target gene expression could not be detected (results not shown). Therefore, we conclude that by using this form of application, DMF is probably not delivered sufficiently to the cells, which attract neutrophils.

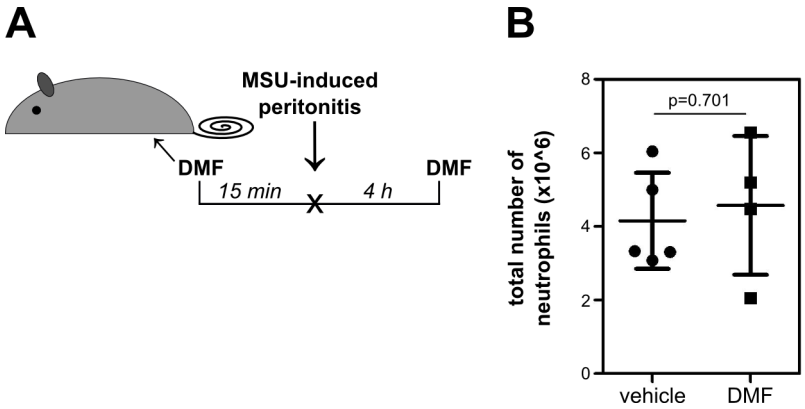

Figure 12: Intraperitoneal injection of DMF does not attenuate peritonitis. Mice were injected i.p. with DMF (500 mg/kg) in PBS containing 10 % DMSO; vehicle-treated mice served as control. DMF was administered 15 min before peritonitis was induced by peritoneal injection of 2 mg MSU crystals in PBS. After 4 h, mice were again injected i.p. with DMF (500 mg/kg) and 6 h after induction of peritonitis, the cellular infiltrate in the peritoneal cavity was analysed and the number of neutrophils was determined by flow cytometry. Statistics: Student's *t*-test.

DMF is used as a drug by oral application. Therefore, we wanted to test a way of administration of Nrf2 activators into mice, which resembles the situation in psoriasis and MS patients treated with DMF. For this purpose, we fed mice via oral gavage with a single daily dose of DMF for seven days. At day 8 we injected MSU crystals i.p. to induce peritonitis (Figure 13A). Oral treatment of mice with DMF resulted in a slight, but non-significant decrease in the number of infiltrating neutrophils (Figure 13B). Furthermore, expression of Nrf2 target genes in the liver was not changed (results not shown). In a second approach we treated mice more extensively with DMF by doubling the daily dose and, indeed, found a clear and significant reduction in the number of infiltrating neutrophils after MSU crystal-induced peritonitis (Figure 4D and E) and upregulated Nrf2 target genes in the liver (Figure 4F).

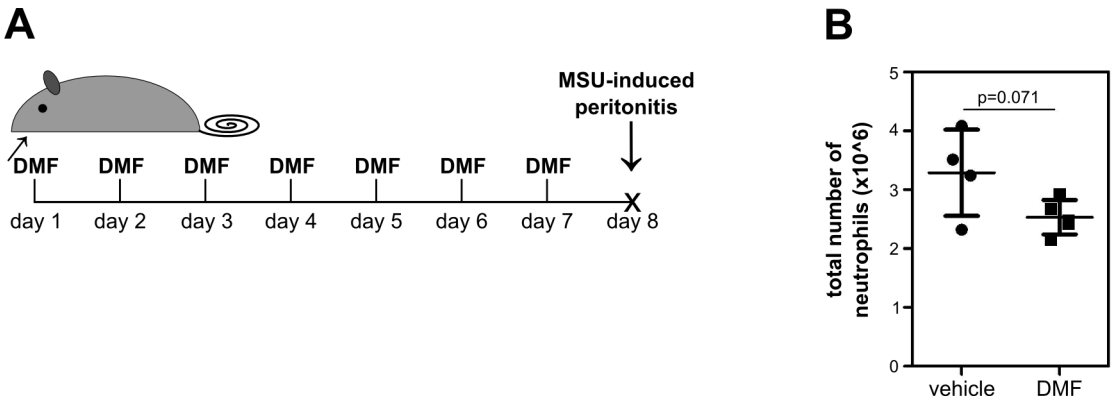

Figure 13: Peritonitis after repeated oral gavage with DMF. Mice were fed daily with DMF (25 mg/kg) in H_2O containing 0.08 % methocel and 10 % DMSO over a time period of 7 d; vehicle-treated mice served as control. At d 8, peritonitis was induced by peritoneal injection of 2 mg MSU crystals in PBS. After 6 h, the cellular infiltrate in the peritoneal cavity was analysed and the number of neutrophils was determined by flow cytometry. Statistics: Student's *t*-test.

