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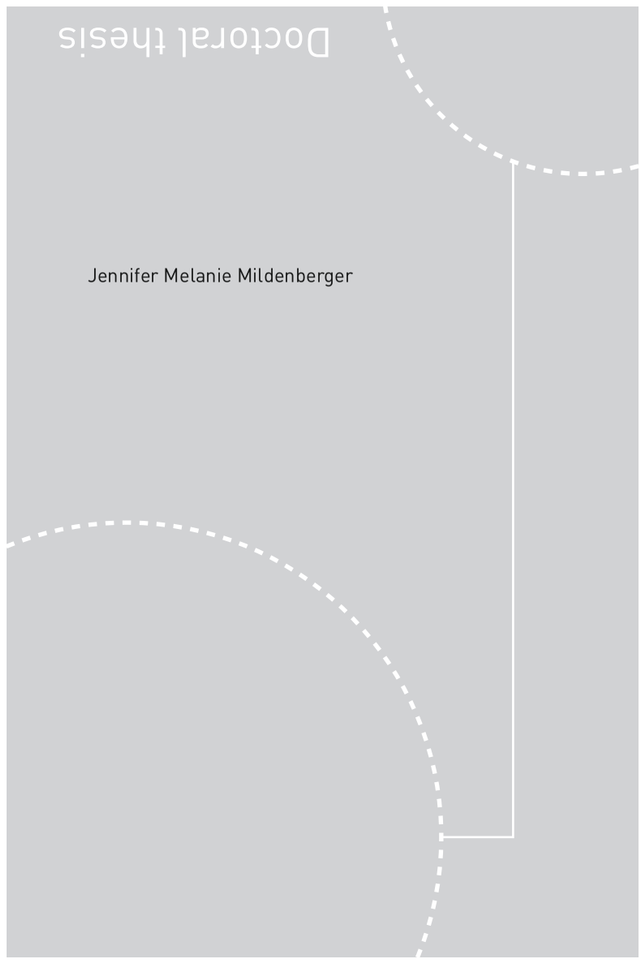
Jennifer Melanie Mildenberger

The Interplay of Oxidative Stress Responses and Autophagy in Inflammation

Doctoral thesis



Jennifer Melanie Mildenberger

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Thesis for the Degree of
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Samspill mellom oksidativt stress og autofagi i betennelsesreaksjoner

For å bevare funksjon og homeostase må cellene stadig stå imot endogen og eksogen trussel. Dette oppnås gjennom forsvar- og vedlikeholdmekanismer som antiokisidantforsvaret, autofagi og betennelsesreaksjonen (inflammasjon). Antioksidantforsvaret minker nivået av reaktive oksygen radikaler (ROS), mens autofagi fjerner og resirkulerer skadde proteiner. Begge prosessene påvirker inflammasjonen som er viktig i forsvaret mot infeksjoner men også årsak til skader. For mye eller for lite aktivitet i antioksidantforsvaret, autofagi eller inflammasjon fører til patologiske forandringer. Det er kjent at svekkede vedlikeholdssystemer og akkumulasjon av skader i våre celler fører til sykdom. Å kunne aktivere vedlikeholdmekanismer vil være til nytte i mange sammenheng, men krever nøyaktig kunnskap om cellenes egne responser. Hensikten med dette arbeidet var å undersøke den vekselsidige regulering av antiokisidantforsvar, autofagi og inflammasjon i forebygging av sykdom og behandling av infeksjoner.

Aldersassosierte nevrodegenerative sykdommer er ofte forbundet med akkumulering av proteinaggregater, som kan skyldes nedsatt funksjon av rense- og vedlikeholds prosesser som autofagi. Aldersbetinget makula degenerasjon (AMD) er den vanligste årsaken til blindhet i eldre og viser proteinaggregater i områder hvor retinaceller og fotoreseptorer er tapt. Cellenes evne til å håndtere stress kan bli påvirket av kostholdet. Omega 3 flerumettede fettsyrer (n-3 PUFA) har vist gunstige effekter på aldersassosierte sykdommer som AMD, men mekanismen er ikke forstått. I kontekst av AMD fant vi at n-3 PUFA dokosaheksaensyre (DHA) induserte en forbigående økning i proteinaggregater, autofagi og aktivering av antiokisidantforsvaret. Fravær av autofagi reseptoren SQSTM1 (sequestosome 1), det essentielle autofagi genet ATG5 (autophagy-related 5) eller regulatoren av antioksidantforsvaret, NFE2L2 (nuclear factor, erythroid 2 like 2) førte til mindre cellevekst ved behandling med DHA. Når disse responsene ikke ble manipulert, men kunne fungere normalt, beskyttet DHA cellene fra skader indusert av feilfoldede proteiner og oksidativt stress.

Underliggende inflammasjon bidrar til utviklingen av mange av de hyppigste sykdommene i vestlige land, mens n-3 PUFA er kjent til å ha en betennelsesdempende effekt. Vi undersøkte om den anti-inflammatoriske effekten til n-3 PUFAs kan skyldes økt fjerning av proteinaggregater eller aktivering av antiokisidantforsvaret som vi hadde sett i retina cellene. Vi foreslår at n-3 PUFA-mediert polymerisering av autofagi reseptoren SQSTM1 som tiltrekker den negative regulatoren av NFE2L2, KEAP1 (kelch-like ECH associated protein 1) er en rask mekanisme for å aktivere antioksidantforsvaret. Samtidig resulterte DHA-behandling i lavere interferon (IFN) signalering. Videre kunne vi vise at n-3 PUFA tilskudd senket nivået av det IFN-stimulerte kjemokinet CXCL10 (C-X-C motif chemokine ligand 10) i pasienter med en krosinsk betennelse. Vi foreslår CXCL10 som en nyttig markør for gunstige effekter av n-3 PUFA tilskudd i inflammasjonsassosierte sykdommer.

Mekanismer som beskytter verten for vevskader kan dempe forsvaret mot infeksjoner. Når immunceller tar opp bakterier (fagocytose) dannes det ROS som er en viktig del av infeksjonsforsvaret. NFE2L2-KEAP1 responsen regulerer oksidativt stress og begrenser ROS produksjon. Vi ville derfor undersøke rollen til KEAP1 i mykobakterie infeksjon. KEAP1 ble rekruttert av ROS produsert ved fagocytosen. Fjerning av KEAP1 i *Mycobacterium avium* infiserte celler førte til høyere nivåer av cytokiner og type I IFNs og økte kjernetranslokeringen av oppstrøms transkripsjons faktorer. Resultatene våre tyder på at KEAP1-ubiquitin ligase komplekset negativt regulerer inflammasjonsresponser ved å ubiquitinere signal proteinet IKKβ (I kappa B kinase beta) og merke det for degradering. Den reduserte inflammasjonsresponsen resulterte i økt intracellulær vekst og overlevelse av *M. avium* i humane makrofager.

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Acknowledgements

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Jennifer Mildenberger
Trondheim, February 2017

List of papers

Paper I

Ida Johansson, Vivi Talstad Monsen, Kristine Pettersen, **Jennifer Mildenberger**, Kristine Misund, Kai Kaarniranta, Svanhild Schønberg and Geir Bjørkøy

The marine n-3 PUFA DHA evokes cytoprotection against oxidative stress and protein misfolding by inducing autophagy and NFE2L2 in human retinal pigment epithelial cells. *Autophagy. 2015 Sep2;11(9):1636-51*

Paper II

Jennifer Mildenberger, Ida Johansson, Eli Kjøbli, Jan Kristian Damås, Trude Helen Flo, Geir Bjørkøy

N-3 PUFAs induce inflammatory tolerance by formation of KEAP1 containing p62-bodies and activation of NFE2L2. *Autophagy, in press, 2017*

Paper III

Jane Atesoh Awuh, Markus Haug, **Jennifer Mildenberger**, Anne Marstad, Chau Phuc Ngoc Do, Claire Louet, Jørgen Stenvik, Magnus Steigedal, Jan Kristian Damås, Øyvind Halaas, Trude Helen Flo

Keap1 regulates inflammatory signaling in M. avium infected human macrophages. *Proc Natl Acad Sci U S A. 2015 Aug 4;112(31):E4272-80*

Abbreviations

This thesis uses the official protein and gene names according to the recommendations of the HUGO gene nomenclature committee (HGNC). Exceptions were made, where the official name strongly differed from the commonly used name, leading to confusion of the reader. In this case, the underlined official name is included with the first appearance of the term.

Abbreviation	Alias	Full name
AA		arachidonic acid (20:4, n-6)
ABIN	TNIP1/2/3	A20 binding and inhibitor of NFκB
AIM2		absent in melanoma 2
AKT1	PKB	AKT serine/threonine kinase 1
ALA		a-linoleic acid (18:3, n-3)
ALIS		aggresome-like structures
AMD		Age-related macular degeneration
AMPK	PRKAA1/2	AMP-activated protein kinase
AP-1		activator protein 1
ARE		antioxidant response element
AREDS		Age-Related Eye Disease Study
ATF6		activating transcription factor 6
ATG		autophagy-related
ATP		Adenosine triphosphate
BACH1		BTB domain and CNC homolog 1
BECN1		Beclin1
BNIP3		BCL2 interacting protein 3
BNIP3L	NIX	BCL2 interacting protein 3 like
BTB		bric-a-brac
BTRC	β-TrCP	beta-transducin repeat containing E3 ubiquitin protein ligase
CASP1		caspase-1
CCL		chemokine ligand
CD14		CD14 molecule
cGAS	MB21D1	cyclic GMP-AMP synthase
CLR		C-type lectin receptor
CREBBP		CREB binding protein
CSNK2		casein kinase 2
CUL3		Cullin3
CXCL10		C-X-C motif chemokine ligand 10
CXCR3		C-X-C motif chemokine receptor 3
DALIS		dendritic cell aggresome-like structures
DAMP		damage associated molecular pattern
DDX		DEAD box
Dectin-2	CLEC6A	DC-associated C-type lectin 2
DHA		docosahexaenoic acid (22:6, n-3)
DNA		Deoxyribonucleic acid
DUB		deubiquitinase
EIF2A	eIF2α	eukaryotic translation initiation factor 2A
EPA		eicosapentaenoic acid (20:5, n-3)
ER		endoplasmatic reticulum
ERAD		ER-related degradation

ERK1/2	MAPK1/3	extracellular signal-regulated kinase
ESAT-6		early secreted antigenic target protein 6)
Fe		iron
FOXO3		forkhead box O3
FUNDC1		FUN14 domain containing 1
GABARAP		GABA type A receptor-associated protein
GAS		gamma interferon activation site
GATA4		GATA binding protein 4
FFAR1	GPR40	free fatty acid receptor 1
FFAR4	GPR120	free fatty acid receptor 4
GPX		glutathione peroxidases
GSH		γ-glutamylcysteinylglycine; glutathione
GSSG		glutathione disulphide
HACE1		HECT domain and ankyrin repeat containing E3 ligase 1
HDAC6		histone deacetylase 6
HDT		host-directed therapies
HIV		human immunodeficiency virus
HMGB1		high mobility group box 1
HMOX1		heme oxygenase 1
HSF1		heat shock transcription factor 1
HSP90		heat shock protein 90
IFI16		interferon gamma inducible protein 16
IFN		interferon
IFNAR1		interferon alpha and beta receptor subunit 1
IFNGR1		interferon gamma receptor 1
IKKα	CHUK	I kappa B kinase alpha
IL1R1		interleukin 1 receptor
IL1RN	IL-1Ra	interleukin 1 receptor antagonist
IMMT	MIC60	inner membrane mitochondrial protein
IRAK		interleukin 1 receptor associated kinases
IRE1	ERN1	inositol-requiring enzyme 1
IRF3		interferon regulatory factor 3
IRGM		immunity related GTPase M
ISG		interferon-stimulated genes
ISG15		ISG15 ubiquitin-like modifier
ISGF3		IFN-stimulated gene factor 3
ISR		integrated stress response
ISRE		interferon-sensitive response elements
ITCH		itchy E3 ubiquitin protein ligase
IκBα		I kappa B alpha; NFKBIA
JAK1		janus kinase 1
JNK	MAPK8	JUN N-terminal kinase
KEAP1		kelch-like ECH associated protein 1
K		lysine
LA		linoleic acid (18:2, n-6)
LBP		lipopolysaccharide binding protein
LIR		LC3 interacting region
LPS		liposaccharide
LRR		leucine rich repeats
LTA4		Lipoxin A_4

LTB4		leukotriene B_4
M		methionine
M. avium		Mycobacterium avium
MAC		Mycobacterium avium complex
MAP1LC3		microtubule associated protein 1 light chain 3
mATG8		mammalian paralogues of yeast ATG8
MD-2	LY96	myeloid differentiation factor-2
MDM		human primary monocyte-derived macrophages
MHC I	MIC	major histocompability complex class I
miRNA		microRNA
Mtb		Mycobacterium tuberculosis
mtDNA		mitochondrial DNA
mTOR		mammalian target of rapamycin
MTORC1		mammalian target of rapamycin complex 1
mtROS		mitochondrial ROS
MYD88		myeloid differentiation primary response 88
n-3 PUFA		omega-3 polyunsaturated fatty acids
NAC		n-acetyl cysteine
NADPH		nicotinamide adenine dinucleotide phosphate
NBR1		NBR1, autophagy cargo receptor (neighbor of BRCA1 gene 1)
NDP52	CALCOCO2	nuclear dot protein 52 kDa
NEDD8		neural precursor cell expressed, developmentally down-regulated 8
NEMO	IKKγ; IKBKG	NF-kappa-B essential modulator
NFE2L2	Nrf2	nuclear factor, erythroid 2 like 2
NFκB		nuclear factor kappa B
NK		natural killer
NLR		NOD-like receptor
NLRP3		NLR family pyrin domain containing 3
NOS2	iNOS	nitric oxide synthase 2
NOX		NADPH oxidase
OA		oleic acid (18:1, n-9)
OPTN		optineurin
p38	MAPK14	P38 mitogen-activated protein kinase
PAMP		pathogen associated molecular pattern
PARK2		parkin RBR E3 ubiquitin protein ligase
PB1		Phox-Bem1
PDK1		pyruvate dehydrogenase kinase 1
PE		phosphatidylethanolamine
PERK	EIF2AK3	protein kinase RNA-like endoplasmic reticulum kinase
PGAM5		PGAM family member 5
PGE2		prostaglandin E_2
PI		phosphatidylinositol
PI3K		phosphatidylinositol-3-kinase
PINK1		PTEN induced putative kinase 1
PIP3		phosphatidylinositol-3,4,5-trisphosphate
PRR		pattern recognition receptor
PTPN1	PTPB1	protein tyrosine phosphatase, non-receptor type 1
RB1CC1	FIP200	RB1 inducible coiled-coil 1
RBX1		ring-box 1
RELA	p65	RELA subunit of NFκB

RIPK1		receptor interacting serine/threonine kinase 1
RLRs		Retinoic acid-inducible gene (RIG)-I-like receptors
RNF216	TRIAD3A	ring finger protein 216
RNS		reactive nitrogen species
ROS		reactive oxygen species
RPE		retinal pigment epithelial
S		serine
SAMM50		sorting and assembly machinery component
SASP		senescence associated phenotype
SIRT1		sirtuin 1
SLR		Sequestosome-like receptors
SOD1		superoxide dismutase
SPM		specialized pro-resolving mediator
SQSTM1	p62	Sequestosome 1
STAT1		signal transducer and activator of transcription 1
STING	TMEM173	stimulator of interferon genes
SUMO		(Small ubiquitin-like modifier protein)
TAB1/2/3		TAK1 binding proteins
TAK1	MAP3K7	TGF-beta activated kinase 1;
TANK		TRAF family member associated NFκB activator
TAX1BP1		Tax1 binding protein 1
TB		tuberculosis
TBK1		TANK binding kinase 1
TFEB		transcription factor EB
TGFβ	TGFB1/2/3	transforming growth factor beta
TIR		Toll/IL-1R homology
TIRAP	MAL	TIR domain containing adaptor protein
TLR		Toll-like receptor
TNFAIP3		(TNF alpha induced protein 3; A20).
TNFRSF		TNF superfamily receptor
TOLLIP		toll interacting protein
TRADD		TNFRSF1A associated via death domain
TRAF6		TNF receptor associated factor 6
TRAM		translocation associated membrane protein
TRIF	TRIM69	TIR-domain-containing adapter-inducing IFNB
TRIM		tripartite motif containing
TYK2		tyrosine kinase 2
UBA		ubiquitin-associated domain
UBD		ubiquitin-binding domains
ULK1/2		unc-51 like autophagy activating kinase
UPR		unfolded-protein response
UPS		ubiquitin-proteasome system
USP		ubiquitin specific peptidase
XBP1		X-box binding protein-1

Abstract

To assure homeostasis, cells have to continuously adapt to exogenous and endogenous threats. This is achieved by response mechanisms like the oxidative stress response, autophagy and inflammation. The oxidative stress response limits the level of damaging reactive oxygen species (ROS), while autophagy removes and recycles damaged proteins. Both processes modulate inflammation, which is essential for cellular defense but also a cause of harm. Exaggerated or insufficient activity of these processes leads to pathologic changes. It is acknowledged that impairment of the maintenance systems and accumulation of damage leads to pathologic changes. The appropriate targeting of cellular maintenance mechanisms could be beneficial for a broad range of pathologies, but demands an in-depth knowledge of endogenous responses. The objective of this work was to study the complex interaction of autophagy, oxidative stress and inflammation and their modulation in disease prevention and infection therapy.

Age-related neurodegenerative diseases are often characterized by the accumulation of protein aggregates, likely as a result of declining clearance mechanisms. Age-related macular degeneration (AMD) is a common cause of blindness in elderly and associated with aggregate deposits in areas where retinal pigment epithelial (RPE) cells and consequently photoreceptors are lost. The cells ability to cope with stress can be influenced by the diet. Omega 3 polyunsaturated fatty acids (n-3 PUFA) have shown beneficial effects on AMD, however, the mechanisms are not understood. We here describe the transient increase of protein aggregation, autophagy and activation of the oxidative stress response by the n-3 PUFA docosahexaenoic acid (DHA). Knockdown of the autophagy receptor SQSTM1 (sequestosome 1), the essential autophagy gene ATG5 (autophagy-related 5) or the regulator of the anti-oxidative response NFE2L2 (nuclear factor, erythroid 2 like 2) resulted in limited cell proliferation during treatment with DHA. Finally, DHA protected RPE cells from cell cycle arrest induced by misfolded proteins or exogenous oxidative stress.

Underlying low grade inflammation contributes to the most common diseases in Western countries and n-3 PUFAs are acknowledged to have anti-inflammatory effects. We investigated if the increase of protein aggregation, autophagy and activation of the oxidative stress response that we had seen in RPE cells, contributes to anti-inflammatory effects of n-3 PUFAs. Our results indicate that n-3 PUFA treatment in macrophages mediated polymerization of the autophagy receptor SQSTM1 (sequestosome 1) and recruitment of KEAP1 (kelch-like ECH associated protein 1), which is the repressor of NFE2L2, as a fast mechanism to activate the anti-oxidative response. Simultaneously, DHA strongly limited IFN (interferon)-dependent signaling. We further show decreased levels of the IFN-stimulated chemokine CXCL10 (C-X-C motif chemokine ligand 10) in n-3 PUFA supplemented patients with chronic inflammation and suggest CXCL10 as a valuable marker for beneficial effects of n-3 PUFAs in inflammation-related diseases.

Responses that protect the host from tissue damage can favor pathogens and dampen the defense against infections. ROS are produced during the uptake of bacteria by immune cells (phagocytosis). ROS represent an important part of defense, while the NFE2L2 -KEAP1 system regulates oxidative stress and limits ROS production. We investigated the role of the ROS sensor KEAP1 in mycobacterial infection. ROS production during phagocytosis recruited KEAP1. Knockdown of KEAP1 in *Mycobacterium avium* infected cells increased expression of cytokines and type I IFNs and augmented nuclear translocation of upstream transcription factors. Our results indicate that the KEAP1-ubiquitin ligase complex ubiquitinates the signaling protein IKKβ (I kappa B kinase beta) and marks it for degradation. The down-regulated inflammation resulted in increased intracellular growth and survival of *M. avium* in human macrophages.

1. Introduction

Health at its molecular level means functionality of our cells. To maintain homeostasis, cells have to withstand exogenous and endogenous threats like damage, mutations, infection, starvation or oxidative stress. Multiple response mechanisms have evolved to encounter different kinds of menace. While some of these responses will reestablish homeostasis, others lead to adaptation and altered cellular function or to cell death if the damage cannot be repaired [1]. The ability of cells to cope with and adapt to stress greatly varies due to environmental and genetic differences. Importantly, many of the most frequent diseases in the Western world are associated with dysregulation of cellular homeostasis [2,3]. To be able to manipulate and possibly restore a functional network of stress responses, in-depth knowledge of the cellular systems of homeostasis and maintenance is required.

Specific coping mechanisms, like the oxidative stress response, the heat-shock and unfolded protein response, autophagy and inflammation act as highly intertwined mechanisms in the cytosol to maintain cellular homeostasis. Importantly, stress responses also contribute to cell-autonomous pathogen defense [4]. Many cellular stresses lead to protein misfolding, activating the heat shock response and inducing chaperones to increase correct protein refolding [5]. One continuously encountered source of stress is reactive oxygen species (ROS) that mainly are a byproduct of mitochondrial respiration and energy generation. ROS function as signaling molecules in the cell, but have to be tightly regulated to prevent damage. During oxidative stress, multiple detoxifying enzymes are induced to protect the cell [6]. At the same time, degradation by the proteasome is increased to clear misfolded proteins. Especially autophagy as a lysosomal recycling mechanism contributes to removal of damaged and aggregated proteins and the maintenance of the cytosol. Stress-responses are highly connected to cell death mechanisms and can initiate cell death if the cellular defense is not successful. Impairment of degradative pathways or overwhelming stress can cause accumulation of protein aggregates, which are observed in several pathologies and especially neurodegenerative diseases [7,8].

Damage occurs all the time and only the continuous removal by proteasomes and autophagy assures proper cellular function. Also dysregulated induction or termination of ROS and inflammatory signaling increases cellular stress and contributes to the development of pathologies [9,10]. Inflammation is important in the defense to pathogens, but needs to be tightly regulated to prevent overwhelming (sepsis) or chronic inflammation. Downregulation

of inflammation may increase susceptibility to opportunistic pathogens. This illustrates the importance of a functional network and strict regulation of oxidative stress, autophagy and inflammation in disease prevention.

To gain more knowledge about the interplay of oxidative stress, autophagy and inflammation we first focused on mechanisms of disease prevention by omega-3 polyunsaturated fatty acids (n-3 PUFA).

Age-related macular degeneration (AMD) is a neurodegenerative pathology of the retina and a leading cause of blindness in the elderly in the Western world [11]. Characteristics of the disease include oxidative stress and protein aggregates in retinal pigment epithelial (RPE) cells, apoptosis of RPE cells and loss of the photoreceptor [12]. N-3 PUFAs have shown preventive effects on the progression of AMD [13–15]. Changes in autophagy have been reported in cancer cells in response to n-3 PUFAs [16,17] and deficiency in autophagy leads to formation of inclusion bodies or aggregates [18–20]. Therefore, we asked if n-3 PUFAs improve cellular homeostasis by increasing autophagy in RPE cells, where the initial phase of AMD occurs.

Further, as n-3 PUFAs also are acknowledged for their anti-inflammatory effects [21], we examined the effect of n-3 PUFAs on oxidative stress and autophagy in macrophages and the involvement of these mechanisms in regulation of inflammatory signaling.

Balancing inflammation does not only consist in limitation of excessive signaling. Boosting of deficient immune responses might allow for efficient clearance of pathogens. Infection causes oxidative stress and ROS are needed during phagocytosis for proper killing of bacteria [22]. At the same time ROS have to be locally limited to prevent damage. The oxidative stress sensor KEAP1 (kelch-like ECH associated protein 1) is an important player in the intricate balance of signal induction and damage prevention; however its role in regulating inflammation is poorly understood. Thus, we investigated the role of KEAP1 in inflammatory responses towards mycobacterial infection.

1.1 The oxidative stress response

ROS are unavoidable byproducts of energy production by mitochondrial respiration and represent a frequent source of damage [23]. Phagocytosis of pathogens activates a NADPH (nicotinamide adenine dinucleotide phosphate) oxidase (NOX) complex, giving rise to local ROS that participate in signaling and defense [24]. Reactions by enzymes like cytochrome p450 or lipoxygenases produce ROS intermediates and ROS can arise from external threats like ionizing radiation and carcinogens [6]. Generation of ROS is necessary for normal

homeostasis including receptor signaling of growth factors [25] and inflammatory signaling [26]. ROS are short lived species produced by reduction of oxygen to form free radicals as superoxide anion (O_2^-) or hydroxyl radical (•OH) and reactive molecules like hydrogen peroxide (H_2O_2) [27]. Balanced ROS are essential for normal physiology, but since ROS also cause oxidative damage on proteins or DNA, good strategies and a robust buffering system is needed to disarm excessive ROS.

First, the specific localization of ROS producing enzymes keeps ROS locally confined. SOD1 (superoxide dismutase) in the cytoplasm or SOD2 in mitochondria convert two O_2^- to H_2O_2, which peroxiredoxin, catalases or GPX (glutathione peroxidases) can degrade to H_2O and O_2 or alcohols [23]. Importantly, mice lacking SOD1 show accelerated aging phenotypes and decreased lifespan, illustrating the importance of the anti-oxidative defense [28]. The most abundant small molecule ROS scavenger in cells is GSH (glutathione; γ-glutamylcysteinylglycine). GSH can act as electron donor, is oxidized to GSSG (glutathione disulphide) and either reduced by NADPH-dependent reductases or exported from the cell. Besides directly scavenging ROS, GSH can regenerate other non-enzymatic anti-oxidants like ascorbic acid (Vitamin C) and α-tocopherol (Vitamin E) [29].

1.1.1 The NFE2L2-KEAP1 axis

In addition to ROS scavenging, anti-oxidative programs take place in the cell. The primary initiator of an anti-oxidative response, transcription factor NFE2L2 (nuclear factor, erythroid 2 like 2; Nrf2), is activated by redox regulation allowing local and specific activity [30,31]. In a quiescent state, KEAP1 in complex with the ubiquitin E3 ligases CUL3 (Cullin3) and RBX1 (ring-box 1) constantly subjects NFE2L2 to ubiquitination and proteasomal degradation [32–34] (Figure 1). Mice deficient of KEAP1 have constitutively activated NFE2L2, which is lethal at weaning stage [35]. NFE2L2 has two binding motifs for KEAP1 and homodimerization of KEAP1 via its BTB (bric-a-brac) domain allows binding of both domains, necessary for ubiquitination of NFE2L2 [36]. The ETGE-motif (“Hinge”) of NFE2L2 is tightly bound by KEAP1, whereas the DLGex-motif (“Latch”) has a lower binding affinity and is designated as “fast on-fast off” binding, or “Hinge-and-Latch” model [36]. Oxidative stress abolishes the repressor function of KEAP1 by oxidation of active cysteines either leading to conformational changes of KEAP1 or dissociation of CUL3 [37]. This liberates the DLGex-motif and stabilizes NFE2L2 in the cytosol with subsequent nuclear translocation. In the nucleus, NFE2L2 dimerizes with Small Maf proteins and binds to ARE (antioxidant response element) sites in genes with anti-oxidative functions like SOD, GPX

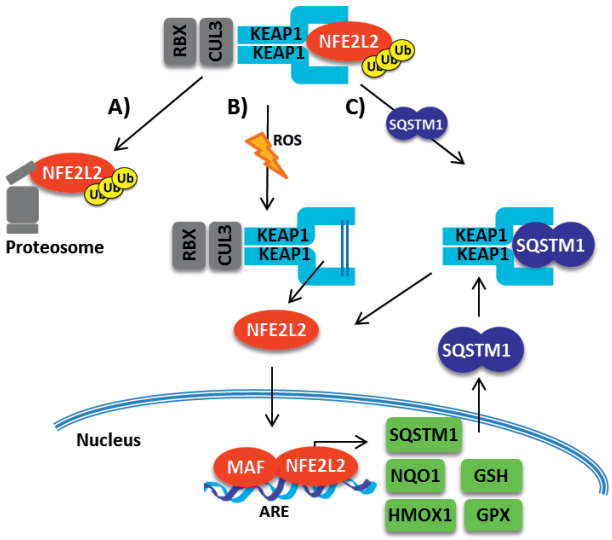

Figure 1: Illustration of the NFE2L2-KEAP1 pathway. A) In a quiescent state, NFE2L2 (nuclear factor, erythroid 2 like 2) is bound to KEAP1, which forms a complex with the ubiquitin E3 ligases CUL3 (Cullin3) and RBX1 (ring-box 1) and mediates ubiquitination and degradation of NFE2L2. **B)** Oxidative stress (ROS) abolishes the repressor function of KEAP1 and stabilizes NFE2L2 with subsequent translocation. In the nucleus, NFE2L2 dimerizes with Small Maf proteins and binds to ARE (antioxidant response element) sites in genes with anti-oxidative and detoxifying functions. **C)** SQSTM1 (sequestosome 1) can bind KEAP1, representing an alternative way of NFE2L2 activation and positive feedback loop. NQO1, NAD(P)H quinone dehydrogenase 1; HMOX1, heme oxigenase 1; GSH, glutathione; GPX, glutathione peroxidase. References are found in the main text.

and most components of the GSH synthesis system. Also several drug-metabolizing enzymes, enzymes of lipid metabolism and enzymes that reduce oxidized protein thiols are transcribed [38]. Generally, NFE2L2 initiates expression of the whole system of necessary co-factors, NADPH regeneration or collaborating detoxifying enzymes. As one example, HMOX1 (heme oxygenase 1) is one of the players in iron metabolism induced by NFE2L2. HMOX1 converts heme to carbon monoxide, the anti-oxidant bilirubin and free Fe(II+) [39]. To prevent Fe(II+) from catalyzing the reaction from H_2O_2 to the reactive •OH, the simultaneously induced NFE2L2 target genes ferritin light and heavy chain convert and store Fe(II+) [38].

1.1.2 Regulation of the NFE2L2 response

NFE2L2 activity can further be regulated. At the transcriptional level, repressor BACH1 (BTB domain and CNC homolog 1) competes with NFE2L2 for the binding on ARE [40]. Further, BACH1 undergoes redox-regulation, allowing transcription by inactivation of BACH1 [41]. In the cytosol, several kinases have been shown to phosphorylate NFE2L2 and thereby influence the stability of the NFE2L2-KEAP1 interaction [42]. Recently, a KEAP1-independent way of NFE2L2 degradation has been described, involving the binding of

NFE2L2 to BTRC (beta-transducin repeat containing E3 ubiquitin protein ligase; β-TrCP) mediating degradation of NFE2L2 via the SKP1–CUL1–RBX1 ubiquitin ligase complex [43,44]. On the other hand, it has been shown that the autophagy receptor SQSTM1 (sequestosome 1; p62) competes with NFE2L2 for the binding site of KEAP1 [45,46]. This interaction liberated NFE2L2 and led to selective degradation of KEAP1 by autophagy [47]. The affinity of SQSTM1 to KEAP1 can be increased by phosphorylation of SQSTM1 at serine (S) 349 or S351 (in mouse or human cells, respectively), representing an alternative route of NFE2L2 activation [48]. Further, nuclear translocation of NFE2L2 directly induces transcription of SQSTM1, representing a positive feedback-loop between NFE2L2 and SQSTM1 levels [46]. In case of aberrant SQSTM1 accumulation, this mechanism can lead to pathologic changes. In mice deficient for autophagy in the liver, instead of being degraded, SQSTM1 levels increase and recruit KEAP1. This causes hyperactivation of NFE2L2 and its target genes, resulting in liver damage [49]. Also in some types of hepatocellular carcinoma, the accumulation of SQSTM1 has been related to persistent activation of NFE2L2, providing cytoprotection for the tumor cells as a cause of the pathology [50]. Importantly, SQSTM1-dependent regulation of NFE2L2 in response to physiologic stimuli has not been described.

In spite of the anti-oxidant system, damage to DNA or proteins can happen and repair or exchange is necessary, or cell death is induced. The DNA damage response or apoptosis will not be discussed here. In the cytosol, chaperones are expressed and upregulated during stress to support refolding of proteins.

1.2 The heat-shock response

In response to protein damage, unfolding and aggregation, the highly conserved program of the heat-shock response is induced. Although first described for heat exposure, this applies to most denaturing stresses [5]. Chaperones assist protein folding in the cell under resting conditions and are induced by stress. In resting state, the chaperone HSP90 (heat-shock protein 90), which is constitutively expressed, interacts with the transcription factor HSF1 (heat shock transcription factor 1). Cellular stress causes liberation of HSF1, which trimerizes, translocates to the nucleus and initiates transcription of further HSPs like HSP70 and HSP27. General transcription is halted while HSPs and HSFs are selectively transcribed and orchestrate the heat-shock response. HSPs function in protein folding and cytoprotection, as well as they can inhibit apoptotic pathways. Finally, HSF1 returns to monomeric form and is kept inactive by interaction with HSPs in the cytosol, terminating the response [7,51,52].

1.3 The unfolded-protein response (UPR)

Protein folding in the endoplasmatic reticulum (ER) is an energy demanding and error prone process, necessary to allow fast translation of secretory proteins to signal intra- and extracellular changes. Proteins that are not correctly folded are sensed and retained in the ER for refolding or degradation. The high demands of protein folding in the ER make it sensitive to changes in cellular homeostasis and extracellular stimuli. Functional alterations of the ER, also referred to as ER-stress, lead to aberrant protein unfolding and accumulation, which activates the unfolded-protein response (UPR) by the transmembrane sensors IRE1 (inositol-requiring enzyme 1; ERN1), PERK (protein kinase RNA-like endoplasmic reticulum kinase; EIF2AK3) and ATF6 (activating transcription factor 6) [53]. PERK phosphorylates EIF2A (eukaryotic translation initiation factor 2A; eIF2α), leading to suppressed translation, except for some specific UPR genes. In this regard, PERK upregulates expression of the transcription factor ATF4. PERK can also activate the anti-oxidative response via NFE2L2 [54]. IRE1 mediates activation of XBP1 (X-box binding protein-1), that together with ATF6 upregulates further UPR target genes [55]. The EIF2A-ATF4 response can also be activated by other stimuli like infection or starvation, termed the integrated stress response (ISR) [56]. Finally, the ER-related degradation (ERAD) by proteasomes is stimulated to degrade the overload of misfolded proteins [57].

1.4 The ubiquitin-proteasome system (UPS)

Degradation is an essential part of maintenance, assuring turnover of damaged proteins or regulation of signaling. There are two catabolic systems in cells: the ubiquitin-proteasome system and lysosomal degradation via autophagy. Both involve ubiquitination, the attachment of ubiquitin molecules to proteins as a signal for degradation. In mammals, the 26S proteasome consists of a 20S core of two α and two β ring subunits forming a central pore that shields its proteolytically active sites from access of folded proteins [58]. Two regulatory 19S subunits are located on each site of the core. In an ATP-dependent manner they recognize ubiquitinated proteins and mediate opening of the core by conformational changes. Ubiquitin removal and unfolding of the target protein, also mediated by the regulatory subunits, allow entry into the proteasome and degradation [59]. The proteasome preferentially recognizes proteins tagged by polyubiquitin chains of at least four ubiquitin molecules linked via lysine 48 (K48) [58]. Proteasomal degradation is a common regulatory mechanism functioning by constant degradation of proteins or transcription factors that can be stabilized under specific circumstances like NFE2L2 [31]. Deficits in proteasomal degradation can manifest in

neurodegenerative age-related diseases like Huntington and Parkinson's disease characterized by accumulation of cytoplasmatic aggregates. A decline in the function of the UPS during aging is acknowledged [60].

If the capacity of the proteasome is exhausted or larger protein aggregates have to be degraded, this happens via autophagy and the lysosome [61].

1.5 Autophagy

Autophagy is an evolutionary conserved recycling process of the cell, depending on lysosomal function for degradation [62]. This mechanism was first described by C. de Duve after his discovery of the lysosome [63,64]. While direct delivery to the lysosome by invagination (microautophagy) and by help of chaperones (chaperone-mediated autophagy) is known, macroautophagy (further referred to as autophagy) is the best described mechanism and most relevant for maintenance of the cytoplasm as a stress response. During autophagy, excessive, damaged or foreign components become engulfed by an isolation membrane to form an autophagosome. The mature autophagosomes then fuse with lysosomes and the enclosed material is degraded and recycled [65] (Figure 2). This liberates amino acids, lipids and carbohydrates for synthesis of new proteins and cell components. Via induction of autophagy, the cell can adapt to starvation or cellular stress. Importantly, autophagy knockout mice die during the neonatal starvation period [66]. In adult mice with inducible autophagy knockout, starvation is detrimental and overall survival is limited to few months due to development of neurodegeneration [67].

Starvation-induced autophagy is regulated via AMPK (AMP-activated protein kinase; PRKAA1/2) and MTORC1 (mammalian target of rapamycin (mTOR) complex 1). AMPK is a sensor of the energy status and is activated by low levels of ATP in relation to AMP and ADP. Under normal conditions, mTORC1 inhibits autophagy and allows protein synthesis [68]. As its name indicates, rapamycin inhibits mTORC1. Rapamycin is an established autophagy inducer and was shown to promote autophagic degradation of aggregated proteins [69]. MTORC1 activity is positively controlled by growth factor and insulin receptor signals via activation of PI3K (phosphatidylinositol (PI) 3-kinase) [70]. PI3K catalyzes the formation of phosphatidylinositol-3,4,5-trisphosphate (PIP_3) at the plasma membrane, leading to recruitment of PDK1 (pyruvate dehydrogenase kinase 1) and AKT1 (AKT serine/threonine kinase 1; PKB). Active AKT1 indirectly activates mTORC1 [71]. Under starving conditions, AMPK becomes activated, inhibits mTORC1 activity and enables autophagy [72]. The autophagic process relies on the coordinated function of several sets of protein complexes. In

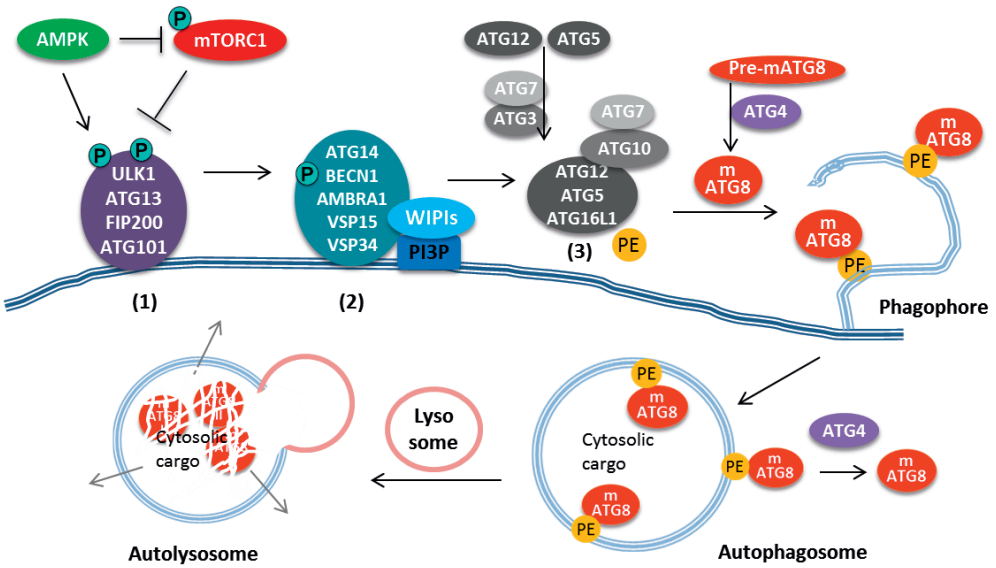

Figure 2: Schematic depiction of autophagy. Under nutrient restriction, autophagy is initiated by activating phosphorylation (P) of ULK1/2 (unc-51 like autophagy activating kinase) by AMPK (AMP-activated protein kinase) or cessation of inhibiting phosphorylation by mTORC1 (mammalian target of rapamycin complex 1). The active ULK1/2 complex **(1)** activates the class III PI3K (phosphatidylinositol (PI) 3 kinase) complex with BECN1 (beclin1), ATG14 (autophagy related 14) and the lipid kinase VSP34 (vacuolar protein sorting 34) **(2)**, producing PIP_3 (PI-3,4,5-trisphosphate). PIP_3 is needed for phagophore formation and recruits WIPIs (WD-repeat protein interacting with PI) involved in membrane elongation. ATG5 and ATG12 are ligated by E1- and E2 like activity of ATG7 and ATG10, respectively, and bind ATG16L1 to form a complex with E3 ligase activity **(3)**. This complex allows phosphatidylethanolamine (PE) conjugation to mATG8 (mammalian paralogues of yeast ATG8) with help of ATG7 (E1) and ATG3 (E2). ATG4 cleaves the precursor form of mATG8 and cleaves mATG8-PE from the outer membrane. The autophagosome closes and fuses with lysosomes, degrading and recycling the enclosed material. FIP200, focal adhesion kinase family interacting protein of 200 kDa; AMBRA1, autophagy and beclin-1 regulator. References are found in the main text.

2016, Yoshinori Ohsumi received the Nobel prize in physiology or medicine for his discoveries of the yeast genes essential for autophagy (autophagy-related genes, ATG) [73]. At that time, this identification and the link to human homologues opened the door for autophagy research [63,74]. In mammals, autophagy is initiated by the ULK1/2 (unc-51 like autophagy activating kinase) complex which is controlled by activating or inhibiting phosphorylation by AMPK and mTORC1, respectively [75,76]. AMPK also phosphorylates and regulates FOXO3 (forkhead box O3) [77], while mTOR phosphorylates TFEB (transcription factor EB) [78], both transcription factors that target ATGs [79,80]. Furthermore, autophagy can be induced independently of AMPK and mTOR [81,82]. The active ULK1/2 complex activates a PI3K class III complex with BECN1 (Beclin1) and ATG14 allowing generation of PIP_3, important for phagophore formation [83]. This also recruits proteins involved in elongation of the autophagosome. An ubiquitin-like conjugation system of ATGs including ATG3, ATG4, ATG7 and ATG10 mediate the formation of an ATG5-ATG12-ATG16L1 complex. This complex has E3 ligase activity and allows

phosphatidylethanolamine (PE) conjugation to the mammalian paralogues of yeast ATG8 (mATG8) like MAP1LC3(A, -B or -C) (microtubule associated protein 1 light chain 3) or GABARAP (or -L1 or -L2) (GABA type A receptor-associated protein). PE conjugation anchors mATG8s to the growing autophagosomal membrane [62]. The autophagosome closes and fuses with lysosomes, degrading and recycling the enclosed material (Figure 2).

1.5.1 Selective Autophagy

Besides basal turnover of cytosol, the sequestration of cargo can be specific, what depends on adaptor molecules. Sequestosome-like receptors (SLR) bind ubiquitinated cargo by their UBA (ubiquitin-associated domain). Their common LIR (LC3 interacting region) domain can then interact with mATG8 family members, targeting ubiquitinated cargo to lysosomal degradation [84] (Figure 3). SQSTM1 was the first autophagy receptor described to directly bind MAP1LC3B and thus mediating selective autophagy [85]. Both, SQSTM1 and MAP1LC3B are degraded by autophagy and recognized markers to monitor autophagic flux by their turnover [86]. By autophagy, misfolded proteins, damaged organelles, activated signaling molecules or pathogens can be selectively degraded, assisting the maintenance of the cell, signal termination [87–89] or pathogen defense [90–92]. One important function of autophagy is mitophagy, which recycles defective mitochondria and thus maintains these important organelles [93]. Similarly, ER-stress not only has been reported to induce autophagy [94], but autophagy also degrades ER [95].

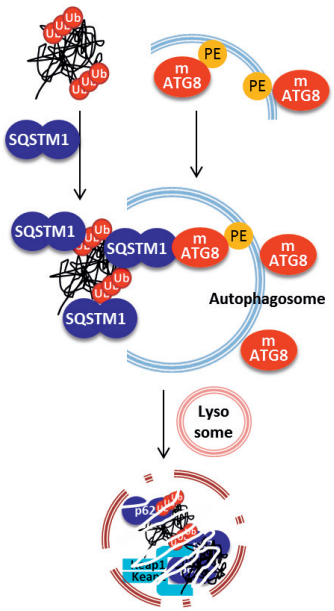

Figure 3: Schematic illustration of selective autophagy.. Sequestosome-like receptors like sequestosome 1 (SQSTM1) bind ubiquitinated (Ub) cargo and interact with mATG8 family members, targeting ubiquitinated cargo to lysosomal degradation. Thereby, misfolded proteins, damaged organelles, activated signaling molecules or pathogens can be selectively degraded. References are found in the main text.

The SLRs, although overlapping in their function, differ in their specificity towards recognized cargo [96]. SQSTM1 preferentially binds to polyubiquitin chains and is important for clearance of ubiquitinated targets, that might include aggregates and mitochondria [97,98]. Accumulation of SQSTM1 in inclusion bodies is found in various proteinopathies and neurodegenerative diseases [99,100]. Interestingly, PB1 (Phox-Bem1) domain-dependent

homopolymerization of SQSTM1 is important for efficient sequestration and turnover of cargo as its UBA has low affinity for one ubiquitin molecule [101]. NBR1 (neighbor of BRCA1 gene 1) similarly binds to ubiquitinated targets, can heterodimerize with the PB1 domain of SQSTM1 and is also found in inclusion bodies [102]. Furthermore, NBR1 has been found to be the main receptor for peroxisome handling [103]. OPTN (optineurin), NDP52 (nuclear dot protein 52 kDa; CALCOCO2) and its close homologue TAX1BP1 (Tax1 binding protein 1) have important roles in xenophagy [104,105] and have been implicated in mitophagy [106,107]. Finally, BNIP3 (BCL2 interacting protein 3), BNIP3L (BCL2 interacting protein 3 like; NIX) and FUNDC1 (FUN14 domain containing 1) have been described as specific mitophagy receptors [96,108]. The latest SLR described is TOLLIP (toll interacting protein), the only SLR so far known to mediate selective removal of aggregated proteins in lower eukaryotes as well as human cells [109].

1.5.2 Aggrephagy

Damaged proteins expose their hydrophobic otherwise hidden parts, leading to aberrant interaction with other proteins and formation of small aggregates [110]. Misfolded proteins are generally sensed by chaperones like HSPs that surveil the cytosol and restore protein folding. HSPs can interact with ubiquitin-conjugating enzymes to direct irreversibly damaged proteins to ubiquitination and degradation by the UPS or autophagy [111]. If not removed by the cellular degradation systems, aggregated and ubiquitinated proteins are recognized by HDAC6 (histone deacetylase 6), an ubiquitin-binding microtubule deacetylase. HDAC6 can transport ubiquitinated cargo along the microtubule to the microtubule-organizing center [112]. There, an aggresome surrounded by filaments is formed which is thought to limit toxic effects of misfolded proteins [113]. In the context of aggregate degradation, HDAC6 also has a role in autophagosome maturation [114].

