THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (PHD)

Modulation of dendritic cell functions by the cell surface autophagy regulator SLAMF5 and by mitochondrial ROS

by Zsófia Agod

Supervisor: Árpád Lányi, PhD

UNIVERSITY OF DEBRECEN
DOCTORAL SCHOOL OF MOLECULAR CELL AND IMMUNE BIOLOGY

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ABBREVIATIONS

5’ppp-dsRNA: 5’-triphosphate containing double-stranded RNA
5’ppp-RNA: 5’-triphosphate containing RNA
7-AAD: 7-aminoactinomycin-D
AMA: Antimycin-A
APC: antigen-presenting cell
Atg: autophagy-related genes
BafA1: bafilomycin A1
cDC: conventional dendritic cell
CLL: chronic lymphocytic leukemia
CLR: C-type lectin receptor
CT: cycle threshold
DALIS: dendritic cell aggresome-like-induced structure
DAMP: damage-associated molecular pattern
DC: dendritic cell
dsRNA: double-stranded RNA
EAT-2: Ewing sarcoma activated transcript-2
EBV: Epstein-Barr virus
ETC: electron-transport chain
GPX: glutathione peroxidase
GRX: glutaredoxin
HCV: hepatitis C virus
IFN: interferon
IL: interleukin
IRF8: interferon regulatory factor 8
ITAM: immunoreceptor tyrosine-based activation motif
ITIM: immunoreceptor tyrosine-based inhibitory motif
ITRM: immunoreceptor tyrosine-based regulatory motif
ITSM: immunoreceptor tyrosine-based switch motif
LC3: microtubule-associated protein light chain 3
LPS: lipopolysaccharide
mAb: monoclonal antibody
MEF: mouse embryonic fibroblast
moDC: monocyte-derived dendritic cell
mTOR: mammalian target of rapamycin
mtROS: mitochondrial ROS
ND: not determined
NLR: NOD-like receptor
NOX: NADPH oxidase
PAMP: pathogen-associated molecular pattern
PBMC: peripheral blood mononuclear cells
pDC: plasmacytoid dendritic cell
PE: phosphatidylethanolamine
PI3K: phosphatidylinositol 3-kinase
PI3P: phosphatydilinositol 3-phosphate
PRR: pattern recognition receptors
PRX: peroxiredoxin
RAPA: rapamycin
RIG-I: Retinoic Acid-Inducible Gene I
RIGL: RIG-I ligand
RLR: RIG-I-like receptor
ROS: reactive oxygen species
SAP: SLAM-associated protein
SH2: Src homology 2
siRNA: small interfering RNA
SLAMF: Signaling lymphocyte activation molecule family
SOD: superoxide dismutase
Tfh: T follicular helper cell
TLR: Toll-like receptor
TRX: thioredoxin
XLP1: X-linked lymphoproliferative 1

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1. INTRODUCTION

Dendritic cells (DCs) are major participants of the immune response, capable of recognizing a wide range of microbes and danger signals. In addition to their sensory function, they coordinate the innate and adaptive arms of the immune system to deliver efficient antimicrobial responses and to ensure that these destructive responses are used solely against pathogens, but not self-structures. To deploy the appropriate response to an enormous number of different environmental challenges DCs must process and integrate signals generated by pattern recognition receptors that are further regulated by various cell surface receptors with immune modulatory functions such as members of the Signaling Lymphocyte Activation Molecule Family (SLAMF) or even by small chemicals e.g. reactive oxidative species (ROS). Our group has a longstanding interest in studying the role of oxidative stress and immunomodulatory receptor signalling as regulators of DC functions contributing to antimicrobial resistance and self-tolerance.

Although mitochondrial ROS (mtROS) have formerly been viewed as harmful by-products of oxidative metabolism, recent studies have established that mammalian cells produce them to modify diverse cellular functions induced in response to disruption of homeostasis by infection or other stress conditions. It is fascinating, how the endosymbiont proteobacteria that evolved into mitochondria support the host to mount optimal responses against pathogens.

Autophagy is another effective mechanism that, beyond its fundamental function in cellular homeostasis, has evolved to protect against pathogens infecting the eukaryotic cell. Beyond serving as cell-autonomous defence mechanism by removing intracellular pathogens, autophagy has been integrated into systemic immune responses by regulating innate and adaptive immunity. The 2016 Nobel Prize in Medicine was awarded to Yoshinori Ohsumi for “discoveries of the mechanisms for autophagy” highlighting the impact of this field on modern medical sciences.

ROS production and autophagy are some of the most ancient mechanisms of innate immunity, yet they are major regulators of the adaptive immune response to intracellular pathogens as well as powerful modulators of the intensity of inflammatory responses. In this dissertation, I present data about the modulatory role of heightened mtROS generation in antiviral responses of plasmacytoid DCs as well as the function of SLAMF5 receptor as a regulator of autophagy in conventional DCs. Considering the well-established role of ROS and autophagy in chronic inflammation, autoimmune diseases and cancer, the novel mechanisms presented in this work should significantly contribute to a better understanding and perhaps management of these debilitating or fatal diseases.

2. THEORETICAL BACKGROUND

2.1. Dendritic cells: key players in the initiation and regulation of the immune response

Dendritic cells (DC) are strategically positioned in nearly all tissues to patrol for infection and for disturbances in cellular homeostasis. They initiate both innate and adaptive immune responses when they sense molecular patterns of microbial origin or signs of tissue damage. Human DCs comprise a heterogeneous population of hematopoietic cells, which based on their tissue localization, phenotypic and functional characteristics can be broadly categorized into two major types: conventional DCs (cDCs) and plasmacytoid DCs (pDCs) [1].

CDCs, located in peripheral tissues, act as sentinels of the immune system continually sampling their environment. They show phagocytic activity whereby they internalize self- and non-self-proteins, and then consequently migrate into the draining lymph nodes via afferent lymphatics. Lymphoid-resident cDCs are located close to lymphoid vessels in lymph nodes and can contact with antigens delivered by the lymph [2]. Alternatively, antigens can be transferred to them from cDCs, which have encountered the antigen in the periphery. In the T-cell rich area of the regional lymph nodes cDCs present peptide fragments of the antigens through major histocompatibility complex (MHC) molecules to T cells.

PDCs significantly differ from cDCs by multiple features, including their tissue localization and function. They are a rare population of circulating cells, which under steady state conditions are absent from peripheral non-lymphoid tissues. They migrate constitutively from the blood into lymph nodes through high endothelial venules. This steady state migration is significantly increased when lymph nodes are exposed to inflammatory conditions [3-5]. Following activation, they accumulate in inflamed tissues to restrict viral propagation locally [6]. Due to their migratory properties, cDCs are often considered the first responders of a host defence against infection, while pDCs, circulating in blood or located in secondary lymphoid tissues, may respond in a delayed fashion.

2.1.1. Sensing microbial invasion and damaged or altered self by pattern recognition receptors

Pathogen sensing by innate immunity is mediated by so-called pattern recognition receptors (PRRs), predominantly expressed by host immune cells. They recognize pathogen-associated molecular patterns (PAMPs), essential for the given microorganism, thus expressed and conserved within certain groups of microbes, but absent from host cells. Thus, PRRs enable DCs to discriminate between foreign and self-structures. PRRs can also detect host-derived materials that are released

during cellular/tissue injury, recently referred to as damage-associated molecular patterns (DAMPs) [7].

In accordance with their distinct functions, cDCs and pDCs have non-overlapping, complementary sensitivities to invading pathogens. CDCs express a wide array of PRRs ensuring the detection of most organisms, such as bacteria, fungi, protozoa and viruses as well as a broad range of damaged self-structures. As key coordinators of antiviral immunity, pDCs possess 'handy' combination of PRRs to recognize viruses invading various cellular compartments [8].

PRR engagement in DCs induces the production of inflammatory cytokines, chemokines and type I interferons (IFNs), important in the early antimicrobial response, but it also impacts on antigen presentation and co-stimulation, thereby is part of the initiation of the adaptive immune response.

The major PRRs include the Toll-like receptors (TLRs), RIG-I-like receptors (RLR), NOD-like receptors (NLR) and C-type lectin receptors (CLR), which survey both the extracellular and intracellular niches to identify microbial pathogens. TLR1, -2, -4, -5 and -6 localize to the cell surface and mainly recognize microbial membrane and cell wall components, such as the TLR4 ligand lipopolysaccharide (LPS). Whilst TLR3, -7, -8 and -9 are predominantly expressed in the endocytic compartment of immune cells and recognize microbial nucleic acids [9]. In contrast to the cell type-specific and limited expression of membrane-bound TLRs, a wide spectrum of immune and non-immune cells are equipped with cytosolic PRRs. RLRs localize to the cytosol and recognize viral RNA. RLRs include RIG-I that senses short double-stranded RNA (dsRNA) and 5'-triphosphate containing RNA (5'ppp-RNA) structures. Since host RNAs are single-stranded and are capped by methylation at the 5' end, RIG-I is able to distinguish self from non-self RNAs [10]. NLRs are cytosolic receptors that recognize a wide variety of microbial PAMPs and endogenous DAMPs. Upon activation, many NLRs form multi-subunit protein complexes termed inflammasomes, the activity of which is crucial for processing the pro-inflammatory cytokine pro-IL-1β into its mature, secreted form [11]. CLRs usually recognize carbohydrate structures on pathogens, however, several CLRs can also bind cell surface-bound self-molecules and have been shown to mediate cellular interactions. Therefore, they are suggested to participate in both pathogen recognition and control of immune homeostasis [12].

2.1.2 Initiation of innate and adaptive immunity by cDCs

2.1.2.1. CDCs are the major professional antigen-presenting cells that prime or anergize T cells

CDCs are the prototype of professional antigen-presenting cells (APC) having the unique characteristics required for priming naïve T cells, thus initiate the adaptive immune response. In the steady state, immature cDCs continuously internalize physiological tissue-derived materials then present these self-antigens in the T-cell area of lymph nodes. In the absence of danger signal-induced

co-stimulation, this process establishes and maintains tolerance to self-components [13]. In response to infection or tissue injury, cDCs become activated, resulting in their transition to a mature cell type with increased co-stimulatory molecule expression. Due to their altered chemokine receptor expression, they migrate to the T-cell area of lymph nodes, where they present antigens and deliver co-stimulatory signals for T cell activation and differentiation. Activation of naïve T cells is highly dependent on the expression of co-stimulatory molecules on cDCs, since a TCR signal in the absence of co-stimulation renders the T cell anergic. CDC maturation leads to upregulation of CD80, CD86 as well as CD70, which bind to CD28 and CD27 initiating important co-stimulatory pathways in naïve T cells. Simultaneous engagement of these receptor/ligand pairs and the TCR is essential for T cell proliferation and survival [14, 15]. Thus, antigen presentation by cDCs, depending on their maturation state, may contribute to both the initiation of a pathogen-specific immune response and to establishment and maintenance of peripheral tolerance.

2.1.2.2. The origin of peptides for antigen presentation; facilitating immunological tolerance by cDC autophagy

In general, antigens synthesized in the cell are loaded onto MHC-I molecules to be displayed for $CD8^+$ T cells, while exogenous antigens captured by endocytosis are presented by MHC-II for $CD4^+$ T cells. However, cDCs have the exquisite capacity to take up antigens of necrotic cells for their subsequent cross-presentation in the context of MHC-I. This process enables the initiation of cytotoxic $CD8^+$ T-cell responses against tumours and intracellular pathogens that do not directly infect cDCs [16]. Moreover, autophagy has been described as an important access route for cytosolic antigens to reach the MHC-II presentation machinery in APCs [17]. This process is termed type 2 cross-presentation [18]. Via autophagy, cells sequester their own components into *de novo* formed double-membrane-bound vesicles; called autophagosomes, to be carried to lysosomes for degradation. This catabolic pathway recycles intracellular components to maintain cellular energy levels and preserves protein and organelle quality through selective elimination of damaged intracellular material whose accumulation would be toxic for the cell [19, 20]. The autophagic vesicles can fuse with endosomes thereby delivering antigenic peptides to the MHC-II-loading compartments [21].

The array of peptides presented to naïve T cells by activated cDCs during the process of priming determines the repertoire of effector T cells, which will survey the surface of cells in target tissues. Therefore, activation of cDCs initiates multiple processes, which limit the display of self-antigens and focus the immune response against an emerging environmental threat. One of the factors that determine the protein potential to become a source of antigenic peptides is the activity of the proteolytic machinery. Autophagy can be influenced by many external cues and thus can adapt antigen presentation and antigen distribution to a particular immune context. Stimulation of cDCs by the TLR4 ligand LPS has been shown to transiently reduce the autophagic process suspending degradation

and MHC-II-restricted presentation of cytosolic antigens by activated cDCs [22]. Reduction of endogenous antigen presentation in response to microbial stimuli has been revealed by the identification of dendritic cell aggresome-like-induced structure (DALIS). DALIS represents a strong aggregation of polyubiquitinated proteins targeted for degradation and its formation is the coordinated consequence of a rapid increase in protein synthesis occurring concomitantly with an autophagy flux reduction upon cDC activation. Since protein aggregates are protected from processing by the proteasome, DALIS assembly retains cytosolic antigens in cDCs [23]. Thus, concomitant with acquiring the license to prime T cells, microbe sensing decreases the ability of cDCs to present self-antigens. Meanwhile, presentation of exogenous antigens by cDCs in the presence of co-stimulatory molecules activates cognate T cells and thus initiates an adaptive immune response against the invading pathogen. These mechanisms allow focusing the immune response on host defence and prevent activation of autoreactive T-cells in the presence of strong co-stimulation that may break peripheral tolerance (**Figure 1**).

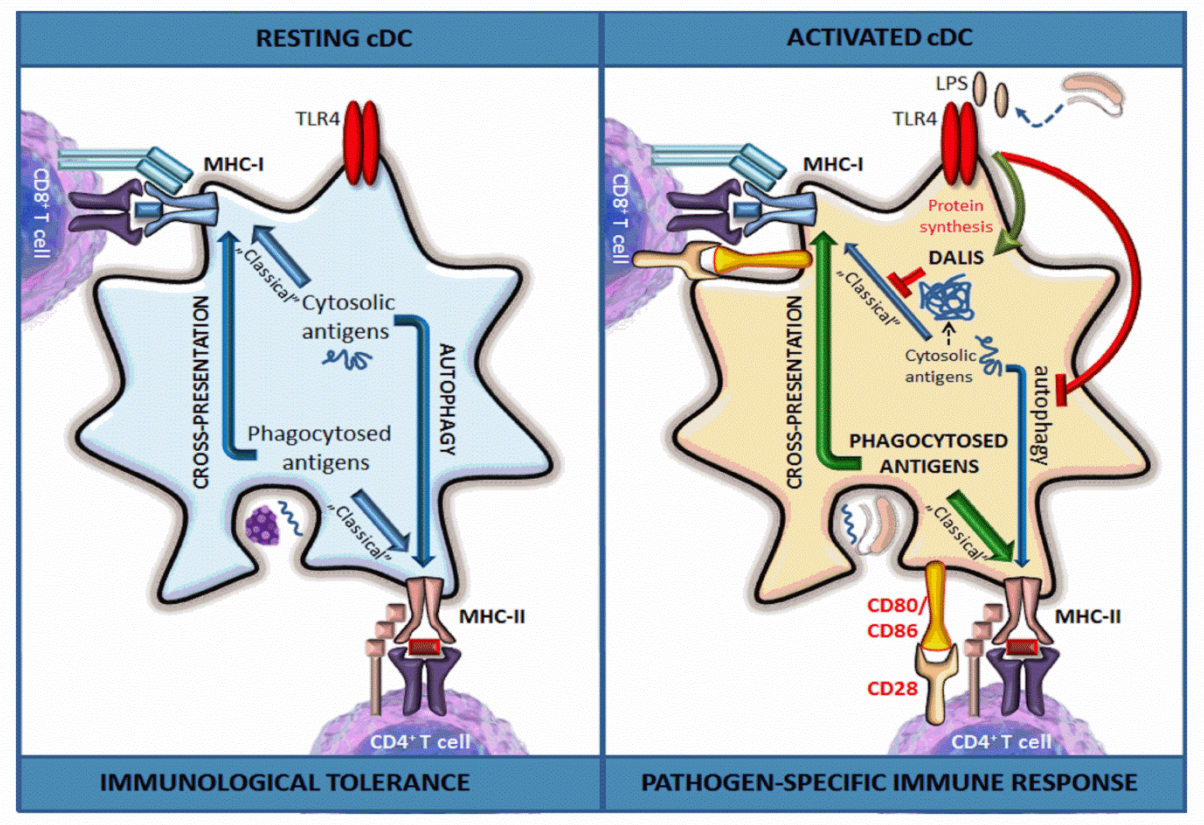


Figure 1. In response to cDC activation, a shift in the array of presented peptides towards phagocytosed antigens occurs due to autophagy flux reduction.
Under steady state conditions cytosolic antigens can be degraded by both the proteasome and via autophagy leading to MHC-I- and MHC-II-dependent presentation of self-antigens, respectively. In response to TLR4 signals, autophagy is inhibited resulting in formation of dendritic cell aggresome-like-induced structure (DALIS) and preferential presentation of phagocytosed antigens.

2.1.2.3. Induction of local inflammation by cDCs; anti-inflammatory and antimicrobial effects of autophagy

In addition to the initiation of adaptive immunity, cDCs are also key participant of the inflammatory reaction. Their activation via PRRs leads to inflammatory cytokine and chemokine secretion that attract immune effector cells to the site of cDC activation. Subsequent modulation of these cells via cytokines or direct cell-cell interactions triggers the inflammatory cascade. The resulting pro-inflammatory state is necessary for the generation of a rapid and robust antimicrobial response and for the proper activation of the adaptive immune response. A complex, continuous communication and information exchange between tissue-resident and recruited immune cells governs both the regulation of direct killing of pathogens, and the resolution of the inflammatory process. As all immune responses have the potential to convey damage to host tissues, tight control of these processes is necessary to avoid excessive inflammation and maintain homeostasis.

An increasing number of studies support the view that autophagy is of extreme importance in restraining potentially detrimental innate immune responses. Several mechanisms have been discovered to mediate cyto-protective and anti-inflammatory effects of autophagy [24]. Reactive oxygen species (ROS) are continuously generated in mitochondria. At physiological levels they function as second messengers in a broad range of signalling pathways, however, due to their high reactivity, they have the potential to damage the integrity of cellular components. Thus, by removing oxidized proteins and organelles as well as the cellular machineries, e.g. damaged mitochondria responsible for excessive ROS production, autophagy controls the release of multiple endogenous danger signals fuelling the inflammatory process [25]. Moreover, it was reported that removal of dysfunctional ROS-generating mitochondria serves as a brake on RIG-I and inflammasome signalling [26, 27]. In this way, autophagy is involved in increasing the activation threshold of the immune response thereby preventing untimely activation under steady state conditions. In addition, the autophagy machinery is re-activated following cDC activation in order to counteract exaggerated cytokine production thereby decreasing the magnitude and duration of inflammation [28]. This is supported by the observation that autophagy inhibition in cDCs is associated with enhanced secretion of pro-inflammatory cytokines IL-1β and IL-23 [29-31].

In some contexts, autophagy can also enhance immune responses. Beyond degrading cytoplasmic self-components with the aim of recycling nutrients or eliminating aberrant organelles and protein aggregates, autophagy can also facilitate the degradation of invading microbes that is presumably one of the most ancient forms of immune defence [32]. Apart from direct elimination of intracellular pathogens, autophagy redirects microbial nucleic acids from the cytosol to the lumen of endosomal compartments, where they meet their cognate PRRs. Likewise, it may deliver cytosolic microbial antigens to MHC-II-loading compartments to support the activation of $CD4^+$ T cells [17, 33]. Moreover, circulating monocytes recruited from the bloodstream to the sites of infection are

induced to differentiate into cDCs or macrophages by a program that is also highly dependent on autophagy [34]. The newly differentiated monocyte-derived dendritic cells (moDCs) may function as resting tissue resident APCs or as sources of inflammatory cytokines. In addition, these cells may migrate to secondary lymphoid organs and maintain the activation of naïve T lymphocytes [35, 36].

Beyond the recognition of microbe that tailors the innate immune response to neutralize the actual environmental threat, additional cues from the surrounding cells also modulate the response. Phagocyte functions during microbial invasion are associated with oxidative stress. On one hand, they release ROS to eradicate the infection; on the other hand, they produce cytokines such as TNF, which may stimulate ROS formation in neighbouring cells altering the redox state in their cytoplasm [37]. In addition to the oxidative stress generated by local inflammation in response to microbial infection, increasing evidence suggests that viruses can also directly regulate cellular ROS production in host cells to manipulate signalling pathways supporting their replication [38-40]. Thus, in the process of microbial eradication, surrounding tissues/cells are under the influence of cytokines and excess ROS, which alters the function of nearby cells. The next part will discuss how this microenvironment can influence responsiveness of infiltrating pDCs and production of the key antiviral cytokines, type I IFNs, important in the fight against intracellular pathogens.