Our experiments demonstrate that both, SFN and DMF, when administered orally, can inhibit inflammasome-dependent inflammation *in vivo*. To test an additional Nrf2 activator, we fed mice by oral gavage with butylated hydroxyanisole (BHA). BHA is commonly used as an antioxidant and preservative in food and was shown to induce a number of Nrf2 target genes [7]. The potent Nrf2 activator tBHQ is a demethylation product of BHA. Because BHA was described to induce Nrf2 target genes within hours [8], we chose a short treatment regime for the oral application of BHA. We found that the effect of BHA administration on the number of infiltrating neutrophils after MSU-induced peritonitis was only minor (Figure 14). Besides, the expression of Nrf2 target genes was not significantly increased (results not shown). Nevertheless, it may well be that upon a modification of the experimental procedure and/or a higher dosage, BHA may dampen inflammasome-dependent inflammation *in vivo*.

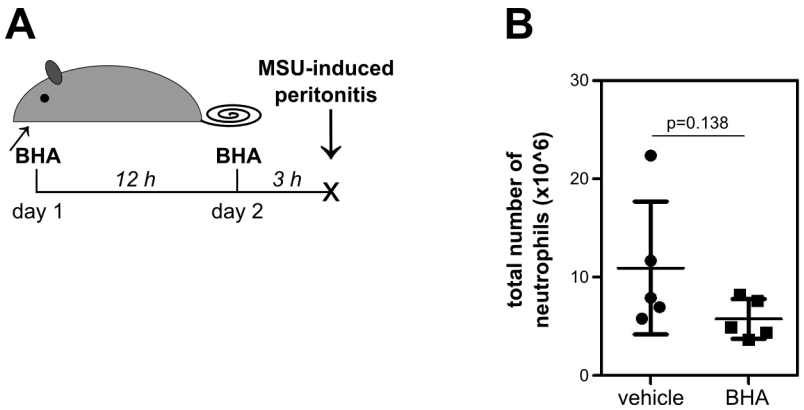

Figure 14: Peritonitis after oral gavage of BHA. Mice were fed twice with an interval of 12 h with BHA (200 mg/kg) in olive oil; vehicle-treated mice served as control. 3 h after the second gavage of BHA, peritonitis was induced by peritoneal injection of 2 mg MSU crystals in PBS. 6 h after induction of peritonitis, the cellular infiltrate in the peritoneal cavity was analysed and the number of neutrophils was determined by flow cytometry. Statistics: Student's *t*-test.

Listeria monocytogenes is a Gram-positive bacterium and a facultative intracellular pathogen that causes severe infection [9]. After being actively internalized by phagocytic cells, Listeria escapes from the phagosome and replicates within the cytosolic compartment. *Listeria*

monocytogenes infection can be detected by multiple receptors in different cellular compartments. It was shown that cytosolic *Listeria monocytogenes* activates caspase-1, which is required for the clearance of the pathogen in murine infection [10]. It was suggested that *Listeria* activates caspase-1 through multiple inflammasomes, including NLRP3, AIM2, and NLRC4; however, there are conflicting reports regarding the relative importance of the individual inflammasomes in *Listeria*-infected macrophages [11, 12].

In order to test Nrf2 activators in an additional *in vivo* mouse model of inflammasome activation, we examined inflammasome suppression by SFN in mice infected with *Listeria monocytogenes* (Figure 15A). We measured bacterial accumulation in the liver and in the spleen of Listeria-infected mice fed with SFN or vehicle. SFN-fed mice had only a mildly increased bacterial load in the liver, which indicates that SFN treatment had only a marginal effect on Listeria infection (Figure 15B). Further efforts are required to optimize the protocol in order to elucidate a possible immunosuppressive role of SFN in this infection model.

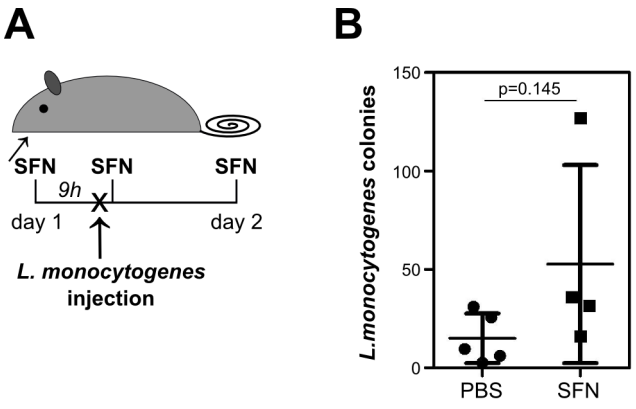

Figure 15: *Listeria monocytogenes* infection upon oral gavage of SFN. Mice were fed with SFN (25 mg/kg) and 9 h later infected with 2 x 10^4 CFUs of *Listeria monocytogenes*, immediately followed by an additional gavage of SFN. 24 h later, SFN was given again. The liver was collected 2 d after infection with *Listeria monocytogenes* and bacterial growth was determined. Statistics: Student's *t*-test.