The autophagic degradation of aggregated proteins has been termed aggrephagy by the group of Per Seglen [115]. Aggrephagy involves the ubiquitin-binding autophagy receptors SQSTM1 and NBR1 to link aggregated and ubiquitinated proteins to the forming autophagosome (Figure 3). One important adaptor for aggrephagy is ALFY (autophagy-linked FYVE domain containing protein; WDFY3). ALFY forms a direct binding scaffold between the autophagy machinery and SQSTM1, necessary for degradation of aggregated cargo [116].

As a physiologic form of aggregation, dendritic cell aggresome-like structures (DALIS) have been described during maturation of professional antigen-presenting cells like dendritic cells and macrophages. DALIS are implicated in antigen presentation by MHC

(major histocompability complex) class I (MIC) molecules [117]. Presence of ubiquitin and SQSTM1 are characteristic for DALIS. Later, it was shown that structures similar to DALIS, namely ALIS (aggresome-like structures), can be formed in various cell types in response to cellular stress like liposaccharide (LPS) stimulation, infection, oxidative stress or proteasomal inhibition [118]. ALIS have been suggested to provide a transient form of storage for unfolded proteins to be destroyed [119]. Since an important component of ALIS is SQSTM1, they have also been referred to as p62-bodies, which might implicate a broader range of structures [111].

Aggregates or inclusions are observed in different tissues with decreased autophagy [19,20]. Similarly, various age-related diseases as AMD and Alzheimer's disease are characterized by aggregates often positive for SQSTM1, implicating that degradative pathways are impaired [120,121]. However, if protein aggregates are causative or simply a consequence of degeneration is still a matter of debate.

1.6 The ubiquitin system

The degradative systems rely on specific recognition of proteins to be degraded. This is assured by modification with ubiquitin [122]. Ubiquitin is a small protein that is found in most organisms and tissues, where it exerts signaling and regulatory functions. Covalent mono- or polyubiquitin conjugation mediates degradation, activation, location or conformational changes of the tagged protein, mostly by recognition through receptors with ubiquitin-binding domains (UBD) [123]. The specificity of the signal is generated by the length of the ubiquitin chain and its linkage-type (K6, K11, K27, K29, K33, K48, K63 or M1), depending on which lysine or the N-terminal methionine (M) the ubiquitin molecules are attached to each other. The most studied conformations are K48-linkage mediating proteasomal degradation [122] and K63-linkage which can induce conformational changes or protein complex formation to activate signaling [124]. SLRs can recognize K63 ubiquitination, leading to autophagosomal degradation. Linear M1 chains have been shown to be involved in pro-inflammatory NFκB (nuclear factor kappa B) complex activation, while most other chain types are just on the way of being elucidated [125].

The covalent attachment of ubiquitin is done by an enzymatic cascade. The E1-ubiquitin activating enzyme activates ubiquitin in an ATP-dependent manner and transfers it onto the E2-conjugating enzyme, which brings it in place for the E3-ubiquitin ligase that forms an isopeptide bond. There are two known E1, about 40 E2 and more than 600 E3 enzymes in mammals, enabling an enormous amount of specific conjugation on different

targets, different linkages, different lengths and even branched chains [126]. Similar to the function of phosphatases in the kinase network, ubiquitination can be reverted by the action of deubiquitinases (DUBs). Compared to E3 ligases, there are only about 80 genes encoding DUBs, indicating broader target specificity. It is thought that localization and expression play an important role to convey specificity to deubiquitination like by mitochondrial USP30 (ubiquitin specific peptidase 30) that deubiquitinates mitochondria and inhibits mitophagy [127,128]. A well-known example of a DUBs is TNFAIP3 (TNF alpha induced protein 3; A20). TNFAIP3 is an ubiquitin-editing enzyme which can remove activating K63 ubiquitin chains [129] and with help of the autophagy receptor and adaptor protein TAX1BP1 [130], the associated E3-ligases ITCH (itchy E3 ubiquitin protein ligase) [131] and RNF11 (ring finger protein 11) [132] add K48 chains, mediating degradation of inflammatory signaling proteins [133].

Besides ubiquitin, there are other ubiquitin-like modifiers with similar conjugation systems like SUMO (Small ubiquitin-like modifier protein) and ISG15 (IFN-induced ubiquitin-like modifier) [123]. Also mixed chains of ubiquitin and ISG15 have been described [134]. Finally, ubiquitin and ubiquitin-like modifiers can undergo posttranslational modifications like acetylation or phosphorylation [135,136].

Thus, ubiquitin and ubiquitin-like modifications form a complex system that is involved in the regulation of many cellular processes.

1.7 The immune response and inflammation

Cellular homeostasis includes the defense against exogenous insults and pathogens. The immune response and inflammation not only assure removal of pathogens, but also support tissue regeneration and maintenance, thus assuring normal function of the organism [1,137]. The immune system of vertebrates can be divided into innate and adaptive immunity. Innate immunity functions non-specific and is crucial for the fast and initial defense in which acute inflammation plays a major role [138], whereas adaptive immunity assures specific elimination and immunologic memory. However, memory functions were also described in innate immune cells [139]. Only the immune system of vertebrates includes both, innate and adaptive immunity, underlining the importance of the innate immune system [140]. In mammals, the mechanical barrier of the skin, chemical barriers as mucous and saliva and a humoral and cellular component convey different layers of protection. Intracellular compartmentalization as cell-autonomous mechanism contributes to pathogen defense [141]. The humoral component includes plasma molecules that facilitate recognition and clearance

of pathogens, like the complement system, pathogen binding proteins and acute phase proteins [138]. The cellular component includes phagocytic cells like macrophages, natural killer cells and dendritic cells, granulocytes as neutrophils and mast cells. Non-professional immune cells of non-hematopoietic origin are epithelial and endothelial cells. The cellular compartment reacts or is recruited to the site of insult. The coordinated action of infiltrating cells is perceived as inflammation, the local redness, swelling, pain and loss of function described by the Roman academic Celsus [142]. Tissue insults are most often first perceived by local endothelium and resident macrophages, directing the recruitment of neutrophils, mast cells and monocytes. These act in acute inflammation and elimination of the trigger [143]. Lipid mediators as the prostaglandins PGE_2 and PGI_2 and the leukotriene LTB_4, are produced by the involved immune cells, facilitating blood vessel permeability and further cell recruitment [144]. The main sensing mechanism in the cellular compartment is pattern recognition achieved by pattern recognition receptors (PRR) situated on cell membranes, in endosomes and in the cytosol [145]. Depending on cell types, different constellations of PRRs are expressed. The PRRs recognize pathogen associated molecular patterns (PAMPs) e.g. bacterial wall components or single stranded RNA that are unique to the pathogen and not found endogenously in the host [146–148]. More recently, vitaPAMPs have been described, special prokaryotic mRNA molecules that are associated with living pathogens and elicit a unique innate response [149]. PRRs are further able to recognize damage associated molecular patterns (DAMP). DAMPs are non-infectious molecules that induce a sterile inflammatory response and include endogenous molecules released from damaged cells like HMGB1 (high mobility group box 1) or ATP [150]. In arteriosclerosis, cholesterol crystals have been shown to activate inflammasomes leading to hyperactivation of immune cells and aggravating vessel occlusion [151]. Exaggerated inflammation leads to tissue damage and release of further DAMPs maintaining chronic inflammation in diseases like arthritis, diabetes and cancer [152]. However, DAMPS also assure surveillance and restriction of tumor cells [153]. Importantly, tissue resident macrophages recognize dead cells and initiate subsequent responses depending on the cause of cell death [1]. Cell death by infection will trigger an immune response, whereas stress and injury lead to tissue-repair, assuring homeostasis in each case. Recruited neutrophils undergo programmed cell death and their phagocytic removal has a direct immunosuppressive outcome [154]. Importantly, the role of inflammation in stimulating regeneration is evolutionary preserved [1,137].

The main types of PRRs are TLRs (Toll-like receptors), CLRs (C-type lectin receptors), NLRs (NOD-like receptors) and RLRs (Retinoic acid-inducible gene (RIG)-I-like receptors).

Additionally, several cytosolic receptors like cGAS (cyclic GMP-AMP synthase; MB21D1) or IFI16 (interferon gamma inducible protein 16) sense viral and bacterial dsDNA [155]. TLRs include a wide range of membrane bound receptors, each sensing specific ligands of bacterial, viral or endogenous origin. They are especially expressed on innate immune cells, which often play a role in the first contact with pathogens. CLRs are also found on the plasma membrane and act in the defense against fungi, mycobacteria and viruses by their mannose specificity [156,157]. Cytoplasmic receptors like NLRs and RLRs are important for the recognition of pathogens inside infected cells. RLRs are involved in the recognition of viruses, mostly viral RNA, and are also present in epithelial and endothelial cells. NLRs are cytoplasmic receptors, of which some bind ligands of bacterial origin, while others are activated by diverse stimuli, forming inflammasomes [158,159]. The activation of PRRs leads via a signaling cascade of adaptors, kinases and transcription factors to the induction of cytokines, IFNs and antimicrobial peptides. These mediators shape the intercellular signaling in all defense processes.

Besides well-established PRR signaling, cell-autonomous processes like the here described stress responses UPR and especially autophagy contribute to innate immune defense by modulating responses towards pathogens [4]. How stress responses could be targeted to modify PRR signaling is not fully elucidated.

1.7.1 The TLR response

The TLRs represent a family of well-studied PRRs that recognize extracellular, endosomal or lysosomal PAMPs and self-ligands [148]. They consist of N-terminal leucine rich repeats (LRR), a transmembrane domain and a cytoplasmatic Toll/IL-1R homology (TIR) domain. TLRs are expressed in both immune and non-immune cells like fibroblasts and endothelial cells [160,161]. However, macrophages and dendritic cells play a particular role as integrators of extrinsic signals. Ten TLRs are identified in humans and twelve in mice. Their localization groups these PRRs into cell surface TLRs (TLR1, TLR2, TLR4, TLR5, TLR6 and TLR10 (human only)) and intracellular, endosomal TLRs (TLR3, TLR7, TLR8, TLR9 and only murine TLR11, TLR12 and TLR13). TLR4 can further travel from the plasma membrane to the endosomal compartment, initiating differential signaling from both localizations [147]. The cell surface TLRs mainly recognize bacterial ligands like LPS from the outer wall of gram negative bacteria by TLR4 or lipoproteins, peptidoglycans and mannan by TLR2 associated with TLR1 or TLR6. The endosomal TLRs detect mostly nucleic acids from bacteria, viruses or from pathogenic self [148]. TLR signaling depends on the recruitment of

TIR-domain containing adaptors that can bind to the cytoplasmatic tail of the TLR. The mediated signaling can be classified by the recruitment of MYD88 (myeloid differentiation primary response 88) or TRIF (TIR-domain-containing adapter-inducing IFNB; TRIM69). Recruitment of MYD88 leads to activation of NFκB and expression of pro-inflammatory cytokines and recruitment of TRIF activates IFN signaling [162]. MYD88 is recruited to all TLRs except TLR3 in a TIR-mediated manner which additionally can depend on TIRAP (TIR domain containing adaptor protein; MAL) [163]. TRIF is recruited to TLR3 and via interaction with the co-adaptor TRAM (translocation associated membrane protein) to endosomal TLR4 [164]. By signaling from the plasma membrane and the endosome, TLR4 establishes a broad response via both MYD88- and TRIF-dependent pathways. The recognition of LPS involves binding of MD-2 (myeloid differentiation factor-2; LY96) to TLR4 and dimerization of TLR4 [165]. In addition, LBP (lipopolysaccharide binding protein) can extract LPS from bacterial membranes and transfer it via CD14 to TLR4, thus LBP and CD14 enhance LPS signaling [166].

MYD88 dependent signaling attracts IRAKs (interleukin 1 receptor associated kinases), forming multimeric complexes called Myddosome [167]. After auto-phosphorylation, IRAK1 dissociates to interact with the E3-ligase TRAF6 (TNF receptor associated factor 6). TRAF6 mediates K63 ubiquitination of itself and the MAPK (mitogen-activated protein kinase) family member TAK1 (TGF-beta activated kinase 1; MAP3K7) [168]. Via ubiquitin chains, TAK1 interacts with its regulatory subunits TAB1, TAB2 and TAB3 (TAK1 binding proteins) leading to activation of TAK1 and initiation of the MAPK and the canonical IKK (I kappa B kinase)-NFκB pathway [169]. Also free ubiquitin chains generated by TRAF6 activate TAK1 and IKKs [170]. Phosphorylation of TAK1 or ubiquitination at K562 further fine-tune the differential activation of MAPK or IKK pathways [171]. In the MAPK pathway, a cascade of MAP kinases finally activates p38 (MAPK14), JNK (JUN N-terminal kinase; MAPK8) and ERK1/2 (extracellular signal-regulated kinase; MAPK1/3), all mediating translocation of AP-1 (activator protein 1) transcription factors [172]. In the IKK-NFκB pathway, TAK1 ubiquitin-dependently binds to the complex of IKKα (CHUK), IKKβ (IKBKB) and NEMO (IKKγ; IKBKG) and phosphorylates IKKβ, which also initiates further auto-phosphorylation and activation [173]. In resting state, the nuclear import sequence of NFκB is hidden by its inhibitory protein IκBα (I kappa B alpha; NFKBIA) [174]. Phosphorylation of IκBα by the IKK complex with subsequent K48-ubiquitination results in proteasomal degradation of IκBα and liberation and translocation of NFκB [175] (Figure 4).

Also linear ubiquitination of and recognition by NEMO is necessary for NFκB activation [176,177].

TRIF-dependent signaling involves recruitment of both TRAF6 and TRAF3. TRAF6 can via RIPK1 (receptor interacting serine/threonine kinase 1) interact with TAK1 and finally

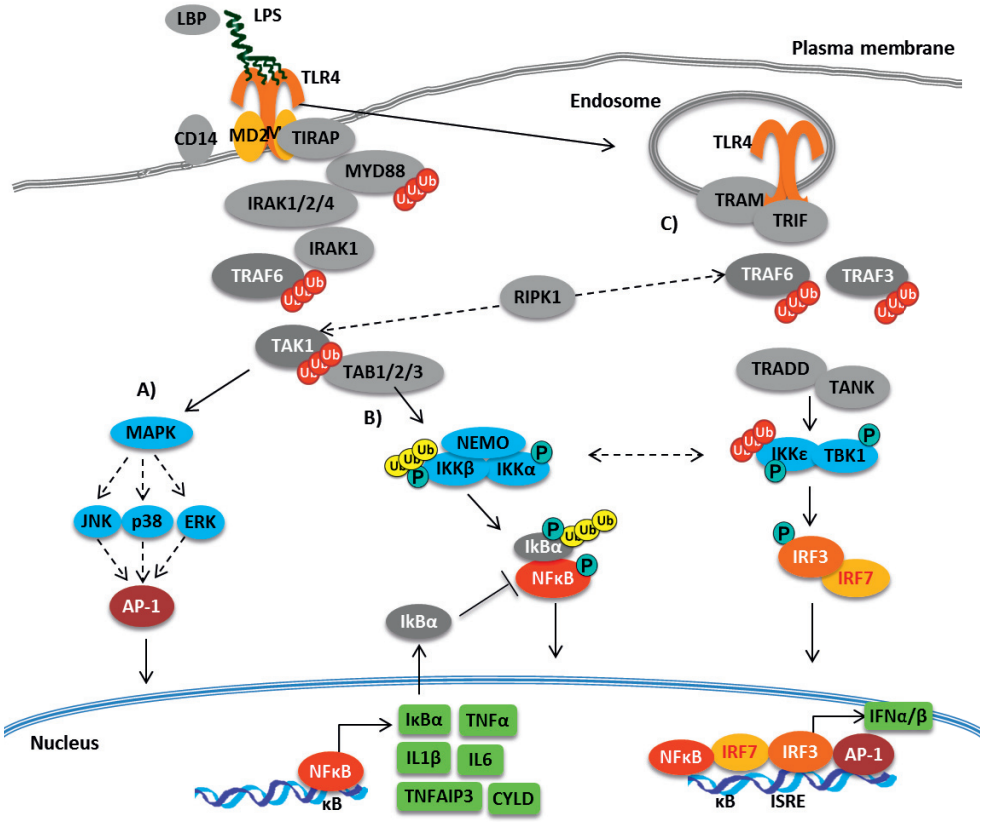

Figure 4: Schematic depiction of TLR4 signling. LBP (lipopolysaccharide binding protein) can extract LPS (lipopolysaccharide) from bacterial membranes and transfer it via CD14 to TLR4 (Toll-like receptor 4) that binds MD-2 (myeloid differentiation factor-2) and dimerizes. Via TIRAP (TIR domain containing adaptor protein) MYD88 (myeloid differentiation primary response 88) is recruited and attracts IRAKs (interleukin 1 receptor associated kinases). After auto-phosphorylation (P), IRAK1 dissociates to interact with the E3-ligase TRAF6 (TNF receptor associated factor 6) which further mediates K63 ubiquitination (Ub) of itself and TAK1 (TGF-beta activated kinase 1). Via ubiquitin chains, TAK1 interacts with TAB1, TAB2 and TAB3 (TAK1 binding proteins and **A)** initiates a cascade of MAPKs (mitogen activated kinases) finally activating p38, JNK (JUN N-terminal kinase) and ERK1/2 (extracellular signal-regulated kinase) and mediating nuclear translocation of AP-1 (activator protein 1). **B)** TAK1 can ubiquitin-dependently bind to the IKK (I kappa B kinase) complex of IKKα, IKKβ and NEMO (NF-kappa-B essential modulator) and phosphorylate IKKβ. Phosphorylation of IκBα (inhibitor of kappa B alpha) by the IKK complex results in ubiquitination and proteasomal degradation of IκBα, nuclear translocation of NFκB (nuclear factor kappa B) and transcription of inflammation-related genes. **C)** TLR4 can be endocytosed and recruit TRAM and TRIF that associate with TRAF6 and TRAF3. TRAF6 can via RIPK1 (receptor interacting serine/threonine kinase 1) interact with TAK1 and finally activate AP-1 and NFκB pathways. TRAF3 undergoes auto-ubiquitination, association with TRADD (TNFRSF1A associated via death domain) and TANK (TRAF family member associated NFκB activator) and recruits the IKK-related kinases IKKε and TBK1 (TANK binding kinase 1). There is extensive crosstalk between the IKK complex and the IKK-related kinases. IKKε and TBK1 phosphorylate IRF3 (interferon regulatory factor 3), leading to nuclear translocation. AP-1, NFκB and IRF3 or IRF7 form the IFN (interferon) enhanceosome and induce expression of IFNs. TNF, tumor necrosis factor; IL1β, interleukin 1 beta; TNFAIP3, TNF alpha induced protein 3; CYLD, CYLD lysine 63 deubiquitinase; kB, kappa B element; ISRE, IFN-sensitive response element. References are found in the main text.

activate AP-1 and NFκB pathways, mediating pro-inflammatory cytokine signaling [178]. TRAF3 on the other hand initiates antiviral responses by auto-ubiquitination, association with the scaffold proteins TRADD (TNFRSF1A associated via death domain) and TANK (TRAF family member associated NFκB activator) and recruitment of the IKK-related kinases IKKε (IKBKE) and TBK1 (TANK binding kinase 1) [179]. It has further been proposed that NEMO can associate with TANK, linking TRAF3 to NFκB activity, but also facilitating recruitment of IKKε and TBK1 [180]. Importantly, there is extensive crosstalk between the IKK complex and the IKK-related kinases [181]. TANK has been shown to mediate an inhibitory effect of IKKε and TBK1 activation on the canonical IKK pathway [182]. IKKε and TBK1 then phosphorylate and activate IRF3 (interferon regulatory factor 3) [183,184]. AP-1, NFκB and IRF3 or IRF7 together with the co-activator CREBBP/p300 (CREB binding protein) [185] can form the IFN enhanceosome and induce expression of IFNβ (IFNB1) [186,187] (Figure 4). Of note, IRF7 is an ISG and activator of IFN production, thus creating a positive feedback loop on the synthesis of antiviral cytokines [188]. Additionally, IRF7 can form heterodimers with IRF3 or IRF5 to induce transcription of IFNα and IFNβ [189,190]. IFNβ has both, a direct immunomodulatory effect on innate and adaptive immune cells and the capability to induce further cytokines.

1.7.2 The Interferon response

The interferon response is a major defense program during viral infection, inhibiting viral replication, supporting host cell death and stimulating the adaptive immune response [191,192]. IFNs also play a role in bacterial infections, however, IFN signaling can limit or boost host defense depending on the type of bacteria and infection [193]. Importantly, both chronic inflammation and age-related pathologies have been linked to unbalanced IFN signaling [194,195].

IFNs can be divided into type I IFNs with IFNα (IFNA2) and IFNβ as most common members, type II IFNs with the single member IFNγ (IFNG) and type III with 4 subtypes of IFNλ (IFNL). Type I IFNs are expressed in most cells and induce IFN-stimulated genes (ISG) to establish an overall antiviral state [196]. Type II IFNs are mainly secreted from natural killer (NK) cells and T-cells in response to IL2 and IL12B [197]. Type II IFNs have an antiviral function but also support macrophage activation and phagocytosis important in mycobacterial defense [198]. Type III IFNs are ISGs and have a more locally confined antiviral action as they protect mucosal cells from infection [199]. IFN-induced mechanisms also include inducible cell-autonomous defense like ROS production and autophagy [200].

IFN I expression is initiated by IRFs as result of PRR activation. A main source of IFN production in non-professional immune cells is sensing of viral RNA by the RIG-I family [201]. Further, several sensors in the cytosol like DDX (DEAD box) helicases, cGAS, STING (stimulator of interferon genes; TMEM173), or the AIM2 (absent in melanoma 2) inflammasome can specifically recognize viral or bacterial RNA or DNA [155]. Endosomal TLRs additionally recognize self-nucleic acids as danger signals, finally activating the TBK1-IRF3 axis. In immune cells, several other IRFs like IRF5 by TLR8 activation [202] can induce IFN expression. Plasmacytoid dendritic cells secrete an important amount of IFNα from activation of endosomal TLR7 and TLR9 and IKKα mediated phosphorylation of IRF7 [203].

The type I IFNs IFNα or IFNβ signal through the heterodimeric receptors IFNAR1 (interferon alpha and beta receptor subunit 1) and IFNAR2. Receptor associated JAK1 (Janus kinase 1) and TYK2 (Tyrosine kinase 2) become autophosphorylated and phosphorylate cytoplasmic STAT1 (signal transducer and activator of transcription 1) and STAT2 [204]. STAT1, STAT2 and IRF9 form the ISGF3 (IFN-stimulated gene factor 3) complex that can translocate to the nucleus. ISGF3 binds to ISRE (interferon-sensitive response elements) of hundreds of specific ISGs, inducing an antiviral response [205,206]. Homodimers of STAT1 bind to GAS (gamma interferon activation site) elements inducing other sets of ISGs like CXCL10 or NOS2. STAT1 homodimers are also induced by IFNγ via IFNGR1 (interferon gamma receptor 1) [207]. STAT3 can be phosphorylated downstream of type I IFNs, contributing to transcription of GAS containing genes [208,209]. STAT3 has also been reported to suppress some parts of the immune response by mediating anti-inflammatory effects of IL10 [210] or by interfering with the formation of STAT1 homodimers [211]. Apparently, the distribution of ISRE and GAS elements in target genes, the usage of different receptors and various STAT combinations allow differential expression of genes in numerous conditions [212]. Importantly, low levels of IFNβ secretion maintain high basal levels of STAT1 and IRF9 that are ISGs themselves, assuring a fast response to IFNAR activation [213] (Figure 3).

One much studied ISG is the antiviral ubiquitin-like modifier ISG15, whose conjugation to viral proteins inhibits viral replication. Recently, different modes of function in humans and mice have been pointed out [214]. Human ISG15 is necessary to stabilize USP18, an ISG15 specific DUB, to negatively regulate IFN type I signaling. Free ISG15 can act as a cytokine [215] and activate IFNγ in T-cells and NK cells [216]. In ISG15 deficient patients, a high IFN I signature maintains viral defense, but failure to induce IFNγ augments susceptibility to mycobacterial infection [217].

Also the chemokines CXCL9 (C-X-C motif chemokine ligand 9), CXCL10 and CXCL11 are ISGs highly inducible by IFNγ and signal through their common receptor CXCR3 (C-X-C motif chemokine receptor 3) to attract T-cells [218,219]. CXCL10 is the best studied of these and is implicated in diseases with underlying inflammation [220]. Besides IFN-and STAT-mediated induction, NFκB, IRF3 and IRF1 can bind the CXCL10 promotor, leading to direct expression downstream of PRRs [221,222].

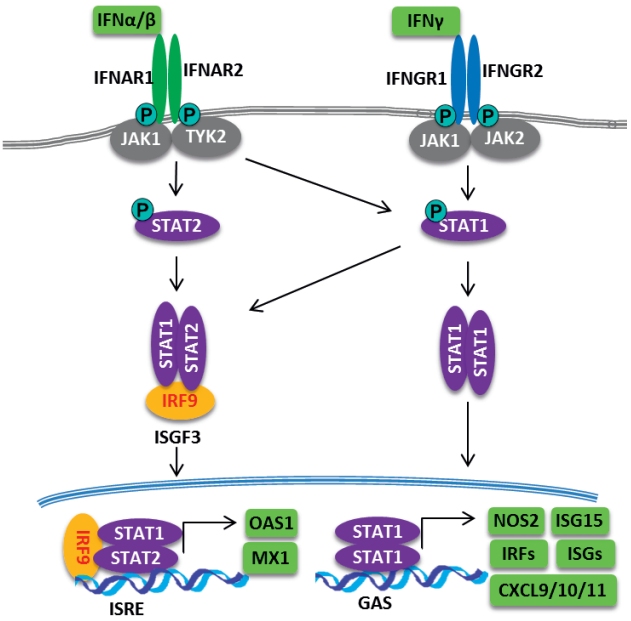

Figure 5: Illustration of the interferon response. IFNα or IFNβ (type I interferons) bind to the heterodimer of IFNAR1 (interferon alpha and beta receptor subunit 1) and IFNAR2. Auto-phosphorylation recruits and activates JAK1 (Janus kinase 1) and TYK2 (Tyrosine kinase 2) which then phosphorylate cytoplasmic STAT1 (signal transducer and activator of transcription 1), STAT2 and STAT3. STAT1 and STAT2 heterodimerize and recruit IRF9 (interferon reglated factor 9), forming the ISGF3 (IFN-stimulated gene factor 3) complex that translocates to the nucleus, binds to ISRE (IFN-response elements) and induces antiviral ISGs (IFN-stimulated genes). STAT1 homodimers bind to GAS (gamma interferon activation site) elements, inducing other sets of ISGs. OAS1, 2'-5'-oligo-adenylate synthase; MX1, MX Dynamin Like GTPase 1; NOS2, nitric oxide synthase 2; CXCL9, C-X-C motif chemokine ligand 9. References are found in the main text.

1.7.3 The inflammasome

Inflammasomes are main processors of active and highly pro-inflammatory IL1β (interleukin 1 beta; IL1B). Thus, inflammasome activity is important in inflammation, but also linked to chronic inflammatory diseases [223]. Recent discoveries extend the involvement of inflammasomes to induction of lipid mediators, autophagy and metabolism [224]. Upon activation, the NLRs recruit adapter molecules and form large cytosolic complexes that recruit and activate CASP1 (caspase-1) by cleavage. Active CASP1 then processes the progenitor form of IL1β and IL18 and after an unconventional secretion, the mature cytokines mediate

local and systemic inflammation [159]. Inflammasome activation is normally dependent on two stimuli, one priming event that increases transcription of NLRs and progenitor cytokines, typically by activation of PRRs, and one activating stimuli that leads to inflammasome assembly. The NLRP3 (NLR family pyrin domain containing 3) and AIM2 inflammasome are the most extensively studied. The NLRP3 inflammasome responds to a variety of PAMPs and DAMPs [225] while the AIM2 inflammasome responds to cytoplasmic DNA [226]. Importantly, all of the stimuli activating the NLRP3 inflammasome involve mitochondrial ROS (mtROS) and release of mitochondrial DNA (mtDNA) as a common mechanism [98,227,228].

1.7.4 Regulation and resolution of the immune response

To assure efficient defense, but avoid detrimental activation and tissue damage, the immune response has to be tightly regulated. Positive feedback mechanisms exist to amplify cytokine production and mount a protective immune response. On the other hand, negative feedback keeps signaling in balance and there are numerous antagonists that are induced together with active inflammatory signaling [229]. This includes upregulation of secreted, soluble decoy receptors like IL1RN (interleukin 1 receptor antagonist; IL-1Ra) [230]. IκBα is upregulated by NFκB activation, sequestering NFκB in its inactive state [231]. Furthermore, microRNAs (miRNA) are induced and mi-R146 inhibits NFκB signaling [232]. Inflammatory signaling involves selective increase in protein phosphorylation and ubiquitination that can be reverted by upregulation of phosphatases like PTPN1 (protein tyrosine phosphatase, non-receptor type 1; PTPB1) [233], K63 DUB CYLD [234] or ubiquitin-editing enzyme TNFAIP3 [235]. The importance of this regulation is underlined by TNFAIP3-deficient mice dying of hyperinflammation [236]. The proteasome and autophagy are important mechanisms to degrade ubiquitinated signaling proteins and stop acute inflammation. For example, TLR4 and TLR9 can become K48-ubiquitinated by RNF216 (TRIAD3A) targeting them for proteasomal degradation [237] and ubiquitinated inflammasomes have been suggested to be degraded by autophagy [89].

Many ISGs are not induced to support antiviral defense but restrict inflammatory signaling. SOCS (Suppressors of cytokine signaling) are expressed by an elegant mechanism downstream of the IFN-STAT1 axis and suppress TLR signaling [238]. Subsequent to the amplification of PRR signaling by IFNs and IFNAR1, TAM tyrosine receptor kinases become expressed on the plasma membrane. Stimulation of TAMs in concert with IFNAR1

selectively induces STAT1-dependent transcription of SOCS1 and SOCS3, which inhibit TIRAP or TRAF3 and TRAF6 respectively [239,240].

Inflammation is needed to clear insults but once accomplished resolution and repair have to take place. By the anti-inflammatory mechanisms described here, the start of inflammation already assures its end [241]. Decreased cytokine signaling, less recruitment of neutrophils and the phagocytic removal of dying neutrophils mediate the end of acute inflammation [242]. NFκB is necessary for induction of anti-inflammatory mediators and has been shown to be equally important in the induction of inflammation as in resolution. Inhibition of NFκB during resolution impairs neutrophil apoptosis and clearance [243]. This leads to decreased secretion of immune suppressive cytokines from macrophages that have ingested apoptotic cells, thus affecting resolution [244]. Importantly, RIPK1 is positively involved in NFκB signaling when ubiquitinated [245], but also mediates the switch to CASP8 dependent apoptosis by its kinase activity after deubiquitination [246,247]. Further, in absence of CASP8, RIPK3 is recruited, inducing necroptosis [248], a highly inflammatory cell death, boosting the immune response [249]. Thus RIPK1 is an important point of modulation and control. With the end of acute inflammation, the production of lipid mediators switches from pro-inflammatory eicosanoids to specialized pro-resolving mediators (SPM) as lipoxins and resolvins [241,242]. Different SPMs can induce further resolution mediators, likewise LTA_4 (Lipoxin A_4) augments IL10 production [250]. Finally, inflammatory signaling initiates repair and proliferation to restore damaged tissue and finally return to homeostasis [137].

1.7.5 Mycobacterial infection

Phagocytosis of extracellular bacteria by professional phagocytes with subsequent degradation through lysosomal enzymes is an important part of defense in bacterial infection. However, some bacteria such as mycobacterial species can reside inside macrophages and evade killing. How this is accomplished is still not completely understood, but mycobacteria can interfere with the host immune response to skew it to anti-inflammatory signaling or benefit from host intrinsic pathways that limit inflammation [251].

Mycobacterium tuberculosis (Mtb) as the cause of tuberculosis (TB) is a global threat with about one third of the world's population harboring latent infection. Due to limited vaccination and treatment success TB still remains a challenge [252]. *Mtb* transmission happens via aerosol inhalation that, by the interaction of T-cells and macrophages, mostly leads to formation of confined granulomas in the lung in which the bacteria hibernate.

Circumstances like T-cell impairment can lead to granuloma cessation and active disease [253]. Similar to *Mtb*, the *Mycobacterium avium (M. avium)* complex (MAC), a group of nontuberculous mycobacteria, may give rise to pulmonary pathology. However, MAC are opportunistic pathogens mostly affecting elderly and immunocompromised people [254]. Similarly, the risk for active TB is higher in HIV (*human immunodeficiency virus*) infected patients [255].

Mycobacteria have a cell wall with a thin protective layer of peptidoglycan at the base of polysaccharides forming a link to a dense layer of mycolic acids, long chain beta-hydroxy fatty acids. The complex cell wall makes mycobacteria largely resistant to damage and drugs [256]. Detection and phagocytosis of mycobacteria happens via engagement of PRRs. CLRs, beside scavenger and other cell surface receptors play an important role in recognition and phagocytosis of mycobacteria and can collaborate with TLR signaling [157]. TLR2 recognizes lipopeptides of the mycobacterial cell wall and makes a prominent contribution in mycobacterial infection. In the context of mycobacteria, TLR4 can detect glycoproteins and secreted proteins, but interacts mostly with acetylated lipomannan [257]. TLR9 in endosomes can detect bacterial CpG DNA [258]. Finally, NOD-like receptors in the cytosol recognize dsDNA and cell wall components, which can lead to inflammasome activation [259,260].

To survive inside macrophages, bacterial proteins can manipulate cytokine production. ESAT-6 (early secreted antigenic target protein 6) secreted by Mtb interacts with TLR2 and prevents Myddosome assembly. Innate recognition by PRRs triggers secretion of TNF, IL1, IL6, IL12, CXCLs, CCLs (chemokine ligands) and other cytokines, chemokines and antimicrobial peptides [261–263]. Induction of an immune response also triggers negative regulators to protect the host from tissue damage, but that might be beneficial for the pathogen. For example, TLR2 activation stimulates secretion of the anti-inflammatory IL10 [264] interfering with production of IL12 [265]. IL12 dependent activation of CD4+ T-cells and production of IFNγ is essential to limit *M. avium* in vivo [266–268].

As an important part of the host-pathogen interaction during phagocytosis, ROS are produced by the NOX complex, while reactive nitrogen species (RNS) are generated by NOS2 (nitric oxide synthase 2; iNOS). ROS and RNS exert anti-microbial actions in the phagosome [22,269]. In line with this, NOS2 and NOX polymorphisms increase the susceptibility for TB [270,271]. Surprisingly, it has also been suggested that the restriction of *M. avium* does not depend on RNS or the respiratory burst, since susceptibility to *M. avium* infection was not increased in NOS2 knockout mice [272].

The normal route of defense by phagocytosis is maturation of the phagosome with increasing acidification to finally form a phagolysosome where the content is degraded. This liberates PAMPS and activates further inflammatory signaling in the cytosol. However, some intracellular bacteria such as mycobacteria are able to block or escape degradation by the phagolysosome. For *Mtb* and *M. avium* a compartment that supports replication of the bacteria can be established by interference with membrane trafficking and phagosome maturation [273]. It has also been speculated that this mycobacterial residence compartment emerges from the phagolysosome [251]. Interestingly, *M. avium* keeps access to nutrients via fusion of transferrin-Fe(III) providing endosomes with the mycobacterial compartment [274].

As another way towards lysosomal degradation, autophagy plays a role in the elimination of intracellular mycobacteria and is induced under infection [275,276]. Permeabilisation of the mycobacterial compartment or evasion of mycobacteria from phagosomes triggers ubiquitination and autophagic removal of the pathogen [277,278]. In line with a microbicidal role of autophagy, in ATG5 knockout mice the growth of *Mtb* was increased [279] and ULK polymorphisms are associated with latent *Mtb* infection [280]. However, these protective effects of basal autophagy could not be reproduced by deficiency of other ATGs [281]. Also, it seems like pathogen-induced autophagy is not sufficient to limit infection, as *M.avium* was reported to use the autophagy-apoptosis pathway of host cells for spreading [282] and some mycobacterial species dampen autophagy by mTOR activation [283] or metabolic changes in the host [284]. On the other hand, exogenous stimulation of autophagy by starvation or IFNγ has been reported to overcome the maturation arrest of phagosomes and induce degradation of mycobacteria [285]. Also *Mtb*-induced cytokines were differentially modulated by autophagy inhibition or induction [286]. Importantly, the IFNγ-induced GTPase IRGM (immunity related GTPase M) plays a crucial role in the defense against mycobacterial infection as IRGM knockout mice are susceptible to *Mtb* and *M.avium* infection [287] and human polymorphisms of IRGM are associated with higher susceptibility for TB [288]. The function of murine and human IRGM differs and human IRGM acts via mitochondrial depolarization to induce autophagy [289]. More recently, it was shown that IRGM directly interacts with BECN1 and ULK1 to induce autophagy [290]. Similarly, the ubiquitin binding adaptor UBQLN1 (ubiquilin 1) was proposed to promote IFNγ-induced xenophagy of *Mtb* by the recruitment of the autophagy machinery [291]. SLRs are important to link ubiquitinated pathogens to the autophagosome and SQSTM1 has been shown to be necessary for autophagic control of mycobacteria and cargo that SQSTM1 brings to the phagolysosome can be cleaved into antimicrobial peptides [292].

Although, there are many defense mechanisms activated during mycobacterial infection, these pathogens remain a challenge for the host. As vaccination possibilities are poor and treatment with antibiotics difficult, new strategies towards host-directed therapies (HDT) are emerging and aim to boost or unleash host intrinsic defenses. This can only be successful with a good understanding of the host response and the strategies of evasion and manipulation by the pathogen [293]. ROS are important in bacterial defense, but might be limited by NFE2L2 activation. NFE2L2 deficient mice showed decreased mycobacterial burden [294] and inhibition of the NFE2L2 target gene HMOX1 in a human macrophage cell line led to limited mycobacterial growth [295]. Although the role of KEAP1 in NFE2L2 activation is well established, the function of KEAP1 during infection has not been clearly elucidated.

1.8 Autophagy and inflammation

Importantly, there are several links between autophagy and inflammation [296]. Basal and IFN-induced autophagy, ER-stress and the connection between both processes have been described as cell-autonomous mechanisms important in innate immune defense, as they complement PRR-elicited responses and modulate responses towards pathogens and production of cytokines [4,141]. Basal autophagy continuously removes damaged intracellular components and reduces cellular stress and ROS production that can be activators of inflammatory signaling [224,227]. Upon inflammation, autophagy is induced both to fight intracellular pathogens [275] and to remove damaged cellular components that might be formed as a result of cell activation. Resolution mediators have been shown to activate NFE2L2 and induce autophagy, leading to restoration of tissue homeostasis [297].

More precisely, TLR-stimulation can induce autophagy [298]. One proposed mechanism is the interaction of TRIF and MYD88 with BECN1, allowing it to function in autophagy induction [299]. Another proposed pathway for TLR-induced autophagy is the TRIF-RIPK1-p38 axis [300]. Fujita et al. have shown that TLR4 activation induces p38-, ROS- and NFE2L2-dependent SQSTM1 accumulation and ALIS-formation, that might be implicated in autophagy induction and removal of pathogens [301]. The role that ALIS formation might play in this context is shown by *Salmonella*, encoding a DUB which prevents formation of ubiquitin-bodies, contributing to immune evasion and favoring the pathogen [302].

The IKK complex has been proposed to be involved in autophagy induction by several stresses including starvation, linking also non-immune autophagy to immune signaling [303].

Finally, IFN signaling can induce autophagy, contributing to protection against mycobacterial infection [285,291,304].

Some ATGs have direct regulatory functions on signaling pathways in inflammation [305]. In this context, the ATG5-ATG12 complex interferes with RIG-I signaling [306]. IRGM has not only been shown to play a major role in coordinating the autophagic machinery in mycobacterial infection [290], but also in limiting TLR induced inflammation and preventing sepsis [307]. The relevance of IRGM in immunity and induction of autophagy is further underlined by the fact that several viruses subvert autophagy by interference with IRGM [308].

SLRs represent an important autophagy tool in immune control as selective targeting can remove ubiquitinated pathogens and signaling components. Correspondingly, NDP52 and OPTN have been implicated in degradation of *Salmonella*, a process that was further dependent on the kinase activity of TBK1 [90,92]. As one pathway, LGALS8 (Galectin 8) recognizes host glycans, exposed by membrane rupture of *Salmonella* containing vesicles, and mediates ubiquitin-dependent recruitment of NDP52 [309]. There is a reasonable amount of reports showing the involvement of OPTN in modulation of IFN production. Interestingly, OPTN seems to play a role in positive as well as negative regulation of this response, dampening the antiviral response [310,311] but being necessary for optimal activation of TBK1-IRF signaling [312]. OPTN has further been shown to negatively regulate NFκB activation by competing with NEMO binding of RIPK1 and binding of linear ubiquitin chains on NFκB [313], suggesting a dual role in the degradation of pathogens but also the ability to induce an immune response.

SQSTM1 has been implicated in the negative regulation of the immune response by sequestration of bacteria [91]. Ubiquitination of the NLRP3 inflammasome and SQSTM1-dependent delivery to autophagosomes was suggested to hamper cytokine maturation [89]. Also SQSTM1-dependent mitophagy in the context of TLR signaling was lately suggested to be important to limit inflammasome activation [98]. Recently, the concept of precision autophagy has been proposed [314]. TRIM (tripartite motif containing) ubiquitin ligases directly bind signaling components and deliver them to selective degradation by cooperating with the autophagy machinery [315]. IFNγ-induced TRIMs were shown to mediate IFNγ-stimulated autophagy. In this context, TRIM20 bound inflammasome components while TRIM21 interacted with IRF3, targeting them to autophagosomal degradation, inhibiting inflammasome and IFN signaling, respectively [316].

Although, SQSTM1 has been directly implicated in negative regulation, it has been shown that SQSTM1 also plays a role in the induction of the immune response. SQSTM1 contains a TRAF6 binding domain and by its homopolymerization and ubiquitin-binding abilities it might function as a scaffold for the ubiquitin-dependent complexation downstream of TLRs leading to NFκB complex activation [317,318]. Also stabilization of the PRR NOD2 by formation of a bigger complex with SQSTM1 has been shown, leading to increased downstream signaling [319]. Another scaffolding function was shown in RIPK1-mediated necroptosis that was dependent on SQSTM1. In absence of SQSTM1 the mode of cell death did not proceed to necroptosis but was shifted to apoptosis [320]. Overall, similar to OPTN, SQSTM1 shows a dual role in the induction but also limitation of inflammation, where the outcome might depend on kinetics and specific circumstances.

Autophagy can be modulated by the ubiquitination machinery involved in inflammation. The E3-ligase TRAF6 can K63-ubiquitinate BECN1, liberating it from the apoptosis regulator BCL2 to release its function in autophagy initiation. The ubiquitination of BECN1 is counteracted by the DUB activity of TNFAIP3 [321]. At the same time, TNFAIP3 can remove ubiquitin chains from NDP52 which otherwise is ubiquitinated by TRAF6, mediating the degradation and limited signaling of TRAF6 in absence of TNFAIP3 [322]. Finally, TNFAIP3 can be degraded by autophagy in some macrophage lines, boosting inflammatory signaling in fungal infections [323]. Also other DUBs modulate inflammatory signaling by means of autophagy. USP14 is recruited by IFN-inducible TRIM14 and cleaves K48-ubiquitin chains from cGAS to prevent selective autophagic degradation by SQSTM1 and allow IFN production. TRIM14 deficiency leads to defective IFN production [324]. Thus ubiquitination is a versatile tool in the interplay of autophagy and inflammation that can be utilized and modified by both players, fine-tuning each other.

1.9 The NFE2L2-KEAP1 system and inflammation

A number of studies have shown the involvement of NFE2L2 in regulation of inflammation. It is demonstrated that the induction of its numerous target genes collaboratively and especially via HMOX1 mediate diverse anti-inflammatory effects [325,326]. NFE2L2 knockout-mice show increased mortality and elevated levels of inflammatory markers during septic shock [327,328]. In humans, NFE2L2 polymorphisms were associated with the development of gastric mucosal inflammation [329] and increased risk for acute inflammatory lung injury [330]. Also, NFE2L2 activation and expression of HMOX1 have been implicated in the development of a macrophage phenotype with lower inflammatory signature. However

this phenotype is thought to contribute to progression of chronic inflammatory atherosclerosis [331–333]. Thus, although a general anti-inflammatory effect of NFE2L2 and HMOX1 is acknowledged, prolonged activation of this system seems to skew the inflammatory response towards exaggerated repair mechanisms and chronic low levels of inflammatory mediators that might confer adverse effects [334].

Intensive crosstalk exists between NFE2L2 and the activity of the NFκB complex, although it is not fully understood. Gene transcription by NFκB produces mediators with strong electrophilic properties that can inactivate KEAP1 and mediate NFE2L2 activation [335]. Secondly, the p65 (RELA) subunit of NFκB has been suggested to deprive the NFE2L2 transcriptional complex from its co-activator CREBBP and to facilitate the interaction with the transcriptional repressor HDAC3, leading to limited transcriptional activity of NFE2L2 [336]. Importantly, in contrast to mice, human IKKβ has an ETGE motive and has been shown to compete with NFE2L2 for the KEAP1 interaction. This might lead to KEAP1 complex-mediated ubiquitination and proteasomal degradation of IKKβ [337] or steric inhibition of IKKβ phosphorylation and autophagic degradation [338], in any case dampening NFκB signaling.

Besides the transcription of SQSTM1 whose accumulation can lead to autophagy induction [301], NFE2L2 has recently been demonstrated to transcriptionally regulate further genes involved in autophagy [339], possibly also accounting for anti-inflammatory effects of NFE2L2. However, a role of SQSTM1 in activating anti-inflammatory effects of NFE2L2 in a physiologic context is poorly described.

1.10 Nutrition

It is widely acknowledged that nutrition is one of our environmental factors that has a high impact on homeostasis, pathology development and health. For humans, nine amino acids, two fatty acids and several vitamins and minerals are considered essential nutrients, meaning that they cannot be synthesized endogenously, but have to be taken up by the diet to assure proper function of the organism [340]. Thus, dietary intake plays an important role for a functioning organism. Western diet, characterized by increased levels of saturated fat, is associated with a higher risk for metabolic but also inflammatory and age-related diseases, indicating that unfavorable diet contributes to morbidity [341].