2.1.3. PDCs are the major type I IFN producing cells

In humans, the type I IFN family consists of 13 IFNα subtypes, IFNβ, IFNε, IFNκ and IFNω, all of which signal through the receptor IFNAR. Nearly every cell is capable of producing type I IFNs, however, pDCs are their main source. Most cells produce IFNβ, while pDCs predominantly produce large amounts of IFNα. Type I IFNs induce resistance to viral replication in all types of nucleated cells, providing a powerful defence mechanism against viral spreading [41]. Type I IFNs produced by pDCs also contribute to the expansion of an antiviral immune response by enhancing the activity of other immune cells including T cells, B cells, NK cells, and cDCs. They promote long-term T-cell survival, polarization of memory T cells towards Th1 and enhance the cytotoxicity and IFNγ production of $CD8^+$ T cells as well as NK cells. In addition, they facilitate antibody production and promote immunoglobulin class switching in B cells. Furthermore, they enhance antigen cross-presentation and maturation of cDCs [42]. Albeit less efficiently than cDCs, pDCs present antigen to $CD4^+$ T cells and are capable of cross-priming $CD8^+$ T cells [43, 44].

2.1.3.1. Dual control of type I IFN production of pDCs via endosomal TLRs and cytosolic RLRs

PDCs express a unique combination of virus specific TLRs, namely TLR7 and TLR9, which are endosomal sensors of microbial nucleic acids. In the early phase of viral infection, when they

circulate in blood or are localized to secondary lymphoid tissues, they engulf non-infectious viral particles or apoptotic bodies from infected cells [45]. Due to the vesicular localization, the ingested viral debris at this point is recognized via these endosomal receptors. Upon TLR-driven activation, pDCs can release type I IFNs rapidly into the circulation, up to 1000 times more than other cell types. The molecular basis of their huge type I IFN secretion capacity can be attributed to at least two factors. On one hand, to their high constitutive levels of IRF7 that is required for TLR7/9-induced IFNα production. On the other hand, to a unique endosomal trafficking mechanism that retains TLRs and their ligands in the early endosomes, where they can induce a robust IFNα production [46]. Concomitant with triggering the production of type I IFNs, TLR7/9-mediated signals prepare pDCs for sensing cytosolic viral nucleic acids by up-regulating RIG-I expression [47].

When activated, pDCs leave the bloodstream and accumulate in the peripheral tissue guided by chemokines released from the site of active inflammation [48]. At this point, they might become infected by viruses, and nucleic acids produced during viral replication can be detected in the cytoplasm by RIG-I. Furthermore, as material delivery from the endosomal compartment to the cytosol occurs in pDCs to facilitate cross-presentation, it could be presumed that viral nucleic acids can come into contact with cytosolic RIG-I upon phagocytosis of the debris of infected cells [49]. RIG-I-dependent viral detection then leads to IRF3 activation that contributes to a second wave of type I IFN production by recruited pDCs at the site of infection [50].

2.1.3.2. A possible role for the altered redox state of pDCs in the first and second waves of type I IFN production

The common endpoint of pDC activation is induction of type I IFNs. The additional signals to which pDCs are exposed when they enter the inflamed peripheral tissues could unravel the significance of the RIG-I-mediated cellular responses. The environments in which pDCs circulate in steady state conditions and migrate during inflammatory responses vary regarding their redox state. Infection and inflammation are characterized by high levels of ROS released by phagocytes or injured cells. Earlier our group has shown that endosomal TLR-mediated antiviral responses of pDCs are highly sensitive to elevated levels of ROS. This is underscored by the observation that pDCs exposed to low dose of H_2O_2 are impaired in their capacity to produce type I IFNs in response to TLR7 stimulation [51]. Although TLR-mediated signalling pathways are relatively well characterized in human pDCs, the functional importance of RIG-I in this cell type remains poorly described. Interestingly, data from mouse embryonic fibroblasts (MEF) suggest that ROS are positive modulators of RIG-I signalling. It has been shown that Atg5-deficient MEFs, which accumulate dysfunctional mitochondria thus, have increased mitochondrial ROS (mtROS) production, display enhanced RIG-I- and MAVS-dependent type I IFN production and resistance to infection with vesicular stomatitis virus. Antioxidant treatment blocked the excess type I IFN production, confirming that ROS were

largely responsible for the enhanced RLR signalling. Moreover, enhancing mtROS level by treating wild type cells with rotenone, a well-known inhibitor of the electron transport chain, was sufficient to amplify RLR signalling [26]. These findings implicate mtROS as an important component of antiviral responses.

Given the strong activating effect of type I IFNs on a wide range of immune cells, it is clear that type I IFN production by pDCs has to be under tight control. Of note, the RIG-I mediated second phase of type I IFN production of pDCs is much lower compared to the TLR-initiated first phase of IFN secretion [47]. It is tempting to speculate that in inflamed tissues where ROS are constantly present and suppress the endosomal TLR-mediated pathways, this moderate, RIG-I-mediated production may be the main mechanism to support potent antiviral responses by pDCs.

2.2. Fine-tuning DC responses via mtROS-mediated redox signalling and immunomodulatory receptors

Temporal and spatial regulation of DC functions is essential to steer the immune response into the desired direction. DCs are activated through the concerted action of many signalling pathways acting within and between the responding DC and other immune cells. Whereas some receptors or ligands or even inorganic molecules have “primary” roles in the initiation of the immune response, others have “secondary”, modulatory, albeit still critical functions in the regulatory process.

2.2.1. Immune modulatory role of mtROS

Over the past decades several studies have demonstrated that dedicated cellular ROS producers such as NADPH oxidases (NOX) participate in cellular signalling pathways [52]. It was eventually shown that mtROS, which were once considered as ill-fated by-products of cellular respiration, are also involved in the regulation of signalling processes, thereby being capable of altering the activation and duration of the inflammatory processes [53]. Mitochondrial production of ROS is increased in the presence of infectious agents as well as under stress conditions associated with cellular damage. ROS are chemically reactive molecules that can oxidize proteins, lipids and DNA and indiscriminately damage cell constituents at high levels. When tightly controlled, however, like other post-translational modifications, reversible oxidation of specific targets can alter protein function, allowing cellular adaptation in response to changes in the intracellular and extracellular environment. As the level of mtROS production is a potential redox signal in inflammatory conditions, it is important to consider how this might be modified and harnessed for treatment of infections or autoimmune diseases.

2.2.1.1. Sources and regulation of mtROS signal

The mitochondrial electron-transport chain (ETC) is a major source of cellular ROS. The orderly flow of electrons down the mitochondrial ETC to complex IV results in their final deposition into molecular oxygen. However, leakage of electrons mainly at complexes I and III of the ETC leads to partial reduction of oxygen, resulting in the formation of superoxide [54]. Once formed, the superoxide is rapidly converted to H_2O_2 by the action of superoxide dismutase (SOD). Unlike superoxide, H_2O_2 can diffuse through membranes into the cytoplasm and the extracellular milieu, thereby potentially acting as a redox signal. Although complex I releases ROS only into the matrix, complex III can produce ROS on both sides of the mitochondrial inner membrane. Unlike ROS produced by complex I, intermembrane space ROS, generated by complex III, may act as an efficient cytosolic signalling molecule having to pass only the mitochondrial outer membrane [53] (**Figure 2**).

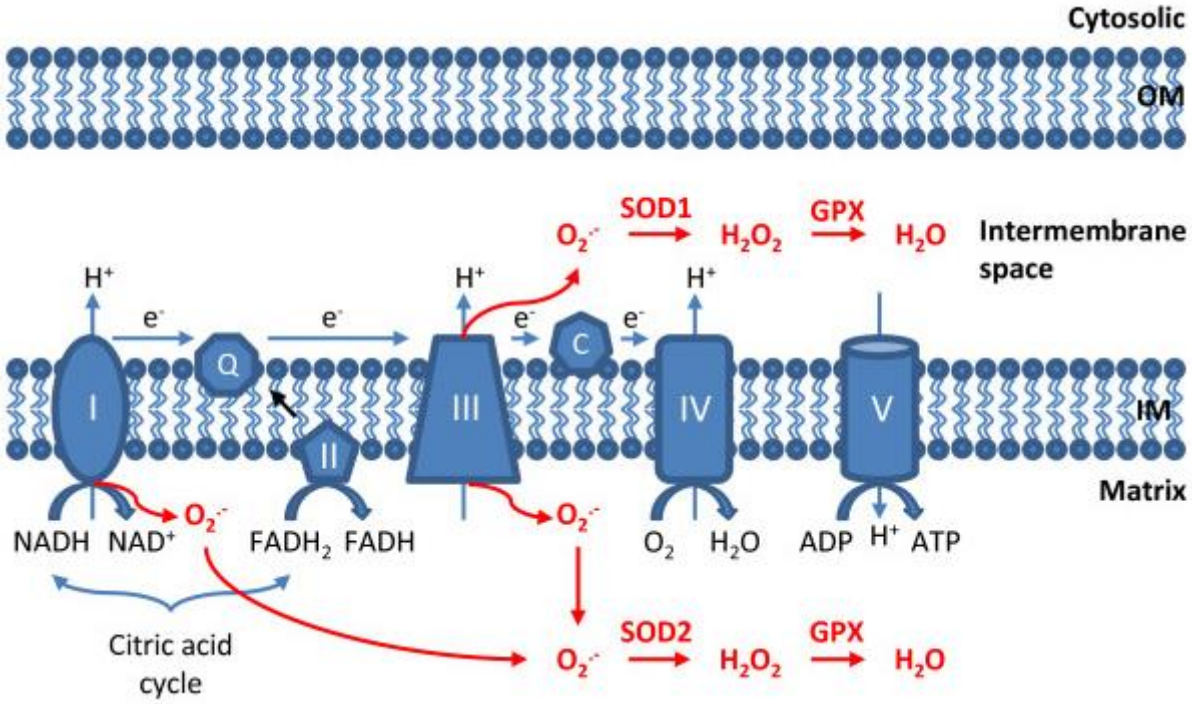


Figure 2. Generation and disposal of mtROS. *(Li et al, Journal of Hematology & Oncology 2013, 6:19)*
A major endogenous source of ROS is the mitochondrial electron transport chain, where continuous electron leakage to O_2 occurs during aerobic respiration. While complex I produces ROS only into the matrix, complex III can release ROS towards both sides of the mitochondrial inner membrane. Superoxide anion (O_2^-) is the first mtROS to be formed when single electron reacts with molecular oxygen, and it is subsequently converted to another type of ROS, hydrogen peroxide (H_2O_2), by superoxide dismutase (SOD). H_2O_2 is fully reduced to water by glutathione peroxidase (GPX). As it is theoretically easier for mtROS in the intermembrane space to reach the cytosol than those in the mitochondrial matrix, it can be hypothesized that complex III is the predominant source of mtROS that influence cellular signalling pathways in the cytoplasm.

The level of mtROS can be regulated via control over production and degradation. Several exogenous stimuli can increase mtROS levels including exogenous ROS and pro-oxidant cytokines, such as TNF generated upon phagocyte activation [55, 56]. Microorganisms and their components can also enhance mtROS generation. Apart from the replicating viruses in the host cells, sensing of PAMP by immune cells can also enhance mtROS formation. Stimulation of macrophages via cell surface TLRs (TLR1, TLR2 and TLR4) but not of endosomal TLRs (TLR3, TLR7, TLR8 and TLR9) results in mitochondrial translocation of TRAF6 that interacts with ECSIT, a protein that has been implicated in mitochondrial respiratory complex I assembly, leading to increased mtROS that aid in the destruction of phagocytosed bacteria [57].

To prevent and combat the excess ROS and maintain cellular homeostasis, there is a robust antioxidant system. Glutathione peroxidase (GPX) and peroxiredoxin (PRX) quickly reduce H_2O_2 to water in the cytosol, thereby regulating H_2O_2 tone in cells [58].

2.2.1.2. Concept of redox signalling

To serve as an efficient regulatory system the modification needs to be specific, reversible and fulfil some physiological role. Due to the lack of regulation of their generation and scavenging as well as their high reactivity towards a wide range of unspecific targets, not all type of ROS act as signalling molecules [59]. H_2O_2, however, has selective reactivity towards particular cysteine residues in proteins. Contrary to previous understanding, H_2O_2 usually cannot oxidize thiol groups of target proteins directly. Due to its high abundance and reactivity, H_2O_2 is more prone to first oxidize a PRX molecule than other target proteins that can lead to the scavenging of signalling-associated H_2O_2. However, PRXs not only participate in elimination of H_2O_2, they can also mediate the redox pathway between H_2O_2 and a target protein. PRXs contain an active site with a redox-sensitive cysteine, which is oxidized by H_2O_2. They may transmit oxidation signal by serving as a redox relay, where the disulphide formed on PRXs is subsequently transferred to a target protein [58] (**Figure 3**). The unparalleled features of this mechanism are specificity and efficiency provided by protein-protein interactions. For instance, in mammalian cells PRX2 forms a redox relay with STAT3 and transmit the oxidative signal to the redox-regulated transcription factor. Thereby PRX2 catalyses the generation of disulphide-linked STAT3 oligomers, which are compromised in their transcriptional activity [60].

A second scenario is described by the floodgate model. This theory is based on the ability of PRXs to undergo reversible hyperoxidation by a second H_2O_2 molecule that transiently inactivates the protein. It may lead to local building up of H_2O_2 inside the cell, which could then attack other targets that in basal conditions would be outcompeted by PRXs [58, 61]. Most probably both mechanisms operate for different signal transduction proteins. Much remains unknown regarding how the cells spatially and temporally channel H_2O_2 into specific signalling pathways to achieve the desired cellular

outcomes. However, it is becoming increasingly clear that signal transduction by ROS molecules occurs in a previously unsuspected sophisticated manner.

Switching off the oxidation signal is mediated by thioredoxin (TRX) and glutaredoxin (GRX), which restore protein function by facilitating the oxidized proteins to return to their reduced state [58].

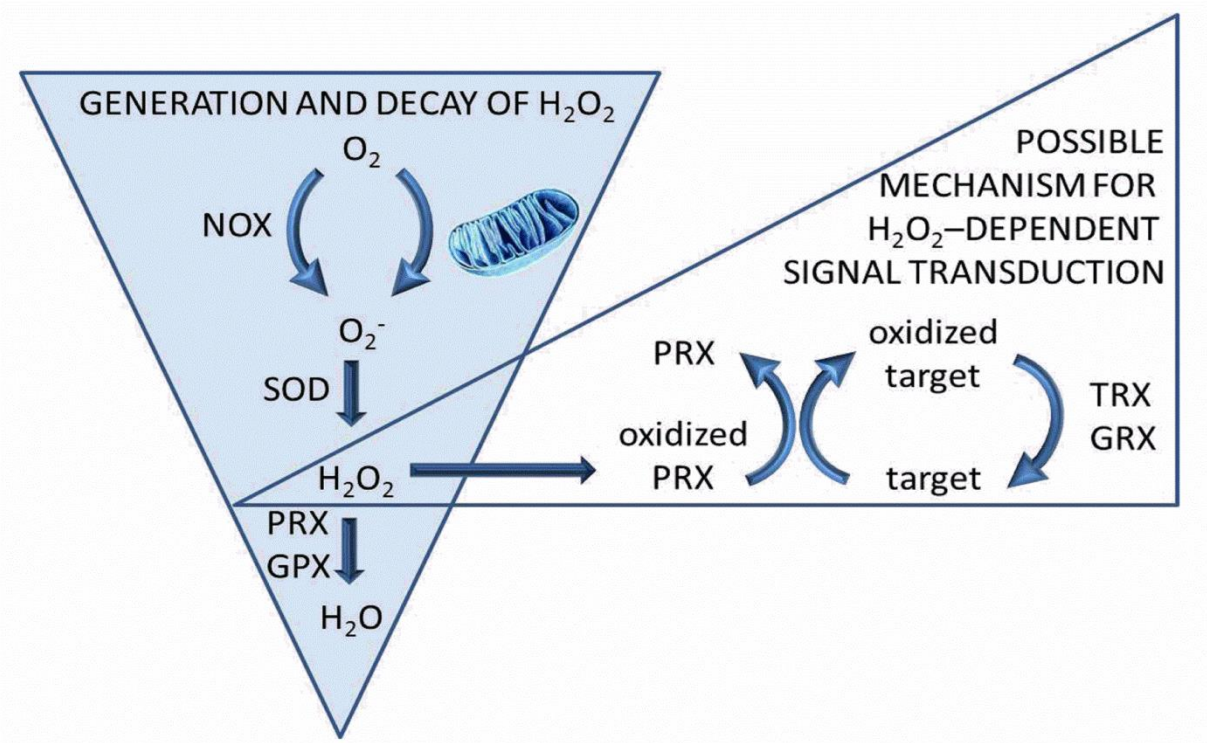


Figure 3. Formation, transmission and termination of the oxidation signal. *(modified from Reczek et al., Curr Opin Cell Biol, 2015.* ***33****: p. 8-13.)*
Superoxide (O_2^-), primarily produced by NADPH oxidase enzymes (NOXs) and mitochondria, is rapidly converted into hydrogen peroxide (H_2O_2) by superoxide dismutases (SOD). H_2O_2 can either be detoxified to water (H_2O) by peroxiredoxin (PRX) and glutathione peroxidase (GPX) or act as a signalling molecule. According to the redox relay model, a scavenging enzyme such as PRX transduces the H_2O_2 signal and oxidizes the target protein. This modification is reversible by the actions of thioredoxin (TRX) and glutaredoxin (GRX).

Redox modifications can impact on cellular signalling by altering protein-protein interactions, the DNA binding activity of transcription factors, and the catalytic activity of enzymes. Interestingly, redox modifications also affect other posttranslational modifications, essential for signal transduction, for instance, phosphorylation. An important class of ROS targets is phosphatases. These enzymes oppose the activity of protein kinases and possess a reactive cysteine in their catalytic domain that is required for enzymatic activity but also makes them a target for oxidation by ROS. As oxidation of phosphatases results in their enzymatic inactivation, cytosolic H_2O_2 can enhance phosphorylation of target proteins, thereby rendering many kinase cascades redox sensitive [62].

Redox signalling is triggered by specific stimuli and is localized to certain compartments or confined areas within a cellular compartment. The signal induces the production and release of redox active molecules such as H_2O_2 that can influence the outcome of signal transduction pathways. An

extensive body of literature has established ROS-mediated cross-regulation of PRRs [63-65]. Such cross-regulation can promote cell activation or inhibition in a context-dependent manner. The role of mtROS in RIG-I receptor-mediated pathway is prominent, as mitochondria serve as a platform for RIG-I-mediated signalling, with the adaptor molecule MAVS linked to its outer membrane [66]. Consequently, components of the RIG-I signalling pathway are proximal in location to the oxidant-generating system. However, as mentioned above, the RIG-I/MAVS signalling pathway becomes functional in pDCs only following TLR stimulation, which receptors have distinct subcellular localization and downstream signalling. Since the early phase of IFN response in pDCs is mediated by endosomal TLRs, while the late phase of IFN response can also be triggered by cytosolic RIG-I, pDCs provide an ideal model to study the impact of elevated mtROS on the antiviral signalling pathways initiated by receptors with distinct subcellular localization. Due to the limited availability of these cells from human peripheral blood, to investigate the role of mtROS in the TLR9- and RIG-I-signalling pathways driving type I IFNs secretion in pDCs, we used the GEN2.2 human pDC cell line [67]. Our results obtained using the GEN2.2 cell line were subsequently validated in primary human pDCs isolated from the peripheral blood of healthy blood donors.

2.2.2 Tuning DC responses by immunomodulatory receptors

Immune cells are activated as a result of signals transmitted by different classes of cell surface receptors, many of them bearing cytoplasmic tyrosine-phosphorylation sites called immunoreceptor tyrosine-based regulatory motifs (ITRM). ITRMs include activation (ITAM), inhibitory (ITIM) and switch (ITSM) motifs, which become phosphorylated by Src family kinases upon receptor engagement, then serve as docking sites to recruit downstream signalling proteins containing Src homology 2 (SH2) domains [68]. Association of SH2-domains with ITAM and ITIM sequences involves two contact sites: a phosphotyrosine-binding pocket and a “specificity determining” region that interacts with residues located C-terminal to the tyrosine [69].

The initial characterization of ITRM signalling and function led to the paradigm that ITAM-coupled receptors synergize with other activating stimuli and transmit signals that lead to cell activation. The ITAM motif is characterized by two Y-x-x-I/L (“x” denotes any amino acid) motifs separated by a spacer sequence. Upon ligand recognition and receptor clustering, tyrosine residues are phosphorylated by Src family kinases. Dual-phosphorylated ITAM serves as docking sites for Syk family kinases, which propagate tyrosine phosphorylation signals [70]. Cell activation is induced when positive signals are not counter-balanced by a different set of receptors bearing ITIM motifs in their cytoplasmic domains with the consensus sequence V/L/I/S-x-Y-x-x-V/L/I (“x” denotes any amino acid). Ligand engagement of these inhibitory receptors results in phosphorylation of their ITIM motifs by Src family kinases and recruitment of tyrosine phosphatases, such as SHP-1 and SHP-2 or the inositol-phosphatase, SHIP, which dephosphorylate signalling intermediates in the activating pathway

leading to the termination of signalling [71]. ITIM-containing molecules, constraining the effects of PRR signalling, may determine the activation threshold, regulate or terminate immune cell activation, and hence greatly contribute to the immune homeostasis. Most known ligands of inhibitory receptors are cell-bound, implying a role in cell–cell interactions.