Nrf2 ablation, Nrf2 activators and inflammasome-dependent inflammation in vivo

Using BMDCs derived from Nrf2 KO and wild-type control mice treated with SFN or vehicle, we could demonstrate that Nrf2 ablation and SFN inhibit inflammasome activation to a different extent and, therefore, most likely by different molecular mechanisms (Figure 6D and E). To address the question, whether also *in vivo* Nrf2 activators inhibit inflammasome activation independently of Nrf2 expression, we fed Nrf2 KO and wild-type control mice with SFN or vehicle and subjected them to MSU crystal-induced peritonitis (Figure 16A). As we already experienced in earlier experiments (Figure 7), neutrophil influx was not significantly reduced in Nrf2 KO mice compared to wild-type animals. Furthermore, in this experiment the inhibitory effect of SFN on inflammasome activation was only marginal (Figure 16B). Since we also found

high variations among individual mice, we speculate that probably more mice and an optimization of the protocol are required to address the question, whether SFN inhibits inflammasome activation independently of Nrf2 *in vivo*.

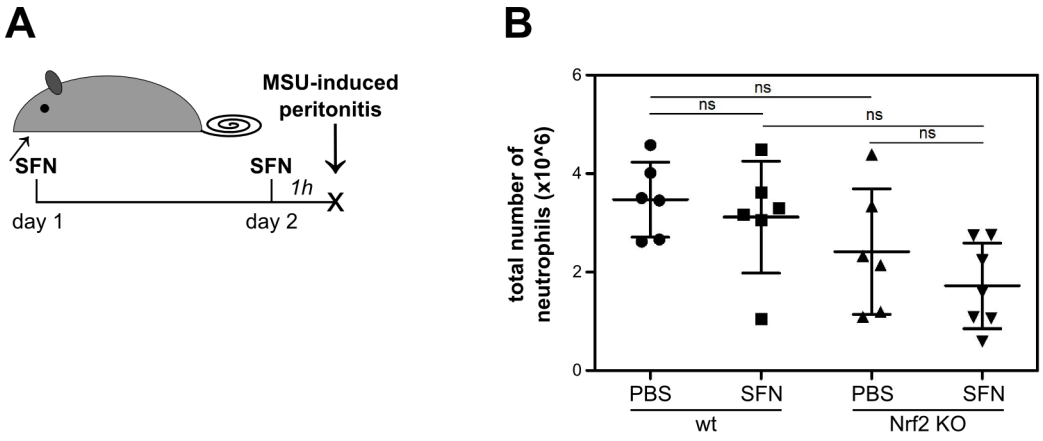

Figure 16: Peritonitis in Nrf2 KO and control wild-type mice after oral gavage of SFN. Mice were fed with SFN (25 mg/kg) in PBS. 1 h after the second gavage of SFN at d 2, peritonitis was induced by peritoneal injection of 2 mg MSU crystals in PBS. 6 h after induction of peritonitis, the cellular infiltrate in the peritoneal cavity was analysed and the number of neutrophils was determined by flow cytometry. Statistics: One-way ANOVA.

Mechanistic insights into downregulation of Nrf2 expression upon inflammasome activation in HPKs

Our results indicate that NLRP3 inflammasome activation induces Nrf2 protein degradation (Figure 5). Using THP-1 cells, we could show that 1 h after nigericin treatment Nrf2 protein expression is strongly reduced (Figure 5D and I). However, when the proteasome inhibitor MG132 is added 1 h after nigericin treatment, Nrf2 protein expression can be restored to a level comparable to untreated controls (Figure 5I). This result indicates on the one hand, that NLRP3 inflammasome activation by nigericin most likely induces Nrf2 protein degradation, and on the other hand, that the degradation is mediated by the proteasome, since addition of MG132 inhibits the degradation.

Since we detected inflammasome activation-induced Nrf2 degradation in both, HPKs and THP-1 cells, we wanted to test whether treatment with MG132 can also restore Nrf2 protein expression after inflammasome activation of HPKs. Nrf2 protein expression in HPKs was strongly reduced 1.5 h after irradiation with UVB and barely detectable 1.5 h after treatment with nigericin (Figure 17A). Addition of MG132 1.5 h after irradiation of cells restored Nrf2 protein expression only to a minimal extent, whereas MG132 had no effect on nigericin-treated HPKs. Interestingly, when nigericin was removed from HPKs 1.5 h after treatment, MG132 was able to restore the protein level of Nrf2 (Figure 17B). This indicates, that in the presence of nigericin Nrf2 protein is completely depleted, which cannot be reversed by inhibition of the proteasomal degradation pathway.