Epidemiological studies have shown beneficial effects of n-3 PUFAs on several aspects of health as for example in cancer prevention, metabolic syndrome, arteriosclerosis or arthritis and these effects might be related to autophagy induction [16,17,342]. N-3 PUFAs

are mainly found in fish and algae [343]. The most important n-3 PUFAs in human diet are eicosapentaenoic acid (EPA 20:5, n-3) and docosahexaenoic acid (DHA 22:6, n-3). Another n-3 PUFA found in canola and flaxseed is α-linoleic acid (ALA, 18:3, n-3), which is also the precursor for EPA and DHA. Further, n-6 PUFAs are essential nutritional components represented by linoleic acid (LA, 18:2, n-6) that is found in plant oil, eggs and cereals. LA can be metabolized to arachidonic acid (AA, 20:4, n-6), an abundant component of cellular membranes. The most common monounsaturated fatty acid in human diet is the non-essential oleic acid (OA, 18:1, n-9). Importantly, PUFAs are highly reactive and easily oxidized molecules [344]. Activation of NFE2L2 has been reported for oxidized PUFAs [345], suggesting that the anti-oxidative response system might be involved in some n-3 PUFA mediated effects. Autophagy was reported to be increased by n-3 PUFA treatment in some cancer cell lines [16,17]. However, n-3 PUFA mediated induction and function of autophagy in non-transformed cells is not fully understood. Also the interplay of n-3 PUFA induced autophagy and NFE2L2 activation has not been clearly investigated.

More recently, many plant derived compounds like curcumin, resveratrol and others have been under investigation and shown potent anti-oxidant but also NFE2L2 activating effects that might be beneficial in inflammation related diseases [326,346,347].

1.10.1 Anti-inflammatory mechanisms of n-3 PUFAs

It is largely acknowledged that n-3 PUFAs have anti-inflammatory effects and different mechanisms of their function are proposed. PUFAs are precursors of eicosanoids, important mediators of pain, inflammation and resolution. While n-6 PUFAs give rise to inflammatory eicosanoids as the prostaglandins PGE_2 and PGI_2 and the leukotriene LTB_4, n-3 PUFAs can be metabolized to the anti-inflammatory SPMs resolvins (Rvs), protectins and maresins [348,349]. By the action of lipoxygenase or cyclo-oxygenase-2, DHA can be metabolized to D-series Rvs like RvD1-RvD6 and EPA can be metabolized to E-series Rvs like RvE1-RvE3, respectively [350,351]. Treatment with SPMs has shown beneficial effects in many disease contexts, among others in patient study of arthritis [352]. Thus, an increased ratio of n-3 to n-6 PUFAs might be able to influence the contribution of inflammatory eicosanoids and SPMs, defining acute inflammation and resolution. Similarly, PUFA administration affects membrane fluidity in immune cells, leading to downregulated synthesis and secretion of pro-inflammatory cytokines [21]. Lipid rafts in which inflammatory receptors are concentrated, have a high content of AA, serving as a source for pro-inflammatory eicosanoid synthesis. High contents of n-3 PUFAs can interfere with lipid rafts and skew mediator synthesis

towards Rvs and protectins. More mechanistically, it has been shown that n-3 PUFAs signal at least partially via free fatty acid receptor (FFAR) 4 (GPR120) and FFAR1 (GPR40), leading to inhibition of NFκB and the NLRP3 and NLRP1b inflammasome [353,354]. Also NFE2L2 activation by n-3 PUFAs was reported to be involved in decreased cytokine signaling [355]. On the other hand, saturated fatty acids were shown to induce pro-inflammatory signaling via reduced AMPK activity and decreased autophagy, which could be reversed by AMPK activators [356]. In the same context, another publication suggested that n-3 PUFAs activate AMPK, which would oppose the effect of saturated fatty acids [357].

According to these findings, n-3 PUFAs dampen cytokine signaling in immune cells by many actions. However, the effects on cytokine levels vary depending on studies and systems and are sometimes contradictory. Presently, there is no robust biomarker for anti-inflammatory effects of n-3 PUFAs that could be used to follow patients during n-3 PUFA supplementation.

1.11 Age-related macular degeneration (AMD)

AMD is a neurodegenerative eye-disease and main cause of blindness in the elderly in developed countries [11]. Macular degeneration means that the macular, the spot in the retina where the highest density of photoreceptors assures sharp central vision, becomes disturbed and dysfunctional. The exact mechanism is not understood, but genetic and environmental factors, oxidative stress and inflammation promote development of AMD [358,359]. In early AMD, extracellular protein aggregates of acute phase inflammatory and oxidative-stress proteins, called drusen, can be observed in the RPE layer. Together with the deposit of intracellular auto-fluorescent lipofuscin, which is composed of lipids and proteins accumulating in dysfunctional lysosomes, this marks the start of the disease [360–362]. As drusen and lipofuscin accumulation indicate disturbed degradation pathways, loss of homeostasis in RPE cells seems to play a role in disease initiation. Advanced AMD involves vision impairment and can be classified as geographic atrophy (“dry” AMD) or choroidal neovascularization (“wet” AMD). Hallmark of “dry” AMD is the loss of RPE cells. These cells are located at the base and responsible for maintenance of the photoreceptors by nutrient transport and waste removal. The retina is highly exposed to oxidative stress, due to photosensitizers, light exposure and high energy needs [12] and the RPE cells form a protective layer against oxidative stress. In addition, microglia, resident macrophages in the retina, play a role in maintenance of the photoreceptors [363]. Death of RPE cells leads to loss of the photoreceptor and impairment of central vision [12]. Lipofuscin deposits and proteins

found in drusen are similar to deposits in other age-related diseases such as Alzheimer's disease [364,365]. The "wet" form of AMD is accompanied by abnormal angiogenesis underneath the retina with formation of fragile and leaky vessels that promote inflammatory processes. This is the only subtype of AMD for which treatment targeting the neovascularization exists [366]. Recent research showed that stress-related pathways play an important role in the development of the disease. Importantly, deletion of the ATG16L-associated autophagy protein RB1CC1 (RB1 inducible coiled-coil 1; FIP200) promoted AMD-like retinal degeneration in mice [367]. On the other hand, induction of autophagy by lipofuscin components was shown to associate with lower viability in RPE cells possibly by induction of programmed cell death [368]. Sachdeva et al. also found that the NFE2L2 response is impaired in aging RPE and inflammasome activation was found upregulated [369], indicating a complex involvement of autophagy, oxidative stress and inflammation in the development of AMD. It is still unclear what kind of cell death RPE cells undergo in AMD and this might vary between patients and with the contribution of factors like autophagy and inflammation [370]. Therefore, protection of RPE cells and prevention of cell death is crucial. Importantly, intake of n-3 PUFAs has been suggested to inversely correlate with disease progression of AMD [13–15,371], but the mechanism for preventive effects of n-3 PUFAs is not known.

2. Aims of Study

ROS signaling, autophagy and inflammation are indispensable for cell survival and functionality by assuring homeostasis in a physiological setting and by providing protective mechanisms and host-defense during cellular menace. However, if the complex regulation of these processes is compromised this leads to pathologic changes. While deficiency of autophagy might lead to diseases associated with protein aggregation or cancer, lack of inflammation can lead to systemic infection and insufficient activation of the immune response. Hyperactivity of both processes leads to cell death and tissue destruction. Similarly, ROS are used for signaling purposes but can be harmful if excessive. Therefore, oxidative stress, autophagy and inflammation have to be tightly controlled. In order to gain more detailed understanding of their reciprocal regulation our specific aims were as follows:

1. Epidemiologic studies indicate that n-3 PUFAs prevent AMD, which is characterized by formation of protein aggregates. We aimed to investigate if n-3 PUFAs augment autophagy and thereby protect RPE cells from damage induced by oxidative stress or protein aggregation.

2. As both augmented autophagy and n-3 PUFA supplementation have been reported to limit inflammation, we wanted to investigate if n-3 PUFAs can induce autophagy in macrophages and if this leads to dampened inflammatory signaling.

3. One central protein in the interface of oxidative stress, autophagy and inflammation is KEAP1. However, the role of KEAP1 in infection is poorly understood. Due to the dual role of KEAP1 as oxidative stress sensor and ubiquitin ligase adaptor we aimed to explore the regulatory role of KEAP1 in mycobacterial infection.

3. Summary of papers

Paper I: **The marine n-3 PUFA DHA evokes cytoprotection against oxidative stress and protein misfolding by inducing autophagy and NFE2L2 in human retinal pigment epithelial cells**

Age-related neurodegenerative diseases are often characterized by the accumulation of misfolded proteins and protein aggregates in the epithelial cells of the retina, possibly as a result of declined or defective clearance mechanisms. The neurodegenerative eye disease AMD is a common cause of blindness in elderly and associated with aggregate deposits in areas where retinal cells and consequently photoreceptors are lost. Epidemiological studies indicate an inverse correlation between the intake of n-3 PUFAs and the incidence of AMD, however, the mechanism for the protective effect of n-3 PUFAs is not completely understod. The spontaneously arising diploid RPE cell line Arpe-19 was used to investigate cytoprotection by the n-3 PUFA DHA. Treatment with physiological doses of DHA (70μM) but not n-6 or n-9 PUFAs resulted in a transient increase in cytosolic protein aggregates associated with the autophagy receptor SQSTM1, ubiquitin and MAP1LC3B. Autophagic flux was increased without affecting cell proliferation. Further, DHA-induced transient ROS production lead to activation of NFE2L2 and expression of the target gene HMOX1 and was prevented by addition of the anti-oxidant N-acetyl cysteine (NAC). Both, siRNA mediated downregulation of SQSTM1, the essential autophagy gene ATG5 and NFE2L2 resulted in limited cell proliferation during treatment with DHA, suggesting a role for autophagy and the anti-oxidative response to protect cells from DHA-induced stress. Finally, cell cycle arrest induced by misfolded proteins or exogenous oxidative stress was prevented in cells pretreated with DHA. These results suggest that DHA induces transient oxidative stress and protein aggregation, which stimulate the endogenous anti-oxidative response and autophagy and confer further cytoprotection.

Paper II: **N-3 PUFAs induce inflammatory tolerance by formation of KEAP1 containing p62-bodies and activation of NFE2L2**

Inflammation is essential in the defense against infection but has to be tightly controlled to prevent tissue damage and restore homeostasis. Our diet can influence the inflammatory state of the body and n-3 PUFAs are acknowledged to have anti-inflammatory effects. The balance of pro- and anti-inflammatory processes is coordinated by macrophages and autophagy has

emerged as a cellular process that dampens inflammation. In paper I, we reported that physiological relevant doses of the n-3 PUFA DHA transiently elevated levels and punctuated structures of SQSTM1 and increased autophagic turnover of polyubiquitinated proteins in Arpe-19 cells, possibly reducing the risk of AMD. We thus hypothesized that n-3 PUFAs affect autophagy to mobilize an anti-inflammatory effect in macrophages. In paper II, we show that DHA transiently induced SQSTM1-positive cytosolic speckles (described as p62-bodies or ALIS) in human primary macrophages (MDM) and the mouse macrophage cell line RAW264.7 in a lipid selective manner. P62-bodies were degraded by selective autophagy, since turn-over of SQSTM1, but not MAP1LC3B, was increased and removal of p62-bodies was prevented by lysosomal inhibition. Treatment with DHA led to transient ROS generation and activation of NFE2L2. In addition, DHA induced posttranslational modifications of SQSTM1 that are in line with polymerization of this autophagy receptor. During presence of p62-bodies, DHA strongly dampened the induction of pro-inflammatory genes including CXCL10. Induction of CXCL10 as well as further down-regulation by DHA, were reduced in cells with downregulated ROS or SQSTM1 levels. KEAP1 could be identified in DHA-induced p62-bodies and depletion of KEAP1 interfered with the ability of DHA to suppress CXCL10 expression. These results suggest that the DHA-induced formation of p62-bodies with association of KEAP1 represents a fast mechanism of NFE2L2 activation, mediating inflammatory tolerance. Finally, reduced CXCL10 levels were related to the improved clinical outcome in n-3 PUFA supplemented heart-transplant patients and indicating CXCL10 as a robust marker for the clinical benefits mobilized by n-3 PUFA supplementation.

Paper III: **Keap1 regulates inflammatory signaling in *Mycobacterium avium*-infected human macrophages**

Infections with *M. avium* are a threat for elderly and immunocompromised people, where mycobacteria are able to establish chronic infection further contributing to morbidity. A better understanding of the host immune response against the pathogen might shed light on pathways that could be targeted to kill the pathogen. The oxidative stress response and inflammation are tightly connected and KEAP1 plays a central role in sensing of oxidative stress. In paper III, we report a role of KEAP1 in regulating inflammation induced by infection with *M. avium* in MDMs. KEAP1 was recruited to mycobacterial phagosomes in a ROS- and time-dependent manner. Knockdown of KEAP1, as well as other members of the CUL3 ubiquitin ligase complex, increased *M. avium*-induced cytokines and type I IFNs. Accordingly, nuclear translocation of NFκB, IRF1 and IRF5 was augmented by

downregulation of KEAP1. We show evidence of a mechanism whereby KEAP1, as part of an ubiquitin ligase complex, facilitates ubiquitination and degradation of IKKβ thus terminating IKK and TBK1 activity upstream of the activation of NFκB, IRF1 and IRF5. Finally, increased inflammatory responses in KEAP1-silenced cells contributed to decreased intracellular growth of *M. avium.* The growth of *M. avium* was rescued in presence of IKKβ or TBK1 inhibitors. Taken together, the results of paper III indicate that KEAP1 negatively regulates inflammatory signaling in *M. avium*-infected MDMs, illustrating the intricate balance between prevention of overwhelming inflammation and facilitated growth of pathogens like *M. avium* inside macrophages.

4. Discussion

Damage to proteins and DNA occurs constantly and only maintenance by stress responses or degradation systems can assure cellular functionality. The organism is capable to adapt its homeostatic range in response mechanisms to varying conditions during nutritional changes and environmental stresses. However, the drawback of this plasticity is vulnerability for pathologic changes when the homeostatic set points are constantly deviated as might occur in Western lifestyle. Inflammation, as an acute mechanism to restore homeostasis, might be constantly activated in these settings [372]. The contribution of inflammation to age-related diseases, especially in the context of Western lifestyle, has been coined as inflammaging, illustrating this vicious cycle [341].

In the three papers presented here, we elucidated several aspects of the interplay between oxidative stress, autophagy and inflammation that might not only influence the conditions mentioned above, but also provide targets and new concepts in the understanding of todays "lifestyle" and age-related diseases.

4.1 Aging and disease

With increasing life expectancy, age-related diseases are becoming more prevalent and pose a challenge to health systems and society. It is acknowledged that during aging oxidative damage accumulates, impairing the maintenance systems UPR, anti-oxidative response and autophagy and thus establishing a vicious cycle of cellular deterioration [9,373,374]. This condition lays the base for various age- and inflammation-related diseases. As this is a complex condition, the hallmarks of aging have been defined, including several points related to the work discussed here, like loss of proteostasis, mitochondrial dysfunction and altered intercellular communication [375]. Over recent years it became clear, that "lifestyle" conditions further contribute to diseases like atherosclerosis, arthritis, cancer, Alzheimers' disease, AMD, diabetes type II and many others.

Autophagy has an acknowledged role in the prevention of age-related diseases, often associated with protein aggregation [2]. Genetically modified mice lacking central autophagy genes display neurodegenerative diseases associated with aggregate accumulation [18,376]. Calorie restriction inducing autophagy is the only known intervention so far that prolonged life span and retarded age-associated changes in rodents and monkeys [377–381]. Also in humans, a very recent report suggested, that calorie restriction activates autophagy that can be

measured in peripheral blood neutrophils [382], while limited inflammation during low calorie intake has been shown in the study of Meydani et al. [383], suggesting that similar mechanisms exist in humans. For a long time, a group of deacetylases, the sirtuins, have been known for their life prolonging function in yeast, flies and *C.elegans* [384,385] and recently similar effects of SIRT1 (sirtuin 1) and SIRT6 have been shown in mice [386]. SIRT1 overexpression or activation was further protecting mice from aggregate prone diseases like Alzheimer's and Huntington's disease [387,388]. At the same time, sirtuins have been reported to be induced by nutrient deprivation and AMPK activity [389,390]. Further, SIRT1 was shown to induce autophagy [391]. Starvation-induced activation of SIRT1 lead to deacetylation of nuclear MAP1LC3B and was necessary for export of MAP1LC3B and initiation of autophagy in the cytosol [392]. Plant compounds as resveratrol have been shown to activate SIRT1, induce autophagy or increase lifespan [393,394]. The same approaches are among the suggested interventions in human geroscience, aiming to target underlying processes of aging instead of individual pathologies [3]. Intriguingly, also n-3 PUFAs have been associated with protective effects for several age-related diseases [395,396] and even telomere shortening [397]. N-3 PUFAs have also been shown to induce autophagy in some cells [16,17] and to activate the SIRT1/AMPK pathway in macrophages [357]. Thus, instead of calorie restriction, beneficial effects in prevention medicine might also be obtained by appropriate use of essential nutrients like n-3 PUFAs as supported by **paper I and II**. Of note, lowered levels of n-3 PUFA-derived resolving mediators have been observed during aging and chronic disease [348,398]. SPMs have been shown beneficial in inflammation-related settings [399,400] and did induce autophagy in macrophages [297]. It was proposed that these mediators should obtain a more central place in clinical practice, using the organisms own resolution strategy [401]. As it was also suggested that SPMs could have a more specific function than fish oil [402], it would be interesting to investigate the contribution of SPMs in the effects shown for n-3 PUFAs in **paper I** and **paper II**.

Noteworthy, infection with *M.avium* poses problems in immunocompromised and elderly people, in line with declining cellular defense, uncoordinated cellular communication and diminished responsiveness to cytokines during aging [195,375]. Generally, aging is an important factor in the vulnerability for disease. Especially, since autophagy and UPR contribute to the defense of the cytoplasm, lowered activity of these systems during aging might increase infections. Thus, also infection could be considered as age-related disease and its treatment in elderly might benefit from improvement or reestablishment of cellular response systems. If the role of KEAP1 as described in **paper III** could be targeted in this

context has to be critically assessed since generally increased inflammatory signaling might contribute to clearance of *M.avium* but could aggravate other inflammation-related diseases.

Changes during aging have their base at the cellular level and one of the hallmarks of aging is cellular senescence [375]. Senescent cells start a program of increased secretion of various cytokines, termed senescence associated secretory phenotype (SASP) that is normally meant to attract macrophages and lead to phagocytosis of cells undergoing SASP. However, cellular senescence and SASP have been suggested to be an origin of chronic inflammation and age-related diseases [403,404]. Intriguingly, elimination of senescent cells in mouse models was able to retardate age-associated changes and disease [405,406]. For neoplastic cells, exaggerated secretion of cytokines has been found to contribute to tumor development and metastasis. Also diabetes type II development has been linked to inflammation promotion by the SASP phenotype [407]. Interestingly, SASP seems to rely on the JAK-STAT axis, which becomes deregulated with progressing age [408]. Blocking of IFN-I dependent signaling in the aging brain was able to partly restore cognitive function [409]. In this aspect, modulating IFN signaling by n-3 PUFAs as shown **in paper II** could over time have a preventive effect on the occurrence of SASP and thus oppose the development of age-related diseases. Recently, it has been shown that senescence is regulated by selective autophagy. In aging cells, a decrease in the SQSTM1-mediated degradation of the transcription factor GATA4 (GATA binding protein 4) allowed NFκB to promote inflammation and caused the SASP phenotype [410]. However, earlier, it was shown that basal autophagy provides recycled amino acids to allow synthesis of the secreted SASP factors [411]. Thus basal and selective autophagy might have differing outcomes [412], what should be considered when pharmacologically targeting autophagy. Overall there are complex links between autophagy, oxidative stress, inflammation and aging and further knowledge on the regulation of these processes would be valuable in prevention medicine.

4.2 Stimulation of endogenous responses in contrast to exogenous supply

In many areas, treatment strategies are evolving apart from antagonizing, interfering or suppressing drugs towards support and stimulation of endogenous coping mechanisms. This is maybe most evident for the immune system, ranging from the use of physiologic resolution mediators in inflammatory diseases [401] to engineered immune cells in cancer treatment [413] or host-directed therapies in mycobacterial infections [293]. We propose a beneficial effect of the stimulation of the endogenous anti-oxidative response by DHA to protect RPE cells from further damage as well as to limit pro-inflammatory signaling in macrophages. This

mechanism might be relevant for other related stimuli acting on endogenous stress responses. The ARED (Age-Related Eye Disease) study I and II have found beneficial effects of exogenous anti-oxidants in the development of AMD, but no further effects by addition of n-3 PUFAs [414]. Our results in **paper I** indicate that n-3 PUFA effects depend on the induction of intrinsic anti-oxidants and can be abolished by addition of exogenous anti-oxidants. In the context of AMD prevention, it would be interesting to compare separate or sequential instead of combined n-3 PUFA and anti-oxidant supplementation. It might also be speculated that supplementation with exogenous anti-oxidants could transiently show similar effects to DHA-induced responses, but over time worsen the ability to efficiently trigger intrinsic systems. This is in line with the theory of Forman et al. that anti-oxidants beneficial for human health truly act as electrophiles, increasing the "nucleophilic tone" and activating the NFE2L2 system [10]. Already in 1942, the concept of hormesis was introduced by Southam and Ehrlich, suggesting that exposure to stresses establishes resistance to further stimuli [415–417]. This corresponds to the described mechanism in **paper I**, where we show that transient and mild oxidative and proteostatic stress induced by DHA protects Arpe-19 cells from further stress induced by peroxide or puromycin-induced protein aggregates. Noteworthy, the heat-shock response with activation of HSF1 has newly been shown to play a role in stimulating aggregate removal by SQSTM1 in response to various stresses, illustrating the complex interaction of cellular stress response systems [418]. Similarly, repeated exposure to LPS has been shown to induce endotoxin tolerance, limiting further inflammatory signaling in septic patients [419] and monocytes [420]. The induction of antagonists by PRR activation is suggested to contribute to this effect, thus preparing the tissue for the insult by mounting the endogenous antagonistic mechanism. **In paper II** we show that also stress induced by DHA can limit subsequent LPS responses, suggesting overlapping adaptation mechanism for different stress responses.

Overall, **paper I and II** show protective effects of transient stress induced by DHA, improving the cellular ability to cope with further challenges.

4.3 DHA induces polymerization of SQSTM1 and formation of p62-bodies

Most processes encountered here, cannot be solely explained by protein levels but rely on modifications or localization. An important aspect of selective autophagy is the regulation of cargo receptors. It is an exciting concept to think of modulated polymerization of for example SQSTM1 to increase selective autophagic degradation independent of increased basal autophagy. It has already been shown that the polymerizing capability of SQSTM1 is

necessary to target cargo to the autophagosome, while binding of MAP1LC3B was not [421]. We observe a modified form of SQSTM1 that likely is due to covalent dimer formation as described before [345,422]. Donohue et al. claim reduced ubiquitin binding and impaired degradation when covalent SQSTM1 dimers are formed under treatment with Verteporfin. In **paper I and II**, we observed small amounts of total SQSTM1 to form high molecular weight forms in response to physiologic amounts of n-3 PUFAs and our data suggest that these are readily degraded by autophagy. One might speculate that few crosslinked SQSTM1 molecules stabilize tightly packed aggregates and facilitate degradation while overall covalent dimerization interferes with normal function. Our data further suggest that DHA-induced crosslinking of SQSTM, leads to a transient storage of damaged proteins that can be resolved by new-synthesis of unmodified SQSTM1. This might be a mechanism to handle transient overload of degradative systems and to allow efficient removal of aggregated proteins after restoration of homeostasis.

Altered binding capacity of cargo receptors that might further regulate the efficiency of selective autophagy has been shown in several studies. Phosphorylation of OPTN by TBK1 did increase the selective autophagic removal of *Salmonella* [92], while phosphorylation of another serine in OPTN played a role in mitophagy [423]. SQSTM1 can be phosphorylated on S403 in the UBA domain by the pleiotropic protein kinase CSNK2 (Casein kinase 2), leading to higher affinity to polyubiquitin chains and increased autophagic flux [424]. This seems however from the kinase point of view to be the basal state, as CSNK2 is a constitutively active kinase with no stimulated regulation known [425]. Also more specific kinase activity has been shown, with TBK1 phosphorylating S403 in SQSTM1 to regulate degradation of bacteria or mitochondria [426,427] and ULK1 phosphorylating the same and another serine, regulating the clearance of aggregates under energetic or proteotoxic stress [428,429]. Finally, ubiquitination of OPTN by the E3 ubiquitin ligase HACE1 (HECT domain and ankyrin repeat containing E3 ubiquitin protein ligase 1) was proposed to increase autophagic flux by targeting OPTN to autophagic destruction [430]. In **paper II** we describe high molecular weight forms of SQSTM1 in response to DHA that are readily degraded by autophagy. High molecular weight forms of SQSTM1 might represent crosslinked dimers. Crosslinking effects on SQSTM1 in response to oxidation have been shown [345,422], suggesting that this could be a mechanism to increase autophagic turnover during stress. If oligomerization of SQSTM1 in **paper I and II** is induced by ROS or directly by DHA as a reactive molecule has not been clarified and might occur simultaneously. Of note, more than half of the lysines in SQSTM1 have been found ubiquitinated by large scale proteomics (14 of 20 lysines, PhosphoSitePlus®

[431]). Preliminary data from our laboratory suggests that some of these sites may be ubiquitinated in response to DHA and could contribute to formation of p62-bodies. In contrast, ubiquitination of SQSTM1 by TRIM21 has been reported to disrupt SQSTM1 oligomerization and KEAP1 activation [483]. Thus, modification of autophagy receptors, like DHA-mediated crosslinking or ubiquitination of SQSTM1, might be a concept to regulate selective targeting of cargo. Besides DHA, plant derived electrophilic compounds have recently been described for their anti-oxidative and anti-inflammatory effects [326,346,347]. It would be interesting if polymerization of SQSTM1 also plays a role for NFE2L2 activation in this context.

There are extensive reports on SQSTM1 positive inclusion bodies, also referred to as p62-bodies, aggregates, ALIS and similar [111,118]. They can be observed in tissues in the context of impaired degradation mechanism like in conditional Atg5 knockout mice, which develop SQSTM1-inclusions and pathology in the liver. There, additional knockout of SQSTM1 limits liver damage and tumor formation [432]. Aggregates positive for SQSTM1 can also be observed in relevant tissues in neurodegenerative diseases [99,100]. However, these are conditions with blocked autophagy or most likely decreased catabolic pathways. On the other hand, ALIS have been described as features of the stress response towards oxidative compounds, infection and TLR stimulation or more recently heme and free FeIII [433]. These are mostly considered as protective form of transient storage for misfolded proteins. Thus, in case of functional autophagy, aggregation of proteins and association with SQSTM1 might exert a beneficial effect by induction of selective autophagy. In contrast to a pathological role in the liver [432], deficiency of SQSTM1 did not improve neurotoxicity of prostaglandin-induced protein ubiquitination [434] and increased α-synuclein pathology in mice [435]. Soluble ubiquitinated aggregation-prone proteins are considered more harmful as compared to ubiquitinated aggregates [436]. In line with this, it has recently been proposed that sequestration of ubiquitinated proteins by SQSTM1 can have a protective effect in atherosclerosis. In cells stimulated with fatty acids that cause atherosclerosis (oxLDL) and mice fed with a high fat diet, additional deletion of SQSTM1 had worse outcomes [437]. The authors concluded that in a setting of decreased degradation due to oxLDL, the formation of inclusion bodies by means of SQSTM1 protected the cells from diffuse distribution of ubiquitinated and misfolded proteins. Importantly, in **paper I and II** we show that this protective mechanism can be induced by physiologic stimuli like DHA.

Autophagy is considered as the degradation route of choice for aggregates as it can engulf large components as organelles while the proteasome degrades proteins one and one.

Indeed, autophagy is stimulated by proteasome overload to cope with accumulating protein aggregates [61]. It has recently been shown that there is also a functional way from aggregates to the proteasome. Ubiquilins, a family of specialized chaperones can liberate parts of aggregated proteins and shuttle them to the proteasome for degradation. This mechanism was necessary for cellular integrity after heat-shock and it remains to assess the relevance of this pathway for other stress conditions [438]. Another exciting mechanism to handle proteotoxic stress was described as unconventional secretion of misfolded proteins, involving deubiquitination at the ER and export in late endosome structures [439]. Lately, also a non-degradative function of autophagy, termed secretory autophagy, has been discussed by which autophagic cargo might undergo unconventional secretion [440]. This might include extracellular delivery of cytosolic proteins, expulsion of organelle content or pathogens as well as removal of aggregate-prone proteins [441]. These pathways might be involved in the appearance of extracellular aggregates in neurodegenerative diseases. It might further be interesting to determine the relation between transient formation of intracellular aggregates in a physiologic setting and their potential expulsion.

Of note, in **paper II** we could identify autophagy receptor TAX1BP1 in DHA induced p62-bodies. Intriguingly, TAX1BP1 is also the adaptor for TNFAIP3 and necessary for the anti-inflammatory function of TNFAIP3. As not only NFκB but also IRF3-dependent signaling is negatively regulated by TAX1BP1 and TNFAIP3 [442,443], recruitment of this complex could play a role in limited activity of IFN-dependent signaling. The cargo receptor and ubiquitin-editing function of TAX1BP1 and TNFAIP3, respectively, could play a role in formation or autophagic degradation of ALIS. Knockdown studies in our system might not have been efficient enough to investigate a role of TAX1BP1 and TNFAIP3 in DHA-mediated anti-inflammatory effects. A functional contribution of ALIS-associated proteins on the regulation of CXCL10 corresponds to the concept of SLRs being able to induce an inflammatory response in case of altered degradation [296]. Likewise, transient accumulation of SQSTM1 could not only connect to inflammatory signaling through TRAF6 but also recruit the anti-inflammatory machinery, preventing excessive inflammation. An interesting question is also how up-regulation of SQSTM1 and formation of p62-bodies affects mechanisms of precision autophagy, like TRIM21-mediated degradation of IRF3 that involves SQSTM1-binding [316]. Overall, this adds to the consideration of aggregates not as passive structures but potentially scaffolds involved in signaling processes.

Interestingly, DUBs relying on reduced cysteines to cleave ubiquitin isopeptide bonds can be transiently inhibited by ROS-dependent oxidation [444–446]. During ROS-producing

stresses, oxidation of cysteines allows the transient halt of negative regulation by DUBs leading to fast accumulation of ubiquitinated proteins and changes in the signal transduction. This might also represent a mechanism of PRR activation where local ROS production could transiently inhibit DUBs and phosphatases to let the signal pass, but assuring subsequent reestablishment of homeostasis by restoration of DUB and phosphatase activities. A study in yeast has shown that oxidative stress leads to selective accumulation of K63-linked ubiquitin chains by DUB inhibition, while accumulation of K48-ubiquitin chains was induced by several stress stimuli [447]. In the context of n-3 PUFA supplementation, transient ROS production might interfere with DUB activity to allow accumulation of ubiquitinated proteins and recruitment of SQSTM1. It would further be an interesting question whether or how DHA modulates DUB activity as DUBs have been proposed as targets for both cancer and inflammatory diseases [448,449]. Importantly, the ubiquitination changes might strongly depend on localization. The increased ubiquitination of aggregated proteins by DHA treatment might also affect the ubiquitination of inflammatory signaling proteins. Ubiquitination of TANK, a scaffold needed for TBK1 activation [179], and IRF1 [450] has been shown to be important for activation of inflammatory signaling. Limited ubiquitination of TANK or IRF1 in presence of p62-bodies could have an impact on IFN signaling and CXCL10 levels and would be a target for further studies.

Also from the molecular understanding of Paget disease of the bone, a functional sequestration of ubiquitinated proteins by SQSTM1 appears to be connected to the appropriate regulation of inflammatory signaling. Paget disease of the bone is associated with mutations in the UBA domain of SQSTM1 and patients with Paget disease show multiple points of bone destruction caused by hyperactivation of osteoclasts [451]. Even though those mutations are dominant, development of Paget disease depends on additional factors and bone destruction happens only locally. It has been proposed that paramyxoviral infections, that were observed in osteoclasts of patients, could contribute to the development of the disease [452]. Our results of **paper II** suggest that formation of p62-bodies associated with ubiquitinated proteins are involved in the regulation of aberrant IRF signaling. Viral infection in the bone marrow could trigger IFN signaling that has to be regulated. Impairment of the UBA-domain of SQSTM1 would abrogate the formation of multiprotein structures of SQSTM1 and increase IRF signaling and osteoclast activity in Paget disease of the bone.

Importantly, the group of Michael Karin has recently suggested that SQSTM1 is recruited to mitochondria in response to NFκB activation, leading to mitochondrial degradation and decrease of mtROS. This mediated lower inflammasome activation by

subsequent stimulation than in absence of SQSTM1 [98]. Our results of **paper II** indicate that SQSTM1 plays an important role in inflammation and that induction of ROS, SQSTM1 and activation of NFE2L2 might limit responses to following inflammatory stimuli. Of note, mass spectrometry analysis of DHA-induced partners of SQSTM1 in **paper II**, also identified two proteins of the inner mitochondrial membrane, IMMT (inner membrane mitochondrial protein, MIC60) and SAMM50 (sorting and assembly machinery component). Enhanced ROS and SQSTM1 levels resulting from DHA treatment might be able to induce mitophagy to regulate inflammatory signaling. It would further be interesting to try to distinguish the contribution of mitophagy and aggrephagy in both DHA-induced effects and TNF-mediated mitophagy.

On the other hand, intriguingly, the mitochondrial phosphatase PGAM5 (PGAM family member 5, mitochondrial serine/threonine protein phosphatase) has been shown to be another substrate of KEAP1 [453], also directing NFE2L2 to mitochondria [454]. PGAM5 has been suggested to dephosphorylate the mitophagy receptor FUNDC1 and thereby activating mitophagy [455]. Likewise, loss of PGAM5 inhibited mitophagy and promoted a Parkinson's-like disorder in vivo [456]. Thus KEAP1-mediated degradation of PGAM5 might limit, while redox inactivation of KEAP1 promote mitophagy and the same might apply for SQSTM1-mediated recruitment of KEAP1.

4.4 DHA might not be beneficial in mycobacterial infection

In **paper II**, we have examined how n-3 PUFAs exert anti-inflammatory effects. These lipids are recognized as beneficial supplements in arthritis and arteriosclerosis and are associated with better outcome under conditions of persistent inflammation as in the heart-transplant patients. However, use of n-3 PUFAs and limited inflammatory signaling is not appropriate in all situations. In **paper III**, we propose that KEAP1 limits inflammatory signaling in macrophages, facilitating growth of *M. avium*. IFNγ plays a role in restriction of mycobacterial growth, while we show in **paper II** that especially IFN signaling and ISG expression is limited by n-3 PUFAs. In fact, it has been shown that supplementation with n-3 PUFAs impairs macrophage defense against *Mtb* [457–460]. Of note, suppression of IFN responses seems to be a pathogenic strategy of *Mtb* [461] and patients with inherited ISG15 deficiency, resulting in reduced IFNγ production, were more susceptible to mycobacterial disease [216]. Also other pathogens have developed mechanisms to interfere with IFN signaling to evade host immunity [462,463]. Likewise, secretion products of *Spirometra erinaceieuropaei plerocercoids* inhibit RELA and ISRE dependent transcription and CXCL10

expression [464]. Furthermore, CXCL10 seems to be especially important in the defense against *Toxoplasma gondii* as neutralization of CXCL10 impaired T-cell recruitment and survival of infected mice [465]. Thus, it becomes clear that also the use of physiologic anti-inflammatory compounds should be critically assessed and possibly avoided under certain conditions. On the other hand, it might be asked if persistent mycobacterial infection is associated with inflammation-related diseases. Similar to other pathogens that establish chronic infection, mycobacterial infection has recently been linked to prevalence of arteriosclerosis and cardiovascular diseases [466,467], making it especially challenging to modulate inflammation in between insufficient mycobacterial killing and exaggerated macrophage activation in inflammation-related diseases. On the other hand, it might also be worth considering that **paper I and II** have shown upregulation of SQSTM1 by DHA. Formation and increased degradation of ALIS is a mechanism thought to contribute to pathogen defense [301,302] and transient activation of this system might be beneficial.

NFE2L2 plays a central role in the interface of anti-oxidative and anti-inflammatory effects as **paper I and II** underline. There has been an interest in the development of small molecule inhibitors for NFE2L2 in the context of cancer treatment [468] or activators of NFE2L2, which might be of use in inflammation-related diseases. The latter target either KEAP1 by covalent modification or interfere with the KEAP1-NFE2L2 interaction [469,470]. Recently, also a potent protein-protein interaction inhibitor of the association between phosphorylated SQSTM1 and KEAP1 was identified, providing a tool to inhibit this alternative way of NFE2L2 activation [471,472]. This promises advances in cancer treatment, since SQSTM1-mediated activation of NFE2L2 is a common mechanism promoting tumor growth [37,48,432]. Mutations in KEAP1 and the E3 complex leading to increased IKK expression have been linked to cancer [473]. Surprisingly, none of these studies discusses the potential of the mentioned protein-protein interaction inhibitors to disrupt the KEAP1-IKKβ interaction, although KEAP1 recognizes comparable domains in NFE2L2 and IKKβ [36,337]. **Paper III** suggests that targeting the interaction between KEAP1 and IKKβ in *M.avium* infection might be an option to improve the cellular defense against this infection. Simultaneous inhibition of NFE2L2 and IKKβ degradation might, at least from the inflammation point of view, limit each other's effects, what could be beneficial to prevent tissue damage. While boosting inflammatory pathways to increase immune defense it might be a good strategy to also activate anti-inflammatory mechanisms to limit side effects.

It would further be interesting to examine how formation of p62-bodies, recruitment of KEAP1 and activation of NFE2L2 as described in **paper I and II,** influences KEAP1-

mediated degradation of IKKβ shown in **paper III**. It could be speculated that recruitment of KEAP1 to SQSTM1 not only stabilizes NFE2L2 but also IKKβ. Intriguingly, NFE2L2 activation did not seem to have an impact on the increased cytokine expression in absence of KEAP1 in **paper III**. In **paper II**, phosphorylated IKKβ and cytokine signaling were lowered after DHA-mediated formation of p62-bodies and NFE2L2 activation. This might reflect that the distribution of KEAP1 between different clients changes according to the conditions and most effect will be seen on the mechanism with most KEAP1 complex activity. This might be IKKβ degradation during *M.avium* infection in **paper III** and NFE2L2 activation in **paper II**. Also slight differences in the binding of KEAP1 to its substrates might influence the rate of ubiquitination and speed of degradation, similar to different ubiquitination of substrates by ANAPC (anaphase promoting complex; APC) assuring ordered progression of the cell cycle [474].

4.5 The relevance of biomarkers for anti-inflammatory effects of DHA

There is an overwhelming literature about anti-inflammatory effects of n-3 PUFAs with widespread effects on cytokine levels but little consensus. Also, several modes of action have been proposed, not all of them which relate to each other. This leads to a very complex network of effects that might vary in different systems and conditions. **In paper II**, we proposed CXCL10 as a robust marker of anti-inflammatory effects of n-3 PUFAs, as its direct and secondary induction depends on some of the most important transcription factors in inflammatory signaling. Thus, also with slightly differing upstream signaling, the outcome on CXCL10 levels seems highly reproducible. This led us to the conclusion that especially conditions with hyperactive CXCL10 signaling such as transplantation [475], arteriosclerosis [476] and inflammatory bowel disease might benefit from n-3 PUFA supplementation. At the same time, the effect of n-3 PUFA supplementation could be monitored by CXCL10 levels. Interestingly, CXCL10 has also been suggested as biomarker for the diagnosis of early AMD [477], thus CXCL10 could be a valuable read-out for beneficial effects of n-3 PUFAs in AMD and several other contexts.

On the other hand, our initial screen **in paper II** also showed inflammatory genes with increased expression by DHA treatment like CXCL2, CXCL1, IL1α, IL8 and IL6. Although these changes have not been validated in more than the two donors of the screen, these results indicate that various effects on inflammatory signaling occur simultaneously, might differ over time and might not all represent anti-inflammatory effects. CXCL1 and CXCL2 have clear κB elements [478] and lack regulation by IFNs shared by CXCL9, CXCL10 and

CXCL11, possibly explaining differential regulation or at least different kinetics. However, it would be of interest to investigate this upregulation further on protein level, over time or for its functional outcome, since CXCL1 and CXCL2 are considered as pro-inflammatory chemokines implicated in neutrophil recruitment [479]. Modulation of different chemokines might have distinct effects on the recruitment of the corresponding cell types, which could be involved in initiation or resolution of inflammation. Thus, it might not be possible to predict the real systemic outcome of DHA supplementation by measurement of some markers, but overall disease parameters should also be considered. However, overall our data on CXCL10 expression correlate well with the previously reported clinical progression of the analyzed heart-transplant patients [480,481].

It would further be interesting to examine if FFAR4 engagement, which has been shown to limit NFκB signaling [353], is involved in the DHA-mediated generation of ROS and activation of NFE2L2 as observed by us and others [482].

Generally, targeting maintenance mechanisms in age- and inflammation-related diseases, in particular proteostasis and anti-oxidative defense could be beneficial for a broad range of pathologies. However, all of the involved systems need to be accurately balanced and precise knowledge is necessary to obtain advantageous modulation. Progress has been made in the understanding of the maintenance mechanisms, to which the work presented here contributes, but more research is still needed to be able to mimic and restore intrinsic responses.

5. Further perspectives

The clearly common patterns we see in age- or inflammation-related diseases suggest their shared etiology and mechanisms. This obviously pushes to the identification of general underlying principles and development of more profound strategies aiming at the origin of dysfunction. At the same time, the concept of personalized medicine adapts therapies to individuals in many areas. We here show different aspects of how some of the basic stress response systems might be modulated to improve their function in maintenance and defense and we obtained some hints how to follow the effects of n-3 PUFAs for individual patients. Still, there is a gap between research and application. Measurement of autophagy in tissue is still not possible, since protein levels alone do not allow extrapolation of autophagic flux. The group of G. Kroemer was recently able to measure autophagy induction after starvation in peripheral blood cells [382]. However, this was limited to neutrophils and represents a cumbersome method. New tools are needed to allow measurement of autophagy in patient material.

To translate our findings to clinical practice, the effects of mediators derived from DHA and other n-3 PUFAs should be separately examined. The broad range of compounds related to n-3 PUFAs might explain the variation seen in different studies, depending on the model system and type of supplementation. Molecules like SPMs that have been associated with autophagy induction and disease prevention, might provide more specific tools to modulate mechanisms targeted by n-3 PUFAs.

The potential of protein-protein interaction inhibitors could further be explored for the disruption of the KEAP1-IKKβ interaction that might be beneficial in the clearance of bacterial infection. Also investigation of regulating mechanisms for the association of KEAP1 with different substrates might provide further targets in infection treatment. Importantly, the possible influence on sepsis development in absence of KEAP1 should be carefully examined.

The maintenance responses described here are a reasonable target for prevention and treatment of many pathologies. However, better knowledge is still needed for their appropriate modulation to improve human health without aggravating side effects.

6. Conclusions

Cellular homeostasis is maintained by intrinsic response systems, including the oxidative stress response and autophagy. If genetic predisposition or environmental factors lead to dysregulation of these mechanisms, susceptibility for disease increases and is further aggravated by compensatory inflammation.

We here describe the modulation of protein aggregation, autophagy and the oxidative stress response by n-3 PUFAs as a protective mechanism for cellular homeostasis. The results presented here show the importance of n-3 PUFA-induced autophagy and the anti-oxidative response to rescue retinal cells from stress-induced cell-death. In macrophages, our data indicate n-3 PUFA-mediated polymerization of SQSTM1 as a fast mechanism to activate the anti-oxidative response and strongly limit IFN-dependent signaling. Finally, we have characterized a role of the ROS sensor KEAP1 in limiting cytokine signaling in mycobacterial infection, possibly protecting the host from tissue damage but favoring the pathogen.

Overall, we propose that our findings add in general to the understanding of the interplay between oxidative stress response, autophagy and inflammation and in particular to the functions of n-3 PUFAs in disease prevention as well as KEAP1-mediated mechanisms in mycobacterial infection. The findings contribute to future design of new strategies for prevention of age- and inflammation-related diseases and for appropriate modulation of inflammatory signaling during infection.

Conclusions

7. References

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Paper I

BASIC RESEARCH PAPER

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The marine n-3 PUFA DHA evokes cytoprotection against oxidative stress and protein misfolding by inducing autophagy and NFE2L2 in human retinal pigment epithelial cells

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Abbreviations: AA, arachidonic acid; AMD, age-related macular degeneration; AREDS, Age-Related Eye Disease Study; ATF4, activating transcription factor 4; ATG4, autophagy-related 4; BafA1, bafilomycin A_1; CHX, cycloheximide; CREB, cAMP responsive element binding protein; DCF, 5-(and-6)-carboxy-2',7'-dichlorodihydrofluorescein diacetate; DHA, docosahexaenoic acid; ER, endoplasmatic reticulum; HMOX1, heme oxygenase 1; KEAP1, kelch-like ECH-associated protein 1; MAP1LC3B, microtubule-associated protein 1 light chain 3 β; MEF, mouse embryonic fibroblast; NAC, N-acetyl cysteine; NQO1, NAD(P)H dehydrogenase, quinone 1; NFE2L2, nuclear factor, erythroid derived 2, like 2; OA, Oleic acid; POS, photoreceptor outer segment; PPARA, peroxisome proliferator-activated receptor α; PUFA, polyunsaturated fatty acid; qRT-PCR, quantitative real-time polymerase chain reaction; ROS, reactive oxygen species; RPE, retinal pigment epithelial; SLC7A11, solute carrier family 7 (anionic amino acid transporter light chain, xc- system), member 11; SRXN1, sulfiredoxin 1; SQSTM1, sequestosome 1; TFEB, transcription factor EB; UBA, ubiquitin associated domain; WT, wild type.

Accumulation and aggregation of misfolded proteins is a hallmark of several diseases collectively known as proteinopathies. Autophagy has a cytoprotective role in diseases associated with protein aggregates. Age-related macular degeneration (AMD) is the most common neurodegenerative eye disease that evokes blindness in elderly. AMD is characterized by degeneration of retinal pigment epithelial (RPE) cells and leads to loss of photoreceptor cells and central vision. The initial phase associates with accumulation of intracellular lipofuscin and extracellular deposits called drusen. Epidemiological studies have suggested an inverse correlation between dietary intake of marine n-3 polyunsaturated fatty acids (PUFAs) and the risk of developing neurodegenerative diseases, including AMD. However, the disease-preventive mechanism(s) mobilized by n-3 PUFAs is not completely understood. In human retinal pigment epithelial cells we find that physiologically relevant doses of the n-3 PUFA docosahexaenoic acid (DHA) induce a transient increase in cellular reactive oxygen species (ROS) levels that activates the oxidative stress response regulator NFE2L2/NRF2 (nuclear factor, erythroid derived 2, like 2). Simultaneously, there is a transient increase in intracellular protein aggregates containing SQSTM1/p62 (sequestosome 1) and an increase in autophagy. Pretreatment with DHA rescues the cells from cell cycle arrest induced by misfolded proteins or oxidative stress. Cells with a downregulated oxidative stress response, or autophagy, respond with reduced cell growth and survival after DHA supplementation. These results suggest that DHA both induces endogenous antioxidants and mobilizes selective autophagy of misfolded proteins. Both mechanisms could be relevant to reduce the risk of developing aggregate-associate diseases such as AMD.