More recently, it has become clear that ITAM-associated receptors can also dampen signalling by other receptors and it is the ligand density that determines whether positive or negative signal is generated. The negative regulatory mechanisms are provided by tonic low-level ITAM signalling, which has opposing effects compared to the acute and high-avidity ligation of ITAM-associated receptors. The resulting pattern of partial versus complete ITAM phosphorylation leads to recruitment of different signalling molecules. Higher level of ligands (extensive cross-linking) induces dual phosphorylation of the tandem ITAM tyrosines, which provide a binding site for the two SH2 domains of Syk family kinases favouring activation. Meanwhile, lower levels of available ligands induce partial phosphorylation that preferentially recruits phosphatases and mediate inhibitory signalling [72]. Typically, activation of ITAM-coupled receptors alone is not sufficient to induce full activation of the cell. However, many microbial products and complex inflammatory stimuli provide ligands for both ITAM-associated receptors and TLRs and activate the two receptor systems in tandem [73]. This synergy may function to fine-tune the amplitude and determine the quality of the induced cellular responses. The inhibitory effect of ITAM-coupled receptors may be an important mechanism to set an immune activation threshold and it may add another level of complexity to the signalling networks and cross-talk between signal transduction pathways regulating a balanced, physiological immune response.

The third type of signalling motif, called ITSM, can convey either activating or inhibitory signals depending on the bound SH2-domain containing protein. The ITSM motif T-V/I-Y-x-x-V/I has high affinity for two small, single SH2-domain proteins, SAP and EAT-2. Unlike most other known SH2 domain-mediated associations that involve two contact sites, they make an additional contact with residues N-terminal to the tyrosine. These single-domain adaptor proteins have dual function. They may act as competitors to interfere with binding of other SH2-domain proteins e.g. the phosphatases SHP-1 and SHP-2 to the phosphorylated tyrosine of the ITSM motif or act as true adaptor proteins and recruit Src family kinases to receptors. Their dual function of being competitors of phosphatases and adaptors of kinases inspired naming their binding motif as a switch motif. The major group whose signalling is mediated by SAP-related adaptors is the SLAM family (SLAMF) [74].

2.3. The role of SLAMF receptors in regulating immunity and tolerance

Engagement of SLAMF receptors results in signalling events that modulate (positively or negatively) multiple phases of the innate and adaptive immune response. The functions mediated by this family of receptors are complex and dependent on the temporal changes in their expression levels, the presence or absence of the adaptor molecules SAP and EAT-2, the cell type, the activation state and location of immune cells [75].

2.3.1. Signal transduction by SLAMF receptors

From the 9 receptors of the SLAM family 6 (SLAMF1, -3, -4, -5, -6, -7) carry one or more copies of ITSM in their cytoplasmic domains. Except for SLAMF1, tyrosine phosphorylation of the ITSM is required for SAP binding to SLAM family members. SAP is expressed primarily in lymphocytes, specifically T cells, NK clls and NKT cells [76]. However, SLAM family members function in multiple cell types, including cells that do not express SAP. Some of these are associated with functions of the related adaptor molecule EAT-2, which is expressed by NK cells, $CD8^+$ T cells and some APCs, while other functions are independent of these adaptors [77].

SLAMF receptors can either promote or inhibit the cellular events triggered by other receptors. These alternative activities are controlled by changes in expression levels of components of their signalling pathways, by which the relative level of adaptor proteins and receptors is altered. Via SAP family adaptors, the SLAM family usually mediates stimulatory signals that promote immune cell activation or differentiation. SAP and EAT-2 couple SLAMF receptors to Src family kinases to transmit tyrosine phosphorylation signals activating other signalling proteins [77]. In the absence of SAP family adaptors, though, SLAMF receptors switch their function and convey signals of inhibitory effectors such as SHP-1, SHP-2 and SHIP-1 phosphatases, thereby suppressing immune cell functions [78] (**Figure 4**). A good example of a switch from an activating to an inhibitory receptor is provided by immune cells lacking SAP family adaptors. SLAMF receptors (SLAMF4, -6, -7) in mouse NK cells deficient in all SAP family adaptors not only lose their activating function, but also are converted into potent inhibitory receptors suppressing the function of other activating NK-cell receptors, thereby leading to a marked suppression of NK-cell responsiveness toward hematopoietic target cells [79]. As the expression of SAP family adapters can be dynamically regulated, the ‘switch’ may also occur under physiological circumstances. For instance, naïve human NK cells do not express SAP, but up-regulate it in response to IL-2 or IL-12 stimulation, whereupon they acquire the potential to kill target cell by SLAMF4 ligation [80]. SLAMF receptors can also transmit inhibitory signals under physiological conditions, for which uterine NK cells that express low amounts of SAP, provide a good example. These cells fail to kill target cells expressing SLAMF2, the ligand of SLAMF4, whereby

SLAMF4 presumably contributes to the protection of the embryo from the maternal immune system [81].

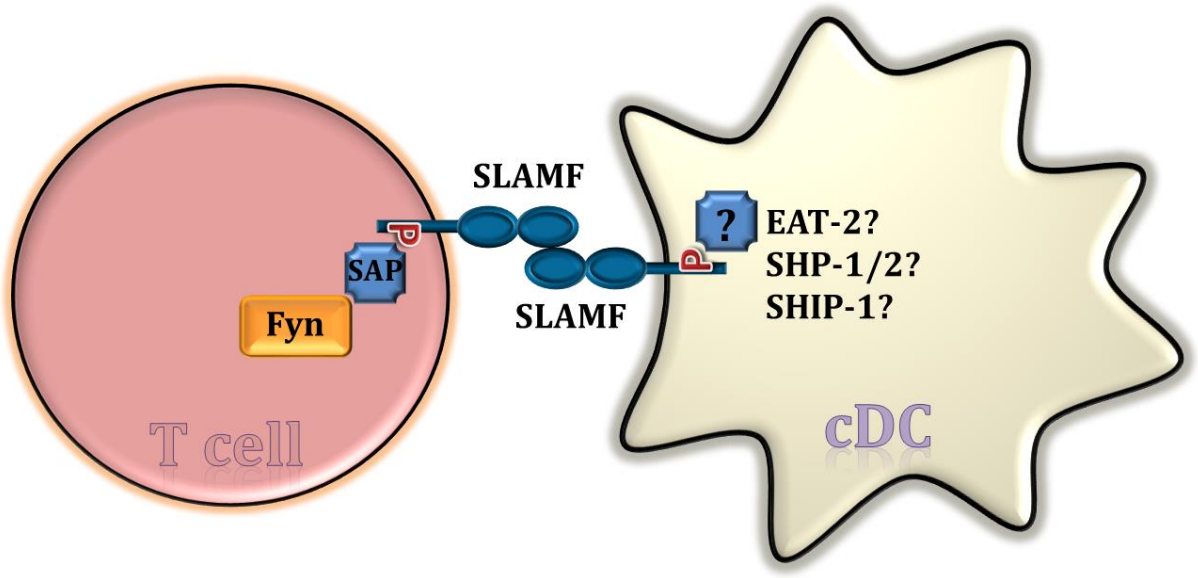
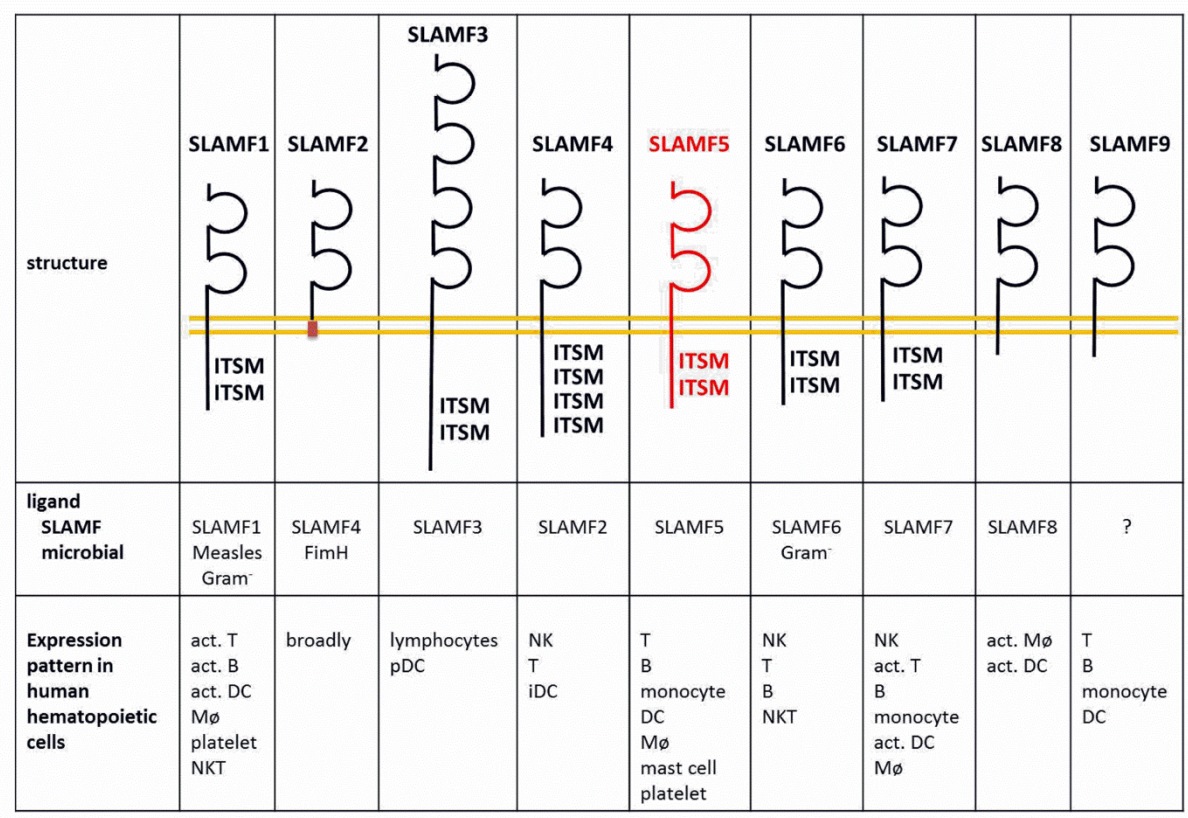


Figure 4. Signal transduction by SLAMF receptors.
Binding of SLAM family members occurs during the interaction of hematopoietic cells. Upon their stimulation by their ligands, ITSM motifs in their cytoplasmic tails become phosphorylated. This recruits various SH2-domain containing proteins to the receptors giving rise to different signals that modulate cell activation. The adaptor molecules SAP and EAT-2 block the binding of inhibitory phosphatases (SHP-1, SHP-2, SHIP-1) and recruit Src family kinases (including Fyn) to SLAM family members. Their signal transduction pathway in cDCs, however, is unknown.

Although SAP and EAT-2 drive a major part of the SLAMF signalling, an increasing amount of data demonstrates alternatively recruited regulatory molecules to members of the family. For instance, in macrophages, SLAMF1 signals through the autophagy macrocomplex that contains Beclin-1 as scaffold protein and the class III phosphatidylinositol 3-kinase (PI3K) Vps34 among other molecules [82]. SLAMF1 uses this complex as a universal signalling pathway to employ its function in more than macrophages [83]. SLAMF4 was also shown to associate with the Beclin-1/Vps34 complex [84]. Furthermore, in macrophages, SLAMF7 interacts with the integrin Mac-1 that can bind the ITAM-containing adapter FcRgamma and DAP12, which mediate immune cell activation via protein tyrosine kinases. Cooperation of SLAMF7- and integrin receptor-mediated signals then initiates phagocytosis, which provide a good example of signal integration and processing of different receptors to achieve specific cellular functions [85].

2.3.2. SLAMF receptors perform functions by homotypic interactions or by recognition of microbial structures

Different combinations of SLAMF receptors are expressed on the surface of hematopoietic cells acting mostly as self-ligands through homotypic interactions. Exceptions to this rule are SLAMF2 and SLAMF4, which associate with each other (**Figure 5**). Because of these features, SLAMF engagement may occur between homotypic cells, but also between different cell types, by which SLAMF receptors both strengthen adhesion and facilitate communication between hematopoietic cells. Differences in their binding affinities, which span at least three orders of magnitude, may contribute to the functional differences exhibited by the individual family members [86]. Platelet aggregation, stable association of $CD8^+$ T cells or NK cells with their target cells and T-B cell interactions provide examples in which cell-cell interactions mediated by integrins are associated with secondary contacts and signalling mediated by SLAM family members [87-90].



ligand SLAMF microbial	SLAMF1 Measles Gram⁻	SLAMF4 FimH	SLAMF3	SLAMF2	SLAMF5	SLAMF6 Gram⁻	SLAMF7	SLAMF8	?
Expression pattern in human hematopoietic cells	act. T act. B act. DC Mø platelet NKT	broadly	lymphocytes pDC	NK T iDC	T B monocyte DC Mø mast cell platelet	NK T B NKT	NK act. T B monocyte act. DC Mø	act. Mø act. DC	T B monocyte DC

Figure 5. SLAMF receptor family.
The SLAMF receptors are part of the Ig-superfamily. Most of them are homophilic ligands, while SLAMF2 and SLAMF4 bind each other. Three of them (SLAMF1, -2 and -6) have also been shown to possess microbial binding capacity. Six of the SLAMF receptors have docking domains (ITSM) for SAP and EAT-2. SLAMF2 is anchored to the plasma membrane by a GPI-anchor. SLAMF receptors are widely expressed in hematopoietic cells.
act: activated, iDC: immature DC

Furthermore, some SLAMF receptors function as microbial sensors. SLAMF1 and SLAMF6 are able to recognize protein structures on the outer cell membrane of several Gram-negative bacteria [91, 92]. In addition, SLAMF1 is exploited by Measles virus in that it allows viral entry, thereby facilitating infection [93]. SLAMF2 can interact with the bacterial lectin FimH, present on the pili of many Gram-negative bacteria [94]. Of note, no specific interactions of SLAMF5 with bacterial entities have so far been reported.

2.3.3. Functions of SLAMF receptors in lymphocytes; lessons learnt from SAP deficient humans and mice

The first indications that the SLAM family is involved in immune regulation were provided by experiments in which SLAMF receptors were triggered using specific antibodies. The results of these manipulations however, need to be interpreted with caution, as antibodies can mimic or block the effects of physiological engagement of SLAMF receptors and, at times, can evoke non-physiological responses [95]. For these reasons, studies of genetically modified mice are crucial for the evaluation of the physiological receptor activities. Due to the functional redundancy and compensation that exists between SLAM family members, studies based on deficiencies of individual SLAMF members may mask their phenotype and functions [89, 96]. Because SAP, their shared intracellular binding partner, interacts primarily with SLAMF receptors, analysis of SAP-deficient humans and mice can help to comprehend the functions of this family. Although, it is likely that part of the phenotypes associated with SAP deficiency is due to alternate (phosphatase or EAT-2) signals rather than to loss of SLAMF receptor functions.

Individuals with deficient SH2D1A gene (the gene encoding the SAP protein) develop an immunodeficiency syndrome: X-linked lymphoproliferative 1 (XLP1) disease [97-99]. For several clinical manifestations of XLP1, the Epstein-Barr virus (EBV), that preferentially infects B cells, acts as a potent trigger. While in most immunocompetent individuals EBV infection at an early age is usually asymptomatic, in a high proportion of XLP1 patients develop life-threatening condition called fulminant infectious mononucleosis or lymphoproliferative disease including B-cell lymphoma as well as hypogammaglobulinemia. However, patients can also develop hypogammaglobulinemia and B-cell lymphoma independently of exposure to EBV [100].

The immune compromised state in XLP1 patients comes from defective interactions between lymphocytes, causing multiple defects, namely impaired cytokine secretion and cytotoxic responses of $CD8^+$ T cells and NK cells, a complete lack of NKT cells and defects in T cell-dependent humoral immune responses [101] (**Figure 6**). Exposure of XLP1 patients to EBV induces a vigorous and uncontrolled immune response suggesting a defect in immune feedback mechanisms. Despite such fulminant immune activation, XLP1 patients fail to effectively control EBV-infected B cells due to

ineffective $CD8^+$T cell and NK cell functions upon engagement with EBV-positive target cells. The cytotoxic functions are inhibited through SLAMF4 and SLAMF6 interacting with their ligands on target cells, since SAP deficiency converts these family members from activating to inhibitory receptors [102-104]. Another characteristic feature of XLP1 patients is the impaired development of NKT cells. The positive selection of these cells is dependent on TCR interactions with lipid antigens presented in the context of CD1d molecules by neighbouring lymphocytes [105]. A secondary signal from either SLAMF1 or SLAMF6 is required to induce their differentiation and expansion [96]. The deficiency in cytotoxicity of $CD8^+$ T cells and NK cells, as well as the near-absence of NKT cells are responsible for the compromised immunity against EBV and, at least in part, for the higher incidence of B-cell lymphoma in XLP1 patients.

As a result of defective clearance of EBV-transformed B cells, T cells are continuously activated by EBV^+ B cells. In the course of a normal EBV-induced response, at the peak of the immune response, when antigen and IL-2 are still abundant, repeated stimulation through the TCR triggers activation-induced apoptosis preventing exuberant T cell expansion. XLP1 patients that suffer fulminant mononucleosis typically lack this T-cell restricting mechanism. Indeed, it was elegantly shown by the Leonardo group that the SLAMF6-SAP pathway is required for re-stimulation-induced cell death [106]. Thus, SLAMF receptors control both the extent of the $CD8^+$ T-cell expansion and the cytotoxicity of these cells, thereby mediating both protections from EBV virus as well as from exacerbated immune pathology caused by the overexpansion of EBV-specific $CD8^+$ T cells. Consequently, in XLP1 the massive expansion of reactive T cells as well as EBV^+ B cells can infiltrate the liver and bone marrow, which cause extensive tissue damage often resulting in organ failure followed by death.

The hypogammaglobulinemia in XLP1 stems from inappropriate B-cell help by $CD4^+$ T cells. SLAMF5 and SLAMF6 participate in the formation of conjugates between activated cognate $CD4^+$ T cells and B cells that guide the differentiation of T follicular helper cells (Tfh) and germinal center (GC) B cells [89]. Moreover, the SLAMF1-SAP signalling contributes to IL-4 production by Tfh cells that influences GC formation [107]. Thus, the Tfh defect in XLP1 leads to severely compromised GC formation and, hence, reduced numbers of memory B cells and long-lived plasma cells [108].

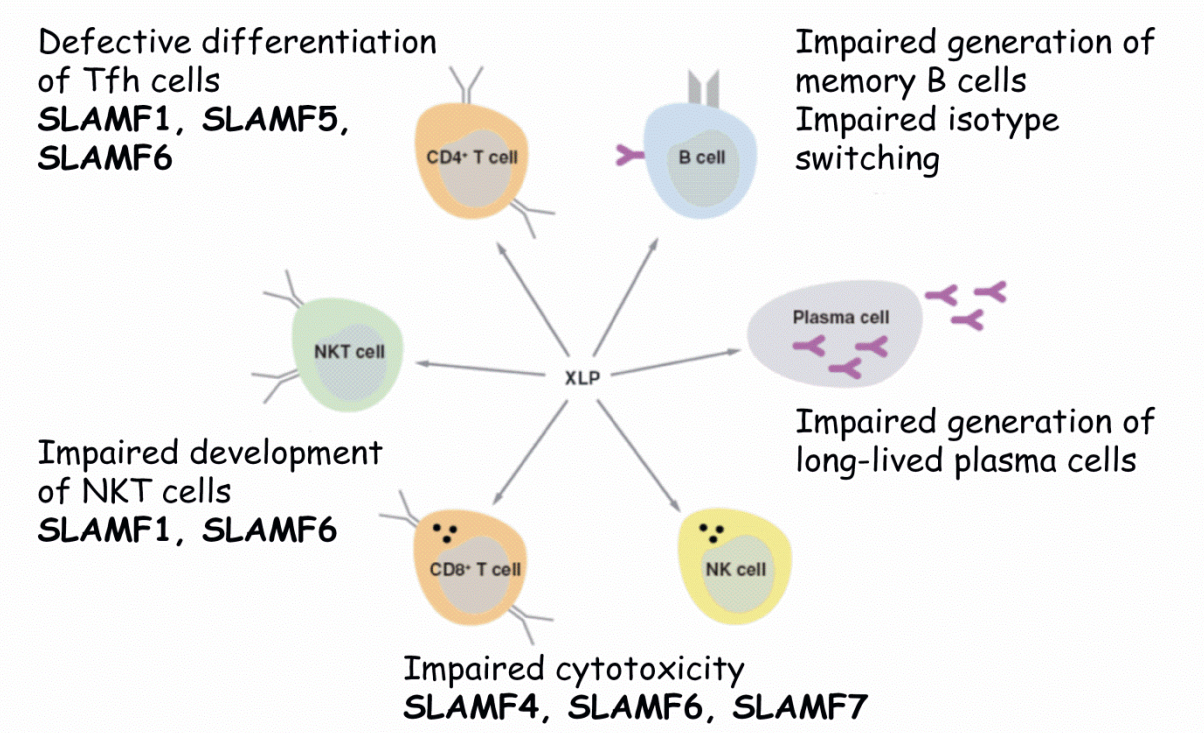


Figure 6. Cellular defects in XLP1 due to aberrant SLAMF signalling. *(modified from Ma et al., Annu Rev Immunol. 2007;25:337-79.)*
The absence of their common adapter molecule, the SAP protein, results in altered signalling of multiple SLAMF receptors. Due to the impaired T-B cell adhesion, differentiation of Tfh cells is defective. In the absence of T-cell help (delivered by Tfh cells), germinal centers fail to develop compromising isotype switching, generation of memory B cells and long-lived plasma cells. Furthermore, target cell lysis by NK cells and $CD8^+$ T cells is impaired and the development of NKT cells is defective.