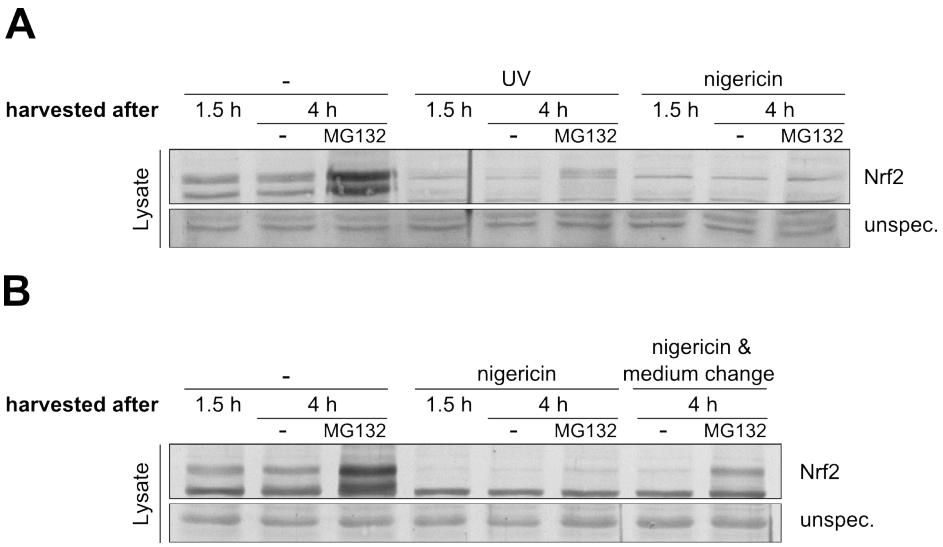

Figure 17: Nrf2 degradation upon inflammasome activation in HPKs. (**A**) HPKs were irradiated with UVB or treated with nigericin (5 µM). After 1.5 h, cells were harvested directly or treated with MG132 (1 µM) or mock-treated. (**B**) Alternatively, 1.5 h after nigericin treatment, nigericin was removed from the cells and fresh medium containing MG132 (1 µM) was added. (**A**, **B**) Cells were harvested 4 h after inflammasome activation and analysed for the expression of Nrf2 by Western blot. An unspecific background band served as loading control.

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Chapter 4 – Conclusion and Discussion

Inflammasomes are an important part of the innate immune system and are activated by a wide range of different PAMPs and DAMPs, which can be exogenous stimuli but also endogenous stress factors. When these protein complexes are activated, they process the pro-inflammatory cytokines pro-IL-1β and -18 and their secretion induces an inflammatory response. Moreover, inflammasome activation also causes pyroptosis, a lytic form of cell death, which is thought to support inflammation.

Nrf2 is a central regulator of cytoprotection and is activated by oxidants and electrophiles, which cause cellular stress. Engagement of the Nrf2 pathway results in an up-regulation of Nrf2 target genes, which code for antioxidant proteins and detoxifying enzymes and help the cell to combat harmful stressors. Thereby, Nrf2 provides a cellular survival mechanism.

Oxidative stress and ROS downstream of PAMPs and DAMPs have been implicated in NLRP3 inflammasome activation. Since Nrf2 activation induces the expression of ROS-detoxifying enzymes, it seems reasonable to assume that inflammasomes and Nrf2 are antagonistic pathways.

Unexpectedly, Nrf2 expression was described to be required for inflammasome activation [1, 2], although this is controversially discussed. Nrf2 KO mice display reduced inflammasome activation and according to the function of Nrf2 as transcription factor, a role for Nrf2 target genes in inflammasome activation was suggested; however, the underlying molecular mechanisms are still unknown. In contrast, more recent publications report that certain Nrf2 activating compounds, which induce the expression of Nrf2 target genes, block inflammasome activation [3-6].

Using murine and human cells, we could confirm a requirement of Nrf2 expression for inflammasome activation (Figure 1). Inflammasome activation was impaired in BMDCs derived from Nrf2 KO mice, as well as in HPKs upon siRNA-mediated knock-down of Nrf2 expression. Nrf2 was reported to be required for NLRP3, AIM2, but not NLRC4 inflammasome activation in mice [2]. Our data are in accordance with the published data, although we did not test NLRC4 inflammasome activation. The literature describes a requirement of Nrf2 expression for inflammasome activation only in murine cells *in vitro* and *in vivo* [1, 2]. Our results demonstrate that Nrf2 expression is also required for inflammasome activation in human cells.