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Introduction

Damaged proteins may have deleterious effects on cellular functions, and accumulation of misfolded proteins is the hallmark of several neurodegenerative diseases such as Huntington, Parkinson, and Alzheimer diseases and other age-related disorders.[1-6] Age-related macular degeneration (AMD) is a neurodegenerative disease of the eye and the leading cause of central blindness in western countries.[7,8] Currently, for 80% to 85% of the 30 to 50 million AMD patients worldwide there are no effective treatment alternatives.[9] Therefore, one major public health challenge is to devise an effective primary prevention of AMD and to improve current treatments.

The pathogenesis of AMD is initiated by degeneration and death of retinal pigment epithelial (RPE) cells followed by loss of the overlying photoreceptor neurons rod and cones.[10] Increased accumulation of intracellular auto-oxidative and autofluorescent lipofuscin in the lysosomes of RPE cells, as well as drusen formation in the extracellular space between the RPE and the Bruch membrane are hallmarks of AMD.[8,11-13] Lipofuscin is a brown-yellow, electron-dense, age-related pigment composed of a complex heterogeneous mixture of lipid–protein aggregates.[14] In drusens various acute phase inflammatory markers and oxidative stress-related proteins have been characterized.[15-17] Oxidative processes have been proposed to play a contributing role in AMD. RPE cells are exposed to chronic oxidative stress due to constant exposure to sunlight and relatively high oxygen tension, and high concentration of lipid peroxidation products from the ingested photoreceptor outer segments (POS).[10,18,19] Oxidatively damaged proteins post-translationally modified e.g. with carboxyethylpyrrole, malondialdehyde, 4-hydroxynonenal, and advanced glycation end products, accumulate in the macular area and serve to further elevate oxidative stress.[20] It is unclear whether accumulation and aggregation is the cause or the consequence of the disease. However, high amounts of deposits predict AMD progression and severity and it is thought that this aggregation of misfolded proteins occurs after failure of the cellular protein quality control mechanisms of the cells.[21] However, the role of aggregates in pathologies is controversial and may even protect the cells by sequestering putatively harmful soluble misfolded proteins. In Huntington disease it has been suggested that formation of aggregates may serve a cytoprotective role.[22] In addition, aggregates may also facilitate the clearance of the toxic materials.[21]

Two major proteolytic systems are responsible for maintaining the cellular function: the proteosomal and lysosomal system. Both systems remove irreversibly damaged proteins and recycle amino acids for protein synthesis. The activity of these systems decline upon aging.[23] Macroautophagy (hereafter referred to as autophagy) is a catabolic process that removes damaged and foreign intracellular components by lysosomal degradation.[24] During autophagy, targeted cytoplasmic proteins and organelles are sequestered by a growing double membrane that forms an autophagosomal vesicle where the content is degraded after fusion with a lysosome. The rate of cellular turnover by autophagy increases in response to cellular stresses like starvation, endoplasmatic reticulum (ER) stress, and elevated levels of reactive oxygen species (ROS) for maintenance of cellular homeostasis.[25-27] The SQSTM1/p62 protein (hereafter referred to as SQSTM1) binds both to ubiquitinated cargos, such as misfolded proteins and protein aggregates via its ubiquitin-associated (UBA) domain and also to the mammalian orthologs of yeast Atg8, located on the phagophore membrane.[28] In this way, SQSTM1 selectively targets ubiquitinated protein aggregates to lysosomal degradation.[28,29] A specific binding of SQSTM1 to misfolded and ubiquitinated proteins, and its presence in cytoplasmic inclusions in diverse human diseases have suggested a general role of SQSTM1 in diseases associated with protein aggregates.[5]

Tissue-specific knockout of autophagy genes in neurons or hepatocytes results in early onset neurodegeneration and liver failure, respectively, accompanied by accumulation of misfolded protein aggregates and ubiquitinated proteins.[30,31] Inducible knockout of autophagy in mice limits survival to 2 to 3 mo due to development of severe neurodegeneration.[32] These findings suggest that autophagy is an important cytoprotective mechanism that counteracts the development of several age-related diseases, especially proteinopathies, by clearence of damaged proteins and organelles.[33] Lysosomal-mediated clearance is also important in RPE cells[34] supporting the idea that also in RPE cells, autophagy is critical to maintain cellular homeostasis. Whether autophagy could be induced as a disease preventive mechanism in these cells is still uncertain. However, caloric restriction and compounds like resveratrol can extend life span in different model organisms possibly due to increased autophagy.[35,36]

Epidemiological studies indicate an inverse correlation between dietary intake of fish and the risk of developing AMD.[37-42] The disease preventive effects of increased fish intake have been associated with the content of marine omega-3 polyunsaturated fatty acids (n-3 PUFAs).[43] Deficiency of n-3 PUFAs in photoreceptors is associated with the development of AMD.[44] Increased intake of marine n-3 PUFAs has also been suggested to reduce the risk of other age-related disorders such as neurodegenerative diseases, different types of cancer, and heart and circulatory diseases.[45-47] However, the disease preventive mechanism(s) mobilized by dietary n-3 PUFAs are not completely understood. Previously, several reports have suggested a change in autophagy in cancer cell lines that are sensitive and display cytotoxic and/or cytostatic responses to physiological doses of PUFAs.[48-53] We explore whether increased autophagy is a part of the cellular response to PUFAs also in spontaneously arising ARPE-19 cells. If so, we hypothesize a correlation between the disease preventive effects of n-3 PUFAs and stimulation of autophagy in retinal pigment epithelial cells where the initial phases of AMD occurs.

Here we report that the n-3 PUFA docosahexaenoic acid (DHA, 22:6, n-3), in contrast to the n-6 PUFA arachidonic acid (AA, 20:4, n-6) and the n-9 fatty acid oleic acid (OA, 18:1, n-9), induces a transient increase in reactive oxygen species (ROS) in spontaneously arising retinal pigment epithelial cells. This mild, subtoxic stress is counteracted by activation of the antioxidant stress response transcription factor NFE2L2/NRF2 (nuclear factor, erythroid derived 2, like 2) that controls the transcription of

a number of genes encoding endogenous enzymatic and nonenzymatic antioxidants. DHA also causes a selective rise in SQSTM1 mRNA and protein levels in an NFE2L2-dependent manner. Further we observe a transient increase in sequestration of misfolded proteins into aggregates after DHA that coincides with an increase in autophagy that could facilitate clearance of the protein aggregates. In line with a mobilization of a protective response, we find that DHA increases the tolerance for oxidative stress and misfolded proteins in retinal pigment epithelial cells.

Results

The n-3 PUFA DHA induces protein aggregation and autophagy in ARPE-19 cells

n-3 PUFAs have been suggested to mobilize disease preventive effects for several age-related diseases. Since insufficient autophagy has been proposed to contribute in the development of several of the same diseases, we asked if n-3 PUFA supplementation could induce autophagy. Cellular responses to lipids were determined in the presence of serum to mimic the in vivo situation. The n-3 PUFA DHA (22:6, n-3) was used in final concentrations of 70 μM and 140 μM in the cell culture experiments which is well within physiological relevant levels found in serum of healthy individuals.[54] The diploid, spontaneously derived ARPE-19 human retinal pigment epithelial cells[55] were used as a model system since these cells are relevant for the development of AMD. To determine the basal autophagy flux in these cells, cell extracts were analyzed by immunoblot for accumulation of lipidated microtubule-associated protein 1 light chain 3 β (MAP1LC3B-II/LC3B-II) and SQSTM1 in the absence or presence of the autophagy/lysosomal inhibitor bafilomycin A_1 (BafA1) for different time points. In exponentially growing ARPE-19 cells, both SQSTM1 and MAP1LC3B-II protein levels displayed a linear increase with time throughout the experiment. The doubling time for SQSTM1 and MAP1LC3B-II was determined to be approximately 7 h and 5 h, respectively (**Fig. S1**). Since SQSTM1 is associated with protein aggregates that might be resistant to

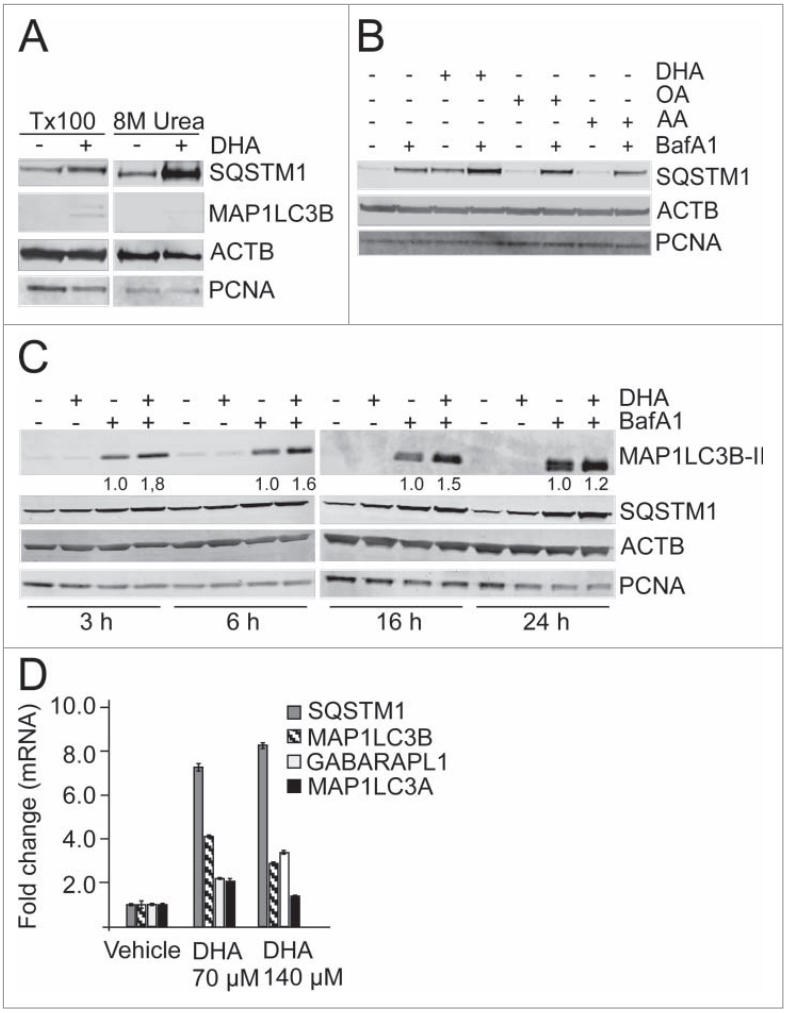

Figure 1. The n-3 PUFA DHA increases protein level of SQSTM1 and induces autophagy in ARPE-19 cells. (**A**) Cells were treated with DHA (70 μM) for 24 h and lysed in Triton X-100 (Tx100) buffer. Equal amounts of protein (20 μg) from T × 100 fraction were centrifugated at 10,000 x g and the pellet was dissolved in the same volume of 8 M urea buffer before loading on the gel. The membrane was immunoblotted for SQSTM1 and MAP1LC3B. β-actin (ACTB) and PCNA are used as loading controls. (**B**) The cells were treated with DHA, OA or AA (70 μM) with or without BafA1 (100 nM) for 16 h. Total cell extracts were immunoblotted for SQSTM1. ACTB and PCNA are used as loading controls. (**C**) Protein levels of SQSTM1 and MAP1LC3B determined by immunoblotting of cells treated with DHA (70 μM), BafA1 (100 nM) or a combination of DHA and BafA1 for the indicated time points. The numbers below the MAP1LC3B-II bands represent fold change relative to BafA1 for each time point normalized to PCNA intensity. ACTB and PCNA are used as loading controls. (**D**) The mRNA levels of *SQSTM1, MAP1LC3B, MAP1LC3A,* and *GABARAPL1* relative to *ACTB* after DHA (70 and 140 μM) supplementation for 16 h determined by quantitative real-time PCR. qRT-PCR data displayed are representative for 2 independent experiments. Mean fold change from triplicate wells ± SD is displayed. Data shown are representative of 3 or more independent experiments, unless otherwise stated.

detergents, the pellets that remain after Triton X-100 protein extraction and centrifugation of the lysates, were resolved in a buffer containing 8 M urea to also solubilize also detergent-resistant proteins. A very clear increase in SQSTM1 protein level was observed in response to DHA in the detergent-resistant pellet while very little MAP1LC3B could be detected (**Fig. 1A**). During the rest of the study, cells were lysed directly in a buffer containing 8 M urea to avoid losing part of the cellular pool of SQSTM1. We then tested whether other lipids also could induce the level of SQSTM1. Interestingly, whereas stimulation with DHA clearly increased the protein level of SQSTM1, that was further elevated when combining DHA and BafA1 (**Fig. 1B**), no increase was observed after treatment with OA nor AA (**Fig. 1B**). The SQSTM1 gene is induced in response to different types of cellular stresses and the protein is continously turned over by autophagy. Since combining the DHA stimuli and the lysosomal inhibitor caused an additive effect, this suggests an increased autophagic turnover of SQSTM1 in response to DHA. Consistently, supplementation with DHA also increased the level of MAP1LC3B-II when combined with lysosomal inhibition (**Fig. 1C**). This observation indicates an autophagy-inducing activity of DHA in the ARPE-19 cells. To determine the time needed for DHA to induce autophagy and increase the level of SQSTM1, cell extracts were prepared after 3, 6, 16, and 24 h supplementation with DHA and BafA1. Already after 3 h with DHA supplementation, the turnover of both SQSTM1 and MAP1LC3B-II was induced, and the effect lasted for the duration of the experiment (**Fig. 1C**). The additive effect of DHA supplementation and inhibition of autophagic degradation by BafA1 suggests lipid-induced synthesis of the 2 proteins. In line with this notion, quantitative real-time PCR (qRT-PCR) analyses revealed a more than 7-fold increase in *SQSTM1* mRNA and more than 4-fold increase in *MAP1LC3B* mRNA levels in response to 16 h DHA treatment (**Fig. 1D**). Interestingly, among the mammalian orthologs of yeast Atg8, the induction of MAP1LC3B seems selective since only minor changes could be detected in mRNA levels of *MAP1LC3A* and *GABARAPL1*. Together, these data suggest that the synthesis of SQSTM1 and MAP1LC3B is induced and autophagy increased in response to DHA in a lipid-specific manner.

Since SQSTM1 was found in the detergent-resistant fraction after DHA supplementation, the cells were immunostained for SQSTM1 and MAP1LC3B. In response to DHA, a transient increase in number and size of SQSTM1-positive punctate cytosolic structures was observed (**Fig. 2A**). The number of SQSTM1-positive structures increased with time up to 16 h. A partial colocalization with MAP1LC3B was observed, which might represent autophagosomes. To quantify the number of punctate SQSTM1-positive structures per cell, more than 500 cells per condition were analyzed using automated imaging. Consistent with the manual inspection, automated image analyses demonstrated that the average number of SQSTM1 punctate structures increased with time after DHA supplementation (**Fig. 2B**). The average number of SQSTM1-positive speckles increased from less than 10 per cell in untreated cells to approximately 50 per cell in cells treated with DHA for 16 h. Interestingly, the number of SQSTM1 speckles that colocalized with MAP1LC3B decreased from approximately 60% in the untreated cells to less than 30% in the cells treated with DHA for 16 h. By extending the treatment time to 24 h, the number of punctate SQSTM1 structures was reduced, and the frequency of colocalization with MAP1LC3B increased (**Fig. 2C**). Together, these data indicate that cells respond to DHA by inducing a transient increase in SQSTM1-positive speckles. The reduction in the number of these speckles coincides with an increased turnover of MAP1LC3B-II and elevated colocalization between SQSTM1 and MAP1LC3B.

DHA induces a transient increase in ROS and activation of NFE2L2 in ARPE-19 cells

PUFA supplementation causes a rise in the level of reactive oxygen species (ROS) in different cell types,[56] and to induce oxidative stress response genes in colon cancer cells.[57] In response to DHA (70 μM and 140 μM) there was a significant increase in ROS levels at 3 h and then the level was reduced with time (**Fig. 3A**). Interestingly, 24 h after adding DHA (140 μM) the level of ROS was lower compared to control cells. The DHA-induced increase in ROS levels could be counteracted by pretreating the cells with the exogeneous antioxidants N-acetyl-cysteine (NAC) or vitamin E (**Fig. 3B**). DHA treatment for 3 h resulted in significantly higher levels of ROS compared to treatment with AA or OA for the same time-period (**Fig. 3C**). Also, no further increase in ROS levels was observed after 6 h and 24 h supplementation with OA or AA (data not shown). Increased levels of ROS represent a stress situation that is counteracted by numerous cellular responses including changes in gene expression coordinated by the transcription factor NFE2L2. In response to ROS, NFE2L2 is stabilized and translocated to the nucleus.[58] Consistent with the ROS measurements, immunofluorescent analyses demonstrated a clear increase in nuclear localization of NFE2L2 in response to DHA while AA and OA did not affect the level of nuclear NFE2L2 significantly (**Fig. 3D**). The immunostaining approach for evaluating NFE2L2 activation was specific since *NFE2L2* siRNA caused a loss in intensity (data not shown). In line with a ROS-mediated activation of NFE2L2 after DHA supplementation, pretreatment with NAC counteracted nuclear translocation of NFE2L2 (**Fig. 3E**) and induction of HMOX1 (heme oxygenase 1) (**Fig. 3F**), representing one of the typical NFE2L2 targets induced by oxidative stress.[59] Immunoblot analyses also demonstrated a clear increase in NFE2L2 protein level in response to DHA consistent with a stabilization of the protein (**Fig. 3H**). Induction of HMOX1 was further analyzed after adding different lipids and found to be selective for DHA (**Fig. 3G**). Further, induction of NFE2L2 was validated by microarray gene expression analyses Using MetaCore for pathway analyses, it was revealed that NFE2L2-associated stress responses were significantly activated after 12 h DHA treatment ($P < 10^{-6}$). The identified, upregulated NFE2L2 target transcripts included *HMOX1, NQO1* (NAD[P]H dehydrogenase, quinone 1), *SRXN1* (sulfiredoxin 1), *ATF4* (activating transcription factor 4), and *SLC7A11* (solute carrier family 7 [anionic amino acid

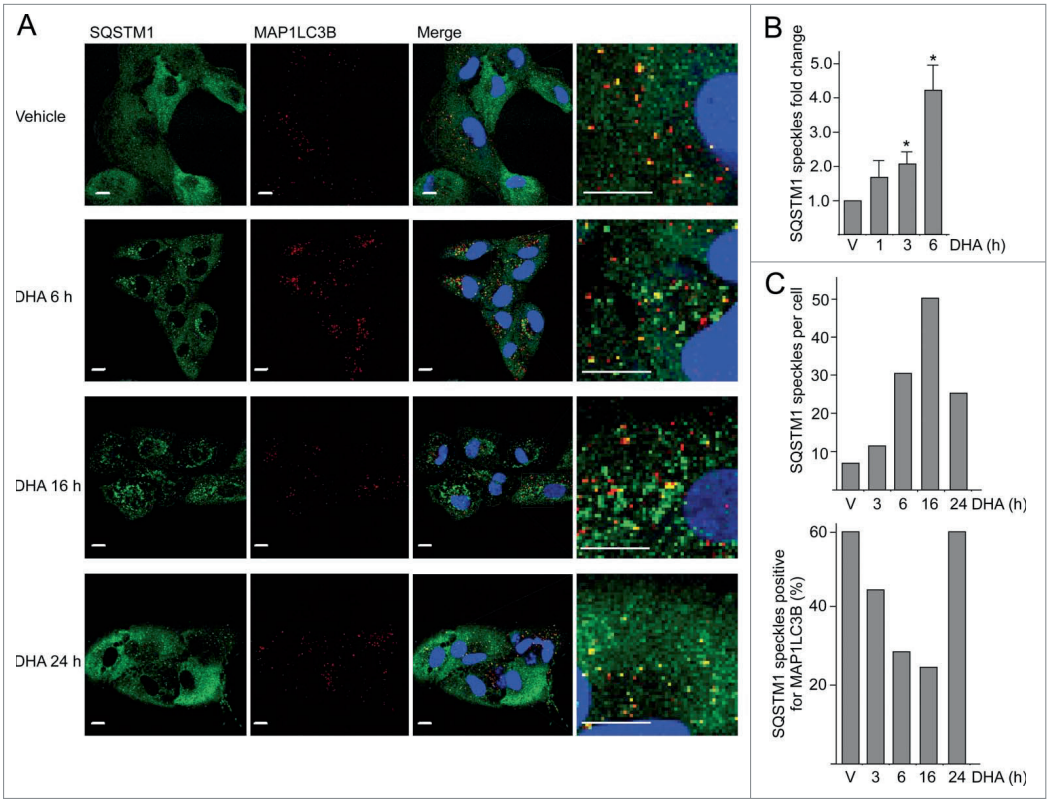

Figure 2. The number of SQSTM1-positive protein speckles in ARPE-19 cells increases after DHA supplementation. (**A**) Immunostaining for SQSTM1 and MAP1LC3B after DHA (70 μM) treatment for indicated time points. Nuclear DNA was stained using Draq5 (5 μM). Scale bar: 10 μm. (**B**) Cells were treated with vehicle (V) or DHA (70 μM) for 1, 3, and 6 h. The SQSTM1-positive speckles were automatically quantified using ScanR automated image acquisition. The quantification displayed are representative for 3 independent experiments from where 2 are automatically quantified for more than 1,000 cells per condition and one is manually counted. *) indicates significantly different from control, Student *t* test $P < 0.05$. (**C**) The number of SQSTM1-positive speckles per cell (upper panel) and SQSTM1 speckles positive for MAP1LC3B (lower panel) in ARPE-19 cells supplemented with vehicle (V) or DHA (70 μM) for the indicated time points. The quantification displayed was performed manually for more than 100 cells per condition from one representative experiment. This quantification is representative for 3 independent experiments.

transporter light chain, xc- system], member 11). In line with these findings, the mRNA level of *SQSTM1*, another NFE2L2 target gene, was highly increased after 16 h DHA supplementation determined by qRT-PCR (**Fig. 1D**). In summary, these data are consistent with a lipid selective induction of ROS that results in elevated transcription of NFE2L2 controlled genes in response to DHA in ARPE-19 cells.

The DHA-induced increase in the protein level of NFE2L2 corresponded with a slight reduction in the protein level of one of its negative regulators KEAP1 (kelch-like ECH-associated protein 1) (**Fig. 3H**). This reduction was blocked by BafA1, supporting the notion that KEAP1 is degraded by selective autophagy.[60] Under resting conditions, KEAP1 sequesters NFE2L2 and targets it for proteosomal degradation.[61] Elevated cellular levels of ROS cause KEAP1 to dissociate from NFE2L2.[62] In addition, SQSTM1 can sequester KEAP1 and the affinity increases upon phosphorylation of SQSTM1 at serine 351 (Ser351) and this mechanism represents an alternative route to activate NFE2L2.[63-66] Interestingly, using an antibody specific for SQSTM1 phosphorylated at Ser351, a clear raise was observed in response to DHA, but not to AA or OA (**Fig. 3I**). Inhibition of lysosmal degradation also caused an increase in the cellular level of this modified form of SQSTM1 that was further enhanced by cotreatment with DHA. Together, these data is consistent with DHA-induced ROS and a resulting stabilization and nuclear translocation of NFE2L2. However, we cannot exclude that phosphorylation of SQSTM1 at Ser351 also contributes to the observed activation of NFE2L2. Activation of NFE2L2 results in elevated mRNA and protein levels of SQSTM1 and HMOX1. Prolonged exposure to DHA results in cellular ROS

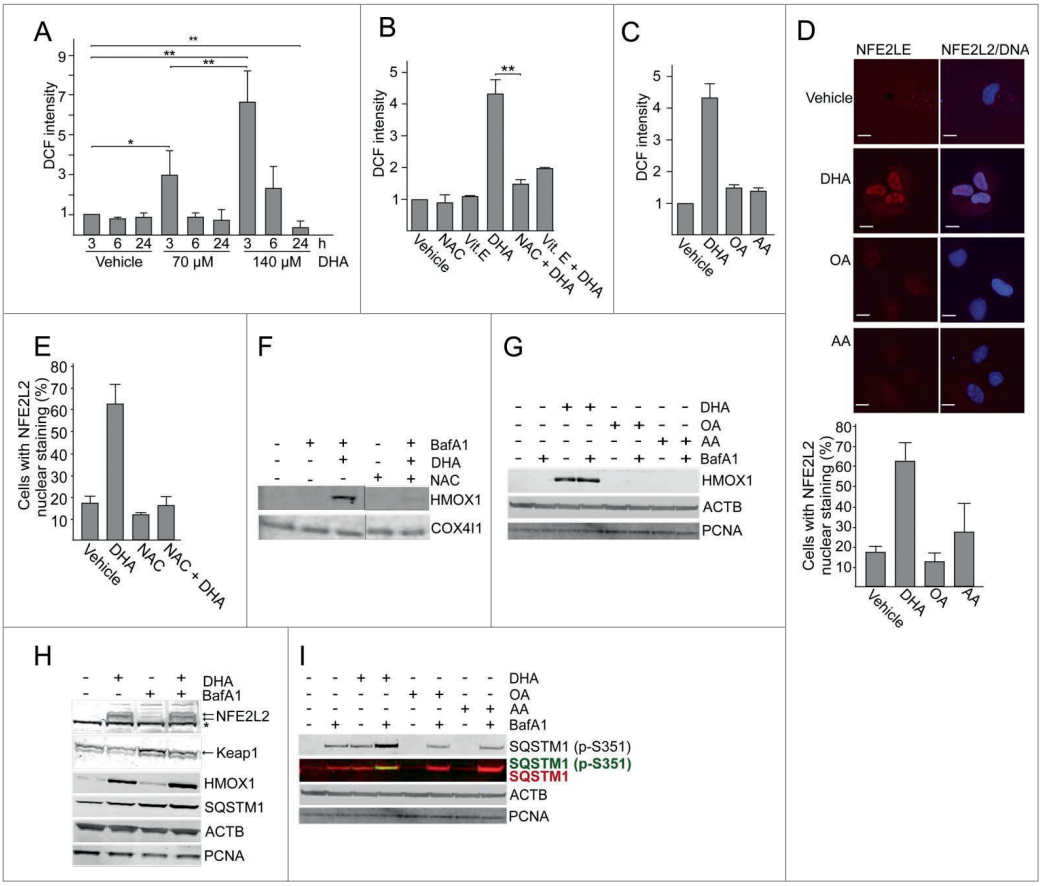

Figure 3. DHA induces a transient increase in ROS and induce NFE2L2 cytoprotective genes. (**A**) Changes in ROS levels measured at different time points after DHA (70 and 140 μM) using a fluorescent ROS DCF probe. The data represent the mean fold change ± SD for 6 independent experiments for 3 h and 3 independent experiments for 6 h and 24 h. Each experiment was performed in duplicates where the mean intensity of 10,000 cells per well ± SD was measured. *) indicates significantly different from control, Student *t* test $P < 0.05$ and **) $P < 0.01$. (**B**) Where indicated the cells were pretreated with antioxidants (5 mM N-acetyl-cysteine (NAC) or 150 μM vitamin E) for 16 h before further stimulations with 140 μM DHA for 3 h. The data represent the average of 3 independent experiments for the DHA and NAC treatments and the average of 2 independent experiments for the Vitamin E treatment. Each experiment was performed in duplicates where the mean intensity of 10,000 cells per well ±SD was measured. **) indicates significantly different from DHA, Student *t* test $P < 0.01$. (**C**) Changes in ROS levels measured 3 h after DHA, OA or AA (140 μM) supplementation using a DCF fluorescent probe. The data represent the average of 3 independent experiments ±SD for DHA treated samples and 2 independent experiments ±SD for AA and OA treated samples. Each experiment was performed in duplicates where the mean intensity of 10,000 cells ±SD per well was measured. (**D**) Immunostaining of NFE2L2 after 70 μM DHA, OA, AA for 6 h. Nuclear DNA was stained using Draq5 (5 μM). Scale bar: 10 μm. The results are representative for 3 independent experiments. Nuclear NFE2L2 staining from one representative experiment was automatically quantified using ScanR automated image acquisition of more than 3,000 cells. Each experiment was performed in duplicates and the data are presented as average percentage number of cells with NFE2L2 nuclear staining ± SD. (**E**) The cells were pretreated with NAC (5 mM) for 1 h prior to further stimulation with DHA (70 μM) in combination with NAC for 6 h. After fixation, the cells were immunostained for NFE2L2. Data are representative for 2 independent experiments. The percentage of cells with NFE2L2 nuclear staining from one representative experiment was automatically quantified using ScanR automated image acquisition. Each experiment was performed in duplicate and the data displayed represent the average percentage number of cells with NFE2L2 nuclear staining ± SD. (**F**) ARPE-19 cells were pretreated with NAC (5 mM) for 1 h following stimulation with DHA (70 μM) for 6 h and BafA1 (100 nM) the last 2 h. Levels of HMOX1 was determined by immunoblotting. COX4I1 was used as loading control. (**G**) The ARPE-19 cells were treated with DHA, OA or AA (70 μM) with or without BafA1 (100 nM) for 16 h before immunoblotting for HMOX1 (100 μg protein loaded). ACTB/β-actin and PCNA were used as loading controls. (**H**) Immunoblot of NFE2L2, HMOX1, KEAP1 (100 μg protein loaded), SQSTM1, ACTB and PCNA (loaded 20 μg protein) after DHA (70 μM) with or without BafA1 (100 nM) for 16 h. Arrows represent NFE2L2 and KEAP1 bands while *) represents a nonspecific NFE2L2 band. ACTB and PCNA were used as loading controls. (**I**) Cells were treated as in (**G**) and immunoblotted for phosphorylated SQSTM1 (Ser351) and total SQSTM1 (100 μg protein loaded). ACTB and PCNA were used as loading controls. Data shown are representative of 3 or more independent experiments, unless otherwise stated.

levels that are lower compared with control cells indicating an induction of endogenous antioxidants by DHA. Interestingly, pretreating the cells with exogenous antioxidants counteracted the DHA-induced ROS levels as well as nuclear translocation and activation of NFE2L2, indicating that ROS is clearly involved in the DHA-dependent activation of NFE2L2.

NFE2L2, SQSTM1, and ATG5 are important in the cellular responses to DHA by limiting oxidative stress and mediating cell survival

To evaluate the importance of NFE2L2, SQSTM1 and autophagy in the cellular responses toward DHA, the cells were transfected with targeted siRNAs. More than 60% reduction in ATG5 protein level was observed in cells transfected with *ATG5* siRNA. However, the cells with reduced ATG5 protein levels did not display any reduced ability to form MAP1LC3B-II or degrade SQSTM1 (**Fig. 4A**), indicating that the remaining ATG5 protein provides sufficient catalytic activity to maintain autophagy largely unchanged. This is in line with previous findings reporting that very low levels of ATG12–ATG5 might be sufficient for maintaining autophagy.[67] Even though no clear reduction in autophagy could be observed in the *ATG5* siRNA tranfected cells, we could still observe a tendency of potentiation of ROS levels in response to DHA compared to the control siRNA-transfected cells (**Fig. 4B**). In addition, downregulation of ATG5 protein levels affected the growth of the cells treated with DHA compared to the control-transfected cells (**Fig. 4C**). These relative differences in sensitivity toward DHA were also observed by counting the number of viable cells 48 h after adding DHA (data not shown). However, since we were unable to establish a clear reduction in autophagy in the ARPE-19 cells after downregulation of ATG5, we are uncertain how these effects relate to autophagy. We therefore analyzed ROS levels and cell survival after DHA supplementation in wild-type (WT) and *atg5*-deficient mouse embryonic fibroblasts (MEFs). Compared to the WT MEFs, the *atg5*$^{-/-}$ MEFs displayed an elevated basal level of ROS (**Fig. 5A**) and NFE2L2 (**Fig. 5B**) in line with previous notions.[26] In response to DHA, the level of both ROS and NFE2L2 increased to higher levels in *atg5*$^{-/-}$ MEFs (**Fig. 5A and B**) and the autophagy-deficient cells were found more sensitive to DHA (**Fig. 5C**). Together, these findings indicate a cytoprotective role of autophagy in the cellular responses toward DHA.

Using well-established siRNA probes targeting *NFE2L2*,[28,64] we estimated NFE2L2 to be approximately 70% downregulated by immunostaining (data not shown) and SQSTM1 was reduced more than 50% after cotreatment with DHA and BafA1 (**Fig. 6A**). Also, the DHA-induced level of HMOX1 was clearly reduced in the *NFE2L2* siRNA-transfected cells compared to the cells transfected with control siRNA (**Fig. S2**). These results are consistent with a important role of NFE2L2 in the regulation of both HMOX1 and SQSTM1 in response to DHA. The clear reduction in SQSTM1 and HMOX1 indicates an efficient and functional downregulation of NFE2L2. Targeting *SQSTM1* with siRNA caused a more than 90% reduction in the protein level of SQSTM1 (**Fig. 6A**). Interestingly, the level of MAP1LC3B-I

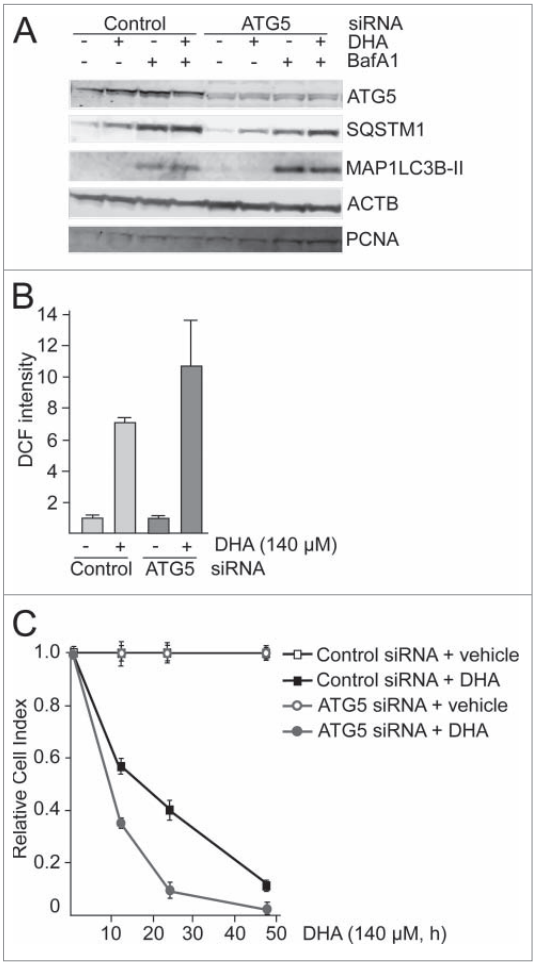

Figure 4. ATG5 is important in the cellular responses to DHA. (**A**) ARPE-19 cells were transfected with control siRNA or *ATG5* siRNA (100 nM) and left for 24 h before reseeding. Following incubation for 24 h, the cells were added DHA (70 μM) or BafA1 (100 nM) for 24 h and immunoblotted for ATG5, SQSTM1, and MAP1LC3B. ACTB and PCNA were used as loading controls. (**B**) The cells were siRNA-transfected as in (**A**). After DHA (140 μM) treatment for 3 h changes in ROS levels were measured using a fluorescent ROS DCF probe. The results are representative for 2 independent experiments. Each experiment was performed in duplicates where the mean intensity ±SD of 10,000 cells per well was measured. The control is normalized to one and the relative fold changes are shown. (**C**) Relative cell index after transfection with control or *ATG5* siRNA (100 nM) after vehicle or DHA (140 μM) based on real-time monitoring using the xCELLigence instrument. The cell index for each treatment was normalized to one at the start of the experiment. For each time point the cell index of control samples (Control siRNA + vehicle and *ATG5* siRNA + vehicle) was normalized to 1. The effect of DHA treatment after transfection with either Control siRNA or *ATG5* siRNA is shown relative to the corresponding controls. Mean normalized cell index with standard deviation of triplicate wells of vehicle or DHA treated cells is displayed. Data shown are representative for 2 independent experiments.

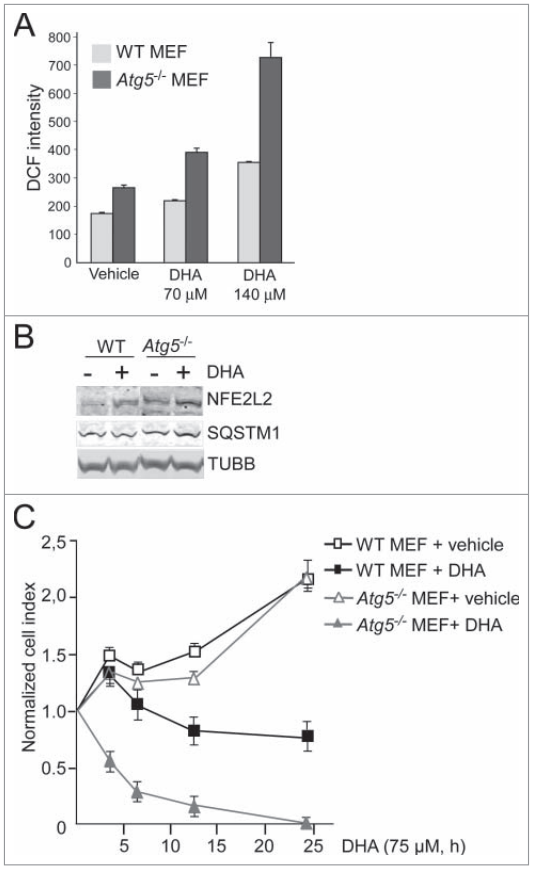

Figure 5. The *atg5* knockout MEFs are more sensitive to DHA compared to wild-type MEFs. (**A**) The levels of ROS were measured in wild-type (WT) and *atg5*$^{-/-}$ MEFs after 3 h DHA treatment (70 and 140 μM) using the fluorescent DCF probe. The data from one representative experiment of 3 independent experiments are displayed. Each experiment was performed in triplicate wells where the mean intensity ±SD of 10,000 cells per well was measured. (**B**) The levels of NFE2L2 and SQSTM1 after vehicle or DHA (70 μM, 16 h) treatment in wild-type and *atg5*$^{-/-}$ MEFs (85 μg protein loaded). TUBB/β-tubulin was used as loading control. The immunoblot is representative for 3 independent experiments. (**C**) Wild-type and *atg5*$^{-/-}$ MEFs were exposed to DHA (75 μM) and cellular responses observed over time using the xCELLigence real-time monitoring system. The cell index was normalized to one at the start of the experiment. Mean normalized cell index ±SD of triplicate wells of vehicle or DHA treated cells are displayed. The results are representative for 5 independent growth experiments scored by cell index using xCELLigence.

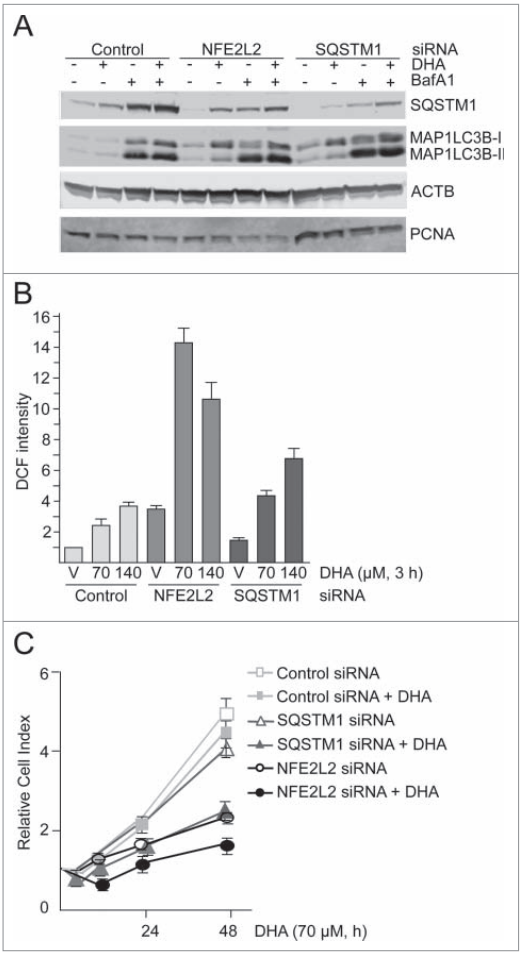

Figure 6. NFE2L2 and SQSTM1 are important in the cellular responses to DHA in ARPE-19 cells. (**A**) Cells were transfected with control, *NFE2L2* and *SQSTM1* siRNA (25 nM) and left for 24 h before reseeding. Following incubation for 24 h, the cells were added DHA (70 μM) or BafA1 (100 nM) for 24 h. Immunoblot for SQSTM1 and MAP1LC3B. ACTB/β-actin and PCNA were used as loading controls. (**B**) The cells were siRNA-transfected as in (**A**). After vehicle (V) and DHA (70 and 140 μM) treatment for 3 h changes in ROS levels were measured using a fluorescent ROS DCF probe. The data are representative for 2 independent experiments both performed in duplicates. The data represent the mean intensity ±SD of 10,000 cells per well and is displayed as relative DCF intensity. (**C**) Relative cell index after transfection with control, *NFE2L2* or *SQSTM1* siRNA (25 nM) after vehicle and DHA treatment (70 μM) based on real-time monitoring using the xCELLigence instrument. The cell index was normalized to one at the start of the experiment. Mean normalized cell index with standard deviation of triplicate wells of vehicle and DHA treated cells is displayed. Data shown are representative for 3 independent experiments.

was elevated in response to both *NFE2L2* and *SQSTM1* siRNA transfection compared to control siRNA-transfected cells. The induced level of MAP1LC3B-I was evident in the untreated control and further increased in response to DHA, indicating that the cells compensate for reduced levels of NFE2L2 and SQSTM1 by inducing the synthesis of MAP1LC3B protein and activation of autophagy. Consistent with an important role of NFE2L2 in the cellular responses to DHA, a more than 3-fold increase in

basal ROS levels was observed that was further strongly increased by DHA in the *NFE2L2* siRNA-transfected cells (**Fig. 6B**). Downregulation of SQSTM1 also resulted in a slightly increased basal ROS level and a further elevation after DHA supplementation compared to control siRNA-transfected cells. Since SQSTM1 is only one of a number of different cytoprotective genes controlled by NFE2L2, the data demonstrate that downregulation of NFE2L2 is more severe in terms of basal and induced levels of ROS compared to siRNA-mediated downregulation of *SQSTM1*. Accordingly, real-time monitoring of cells transfected with siRNA against *NFE2L2* and *SQSTM1* displayed a reduced cell growth in the presence of 70 μM DHA, consistent with a cytostatic effect. Again, siRNA mediated downregulation of *NFE2L2* was found more severe than downregulation of SQSTM1 after DHA supplementation (**Fig. 6C**). By increasing DHA concentration (140 μM), the sensitivity was further increased (data not shown). Together, these results indicate that NFE2L2, SQSTM1 and autophagy cooperate in regulating the cellular responses toward DHA; interfering with any of these processes turns a transient, mild increase in ROS into a cytotoxic stress condition.

DHA mobilizes cytoprotection toward protein aggregates and oxidative stress

Protein aggregates are involved in aging, diseases, and cell death, and autophagic degradation of these aggregates is important for cell survival. We observed that DHA stabilizes and activates NFE2L2, and causes a subsequent increase in SQSTM1 mRNA and protein levels. SQSTM1 are involved in sequestration of misfolded, ubiquitinated proteins into protein aggregates, and ensures selective degradation of these by autophagy.[29,68] Therefore, we wanted to investigate if DHA makes the cells more resistant to accumulation of protein aggregates or subsequent oxidative stress. Treating the cells with puromycin, a protein synthesis inhibitor that causes release of premature and misfolded proteins during translation,[28,69] led to a clear increase in mono- and polyubiquitinated protein aggregates (**Fig. 7A**). In contrast, no such increase could be observed in response to the translational inhibitor cycloheximide (CHX) for the same period of time.

After pretreating the cells with DHA, the cells were further treated with puromycin or cycloheximide for 4 h. The drugs were then removed and the cells were incubated for a further 16 h in the presence of the tymidine analog 5-ethynyl-2′-deoxyuridine (EdU) to evaluate the frequency of cells performing S

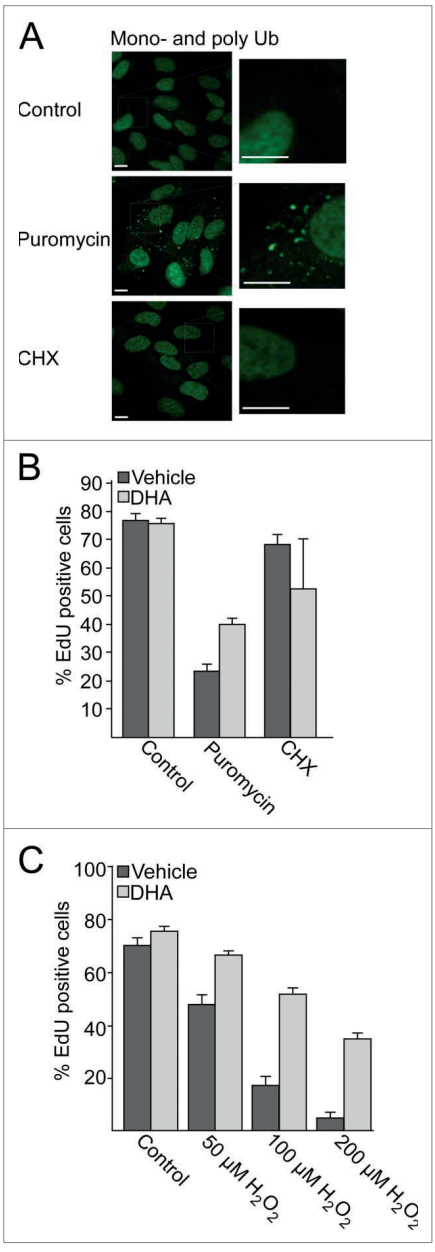

Figure 7. DHA pretreatment protects ARPE-19 cells against protein aggregates and oxidative stress. (**A**) Immunostaining for mono- and polyubiquitinated proteins in ARPE-19 cells treated with puromycin (10 μM) or cycloheximide (CHX, 10 μg/ml) for 4 h. Scale bar: 10 μm. (**B**) The cells were pretreated with vehicle or DHA (70 μM) for 16 h before stimulation with puromycin (10 μM) or cycloheximide (CHX, 10 μg/ml) for 4 h. Following washout and EdU labeling for 16 h the percentage of EdU-positive cells were quantified automatically for more than 3,500 cells using ScanR automated image acquisition. Data shown are representative for 2 independent experiments. (**C**) After pretreatment with vehicle or DHA for 16 h, the cells were stimulated with hydrogen peroxide (H_2O_2) in the indicated concentrations. After washing and EdU labeling for 16 h the percentage of EdU-positive cells were quantified automatically using ScanR automated image acquisition of more than 3,000 cells. Data shown are representative for 3 independent experiments.

phase during this time.[70] By automated imaging and image analyses of more than 3,500 cells, it was found that more than 75% of the untreated cells displayed nuclei positive for EdU (**Fig. 7B**). Pretreatment with DHA did not affect the frequency of cells that performed S phase. In response to a 4 h treatment with puromycin, but not cycloheximide, the number of nuclei positive for EdU was clearly reduced compared to control. Interestingly, pretreating the cells with DHA partially rescued the cell proliferation following a puromycin challenge. These results indicate that under conditions where SQSTM1 is induced, there is also an improved tolerance for misfolded proteins formed in response to puromycin.