2.3.4. Functions of SLAMF receptors in macrophages and cDCs

Despite of the presence of multiple SLAM family members, their function in myeloid cells has hardly been explored. Their expression depends on the activation state of cells and given the timing of their surface expression, they can modulate the extent of inflammation at distinct stages of an infection. SLAMF2 and SLAMF5 are constitutively expressed on human macrophages and cDCs, thus ideally placed to modulate their functions in various phases of the immune response, including the control of immune homeostasis. Meanwhile, SLAMF1, SLAMF7 and SLAMF8 are highly expressed during an ongoing infectious inflammation. As SAP related adaptors are absent or minimally expressed in these cell types presumably other mediators drive SLAMF receptor functions [109].

Accumulating evidence suggests that members of the SLAM family have divergent or even opposing functions in the regulation of phagocytic cells. Clearance of dying cells and pathogens is one of the key functions of phagocytes and is achieved by phagocytosis. A recent study showed that the mechanism by which macrophages engulf unwanted hematopoietic cells, a process crucial for cancer control, is dependent on SLAMF signalling. This function required a single SLAM family member,

SLAMF7 that synergizes with the integrin Mac-1 both to recognize ligands on target cells and to generate signals leading to phagocytosis [85].

SLAMF1 can bind to and facilitate internalization of Gram-negative bacteria into macrophages. Once SLAMF1 engages the pathogen, it is translocated into the developing phagosome, where it induces the production of phosphatydilinositol 3-phosphate (PI3P) by recruiting the Beclin-1/Vps34 complex. The SLAMF1-enhanced production of PI3P initiates two important microbicidal functions in the phagosome. First, it promotes the production of free radicals by the classical phagocytic NADPH oxidase (Nox2) complex. Second, as a docking lipid, PI3P positively regulates phagolysosomal fusion. Thereby, SLAMF1 facilitates elimination of trapped bacteria via ROS production and degrading enzymes [91].

In contrast to SLAMF1, SLAMF8 negatively regulates ROS production by inhibiting Nox2 activity in bone marrow-derived macrophages. SLAMF8 is expressed upon activation of macrophages by IFNγ and bacteria indicating that this receptor functions in late responses to innate stimuli, operating to dampen an ongoing innate immune response [110].

The opposite effects on ROS production displayed by SLAMF1 and SLAMF8 receptors were shown to influence cell motility. SLAMF1 positively regulates migration of cDCs to lymph nodes and monocytes/macrophages to sites of ongoing inflammation, whilst SLAMF8 has a negative effect on cell motility. Their differential expression and induction of Nox2 activity in response to innate stimuli may allow fine-tuning the extent of infiltration of inflammatory cells. Initial inflammatory signals may trigger SLAMF1-mediated Nox2 activation resulting in a spike in ROS that contributes to the migratory activity of SLAMF1-expressing cells. Subsequent inflammatory mediators (IFNγ) induce SLAMF8 expression, which suppresses the production of ROS and hence reduces additional migratory infiltration [111].

The production of pro-inflammatory cytokines was shown to be impaired by SLAMF deficiency. LPS stimulation of SLAMF1-deficient macrophages resulted in reduced IL-12 and TNFα production [112]. Similarly, SLAMF5 knockdown in murine bone marrow-derived macrophages was found to decrease LPS-induced TNFα and IL-6 production [113].

SLAMF receptors also ensure direct interactions between cells of the innate and adaptive immune system. So far, I have described how cDCs affect T cell responses but of course, T cells also influence cDC functions. During antigen presentation, T cells modulate cDCs by the release of IFNγ and by CD40 ligand expression (CD40L), which is induced on the surface of $CD4^+$ T cells activated via the TCR. SLAMF1 is expressed on the surface of activated APCs as well as on the surface of memory and recently activated $CD4^+$ T cells. The homophilic interaction of SLAMF1 reduced IL-12 and TNFα production from CD40L-stimulated cDCs. Consequently, cDCs that were previously co-stimulated with CD40L- and SLAMF1-bearing cells inhibited the differentiation of naïve $CD4^+$ T cells into IFNγ-producing Th1 effector cells, establishing SLAMF1 as a negative regulator of cDC inflammatory responses [114]. These results seem to be in contradiction with the positive role of

SLAMF1 on LPS-mediated cytokine production by murine macrophages [112]. It is plausible that SLAMF1 has two opposing effects, depending on the stimulus. Indeed, SLAMF1 engagement on human cDCs slightly increased LPS-mediated cytokine production. These findings suggest that SLAMF1 does not interfere with inflammatory responses induced by bacteria, while at later stages of the immune response, SLAMF1 engagement that takes place between activated cDCs and $CD4^+$ Th1 cells, acts as an inhibitory feedback loop to limit production of excessive amounts of inflammatory and Th1-polarizing cytokines by cDCs [114].

2.3.5. Involvement of SLAMF receptors in pathogenesis of human diseases and their effects on autophagy that may alter disease outcomes

In line with the observation that SLAMF receptors modulate inflammatory effector functions of phagocytes, the cells that are the first to react to invading pathogens, they also affect the pathogenesis of colitis. A characteristic of an early inflammatory landscape in the colon is an increased recruitment of monocytes, which are skewed to become inflammatory mononuclear phagocytes instead of hypo-responsive resident macrophages. SLAMF1-SLAMF1 interactions on the cell surface of macrophages/monocytes enhance their migratory capacity to sites of ongoing inflammation. Additionally, modulation of cytokine production by SLAMF1 may also influence the severity of colitis. Furthermore, enhanced phagosomal maturation and ROS production that results from the interaction of SLAMF1 with *E. coli* and the subsequent recruitment of the autophagy macrocomplex Beclin-1/Vps34 could lead to a higher activation state of the phagocytes in the lamina propria of the colon [115].

In a clinical context, there is already evidence to suggest abnormalities in SLAMF expression during various immune pathologies. A considerable degree of genetic polymorphism as well as differential usage of isoforms has been identified for many of the genes encoding SLAM family members [116]. These genetic changes can render the proteins to elicit different downstream signalling pathways or could influence the stability and duration of homophilic interactions. Indeed, certain polymorphisms that affect protein function or expression of a SLAM family member were reported to regulate susceptibility to systemic lupus erythematosus (SLE) and rheumatoid arthritis (RA) in humans [117, 118]. Likewise, a correlation between SLAMF protein expression levels and changes in autophagy has also been observed [84]. Cellular genome-wide association study revealed that high expression of SLAMF4 in human lymphoblastoid cells is associated with lower autophagy induction in response to rapamycin, an inhibitor of mammalian target of rapamycin (mTOR). Consistent with this, receptor activation inhibits starvation- and rapamycin-induced autophagy in the human monocytoid THP1 cells and in mouse bone marrow-derived macrophages. Ligand-bound SLAMF4 associates with the autophagy complex proteins Beclin-1 and Vps34 reducing its lipid kinase activity. Decreased Vps34 activity in turn decreases generation of PI3P, a phospholipid

essential for the initiation of autophagy [84]. As dysregulation of autophagy has been implicated in various autoimmune disorders, it is plausible that lower expression of SLAMF4 increases the risk and severity of some autoimmune diseases by enhancement of autophagy.

It was recently demonstrated that maintained SLAMF1 expression in chronic lymphocytic leukemia (CLL) cells is associated with a favourable prognosis. SLAMF1 is lost in a subset of patients with an aggressive CLL that associates with a shorter time to first treatment and reduced overall survival. SLAMF1 was found to activate the autophagic flux by governing dissociation of Beclin-1 from its inhibitor BCL2, and stabilization of the Beclin-1/Vps34 autophagy macrocomplex. SLAMF1-deficient CLL cells are resistant to autophagy-activating therapeutic agents, signifying that SLAMF1 is an important marker for patient management [83].

Based on the known effect of triggering SLAMF receptors on other immune cells, one possible mechanism for them to modulate cDC function is via regulation of the process of autophagy.

2.4. Regulation of autophagy in cDCs

Upon sudden and substantial changes in their environment, cells mount specific cyto-protective responses for survival. Autophagy is an essential adaptation mechanism against various cellular stress conditions, such as starvation or oxidative stress, maintaining the homeostatic balance at the level of both the cell and the organism. As such, it is regulated at multiple levels.

Autophagy induction is controlled by specialized Atg proteins that promote the genesis of autophagosome, and a complex network of regulatory factors that rules this process. It follows a well-ordered sequence of events, in which one of the earliest steps involves ULK1 and Vps34 complexes, acting as important gatekeepers. Inhibition of mTOR allows the ULK1 kinase to phosphorylate Beclin-1, thus activate the Vps34 complex that subsequently initiates autophagosomal membrane nucleation [119]. Vps34 is a class III PI3K that generates PI3P-rich subdomains at regions associated with the endoplasmic reticulum, which serve as anchor for recruiting PI3P binding proteins [28]. Vps34 is functional in autophagy only when it interacts with Beclin-1, a protein bound to and inhibited by BCL2. Thus, signals that affect the interaction between Beclin-1 and its inhibitory partner has the potential for modulating autophagy [120]. Among others, SLAMF1 and SLAMF4 receptors have been shown to regulate autophagy via interaction with Beclin-1 [83, 84].

In the process of autophagosomal elongation, microtubule-associated protein light chain 3 (LC3) is covalently coupled with phosphatidylethanolamine. Via this lipid modification, LC3 is targeted to the forming autophagic membrane. LC3 participates in membrane expansion and size regulation of autophagosomes and it was also shown to guide the transport of materials to the autophagosome [121].

Proper assignment of cytosolic structures for degradation via autophagy is facilitated by ubiquitin-mediated targeting [122]. Unwanted self-proteins and invading pathogens conjugated with ubiquitin are escorted to autophagosomes through ubiquitin-binding adaptor proteins, which provide anchors for the vesicle assembly around the selected cargo. The autophagosomes next undergo maturation into autolysosomes by fusion with lysosomal organelles, followed by degradation of the inner of two membranes together with the captured materials by lysosomal enzymes [123, 124].

A well-studied physiological condition in which autophagy is induced is starvation. Under extended periods of starvation, autophagy becomes the major source of nutrient supply for the cell. Nutrient deprivation is a potent inhibitor of the mTOR protein kinase that has long been known as one of the master regulators of autophagy. Under nutrient-rich conditions, however, mTOR is active and supports cell growth and proliferation by enhancing anabolic processes including protein, lipid and nucleotide synthesis, mainly through phosphorylation of its downstream effector ribosomal protein S6 kinase (p70S6K). Importantly, at the same time mTOR is a potent repressor of catabolic programmes via inhibiting ULK1, thereby blocking autophagy [125].

Beyond nutrient availability, mTOR is able to integrate signals induced by various growth factors and TLR ligands [126]. As mentioned earlier, autophagy plays an important role in cDC functionality. The particularly high basal autophagic flux of cDCs assists in the clearance of intracellular pathogens and fuels antigen processing and presentation. Following stimulation of the TLR4 pathway by LPS, autophagic flux is transiently reduced in activated cDCs. This effect has been attributed to mTOR activation that, by inducing the phosphorylation of p70S6K as well as ULK1 kinase, increases protein synthesis and reduces autophagy, respectively [22]. Consequently, autophagy-dependent MHC-II presentation is reduced that in turn focuses the adaptive immune response on captured exogenous antigens. In the later phase of cDC activation, autophagy is restored. In contrast to starvation-induced autophagy that is highly dependent on mTOR inhibition, IFNγ/TLR stimulation is linked to up-regulated mTOR activity, indicating that recovery of autophagy following IFNγ/TLR treatment may not be directly controlled by mTOR.

Besides the massive impact of mTOR on autophagy, in macrophages and cDCs the process is also regulated by the IRF8 transcription factor. IFNγ/TLR treatment stimulates the expression of many autophagy genes along with that of IRF8 itself, indicating that some autophagy factors are turned over then replenished with newly synthesized components in cDCs during infection. Microarray analyses with murine bone marrow derived DCs revealed that IRF8 stimulates transcription of many autophagy genes both in the steady state and after IFNγ/TLR stimulation [127] (**Figure 7**). Beyond transcription, the amount of the IRF8 protein within the cell is modulated via ubiquitination by at least two E3 ubiquitin ligases (TRIM21 and c-Cbl) and a deubiquitinase enzyme (USP4) [128-130]. While monoubiquitination of IRF8 facilitates its ability to induce the transcription of target genes, its polyubiquitination affects the stability of the protein by targeting it for proteasomal degradation [131].

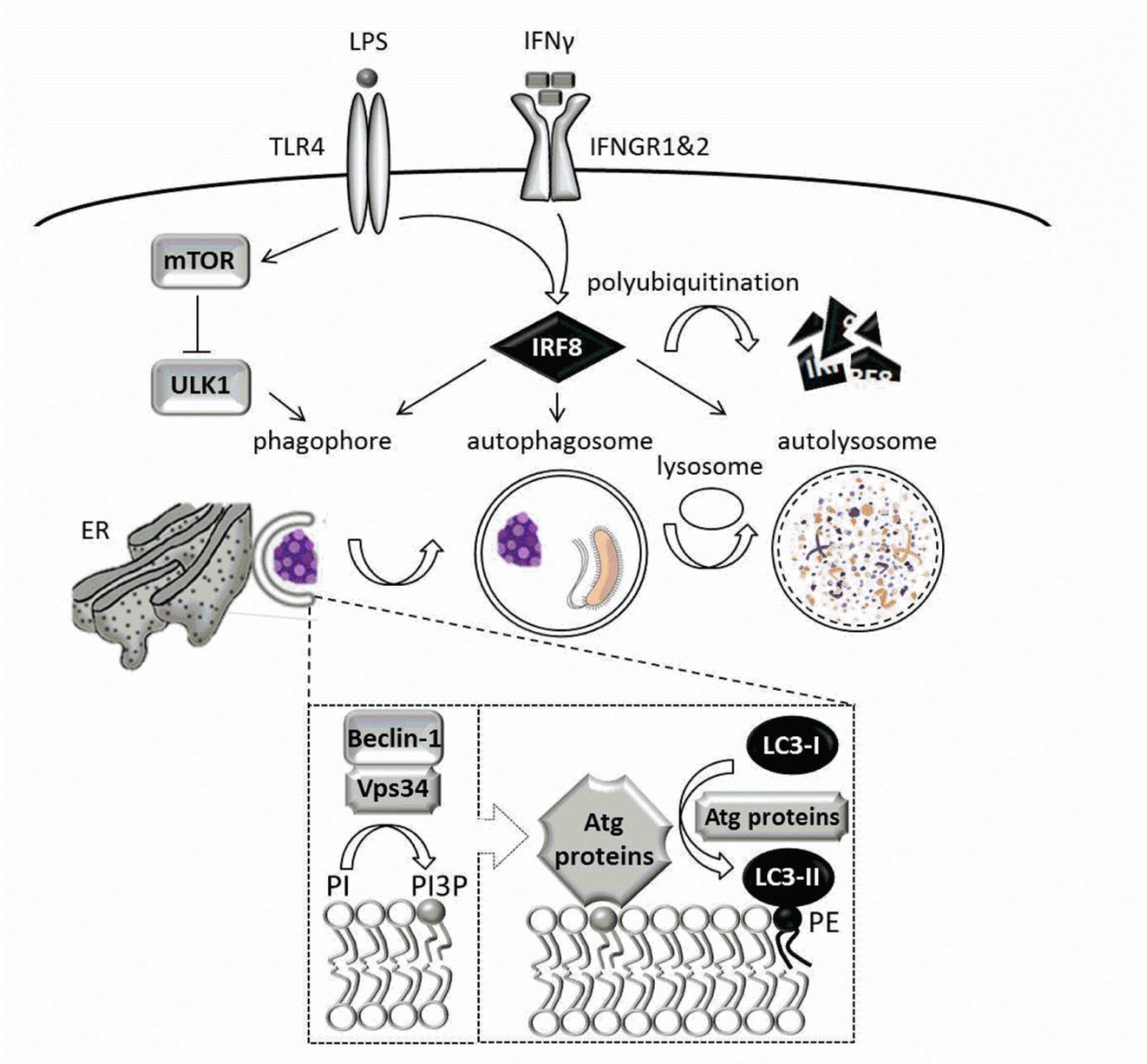


Figure 7. A simplified model of the autophagy pathway and its regulation in cDCs.
The type III phosphatidylinositol 3-kinase Vps34 together with Beclin-1 forms the Vps34 macrocomplex. This complex in turn creates phosphatidylinositol 3-phosphate (PI3P)-rich regions on the surface of endoplasmic reticulum (ER) that leads to the activation of downstream autophagy-related (Atg) proteins. Autophagic membrane is elongated based on two protein and lipid conjugation systems, which attach LC3 to phosphatidylethanolamine (PE) and to the autophagic membrane. The elongating autophagic membrane, known as phagophore, envelops its targets and eventually closes to form a double membrane vesicle, called autophagosome. Its subsequent fusion with the lysosome leads to the dissolution of the internal membrane and formation of autolysosome, where the degradation of the captured material occurs. Upon TLR4 stimulation, mTOR negatively regulates the autophagy initiation factor ULK1 kinase, a protein promoting formation of the Vps34 macrocomplex. A recently identified key factor regulating autophagy in cDCs is IRF8 that is activated in response to LPS/IFNγ and promotes the expression of a series of autophagy genes involved in various steps of autophagy, promoting autophagosome formation and lysosomal fusion. IRF8 activity and its stability were shown to be regulated by polyubiquitination and subsequent proteasomal degradation.

The above discussed findings indicate that cDC autophagy is regulated at multiple level. Autophagy regulation by PRR might be fine-tuned by activating and inhibitory signals given by different receptors. Identification of cell surface-expressed autophagy regulatory factors, readily

accessible for antibodies, may provide excellent targets to control autophagy in various disease states. The known autophagy regulatory functions of SLAMF receptors make them potential candidates for this purpose. Our focus has been on SLAMF5 that is expressed at high levels on immature DCs and its expression is increased following maturation stimuli [132]. We set out to determine whether this receptor would affect cDC autophagy as well as the inflammatory responses of cDCs and if so, via what molecular mechanisms. We modelled human cDCs by differentiating primary human monocytes into moDCs *in vitro,* in the presence of GM-CSF and IL-4 for 5 days [133].

2.5. Aims of the study

Aim 1. Clarify how pDC-controlled antiviral responses are affected by mtROS via:

- exploring how mtROS influence type I IFN production in response to ligands that activate TLR9 or RIG-I,
- identification of molecular targets and mechanism by which mtROS may affect the TLR9 and RIG-I signal transduction pathways.

Aim 2. Assess the specific involvement of SLAMF5 receptor, a member of the SLAM family of cell surface receptors, in cDC functions by

- studying the impact of SLAMF5 on the phenotype and the functional properties of LPS/IFNγ-treated cDCs,
- examining the function of SLAMF5 in the autophagic process,
- identification of some of the underlying molecular mechanisms via examining the effect of SLAMF5 on known regulatory pathways of autophagy.

3. MATERIALS AND METHODS

3.1. Isolation of primary human pDCs and monocytes

Peripheral blood mononuclear cells (PBMCs) were separated from human heparinized leukocyte-enriched buffy coats by Ficoll-Paque (GE Healthcare) density gradient centrifugation. PDCs were purified by magnetic cell sorting using the human CD304 (BDCA-4/Neuropilin-1) MicroBead Kit (Miltenyi Biotech). After separation on VarioMACS magnet, pDCs were cultured in 48-well cell culture plates at a density of $5x10^5$ cells ml^{-1} in RPMI 1640 medium (Sigma-Aldrich) supplemented with 10% heat-inactivated FBS (Life Technologies), 2 mM L-glutamine, 100 U ml^{-1} penicillin, 100 µg ml^{-1} streptomycin (all from Sigma-Aldrich), and 50 ng ml^{-1} recombinant human IL-3 (Peprotech).