To address the question, whether Nrf2 is a positive regulator of inflammasome activation *in vivo*, the MSU crystal-induced peritonitis model was used [1, 2]. With the same model, which is known to be dependent on inflammasome activation [7, 8], we also measured reduced inflammation in Nrf2 KO mice compared to wild-type control mice. However, the reduction was in several experiments repeatedly not significant (Figure 7). Unfortunately, we were limited in the number of mice available for a single experiment. It may be that with a bigger cohort of

animals, MSU-induced peritonitis is consistently dependent on Nrf2 expression. In addition, the low number of animals we used in a different mouse model for inflammasome activation, the mouse ear-swelling model, can be the reason that also these results were not statistically significant (Figure 8). Our experiments might also suggest that Nrf2 expression only supports inflammasome-dependent inflammation *in vivo*, but is not strictly required for this process.

Several Nrf2 activating compounds were described to block inflammasome activation [3-6]. Our results demonstrate that Nrf2 activators inhibit inflammasome activation in THP-1 cells, as well as in HPKs and in freshly isolated PBMCs from human blood (Figure 3). Therefore, Nrf2 activators are able to block inflammasome activation in different cell types indicating a general mechanism. Furthermore, we extended the list of Nrf2 activators, which dampen inflammasome-dependent inflammation *in vivo*. SFN and 15d-PGJ_2 are known to reduce MSU crystal-induced peritonitis [5, 6]. We show that also DMF has this effect; however, mice need to be treated more extensively with DMF (Figure 4D and E and Figure 13). We hypothesize that the reason for this might be the reduced stability of DMF compared to SFN. This observation is in accordance with the treatment regime of the drug DMF, where patients need to take the medication for an extended time period in order to benefit from the treatment [9]. In particular, the finding that DMF blocks inflammasome activation is very interesting regarding the fact that this Nrf2 activator is already approved as a drug for psoriasis and MS patients. In both diseases a role of inflammasomes is discussed [10-15]. If DMF indeed exerts its therapeutic activity in psoriasis and in MS - at least in part - via inflammasome inhibition, then the drug may also have positive effects for patients suffering from different diseases with an involvement of inflammasomes (see below).

Apart from SFN and DMF, we wanted to test other Nrf2 activators for inflammasome inhibition *in vivo*. Furthermore, we wanted to determine whether SFN and DMF can also block inflammasome activation by using different ways of application and by applying a different mouse model.

In our hands, BHA-fed mice did not show significantly reduced inflammation *in vivo* in MSU-induced peritonitis (Figure 14). We speculate that probably a longer feeding regime and/or a higher dose of BHA is required to receive a significant result.

We demonstrate that feeding of mice via oral gavage with SFN or DMF results in reduced inflammasome activation *in vivo* (Figure 4). In contrast, the publications showing inflammasome inhibition by SFN and 15d-PGJ_2 report a different way of application, namely i.p. injection [5, 6]. By injecting DMF i.p. before MSU-induced peritonitis, we did not see any changes in inflammasome-dependent inflammation *in vivo* (Figure 12). We speculate that as experienced before, the instability of DMF might be an issue in this experiment. It would be interesting to test

a higher dose or a longer application of DMF in this experimental set-up. However, inflammasome inhibition by oral application of Nrf2 activators is more relevant for pharmacological applications.

In addition, we examined inflammasome suppression by SFN in mice infected with *Listeria monocytogenes*. In contrast to MSU injection to the peritoneum, which only activates the NLRP3 inflammasome, *Listeria* was described to activate multiple inflammasomes, including NLRP3, AIM2, and NLRC4 [16, 17]. Unfortunately, we are not able to answer the question, whether SFN can also block inflammation in this model, because our data from this experiment are not statistically significant (Figure 15).

Nrf2 has a protective role in a number of chronic non-malignant diseases [18-27]. In mouse models of these diseases, Nrf2 KO mice are usually more vulnerable and present with a worse disease pattern compared to wild-type control animals. Accordingly, treatment with Nrf2 activators is often shown to ameliorate symptoms or to prevent the disease [28].

Interestingly, for many disorders, in which Nrf2 is believed to have a protective role, also aberrant inflammasome activation was implicated. Examples are neurodegenerative disorders, like Alzheimer's disease (AD) [29, 30], Parkinson's disease (PD) [31, 32], amyotrophic lateral sclerosis (ALS) [33], and multiple sclerosis (MS) [12-15]; and metabolic disorders like obesity and diabetes [34-39]. At first glance, this correlation seems contradictory: Nrf2 is required for inflammasome activation, which contributes to pathology in the disorders mentioned above. Yet, Nrf2 KO mice present with worse disease symptoms and Nrf2 activation is beneficial.