To test whether activation of NFE2L2 observed after DHA stimulation makes the cells more resistant to subsequent exposure to oxidative stress, ARPE-19 cells were pretreated with DHA for 16 h following a 30 min challenge with hydrogen peroxide (H_2O_2). After washing, cells were incubated for 16 h in the presence of EdU. Again, pretreatment with DHA partially rescued proliferation of the cells (**Fig. 7C**). Together, these results indicate that DHA induces cellular responses that mobilize resistance to misfolded proteins and oxidative stress.

Discussion

We here demonstrate that the n-3 PUFA DHA selectively induces a transient increase in cellular ROS levels that is counteracted by activation of NFE2L2 and induction of oxidative stress response genes and proteins in ARPE-19 cells. In addition, DHA stimulates the synthesis of SQSTM1 and elevates autophagy. Pretreatment with DHA counteracted cell cycle arrest induced by misfolded proteins or oxidatives stress. Together our data indicate that DHA induces an interplay between NFE2L2 activation and autophagy in retinal pigment epithelial cells that makes the cells more tolerant to misfolded proteins and oxidative stress. These responses could represent putative disease preventive mechanisms mobilized after a mild, transient oxidative stress induced by DHA in a lipid selective manner.

Elevated levels of ROS are implicated in the pathogenesis of a number of neurodegenerative diseases, including AMD.[18,71] NFE2L2 is the main regulator of the expression of genes encoding proteins that controls cellular redox status.[58,72] Upon aging, *nfe2l2*-knockout mice have an increased risk of developing AMD-like phenotype,[73] emphasizing the important cytoprotective role of the cell's endogenous antioxidative system. Physiologically relevant doses of DHA caused a transient increase in ROS followed by increased protein levels and nuclear translocation of NFE2L2. Subsequently, increased expression of NFE2L2-modulated genes was detected by gene-expression arrays, and as increased protein levels of the NFE2L2 regulated proteins HMOX1 and SQSTM1. The n-6 PUFA arachidonic acid and the monounsaturated oleic acid did not cause a similar increase in ROS or activation of NFE2L2. Currently, the mechanisms underlying this lipid selectivity is incompletely understood. However, these lipids serve as precursors for different types of lipid-derived signaling compounds resulting from both nonenzymatic and enzymatic reactions.[74] The ARPE-19 cells did not display any changes in cell growth or survival after adding any of the lipids (70 μM). Nevertheless, in cells where NFE2L2 was downregulated, both the basal and DHA-induced levels of ROS were increased consistent with a central role of NFE2L2 in redox balance. Interestingly, in cells depleted for NFE2L2 the growth rate was clearly affected even under normal growth conditions. After DHA supplementation the cytostatic response was further potentiated. These data are consistent with previous reports demonstrating that DHA directly or indirectly induces NFE2L2.[74,75] Also, we found that DHA pretreatment protected the ARPE-19 cells from a cytostatic effect upon a subsequent challenge with hydrogen peroxide. Together, these results indicate that DHA induces activation of NFE2L2 and an increased buffer capacity for oxidative stress in retinal pigment epithelial cells.

Autophagy has emerged as a cellular process for selective clearance of damaged proteins and organells, and is crucial to avoid the development of several age-related diseases, including different types of neurodegeneration.[76-78] Mice genetically modified to lack autophagy in a tissue-specific manner display early onset neurodegeneration accompanied by accumulation of misfolded protein aggregates and ubiquitinated proteins.[30,31] Inducible, systemic knockout of autophagy restricts lifespan in mice to 2 to 3 mo due to the development of severe neurodegeneration.[32] Interestingly, deletion of *ATG5* in the lens has been reported to result in age-related cataract accompanied by accumulation of polyubiquitinated and oxidized proteins and SQSTM1.[79] ATG5 is also required for lysosomal fusion of phagosomes containing photoreceptor outer segments (POS) important in renewal of photoreceptors in RPE cells and thus optimal vision.[80] Also, it was recently reported that prolonged use of the autophagy inhibitor chloroquine in treatment of malaria, could cause chloroquine-associated visual loss due to degeneration of RPE cells.[81] AMD develops with age and a marked reduction in autophagy activity in the retina of aged mice have been suggested to be involved in age-associated visual loss and retinal dystrophy.[82] Together, these findings suggest that elevation of autophagy could protect from development of AMD as well as other neurodegenerative diseases. In this context it is interesting that physiologically relevant concentrations of DHA increases autophagy in the ARPE-19 cells. In addition to increased autophagy, we also observed an increased level of *SQSTM1* mRNA and SQSTM1 protein level. SQSTM1 selectively targets misfolded, ubiquitinated proteins for lysosomal degradation by binding both to ubiquitinated cargos, via its UBA domain, and to mammalian orthologs of yeast Atg8 on the growing phagophore membrane.[28] In this way, the elevated levels of SQSTM1 observed after DHA might enhance the cell's capacity to sequester damaged and ubiquitinated proteins into aggregates. In accordance, a transient increase in the number and size of cytosolic protein aggregates positive for SQSTM1 was observed after DHA. The reduction in the number of these speckles coincides with an increased turnover of MAP1LC3B-II and elevated colocalization between SQSTM1 and MAP1LC3B, which may indicate selective removal of these structures by increased autophagy.

Intriguingly, we observe DHA pretreatment to protect ARPE-19 cells from cell cycle arrest induced by misfolded proteins released during protein translation in the presence of puromycin. We speculate that this may be due to DHA-induced increase in SQSTM1 and subsequent aggregation and autophagic clearance. Such a model would be consistent with the protective role of ubiquitination and aggregation of mutant aggregate-prone huntingtin as a mechanism for cell survival,[22,83] and autophagy as the mechanism responsible for the clearance of these huntingtin aggregates.[29,84] Also, others have reported that DHA-derived ROS is involved in protein quality control by regulating aggregation and further autophagic degradation of misfolded apolipoprotein B in hepatocytes.[85]

Interestingly, both the basal and DHA-induced level of MAP1LC3B-I were elevated in cells depleted for NFE2L2 and SQSTM1. This increase in MAP1LC3B-I could indicate a compensatory role of autophagy under conditions of increased ROS levels. Downregulation of SQSTM1 did not influence cell growth under normal conditions but resulted in a prominant cytostatic response when combined with DHA treatment. As expected, the cytostatic responses toward DHA after downregulating SQSTM1 were not as clear compared to cells with downregulated NFE2L2. These differential effects likely reflect that NFE2L2 controls a number of mechanisms that counteract oxidative stress where SQSTM1-mediated targeting of misfolded proteins for autophagic degradation is only one of these. The complex interplay between oxidative stress and autophagy is also illustrated by the finding that ROS levels are increased in cancer cells lacking autophagy.[86] In addition, underscoring the complexity of this interplay, NFE2L2 controls the expression of the autophagy cargo receptor SQSTM1, but at the same time, phosphorylated SQSTM1 partly controls the activity of NFE2L2 by sequestering its negative regulator KEAP1.[58,64-66] NFE2L2 transcriptionally cooperates with several cellular stress response pathways, including ATF4-regulated stress responses,[87] that further regulates SQSTM1,[88] and autophagy.[89] Also, in addition to TFEB (transcription factor EB),[90] recent studies have pointed to CREB (cAMP responsive element binding protein) and PPARA (peroxisome proliferator-activated receptor α) important for the transcriptional regulation of autophagy.[91,92]

The pathogenesis of AMD is strongly associated with oxidative stress.[10,18,20,93,94] Currently, there is no established prevention or therapy for early AMD. The Age-Related Eye Disease Study (AREDS) is among the largest and most robust randomized clinical trials designed to investigate the role of daily oral supplementation of antioxidant vitamins and minerals.[13] The AREDS study demonstrated that daily supplementation with antioxidant reduced the risk of developing advanced AMD by 25% at 5 y. Epidemiological studies have suggested a protective role of n-3 PUFAs for developing AMD.[37-42,95-97] Also, subgroup analysis from the Nutritional AMD Treatment 2 Study from 2013, revealed that high levels of n-3 PUFAs in red blood cells can prevent AMD progression.[98] To assess if antioxidants and n-3 PUFAs could induce additive effects, a second AREDS2 study was designed to evaluate if inclusion of n-3 PUFAs to the AREDS formulation further reduced the risk of progression to advanced AMD. However, no additional effect was observed in preventing AMD progression.[99] Our results demonstrate that DHA potently induces the endogenous oxidative stress defense coordinated by NFE2L2 in ARPE-19 cells. The DHA-induced activation of NFE2L2 was abolished by cotreatment with exogenous antioxidants. Thus, it is possible that the 2 approaches to prevent AMD, either by reducing ROS via exogenous antioxidants, or by n-3 PUFAs to mobilize the endogenous ROS scavenging systems, neutralize each other. Based on our results, it would be interesting to determine if additional effects of the 2 approaches could be present if antioxidants and n-3 PUFAs are sequentially supplemented. However, further research is needed in order to determine the kinetics of the 2 responses in relevant cell types.

Further studies are needed to determine if aggregation, autophagy and NFE2L2 activation are part of the physiological responses to DHA also in vivo. Our data indicate that activation of NFE2L2 and elevated autophagy could represent markers for the disease preventive effects of DHA supplementation. Interestingly, our results emphasize that exogenous antioxidants may interfere with and counteract some of the putative positive effects of DHA, including the activation of NFE2L2. The current study shows that DHA induces the cellular antioxidant responses controlled by NFE2L2 and stimulate protein quality control and autophagy. These cellular mechanisms harbor a number of putative biomarkers that may be utilized in the future to defined and improve disease preventive effects of marine n-3 PUFAs.

Materials and Methods

Cell lines and reagents

ARPE-19 were obtained from ATCC (CRL-2302) and cultured in DMEM:F12 medium (Sigma, D8437), supplemented with fetal bovine serum (10%) (Gibco, 10270-106) and gentamicin (0.05 mg/mL; Gibco, 15710049). Immortalized wild-type (WT) and *atg5*$^{-/-}$ MEFs were a kind gift from Noboru Mizushima and were grown in DMEM (Sigma, D5796) supplemented with fetal bovine serum (10%) (Gibco, 10270-106) and gentamicin (0.05 mg/mL; Gibco, 15710049). All cell lines were maintained in a humidified atmosphere of 5% CO_2; 95% air at 37°C. All experiments were performed in subconfluent, exponentially growing cells that never exceeded passage number 25.

Docosahexaenoic acid (DHA; Cayman, 90310), oleic acid (OA; Cayman, 90260) and arachidonic acid (AA; Cayman, 90010) were added to prewarmed complete medium to the final desired concentration and vortexed at full speed before added to the cells. Vehicle-treated samples were added to the same volumes of absolute ethanol and was used as control throughout all experiments.

Other reagents used: bafilomycin A_1 (BafA1; Sigma, B1793), puromycin (Sigma, P9620), cycloheximide (CHX; Sigma, C4859), and hydrogen peroxide (H_2O_2; Merck Millipore, 108600).

The following antibodies were used: anti-SQSTM1/p62 (Progen, GP62-C); anti- NFE2L2/NRF2 (Santa Cruz Biotechnology, sc-13032); anti-MAP1LC3B/LC3B (Cell Signaling Technology,

D11); anti-HMOX1 (Enzo, ADI-OSA-110), anti-ATG5 (Novus Biologicals, NB110-53818), anti-ACTB/β-actin (Abcam, ab6276), anti-KEAP1 (Santa Cruz Biotechnology, E20), anti-phospho-SQSTM1/p62 (Ser351; MBL, PM074), anti–mono- and polyubiquitininated conjugates (clone FK2; Biomol, PW8810), anti-TUBB/β-tubulin (Abcam, ab6046), anti-PCNA (Santa Cruz Biotechnology, sc-7907), anti-COX4I1/COX IV (Abcam, ab33985). All secondary antibodies were from Invitrogen (Alexa conjugates, catalog numbers A-11073, A-21428 and A-11001) or Li-Cor Biotechnology (NIR dye conjugates, catalog numbers 926-32211, 926-32214, 926-32411926-68077, 926-68071, 926-68070).

Microarray gene expression profiling

ARPE-19 cells were treated with vehicle (ethanol) or 70 μM DHA for 1, 3, 6, 12, and 24 h before RNA isolation using High Pure RNA isolation kit (Roche, 11828665001). Microarray gene expression profiling was performed in independent triplicates for all time points using Illumina HumanHT-12 v4 Expression BeadChip according to the manufacturer's protocol (Illumina). The statistical analysis was based on summary expression measures using the raw data (CEL) files performed by the robust multiarray average method. The statistical analysis was performed in R (http://www.r-project.org), using packages Limma from Bioconductor.[100] Differentially expressed genes were selected based on a threshold of 0.05 on the adjusted *P* values. Enrichment analyses was performed in MetaCore™ (Thomson Reuters, UK) a data-mining and pathway analysis tool. All data have been submitted to ArrayExpress with the accession number E-MTAB-3016.

Quantitative real-time PCR

Total RNA was extracted from DHA treated cells using the High Pure RNA isolation kit. Purity and quantity were measured by Nanodrop. 1 μg total RNA was used for cDNA synthesis using the iScript Select cDNA synthesis kit (Bio-Rad, 170-8896). The cDNA was diluted 1:10 before real-time PCR was performed in parallel 25 μl reactions containing 12.5 μl 2X QuantiTect SYBR Green PCR master mix (Qiagen, 204141) and 2.5 μl 10X QuantiTect Primer Assay (Qiagen, catalog numbers Hs_*SQSTM1*_1_SG, Hs_*MAP1LC3B*_2_SG, Hs_*GABARAPL1*_1_SG, Hs_*MAP1LC3A*_1_SG, *Hs_GAPDH*_2_SG, Hs_*ACTB*_2_SG). The cycling conditions for the StepOne plus system (Applied Biosystems, Foster City, CA, USA) were 95°C for 15 min, 40 cycles of 94°C for 15 sec, 55°C for 30 sec and 72°C for 30 sec. Relative RNA transcription levels were transformed into linear form by 2 (−deltadeltaCt). Transcripts were normalized to the quantity of *ACTB* for each condition.

siRNA-mediated knockdown

For *NFE2L2* and *SQSTM1* downregulation cells were transfected using 25 nM siRNA oligo (final concentration) by DharmaFECT transfection reagent 1 (Dharmacon, T-2001-03) and compared to 25 nM control nontargeting siRNA. For *ATG5* downregulation cells were transfected using 100 nM siRNA oligos (final concentration) and compared to the same final concentration of nontargeting siRNA. Following 24 h, the cells were collected by trypsinization and seeded for real-time cell monitoring, ROS measurements and immunoblot analysis. The following smartpool siRNA oligonucleotides were obtained from Dharmacon; control nontargeting siRNA (D-001210-01); *NRF2/NFE2L2* siRNA (D-003755-02), target sequence: 5′-CCAAAGAGCAGUUCAAUGA; *SQSTM1* siRNA (J-010230-06) and *ATG5* siRNA (L-004374-00)

Real-time cell monitoring

Real-time growth curves were obtained using an xCELLigence system (Roche) according to the supplier's recommendations in the presence or absence of DHA (70 μM and 140 μM). Where indicated, the cells were pretreated with siRNA (nontargeting, *NFE2L2, SQSTM1*, or *ATG5*) 24 h before monitoring in real time with or without DHA (70 μM and 140 μM).

ScanR automated image acquisition

The microscope-based imaging platform ScanR (Olympus, Hamburg, Germany) were used to image SQSTM1-positive structures in the presence and absence of DHA (70 μM). Images were taken with a 20 × objective, using the excitation filters (wavelength [nm]/width [nm]): FITC (485/20) and Draq5 (650/13). For emission, a combination filter (440,521,607 and 700 nm) was used for all fluorophores (Chroma Technology Corp, Bellows Falls, VT). For each well, approximately 2000 cells were counted. The images were analyzed by the ScanR Analysis software (Olympus). Using the ScanR analysis software (Olympus) the number of cells was counted (based on the nuclear-stain) and the number of SQSTM1 dots within the cells (nucleus and surrounding cytosol) or Click EdU-positive nuclei.

Cell proliferation assay

Cell proliferation was monitored using Click-iT® EdU Alexa Fluor® 488 Imaging Kit (Invitrogen, C10337) according to the manufacturer's protocol. For EdU incorporation experiments, cells were pretreated with vehicle or DHA (70 μM) for 16 h, washed 2X PBS before further stimulation with puromycin (10 μM), cycloheximide (10 μg/ml) for 4h or hydrogen peroxide (50, 100 or 200 μM) for 30 min. After 2X washing in PBS the cells were added 5-ethynyl-2′-deoxyuridine (EdU) (5 μM) for 16 h and fixated using 4% paraformaldehyde. For the click reaction the cells were washed in 3% BSA (Sigma, A7906) and permabilized using 0.5% Triton X-100 (Sigma, T8787) for 20 min. After additional washing the cells were incubated with Click-It reaction cocktail containing Alexa Fluor 488 azide (Invitrogen, C10337) for 30 min. DNA was stained using 5 μg/ml Hoechst 33342 included in the kit (Invitrogen, C10337). EdU-positive cells were automatically quantified using ScanR automated image acquisition.

Detection of reactive oxygen species

ROS levels were determined using the Image-iT ™ LIVE Green Reactive Oxygen Species Detection Kit (which utilizes 5-(and-6)-carboxy-2′,7′-dichlorodihydrofluorescein diacetate [DCF]; Invitrogen, C6827) and a BD FACS Canto flow

cytometer (BD Biosciences, San Jose, CA, USA). The cells were treated with 70 μM or 140 μM of designated lipids for the indicated time points. When indicated, the cells were pretreated for 16 h with 150 μM vitamin E (Sigma, T3251) and 5 mM N-acetyl cysteine (NAC; Sigma, A9165) before DHA treatment (140 μM) for 3 h. The cells were incubated at 37°C and 5% CO_2 with 0.3 μM DCF for 30 min before intracellular ROS was determined. The experiments were performed in duplicates and the data represent mean intensity of 10,000 cells per well ± SD. The results represent the average of 6 independent experiments for 3 h and 3 independent experiments for 6 and 24 h. For the antioxidant treatments, the results represent the average of 3 and 2 independent experiments for NAC and vitamin E, respectively. For WT and *atg5* knockout MEFs the experiments were performed in triplicates and the data represent the average of 3 independent experiments. *P* values were calculated using the Student *t* test.

Immunostaining

The cell cultures were treated as specified for indicated time points and fixed in 4% paraformaldehyde before immunostaining using indicated antibodies and visualization by fluorescently labeled secondary antibodies. Nuclear DNA was stained using Draq5 (5 μM; Biostatus, DR50200) or Hoechst 33342. Immunostaining was imaged with an Axiovert200 microscope equipped with a 63 × 1.2W objective and the confocal module LSM510 META (Carl Zeiss, Jena, Germany). Images were processed using the LSM software and mounted using Canvas 11 (Deneba). Images are representative of more than 200 randomly selected cells in each condition and of 2 or more independent experiments. All images to be compared were taken with the same settings.

Immunoblotting

After the indicated treatment the cells were harvested by trypsinization and lysed in a urea buffer containing 8 M urea (Merck Millipore, 1084870500), 0.5% (v/v) Triton X-100 (Sigma, T8787), 100 mM DTT (Sigma, 646563), Complete® protease inhibitor (Roche, 1187350001) and phosphatase inhibitor cocktail II (Sigma, P5726) and III (Sigma, P0044). When indicated, cells were lysed in a buffer containing 0.25% Triton X-100 (Sigma, T8787), 1 % NP40 (Sigma, NP40S), 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1 mM EDTA pH 8, Complete® protease inhibitor, and phosphatase inhibitor cocktail II (Sigma, P5726) and III (Sigma, P0044). For wild-type and $atg5^{-/-}$ MEFs, the extracts for NFE2L2 detection were prepared using a nuclear extract kit (Active Motif, 11447358). Total protein concentration in the lysates was determined by BioRad protein assay (Bio-Rad, 500-0006). Equal amounts of proteins (20 μg if nothing else stated) were separated using NuPAGE® Novex® 12% or 4-12% Bis-Tris Gels (Invitrogen). Bound antibodies were imaged by near infrared fluorescence using appropriate fluorescent dye labeled secondary antibodies and an Odyssey Near Infrared scanner (Li-Cor Biosciences, Lincoln, Nebraska, USA). PCNA and COX4I1 were used for normalization purposes. In addition, loading was also detected using immunoblotting for ACTB/β-actin and TUBB/β-tubulin. Images were processed using Li-Cor Odyssey software and mounted using the Canvas 11 software (Deneba).

Statistics

Values were expressed as mean ± standard deviation (SD). Statistical analyses were performed by the 2-tailed Student *t* test, 2-sample assuming equal variances. *P* values < 0.05 was considered statistically significant and is labeled with *) and $P < 0.01$ is labeled with **). Error bars for qRT-PCR represent the standard deviation of triplicate wells.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Supplemental Material

Supplemental data for this article can be accessed on the publisher's website.

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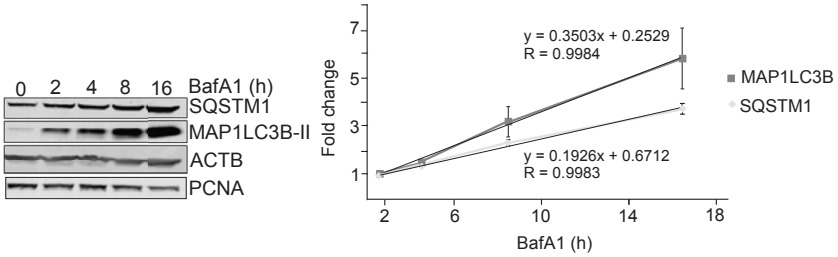

Figure S1. The autophagic turnover rate of SQSTM1 and MAP1LC3B in ARPE-19 cells. (A) The ARPE-19 cells were treated with BafA1 (100 nM) for the indicated time points. Equal amounts of protein (20 μg) was loaded for each condition and immunoblotted for SQSTM1 and MAP1LC3B. ?-actin (ACTB) and PCNA were used as loading controls. (B) The half-lives of SQSTM1 and MAP1LC3B were quantified using the regression line from the average fold change from four independent experiments. BafA1 treatment for 2 h was set to 1. R represents the correlation coefficient.

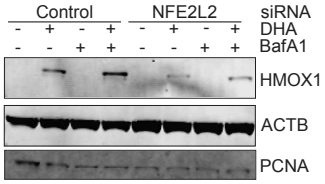

Figure S2. The level of the NFE2L2 target-protein HMOX1 (heme oxygenase 1) is reduced when NFE2L2 is downregulated. The cells were transfected with control or NFE2L2 siRNA (25 nM) and left for 24 h before reseeding. Following incubation for 24 h, the cells were added DHA (70 μM) or BafA1 (100 nM) for 24 h. Immunoblot for HMOX1. ACTB/b-actin and PCNA were used as loading controls.

Paper II

N-3 PUFAs induce inflammatory tolerance by formation of KEAP1 containing p62-bodies and activation of NFE2L2

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Abbreviations: AA, arachidonic acid; ACTB, actin beta; ATG8, autophagy related 8; BafA1, bafilomycin A1; CHUK (IKKα), conserved helix-loop-helix ubiquitous kinase; COX4I1, cytochrome c oxidase subunit 4I1; CXCL10, chemokine (C-X-C motif) ligand 10; DHA, docosahexaenoic acid; DUB, deubiquitinating enzyme; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GPR120, G protein coupled receptor 120; HMOX1, hemeoxygenase 1; IFN, interferon; IFNAR1, interferon alpha and beta receptor subunit 1; IKBKB (IKKβ), inhibitor of kappa light polypeptide gene enhancer in B-cells kinase beta; IRF, interferon regulatory factor; KEAP1, kelch-like ECH-associated protein 1; LIR, light chain 3 (LC3)-interacting region; LMNA, lamin A/C; LPS, lipopolysaccharide; MAP1LC3B, microtubule-associated protein 1 light chain 3 beta; MAPK1/3 (ERK1/2), mitogen-activated protein kinase 1; MDM, monocyte-derived macrophages; n-3 PUFA, omega-3 polyunsaturated fatty acid; NAC, N-acetylcysteine; NFE2L2 (Nrf2), nuclear factor erythroid-derived 2-like 2; NFKB, nuclear factor-kappa B; NFKB2 (p105), nuclear factor kappa B subunit 2; NQO1, NAD(P)H quinone dehydrogenase 1; OA, oleic acid; RELA (p65), PCNA, proliferating cell nuclear antigen; RELA proto-oncogene, NF-kB subunit ; p(I:C), poly I:C; ROS, reactive oxygen species; RPE, retinal pigment epithelial; SLC7A11 (xCT), solute carrier family 7 member 11; SQSTM1 (p62), sequestosome 1; STAT1, signal transducer and activator of transcription 1; TAX1BP1, Tax1 (human T-cell leukemia virus type I) binding protein 1; TBK1, TANK-binding kinase 1; TLR4, toll-like receptor 4; TNFAIP3 (A20), tumor necrosis factor, alpha-induced protein 3; TNF, tumor necrosis factor alpha; Ub, ubiquitin; UBA, ubiquitin-associated domain

Abstract

Inflammation is crucial in the defense against infections but must be tightly controlled to limit detrimental hyper-activation. Our diet influences inflammatory processes and omega-3 polyunsaturated fatty acids (n-3 PUFAs) have known anti-inflammatory effects. The balance of pro- and anti-inflammatory processes is coordinated by macrophages and autophagy has recently emerged as a cellular process that dampens inflammation.

Here we report that the n-3 PUFA docosahexaenoic acid (DHA) transiently induces cytosolic speckles of the autophagic receptor SQSTM1/p62 (sequestosome 1) (described as p62-bodies) in macrophages. We suggest that the formation of p62-bodies represents a fast mechanism of NFE2L2/Nrf2 (nuclear factor, erythroid 2 like 2) activation by quantitative recruitment of KEAP1 (kelch-like ECH-associated protein 1) Further, the autophagy receptor TAX1BP1 (tax1 binding protein 1) and ubiquitin-editing enzyme TNFAIP3/A20 (TNF alpha induced protein 3) could be identified in DHA-induced p62-bodies. Simultaneously, DHA strongly dampened the induction of pro-inflammatory genes including CXCL10 (C-X-C Motif Chemokine Ligand 10) and we suggest that formation of p62-bodies and activation of NFE2L2 leads to tolerance towards inflammatory stimuli. Finally, reduced CXCL10 levels were related to the improved clinical outcome in n-3 PUFA supplemented heart-transplant patients and we propose CXCL10 as a robust marker for the clinical benefits mobilized by n-3 PUFA supplementation.

Introduction

Low grade persistent inflammation is involved in the initiation and progression of age-related diseases.[1] The individual risk of developing inflammation-related diseases clearly varies due to genetic and environmental differences. The diet is an important factor influencing the immune system, and a diet rich in marine n-3 PUFAs possesses anti-inflammatory properties. Beneficial effects of n-3 PUFAs involve a changed membrane lipid composition and the promoted synthesis of anti-inflammatory and pro-resolving eicosanoids.[2] Also, regulatory signaling by n-3 PUFAs has been reported, among others via the selective GPR120 (G-protein coupled receptor 120) leading to reduced activity of NFKB (nuclear factor kappa B) complex and the inflammasome.[3-5]

Macroautophagy (hereafter referred to as autophagy) is a catabolic process that eliminates damaged and excessive intracellular components by lysosomal degradation.[6] Cytoplasmic proteins and organelles are sequestered by a growing double membrane that forms an autophagosomal vesicle where the content is degraded after fusion with a lysosome.[7] The autophagic receptor SQSTM1 binds to ubiquitinated cargos and interacts with mammalian orthologues of yeast *ATG8* (autophagy related 8) on the growing autophagosomal membrane leading to selective degradation.[8]

Homopolymerization of SQSTM1 is important for efficient sequestration and turn-over of cargo.[9] In case of altered protein degradation or in response to cellular stresses, the accumulation of SQSTM1 in ubiquitin (Ub) positive so called p62-bodies (also known as aggresome-like induced structures (ALIS)) has been described and might provide a temporary form of storage for unfolded proteins to be destroyed.[10] Autophagy is important to prevent inflammation prone conditions by

maintaining cellular homeostasis but also directly by selective elimination of invading pathogens (xenophagy)[11-13] and the removal of activated inflammasomes.[14-16]

As for autophagic removal of cargo, several inflammatory responses depend on ubiquitination for complexation, activation or degradation of the signaling proteins.[17] Interestingly, SQSTM1 can also have a pro-inflammatory role as its ubiquitin-binding and polymerizing abilities were necessary for NF-κB complex activation, suggesting a scaffold function in the formation of signaling complexes.[18, 19]

We recently reported that physiological relevant doses of the n-3 PUFA DHA elevated levels and cytosolic structures of SQSTM1 and increased turnover of polyubiquitinated proteins in retinal pigment epithelial cells (Arpe-19), possibly reducing the risk of age-related macular degeneration (AMD).[20] We thus hypothesized that n-3 PUFAs affect autophagy or ubiquitination to mobilize an anti-inflammatory effect in macrophages. We here report that human primary monocyte derived macrophages (MDM) and RAW264.7 mouse macrophages respond to n-3 PUFAs by induction of SQSTM1 mRNA and protein levels followed by a transient rise in the number of ALIS like p62-bodies. While p62-bodies are present, there is a clearly dampened response to lipopolysaccharide (LPS), particularly by a reduced IFN (interferon) type-I response. Consistently, the reduced secretion of the IFN response protein CXCL10 is evident both in macrophage cell cultures and in blood samples from patients on n-3 PUFA supplements.

Results

The n-3 PUFA DHA induces p62-bodies in macrophages

To determine if n-3 PUFAs affect localization of SQSTM1 and ubiquitinated proteins in macrophages, RAW264.7 were supplemented with 70 μM of the n-3 PUFA DHA, the n-6 PUFA arachidonic acid (AA) or the n-9 monounsaturated fatty acid oleic acid (OA). The cells were immunostained for SQSTM1 and conjugated Ub. Confocal inspection showed formation of p62-bodies in response to DHA in a lipid selective manner. Further, the DHA-induced p62-bodies co-localized more stringently with ubiquitinated proteins (**Figure 1A**) than with MAP1LC3B (microtubule associated protein 1 light chain 3 beta) (data not shown), thus representing aggregates rather than autophagosomes. The number of p62-bodies and co-localization with Ub transiently increased up to 16 h and decayed to basal levels after 24 h (**Figure 1B**). Consistently, also the level of SQSTM1 and Ub-conjugated proteins rose in response to DHA supplementation in RAW264.7 in a time and concentration dependent manner (**Figure 1C, S1A and S1B**).

Human primary MDM displayed the same lipid-specific increase in p62-bodies and co-localized ubiquitin after DHA supplementation (**Figure 1D**). Automated image analysis demonstrated that the average number of p62-bodies increased up to 24 h after DHA supplementation and decreased thereafter (**Figure 1E**), thus showing the same transient rise although at a slightly slower kinetics compared to the mouse macrophages. MDMs similarly demonstrated a selective increase in the protein levels of SQSTM1 and conjugated Ub in response to DHA while no increase was observed after treatment with OA or AA (**Figure 1F**)**.** Interestingly, the increase in protein level of SQSTM1 was observed already after 6 h treatment with DHA and was further increased with time up to 24 h (**Figure S1C**).

DHA induces formation of detergent resistant p62-bodies degraded by autophagy and activates NFE2L2

DHA supplementation caused a striking translocation of SQSTM1 from a mainly

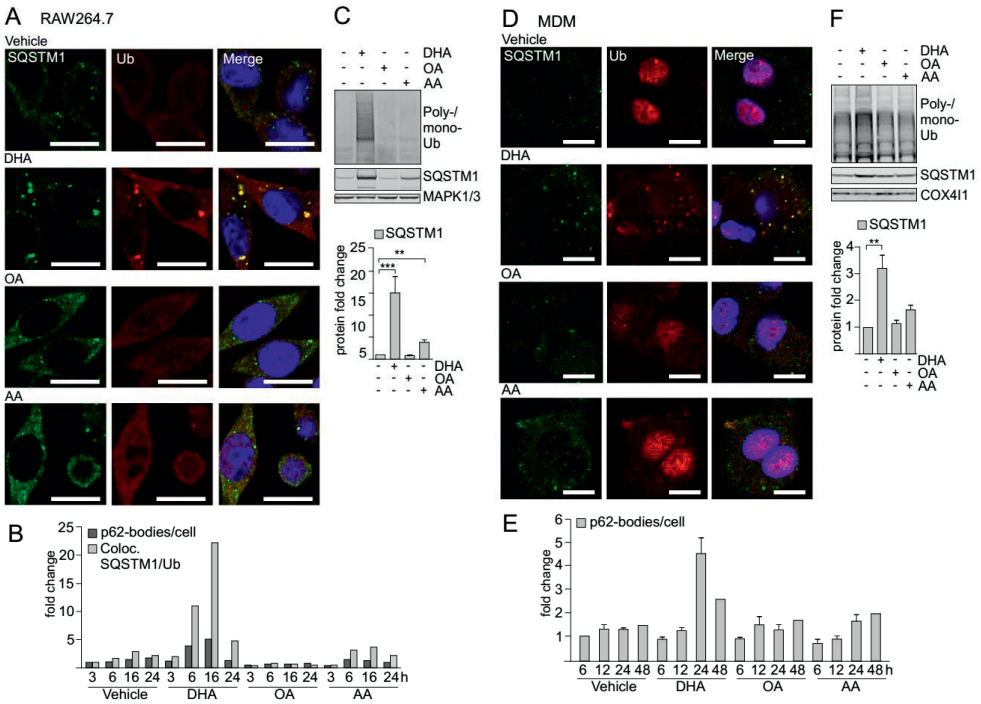

FIGURE 1. The n-3 PUFA DHA induces p62-bodies in macrophages. (**A**) Confocal analysis of RAW 264.7 treated with DHA, OA or AA (70 µM) for 8 h (scale bars, 10 µm) and (**B**) automated quantification of SQSTM1- and Ub-positive speckles (< 7000 cells/condition). Data are representative of three independent experiments of which two were manually counted. (**C**) Immunoblot analysis (IB) and quantification (below) of RAW 264.7 treated with DHA, OA and AA (70µM) for 16 h, n=4, (repeated-measures ANOVA with Dunnett's). (**D**) Confocal analysis of MDMs treated with DHA, OA or AA (70 µM) for 24 h (scale bars, 10 µm) and (**E**) automated quantification of p62-bodies (< 4000 cells/condition), n=3 except for 48 h where n=1. (**F**) IB and quantification (below) of MDMs treated with DHA, OA and AA (70µM) for 16 h, n=5, (Friedman test with Dunn's).

diffuse cytoplasmic location into cytoplasmic bodies associated with ubiquitinated proteins. Such protein deposits or ALIS tend to be resistant to detergents.[21, 22] To determine if DHA-induced p62-bodies were detergent resistant, the pellets that remained after centrifugation in a detergent lysisbuffer were resolved in 8 M urea buffer. Interestingly, the protein level of SQSTM1 in the detergent resistant pellet was clearly elevated after DHA supplementation in RAW264.7 (**Figure 2A**) and increased with centrifugation speed (**Figure S1D**). Thus, for all the rest of the study, cells were lysed directly in 8 M urea buffer to avoid losing part of the SQSTM1 pool.

SQSTM1 is continuously turned over by autophagy.[21] A combined treatment with DHA and the lysosomal inhibitor bafilomycin A_1 (BafA1) caused an additive increase in the protein level of SQSTM1 in the detergent resistant fraction. However, no concurrent increase in the protein level of MAP1LC3B was observed after BafA1 treatment (**Figure 2A**). This shows that autophagy can be elevated as measured by the number of SQSTM1 proteins degraded per cell, without affecting the turnover of

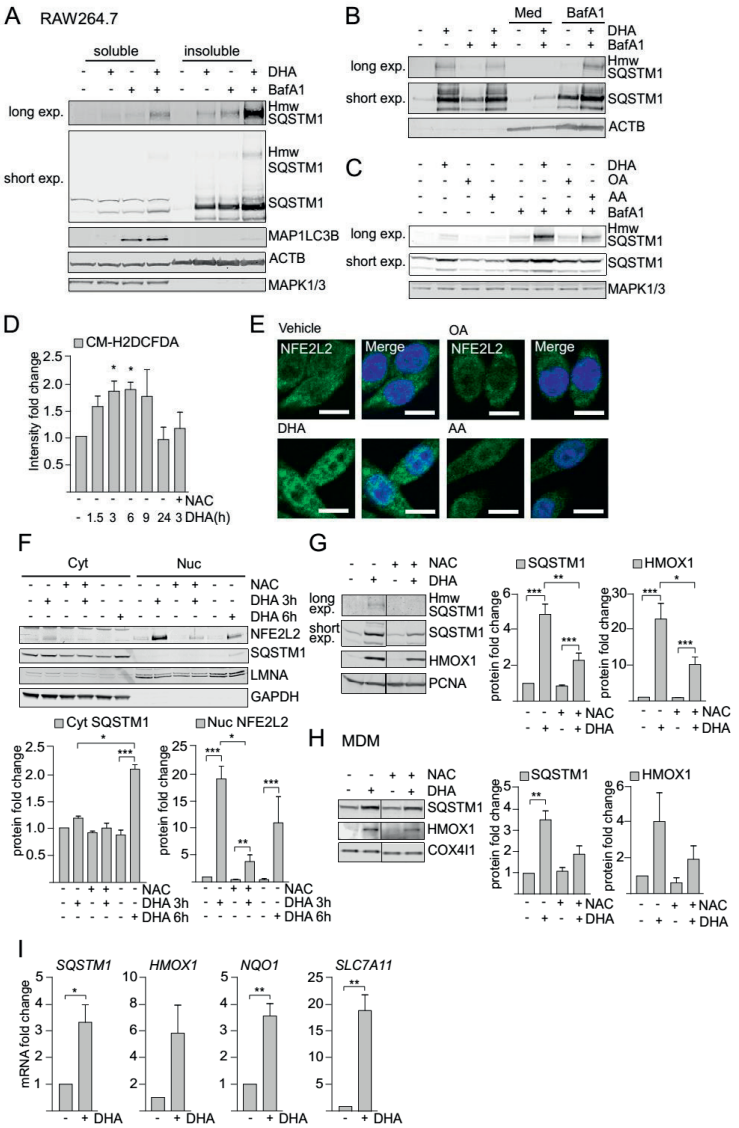

FIGURE 2. DHA induces formation of detergent resistant p62-bodies degraded by autophagy and activates NFE2L2. IB of soluble and insoluble fractions of RAW 264.7 treated with DHA (70 µM) w/wo BafA1 (100 nM) for (**A**) 16 h or (**B**) 8 h followed by 12 h incubation in fresh medium (Med) w/wo BafA1. Hmw SQSTM1 was visualized by long exposure (exp). (**C**) IB of RAW 264.7 treated with DHA, OA or AA (70 µM) w/wo BafA1 (100 nM) for 8 h. Data are representative of three independent experiments. (**D**) FACS analysis of ROS levels in RAW264.7 at different time points after DHA (70 µM) using a fluorescent CM-H2DCFDA probe, n=3, (paired t-test). (**E**) Confocal analysis of RAW264.7 treated with DHA, OA or AA (70 µM) for 3 h (scale bars, 10 µm). Representative images are shown, n=3. (**F**) IB analysis of cytosolic (Cyt) or nuclear (Nuc) fractions after 3 h DHA w/wo addition of 5mM NAC or 6 h DHA and quantification of cytosolic SQSTM1 levels and nuclear NFE2L2 levels (below), n=3, (repeated-measures ANOVA with Dunnett's). (**G**) IB analysis and quantification (right) of RAW264.7 after 3 h DHA w/wo addition of 5mM NAC, n=3, (repeated-measures ANOVA with Dunnett's). (**H**) IB analysis and quantification (right) of MDM after 3 h DHA w/wo addition of 5mM NAC, n=3. (**I**) QRT-PCR analysis of NFE2L2 target genes *SQSTM1*, *HMOX1*, *NQO1* and *SLC7A11* in vehicle and DHA treated MDMs, n=5 (Friedman test with Dunn's).

MAP1LC3B-II. Interestingly, DHA also induced some slower migrating proteins detected by SQSTM1 antibodies in both RAW264.7 and MDMs (**Figure 2A and S1E**) and clearly reduced by siRNA targeting SQSTM1 (**Figure S1E).** The same migration pattern was detected using five different SQSTM1 specific antibodies in Arpe-19 cells (**Figure S1F**). The two most prominent of these additional SQSTM1 bands migrated at approximately 70 kDa and 120 kDa, of which the latter is indicated as high molecular weight (hmw) SQSTM1. Each of the additional bands accounted for about 5% of total SQSTM1 levels in DHA and BafA1 treated cells. The hmw SQSTM1 showed the same concentration- and time-dependent increase as unmodified SQSTM1 (**Figure S1A and S1B**), was especially prominent in the detergent resistant fraction and was strikingly elevated after cotreatment with DHA and BafA1 (**Figure 2A).** Importantly, hmw SQSTM1 could be depleted from the soluble fraction and enriched in the detergent resistant pellet by increasing centrifugation speed (**Figure S1D**). The elevated protein levels of SQSTM1 and hmw SQSTM1 were of transient nature. Removal of DHA and incubation for additional 12 h in fresh medium clearly reversed the DHA-induced effects (**Figure 2B**). The reduction in SQSTM1 protein levels could be blocked by adding BafA1 in the recovery medium, suggesting turnover by autophagy. As for the p62-bodies, hmw SQSTM1 was mainly induced by DHA (**Figure 2C**). Together, these results demonstrate that DHA induces SQSTM1 protein levels, posttranslational modifications and translocation of the protein into detergent-resistant cytoplasmic bodies in a lipid selective manner. Furthermore, the formed structures are of transient nature and degraded by autophagy.

The *SQSTM1* gene is induced in response to cellular stress like reactive oxygen species (ROS) via the transcription factor NFE2L2[23, 24] and ROS are involved in the formation of ALIS.[22] We have previously found that DHA induced transient ROS and NFE2L2 nuclear translocation in Arpe-19 cells.[20] Also in macrophages, DHA induced ROS at time points preceding the increase in SQSTM1 protein levels as measured by a fluorescent ROS DCF probe (**Figure 2D**). Nuclear translocation of NFE2L2 could be observed by confocal microscopy (**Figure 2E**) and immunoblotting of cytosolic (Cyt) and nuclear fractions (Nuc) (**Figure 2F**). Consistent with a NFE2L2-mediated anti-oxidative response, the NFE2L2 target protein HMOX1 (hemeoxygenase 1) showed a similar increase as SQSTM1 in response to DHA (**Figure 2G**). Both the DHA induced ROS increase, translocation of NFE2L2 and rise in SQSTM1 and HMOX1 protein levels was strongly reduced by a pretreatment with the ROS scavenger N-acetyl cysteine (NAC) (**Figure 2D, 2F and 2G**). Similarly, in MDMs, HMOX1 protein levels increased in response to DHA, but clearly less so in the presence of NAC (**Figure 2H and S1C**). Importantly, DHA induced the transcriptional activity of NFE2L2 as measured as increased mRNA levels of its target genes *SQSTM1*, *HMOX1*, *NQO1* (NAD(P)H quinone dehydrogenase 1) and *SLC7A11* (solute carrier family 7 member 11) in MDMs (**Figure 2I**).

DHA strongly reduced expression of CXCL10 by affecting several signaling pathways

Anti-inflammatory effects of n-3 PUFAs have been determined as reduced induction of diverse pro-inflammatory cytokines. To identify putative anti-inflammatory effects of DHA in our system, we determined if macrophages respond differently to LPS when pretreated with DHA for 16 hours. This time point was used since the number of p62-bodies peaked at this time. As an initial screen, NanoString technology was

used to quantify the levels of 579 inflammation-related transcripts in DHA-pretreated MDMs from 2 donors that were stimulated with LPS for 3 hours. Of these genes, 163 were selected as LPS-responsive transcripts as their mRNA levels increased more than 1.5-fold in each donor. Among those, 148 were downregulated and 15 upregulated by DHA pretreatment (**Figure 3A and Table SI**). The clearest reduction was seen for *CXCL9, CXCL10* and *CXCL11*, a subfamily of chemokines that are highly inducible by IFNγ and signal through their common receptor CXCR3 (C-X-C motif chemokine receptor 3)[25] to attract further immune cells. CXCL10 is the best studied of these and is implicated in diseases with underlying inflammation.[26] Of note, classical NFKB target genes including *TNF* (tumor necrosis factor), *IL1B* (interleukin 1 beta) and *IL6*, displayed only minor or opposite changes by DHA pretreatment in this screen. These observations were validated by qRT-PCR as in primary macrophages isolated from five different healthy donors, DHA pretreatment caused an approximately 80% reduction in the LPS-induced expression of *CXCL10* in a lipid selective manner (**Figure 3B and 3C**). The lowered mRNA levels of *CXCL10* were accompanied by reduced secretion of CXCL10 measured by ELISA (**Figure S2A**). Also in RAW264.7 macrophages, we observed a significant decrease in the induction of *Cxcl10* but also *Tnf* mRNA after DHA pretreatment and stimulation with LPS for 3 h (**Figure S2B**). We thus asked if this difference could be explained by different kinetics of *TNF* induction in both systems. Indeed, in MDMs, DHA pretreatment cause reduction in the LPS induction of *TNF* mRNA levels at earlier time points and was more pronounced at the peak of *TNF* expression at 2 h of LPS stimulation (**Figure S2C**). The DHA-mediated decrease in *CXCL10* was not limited to induction by LPS but could also be seen with other inducers of IFN signaling as TLR3 (toll-like receptor 3) ligand poly(p)(I:C) and the synthetic TLR8 ligand pU (**Figure S2D**). Overall, these data show that DHA particularly dampens type-I IFN responses, including CXCL10-induction, in a lipid selective manner in mouse and human macrophages.