Positive selection for monocytes from PBMCs was performed using CD14 antibody-coated magnetic microbeads (Miltenyi Biotech) according to the manufacturer's protocol. *(Experiments were performed by Zsófia Agod and Kitti Pázmándi.)*

3.2. RNA interference and moDC generation

To reduce the level of SLAMF5 in monocytes the following 25-nt Stealth™ RNAi oligonucleotides were ordered from ThermoFisher Scientific:

SLAMF5 sense: 5'- UGGCUAUGUUCUUUCUGCUUGUUCU -3'

SLAMF5 antisense: 5'-AGAACAAGCAGAAAGAACAUAGCCA -3'

negative control for SLAMF5 sense: 5'- UGGUAUGCUUUCUGUUCGUUUCUCU -3'

negative control for SLAMF5 anti-sense: 5'- AGAGAAACGAACAGAAAGCAUACCA -3'

IRF8-(Assay ID: s7100) and TRIM21-(Assay ID: s13462) specific Silencer Select siRNAs and non-targeted Silencer Select Negative Control No 1 siRNA were purchased from ThermoFisher Scientific. The siRNA duplexes were delivered by electroporation using GenePulser Xcell instrument (Bio-Rad Laboratories). Transfected cells were cultured at a density of 10^6 cells ml^{-1} in RPMI-1640 medium, supplemented with 10% FBS (both from ThermoFisher Scientific), 100 U ml^{-1} penicillin, 100 µg ml^{-1} streptomycin (both from Sigma-Aldrich), 80 ng ml^{-1} GM-CSF (Gentaur Molecular Products), and 100 ng ml^{-1} IL-4 (PeproTech) for 5 days to generate moDCs. Culture medium was refreshed on day 2 by removing three-quarters of the supernatant and replacing it by complete medium containing GM-CSF and IL-4. *(Experiments were performed by Zsófia Agod.)*

3.3. Culturing of GEN2.2 cells

The human plasmacytoid dendritic cell line GEN2.2 was grown on a layer of mitomycin C (Sigma-Aldrich)-treated murine MS5 feeder cells in RPMI 1640 medium (Sigma-Aldrich) supplemented with 10% heat-inactivated FBS (Life Technologies), 100 U ml^{-1} penicillin, 100 µg ml^{-1} streptomycin (both from Sigma-Aldrich) and 5% non-essential amino acids (Life Technologies). For

experiments, the GEN2.2 cells were removed from the feeder layer and seeded on 24-well plates in complete RPMI 1640 medium (Sigma-Aldrich). *(It was carried out by Zsófia Agod, Kitti Pázmándi and Marietta M. Budai.)*

3.4. Induction of elevated level of mtROS

MtROS production was monitored by loading GEN2.2 cells with 5 μM MitoSox™ Red mitochondrial superoxide indicator (Life Technologies) according to the manufacturer's recommendations. For enhanced generation of mtROS, cells were conditioned with 0,5 μg ml^{-1} Antimycin-A (AMA; Sigma-Aldrich) for 6 hours. As a control, cells were also treated with MitoTEMPO (300 μM, Sigma-Aldrich), a mitochondria-targeted antioxidant 1 hour prior to and along with the AMA treatment. At the end of the designated treatments the fluorescence intensity of MitoSox™ Red was measured at 580 nm with a FACS Calibur flow cytometer and data were analysed by FlowJo software (Treestar). *(Experiments were performed by Kitti Pázmándi.)*

3.5. Receptor cross-linking

MoDCs suspended at a density of 10^7 ml^{-1} were incubated in complete medium containing 10 μg ml^{-1} anti-SLAMF5 antibody (clone 152-1D5; LifeSpan BioSciences, Cat.No. LS-C134663) or an IgG isotype control antibody (Biolegend, Cat.No. 400124) at 4 ºC for 45 min. Cells were then washed, suspended in complete medium containing 10 μg ml^{-1} anti-$F(ab')_2$ of goat anti-mouse IgG (Jackson ImmunoResearch Laboratories, Cat.No. 115-006-062), re-seeded into 24-well cell culture plates and incubated at 37 ºC for 2 hours in a humidified atmosphere containing 5% CO_2. *(Experiments were performed by Zsófia Agod and Árpád Lányi.)*

3.6. Cell stimulation

For TLR9-mediated type I IFN production, GEN2.2 cells or primary pDCs were treated with 1 μM CpG-A (ODN 2216; Cat.No. HC4037 from Hycult Biotech) for 6 hours. To induce RIG-I receptor expression GEN2.2 cells or primary pDCs were incubated with 0,25 μM CpG-A for 16 hours. The cells were then washed and added back to plates in fresh medium. To achieve stimulation of RIG-I 5'ppp-dsRNA (Cat.No. tlrl-3prna from InvivoGen) was applied through Lyovec transfection reagent (InvivoGen) according to the manufacturer's recommendations. Briefly, 25 μl of the 5'ppp-dsRNA-LyoVec complex containing 1 μg ml^{-1} working concentration of the RIG-I ligand (RIGL) was added to the cells for the indicated time periods in all experiments.

MoDC maturation was induced by simultaneous addition of 100 ng ml^{-1} LPS (Ultrapure lipopolysaccharide from Salmonella minnesota R595, Cat.No. tlrl-smlps) and 10 ng ml^{-1} recombinant human IFNγ (PeproTech, Cat.No. 300-02) for the indicated time periods. For autophagy induction, immature moDCs were exposed to 50 nM rapamycin (Merck, Cat.No. 553210) for 4 hours. In some experiments, cells were incubated with 20 nM bafilomycin A1 (BafA1; InvivoGen, Cat.No. tlrl-baf1)

or 1 μM MG132 (SelleckChem, Cat.No. S2619) for the last 2 hours. *(Experiments were performed by Zsófia Agod, Kitti Pázmándi, Marietta M. Budai and Tünde Fekete.)*

3.7. Western blotting

For protein extraction, cells were lysed in Laemmli buffer. Protein extracts were resolved by SDS-PAGE and transferred electrophoretically onto nitrocellulose membranes (Bio-Rad Laboratories). Non-specific binding sites were blocked with 5% non-fat dry milk or 5% BSA in case of phospho-IRF3. Membranes were probed with the following primary antibodies: anti-RIG-I (Cat.No. 4520), anti-phospho-IRF7 (Ser477; Cat.No. 12390), anti-IRF7 (Cat.No. 4920), anti-IRF3 (Cat.No. 4302), anti-MAVS (Cat.No. 3993), anti-phospho-p70S6K (Thr389; Cat.No. 9206), anti-p70S6K (Cat.No. 9202), anti-IRF8 (Cat.No. 5628S) all from Cell Signaling, anti-SLAMF5 (clone H128), anti-β-actin (Cat.No. sc-47778), anti-Akt1 (Cat.No. sc-5298), anti-ubiquitin (Cat.No. sc-9133), anti-TRIM21 (Cat.No. sc-25351) all from Santa Cruz Biotechnology, anti-EAT-2 (Cat.No. LS-C169054, LifeSpan BioSciences), anti-LC3 (Cat.No. NB100-2220, Novus Biologicals), anti-phospho-Akt (Ser473; Cat.No. AF887, R&D System) or anti-phospho-IRF3 (S386; Cat.No. ab76493, Abcam). Bound primary antibodies were detected with anti-mouse or anti-rabbit horseradish peroxidase-conjugated secondary antibodies (GE Healthcare). Signals were developed by using SuperSignal West Pico or Femto chemiluminescent substrates (Thermo Scientific) and film exposure. Densitometric analysis of immunoreactive bands was performed using the Kodak 1D Image Analysis Software version 3.6. To ensure equal protein loading β-actin served as loading control, while the level of phosphorylation was normalized to the total amount of the same protein present in the samples. *(Experiments were performed by Zsófia Agod, Marietta M. Budai and Dóra Benzce.)*

3.8. RNA extraction, reverse transcription and real-time quantitative PCR

Extraction of total RNA was performed using TRI-Reagent (Molecular Research Center) according to the protocol of the manufacturer. 1 μg of total RNA was treated with DNase I (Thermo Scientific) to exclude amplification of genomic DNA, then reverse transcribed into cDNA using the High Capacity cDNA RT Kit of Applied Biosystems. The cDNA product was used for real time quantitative PCR reactions using Dream Taq DNA polymerase (Thermo Scientific) and the following gene-specific primers according to the manufacturer's instructions: IFNA1 (Assay ID Hs.PT.49a.3184790.g), cyclophilin from Integrated DNA Technologies and IRF8 (TermoFisher Scientific, Assay ID: Hs00175238_m1). Quantitative PCR was performed using the ABI StepOne Real-Time PCR System (Applied Biosystems) and cycle threshold (CT) values were determined using the StepOne v2.1 Software (Applied Biosystems). The relative amount of mRNA ($2^{-\Delta CT}$) was obtained by normalizing to the cyclophilin house keeping gene in all experiments. *(Experiments were performed by Zsófia Agod, Marietta M. Budai, Tünde Fekete and Hyelim Moon.)*

3.9. Flow cytometry

Cell viability was determined by 7-aminoactinomycin-D (7-AAD; 10 μg ml^{-1}; Sigma-Aldrich) staining for 15 min immediately before flow cytometric analysis. Cell surface protein expression was analysed with FITC-labelled monoclonal antibodies against HLA-A, B, C (Sony Biotechnology), HLA-DQ, CD40 (all from BioLegend), PE-tagged monoclonal antibodies against SLAMF5 (BioLegend, clone 1.21), CD14, CD86 (both from R&D Systems), DC-SIGN (Sony Biotechnology) and APC-conjugated monoclonal antibodies against CD1a (BioLegend). Isotype-matched control antibodies were obtained from BioLegend. Measurement of autophagy was performed using the Cyto-ID Autophagy detection kit (Enzo Life Sciences). Cells were incubated with Cyto-ID dye (1:2000) for 30 min at 37 ºC, then were washed and immediately subjected to flow cytometry. Fluorescence intensities were measured with a FACS Calibur cytometer and data were analysed with the FlowJo software (TreeStar). *(Experiments were performed by Zsófia Agod.)*

3.10. ELISA

The concentration of the secreted IL-1β and IL-12 was determined by the BD-OptEIA Human ELISA kits (BD Biosciences). The level of IL-23 was evaluated by the human IL-23 ELISA Ready-Set Go kit (eBioscience). The pre-coated human IFNα ELISA kit was purchased from PBL InterferonSource. Assays were performed according to the manufacturer's instructions. Absorbance measurements were carried out by a Synergy HT microplate reader (Bio Tek Instruments) at 450 nm. *(Experiments were performed by Zsófia Agod and Kitti Pázmándi.)*

3.11. Statistical analysis

Data are expressed as mean ± SD. All results were confirmed in at least three independent experiments. Data were analysed with GraphPad Prism v.6. software. Statistical differences among the experimental groups were determined by Student's unpaired *t* test or ANOVA, followed by Bonferroni *post hoc* analyses for least-significant differences. P-values of <0.05 were considered to be statistically significant. *(It was carried out by Zsófia Agod and Kitti Pázmándi.)*

4. RESULTS

4.1. Regulation of type I IFN responses by mtROS in pDCs

4.1.1. Raising mtROS level in GEN2.2 cells by the complex III inhibitor AMA

Studies aiming at understanding pDC-controlled antiviral responses have been hampered by the paucity of these cells in peripheral blood. To overcome this limitation we used the human pDC cell line GEN2.2, widely accepted by the field as a reliable surrogate for pDCs [67]. Based on previous publications ROS play an essential role in the regulation of the virally-stimulated signalling pathways [134, 135]. To evaluate how changes in the redox state induced by mitochondria influence activation of specific PRRs in pDCs, we treated GEN2.2 cells with Antimycin-A (AMA). AMA is a well-known complex III inhibitor that enhances the release of ROS into the mitochondrial matrix and to the intermembrane space. First, we evaluated the optimal concentration of AMA for inducing accumulation of ROS in GEN2.2 cells. Before exposure to various concentration of AMA, GEN2.2 cells were loaded with the fluorescent dye, MitoSox^{TM} that selectively detects superoxide in the mitochondria of live cells. We found consistently enhanced fluorescence signal of MitoSox^{TM} in GEN2.2 cells treated with 0,5 μg ml^{-1} AMA for 6 hours. To verify that the increased MitoSox^{TM} fluorescence was due to increased mtROS generation, we pre-treated GEN2.2 cells with the mitochondria-targeted antioxidant, MitoTEMPO, before exposure to AMA. The MitoTEMPO treatment significantly reduced fluorescence to almost control levels (**Figure 8**), indicating that the signal indeed resulted from the accumulation of mtROS. To ensure that our treatment did not cause excessive cell death, viability of cells was assessed by flow cytometry. The percentage of viable cells, defined as stained negatively for 7-AAD, was similar to controls up to 24 hours following treatment (data not shown).

From these experiments we could conclude that AMA treatment of GEN2.2 cells is suitable as an *in vitro* model of the *in vivo* stress conditions and/or metabolic changes induced by viral infection.

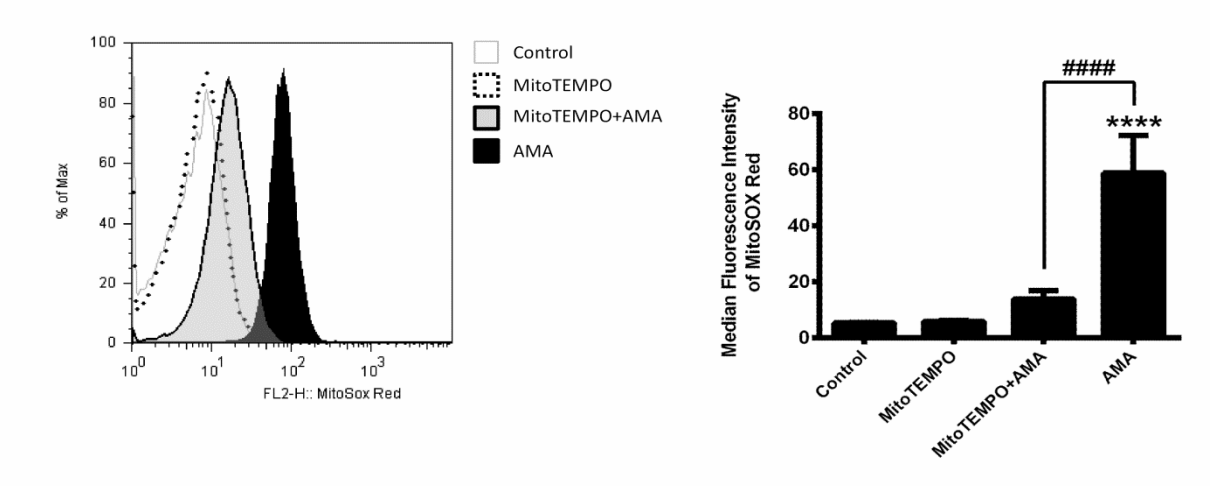


Figure 8. AMA treatment leads to accumulation of mtROS in GEN2.2 cells that can be suppressed by pre-treatment with the antioxidant MitoTEMPO.
GEN2.2 cells were loaded with MitoSox™ Red, a superoxide indicator that accumulates in mitochondria. Its oxidation by superoxide leads to the generation of red fluorescence that can be detected by flow cytometry. To enhance mtROS generation cells were treated with AMA (filled black) for 6 hours or left untreated as a control (thin gray line). To limit mtROS accumulation both AMA-treated (gray shading) and control cells (dotted line) were pre-conditioned with MitoTEMPO. A representative histogram and the means ± SD *of eight independent experiments are shown. ****$p<0.0001$ vs. control (ANOVA); $^{####}p<0.0001$ vs. AMA (unpaired t test)*

4.1.2. MtROS inhibit CpG-A-induced type I IFN production in GEN2.2 cells by reducing the phosphorylation level of IRF7

First, we evaluated the possible consequences of increased mtROS generation in pDCs on the initial wave of IFNα production induced via TLR9. TLR9 is activated by unmethylated CpG motifs prevalent in microbial but not vertebrate genomic DNA. CpG type A is a potent stimulator of type I IFN response, but a weak activator of pro-inflammatory cytokine production [136]. Resting or CpG-A treated cells were incubated with AMA, and 6 hours after stimulation expression of IFNα was measured at both the mRNA and protein levels. As shown in **Figure 9**, AMA-treatment did not alter the baseline expression of IFNA1 gene, and neither unstimulated nor did AMA-treated cells secrete IFNα. Exposure to CpG-A significantly up-regulated IFNA1 mRNA production, strikingly, this effect was abrogated when cells were activated in the presence of elevated levels of intracellular ROS triggered by treatment with AMA (**Figure 9A**). Consistently, the capacity of CpG-A to induce potent IFNα secretion is markedly reduced by concurrent AMA treatment (**Figure 9B**).

In the attempt to understand how mtROS may affect the TLR9 signalling pathway, we analysed the impact of mtROS on IRF7 phosphorylation that is known to play a critical role in TLR-induced type I IFN gene transcription in pDCs [137]. Similar to type I IFN production, AMA-treatment alone had no effect on the phosphorylation of IRF7 in resting cells, whereas, it significantly attenuated the CpG-A-induced phosphorylation event (**Figure 9C**).

Taken together, these experiments showed that excess ROS induction in the mitochondria blocks CpG-A-induced transcription of IFNA1 mRNA and hence IFNα secretion of GEN2.2 cells, presumably via inhibition of IRF7 phosphorylation by mtROS.

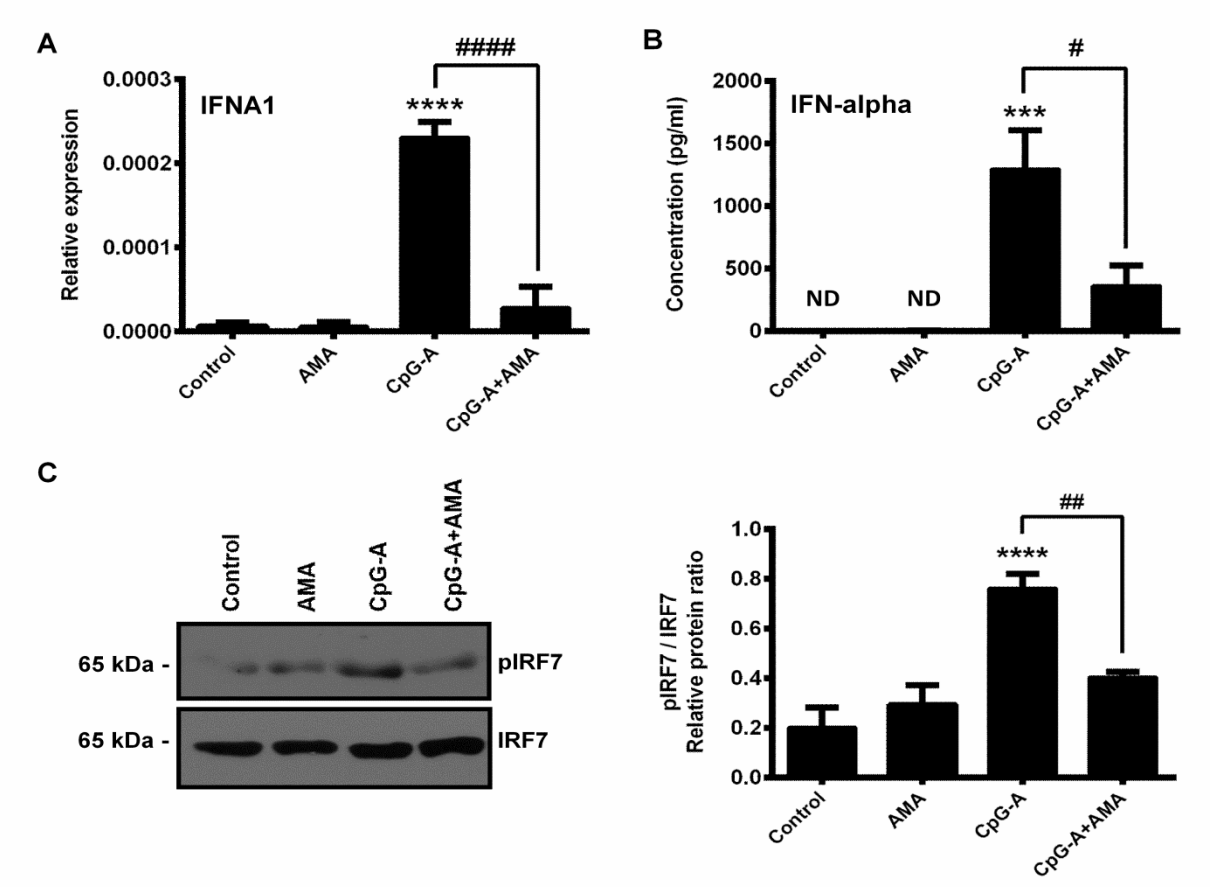


Figure 9. MtROS suppress CpG-A-induced production of IFNα by inhibiting IRF7 phosphorylation in GEN2.2 cells.
*GEN2.2 cells were left untreated (Control) or were treated for 6 hours with AMA (0,5 μg ml^{-1}) or CpG-A (1 μM) alone or simultaneously with CPG-A and AMA. Expression of IFNA1 mRNA (**A**) or the concentration of IFNα in supernatants (**B**) were measured by real-time quantitative PCR analysis and ELISA, respectively. Immunoblot analysis of phosphorylated (p-) and total IRF7 in lysates of GEN2.2 cells stimulated for 30 min as described above (**C**). Bars represent the means ± SD of three independent experiments (**A**, **B**, **C**) and a representative blot is shown (**C**). ***p<0.001, ****p<0.0001 vs. control (ANOVA); $^{\#}$p<0.05, $^{\#\#}$p<0.01, $^{\#\#\#\#}$p<0.0001 vs. CpG-A (unpaired t test)*

4.1.3. Elevated level of mtROS abrogates CpG-A-induced RIG-I expression in GEN2.2 cells

A key molecular feature of pDCs is a very low basal expression of RIG-I under steady state conditions that is rapidly and dramatically up-regulated upon stimulation by endosomal TLR ligands [47]. Based on this previous observation, to address the effect of CpG-A on RIG-I expression in GEN2.2 cells, we first determined by titration a dose of 0,25 μM as the lowest amount of CpG-A required to achieve the maximal induction of RIG-I expression (**Figure 10A**).