One hypothesis to explain this phenomenon is based on the nature of the mouse models used. A lot of mouse disease models are elicited by chemical induction; for example, the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) animal model of PD [18], dextran sulfate sodium (DSS)-induced experimental colitis in mice [22], pulmonary fibrosis induced by bleomycin [24], and hepatotoxin carbon tetrachloride (CCl_4)-induced liver injury [25] – to name a few. These compounds, are often toxic and hence it is not surprising that Nrf2 induction plays a major role in ameliorating the disease. Nrf2 regulates a lot of detoxifying enzymes and therefore, plays a major role in chemical detoxification. However, it is possible that in the actual human disease, which is not elicited by toxic compounds but by aging or metabolic disturbances, Nrf2 expression plays a different role. A lot of animal disease models are being criticised for not being an adequate equivalent of the disease in human [40-42]. One example is EAE, a mouse model for MS. There are different protocols to induce demyelination in EAE, and interestingly, only upon aggressive immunization, a requirement for Nrf2 in EAE was found [21]. Conversely, aggressive immunization of mice was able to induce EAE even in the absence of NLRP3 or ASC, whereas low dose immunization required NLRP3 and ASC for EAE induction [43]. These results suggest that

data based on mouse models of diseases have to be carefully interpreted regarding the similarity to the human disorder.
For sure, Nrf2 is an important regulator of cytoprotection via induction of expression of numerous target genes. Therefore, it may well be that this cytoprotective activity has a stronger positive effect in disease models than inflammasome activation.

Despite the uncertainty regarding the precise role of both - Nrf2 and its target genes on the one hand, and inflammasomes on the other hand - in a number of chronic inflammatory disorders, patients suffering from inflammatory diseases with an involvement of inflammasomes may profit from the treatment with Nrf2 activators, independently of Nrf2 target genes. However, it should be kept in mind that different Nrf2 activators may also have different adverse effects via targeting other pathways than Nrf2 or inflammasomes.
In conclusion, upon treatment with Nrf2 activating compounds, patients suffering from diseases, for which both - aberrant inflammasome activation and a protective effect of Nrf2 - has been described, might benefit from an induction of protective Nrf2 target genes and at the same time from an inhibition of inflammasomes, and thereby so to speak hit two birds with one stone.

Nrf2 activating compounds are considered to have anti-inflammatory effects, which are poorly characterised and – at least in part – Nrf2-independent [44-50]. It was proposed that inhibition of inflammasome activation by the Nrf2 activators SFN and 15d-PGJ_2 constitutes such an anti-inflammatory and Nrf2-independent effect [5, 6]. We convincingly show that, whereas Nrf2 ablation in BMDCs only reduced inflammasome activation compared to control cells, SFN completely abolished secretion of IL-1β, independently of Nrf2 expression (Figure 6D and E). This result indicates that SFN inhibits NLRP3 inflammasome activation – at least in part – independently of Nrf2. Therefore, inflammasome inhibition, on the one hand by Nrf2 ablation, and on the other hand by Nrf2 activators, is caused by different molecular mechanisms.
We also wanted to address this question in an *in vivo* model of inflammasome activation. Therefore, Nrf2 KO and wild-type control mice were subjected to MSU-induced peritonitis after feeding with SFN (Figure 16). Unfortunately, the variations among animals were very high and our results were not statistically significant.
Furthermore, SFN blocks inflammasome activation also in Keap1 knockout THP-1 cells (Figure 5H). Since Nrf2 activators activate the transcription factor upon modification of the Nrf2 inhibitor Keap1, this confirms an Nrf2-independent mechanism underlying the anti-inflammatory activity of SFN.
Nrf2 activators blocked inflammasome signalling, when added shortly before inflammasome activation (Figure 3A-F). Therefore, it is unlikely that induction of Nrf2 target genes by these

compounds is involved in inflammasome inhibition. Congruently, we demonstrate that blocking protein synthesis by the addition of cycloheximide shortly before treatment with SFN, does not interfere with the capacity of SFN to block inflammasome activation (Figure 3G and H). In conclusion, our results strongly suggest that inflammasome inhibition by SFN is – at least in part – independent of Nrf2 and induction of target genes.