The synthesis of CXCL10 is regulated at transcriptional level by the transcription factors NFKB complex, IRF3 (interferon regulatory factor 3), IRF1 and STAT1 (signal transducer and activator of transcription 1).[27, 28] To determine which initial signaling axis DHA affects, phosphorylation of IRF3 (S386) and the NF-κB subunit RELA/p65 (S536) was analyzed. DHA pretreatment led to reduced levels of phosphorylated IRF3 in response to LPS in MDMs while no change in phosphorylation of RELA (S536) was observed (**Figure 3D**). We also checked subunit NFKB1/p105 (S933) of the heterodimeric NFKB complex, which showed a reduced phosphorylation in response to LPS after DHA pretreatment (**Figure S2E**). In RAW264.7, DHA similarly reduced the phosphorylation of IRF3 (S396) and NFKB1 (S933) but not RELA (S536) (**Figure S2F**). We then analyzed the phosphorylated, active forms of IKBKB/IKKβ (inhibitor of nuclear factor kappa B kinase subunit beta) and TBK1 (TANK-binding kinase 1) that are upstream kinases of classical NFKB and IRF3 signaling, respectively.[29] Surprisingly, in MDMs, DHA pretreatment decreased the LPS-induced levels of phosphorylated IKBKB but not TBK1 (**Figure 3E**).

Besides direct induction via IRF3 and NFKB, *CXCL10* is induced as a secondary response gene in an IFN-dependent manner. After TLR4 stimulation, secreted IFNs bind to IFNAR1 (interferon alpha and beta receptor subunit 1) in an autocrine manner and activate JAK2 (janus kinase 2)-STAT1 signaling and synthesis of CXCL10.[30, 31] We found about 50% reduced *IFNB* and *IFNG* mRNA induction after DHA pretreatment in

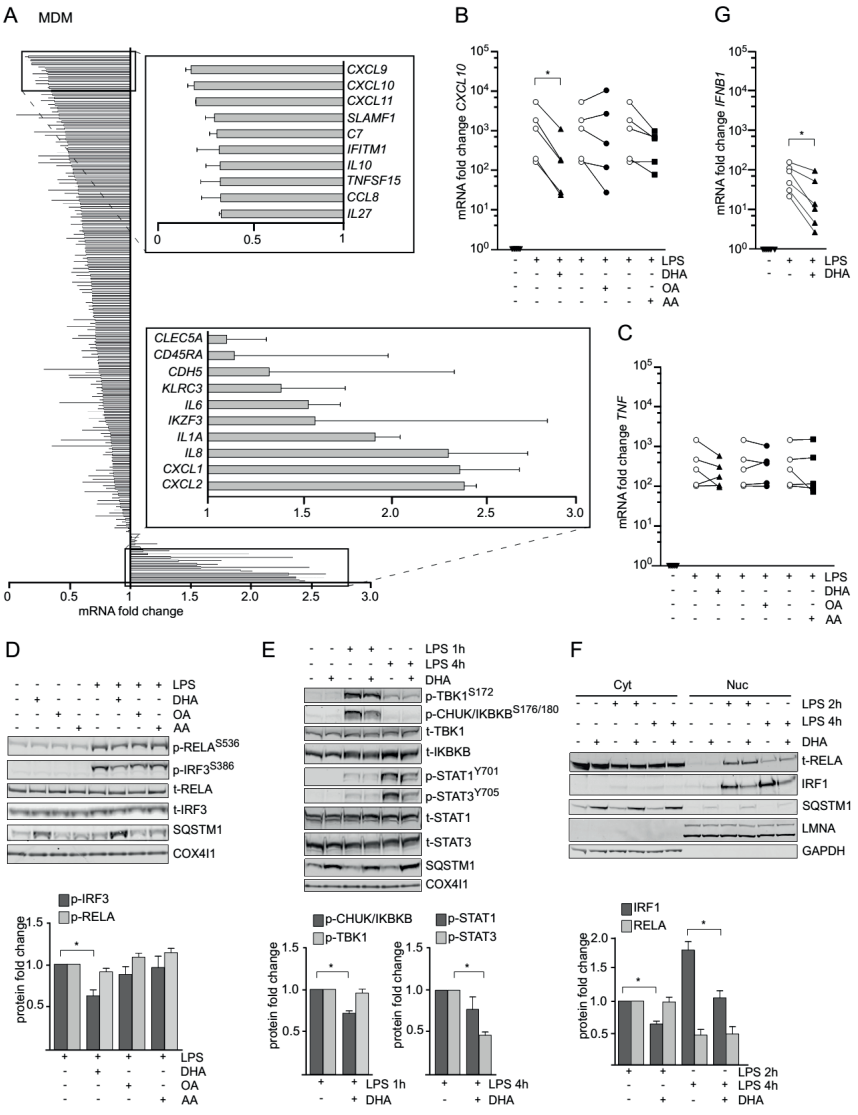

FIGURE 3. The n-3 PUFA DHA strongly reduced expression of CXCL10 by affecting several signaling pathways. (**A**) MDMs were treated with DHA (70 µM) for 16 h before stimulation with LPS (100 ng/ml) for 3 h. Transcript levels of 579 genes (nCounter GX Human Immunology kit assay) were determined. Transcripts that were more than 1.5-times increased by LPS in both donors were selected and the fold change between vehicle- and DHA-treated cells calculated, mean ± SD, n=2. (**B**) QRT-PCR analysis of *CXCL10* and (**C**) *TNF* in MDM treated with DHA, OA or AA for 16 h and LPS for 3 h, n=5 (Friedman test with Dunn's). (**D**) IB analysis and quantification of IRF3 and RELA phosphorylation in MDMs treated with DHA, OA or AA for 16 h and LPS for 1 h, n=5. (Friedman test with Dunn's), of (**E**) TBK1, IKBKB, STAT1 and STAT3 phosphorylation in MDM treated with DHA, OA or AA for 16 h and LPS for 1h or 4h, n=5. (Wilcoxon matched-pairs signed rank test, one-tailed) and of (**F**) IRF1 and RELA in cytosolic (Cyt) and nuclear (Nuc) fractions in MDMs treated with DHA, OA or AA for 16 h and LPS for 2h or 4h, n=5 (Wilcoxon matched-pairs signed rank test). (**G**) *IFNB1* mRNA levels after incubation with DHA for 16 h and LPS for 2 h, n=6 (Wilcoxon matched-pairs signed rank test).

the NanoString data (**Table SI**), and could confirm limited induction of *IFNB* mRNA 2 h after LPS stimulation in DHA pretreated MDMs (**Figure 3G**). Moreover, inhibition of IFNAR1 suppressed LPS-mediated CXCL10-induction similarly to DHA and limited further reduction by DHA (**Figure S2G**), demonstrating that the n-3 PUFA supplementation also dampened autocrine IFN signaling. Consistently, DHA pretreatment decreased the phosphorylation of both STAT1 and STAT3 when induced by 4 h LPS stimulation, however more pronounced and only significantly for STAT3 (**Figure 3E**). Also in RAW264.7 macrophages DHA but not OA or AA pretreatment dampened the activation of STAT1 (**Figure S2F**).

Of note, in the mRNA expression screen DHA pretreatment also reduced the LPS inductions of *IRF1, IRF4, IRF7* and *IRF8* (**Table SI**). IRF1 is strongly induced in response to IFN and other cytokines[32] and has been shown to bind to the *CXCL10* promotor.[33] Therefore, we investigated if nuclear translocation of IRF1 is affected by DHA. Immunoblotting showed a clear increase of nuclear IRF1 levels after 2 h and 4 h of LPS stimulation that was clearly reduced in DHA pretreated MDMs. RELA could also be detected in nuclear lysates after 2 h of LPS stimulation, but was unchanged by DHA-pretreatment (**Figure 3F**). In addition, confocal image analysis showed increased intensity of nuclear IRF1 staining at 2 h of LPS stimulation, which was limited by DHA pretreatment while number, area and maximum radius of p62-bodies were augmented (**Figure S2H**).

The DHA-mediated inflammatory tolerance is related to the formation of p62-bodies

The results presented above demonstrate that DHA selectively induced the formation of p62-bodies or ALIS and reduced LPS-induced CXCL10 expression. To assess the relation between both observations, MDMs were pretreated with DHA for increasing times and finally stimulated with LPS for 1 h. Interestingly, the decrease in phosphorylated IRF3 and IKBKB was clearest when SQSTM1 levels were most induced (**Figure 4A**).

We thus speculated that formation of p62-bodies could play a role in establishing tolerance towards inflammatory signaling. We first checked if impaired formation of p62-bodies would impair downregulation of *CXCL10* by DHA. As shown above, the induction of SQSTM1 by DHA could be prevented by adding the anti-oxidant NAC. Treatment with NAC before stimulation with LPS limited CXCL10 induction to a similar extent to DHA alone, showing little further effect of the combination of NAC and DHA (**Figure 4B**). SQSTM1 is known to be necessary for the formation of ALIS [22]. Thus we speculated that knockdown of SQSTM1 would interfere with the formation of p62-bodies and the downregulation of CXCL10 by DHA. SQSTM1 was shown to have anti-inflammatory[12, 16, 34] but also pro-inflammatory effects itself.[18, 19] In line with the pro-inflammatory role of SQSTM1, the SQSTM1-depleted MDMs displayed reduced expression of *CXCL10* in response to LPS. While the reduction by DHA was less in SQSTM1 depleted cells, CXCL10 levels were similar in DHA pretreated cells in presence or absence of SQSTM1 (**Figure 4C**). SQSTM1-depleted MDMs, also showed decreased levels of phosphorylated IKBKB and IRF3, although not significant for IRF3, and no further decrease by DHA treatment (**Figure 4 D**).These data suggest that the formation of p62-bodies is important for the LPS tolerance induced by n-3 PUFAs like DHA.

KEAP1 is sequestered in DHA-induced p62-bodies and contributes to inflammatory tolerance

The obtained results indicated that the DHA-

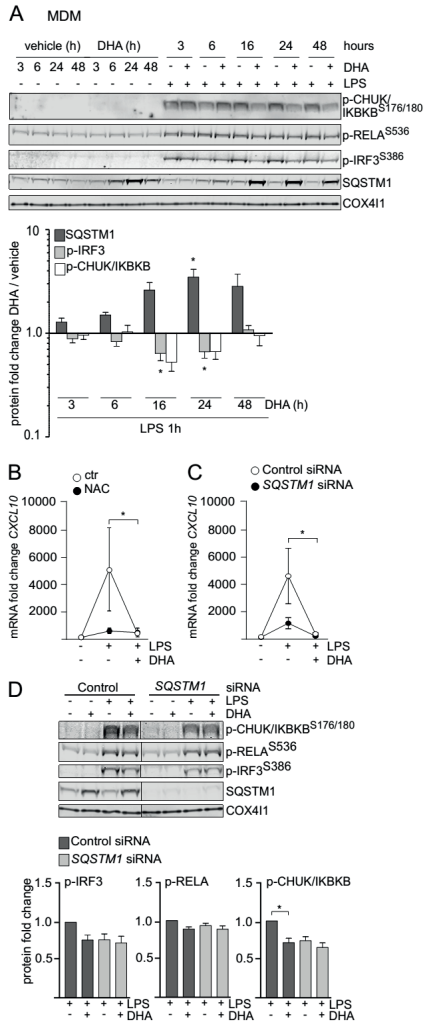

FIGURE 4. The DHA-mediated inflammatory tolerance is related to the formation of p62-bodies. (**A**) IB analysis of MDMs treated with DHA (70 µM) for indicated times followed by LPS for 1 h. The quantification (below) represents mean fold changes ± SEM of DHA + LPS compared to vehicle + LPS on log scale, n=6, (Friedman test with Dunn's). (**B**) QRT-PCR analysis of CXCL10 levels in MDM after 16 h DHA w/wo addition of 5mM NAC followed by 3 h LPS, n=3, (repeated-measures ANOVA with Dunnett's). (**C**) QRT-PCR analysis of *CXCL10* in *SQSTM1* siRNA transfected MDMs treated with DHA for 16 h and LPS for 3 h, n=5, (Friedman test with Dunn's). (**D**) IB analysis of IRF3, RELA and IKBKB phosphorylation in control and *SQSTM1* knockdown MDMs, n=6 (Friedman test with Dunn's).

mediated formation of p62-bodies dampened LPS-induced signaling in macrophages. We therefore aimed to characterize the proteins recruited into the DHA induced p62-bodies that mediate the tolerance towards inflammatory stimuli.

The previously established centrifugation protocol was used for partial purification of DHA-induced p62-bodies from RAW264.7 cells and the isolated proteins were identified by mass spectrometry (MS). Proteins were considered as DHA-specifically enriched in the detergent insoluble fraction if more abundant in DHA treated samples versus vehicle as well as further elevated in samples co-treated with DHA and BafA1 versus BafA1 alone. Using these criteria, SQSTM1 was clearly the most abundant DHA- and BafA1-induced protein in the detergent resistant fraction (**Figure S3A**). Interestingly, TAX1BP1 and KEAP1 were the second and third most prominent proteins recruited into the detergent resistant fraction in response to DHA and clearly further accumulated by inhibiting lysosomal degradation. However, since this analysis was only based on different detergent solubility, the detected proteins could reside in many different compartments and structures. In order to limit the detection of proteins to DHA-induced partners of SQSTM1, we performed immuno-precipitation (IP) of endogenous SQSTM1 from vehicle treated or DHA-stimulated RAW264.7 and identified co-purified interaction partners by MS (**Figure S3B**). KEAP1 and TAX1BP1 were the only proteins that were found both in the detergent resistant fraction as well as co-immunoprecipitated with SQSTM1 and were thus considered as located to SQSTM1 containing protein bodies in a DHA-induced manner**.** These interactions were validated in MDMs where KEAP1 and TAX1BP1 co-immunoprecipitated with SQSTM1 (**Figure 5A**). Apparently, DHA did not change the affinity of TAX1BP1 or KEAP1 for

SQSTM1 but the association increased proportionally with the lipid induced rise in SQSTM1 protein level.

TAX1BP1 is an autophagy receptor involved in xenophagy[35] and could bind SQSTM1 indirectly via ubiquitin. KEAP1 mediates ubiquitination and degradation of NFE2L2 under normal unstressed conditions[36] and has been suggested to contribute to degradation of protein aggregates.[37] Also, the association of KEAP1 with p62-bodies has been shown to stabilize and activate NFE2L2.[38] NFE2L2 activation, besides its anti-oxidative function, has been shown to play an important role in the regulation of inflammation.[39-42] In addition, we recently demonstrated a role for KEAP1 in the regulation of inflammatory signaling, via ubiquitination and degradation of IKBKB.[43] Intriguingly, TAX1BP1 has been implicated as a negative regulator of NFKB signaling due to the recruitment of the ubiquitin-editing enzyme TNFAIP3.[44] Also, IRF3-dependent signaling is negatively regulated by TAX1BP1 and TNFAIP3.[45, 46] Immunostaining of MDMs demonstrated that both KEAP1 and TAX1BP1 are located in DHA-induced p62-bodies (**Figure 5B and S3C**). In contrast to MS data and IPs, by confocal analysis also TNFAIP3 could be detected in p62-bodies (**Figure S3C**) indicating that this protein interacts with the p62-bodies in a more transient manner. Importantly, about 20% of KEAP1 was found in insoluble aggregates and corresponded well with the fraction of SQSTM1 that translocated to the detergent resistant fraction in response to DHA treatment (**Figure 5C**). As the sequestration of KEAP1 by SQSTM1 has previously been shown to lead to NFE2L2 activation, we analyzed the mRNA level of *NQO1* in SQSTM1 knockdown cells treated with LPS and DHA. In line with an important role of SQSTM1 in activation of NFE2L2 in presence of DHA, *NQO1* mRNA (as opposed to CXCL10) was significantly

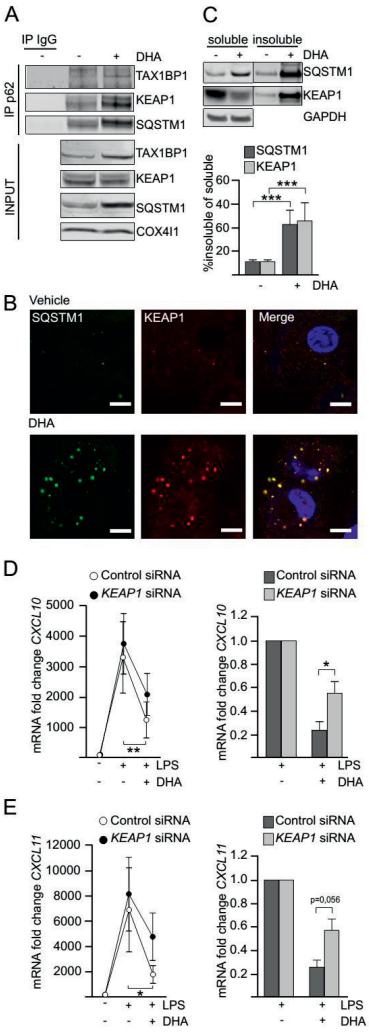

FIGURE 5. KEAP1 is sequestered in DHA-induced p62-bodies and contributes to inflammatory tolerance. (**A**) IP of SQSTM1 from MDMs treated with DHA for 16 h and IB analysis of KEAP1 and TAX1BP1, INPUT (10%). Data are representative of 3 independent experiments. (**B**) Immunostaining for SQSTM1 and KEAP1 in MDMs treated with DHA for 24 h (scale bars, 10 µm). (**C**) IB analysis and quantification (below) of SQSTM1 and KEAP1 in soluble and insoluble fractions from RAW264.7 after 16 h DHA, n=4 (repeated-measures ANOVA). (**D**) QRT-PCR analysis of *CXCL10* or (**E**) *CXCL11* in *KEAP1* siRNA transfected MDMs treated with DHA for 16 h and LPS for 3 h, n=5 (Friedman test with Dunn's). (right) Comparison of the downregulating effect of DHA in control or *KEAP1* siRNA treated MDMs from (**D**) or (**E**) respectively, n=5, (Mann Whitney test).

induced by DHA in a SQSTM1-dependent manner (**Figure S3D**).

We next asked if recruitment of KEAP1, TAX1BP1 or TNFAIP3 into p62-bodies have a repressive function on CXCL10 signaling. MDMs were transfected with siRNA targeting *KEAP1, TAX1BP1* and *TNFAIP3* and decreased protein or mRNA levels were observed, albeit TNFAIP3 was less efficiently reduced (**Figure. S3E, S3F**). As KEAP1 is the repressor of NFE2L2, the expression of NFE2L2 target genes was analyzed in KEAP1-depleted MDMs and showed at least partial activation of NFE2L2 with induction of *NQO1* and *SLC7A11* (**Figure S3G**). Depletion of *TAX1BP1* or *TNFAIP3* did not have any significant effect on *CXCL10* levels (**Figure S3H and S3I**) while depletion of KEAP1 significantly limited the ability of DHA to dampen LPS-induced *CXCL10* expression (**Figure 5D**). When directly comparing the reduction by DHA-pretreatment on LPS-induced CXCL10 expression, there was a significant difference in presence or absence of KEAP1 (76.4% ± 16.4% vs 44.8% ± 22.2% respectively, with less effective DHA pretreatment in KEAP1-depleted cells in all 5 replicates) (**Figure 5D, right**). The same pattern could be seen for mRNA induction of *CXCL11* (**Figure 5E**), another chemokine strongly affected by DHA. Thus, the formation of p62-bodies and sequestration of KEAP1 plays a role in the DHA-mediated inflammatory tolerance in macrophages.

The plasma level of CXCL10 is reduced by n-3 PUFA supplementation but increased in the placebo group in hypertensive heart transplant recipients.

According to our results, we expected n-3 PUFA supplementation to be most effective in conditions with chronically elevated CXCL10 levels. Therefore, we obtained plasma samples of the heart transplant patients in the OmaCor-study, as elevated levels of CXCL9 and CXCL10 are known as causes for transplant rejection.[47, 48] Plasma samples of these patients were taken at baseline after transplantation and following 12 months with daily administration of n-3 PUFA (DHA and eicosapentaenoic acid (EPA)) or placebo (corn oil). This clinical trial documented an improved clinical outcome from intake of n-3 PUFAs compared to the placebo group as measured for example by lowered blood pressure.[49] However, corresponding reduction in any inflammatory mediators in response to the n-3 PUFAs have not been previously found. In fact, TNFα was slightly increased by n-3 PUFA treatment in this study.[50] We measured CXCL10 by ELISA on those patient plasma samples and found the CXCL10 levels in the placebo group increased after transplantation in line with a non-resolved inflammation in these patients. On the other hand, in total agreement with the results from the cell based assays, n-3 PUFA administration significantly decreased CXCL10 levels after transplantation (**Figure 6, left**). In line with our expression analyses, the IL6 levels did not differ between the placebo and the n-3 PUFA group (**Figure 6, right**). These data suggest that n-3 PUFA supplementation dampens conditions driven by sterile inflammation and that a reduced type-I interferon response, including lowered CXCL10, represents potent markers for such beneficial effects.

To relate this observation to our in vitro mechanism, we wanted to assess NFE2L2 activation in our patient material. IL36G has been shown as direct target gene of NFE2L2 and up-regulated by NFE2L2 activation.[51] We measured IL36G levels as read-out for NFE2L2 and found an inverse correlation between CXCL10 and IL36G levels at baseline ($r=-0.57$, $p<0.05$), suggesting that NFE2L2 activity contributes to limit CXCL10 levels.

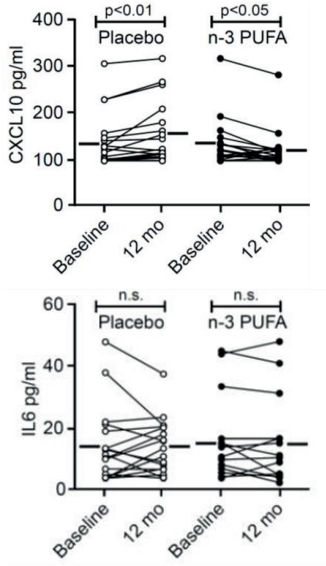

FIGURE 6. The plasma level of CXCL10 is reduced by n-3 PUFA supplementation but increased in the placebo group in hypertensive heart transplant recipients. The concentration of CXCL10 (upper panel) and IL6 (lower panel) was determined by ELISA in plasma of hypertensive heart transplant recipients isolated at baseline and after 12 months of daily supplementation of placebo (corn oil, n=19) or n-3 PUFAs (n=16). Horizontal lines represent mean plasma levels. p<0·01, p<0·05 and non-significant (n.s.) were all vs baseline.

In summary, our data are consistent with an important role of DHA-induced SQSTM1 polymerization and resulting NFE2L2 activation in the reduction of serum levels of CXCL10.

Discussion

With increasing evidence of the health beneficial and anti-inflammatory effects of n-3 PUFAs, these lipids represent a valuable tool to identify cellular mechanisms of maintenance and homeostasis. In response to poly-unsaturated fatty acids, we found that macrophages dampened pro-inflammatory signaling as evident by reduced transcription of a large number of inflammation related genes after LPS stimulation. The effects were clearest for type-I IFN related responses with CXCL10 as the most prominent. Importantly, CXCL10 protein levels were reduced both in the media of cultured macrophages and in blood samples of patients supplemented with n-3 PUFAs. We found that p62-bodies, protein bodies containing the autophagic cargo receptor SQSTM1, are formed in response to the n-3 PUFA DHA. The ROS sensor KEAP1, the autophagy receptor TAX1BP1 and the ubiquitin-editing enzyme TNFAIP3 could be found in p62-bodies. Of these proteins, depletion of KEAP1 led to limited effects of DHA supplementation. We suggest that the DHA-mediated formation of p62-bodies and recruitment of KEAP1 represent a fast mechanism for NFE2L2 activation, playing a role in the regulation of inflammatory signaling in macrophages. Thus, the data point to an important balance where dietary lipids influence endogenous and exogenous antioxidants in controlling inflammatory signaling in macrophages.

SQSTM1 is known to form aggregates and to sequester ubiquitinated proteins into speckles also described as ALIS.[21] These structures were initially shown in dendritic cells during maturation[52] and may occur in response to various cellular stresses like LPS stimulation, infection, oxidative stress or proteasomal inhibition.[53] DHA supplementation led to a transient increase in detergent-resistant p62-bodies associated with ubiquitinated proteins. Sequestration of potentially harmful and misfolded proteins might help in detoxificaiton of the cytoplasm and could disarm inflammation-prone conditions. Interestingly, Sergin et al. reported a protective role of SQSTM1 in the sequestration of ubiquitinated inclusions in atherosclerosis, as deletion of SQSTM1 caused diffuse distribution of ubiquitinated and misfolded proteins and had worse outcomes.[54]

ALIS are reported to be degraded via the autophagic-lysosomal pathway.[8, 22] The increase in p62-bodies observed after DHA

supplementation was transient and reversible in case of functional autophagosome-lysosome fusion, indicating that DHA-induced p62-bodies are handled via lysosomal degradation. Further, our results suggest that the autophagic clearance of tightly packed protein speckles of SQSTM1 and associating proteins can be elevated without any major changes in basal autophagy as measured by the turnover of MAP1LC3B-II. SQSTM1 can homo-polymerize[55] and one molecule of MAP1LC3B likely intereacts with a chain of SQSTM1.[8] Thus, the autophagic turnover of SQSTM1 may increase by stimuli that increase polymerization without any change in MAP1LC3B degradation. How homopolymerization of SQSTM1 is regulated represents an interesting question as the ability of cargo receptors to polymerize may be an important regulatory step for autophagy. In this regard, we observed posttranslational modifications of SQSTM1, as seen by slower migrating protein forms in response to n-3 PUFA supplementation. SQSTM1 can form covalently crosslinked oligomers in response to ROS-inducing drugs in a PB1-dependent manner.[56] We find that DHA stimulates the formation of hmw SQSTM1, which might be explained by the generation of ROS or direct crosslinking of SQSTM1 as DHA is a highly reactive and easily oxidized molecule.[57] Also, a transient increase in hmw SQSTM1 as well as activation of NFE2L2 has been reported for other PUFAs.[58] Thus, covalent crosslinking of SQSTM1 dimers can be an important mode of regulation of protein turnover after oxidative stress also under non-cytotoxic conditions. Also our data indicate that crosslinked SQSTM1 in protein aggregates mediates a transient storage of damaged proteins that are degraded by autophagy after new-synthesis of unmodified SQSTM1. On the contrary, diets containing already oxidized n-3 PUFAs have been shown to increase inflammatory signaling[59], suggesting that the original reactivity of the molecule might be needed for beneficial effects. In this regard, an balanced level of exogenous anti-oxidants in the n-3 PUFA formulation seems important to prevent oxidation of the lipid but also allow activation of endogenous anti-oxidative responses.

We observed activation of NFE2L2 with coinciding induction of its target genes *SQSTM1, HMOX1, NQO1* and *SLC7A11* during DHA treatment in total agreement with what we recently reported also in non-transformed, diploid epithelial cells.[20] The interaction of SQSTM1 and KEAP1 and the sequestration of KEAP1 into p62-bodies has been shown as an alternative way to liberate and activate NFE2L2.[60, 61] We here describe the formation of p62-bodies with quantitative sequestration of KEAP1 in response to the physiologic stimuli DHA as a fast mechanism for NFE2L2 activation. In this line, both depletion of SQSTM1 or KEAP1 modulated NFE2L2 activation and impaired the DHA-mediated anti-inflammatory effects. NFE2L2 has been firmly established as a regulator of inflammation.[40, 62] NFE2L2 knockout-mice showed increased mortality and inflammatory markers like phosphorylated IRF3 during septic shock[41], including augmented expression of CXCL10.[39] In humans, NFE2L2 polymorphisms are associated with the development of gastric mucosal inflammation[63] and increased risk for acute lung injury characterized by inflammation.[64] Also, NFE2L2 and expression of HMOX1 have been implicated in the development of a macrophage phenotype with lower inflammatory signature.[42, 65, 66] Finally, in NFE2L2 knockout mice, the anti-inflammatory effects of DHA have been shown to be NFE2L2-dependent.[67]

Interestingly, exposure to LPS has not only been found to induce ALIS, but also to establish endotoxin tolerance, limiting

further inflammatory signaling in septic patients[68] and monocytes.[69] In addition, Vasconcellos et al. recently demonstrated that free heme and iron, as a result of hemolysis, induce ALIS as a way to maintain homeostasis.[70] These reports suggest that under physiologic conditions transient formation of p62-bodies could represent a beneficial mechanism of NFE2L2 activation. Of interest, a role of KEAP1 in the clearance of p62-bodies has been reported[37] while CXCL10 secretion was increased in autophagy deficient cells.[71] Thus, presence of KEAP1 in p62-bodies might promote their autophagic degradation, limiting CXCL10 expression. Furthermore, we have previously shown that KEAP1 dampens inflammatory signaling by degradation of IKBKB in mycobacterial infection[43], thus KEAP1 might have additional NFE2L2-independent ways of regulating inflammation.

Anti-inflammatory effects of n-3 PUFAs have been established on the expression of many cytokines, but differ between model systems. By taking a broad approach to screen for quantitative differences of more than 500 inflammation gene products, we could identify CXCL10 as the most clearly dampened cytokine after n-3 PUFA treatment. This cytokine was consistently lowered at both mRNA level and as secreted protein in mouse macrophages, primary human macrophages as well as in heart transplant patients in a lipid selective manner. Increased intake of DHA has been found to reduce the level of CXCL10 *in vivo* in a mouse model of stress-induced dry eye.[72] Also, n-3 PUFA supplementation decreased the CXCL10 concentration in hepatitis C patients.[73] Of note, one clinical trial suggested that n-3 PUFAs from flaxseed augmented the expression of NQO1 in cystic fibrosis patients [74], allowing to speculate that the formation of p62-bodies and activation of NFE2L2 as we have shown in macrophages might also play a role to limit CXCL10 during clinical DHA supplementation. This is further supported by the inverse correlation that we found in our patient samples between CXCL10 and the NFE2L2 controlled cytokine IL36G.[51] Importantly, we here show lowered CXCL10 levels to correlate with the better clinical outcome seen in n-3 PUFA supplemented heart transplant patients. This study used an n-3 PUFA formulation with the minimum of exogenous anti-oxidants. Our data show that exogenous anti-oxidants can interfere with the activation of the anti-oxidative response by DHA and might thus prevent health-beneficial effects. Patients with cardiovascular diseases, inflammatory disorders, microbial pathologies and Alzheimer's disease also have increased CXCL10 levels as a consequence of persistent inflammation.[26, 75] Thus, CXCL10 might be a useful marker for anti-inflammatory effects of n-3 PUFAs in these pathologic conditions.

In summary, we identify CXCL10 as a robust marker for the inflammatory tolerance established by DHA in clinical settings with low-grade chronic inflammation. We propose that lipid induced transient activation of the cellular stress response, as seen by activation of NFE2L2 by formation of p62-bodies with association of KEAP1, counteracts the signals that drive the type-I interferon response and in particular the synthesis of CXCL10. The identified effects of n-3 PUFAs on interferon signaling contribute to our understanding of how the risk of inflammation- and age-related diseases can be modulated by dietary lipids.

Materials and methods

Cell culture and stimulation

RAW264.7 (ATCC) were cultured in RPMI (R8758, Sigma) with 10 % FBS (10270-106, Gibco) and gentamicin (0.05 mg/mL) (15710049, Gibco). Arpe-19 (ATCC, CRL-

2302) were cultured in DMEM:F12 (Sigma, D8437). PBMC were isolated from buffy coats (obtained from the blood bank of St.Olavs Hospital, Trondheim, Norway) by a Lymphoprep™ (1114547, AXIS-SHIELD PoC AS) density gradient. Monocytes were selected by plastic adherence and differentiated into monocyte-derived macrophages (MDM) by culture in RPMI with 30 % human serum (HS) for 5 days, following 10% HS during experiments. All cells were maintained in 5 % CO_2 at 37°C.

Docosahexaenoic acid (DHA; 90310, Cayman), oleic acid (OA; 90260, Cayman) and arachidonic acid (AA; 90010, Cayman) were added to prewarmed complete medium (70 μM) before given to the cells. Absolute EtOH was used as control in all experiments.

Other reagents used were: Bafilomycin A_1 (BafA1; B1793, Sigma), LPS (tlrl-3pelps, InvivoGen), pU complexed with pL-Arg at a 1:1 ratio (w/w), p(I:C) transfected with Lipofectamine RNAiMAX (13778150, Invitrogen), N-acetyl-cysteine (NAC; A9165, Sigma).

Ab used were: ATCB/beta actin (ab6276, Abcam); COX4I1/COXIV (ab33985, Abcam); GAPDH (CB1001, Merck Millipore); HMOX1 (ADI-OSA-110, Enzo); IRF3 (sc-9082, Santa Cruz Biotech.); KEAP1 (10503-2-AP, proteintech); KEAP1 (E20, Santa Cruz); LMNA/Lamin A/C (sc-6215, Santa Cruz); mono- and polyUb conjugates (FK2, PW8810, Biomol); SQSTM1 (GP62-C, Progen); phospho-IRF3 (S386, ab76493, Abcam); STAT1 (610116, BD Biosciences); TNFAIP3/A20 (sc-166692, Santa Cruz Biotech.); and Ab used from Cell Signaling Tech. were: IKBKB/IKKβ (8943); IRF1 (4302); IRF3 (4302S), MAP1LC3B (3868); MAPK1/3/p44/42 (9107); RELA/p65 (3034); phospho-CHUK/IKBKB/IKKα/β (S176/180, 2697); phospho-IRF3 (S396, 4302); phospho-NFKB2/p105 (S933, 4806); phospho-RELA/p65 (S536, 3033); phospho-STAT1 (Y701, 7649); phospho-STAT3 (Y705, 9145); phospho-TBK1 (S172, 5483); STAT1 (14994); STAT3 (9139); TAX1BP1 (5105); TBK1 (3013). For IP SQSTM1 (PM045, MBL) was used and as control guinea pig IgG (sc-2711, Santa Cruz Biotech.). To inhibit IFNAR1 signaling, anti-Interferon-α/β Receptor Chain 2 (MAB1155, Merck millipore) was used. SQSTM1 Ab used in supplemental figure 1G were: (610832, BD), (sc-28359,Santa Cruz Biotech.), (H00008878-M03, Abnova), (ab31782, Abcam) and (GP62-C, Progen). All secondary Ab were from Invitrogen (Alexa conjugates), Li-Cor Biotechnology (NIR dye conjugates) or Dako (HRP conjugates).

ROS detection

Cell were stained with 0.4μM of the ROS sensitive probe CM-H2DCFDA (C6827, Invitrogen) for 30min at 37°C and mean fluorescence was analyzed by a BD FACS Canto (BD Biosciences, San Jose, CA, USA). Experiments were performed in duplicates with 10000 cells per condition.

Quantitative real-time PCR (qRT-PCR)

Total RNA was isolated by using RNeasy kit (Qiagen). 1 μg total RNA was used for cDNA synthesis with the High-Capacity RNA-to-cDNA Kit (4387406, Applied Biosystems). Real-time PCR was performed using PerfeCTa qPCR FastMix ROX (QUNT95077-012, Quanta) and TaqMan® Gene Expression Assays (Applied Biosystems). The cycling conditions for the StepOne plus system (Applied Biosystems) were 45ºC for 2 min, 95 ºC for 30 sec, 40 cycles of 95ºC for 1 sec, 60ºC for 20 sec. The following probes were used: *CXCL10* (Hs01124251_g1), *Cxcl10* (Mm00445235_m1), *CXCL11* (Hs04187682_g1), *IFNB1* (Hs01077958_s1), *TNF* (Hs01113624_g1), *Tnf* (Mm00443260_g1), *GAPDH* (Hs99999905_m1), *Gapdh* (Mm99999915_g1), *KEAP1* (Hs00202227_m1), *SQSTM1/SQSTM1*(Hs00177654_m1),

HMOX1 (Hs01110250_m1), *NQO1* (Hs00168547_m1) and *SLC7A11* (Hs00921938_m1). Relative mRNA levels were transformed into linear form by the 2 (-ΔΔCt) method [76]. Transcripts were normalized to GAPDH.

Nanostring

Total RNA was extracted and mRNA transcripts were assessed by digital transcript counting (nCounter GX Human Immunology kit assay, NanoString Technologies, Seattle, WA). Total RNA (100 ng) was assayed on nCounter Digital Analyzer (NanoString) according to the manufacturer's instructions. Data were normalized by scaling with the geometric mean of the built-in control gene probes for each sample.

Immunoblotting

For total protein extracts, 8 M urea lysisbuffer was used (8 M urea, 0.5 % (v/v) TX-100, DTT (100 mM), Complete® protease inhibitor (PI; 11873580001, Roche), and phosphatase inhibitor cocktail (PIC) 2 and 3 (P5726 and P0044, Sigma). Protein concentration was determined by BioRad protein assay (500-0006, BioRad). 50 µg protein were separated using NuPAGE® Novex® Bis-Tris Gels (Invitrogen), transferred to nitrocellulose membranes by Invitrogen's iBlot system. Bound Ab were imaged using appropriate near-infrared (NIR) dye conjugates (Li-Cor) and an Odyssey NIR scanner (Li-Cor Biosciences) or using HRP-linked 2^{ndary} Ab (Dako) and SuperSignal West Femto substrate (34096, Pierce) with a Li-Cor Odyssey Fc System. Image studio 3.1 was used for quantification. For statistical assessment, mean intensity values were used, while bar graphs of quantifications show fold changes.

Soluble and insoluble fractions

Cells were lysed in a detergent buffer (NP-40 (1 %), TX-100 (0.25 %), Tris-HCl (0.05 M), NaCl (0.15 M), EDTA (0.001 M), PI, PIC2 and 3) and lysates cleared by centrifugation (1000 g, 10 min). Supernatants were centrifuged at 10 000 g for 20 min and the new supernatant collected (soluble fraction). The detergent resistant pellet was washed twice and finally resuspended in 8 M urea buffer (insoluble fraction).

Nuclear extraction

Cells were lysed in a hypotonic buffer containing 10 mM HEPES, 1.5 mM MgCl2, 10 mM KCl, 0.5 mM DTT, 0.1 % NP-40, PI, PIC2 and 3. Lysates were vortexed on ice for 15min and centrifuged at 3000 g for 10 min. The nuclear pellet was washed once and finally resuspended in 8 M urea buffer. Cytoplasmic extracts were added 20 % 8 M urea buffer.

Immunostaining

The cells were fixed in 4 % PFA, permeabilized with MeOH, blocked with 3 % goat-serum and stained with primary and fluorescently labeled 2^{ndary} Ab (Alexa conjugates, Invitrogen). Nuclear DNA was stained using Draq5 (5 µM; DR50200, Biostatus) or Hoechst 33342 (5 µg/ml; C10337, Life Technologies). Imaging was done on an Axiovert200 microscope with a 63 x 1.2W objective and the confocal module LSM510 META (Carl Zeiss). Images were processed using the ZEN software (Zeiss) and mounted using Canvas 11 (Deneba).

ScanR automated image acquisition

The microscope-based imaging platform Scan^R (Olympus, Hamburg, Germany) was used to image p62-bodies. Images were taken with a 20x objective, using the excitation filters (wavelength [nm]/width [nm]): FITC (485/20), Alexa647 (640/30) and Hoechst (650/13). For emission, a combination filter (440/521/607/700) was used for all fluorophores (Chroma Technology Corp, Bellows Falls, VT). The images were analysed by the ScanR Analysis software (Olympus).

Small interfering RNA

MDMs were transfected at day 5 and day 7 by Lipofectamine RNAiMAX (13778150,

Invitrogen) using 20 nM siRNA or non-targeting oligo. The following smartpool siRNA oligonucleotides were obtained from Dharmacon: non-targeting (D-001210-01), SQSTM1 (J-010230-06), TAX1BP1 (L-016892-00), TNFAIP3 (L-009919-00) and KEAP1 (LQ-012453-00).

ELISA

CXCL10 in supernatants and serum was quantified using an ELISA human Duo-Set (DY266, R&D, Abingdon, UK) according to the manufacturer's instruction. IL36G in serum was quantified using an Human IL-36G (IL-1F9) ELISA Kit (EHIL36G, Thermo Scientific).

IP and Mass spectrometry (MS) analyses

For IP, Dynabeads Protein A (10002D, Life Technologies) were incubated with Ab for 1 h at room temperature and complexes were crosslinked with BS^3 (21580, Life Technologies) according to the manufacturer's protocol. Cells were lysed in a buffer containing 0.25 % TX-100, 1 % NP-40, 50 mM Tris-HCl (pH 8), 150 mM NaCl, 1 mM EDTA (pH 8), PI, PIC 2 and 3. Lysates were adjusted for concentration and volume and immunoprecipitated at 4°C overnight. Beads were washed 3 times with 400 mM KCl and eluted with LDS at 80°C. For MS, eluates were run into NuPAGE® Gels and stained with SimplyBlue SafeStain (LC6060, Life Technologies). Gel pieces were subjected to in gel reduction, alkylation, and tryptic digestion using 6 ng/µl trypsin (V511A, Promega, Wisconsin, USA) [77]. OMIX C18 tips (Varian, Inc., Palo Alto, CA, USA) were used for sample clean up and concentration. Peptide mixtures containing 0.1 % formic acid were loaded onto a Thermo Fisher Scientific EASY-nLC1000 system and EASY-Spray column (C18, 2µm, 100 Å, 50µm, 15 cm). Peptides were fractionated using a 2-100 % acetonitrile gradient in 0.1 % formic acid over 50 min at a flow rate of 250 nl/min. The separated peptides were analysed using a Thermo Scientific Q-Exactive mass spectrometer. Data was collected in data dependent mode using a Top10 method. The raw data were processed using the Proteome Discoverer 1.4 software. The fragmentation spectra were searched against the Swissprot SwissProt_2011_12 database using an in-house Mascot server (Matrix Sciences, UK). Peptide mass tolerances used in the search were 10 ppm, and fragment mass tolerance was 0.02 Da. Peptide ions were filtered using a false discovery rate (FDR) set to 5 % for peptide identifications.

Patients

Thirty-five clinically stable hypertensive heart transplant recipients were studied 1–12 years after transplantation and randomized in a double-blind fashion to receive either 3.4 g of omega-3 fatty acids daily or placebo (corn oil) for one year in the OmaCor-study (Holm et al., 2001a; Holm et al., 2001b). Plasma was sampled for cytokine analyses after overnight fasting at baseline and after 12 months. The study was approved by the Regional Ethics Committee and all patients gave written informed consent to participation.

Statistical analysis

Statistical analyses were performed in Prism 6 (Graph pad). Values are expressed as mean ± standard error of the mean (SEM) if not otherwise stated. Statistical analyses were performed on mRNA fold changes compared to vehicle or on intensities obtained by densitometry of immunoblots, confocal images or FACS analysis. Data from primary cells was analyzed by non-parametric tests where possible, data from celllines was logtransformed and analyzed by parametric tests as indicated. *P*-value < 0.05 was considered statistically significant and is labeled with *) and $p < 0.01$ is labeled with **), $p < 0.001$ labeled with ***).

Ethics statement

The Regional Committees for Medical and Health Research Ethics at the Norwegian University of Science and Technology

(NTNU) approved the use of PBMCs from healthy adult blood donors after informed consent (identification number 2009/2245–2). All donors provided written informed consent.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed

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Figure S1

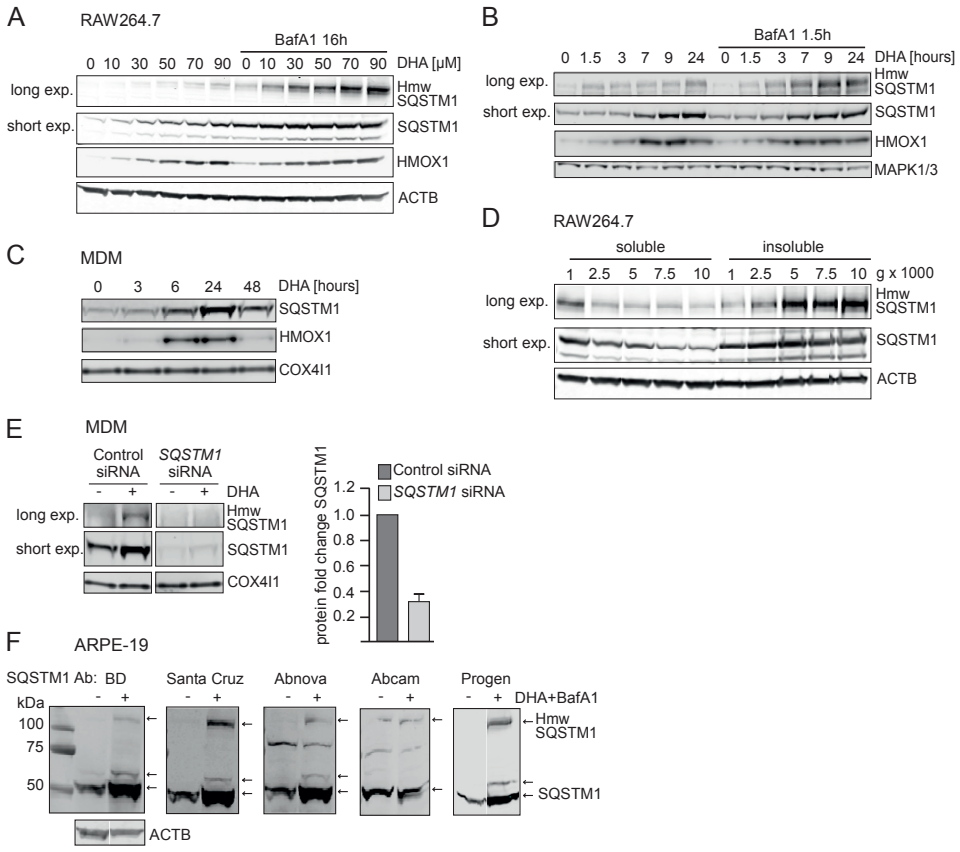

Figure S1. Related to Figure 1 and 2. SQSTM1 protein level and modifications are induced by DHA.
Immunoblot (IB) analysis of RAW 264.7 treated with
(A) indicated concentrations of DHA w/wo BafA1 (100 nM) for 16 h or
(B) DHA (70 μM) for indicated time points and BafA1 for the last 1.5 h. Hmw SQSTM1 was visualized by long exposure (exp).
(C) IB analysis of MDMs treated with DHA (70 μM) for indicated time points.
(D) IB analysis of SQSTM1 in RAW264.7 treated with DHA and BafA1 for 16 h. Soluble and insoluble fractions were separated with increasing centrifugation speed.
(E) IB analysis of SQSTM1 in MDMs transfected with *SQSTM1* siRNA and stimulated with DHA for 16 h. Hmw SQSTM1 was detected by long exposure (exp). Quantification of n=3 (right).
(F) IB analysis with indicated SQSTM1 antibodies in Arpe-19 cells treated with DHA and BafA1 for 16 h.
Arrows represent SQSTM1 migrating as 120 kDa and 70 kDa respectively.