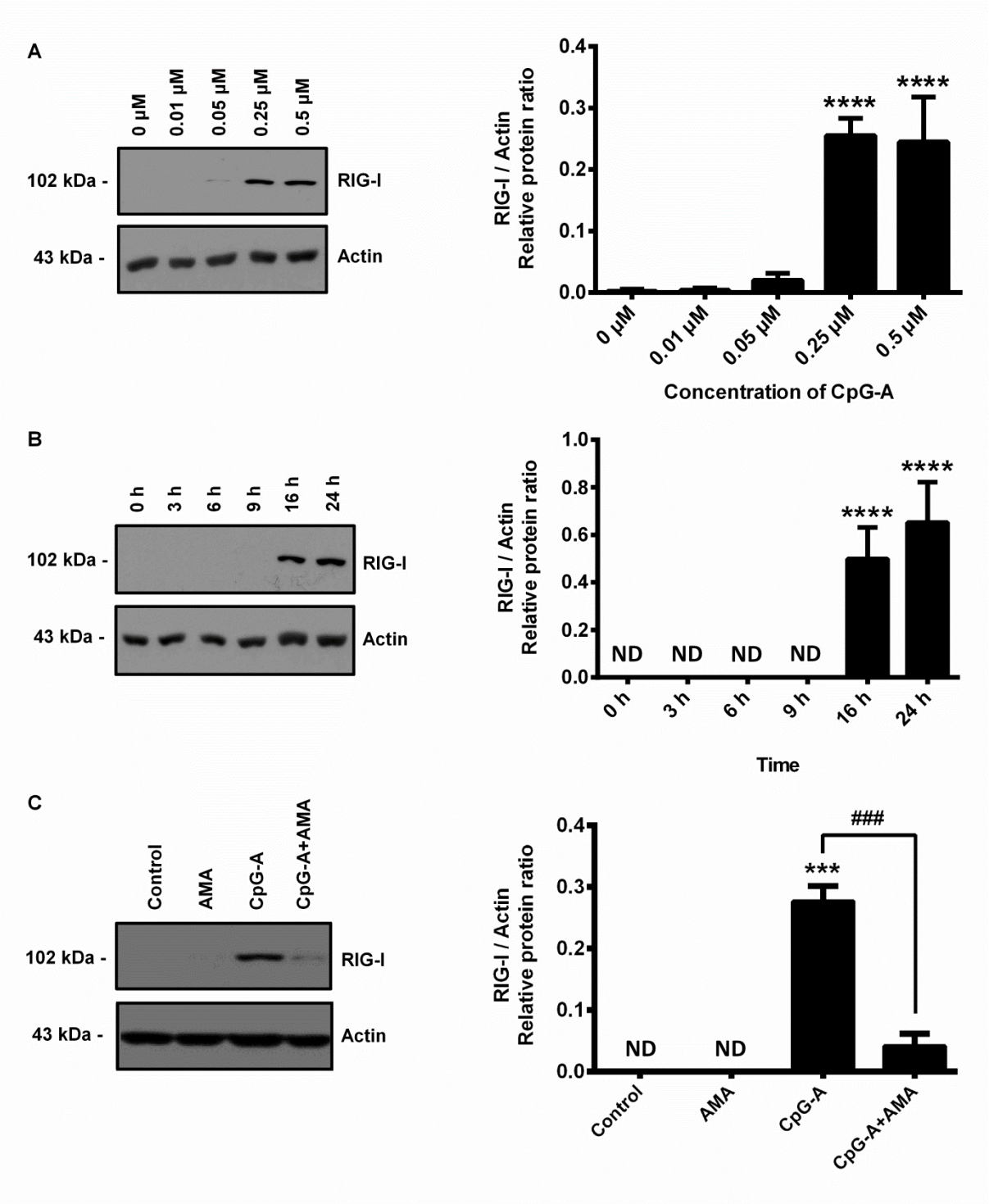


Figure 10. CpG-A-mediated induction of RIG-I expression is inhibited by AMA.
*To determine the optimal conditions for RIG-I induction, first, GEN2.2 cells were exposed to increasing concentration of CpG-A (ranging from 0,01 to 0,5 µM) for 16 hours (**A**). Next, GEN2.2 cells were stimulated with 0,25 µM of CpG-A, then RIG-I expression was measured in different time points (**B**). Afterwards, GEN2.2 cells were treated with 0,25 µM CpG-A in the presence or absence of AMA, then the amount of RIG-I in cell lysates was determined at 16 hours by western blot analysis (**C**). Representative blots and the means ± SD of four independent experiments are shown. ***p<0.001, ****p<0.0001 vs. control (ANOVA); ###p<0.001 vs. CpG-A (unpaired t test)*

Next, we measured the effect of CpG-A treatment on RIG-I expression over time and found that it reached a maximum after 16 hours (**Figure 10B**). Thus, 16 hours exposure in the presence of 0,25 μM CpG-A was used to induce the expression of RIG-I in GEN2.2 cells in all of our further experiments.

In order to evaluate the impact of mtROS on RIG-I expression, stimulation of GEN2.2 cells with CpG-A in the presence and absence of AMA was performed. As shown in **Figure 10C**, AMA-treatment did not influence RIG-I expression in untreated cells, however, it substantially restricted the capacity of CpG-A to increase RIG-I protein level.

Altogether these data point to the essential negative regulatory role of mtROS both in the early wave of type I IFN responses and in the endosomal TLR-induced expression of RIG-I.

4.1.4. MtROS synergize with RIG-I ligand to stimulate type I IFN production in GEN2.2 cells

Although elevated level of mtROS seems to be a strong inhibitory signal for the induction of RIG-I expression, we have no information about the effect of mtROS on RIG-I signalling once RIG-I had been induced prior to the release of mtROS. Thus, we investigated the effects of mtROS on RIG-I-induced IFNα production. To provoke up-regulation of RIG-I, first, GEN2.2 cells were pre-treated with 0,25 μM CpG-A for 16 hours. Afterwards, the culture supernatants were removed and following thorough washing steps the cells were re-seeded in fresh medium. Cells were then re-activated by transfecting 5'ppp-dsRNA, a synthetic RIG-I ligand (RIGL) into the cytosol. As described previously in primary pDCs, RIGL treatment stimulated IFNA1 gene transcription and IFNα protein secretion in GEN2.2 cells as well [47]. Remarkably, an even greater enhancement of IFNA1 mRNA and IFNα protein production was observed when cells were activated by RIGL in the presence of AMA (**Figure 11A, B**). These data suggested a context-dependent regulatory role for mtROS on type I IFN production. Thus, we investigated whether the opposing regulatory effects of elevated mtROS on TLR9- and RIG-I-mediated cellular responses depend on the PRR or the activation state of the cell at the time of mtROS induction. To this end, cells were pre-treated with CpG-A (0,25 μM) as described previously, then, instead of RIGL, they were re-activated with a higher dose of CpG-A (1 μM) in the presence or absence of AMA. Elevated level of mtROS inhibited the type I IFN production induced by CpG-A re-stimulation demonstrating that the regulatory effects of mtROS was PRR specific (**Figure 11C, D**).

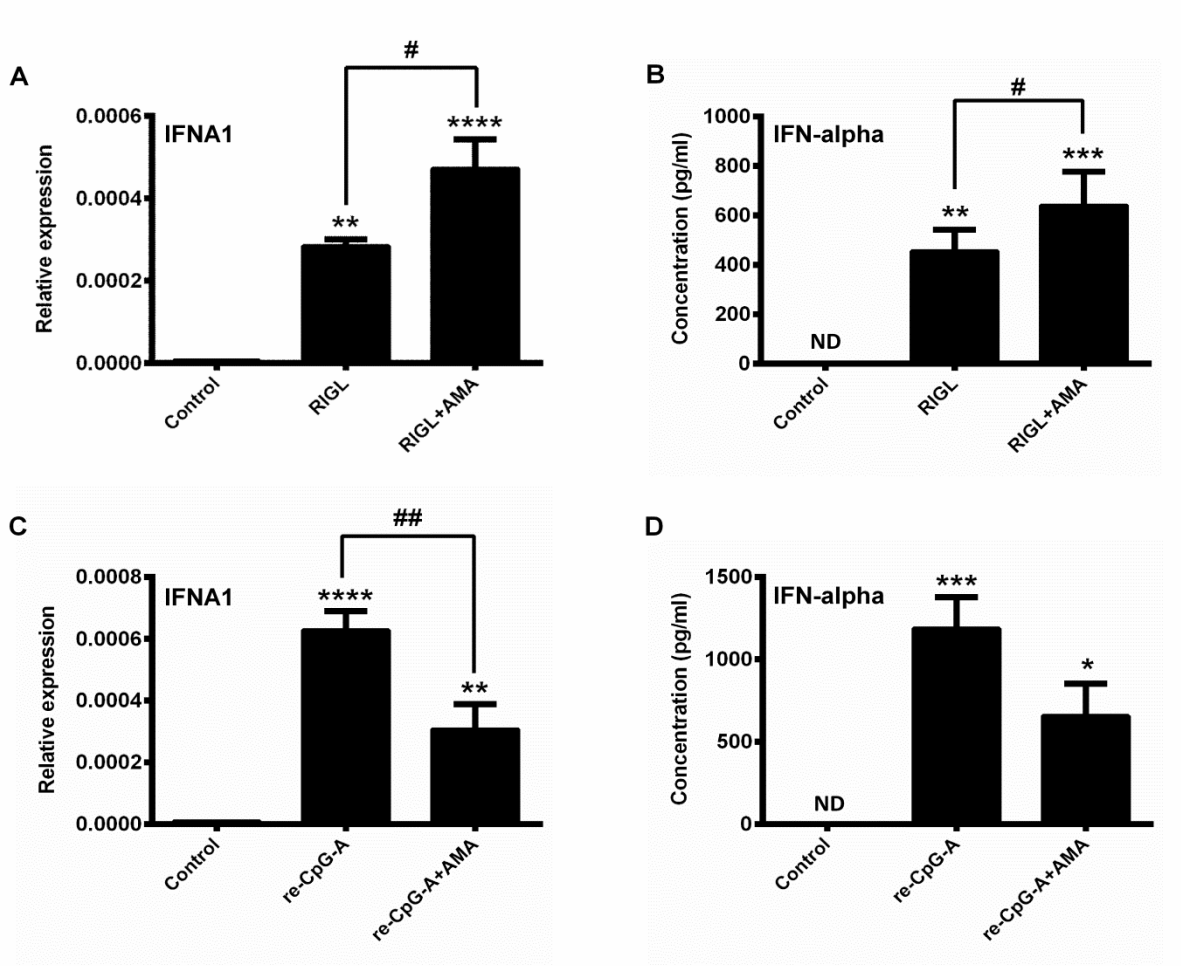


Figure 11. Opposing effects of AMA on type I IFN production of GEN2.2 cells activated by RIG-I or two consecutive stimulations with CpG-A.
*CpG-A pre-conditioned GEN2.2 cells were re-activated with the specific RIG-I ligand (RIGL) 5'ppp-dsRNA in the presence or absence of AMA (**A, B**). In parallel experiments, the re-activation of the cells was carried out with high dose of CpG-A (1 μM) alone or in combination with AMA (0,5 μg ml^{-1}) (**C, D**). The IFNA1 mRNA expression level was determined by real-time quantitative PCR and IFNα protein level was assessed by ELISA. Data are presented as means ± SD of four individual experiments. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 vs. control (ANOVA); $^{\#}$p<0.05 vs. RIGL and $^{\#\#}$p<0.01 vs. CpG-A (unpaired t test)*

4.1.5. MtROS affect type I IFN production of primary pDCs in a PRR-dependent manner

Thus far, we used a human transformed pDC line. To examine whether the phenotype observed with GEN2.2 cells translates to primary pDCs, we isolated primary human pDCs from peripheral blood of healthy donors and activated the cells as described for GEN2.2 cells. These experiments showed that, consistent with our observation made with the GEN2.2 cell line, type I IFN production initiated by CpG-A was abolished by mtROS in primary pDCs, whereas it was significantly enhanced in response to activation with RIGL (**Figure 12**).

These results validate the opposing, context-dependent modulatory role of mtROS in the TLR9- and RIG-I-mediated type I IFN production by human pDCs.

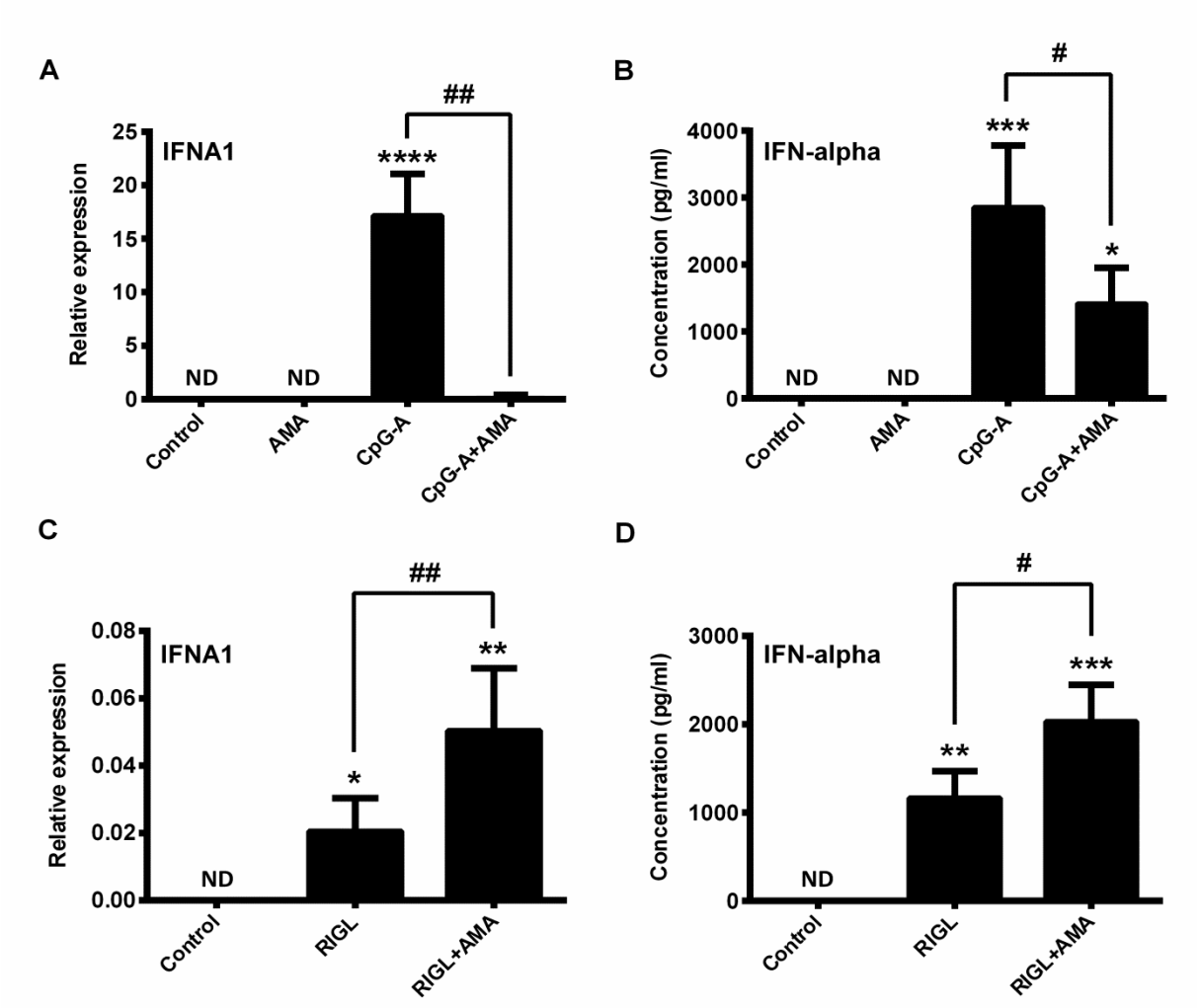


Figure 12. Elevated level of mtROS similarly influences type I IFN production in primary pDCs as in GEN2.2 cells.

*Primary pDCs were treated with AMA; CpG-A; CPG-A and AMA simultaneously or left untreated (Control). Following a 6-hour activation period, expression of IFNA1 mRNA and secretion of IFNα protein were determined by real-time quantitative PCR and ELISA, respectively (**A, B**). CpG-A pre-conditioned primary pDCs were re-activated with the specific RIG-I ligand (RIGL) 5'ppp-dsRNA in the presence or absence of AMA (0,5 μg ml^{-1}). IFNA1 mRNA expression level was determined by real-time quantitative PCR after 3 hours (**C**) and IFNα protein level was assessed by ELISA after 6 hours (**D**). Data are presented as means ± SD of three independent experiments. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 vs. control (ANOVA); $^{\#}$p<0.05, $^{\#\#}$p<0.01 vs. CpG-A or RIGL (unpaired t test)*

4.1.6. MtROS act as an enhancer for the key signalling molecules of the RIG-I pathway

Based on the observation that elevated level of mtROS increases the ability of pDCs to mount an efficient RIG-I-mediated antiviral response, the regulation of signalling molecules by mtROS downstream of RIG-I was investigated. IRF3 is crucial to drive type I IFN production in pDCs in response to RIG-I-activating ligands [50]. We observed that RIGL-induced phosphorylation of IRF3

was augmented by concomitant treatment with AMA, which mirrors the capacity of these cells to secrete IFNα (**Figure 13A**).