Nrf2 is a transcription factor and it is discussed that the reason for impaired inflammasome activation upon ablation of Nrf2, is the reduction of Nrf2 target gene expression [1, 2]. Yet until now, not a single Nrf2 target gene has been described to be critical for inflammasome activation. To address the question, whether Nrf2 target genes play a role in inflammasome activation, we activated the inflammasome in peritoneal macrophages derived from mice expressing constitutively active (ca) Nrf2. Inflammasome activation was not increased, which indicates that Nrf2 does not regulate the expression of a protein, whose amount is limiting for inflammasome activation (Figure 2A-C).
Since HPKs are usually difficult to transfect with expression vectors, we developed a protocol for transduction of HPKs with lentiviral expression constructs, which provides a good transduction efficacy. Overexpression of Nrf2 increased the secretion of mature IL-1β upon UVB-mediated inflammasome activation; however, overexpression of caNrf2, which strongly upregulates expression of Nrf2 target genes, did not enhance inflammasome activation (Figure 2D and E). This result is in line with the experiment using cells from caNrf2 mice described before. Interestingly, a nuclear localization sequence (NLS)-deficient mutant of Nrf2 and a mutant of Keap1, which cannot mediate Nrf2 degradation, strongly increased IL-1β production. Conversely, overexpression of dominant negative (dn) Nrf2 had no detectable impact on IL-1β secretion, although Nrf2 target gene expression was abolished. We concluded from these results that NLRP3 inflammasome activation is not correlated with the expression of Nrf2 target genes, but rather with the abundance of Nrf2 with Keap1 in the cytoplasm. This hypothesis is supported by the finding that Nrf2 expression increases the processing of pro-IL-1β in HEK 293T cells reconstituted with an NLRP3 inflammasome (Figure 11).

Since induction of Nrf2 target genes is not required for inflammasome activation, it is possible that a physical interaction might underlie the requirement of Nrf2 for this process. Indeed, we detected an interaction between overexpressed caspase-1 and all endogenous components of the Nrf2/Keap1/Cul3/Rbx1 complex (Figure 6B). However, we speculate that the interaction between Nrf2 and caspase-1 is indirect and mediated by an unknown protein.

The treatment of cells with inflammasome activators causes cellular stress and it is reasonable to assume that cells would benefit from Nrf2 activation and the resulting expression of cytoprotective genes. Surprisingly, we found that Nrf2 is degraded upon inflammasome activation. Irrespectively of the NLRP3 inflammasome activator we tested - UVB and nigericin in HPKs and nigericin and MSU in THP-1 cells - Nrf2 protein was completely degraded within one to two hours (Figure 5A, B and D). This suggests that Nrf2 expression and availability in the cytoplasm are only critical for the NLRP3 inflammasome at the initial steps of inflammasome activation.

Interestingly, Nrf2 mRNA expression was strongly downregulated upon UVB irradiation, but was not impaired after nigericin treatment (Figure 5C and E). This indicates that NLRP3 inflammasome activation causes Nrf2 protein degradation. We showed that ablation of Keap1 expression, which is the most important mediator of proteasomal degradation of Nrf2, is partially dispensable for degradation upon inflammasome activation (Figure 5F-H); yet, Nrf2 protein degradation after inflammasome activation is proteasome-mediated, because addition of the proteasome inhibitor MG132 interferes with the degradation (Figure 5I). It is known that proteasomal degradation of Nrf2 can be also regulated by Keap1-independent mechanisms, for example by the β-TrCP-Skp1-Cul1-Rbx1 E3 ubiquitin ligase [51, 52] or the Hrd1 E3 ubiquitin ligase [53]. It would be interesting to test in the future, whether one of these complexes is involved in Nrf2 degradation after inflammasome activation.

Moreover, caspase-1 expression is not required for inflammasome activation-induced Nrf2 degradation (Figure 5F and G), but knockout of ASC or NLRP3 blocked the degradation (Figure 5G). These results suggest that Nrf2 degradation is regulated by a mechanism upstream of inflammasome activation, taking place, when the NLRP3 inflammasome assembles. More experimental efforts are required to shed light on the mechanism of Nrf2 degradation upon inflammasome activation.

Besides, in HPKs we found that depending on the NLRP3 inflammasome activator, Nrf2 degradation is differently regulated (Figure 17A). Addition of MG132 after UVB irradiation interfered with Nrf2 degradation, but still a lot of protein was degraded. MG132 had a much weaker effect in HPKs compared to THP-1 cells, which would indicate that in HPKs also non-proteasomal Nrf2 protein degradation after inflammasome activation plays a role. Nigericin treatment in HPKs resulted in a faster degradation of Nrf2 compared to UVB irradiation (Figure 5A and B and Figure 17A) and inhibition of the proteasome by MG132 did not have an obvious effect on Nrf2 protein levels. Nrf2 mRNA expression was not impaired upon nigericin treatment (Figure 5C). Therefore, Nrf2 mRNA is expected to be translated, which would restore Nrf2 protein levels. However, in the presence of nigericin, translation of Nrf2 mRNA seems impaired. In contrast, when nigericin is removed from the cells and subsequently MG132 is added, Nrf2

protein levels can be restored (Figure 17B). There are still open questions regarding the mechanisms of degradation of Nrf2 upon inflammasome activation, which should be addressed in the future.