Figure S2

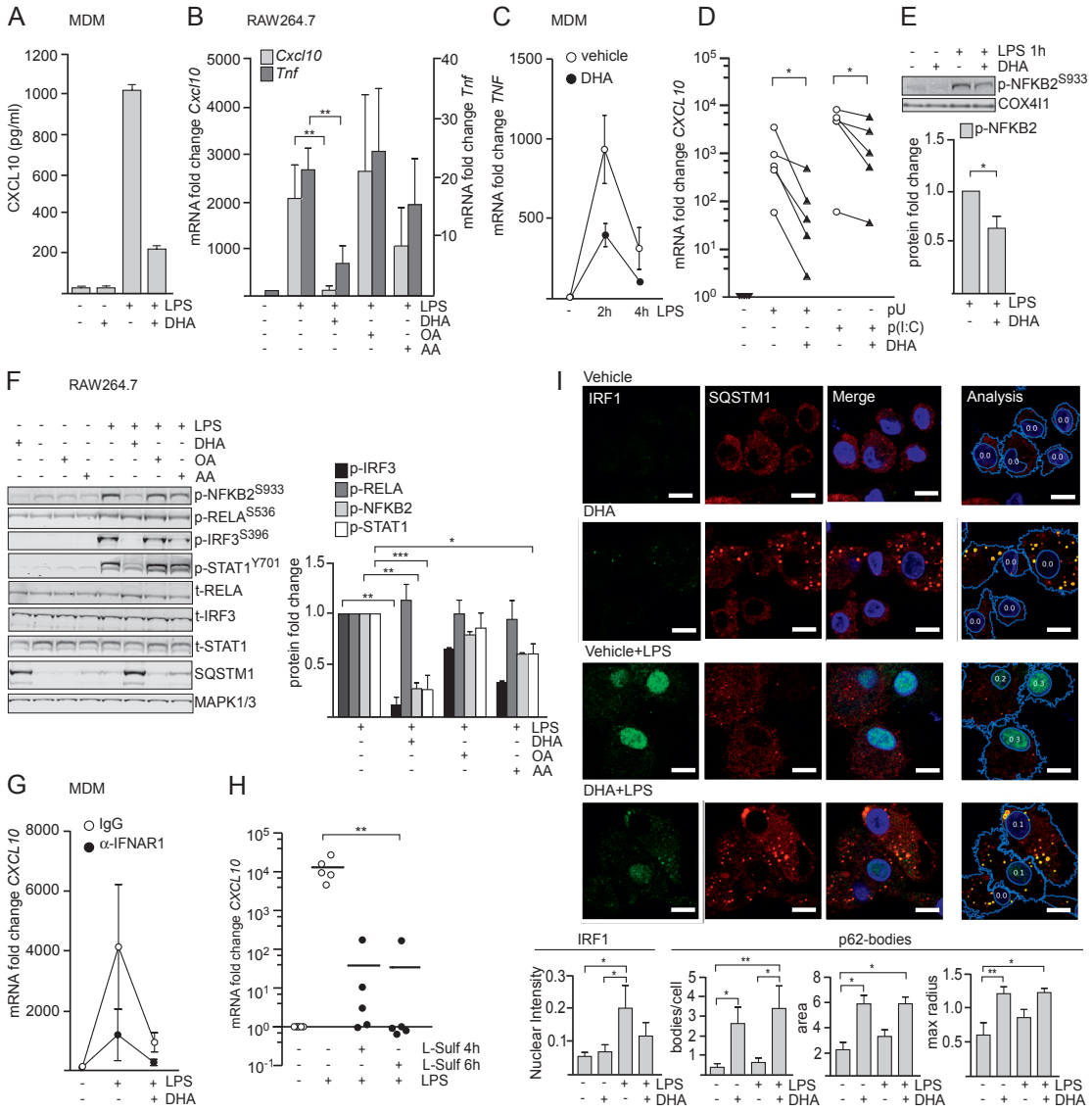

Figure S2. Related to Figure 3 and 4. DHA affects IRF signaling.
(A) ELISA of CXCL10 levels in MDMs treated with DHA for 16 h and LPS for 3 h. Shown is a representative result of three independent experiments (mean ± SD of duplicates).
(B) QRT-PCR-analysis of *Cxcl10* or *Tnf* in RAW 264.7 treated with DHA, OA or AA for 16 h and LPS for 3 h, mean fold change ± SEM, n=3 (**p<0.01, repeated-measures ANOVA with Dunnett's).
(C) QRT-PCR analysis of *TNF* in MDMs treated with DHA for 16 h and LPS for 2 h or 4 h, mean fold change ± SEM, n=5.
(D) QRT-PCR analysis of *CXCL10* in MDMs treated with DHA for 16 h and pU (1µg/ml, complexed to pL-Arg) or p(I:C) (1µg/ml, transfected) for 3 h, mean fold change ± SEM, n=5 (Wilcoxon matched-pairs signet rank test, one-tailed).
(E) IB analysis of phosphorylated NFKB2 (Ser 933) in MDMs treated with DHA for 16 h and LPS for 1 h, mean fold change ± SEM, n=5 (Wilcoxon matched-pairs signet rank test, one-tailed).
(F) IB analysis and quantification (right) of phosphorylated IRF3, RELA and STAT1 in RAW 264.7 treated with DHA, OA or AA for 16 h and LPS for 3 h, mean fold change ± SEM, n=3 (**p<0.01, repeated-measures ANOVA with Dunnett's).
(G) QRT-PCR analysis of *CXCL10* in MDMs treated with anti-IFNAR1 (1µg/ml) or control antibody, DHA for 16 h and LPS for 3 h, mean fold change ± SEM, n=4.
(H) QRT-PCR analysis of *CXCL10* in MDMs treated with L-Sulforaphane (L-Sulf; 25µM) for 4 h or 6 h and LPS for the last 4 h, mean fold change ± SEM, n=5 (**p<0.01,Friedman test with Dunn's)
(I) Immunostaining for IRF1 and SQSTM1 in MDMs treated with DHA for 16 h and LPS for 2 h (scale bars, 10 µm).
Images were analyzed by CellProfiler 2.2 (panel below) for mean intensity of nuclear IRF1staining, mean number of p62-bodies per cell, mean area of p62-bodies and maximum radius of p62-bodies, >150 cells/condition/donor (n=6), mean ± SEM (**p<0.01, *p<0.05, Friedman test with Dunn's).

Figure S3

A

RAW 264.7

	control		BafA1	
Proteins	**vehicle**	**DHA**	**vehicle**	**DHA**
SQSTM1	477.2	2564	8787.9	18286.9
TAXBP1	-	23.8	2522.7	2803.4
KEAP1	-	84.7	1287.6	1695.6
2AAA	46.5	143.8	427.7	865.2
ANGL2	-	20.4	199.8	714.8
FRIH	-	37.2	412.9	633.3
TCPD	72.6	320.7	423.4	490.4
ATPB	-	52.8	144	257.3
FUS	62.6	310.1	52.3	205
MYO5A	108.3	446.9	111.3	188.7
PK1IP	80.7	383.7	-	184.2
ABCF1	28.3	87.3	53.5	146.7
CDC42	-	114.5	39.6	135.3
IMA3	-	61	-	128.7
CPSF2	-	22.1	-	123.9
UBP14	-	31.9	84.9	116.4
SC61B	-	24.9	67.9	111.5
PSD12	-	52.7	69.3	101.9
SHIP1	-	32.3	-	85
AFAD	24	75	66.2	75.4
ARHG7	-	137.6	-	59.2
PSMD5	-	29.1	-	52.3
TUT7	-	133.7	-	50.1
SYWC	-	38.9	44.2	50
UFL1	-	30.9	-	46.3
LIMA1	297	992	24.1	44.9
ABCD3	-	22.7	-	36.7
WDR1	27.1	216.2	-	36.4
MYPT1	38.3	135.5	-	36.1
GSH0	-	39.6	-	31.8
TRM2A	-	26.7	-	31.7
IKZF1	-	40.5	-	29.7
RAVR1	-	39	-	26.3
SVIL	88.4	615.4	-	23.3
TDRD3	-	28.8	-	21.3

B

	Vehicle			DHA		
Protein	**Score**	**Peptides**	**PSMs**	**Score**	**Peptides**	**PSMs**
SQSTM1	8767	22	283	26372	25	785
KEAP1	2124	29	71	5291	34	161
MIC60	1358	24	48	4117	34	135
SAM50	176	7	9	650	14	18
GBB4	47	2	2	59	2	2
TAX1BP1	-	-	-	507	14	17

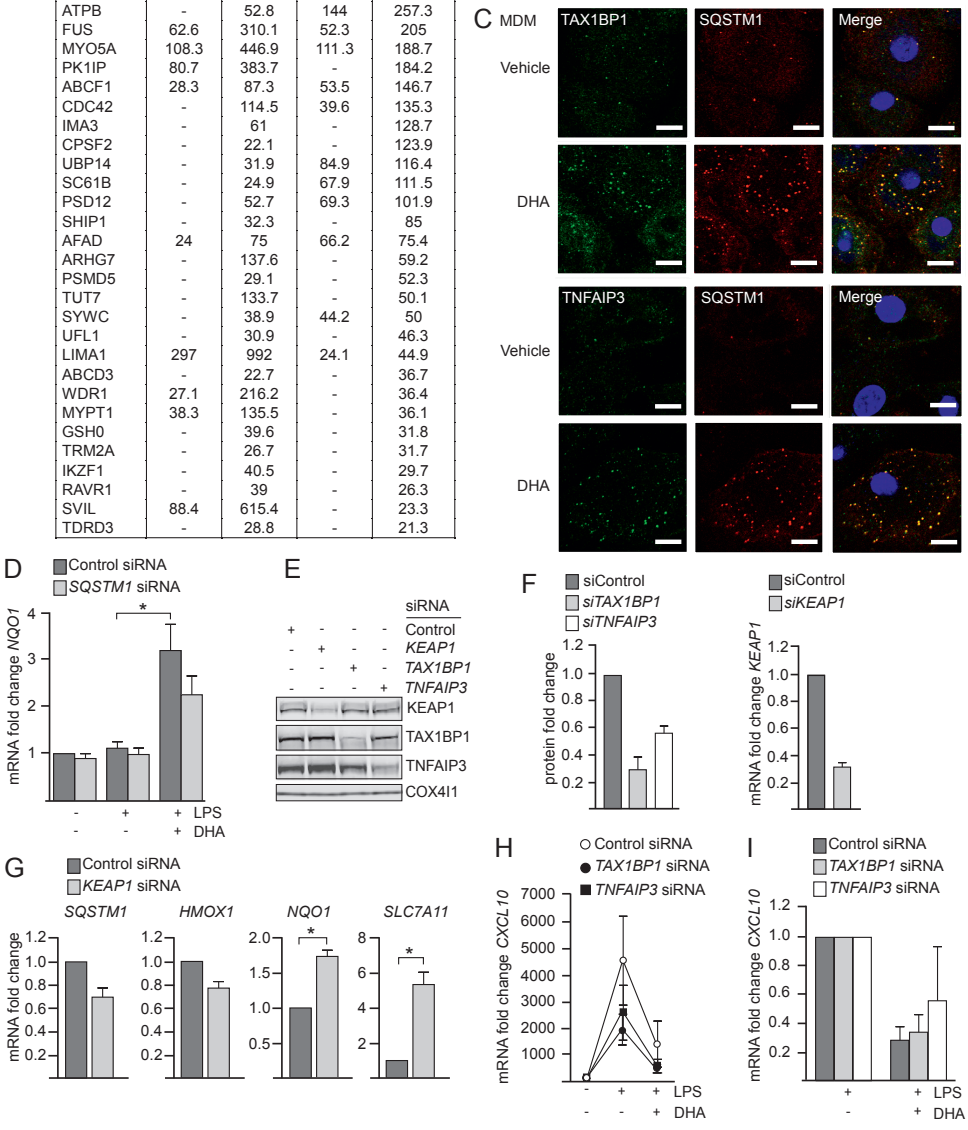

Figure S3. Related to Figure 5. Binding partners in p62-bodies.
(A) Insoluble fractions were prepared from RAW264.7 treated with DHA w/wo BafA1 for 16 h and subjected to MS analysis. Proteins that increased by DHA supplementation in both control cells and BafA1 treated cells were considered as DHA specifically enriched. Cut-off was set at a 3-fold increase by DHA in control cells. The proteins are displayed according to maximum levels in DHA and BafA1 treated samples. Score values are shown.
(B) IP of SQSTM1 from RAW264.7 treated with DHA for 16 h and analysis by MS. Proteins shown were detected 10 times over background and increased by DHA in 3 independent experiments. Score-values of one representative experiment are shown, (PSM, peptide-spectrum match).
(C) Immunostaining for TAX1BP1 and TNFAIP3 in MDMs treated with vehicle or DHA for 24 h (scale bars,10 µm).
(D) QRT-PCR analysis of *NQO1* mRNA in control or *SQSTM1* knockdown MDMs with vehicle or DHA and LPS, n=5 (*p<0.05, Friedman test with Dunn's)
(E) IB analysis and **(F)** quantification of MDMs treated with *TAX1BP1* or *TNFAIP3* siRNA (protein levels) and KEAP1 siRNA (mRNA levels).
(G) QRT-PCR analysis of NFE2L2 target gene mRNA in control or *KEAP1* knockdown MDMs, n=3 (*p<0.05, one-sample t-test).
(H) QRT-PCR analysis of *CXCL10* in *TAX1BP1* or *TNFAIP3* siRNA transfected MDMs treated with DHA for 16 h and LPS for 3 h, n=5 (*p<0.05, Friedman test with Dunn's).
(I) Comparison of the downregulating effect of DHA in control or *TAX1BP1* or *TNFAIP3* siRNA treated MDMs from (H) (Mann Whitney test).

Table S1. Nanostring analysis of genes selected for Figure 3A

MDMs were treated with DHA for 16 h before stimulation with LPS for 3 h. Shown are normalized counts of mRNA transcripts of the nCounter GX Human Immunology kit assay that were more than 1.5-times increased by LPS in both donors. Genes are sorted after most DHA-mediated reduction of LPS-induced expression. Normalized transcript numbers are displayed.

	Donor A				Donor B			
Gene	Vehicle -	DHA -	Vehicle LPS	DHA LPS	Vehicle -	DHA -	Vehicle LPS	DHA LPS
CXCL9	21.4	21.6	539.0	90.0	18.8	13.6	807.8	167.2
CXCL10	8.6	6.2	2893.9	517.9	5.9	10.4	4512.2	1021.9
CXCL11	12.8	13.4	596.5	126.2	13.9	11.5	1076.8	229.5
SLAMF1	15.7	12.3	521.4	144.8	16.8	11.5	396.4	137.9
C7	2.9	5.1	7.4	2.2	2.0	1.0	7.6	2.7
IFITM1	10.0	7.2	363.8	92.2	6.9	12.5	457.9	193.0
IL10	148.4	97.5	851.2	245.8	150.4	129.4	406.1	161.0
TNFSF15	403.7	145.8	13806.9	5758.3	773.0	211.9	4487.4	1207.8
CCL8	18.6	6.2	1533.1	418.1	11.9	14.6	1887.8	788.9
IL27	14.3	15.4	4334.3	1478.0	15.8	16.7	2642.8	941.0
LILRA5	666.2	322.4	1054.5	284.2	749.3	443.6	1670.8	764.0
IL2RA	22.8	17.5	310.8	87.8	11.9	19.8	220.3	103.2
CD70	5.7	2.1	188.5	64.7	2.0	3.1	114.5	49.8
SOCS1	85.6	52.4	1081.0	302.8	30.7	39.7	664.2	331.8
TNFSF10	38.5	28.8	1780.5	604.6	60.4	45.9	2394.4	1084.2
SOCS3	52.8	40.0	6325.4	2370.0	56.4	36.5	4042.4	1849.1
IFIT2	35.7	33.9	3456.5	1335.3	158.4	151.4	19149.5	8562.5
IL20	7.1	11.3	14.7	9.9	8.9	4.2	23.8	4.5
LAG3	12.8	12.3	28.0	8.8	11.9	13.6	22.7	12.5
IDO1	8.6	5.1	2069.2	999.6	4.0	2.1	1819.8	767.6
GBP5	49.9	33.9	2194.4	839.4	46.5	43.8	1606.0	882.3
BATF	67.1	47.2	332.8	141.5	70.3	52.2	360.7	185.0
CCL2	159.8	95.5	727.5	218.4	444.4	167.0	713.9	464.3
NOD2	44.2	44.2	86.9	51.6	81.2	68.9	184.7	66.7
BATF3	31.4	50.3	866.0	386.2	33.7	41.8	422.3	218.8
IRF4	21.4	9.2	122.2	49.4	17.8	19.8	68.0	40.0
CEACAM1	4.3	10.3	11.8	6.6	5.9	11.5	16.2	7.1
TNFSF8	81.3	59.6	172.3	87.8	106.9	60.5	189.0	92.5
IFNB1	4.3	3.1	14.7	8.8	5.9	4.2	15.1	6.2
IFNG	7.1	4.1	25.0	6.6	4.0	7.3	13.0	9.8
IL12B	15.7	9.2	3845.3	2394.2	10.9	8.4	438.5	176.1
JAK3	52.8	23.6	166.4	56.0	38.6	18.8	133.9	93.4
PTGS2	55.6	60.6	11232.6	6854.4	58.4	48.0	4364.3	1932.7
CD7	10.0	8.2	17.7	7.7	6.9	7.3	19.4	12.5
TLR7	41.4	15.4	92.8	53.8	24.7	16.7	82.1	40.9
LAMP3	15.7	18.5	1437.4	739.5	1.0	10.4	577.8	336.2
CDKN1A	1479.4	1171.5	3658.3	1676.6	2500.1	2350.8	5107.3	3396.7
MX1	910.2	630.4	12970.4	6129.2	1002.6	826.7	12714.8	8501.1
TAGAP	209.7	147.9	960.2	537.7	213.8	202.5	927.7	553.2
ITLN2	1.4	4.1	4.4	3.3	1.0	3.1	2.2	0.9
CFB	71.3	48.3	1156.1	544.2	53.5	52.2	657.7	453.6
IL10RA	2784.7	1898.5	10547.8	5965.7	2584.3	2394.6	8443.4	5063.5
LILRB2	336.7	185.8	559.6	206.3	353.4	274.5	676.1	548.8
BCL3	211.1	146.8	2111.9	973.3	302.9	290.2	1909.4	1400.8
LILRB5	17.1	20.5	28.0	14.3	89.1	62.6	140.4	97.0
IFIH1	195.4	163.3	1870.4	1061.0	213.8	200.4	1846.8	1180.3
LCP2	2057.1	1413.8	6022.0	3490.3	2173.5	1719.3	4220.6	2678.9
IFI35	198.3	215.6	958.8	523.4	223.7	237.0	805.7	540.8
TCF4	226.8	134.5	437.4	263.3	198.0	156.6	397.4	244.6
IL5	8.6	7.2	17.7	12.1	5.0	6.3	19.4	10.7
GBP1	1242.5	1057.6	17325.3	10637.7	771.0	632.6	13768.9	8537.6
CCL5	97.0	63.7	11849.7	7101.3	54.4	36.5	7112.9	4523.6
LTA	11.4	15.4	56.0	31.8	12.9	14.6	38.9	26.7
CLEC6A	30.0	21.6	69.2	50.5	19.8	26.1	57.2	30.2
TNFSF13B	833.1	661.2	1880.7	1121.4	713.6	757.9	2021.8	1356.4
JAK2	402.3	321.4	1577.3	887.7	392.9	333.0	1582.2	1120.7
CTLA4_all	10.0	5.1	20.6	13.2	6.9	10.4	14.0	8.9
TAP1	452.2	410.7	1481.6	959.0	502.8	431.1	1341.4	855.6
CCR7	10.0	4.1	854.2	628.7	5.9	7.3	318.6	176.1
TNFRSF9	154.1	96.5	1151.7	792.2	366.2	201.5	1023.8	634.2
CASP1	311.0	221.8	595.0	343.4	514.7	421.7	1071.4	786.3
GPR183	512.1	396.3	3060.4	1958.6	889.8	768.3	3366.4	2268.0
ADA	141.2	118.1	1798.2	1034.7	103.9	109.6	1039.0	777.4
FKBP5	774.6	521.6	1547.9	875.6	601.8	494.8	1094.0	835.2
CCL23	51.4	38.0	347.6	208.5	37.6	23.0	193.3	146.8
CD2	8.6	8.2	17.7	13.2	7.9	10.4	17.3	10.7
IL1RAP	132.7	128.3	350.5	201.9	83.1	67.9	225.7	179.7
STAT5A	399.4	366.6	2328.4	1790.7	372.2	349.7	1428.8	866.3
BCL2L11	25.7	7.2	78.1	59.3	17.8	6.3	65.9	40.9
STAT3	1107.0	962.1	2356.4	1496.6	1132.3	1050.1	2123.3	1600.1
PTPN2	442.2	452.8	874.8	573.9	432.5	364.3	872.6	640.4
TNFRSF17	10.0	10.3	20.6	14.3	7.9	6.3	21.6	15.1
CCL4	216.8	107.8	98648.4	74244.4	86.1	57.4	39489.1	25425.0
STAT4	32.8	26.7	1092.8	727.5	28.7	21.9	479.5	351.3
SLAMF7	2371.0	2019.6	21409.2	15771.7	2555.6	2210.9	16213.0	10821.6
CD40	1185.5	971.3	11703.9	8460.8	779.9	752.6	7994.2	5484.2
TNFAIP6	24.3	28.8	6212.0	4370.3	20.8	23.0	4383.7	3117.4
CCL3	1158.4	797.8	52407.4	43812.6	887.8	701.5	30011.1	17796.4
CFP	18.6	18.5	41.2	13.2	19.8	23.0	40.0	44.5
IL2RB	18.6	19.5	30.9	23.0	16.8	25.1	33.5	23.1
IL23R	11.4	4.1	29.5	16.5	4.0	9.4	16.2	14.2
CD274	303.9	268.0	3569.9	2406.2	286.0	295.4	3362.0	2570.4
EBI3	37.1	25.7	1698.1	1225.6	26.7	23.0	896.4	646.6
CCR8	4.3	2.1	8.8	5.5	3.0	12.5	11.9	9.8
CCL19	11.4	15.4	197.4	116.3	13.9	9.4	246.2	210.8
IRF1	166.9	162.2	2558.2	1828.0	147.5	154.5	1692.4	1239.9
IL9	5.7	1.0	17.7	12.1	5.0	3.1	15.1	11.6
PML	557.8	537.0	4627.3	3203.9	815.6	835.1	4893.5	3713.3
MUC1	21.4	10.3	32.4	20.9	9.9	24.0	25.9	21.4
CCL20	17.1	21.6	29216.2	23023.3	17.8	13.6	11693.2	8027.9
POU2F2	265.3	213.6	466.9	287.5	114.8	140.9	400.7	346.0

Continued:

	Donor A				Donor B			
Gene	Vehicle -	DHA -	Vehicle LPS	DHA LPS	Vehicle -	DHA -	Vehicle LPS	DHA LPS
IRF7	122.7	129.4	1063.3	781.2	201.9	214.0	1612.4	1204.3
IRF8	590.6	588.3	2316.6	1720.5	535.5	565.8	1671.8	1236.3
IL1B	47.1	20.5	35588.7	30491.1	31.7	32.4	16211.9	10234.6
KLRB1	10.0	6.2	17.7	7.7	6.9	15.7	15.1	16.0
PIGR	5.7	5.1	14.7	9.9	5.9	9.4	10.8	8.9
IL23A	31.4	17.5	7337.2	6881.9	17.8	12.5	2394.4	1393.7
FAS	21.4	19.5	131.1	76.8	26.7	23.0	77.8	72.9
STAT2	1272.5	1105.8	3729.0	2751.9	1419.3	1360.2	4016.5	3185.0
IL1R1	47.1	42.1	120.8	99.9	50.5	30.3	76.7	54.3
CD48	3142.7	2609.0	7331.3	5428.0	3252.4	2927.0	6422.8	5100.8
ICAM1	1740.4	1314.2	10463.8	8132.7	1592.5	1241.2	9810.7	7510.3
MCL1	2313.9	1941.6	6291.5	4687.4	2149.8	2019.9	5505.8	4424.9
IL15	22.8	28.8	346.1	289.7	37.6	38.6	324.0	232.1
XBP1	1051.4	869.7	2787.9	2229.6	725.5	530.3	1951.6	1473.8
CCL18	81.3	60.6	126.7	54.9	35.6	36.5	74.5	83.6
IL1RN	26529.8	24162.4	66835.8	54227.6	20596.0	16335.4	60069.6	44683.6
RELA	647.7	590.4	1976.4	1637.1	632.5	614.8	1726.9	1275.4
CLEC4E	152.6	89.3	757.0	497.1	116.8	98.1	613.4	562.1
NFKB2	943.0	734.1	4899.8	3910.6	1068.0	798.6	3749.8	2920.0
CCL24	52.8	24.6	156.1	137.2	29.7	24.0	88.6	62.3
TICAM1	679.1	588.3	1998.5	1518.6	645.3	590.8	1383.5	1153.6
CD44	9325.5	8917.3	60760.7	49954.9	9579.9	9129.6	32569.6	26288.6
PDCD1	15.7	12.3	28.0	25.2	10.9	16.7	30.2	22.2
LCK	11.4	8.2	19.2	18.7	9.9	8.4	23.8	16.0
PDGFB	34.2	35.9	291.6	259.0	99.0	80.4	266.8	209.0
BCL2	234.0	198.2	739.3	591.4	171.2	138.8	412.6	361.1
KIR	7.1	11.3	23.6	13.2	8.9	10.4	15.1	16.9
CD58	1540.7	1387.1	3051.5	2468.8	1417.3	1224.5	2327.4	2039.4
C4BPA	7.1	5.1	17.7	6.6	5.0	4.2	10.8	14.2
DEFB103A	8.6	3.1	42.7	37.3	5.0	2.1	28.1	23.1
CD83	4301.1	3993.0	16438.7	14809.4	3028.7	2621.1	10486.8	8349.9
NFKB1	370.9	247.5	2247.4	1747.9	258.3	213.0	1333.8	1229.2
LITAF	6139.9	6115.3	22163.2	18656.3	7754.8	7989.7	19928.2	17104.5
MAP4K4	982.9	881.0	1930.8	1648.1	754.2	697.3	1131.8	958.8
CSF1	1181.2	891.2	3680.4	3213.8	1632.1	1127.4	3370.7	2798.1
IL18	1194.0	1104.8	5946.9	5163.6	1801.4	1525.1	5546.9	4643.7
NFKBIZ	85.6	34.9	1352.0	1280.5	59.4	28.2	588.6	449.2
RELB	309.6	218.7	773.2	648.5	209.8	198.3	630.7	557.7
C6	25.7	22.6	44.2	31.8	21.8	25.1	34.6	34.7
ETS1	35.7	34.9	297.5	222.7	34.6	24.0	150.1	147.6
GZMA	22.8	20.5	39.8	27.4	14.9	16.7	28.1	29.4
CCRL2	1978.7	2064.8	4926.3	4624.9	2066.6	1969.8	4432.3	3573.7
NFKBIA	2410.9	2035.0	10795.2	9957.4	2168.6	1859.1	9899.3	8153.3
FCAR	11.4	16.4	19.2	12.1	5.9	7.3	18.4	21.4
IL7R	58.5	73.9	3599.4	3059.1	31.7	18.8	2869.6	2719.0
TRAF1	212.6	248.5	7197.3	6929.0	200.9	153.5	4423.7	3753.4
TNF	48.5	52.4	3362.3	3659.3	53.5	50.1	1477.4	1089.5
LEF1	11.4	12.3	30.9	34.0	11.9	9.4	19.4	14.2
CD82	1446.5	1740.3	3207.6	3026.2	1212.5	1324.7	2421.4	2163.1
TAP2	984.3	998.0	1583.2	1551.5	723.5	678.5	1552.0	1375.9
CD80	20.0	24.6	880.7	756.0	38.6	44.9	835.9	853.8
NOTCH1	89.9	82.1	235.6	185.4	101.0	99.2	185.8	205.5
SRC	690.5	839.9	3973.5	3593.4	841.3	1160.8	3046.7	3071.2
ICAM4	48.5	47.2	73.6	72.4	37.6	43.8	76.7	72.0
STAT1	1673.4	1970.3	3178.2	2892.3	1972.6	2252.7	4568.4	4649.9
CSF2	10.0	6.2	73.6	77.9	6.9	10.4	22.7	20.5
ICOSLG	211.1	167.4	795.3	769.2	99.0	91.9	508.7	506.1
IRAK2	95.6	85.2	1064.8	1125.8	117.8	100.2	804.6	764.0
TNFAIP3	814.6	849.1	4206.1	4414.2	1097.7	1034.5	3665.5	3547.0
PTGER4	263.9	216.6	1005.9	1027.0	264.3	193.1	565.9	592.4
TNFRSF4	15.7	16.4	185.6	167.9	21.8	9.4	153.4	178.8
BID	319.6	371.7	704.0	758.2	289.0	335.1	661.0	716.0
CLEC5A	229.7	213.6	559.6	531.1	373.1	418.6	766.8	962.4
CD45RA	2.9	2.1	4.4	7.7	4.0	9.4	9.7	5.3
CDH5	1.4	8.2	5.9	12.1	3.0	4.2	8.6	5.3
KLRC3	8.6	9.2	16.2	18.7	4.0	6.3	7.6	12.5
IL6	8.6	5.1	1976.4	2814.4	5.0	10.4	469.8	785.4
IKZF3	2.9	5.1	4.4	11.0	7.9	6.3	13.0	8.9
IL1A	11.4	19.5	175.3	352.2	15.8	21.9	42.1	76.5
IL8	135.5	98.6	10674.4	27965.2	78.2	102.3	8361.4	16779.8
CXCL1	45.7	38.0	1459.5	3130.4	73.2	78.3	1803.6	4699.7
CXCL2	22.8	23.6	119.3	291.9	85.1	101.3	219.2	515.9

Paper III

Keap1 regulates inflammatory signaling in *Mycobacterium avium*-infected human macrophages

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Several mechanisms are involved in controlling intracellular survival of pathogenic mycobacteria in host macrophages, but how these mechanisms are regulated remains poorly understood. We report a role for Kelch-like ECH-associated protein 1 (Keap1), an oxidative stress sensor, in regulating inflammation induced by infection with *Mycobacterium avium* in human primary macrophages. By using confocal microscopy, we found that Keap1 associated with mycobacterial phagosomes in a time-dependent manner, whereas siRNA-mediated knockdown of Keap1 increased *M. avium*-induced expression of inflammatory cytokines and type I interferons (IFNs). We show evidence of a mechanism whereby Keap1, as part of an E3 ubiquitin ligase complex with Cul3 and Rbx1, facilitates ubiquitination and degradation of IκB kinase (IKK)-β thus terminating IKK activity. Keap1 knockdown led to increased nuclear translocation of transcription factors NF-κB, IFN regulatory factor (IRF) 1, and IRF5 driving the expression of inflammatory cytokines and IFN-β. Furthermore, knockdown of other members of the Cul3 ubiquitin ligase complex also led to increased cytokine expression, further implicating this ligase complex in the regulation of the IKK family. Finally, increased inflammatory responses in Keap1-silenced cells contributed to decreased intracellular growth of *M. avium* in primary human macrophages that was reconstituted with inhibitors of IKKβ or TANK-binding kinase 1 (TBK1). Taken together, we propose that Keap1 acts as a negative regulator for the control of inflammatory signaling in *M. avium*-infected human primary macrophages. Although this might be important to avoid sustained or overwhelming inflammation, our data suggest that a negative consequence could be facilitated growth of pathogens like *M. avium* inside macrophages.

Keap1 | human primary macrophages | infection | *Mycobacterium avium* | inflammation

The *Mycobacterium avium* complex consists of widely distributed opportunistic mycobacteria that cause nontuberculous pulmonary disease in immunocompromised individuals (1, 2). Like the more virulent *Mycobacterium tuberculosis* (Mtb), *M. avium* invades host macrophages and may escape killing by preventing phagosomal maturation while retaining access to essential nutrients (2–4). Initial infection is sensed by various pattern recognition receptors (PRRs) from surface Toll-like receptors (TLRs) and C-type lectin receptors (CLRs), to endosomal TLRs, cytosolic NOD-like receptors (NLRs), and nucleic acid sensors (5–8). Engagement of these PRRs initiates production of inflammatory mediators and type I IFNs through activation of the canonical IκB kinase (IKK)-α/β kinases and the transcription factor NF-κB or the IKK-related kinase TBK1 and IFN regulatory factors (IRFs) (9, 10). Individual PRRs initiate overlapping and distinct signaling pathways in various cell types. Endosomal TLRs act as sensors of foreign nucleic acids and trigger the production of type I IFNs and inflammatory cytokines. In plasmacytoid dendritic cells (DCs), this results from RNA and DNA activation of NF-κB, IRF5, and IRF7, whereas IRF1, rather than IRF7, drives type I IFNs downstream of TLR7 and TLR9 in myeloid DCs (11–14). IFN-β production induced by *Candida albicans* in DCs is largely dependent on the CLR Dectin-1 and signaling through the Syk kinase and IRF5 (15). *Listeria monocytogenes* and Mtb may disrupt the phagosomal membrane of macrophages and trigger type I IFN responses through cytosolic Nod1/Nod2/RIP2 and IRF3 (*L. monocytogenes*) or IRF5 (Mtb) responding to bacterial cell-wall peptidoglycan, or STING/TBK1/IRF3 responding to bacterial DNA (6, 8). There is more evidence that TBK1 and IKKε are also activated by TLR ligands that signal via MyD88, leading to IFN-β production without the activation of IRF3 (16), and we recently showed that IRF5 activation in human monocytes by *Staphylococcus aureus* ssRNA was dependent on IKKβ and not TBK1 (17).

Inflammatory mediators shape the immune response and the outcome of infection (reviewed in refs. 2, 3, 5, 7, 18). Mice deficient in the adaptor MyD88 that conveys signals from TLRs and IL-1 and IL-18 receptors are extremely susceptible to infections with Mtb and *M. avium*. Mice deficient in TNF or IL-1 receptor die rapidly after Mtb infection, and people receiving anti-TNF therapy have increased risk for reactivation of latent tuberculosis (3). On the contrary, mice deficient in type I IFNs actually do better than WT mice, arguing for a role of IFN-α/β in supporting Mtb survival (19). PRR activation induces the production of reactive oxygen species (ROS) via phagosomal NADPH oxidase (NOX) (20). However, excessive oxidative signaling may be damaging to cells; hence, an efficient mechanism for modulation is imperative.

Significance

Inflammatory signaling is a central mechanism controlling host defenses to pathogens. Members of *Mycobacterium avium* complex cause disease in immunocompromised patients and in individuals with predisposing lung abnormalities. We provide evidence of a mechanism in human primary macrophages whereby the oxidative stress sensor Kelch-like ECH-associated protein 1 (Keap1) negatively regulates inflammatory responses and thus facilitates intracellular growth of *M. avium*. Our findings are of high biological and clinical significance, as opportunistic infections with nontuberculous mycobacteria are receiving renewed attention because of increased incidence and difficulties in treatment. Altered Keap1 gene expression may also have vital clinical implications for other inflammation-associated conditions, opening novel research venues for translational research, for instance in the expanding field of host-targeted therapy for infectious diseases.

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Kelch-like ECH-associated protein 1 (Keap1) plays a well-established role as a sensor for ROS for the protection of cells against oxidative damage (21). Keap1 is characterized by several distinct binding domains, allowing its self-interaction and interaction with other proteins such as cullin-ring 3 E3 ubiquitin ligase (Cul3), the transcription factor nuclear factor (erythroid-derived 2)-like 2 (Nrf2), and p62 (22–25). Modification of Keap1 by electrophilic or oxidative stress leads to stabilization of Nrf2. Nrf2 translocates to the nucleus and activates the transcription of cytoprotective genes, including p62, heme oxygenase, and NADPH dehydrogenase, quinone 1. The Keap1–p62 interaction may lead to Keap1 degradation by autophagy, resulting in a noncanonical mechanism of regulation of Nrf2 (24, 26, 27). There is some evidence suggesting a role for the Keap1 E3-ligase complex in the regulation of NF-κB signaling in the context of tumorigenesis (26–28). Although the role of Keap1 in ROS signaling has been well established, its role in regulation of inflammatory signaling has not been clearly elucidated. We show here that Keap1 down-regulates the inflammatory response necessary for the control of *M. avium* infection through the canonical IKK complex and its homolog, TBK1.

Results

***M. avium* Induces ROS and the Recruitment of Keap1 to Its Phagosomes in Human Primary Macrophages.** ROS can be triggered by invading bacteria, and, despite the fact that pathogenic mycobacteria have developed strategies to counter it, ROS modulates antimycobacterial host defenses, including inflammation (29–31). We investigated whether *M. avium* induced ROS production in human primary monocyte-derived macrophages (MDMs). Cells were infected with *M. avium*, and, 1 h after infection, ROS was significantly increased as shown by confocal fluorescence microscopy (Fig. 1 *A* and *B*). Small amounts of ROS were detected in untreated macrophages. When ROS was inhibited by using *N*-acetyl cysteine (NAC), which enhances the production of the antioxidant glutathione, ROS levels were significantly decreased in *M. avium*-infected macrophages (Fig. 1*A*, *Lower*, and *B*), with a similar trend for the positive control, *tert*-butyl hydroperoxide (TBHP). As Keap1 is well described as a sensor for ROS and *M. avium* triggered ROS generation, we wondered if Keap1 was recruited to mycobacterial phagosomes. We found that Keap1 was recruited to CFP-*M. avium* phagosomes 4 h after infection (Fig. 1*C*). This recruitment significantly decreased by approximately 50% after 24 h (Fig. 1 *C* and *D*). In addition, when ROS was inhibited by NAC or diphenyleneiodonium (DPI), which inhibits ROS generated by NOX2, there was a significant reduction of Keap1 association with *M. avium* phagosomes (Fig. 1*E*). However, this recruitment did not seem to depend on p62, a well-known Keap1 interaction partner and a receptor for autophagy (23, 24), as there was no difference in Keap1 association with *M. avium* phagosomes when p62 was knocked down by using siRNA (Fig. 1*F*). These observations show a recruitment of Keap1 to *M. avium* phagosomes that is partly ROS-dependent, and suggest a role for Keap1 in *M. avium* infections.

Keap1 Knockdown Increased Inflammatory Cytokine Production During *M. avium* Infection of Human Primary Macrophages. We next investigated if Keap1 may affect mechanisms that contribute in controlling *M. avium* infections. We knocked down Keap1 in MDMs by using targeting siRNA (siKeap1) and screened for the expression levels of 578 human genes known to be differentially expressed in immunology (nCounter GX Human Immunology Kit; NanoString Technologies) 4 h after infection with *M. avium*. The expression of some of these genes is shown in Table S1. Keap1 knockdown efficiency of 50–80% was achieved at mRNA and protein levels in the MDMs (Fig. 2 *A* and *B*). Most of the genes induced by *M. avium* infection seemed to be regulated by Keap1, such as IL-1β, IL-18, and IRF1 (Table S1), and apparently NF-κB–driven (www.bu.edu/nf-kb/gene-resources/target-genes/). However, several genes were expressed but not further induced by

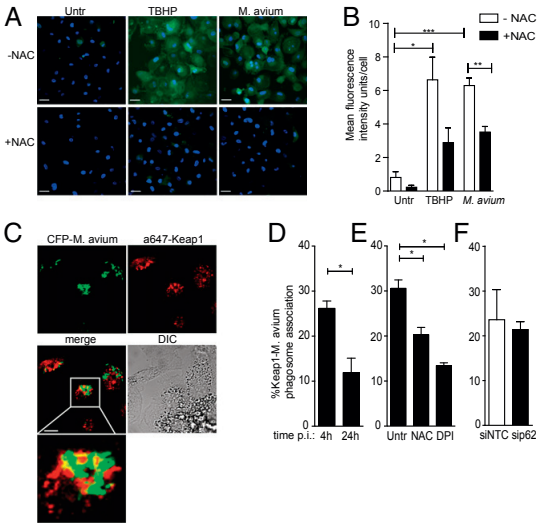

Fig. 1. *M. avium* induced cellular ROS generation and recruitment of Keap1 to its phagosome. (*A*) *M. avium* induction of cellular ROS production in human primary macrophages detected by confocal microscopy. After a 1-h infection, ROS was analyzed by a fluorogenic marker for ROS in live cells, 5-(and-6)-carboxy-2′,7′-dichlorodihydrofluoresceindiacetate (carboxy-H_2DCFDA), and TBHP was used as a positive control. NAC 5 mM was used to inhibit ROS production. (*B*) Quantification of ROS induction per cell by using Imaris Cell module from three independent experiments counting at least 100 cells for each condition. (*C*) MDMs were infected with CFP-*M. avium* for 4 h and fixed in 2% PFA, and localization of Keap1 was assessed by immunofluorescence staining. (*D*) Quantification of Keap1 association with *M. avium* phagosomes at 4 and 24 h after infection by immunofluorescence from three independent experiments, counting at least 50 infected cells per condition. (*E*) Quantification of the effect of ROS inhibition on Keap1 association with *M. avium* phagosomes. Macrophages were pretreated with ROS inhibitors NAC and DPI, 100 μM, for 30 min, and then cells were infected for 4 h, fixed, and stained for Keap1. (*F*) Quantification of the effect of p62 siRNA knockdown (sip62) on Keap1 association with *M. avium* phagosomes. Macrophages were pretreated with siNTC or sip62, 20 nM, for 72 h, and then cells were infected for 4 h, fixed, and stained for Keap1. Untr, untreated. (Scale bars: *A*, 20 μm; *C* 10 μm.) Data shown are mean ± SEM (**P* < 0.05, ***P* < 0.01, and ****P* < 0.001, Student *t* test).

M. avium infection and also not regulated by Keap1, like MyD88 and the scavenger receptor CD36 (Table S1). We selected a few genes important in mycobacterial immune response for further evaluation (2, 18). Infection induced significantly higher fold expression of TNF, IL-6, IL-1β, CXCL10, and IFN-β mRNA levels measured by quantitative PCR in Keap1 knockdown cells than in nontargeting control siRNA (siNTC)-treated cells (Fig. 2*C*). Protein levels of cytokines were measured by ELISA in cell supernatants and showed similarly significant results in fold changes (Fig. 2*D* and Fig. S1*A*). However, CXCL10 and IFN-β induction by infection were often low and highly variable from donor to donor when absolute protein levels were measured (Fig. S1*A*). As expected, Keap1 inhibition also led to increased transcription of some Nrf2 target genes (Fig. S1*B*) (22). We thus show that Keap1 regulates NF-κB signaling and specifically represses inflammatory cytokine and type I IFN expression induced by *M. avium* infection in human primary macrophages.

Keap1 Knockdown Increased Nuclear Translocation of NF-κB-p65 and IRF1, -3, and -5 in Human Macrophages. Having determined that Keap1 inhibits inflammatory cytokines and type I IFNs during *M. avium* infection, we further investigated the point in the signaling

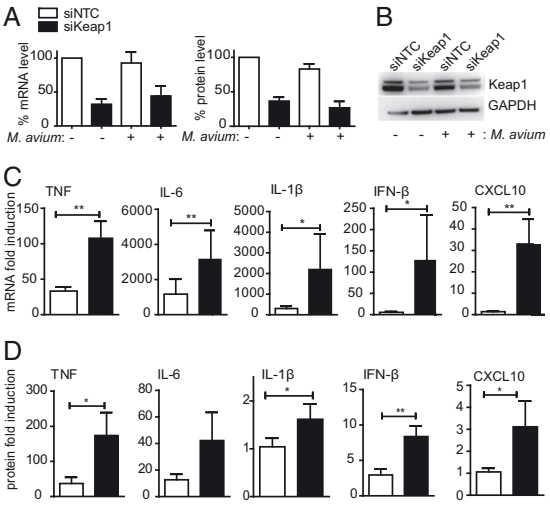

Fig. 2. Keap1 down-regulates inflammatory cytokine expression during *M. avium* infection. (*A* and *B*) MDMs were transfected with 20 nM pooled siRNA against Keap1 (siKeap1) or siNTC. Keap1 knockdown was analyzed by qPCR or immunoblotting and normalized to GAPDH. (*C*) Effect of *M. avium* infection on cytokine mRNA expression after siNTC treatment (white bars) or Keap1 knockdown (black bars) was analyzed in MDMs 4 h after *M. avium* infection. (*D*) Keap1 effect on cytokine protein expression in response to *M. avium* infection as measured by ELISA in supernatants of cells harvested for mRNA analysis (white bars, siNTC-treated MDMs; black bars, siKEAP-treated MDMs). All fold induction values have been calculated relative to uninfected control cells treated with siNTC or siKEAP, respectively. All experiments were repeated $n = 4$–8 times from cells obtained from different blood donors, and data shown are the mean ± SEM (*$P < 0.05$ and **$P < 0.01$, Student t test; Fig. S1 and Table S1).

cascade at which Keap1 may interfere. We first examined Keap1 regulation of transcription factor activation by using a recently developed nuclear translocation assay whereby transcription factors are stained with specific antibodies and nuclear accumulation is assayed (17). We infected cells for 1 h and 4 h and analyzed the nuclear translocation of NF-κB-p65 and IRF1, -3, and -5 (Fig. 3). We saw a strong early and sustained nuclear accumulation of NF-κB (p65, 45% and 30% at 1 and 4 h post infection, respectively) and IRF5 (from 26% to 48% and 62% 1 h and 4 h post infection, respectively) and a later IRF1 translocation (10% and 38% at 1 and 4 h post infection, respectively), whereas IRF3 was hardly detected (1–4% nuclear translocation) upon *M. avium* infection (Fig. 3*A*). In Keap1 knockdown cells, however, and in line with our cytokine observations (Fig. 2), there was approximately a two- to threefold increase in nuclear accumulation compared with control siRNA-treated cells for NF-κB and IRF1 and a twofold increase in IRF5 1 h after infection. IRF3 translocation was still apparently quite low and seemed unaffected by Keap1 knockdown 1 h after *M. avium* infection (Fig. 3*B*). Antibodies to IRF7 and -8 showed high nonspecific staining and were thus unsuitable for use in the nuclear translocation assay. Furthermore, knocking down NF-κB (RelA) reduced *M. avium*-induced TNF and IFN-β, whereas only modest effects were seen with siIRF1 or IRF5 (Fig. S2), possibly as a result of knockdown efficiencies of only 40–70% of messenger levels. These data are indicative of the fact that the cytokines were driven by NF-κB and possibly IRF1 and -5.

Keap1 Inhibits Inflammatory Cytokine Responses during *M. avium* Infection Through the Canonical IKK Complex and TBK1. Upstream of NF-κB and IRFs are central kinases, canonical IKKs and TBK1, respectively, that control signaling and inflammatory cytokine and type I IFN production. Translocation of NF-κB and IRF1 were significantly inhibited at 1 h and 4 h after infection when cells were treated with kinase inhibitors for IKKβ (IKKVIII) or TBK1 (MRT67307 or BX795; Fig. 3*C*). Similar trends were also observed for IRF5, but results were more variable and did not reach significance (Fig. 3*C*). These data suggest that IKKβ and TBK1 are involved in signaling pathways engaged by *M. avium*.