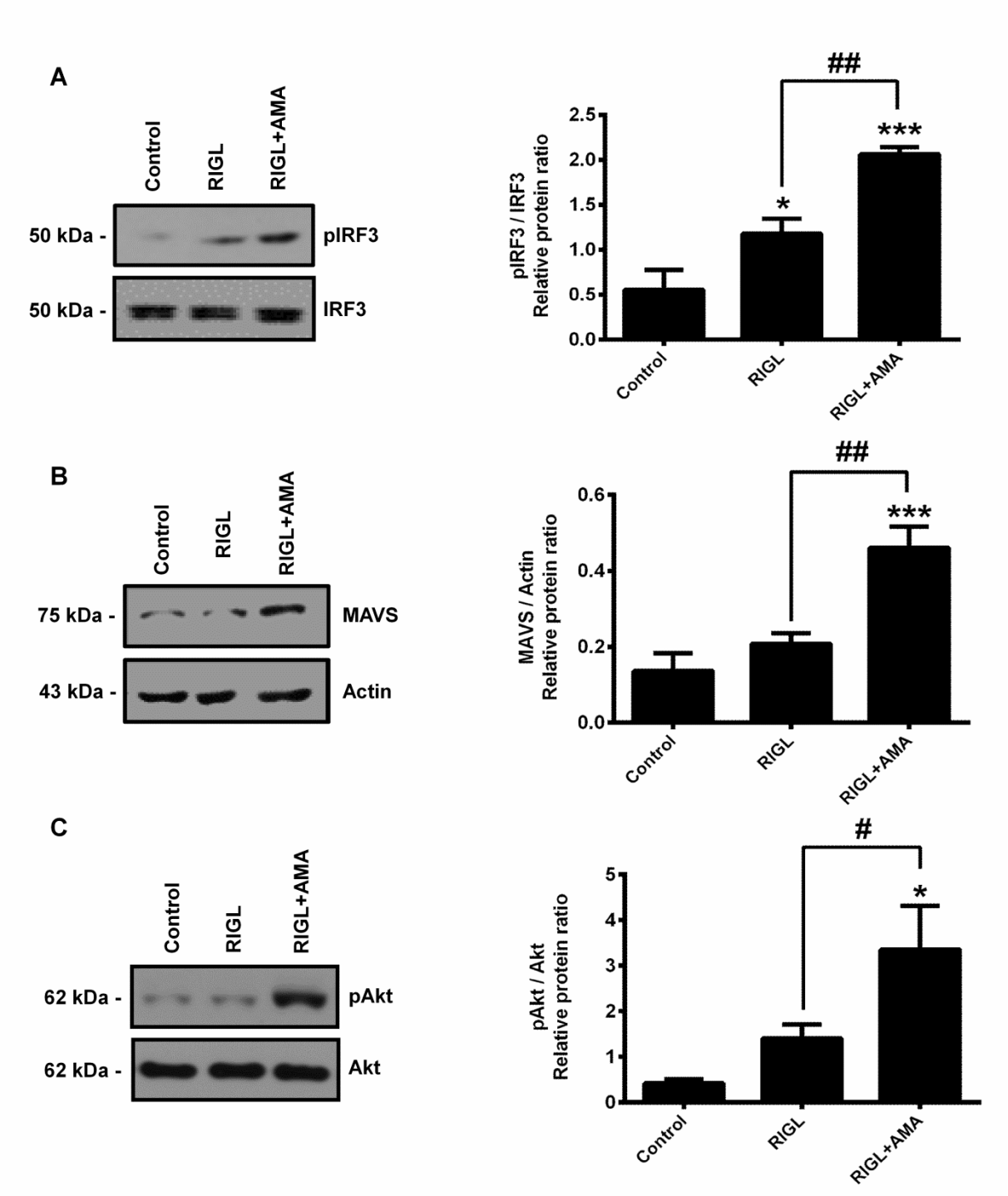


Figure 13. Key components of the RIG-I pathway are positively regulated by elevated level of mtROS.

*CpG-A pre-conditioned GEN2.2 cells were treated with the specific RIG-I ligand (RIGL) 5'ppp-dsRNA in the presence or absence of AMA. Phosphorylation of IRF3 (**A**) and Akt (**C**) were determined by western blotting after a 30 min incubation time, whereas expression of the MAVS adaptor protein (**B**) was measured 2 hours after stimulation. Representative blots and the means ± SD of four independent experiments are shown. *p<0.05, ***p<0.001 vs. control (ANOVA); $^{\#}$p<0.05, $^{\#\#}$p<0.01 vs. RIGL (unpaired t test)*

To start depicting the role of mtROS in the upstream signalling pathways leading to IRF3 phosphorylation, we analysed the expression of MAVS, the adaptor protein of RIG-I that was previously shown to be regulated in a redox-dependent manner [138, 139]. We found similar MAVS protein level in control and RIGL-stimulated cells; however, it was increased significantly when GEN2.2 cells were exposed to RIGL in the presence of AMA (**Figure 13B**).

Previous studies have indicated that Akt participates in RIG-I-mediated immune signalling leading to IRF3 activation and type I IFN production [140]. As the PI3K/Akt pathway was also shown to be redox-sensitive, we investigated the effect of mtROS on Akt phosphorylation [141]. We detected an increase in Akt phosphorylation after co-exposure of GEN2.2 cells to RIGL and AMA (**Figure 13C**). These findings raise the possibility that mtROS enhance RIG-I-induced type I IFN production via their direct, activating effect on the components of the RIG-I signalling pathway.

Taken together, data presented above demonstrate that mtROS possess a context-dependent regulatory function in both the early and the late phases of type I IFN responses of pDCs, depending on the actual viral sensing pathway stimulated. The opposing effect of mtROS on the TLR- and RIG-I-mediated signalling pathways reflects the versatile role of mtROS in fine-tuning the type I IFN-mediated antiviral responses of pDCs (**Figure 14**).

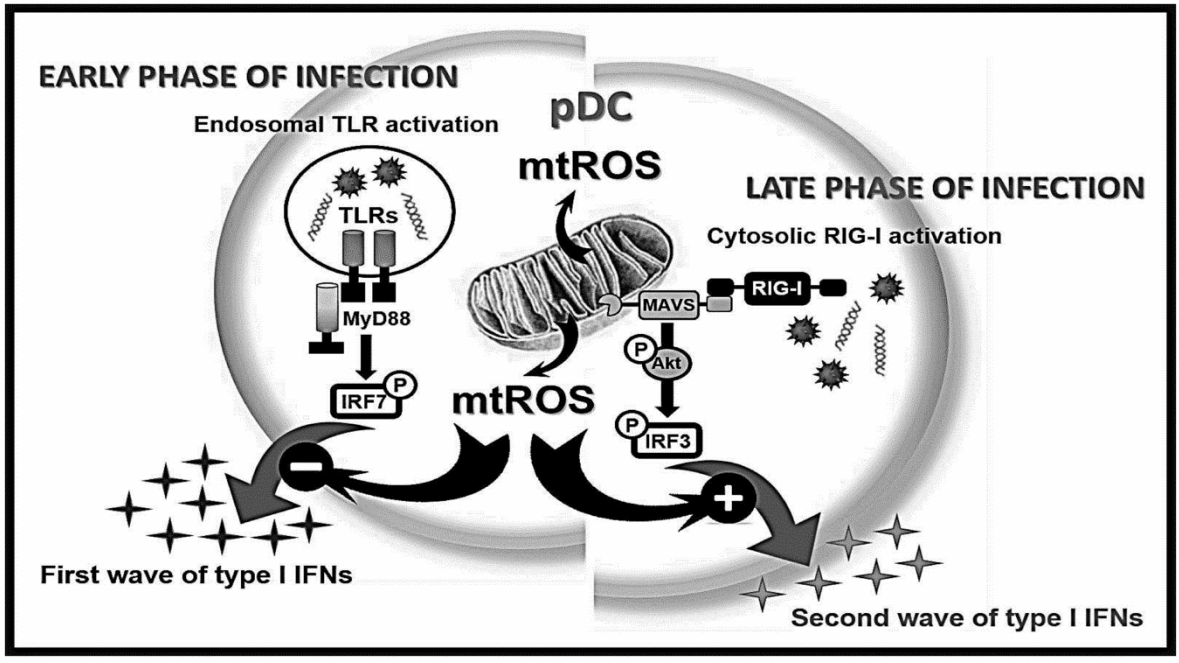


Figure 14. Schematic model of mtROS-mediated modulation of TLR- and RIG-I signalling pathways in pDCs.
Viral infection frequently leads to perturbations in mtROS level that alters signalling processes. We found that activation of TLR9 led to signalling cascades that were inhibited by mtROS, while the TLR-induced RIG-I pathway was boosted in the presence of mtROS. Thus, our data revealed a context-dependent regulatory role for mtROS on the production of type I IFNs by pDCs.

4.2. SLAMF5 enhances autophagy and fine-tunes cytokine response in moDCs via stabilization of IRF8

4.2.1. SLAMF5 is up-regulated during the differentiation and activation of moDCs, but is not required for their survival or phenotypic maturation

Regarding surface proteins with immune modulatory functions a number of alterations are observed during moDC differentiation. Our data show that SLAMF5 is strongly up-regulated during *in vitro* differentiation of human monocytes into immature moDCs implicating SLAMF5 as a regulator of the differentiation process (**Figure 15A**). To assess the function of SLAMF5 in moDCs we performed RNA interference targeting SLAMF5 on freshly isolated monocytes. SLAMF5 depletion was 80-95% complete 5 days after the introduction of siRNA by electroporation, as judged by flow cytometry and western blot (**Figure 15B**). It was published earlier that SLAMF5 served as a survival receptor for chronic lymphocytic leukemia cells and down-modulation of the receptor induced cell death [142]. However, the possibility that SLAMF5 deficiency may change the survival of moDCs was not supported by our 7-AAD-staining experiment. Besides the absence of any detectable change in the survival rate, the overall number of moDCs differentiated from monocytes was also unaltered by SLAMF5 silencing (**Figure 15C**). Next, we measured whether cells lacking SLAMF5 display any apparent defect in differentiation. At this aim, after 5 days of *in vitro* differentiation, we measured the monocyte marker CD14, highly expressed on monocytes but downregulated on moDCs, as well as DC-SIGN, typically expressed by differentiated moDCs. As shown in **Figure 15D, E**, cell surface expression of CD14 and DC-SIGN was identical in SLAMF5-silenced and control moDCs suggesting that SLAMF5 is either not required or is not the major player of moDC differentiation. Alternatively, in the absence of SLAMF5 its function as a regulator of moDC differentiation may be compensated by other SLAMF members.

As simultaneous LPS and IFNγ challenge further increases SLAMF5 expression in moDCs (**Figure 15A**), we decided to analyse the potential role of this receptor as a modulator of moDC activation in response to LPS/IFNγ. To this end, we measured whether SLAMF5-silenced moDCs were impaired in their capacity to enhance expression of cell surface markers associated with activation-induced moDC maturation. We found that upon stimulation, cell surface expression of MHC class I and II increased to the same extent in control and SLAMF5-silenced cells. In addition, the expression of the co-stimulatory molecules CD40 and CD86 was equivalently enhanced in control- and knockdown cells (**Figure 15F**). Thus, the phenotypic maturation of SLAMF5 knockdown moDCs was similar to control cells.

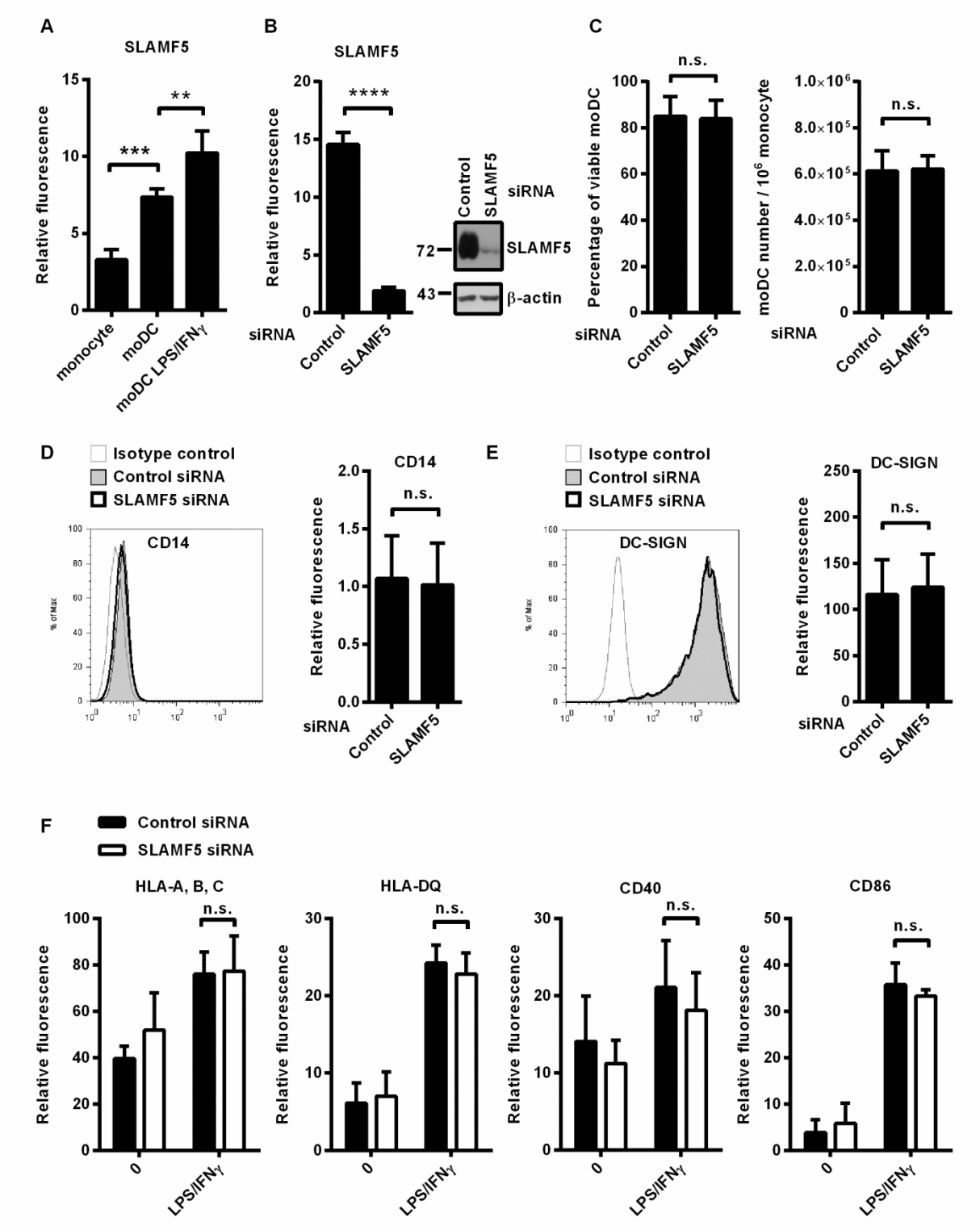


Figure 15. Phenotypic analysis of moDCs revealed no significant differences in response to SLAMF5 silencing.

*Flow cytometric analysis of SLAMF5 expression on monocytes and moDCs on day 5 of in vitro differentiation treated or not with LPS/IFNγ (**A**). Monocytes were transfected with the indicated siRNAs and differentiated into moDCs. On day 5, protein level of SLAMF5 was measured by flow cytometry (left panel) and western blot analysis (right panel) (**B**). The percentage of viable cells that are negative for 7-AAD (left panel) and the total number of moDCs differentiated from 10^6 monocytes (right panel) (**C**). Cell surface expression of CD14 and DC-SIGN on control and SLAMF5-silenced moDCs (**D, E**). Representative histograms show protein expression in control (thin line with gray*

*shading), knockdown cells (bolded black line) and staining with isotype control antibody (thin gray line). Bar graphs display the relative fluorescence intensity values of CD14 and DC-SIGN. MoDCs were exposed to 100 ng ml^{-1} LPS and 10 ng ml^{-1} IFNy for 24 hours (**F**). Expression of HLA-A, B, C, HLA-DQ, CD40 and CD86 was determined by flow cytometry analysis. Values are expressed as relative fluorescence. The results shown are taken from at least three independent donors. Error bars indicate SD. **p < 0.01, ***p < 0.001, ****p < 0.0001, ns: not significant (unpaired t test)*

4.2.2. Manipulation of SLAMF5 signalling changes the intensity of moDC autophagy

Recent publications revealed SLAMF receptors as regulators of the autophagic process in specific immune cells [83, 84]. Therefore, we set out to evaluate the impact of SLAMF5 on moDC autophagy. The most widely used assay to monitor this process is measuring LC3 protein levels by western blotting. In resting moDCs LC3 is present in two forms, corresponding to a cytosolic LC3-I and a lipid-conjugated, autophagosome-associated LC3-II isoforms. The conjugation increases the electrophoretic mobility of the protein, thus LC3-II can be distinguished from the non-modified form that migrates slower in SDS-PAGE. The amount of LC3-II is closely correlated with the number of autophagosomes, serving as a good indicator of autophagosome formation. However, when autophagosomes fuse with lysosomes, inner membrane located LC3-II is degraded along with the cargo. Therefore, the level of LC3-II is determined not only by the rate of generation but by the rate of autophagic degradation as well [143]. Thus, to monitor autophagosome formation, we inhibited LC3-II degradation by treating the cells with bafilomycin A1 (BafA1) that blocks autophagosome-lysosome fusion. Interestingly, even in the absence of any stimulation, we observed a lower LC3-II/β-actin ratio in SLAMF5-silenced moDCs compared to controls that indicated the importance of SLAMF5 in setting the basal level of autophagy in moDCs. As mentioned earlier, LPS/IFNγ treatment transiently reduces autophagy in moDCs [22]. The expected transient, activation-dependent decrease in LC3-II levels was apparent 4 hours past treatment with LPS/IFNγ both in control- and SLAMF5-silenced moDCs. However, in our experiments, 8 hours after stimulation the level of LC3-II recovered only in control cells whereas it failed to do so in SLAMF5-silenced cells (**Figure 16A**).

The above experiment was performed in the absence of BafA1 to ascertain whether SLAMF5 silencing causes blockage of autophagosome-lysosome fusion and lysosomal clearance. Without BafA1 treatment LC3-II would accumulate in greater amounts if its turnover in lysosomes was inefficient. LC3-II levels were again lower in SLAMF5-silenced moDCs suggesting that LC3-II was readily processed by the autolysosomes (**Figure 16B**). This result proved that instead of being involved in autophagosome maturation, SLAMF5 enhances autophagosome biogenesis.

To validate the impact of SLAMF5 on autophagy, we used a fluorescent probe, Cyto-ID that selectively labels autophagic vacuoles in live cells [144]. Consistent with the previous immunoblot data, flow cytometry analysis revealed that the fluorescence intensity of Cyto-ID was significantly lower in SLAMF5-silenced cells than in controls (**Figure 16C**).

If absence of SLAMF5 abrogates autophagy, we reasoned that enhanced SLAMF5 signalling by cross-linking with agonistic antibodies should increase the intensity of autophagy in moDCs. This hypothesis turned out to be correct. Consistently with our earlier findings, ligation of SLAMF5 with anti-SLAMF5 mAb (152.1D5) in conjunction with $F(ab')_2$ fragments of a polyclonal anti-mouse Ab, increased LC3-II levels both in the presence and absence of BafA1 (**Figure 16D, E**). Isotype-matched control antibodies were used to correct for inadvertent stimulation of Fc receptors.

By these experiments, we identified SLAMF5 as a cell-surface expressed regulator protein required for the maintenance of basal autophagy as well as the recovery of autophagy after LPS/IFNγ stimulation.

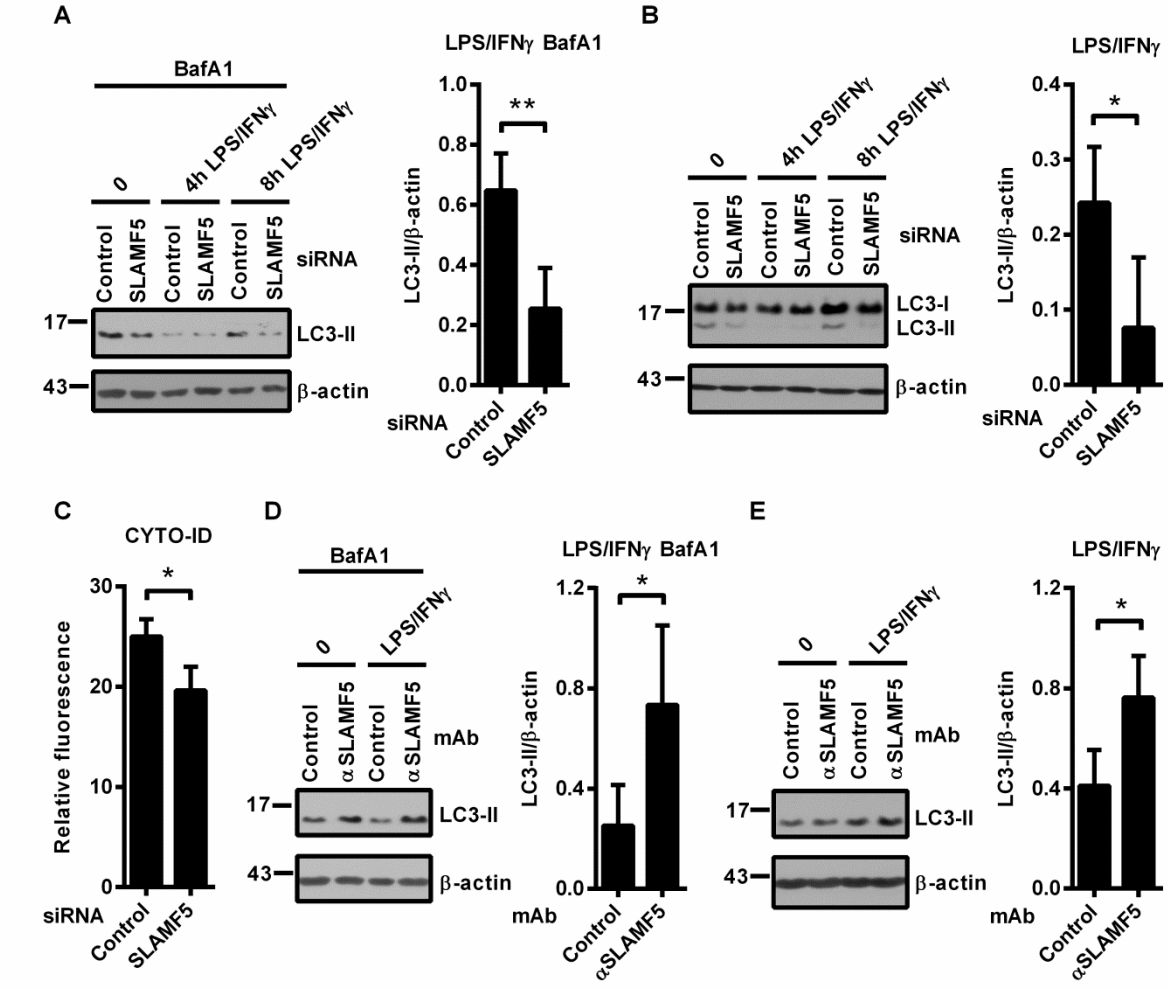


Figure 16. Silencing or cross-linking of SLAMF5 influences moDC autophagy under steady state conditions and in response to LPS/IFNγ activation.