An interesting and important question that remains, is how do Nrf2 activators block the inflammasome independently of Keap1. Nrf2 activators are known to modify cysteine residues of Keap1. It is likely that Nrf2 activating compounds such as SFN also modify cysteine residues of other proteins than Keap1 and that these modifications culminate in inflammasome inhibition. A possible candidate for modification by SFN is caspase-1, which contains some cysteine residues on its surface; however, it was reported that caspase-1 is not a direct target of SFN modification [6]. We aim to further investigate this topic and successive studies are already ongoing.

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Abbreviations and Units

Abbreviations

A/A	Antibiotic-antimycotic
AEBSF	4-(2-Aminoethyl)benzenesulfonyl fluoride
AIM2	Absent in melanoma 2
ANOVA	Analysis of variance
AP	Alkaline phosphatase
APS	Ammonium persulfate
ASC	Apoptosis-associated speck-like protein containing a CARD
ATP	Adenosine triphosphate
BCIP	5-Bromo-4-chloro-3-indolyl-1-phosphate
BMDCs	Bone marrow-derived dendritic cells
BPE	Bovine pituitary extract
BSA	Bovine serum albumine
ca	Constitutively active
CAPS	Cryopyrin-associated periodic syndrome
CARD	Caspase recruitment domain
CHX	Cycloheximide
CIP	Calf intestinal phosphatise
Co-IP	Co-immunoprecipitation
CFU	Colony-forming unit
ctrl	Control
cyt	Cytoplasmic
DAMP	Danger-associated molecular pattern
ddH_2O	Bidistilled water
DMF	Dimethyl fumarate
DMSO	Dimethylsulfoxide
dn	Dominant negative
DNFB	Dinitrofluorobenzene
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphate
ECL	Enhanced chemiluminescence
EDTA	Ethylenediaminetetraacetic acid

EGF	Epidermal growth factor
ELISA	Enzyme-linked immunosorbent assay
ER	Endoplasmic reticulum
FBS	Fetal bovine serum
FITC	Fluorescein isothiocyanate
GFP	Green fluorescent protein
HA	Influenza virus Haemagglutinin
HPKs	Human primary keratinocytes
HRP	Horseradish peroxidase
IFN	Interferon
IKK	Inhibitor of nuclear factor κB kinase
IL	Interleukin
IL-1R	Interleukin-1 receptor
IL-1Ra	Interleukin-1 receptor antagonist
IP	Immunoprecipitation
i.p.	Intraperitoneally
IPAF	Inflammatory protease activating factor
IκB	Inhibitor of nuclear factor κB
LB	Luria-Bertani
LDH	Lactate dehydrogenase
LPS	Lipopolysaccharide
LRR	Leucine-rich repeat
mGM-CSF	Mouse granulocyte-macrophage colony-stimulating factor
mRNA	Messenger ribonucleic acid
MSU	Monosodium urate
NAIP	NLR family apoptosis-inhibitory protein
NBT	Nitro blue tetrazolium
NF-κB	Nuclear factor kappa B
NLRP	NOD-like receptor family, pyrin domain containing 3
NLS	Nuclear localization sequence
NP-40	Nonidet P-40
ns	Not significant
nuc	Nuclear
PAMP	Pathogen-associated molecular pattern
PAS	Protein A Sepharose
PCR	Polymerase chain reaction

PFA	Paraformaldehyde
PMA	Phorbol 12-myristate 13-acetate
Puro	Puromycin
qRT-PCR	Quantitative real-time PCR
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RT	room temperature
scr	scrambled
SDS	Sodium dodecyl sulphate
SFN	Sulforaphane
siRNA	Short interfering ribonucleic acid
SN	Supernatant
tBHQ	*tert*-Butylhydroquinone
TEMED	Tetramethylethylendiamine
TLR	Toll-like receptor
TNF	Tumour necrosis factor
UV	Ultraviolet
UVB	Ultraviolet B
v/v	Volume per volume
VEGF	Vascular endothelial growth factor
w/v	Weight per volume
wt	Wild-type
15d-PGJ_2	15-deoxy-Δ-^{12,14}-prostaglandin J_2

Units

mA	Milliampere
bp	Base pairs
°C	Degree Celsius
cm	Centimeter
dpi	Dots per inch
g	Gram
µg	Microgram
mg	Milligram
ng	Nanogram
h	Hour
J	Joule
mJ	Millijoule
l	Liter
µl	Microliter
ml	Milliliter
m	Meter
µm	Micrometer
mm	Millimeter
nm	Nanometer
min	Minute
M	Molar
µM	Micro-molar
mM	Milli-molar
nM	Nano-molar
OD	Optical density
sec	Second
U	Unit
V	Volt

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