We next measured the effect of Keap1 knockdown in MDMs on the phosphorylated and total protein levels of these kinases and nine other different proteins in the NF-κB and IRF signaling pathways (IKKα, IKKβ, IKKγ, IκB, NF-κB-p65, TBK1, IRF3,

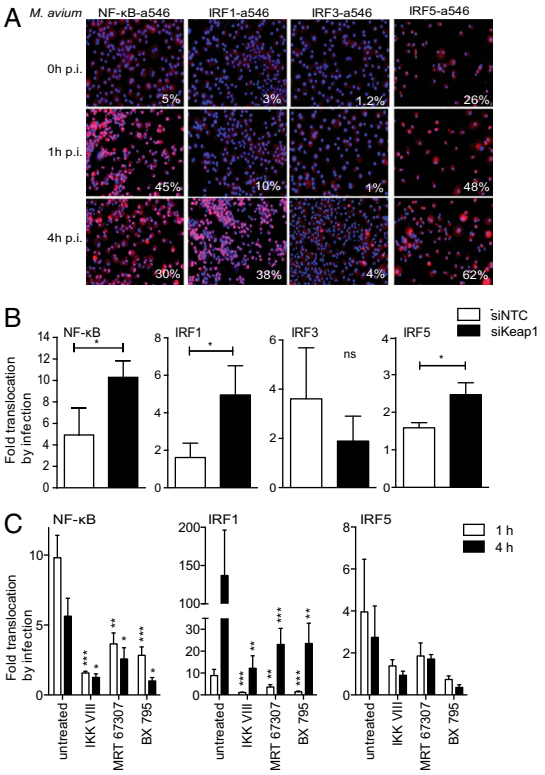

Fig. 3. NF-κB, IRF1, and IRF5 show increased nuclear accumulation in macrophages upon *M. avium* infection during Keap1 knockdown. (*A*) MDMs were infected with *M. avium* for 1 h and 4 h, fixed, and stained for various transcription factors, and Alexa 546 (red) IgG was used as secondary antibody for imaging. Hoechst (blue) was used as a nuclear stain. Images are representative of three independent experiments. (*B*) Macrophages were siRNA-treated for 72 h and infected for 1 h. Cells were then fixed and stained for the different transcription factors. Quantification of translocation in bar charts represents observations from three independent experiments with two replicates in each, and data shown are the mean ± SEM (*$P < 0.05$, Student t test). (*C*) The effect of inhibition of inflammatory signaling by using IKKβ inhibitor VIII and the TBK1 inhibitor BX795 or MRT67307 on the translocation of NF-κB, IRF1, and IRF5. MDMs were pretreated for 30 min with 5 μM inhibitors, then infected for indicated time periods and assessed by immunostaining and Scan^R analysis. Duplicate samples were analyzed in five independent experiments. Inhibitor treatment significantly affected nuclear translocation of transcription factors. Data shown are the mean ± SEM (*$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$, Student t test; Fig. S2).

IRF5, IRF7, IRF8, and p38) subsequent to *M. avium* infection (Fig. 4 and Fig. S3). There was an overall significant increase in total and phosphorylated levels of members of the IKK complex and NF-κB in Keap1 knockdown cells compared with controls and relative to uninfected samples. These regulatory effects were quite rapid, as significant differences could be detected as early as 30–60 min after infection (Fig. 4). Notably, expression of the inhibitor of NF-κB, IκB, was not affected by Keap1 knockdown after infection (Fig. S3*A*). In addition to this, and interestingly so, we also observed a significant increase in phosphorylated (as early as 30 min post infection) and total TBK1 levels in Keap1 knockdown cells after infection (Fig. 4). However, total IRF3, IRF5, IRF7, and IRF8 levels were not affected with Keap1 knockdown (Fig. S3*B*). Expression of the p38 MAPK in response to infection remained unchanged by Keap1 knockdown (Fig. S3*B*). p38 regulates the transcription factors CREB and c/EBPβ and does not use IKK or TBK1 (32). Of important note is the observation that, in Keap1 knockdown cells, changes in the mRNA levels of IKKβ and TBK1 were insignificant (Fig. S3*C*).

Taken together our results show evidence that Keap1 may negatively regulate activation of the IKK complex and TBK1, leading to reduced nuclear translocation of NF-κB, IRF1, and IRF5 driving the expression of inflammatory cytokines and type I IFNs induced by *M. avium* infection.

Disruption of Cul3 and Rbx1 Genes Up-Regulates Inflammatory Cytokine Responses During *M. avium* Infection. Under normal conditions, the Keap1/Cul3-Rbx1 complex constantly facilitates Nrf2 degradation. However, when a cell encounters oxidative or electrophilic stress, Nrf2 dissociates from the complex and translocates into the nucleus, where it activates myriad antioxidant and cytoprotective genes. Along with Keap1, Cul3 and Rbx1 make up the ubiquitin ligase complex that is responsible for the ubiquitination and degradation of Nrf2 (22). We thus investigated if the regulatory effect of Keap1 on inflammation induced by *M. avium* was also observed when the other members of the ubiquitin ligase complex or Nrf2 were disrupted. Indeed, TNF and IFN-β mRNA levels significantly increased at 4 h aftr infection with the knockdown of both Cul3 and Rbx1 (Fig. 5*A*), thus phenocopying Keap1. Specificity of the siRNAs was verified, as they silenced only the target gene and did not affect expression of the other partners (Fig. S4*A*). However, silencing of Nrf2 did not change TNF levels induced by *M. avium* infection (Fig. 5*B*). p62 has also been shown to regulate Nrf2 via the Keap1/Cul3/Rbx1 complex (22, 23). When we knocked down p62, there was rather a trend of decreased TNF mRNA levels (Fig. 5*B*) in response to infection, opposite from what we observed with Keap1 knockdown. These data suggest that Keap1 may regulate the inflammatory response induced by *M. avium* via members of the ubiquitin ligase complex Cul3/Rbx1, and that this effect is independent of Nrf2 and p62, which are other important interaction partners.

Keap1 Plays a Role in the Ubiquitination and Degradation of IKKβ During *M. avium* Infection. Given that Keap1 knockdown led to IKKβ and TBK1 accumulation and Keap1 is an adaptor in the Cul3-ubiquitin ligase complex, we further investigated whether Keap1 might contribute in the ubiquitination and degradation of activated IKKβ and TBK1 to terminate inflammatory signaling. Previous studies in cancer cell lines indicate that IKKβ directly interacts with Keap1 through a conserved motif and that Keap1/Cul3 contributes in regulating IKKβ (27). We infected control siRNA- and siKeap1-treated macrophages from four different donors with *M. avium* in the presence and absence of the proteasome inhibitor MG132, followed by immunoprecipitation of IKKβ or TBK1 and Western blot analyses of ubiquitinated IKKβ and TBK1. *M. avium* infection induced phosphorylation of IKKβ, and ubiquitinated IKKβ accumulated when MG132 was added (Fig. 5*C*). In Keap1-knockdown cells, ubiquitination of IKKβ was significantly lower, even in the presence of MG132, supporting a mechanism whereby Keap1 regulates IKKβ activity through ubiquitination and degradation, and that accumulation of active IKKβ (i.e., p-IKKβ) results in increased translocation of key transcription factors and production of cytokines in Keap1-knockdown macrophages. We also observed an accumulation of TBK1 during Keap1 knockdown suggestive of Keap1 regulation of TBK1. However, unlike IKKβ, TBK1 did not seem to be ubiquitinated during *M. avium* infection in macrophages (Fig. S4*B*). These results suggest that the Keap1-Cul3-Rbx1 ubiquitin ligase complex is specifically responsible

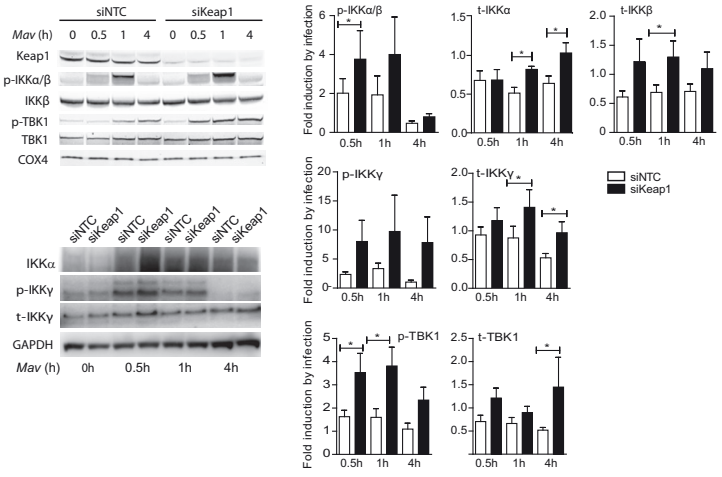

Fig. 4. Keap1 knockdown stabilizes the IKK complex and TBK1 protein levels. MDMs were transfected with siRNA and Keap1 siRNA knockdown levels analyzed by Western blotting compared with siNTC sample 30 min, 60 min, and 4 h after *M. avium* infection. Phosphorylated and total protein levels were examined with anti-phospho (p-) or anti-total (t-) antibodies at the same time points after infection. Cells were infected with *M. avium* 72 h after siRNA treatments, and blots from one representative experiment are shown. Quantification of protein in bar charts represent observations from $n = 6$–8 independent experiments normalized to GAPDH or COX4 using different blood donors. Data shown are the mean ± SEM (*$P < 0.05$, Student t test; Fig. S3).

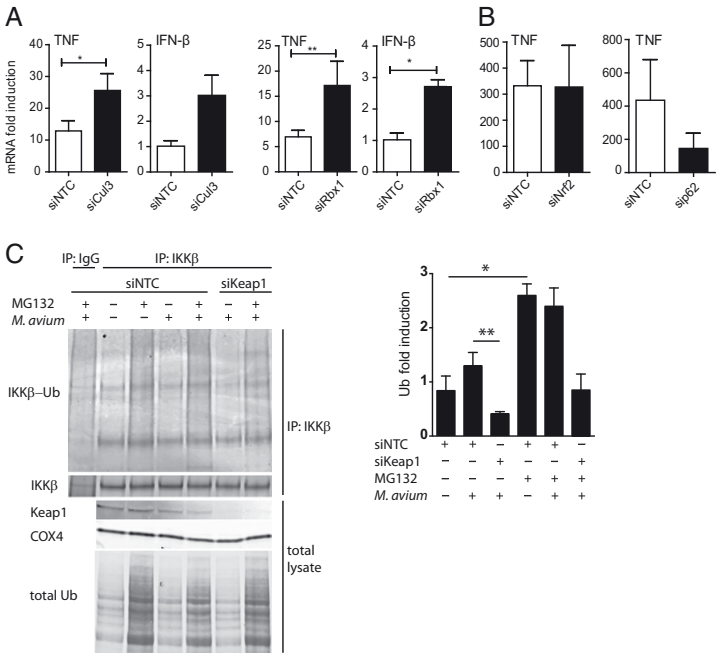

Fig. 5. The Keap1/Cul3-Rbx1 ubiquitin ligase complex regulates *M. avium*-induced cytokine responses through ubiquitination and degradation of IKKβ. MDMs were transfected with 20 nM pooled siRNA against Cul3, Rbx1, Nrf2, and p62 (siCul3, siRbx1, siNrf2, and sip62, respectively) or siNTC. Effect of knockdown on cytokine mRNA expression in siRNA-treated MDMs was analyzed 4 h after *M. avium* infection. As in Fig. 2, results are presented as fold induction in response to infection. (*A*) Effect of Cul3 and Rbx1 knockdown on infection-induced TNF and IFN-β mRNA expression. (*B*) Effect of Nrf2 (siNrf2) knockdown on TNF and p62 knockdown (sip62) on TNF mRNA expression in comparison with siNTC. Results represent at least two independent experiments with two replicates, and data shown are the mean ± SEM (**P* < 0.05, Student *t* test). (*C*) The effect of Keap1 on ubiquitination of IKKs was analyzed by IP of IKKβ and subsequent staining for ubiquitination was performed after Keap1 knockdown and infection for 2 h. Cells were pretreated with or without 10 μM MG132, a proteasome inhibitor. A representative blot is shown, and bar charts are quantifications from four independent experiments. Data shown are the mean ± SEM (**P* < 0.05 and ***P* < 0.01).

for ubiquitin-mediated degradation of IKKβ but not TBK1. Hence, the mechanism of Keap1 regulation of TBK1 may be indirect, possibly mediated through IKKβ (33).

Increased Inflammatory Responses Resulting from Keap1 Knockdown Restrict Intracellular Growth of *M. avium* in Human Macrophages. Our results show that Keap1 negatively regulates inflammation induced by *M. avium*; hence, we wanted to investigate if this affected mycobacterial growth. We knocked down Keap1 and infected MDMs with luciferase-expressing *M. avium*. Bacteria were then enumerated by measuring luciferase enzyme activity over a 72-h period. Keap1 knockdown was done with four individual and pooled siRNA duplexes (Fig. 6*A*). Four hours after infection, all infected cells showed approximately equal level of luciferase activity, indicating an equal uptake of bacteria. Forty-eight and 72 h after infection, bacterial counts from all Keap1-knockdown samples were significantly lower than in controls ($P < 0.01$ and $P < 0.001$, respectively). The effect of Keap1 knockdown on bacterial growth was also confirmed by cfu counts (Fig. S5*A*). To investigate the contribution of the inflammatory response in controlling infection during Keap1 knockdown, we used inhibitors for IKKβ (IKKVIII) and TBK1 (MRT67307 and BX795), and an inactive MRT67307 analog, MRT166. As shown in Fig. 6*B*, both IKKβ and TBK1 inhibitors significantly increased bacterial growth ($P < 0.05$) in Keap1-knockdown samples.

We conclude that Keap1 is a negative regulator of IKK- and TBK1-mediated inflammatory signaling, which is important to avoid chronic or overwhelming inflammation. A negative consequence seems to be facilitated growth of *M. avium* in human primary macrophages.

Discussion

Inflammatory signaling is central in controlling host defenses to intracellular pathogens. Here, we have pioneered a role of the oxidative stress sensor Keap1 in regulating inflammatory signaling and intracellular survival of *M. avium* in human primary macrophages. Silencing of Keap1 enhanced *M. avium*-induced inflammatory cytokine and type I IFN responses, resulting in better control of mycobacterial growth. We show evidence of a mechanism whereby the Keap1/Cul3-Rbx E3 ubiquitin ligase complex regulates IKKβ activity through ubiquitination and degradation, and that accumulation of p-IKKβ results in increased translocation of transcription factors NF-κB, IRF1, and IRF5 and production of target gene expression in Keap1-knockdown macrophages. We also observed Keap1 regulation of TBK1, shown by increased TBK1 and p-TBK1 upon Keap1 knockdown in *M. avium*-infected macrophages. However, unlike IKKβ, TBK1 did not seem to be ubiquitinated, suggesting the mechanism may be indirect, possibly mediated through IKKβ.

We found that *M. avium* can induce intracellular ROS generation and observed, to our knowledge for the first time, that Keap1 was recruited to mycobacterial phagosomes in an ROS-dependent manner. Komatsu and coworkers have recently shown the recruitment of Keap1 to *Salmonella* phagosomes in a

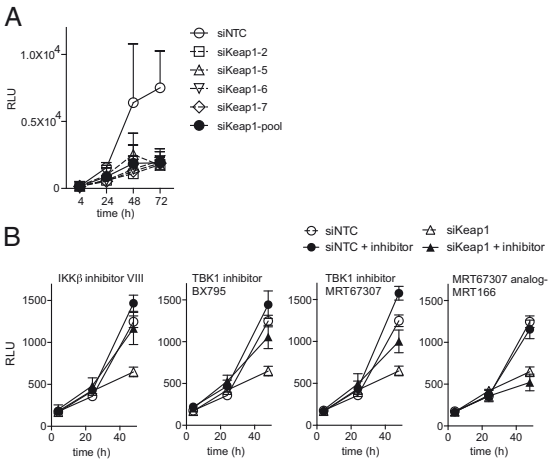

Fig. 6. Keap1 knockdown restricts the growth of *M. avium* in MDMs. (*A*) Single siRNA duplexes against Keap1 and the pooled duplexes were transfected into MDMs before cells were infected with luciferase-expressing *M. avium*. Bacterial survival was quantified 4 h, 24 h, 48 h, and 72 h after infection by analyzing luciferase activity of bacteria as a measure of survival (RLU, relative luciferase unit). (*B*) The effect of inhibition of inflammatory signaling using IKKβ inhibitor VIII (2 μM), the TBK1 inhibitor MRT67307, and its inactive analog, MRT166 (both 1 μM). siRNA-treated cells were pretreated for 30 min with inhibitors and then infected for 4, 24, and 48 h. Triplicate groups were analyzed in three independent experiments. Differences between siNTC and siKeap1 were highly significant at $P < 0.05$ at 48 and 72 h after infection. Data shown are the mean ± SEM (Fig. S5).

p62-dependent fashion (34). However, a physiological significance of this association remains to be determined. We did not find in the present study that Keap1 recruitment to mycobacterial phagosomes depended on p62, and thus the identity of the recruiting partner protein remains elusive. Nonetheless, we addressed the possible outcomes of Keap1 interactions and recruitment to mycobacterial phagosomes, in particular because the formation of signaling complexes on endomembranes seems to be a common theme for inflammatory signaling.

Cytokines play important roles as effectors and regulators of mycobacterial immunity, although their mechanism of regulation is complex and continues to be poorly understood. We observed that Keap1 down-regulated NF-κB–driven cytokines and show evidence that Keap1 also inhibits type I IFN, IFN-β, and the IFN-inducible gene CXCL10 during *M. avium* infection. Similar results were observed with Cul3 and Rbx1, members of the Keap1/Cul3-Rbx1 E3 ubiquitin ligase complex. Notably, there were usually quite low amounts of IFN-β and CXCL10 induced by *M. avium* that varied widely among blood donors. Nonetheless, in addition to our findings, several studies are also highlighting a role of the type I IFN response in bacterial infections, including *Escherichia coli* (35) and Mtb (6, 36–38). There is mounting evidence of association of excessive production of type I IFNs and exacerbated Mtb infections in mouse models and humans, possibly via an eicosanoid imbalance (19, 39), although the mechanisms behind this are not well understood. In Mtb-infected macrophages, production of cytokines such as TNF, IL-12, and IL-1β was inhibited by recombinant IFN-β (40), and, indeed, when we added recombinant IFN-β to *M. avium*-infected macrophages, we observed a trend of increased bacterial growth (Fig. S5*B*). It might thus be important to maintain low IFN-β levels in macrophages particularly early in infection, as observed in our experiments, to curtail the inhibitory effects on the other rather host-protective cytokines.

The present results further clearly implicate Keap1 in the control of the IKK complex and TBK1, suggesting a role for Keap1 in inhibiting the activation of infection-induced inflammatory signaling and type I IFNs. Our results are in line with reports citing a role of Keap1 in the regulation of NF-κB signaling and showing a consequence thereof in tumorigenesis (26, 27). These studies showed that Keap1 regulated TNF-induced NF-κB activity via IKKβ. The E(T/S)GE motif, which is found only in the IKKβ subunit of the IKK complex, was essential for direct interaction with the C-terminal Kelch domain of Keap1 (27, 41). However, we show evidence that the entire IKK complex, along with its homolog TBK1, is significantly affected by Keap1, even though only IKKβ seemed to be ubiquitinated and possibly degraded through Keap1. The suggested mechanism is further supported by the fact that similar effects as for Keap1 on inflammation were observed with Cul3 and Rbx1. The regulation of TBK1 and the other IKK homologs by Keap1 has not been shown before to our knowledge, and could be indirect. IKKs and their homologs have been described to regulate each other through the phosphorylation of their catalytic and regulatory subunits during an innate immune response (16, 42). Sustained kinase activity of IKKβ in Keap1-silenced macrophages could thus impact the activity and phosphoprotein levels of TBK1 and the other IKKs, but that remains to be further elucidated in our settings. Although we did not find ubiquitination of TBK1, other studies have described regulation of IFN signaling through the degradation of TBK1 by the PRR NLRP4 by using another E3 ligase, deltex homolog 4, in response to viral ligands (43). Alternatively, because of the inherent difficulties in immunoprecipitation (IP) and ubiquitin blotting for endogenous proteins in primary macrophages (low levels, high background), we cannot conclude that TBK1 is not directly regulated by Keap1 similar to IKKβ. However, TBK1 does not have the proposed Keap1 interaction domain (D/N)XE(T/S)GE as found in IKKβ, and could be indirectly regulated through IKKβ. Our study thus clearly places the IKKβ/NF-κB signaling axis as the central pathway that is regulated by Keap1.

The main pathways leading to IFN-α/β induction during mycobacterial infections remain unclear and quite varied. Pandey et al. (6) demonstrated that phagosomal Mtb peptidoglycan triggers the expression of type I IFNs in a mainly TBK1/IRF5-dependent manner. Others have shown a requirement of the TBK1/IRF3 or -5 signaling axis in response to cytosolic Mtb DNA (8, 13). In our hands, *M. avium* induced a generally low IFN-β response and no IFN-α. In addition to NF-κB, nuclear translocation assays suggested that IRF1 and -5 were activated and also further increased by Keap1 knockdown. IRF1 is shown to be involved in IFN-β production by TNF (44). Chemical inhibition of IKKβ or TBK1 both reduced *M. avium* induced nuclear translocation of NF-κB, IRF1, and possibly IRF5. We could not detect significant activation of IRF3 in our experiments, and, because of unspecific staining by available antibodies, we could not address the role of IRF7 and -8. To address the role of transcription factors in driving cytokine responses, we individually silenced NF-κB (RelA), IRF1, and IRF5 before infection with *M. avium* and measured cytokine mRNA transcription. These experiments proved challenging, with highly variable knockdown of 40–70% and low induction of IFN-β. siRelA inhibited *M. avium*-induced TNF and IFN-β mRNA, and, although there was a tendency of reduced IFN-β by siIRF1 and siIRF5, results were not significant. Together, we show evidence that IKKβ and TBK1 are involved in *M. avium*-induced activation of NF-κB, IRF1, and possibly IRF5. NF-κB is driving TNF and IFN-β expression, but the role of IRF1 and IRF5 are less clear and need further elucidation. Some studies also suggest a coregulation of IFN-β production by NF-κB and IRF5 (14), and we recently showed an IKKβ/IRF5-dependent

pathway in response to *S. aureus* ssRNA (17). Our data indicate that IKK and TBK1 regulation by Keap1 might have even broader consequences in the innate immune response to mycobacterial infections, as these kinases play important roles in different arms of the immune response to infection.

To determine if the role of Keap1 in inflammation was linked to its recruitment to *M. avium* phagosomes, we investigated the role of p62 through siRNA knockdown. p62 is well known for its role in protein aggregation and trafficking of ubiquitinated cargo to autophagy, and intricately linked to Keap1 (23, 24, 45). p62 is also reported to regulate various signaling events, including those activated by TNF, IL-1β and nerve growth factor receptors through scaffolding TRAF6 and atypical protein kinase C with these receptors in different cell types (46–48). Keap1 interacts with p62 (23–25), and p62 and LC3 are recruited to bacterial phagosomes to mediate autophagic clearance of the bacteria (49–51). However, Keap1 recruitment to mycobacterial phagosomes seemed independent of p62 in the present study. We also found that knockdown of p62 showed a trend of reduced expression of TNF in line with other studies (47, 48, 52), and opposite from what we observed with Keap1 knockdown. We thus found no evidence that suggests that the effect seen on NF-κB signaling by Keap1/Cul3-Rbx1 might be dependent on p62. Finally, we observed that silencing of Keap1 reduced bacterial growth in MDMs. The mechanism behind it may be more complex but can be explained at least in part by the role of Keap1 in modulating the inflammatory responses, as inhibition of IKKβ and TBK1 complemented the growth impairment observed with Keap1 silencing. Inhibiting inflammatory responses improved bacterial growth even in mock-treated cells. However, pretreatment of infected cells with recombinant TNF and IL-1β showed no effect on mycobacterial growth, and IFN-β showed increased bacterial growth (Fig. S5*B*). Results could thus not be ascribed to auto- and paracrine activity of individual cytokines despite the well-characterized importance of these cytokines in mycobacterial defenses (2, 3, 7, 18, 39, 40). We therefore propose that Keap1 acts as a negative regulator for controlling inflammatory signaling in *M. avium*-infected human primary macrophages. Although this might be important to avoid sustained or overwhelming inflammation, our data suggest that a negative consequence could be facilitated growth of pathogens like *M. avium* inside macrophages.

Recent studies have reported frequent loss-of-function mutations in the *Keap1-Cul3-Rbx1* complex in several human cancers that could be associated with pathological NF-κB activation in addition to increased transcription of Nrf2 cytoprotective target genes (21, 28). Our results indicate that altered Keap1 gene expression may have vital clinical implications also for other inflammation-associated conditions, including mycobacterial infections, which opens novel research venues for translational research, for instance in the expanding field of host-targeted therapy for infectious diseases.

Materials and Methods

Materials. Rabbit polyclonal antibody against Keap1 (cat. no. 10503–2-AP) was obtained from ProteinTech, and Keap1 antibody (sc-15246) was obtained from Santa Cruz Biotechnology. Anti-rabbit Alexa 647 and 546 was from Molecular Probes, and nitrocellulose membrane was from GE Healthcare Life Science. Cell culture media including RPMI and OptiMEM were obtained from Sigma and Invitrogen, respectively. Protease inhibitor mixture was from Roche. GAPDH, IRF5, and IRF7 antibodies were from Abcam; IRF8 antibody was from Atlas Antibodies; and all other antibodies, including anti-IRF1, anti-IRF3, anti–NF-κB-p65, anti–phospho-NF-κB-p65, anti-IKKβ, anti–phospho-IKKα/β, anti-TBK1, and anti–phospho-TBK1 were purchased from Cell Signaling Technology. Ubiquitin antibody α-Ub FK2 was from Enzo (BML-PW8810). COX IV antibody (ab33985) for blotting and controlling for loading control in immunoprecipitation assays was obtained from Abcam. Proteasome inhibitor MG132 was from Sigma (C2211). Nuclear stains DRAQ5 and Hoechst 33342 were from Alexis Biochemicals and Life Technologies, respectively. NAC, DPI, MRT67307, and MRT166 were purchased from Sigma. BX795 was from Axon MedChem and IKK inhibitor VIII was from Merck Millipore. RNA was extracted from cells using RNeasy Mini Kits (Qiagen) and reverse-transcribed to cDNA by using the High-Capacity cDNA Reverse Transcription Kit (Life Technologies). Transfection reagents used were Lipofectamine RNAiMax (Invitrogen) and siLentFect Lipid Reagent for RNAi (Bio-Rad). All primer/probes were from Applied Biosystems, and qPCR was done by using the StepOne Plus system from Applied Biosystems.

Methods.

Cells. Human peripheral blood mononuclear cells (PBMCs) were isolated from buffy coats obtained from the blood bank of St Olavs Hospital (Trondheim, Norway). MDMs were generated from monocytes enriched from total PBMCs by plastic adherence and maintenance in RPMI 1640 medium (Gibco) supplemented with 30% (vol/vol) (before stimulation) or 10% (vol/vol) (after stimulation) human serum (blood bank of St Olavs Hospital, Trondheim, Norway).

ROS detection assay. The Image-iT LIVE Green ROS detection kit (Molecular Probes) was used to detect ROS in human macrophages infected with luciferase-expressing *M. avium*. The manufacturer's protocol was followed, and control and test samples were incubated for 1 h before detection of ROS. ROS inhibition was achieved by pretreating cells with NAC or DPI for 30 min before infection. Fluorescence intensity per cell was measured by using the Bitplane Imaris Cell module.

Mycobacteria and macrophage infection. Transformants of the virulent *M. avium* clone 104 expressing firefly luciferase or CFP are described elsewhere (4). Mycobacteria were cultured in Middlebrook 7H9 medium (Difco/Becton Dickinson) supplemented with glycerol, Tween 80, and albumin dextrose catalase (ADC). Single-cell suspensions of mycobacteria from log-phase broth cultures were added to macrophages differentiated from human PBMCs at a multiplicity of infection of 10 for appropriate times. In some experiments, macrophages were pretreated with proteasome inhibitor, MG132, inhibitors MRT67307 (MRT67307 analog; MRT166) or BX795 (TBK1, Sigma, and Axon MedChem, respectively), or IKK inhibitor VIII (Merck Millipore) for 30 min before infection and throughout the entire time course of infection. Mycobacterial survival was assessed by colony counting after plating on 7H10/oleic acid ADC agar or by luciferase activity (Promega).

Immunofluorescence Assays. Macrophages grown on glass-bottomed dishes or plates (MatTek) were fixed with 2% paraformaldehyde, and then saponin-based permeabilization buffer or ice-cold methanol was used for cell permeabilization. Unspecific binding was blocked with 20% human serum. Cells were stained with Keap1 and transcription factor-specific primary and Alexa-fluorochrome secondary antibodies. The cells were imaged by using a Zeiss LSM 510 META microscope (Carl Zeiss Microimaging) equipped with a 63×/1.45 oil-immersion objective. Quantitative protein recruitment analysis from confocal images was done with Bitplane Imaris software. In the nuclear translocation assay, human primary macrophages were prepared and stained as described earlier, also including nuclear stain Hoechst 33342. Cells were then imaged by using the Olympus Scan^R microscope, and nuclear translocation was quantified as overlapping fluorescence of transcription factor and nuclear stain by using the accompanying software (Olympus) (17). The specificity of the transcription factor staining was verified by using specific siRNAs.

siRNA Transfection Assay. Transfection with siRNA was performed by using Lipofectamine RNAiMAX transfection reagent or siLentFect Lipid Reagent for RNAi according to the manufacturer's protocol at 0 and 48 h. Gene knockdown was evaluated by quantitative real-time PCR or Western blotting. Keap1 pooled HP Validated siRNA (a pool of four duplexes; Qiagen) and ON-TARGETplus human siRNAs (Dharmacon/Thermo Scientific) were used to target Keap1, p62, IKKβ, TBK1, and Nrf2. In case of siKeap1, individual siRNAs were tested and found equally efficient in silencing Keap1, with resulting increased inflammation and reduced mycobacterial growth (Fig. 5). Nontargeting control siRNAs from both manufacturers (Qiagen and Dharmacon/Thermo Scientific) were also included. Cells were treated for a total of 72 h with 20 nM siRNA.

RNA Extraction and qPCR Assessment of mRNA Levels. Total RNA was extracted from cells by using RNeasy Mini kits including DNase digestion with the QIAcube robot (all from Qiagen). cDNA synthesis was performed with the High-Capacity RNA-to-cDNA Kit according to the recommended protocol.

A total of 578 immunology-related human genes and 14 internal reference genes were included in the digital transcript counting (nCounter GX Human Immunology kit assay; NanoString Technologies). Total RNA (100 ng) was assayed on nCounter Digital Analyzer (NanoString) according to the manufacturer's instructions. Data were normalized by scaling with the geometric mean of the built-in control gene probes for each sample. Gene expression was analyzed by using the accompanying nSolver software. qPCR was performed with the

StepOnePlus System, TaqMan Gene Expression Assays, and TaqMan Universal Master Mix (ABI). The level of GAPDH mRNA was used for normalization. All primer/probe sets used for qPCR were obtained from and validated by Qiagen and used according to the manufacturer's recommendations.

Assessment of Protein Levels. Inflammatory mediators in cell supernatants were analyzed by ELISA according to the manufacturer's protocols (R&D Systems). IFN-β ELISAs were from PBL Assay Science. Western blots were performed following standard procedures. Protein lysates were quantified using the bicinchoninic acid assay (Fisher Scientific) and run in 10% NuPAGE mini gels (Invitrogen), then transferred to nitrocellulose membrane by using Invitrogen iBlot system and incubated with primary antibody at 4 °C for 24–48 h. The blots were developed with SuperSignal West Femto (Thermo Scientific) and visualized with Image Estimation 2000R (Kodak). The Kodak 1D Image Analysis software was used for band intensity quantification.

For IP and detection of ubiquitination, after Keap1 knockdown, macrophages were pretreated with 10 μM MG132 for 30 min before infection for 2 h. Macrophages were lysed in RIPA buffer [50 mM Tris·HCl, 200 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.05% SDS, 10 mM EDTA, 10 mM EGTA, phosphatase inhibitor mixture 2 and 3 (Sigma), and complete protease inhibitor mixture (Roche)]. Dynabeads Protein A (Life Technologies) were incubated with anti-IKKβ or normal rabbit IgG (R&D Systems) for 1 h at room temperature. Dynabead/antibody complexes were crosslinked with BS^3 reagent (Life Technologies) for 15 min according to the manufacturer's protocol. Lysates were adjusted for similar concentration and volume and immunoprecipitated with the prepared beads at 4 °C overnight. After IP, beads were washed three times with RIPA buffer and once with 1 M urea, transferred to new tubes, and eluted by denaturation with LDS sample buffer. Eluates were run in 10% NuPAGE mini gels, transferred to nitrocellulose membranes using Invitrogen's iBlot system, and then incubated with primary antibodies at 4 °C for 24 h. Membranes were then incubated with IRDye secondary antibodies and visualized by the Odyssey Imaging System (Licor). Image Studio 3.1 software was used for quantification.

Statistical Analysis. When cells were counted by microscopy, at least 50 cells were counted for each condition in each experiment. Unless otherwise stated, at least three independent experiments were performed for all figures. The means ± SD or SEM is shown in the figures, and P values were calculated by using a paired two-tailed Student t test of log-transformed data with GraphPad Prism software. Statistical significance was set at $P < 0.05$.

Ethics Statement. The Regional Committees for Medical and Health Research Ethics at Norwegian University of Science and Technology (NTNU) approved use of PBMCs from healthy adult blood donors after informed consent (identification number 2009/2245–2). All donors provided written informed consent.

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Supporting Information

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SI Methods

For IP and detection of ubiquitination, macrophages were pretreated with 10 μM MG132 for 30 min before infection for 2 h (Fig. S4). Macrophages were lysed in RIPA buffer (50 mM Tris·HCl, 200 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.05% SDS, 10 mM EDTA, 10 mM EGTA, phosphatase inhibitor mixture 2 and 3 (Sigma), and complete protease inhibitor mixture (Roche). Dynabeads Protein A (Life Technologies) were incubated with anti-IKKβ, anti-TBK1, or normal rabbit IgG (R&D Systems) for 1 h at room temperature. Dynabead–antibody complexes were crosslinked with BS^3 reagent (Life Technologies) for 15 min according to the manufacturer's protocol. Lysates were adjusted for similar concentration and volume and immunoprecipitated with the prepared beads at 4 °C overnight. After IP, beads were (*i*) washed three times with RIPA buffer, (*ii*) blocked with casein for 1 h and then washed three times with RIPA buffer, or (*iii*) washed three times with RIPA buffer and then once with 1 M urea, transferred to new tubes, and eluted by denaturation with LDS sample buffer. Eluates were run in 10% NuPAGE mini gels, transferred to nitrocellulose membranes by using an Invitrogen iBlot system, and then incubated with primary antibodies at 4 °C for 24 h. Membranes were then incubated with IRDye secondary antibodies and visualized by the Odyssey Imaging System (Licor). Image studio 3.1 software was used for quantification.

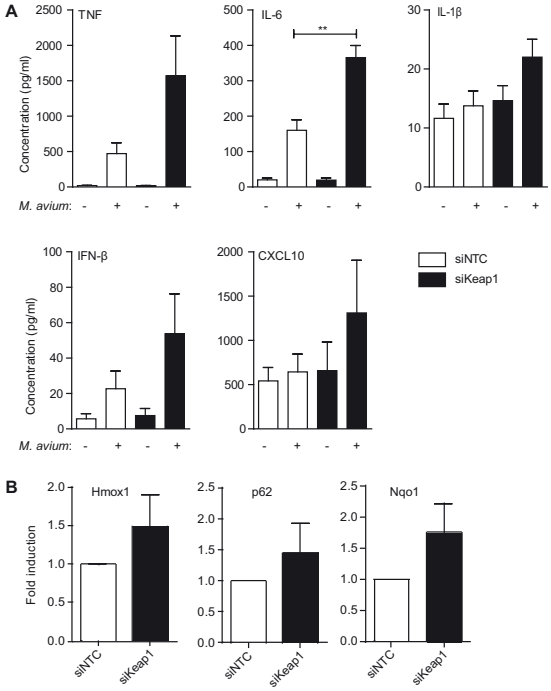

Fig. S1. Keap1 knockdown increased *Mycobacterium avium*-induced cytokine production and transcription of Nrf2-driven cytoprotective genes. MDMs were pretreated with siKeap1 or siNTC. (*A*) *M. avium* infection for 4 h. TNF, IL-6, IL-1β, IFN-β, and CXCL10 protein levels were measured by ELISA in supernatants of cells harvested for mRNA analysis (Fig. 2). Results show absolute values of cytokines in uninfected and infected cells. (*B*) Heme oxygenase 1 (Hmox1), p62, and NAD(P)H dehydrogenase (quinone 1, Nqo1) transcripts in uninfected cells. Results are shown as fold induction. All experiments were repeated $n = 4$–8 times from cells obtained from different blood donors, and data shown are the mean ± SEM (*$P < 0.05$ and **$P < 0.01$, Student *t* test).

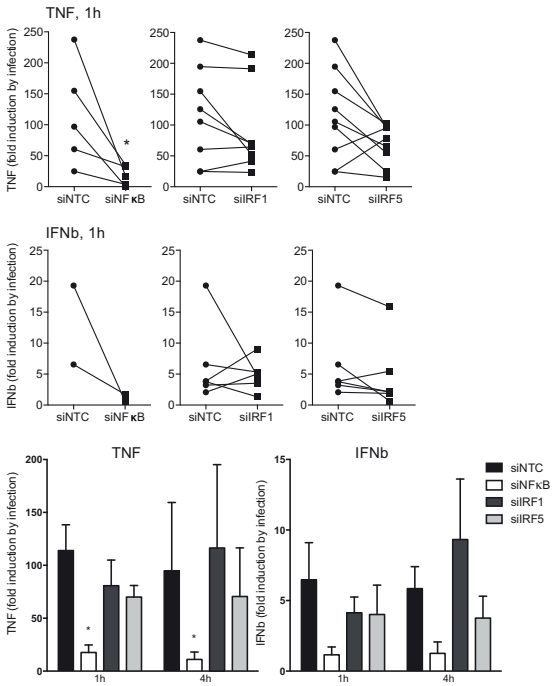

Fig. S2. Knockdown of NF-κB, IRF1, and IRF5 inhibited inflammatory cytokine expression during *M. avium* infection. MDMs were transfected with 20 nM pooled siRNA against the different transcription factors or siNTC. Knockdown was analyzed by qPCR and normalized to GAPDH. Fold effects on TNF and IFN-β mRNA expression after siRNA treatment 4 h after *M. avium* infection was calculated relative to uninfected controls. Data are shown for $n = 2–9$ independent experiments with different donors as the mean ± SEM (*$P < 0.05$).

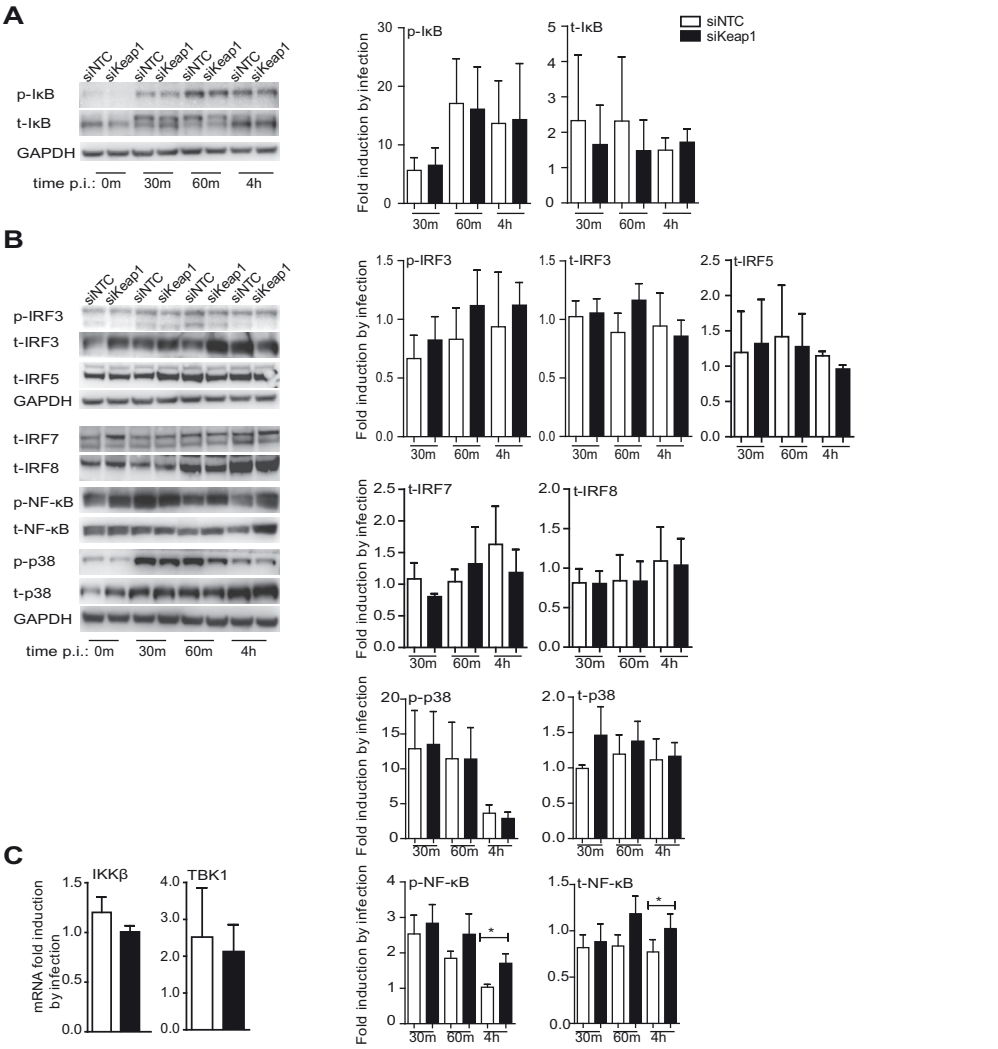

Fig. S3. Keap1 knockdown showed no effect on IκB and IRF protein levels and IKKβ and TBK1 mRNA expression during *M. avium* infection. MDMs were transfected with siKeap1, and knockdown levels were analyzed by Western blotting compared with siNTC samples 30 min, 60 min, and 4 h after *M. avium* infection. (*A*) Phosphorylated and total protein levels were examined with anti-phospho (p-) or anti-total (t-) antibodies at the same time points after infection for IκB. (*B*) Protein levels for some members of the IRF family. (*C*) IKKβ and TBK1 mRNA expression was analyzed by qPCR 4 h after infection. All fold induction values have been calculated relative to uninfected controls. All experiments were repeated independently $n = 4$–6 times from cells obtained from different donors, and data shown are the mean ± SEM. *P* values obtained by Student *t* test were not significant.

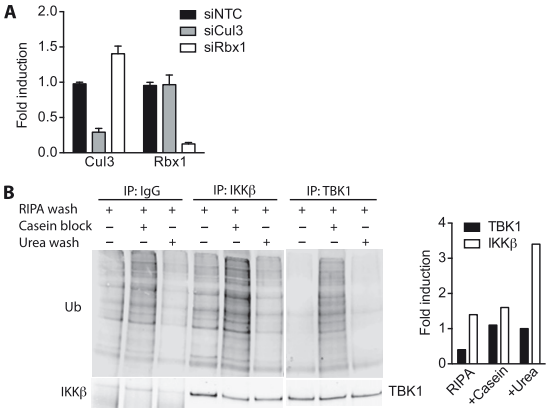

Fig. S4. IKKβ but not TBK1 is ubiquitinated following *M. avium* infection of MDMs. (*A*) MDMs were transfected with siNTC, siCul3, or siRbx1. Knockdown levels of Cul3 and Rbx1 were analyzed by real-time PCR and are presented as fold induction relative to siNTC-treated controls. (*B*) MDMs were pretreated with 10 μM of the proteasome inhibitor MG132 before *M. avium* infection for 2 h. Ubiquitination of IKKs was analyzed by immunoprecipitations of IKKβ or TBK1 and subsequent staining for ubiquitin. A representative blot from one of two experiments is shown.

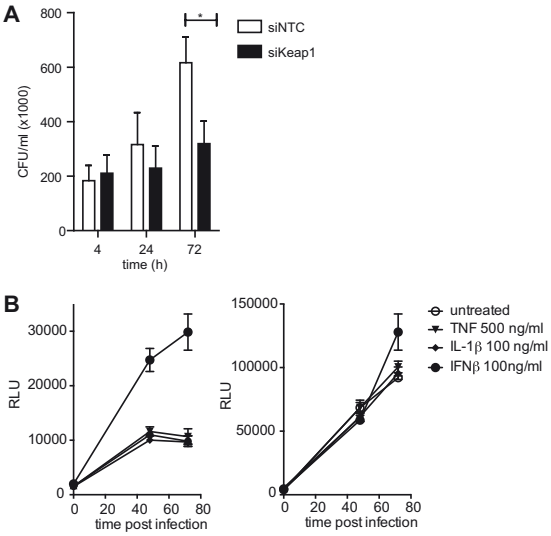

Fig. S5. The effect of Keap1 knockdown, ROS inhibitors, or addition of cytokines on intracellular growth of *M. avium* in MDMs. (*A*) MDMs were pretreated with siKeap1 or siNTC before infection with luciferase-expressing *M. avium*. Bacterial numbers were quantified over time by cfu counts. Bars represent data from two independent experiments with cfu counts analyzed in triplicate. Data shown are the mean ± SEM (*$P < 0.05$, Student *t* test). (*B*) MDMs were pretreated with recombinant TNF (500 ng/mL), IL-1β (100 ng/mL), or IFN-β (100 ng/mL) for 30 min before infection with luciferase-expressing *M. avium*. Cytokines were from R&D Systems. Bacterial growth was quantified by luciferase activity (RLU, relative luciferase units) over time. Data shown are the mean ± SEM (triplicate cell parallels each assayed in duplicate for luciferase activity) from two donors.

Table S1. Keap1 regulates *M. avium*-induced proteins in human primary macrophages

	Fold induction by *M. avium* infection	
Gene	siNTC	siKeap1
CCL4	140.4	168.7
IL-1β	98.0	343.8
PTGS2 (COX2)	42.1	45.9
TRAF1	14.3	11.5
CXCL2	11.5	19.4
CCR7	7.9	9.8
IL-18	6.9	10.9
TNFAIP3 (A20)	5.1	7.9
CD274	5.1	6.0
CD40	3.4	4.1
IRF1	2.7	5.0
CLEC4E (Mincle)	2.6	2.8
NFKB2	2.6	3.5
GBP1	2.3	4.0
CD83	1.6	2.8
SLAMF7	1.6	1.9
GPR183	1.5	1.5
CD36	1.0	1.0
MYD88	0.9	1.0
HLA-B	0.9	1.1
HLA-C	0.8	1.0
HLA-A	0.8	1.1
CD81	0.8	1.0
IRAK1	0.7	0.9

Human primary macrophages were treated with siKeap1 or siNTC control and infected with *M. avium* for 4 h. Induction of immunology-related genes using NanoString Technology. Excerpt of genes from a 578-gene array is shown as fold induction by infection.