*Control and SLAMF5-silenced moDCs were stimulated or not with LPS/IFNγ for the indicated hours in the presence (**A**) or absence (**B**) of 20 nM BafA1 applied for the last 2 hours. LC3 conversion was measured by western blotting. The immunoblot on the left is a typical example of four independent experiments, and the ratio of LC3-II and β-actin analysed by densitometry is shown on the right. Control and SLAMF5-depleted moDCs were stained with Cyto-ID, then fluorescence intensity was analysed with flow cytometry (**C**). Graph displays the relative fluorescent intensity of Cyto-ID summarizing results of four donors. MoDCs were incubated with 10 μg ml^{-1} control- or anti-SLAMF5 antibodies followed by cross-linking with $F(ab')_2$ fragment of goat anti-mouse IgG. After 2 hours, cells*

*were treated with LPS/IFNy for 8 hours in the presence (**D**) or absence (**E**) of 20 nM BafA1 applied for the last 2 hours of the experiment. LC3-II and β-actin levels were analysed by western blotting, graphs depict the mean ratios of LC3-II to β-actin obtained from three independent experiments. *p < 0.05, **p < 0.01 (unpaired t test)*

4.2.3. SLAMF5 regulates autophagy by a mechanism independent of mTOR

We examined whether SLAMF5 exerts its function on autophagy via interfering with the signalling of mTOR, the key negative regulator of autophagy [145]. If the reduced autophagy flux in the absence of SLAMF5 is the consequence of increased mTOR activity, blocking mTOR is expected to reverse the effect of SLAMF5 silencing. To examine this scenario, we achieved mTOR suppression by culturing control and SLAMF5-silenced moDCs in the presence of rapamycin for 4 hours. Our observation that rapamycin treatment did not modify the extent of autophagy defect elicited by SLAMF5 silencing made unlikely that SLAMF5 modulates autophagy through the mTOR pathway (**Figure 17A, B**).

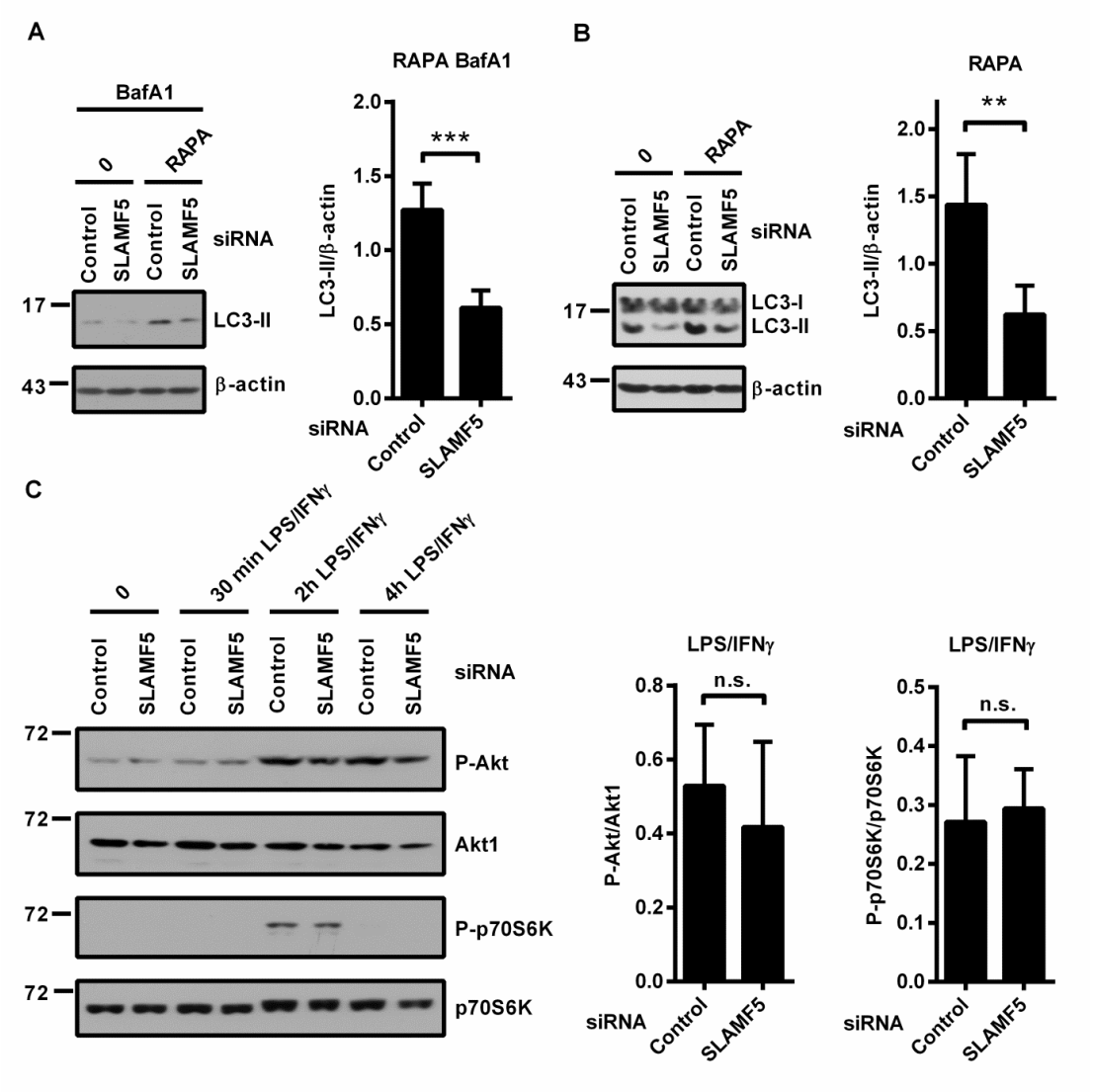


Figure 17. Autophagic block in SLAMF5-silenced cells is not mediated by activation of the autophagy-inhibitory mTOR signalling.
*SLAMF5-silenced or control moDCs were conditioned with the autophagy-inducer rapamycin (RAPA) for 4 hours in the presence (**A**) or absence (**B**) of 20 nM BafA1 applied for the last 2 hours. LC3 conversion was analysed in cell lysates by western blotting. A representative blot is shown on the left and densitometry of LC3-II to β-actin ratios of four donors is displayed on the right. SLAMF5 knockdown or control moDCs were stimulated with LPS/IFNγ and phosphorylation of Akt and p70S6K were determined by western blotting (**C**). Representative blots of four independent experiments are shown. Bars illustrate the mean ratios of the phospho-proteins (p-) to the total amount of the same proteins. Data are expressed as mean ± SD. **p < 0.01, ***p < 0.001, ns: not significant (unpaired t test)*

To further test this hypothesis, we examined the phosphorylation of Akt, a protein activated upstream of mTOR as well as the mTOR substrate p70S6K in response to LPS/IFNγ within a 4-hour time period. If SLAMF5 interferes with mTOR signalling, SLAMF5 silencing is expected to increase the phosphorylation events within the Akt/mTOR/p70S6K pathway. However, we observed that the phosphorylation of Akt and p70S6K was unaffected by SLAMF5 depletion (**Figure 17C**).

These results together suggest that SLAMF5 regulates autophagy independently of mTOR.

4.2.4. SLAMF5 enhances autophagy by blocking TRIM21-dependent proteasomal degradation of IRF8

In search of the molecular mechanism by which SLAMF5 promotes autophagy, we next focused on the IRF8 transcription factor on the basis of its central role in autophagy. Studies on murine bone-marrow derived DCs show that under steady state conditions IRF8 is expressed at low levels while in response to LPS/IFNγ its expression is strongly stimulated, which in turn activates many genes involved in all phases of autophagy [127]. To determine whether IRF8 affects autophagy in human moDCs its expression was silenced using an IRF8-specific siRNA (**Figure 18A**). The significant decrease in LC3-II levels (**Figure 18B, C**) and in the fluorescence intensity of the autophagy specific probe, Cyto-ID (**Figure 18D**) detected in the absence of IRF8 imply that similar to murine bone-marrow derived DCs, human moDCs also use IRF8 for the regulation of autophagy. Next, to determine whether SLAMF5 and IRF8 are part of the same or different autophagy regulatory pathways SLAMF5 was cross-linked with the above described 152.1D5 mAb antibody on moDCs transfected with control or IRF8-specific siRNAs. As shown in **Figure 18E**, cross-linking of SLAMF5 significantly increased autophagy in moDCs transfected with the control oligonucleotides, however this induction was dependent on the presence of IRF8. This observation established IRF8 as part, and a downstream element of the SLAMF5 autophagy regulatory pathway. To gain more insight into the operation of the SLAMF5-IRF8 pathway, protein levels of IRF8 in control and SLAMF5-silenced cells were determined at various time points following activation with LPS/IFNγ. As shown in **Figure 18F**, we observed greatly reduced amount of IRF8 protein in SLAMF5-silenced cells compared to controls.

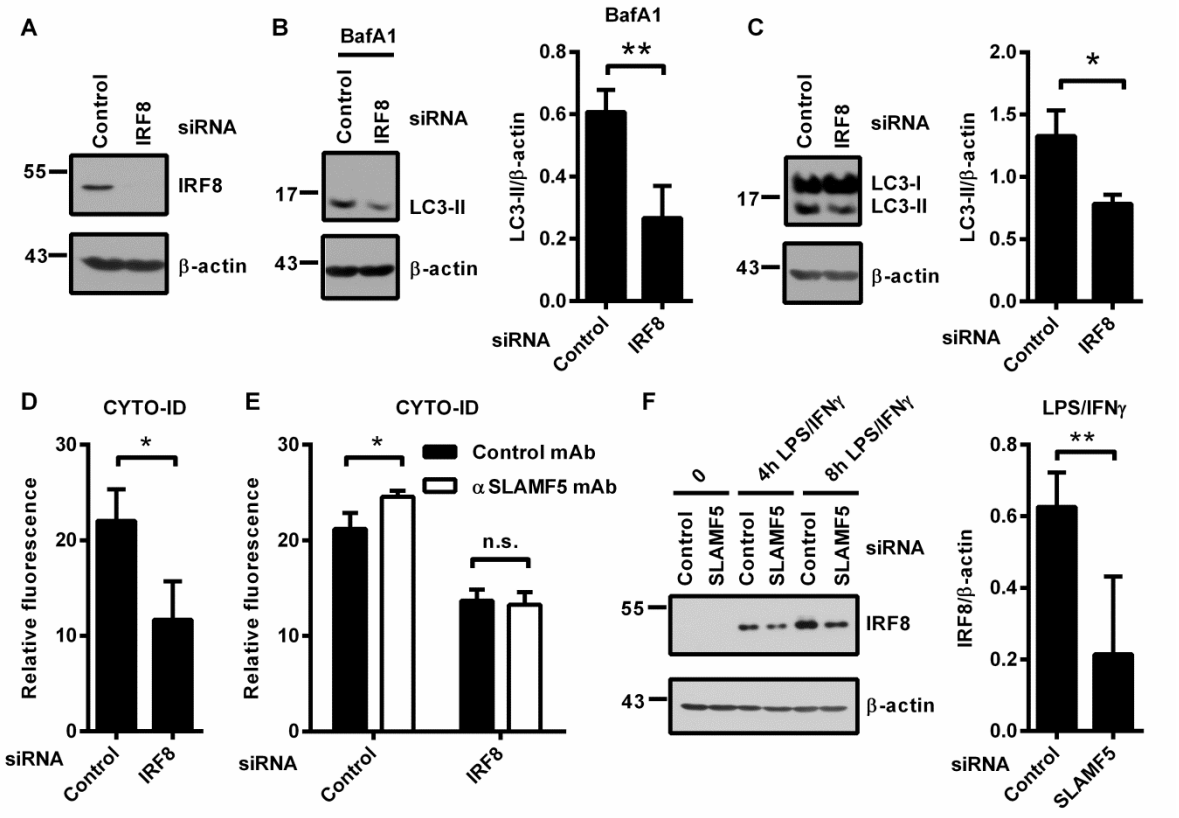


Figure 18. The level of the autophagy regulator IRF8 is controlled by SLAMF5 in moDCs.
*Monocytes were transfected with control or IRF8-specific siRNAs and differentiated into moDCs. The efficiency of IRF8 knockdown was evaluated on day 5 by western blot analysis (**A**). LC3 conversion was determined in control and IRF8-silenced moDCs by western blotting in the presence (**B**) or absence (**C**) of BafA1. One representative of three experiments is shown. Bar graphs depict the ratio of LC3-II/β-actin. Control and IRF8-silenced moDCs were labelled with Cyto-ID, then analysed by flow cytometry (**D**). Graph displays the relative fluorescent intensity of Cyto-ID obtained in three independent experiments. Cyto-ID staining of control and IRF8-silenced moDCs in which SLAMF5 was cross-linked with the agonistic 152.1D5 antibody (**E**). Control or SLAMF5 knockdown moDCs were stimulated with LPS (100 ng ml^{-1}) and IFNγ (10 ng ml^{-1}) for the indicated time periods. IRF8 protein level was analysed by western blotting and the ratio of IRF8/β-actin was quantified from five independent experiments (**F**). *p < 0.05, **p < 0.01, ns: not significant (unpaired t test)*

To determine the mechanism by which SLAMF5 regulates IRF8 protein levels, first, we measured the amount of IRF8 mRNA. Interestingly, we found no difference between SLAMF5-silenced and control cells (**Figure 19A**). Given the fact that IRF8 activity is modulated by posttranslational modifications such as polyubiquitination and subsequent proteasomal degradation, in the following experiments we investigated whether SLAMF5 affects the degradation of IRF8. For this purpose, we activated the cells in the presence of the proteasome inhibitor MG132, which restored the availability of IRF8 in SLAMF5 knockdown moDCs. In control experiments we noted that ubiquitin-conjugated proteins are increased in MG132-treated samples (**Figure 19B**). The TRIM21 E3 ubiquitin

ligase has been shown to catalyse the ubiquitination of IRF8 in murine macrophages, leading to its proteasomal destruction [128]. We therefore performed experiments to define the possible role of TRIM21 in the signalling events leading to IRF8 degradation in the absence of SLAMF5. To this end, the expression of SLAMF5, TRIM21 (**Figure 19C**) or both proteins was reduced by the introduction of relevant siRNAs via electroporation. We observed that IRF8 degradation in SLAMF5-depleted moDCs was blocked when cells were co-transfected with the TRIM21-specific, but not with the control oligo (**Figure 19D**). This result confirmed the participation of TRIM21 in the degradation of IRF8 in SLAMF5-silenced cells.

EAT-2 is currently the only known adapter protein of SLAMF receptors in APCs [146]. Beyond that, a recent report described it as an enhancer of the autophagy process [84]. Based on these, we examined whether EAT-2 partakes in transmitting SLAMF5 signals in moDCs. We found that although EAT-2 is expressed in human monocytes, it is promptly down-regulated in response to the used DC differentiation signals and it is undetectable both in resting and LPS/IFNγ-activated moDCs (**Figure 19E**). Therefore, the SLAMF5-driven autophagy in moDCs is presumably an EAT-2 independent process.

According to these results, although SLAMF5-silenced moDCs are capable of up-regulating IRF8 synthesis, have nevertheless decreased IRF8 protein level due to the increased proteasomal degradation of this protein. This process appears to be dependent on TRIM21 but independent of the SLAM family-specific adaptor EAT-2. Based on this, a possible mechanism connecting SLAMF5 to autophagy relies on sustained activity of IRF8, a major transcription factor of autophagy-related genes.

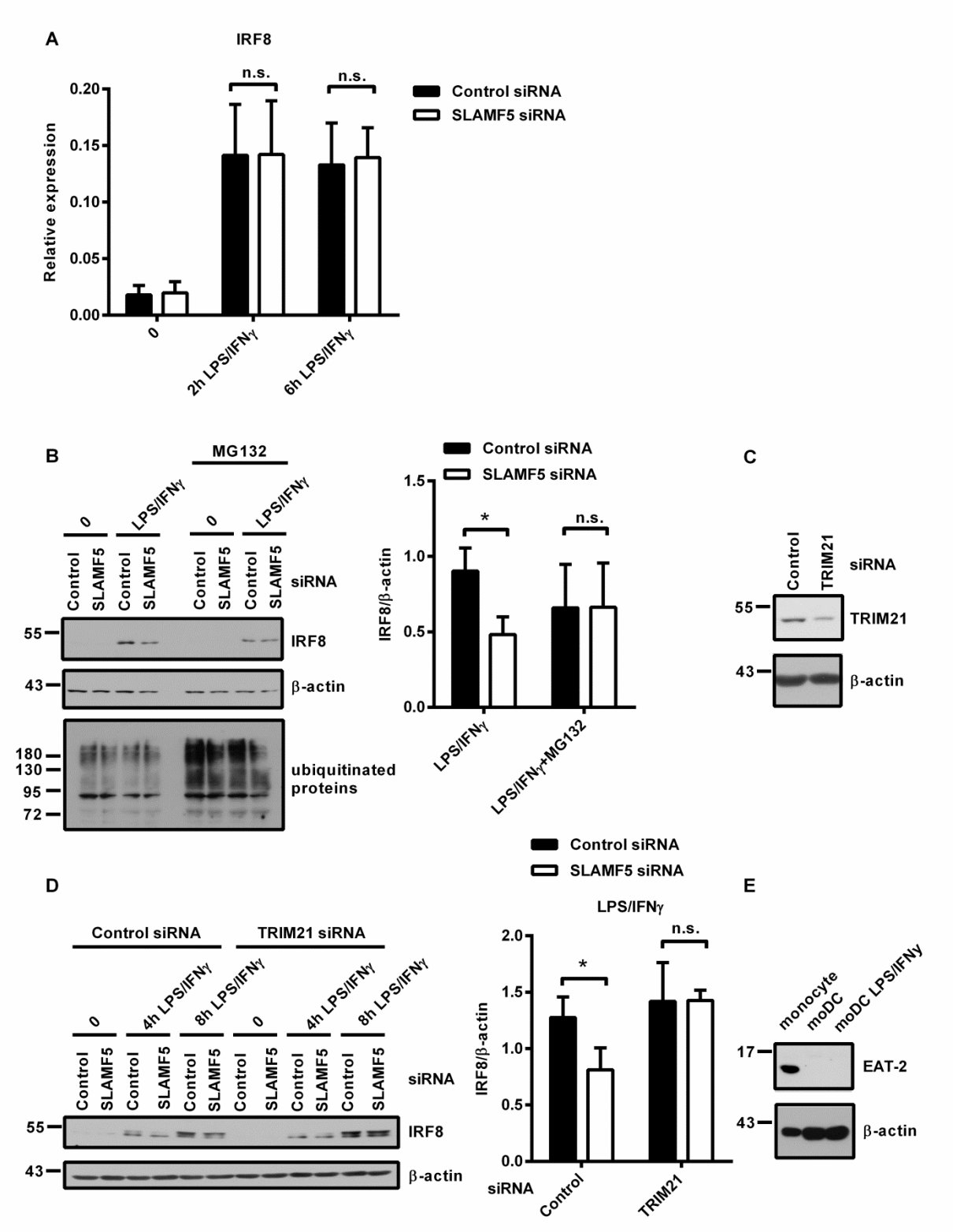


Figure 19. Proteasomal degradation of IRF8 in response to SLAMF5 silencing is mediated by TRIM21.

*Control and SLAMF5 knockdown moDCs from five donors were stimulated with LPS/IFNγ for the indicated time periods, and then mRNA expression of IRF8 was analysed by real-time quantitative PCR (**A**). Control- and SLAMF5-silenced moDCs were treated with LPS/IFNγ for 8 hours and, where indicated, 1 μM MG132 was applied for the last 2 hours. IRF8 protein levels were determined by western blot analysis (**B**). A representative blot is shown on the left together with β-actin as loading*

*control. An anti-ubiquitin blot confirms the activity of the proteasome inhibitor MG132 in our experimental system. Densitometric analyses of IRF8 to β-actin ratios from four donors are depicted on the right panel. Efficiency of TRIM21 silencing was established on day 5 by western blotting (**C**). The expression of SLAMF5 alone or in combination with TRIM21 was silenced by transfecting monocytes with the indicated set of gene-specific or control siRNAs. On day 5, transfected moDCs were treated with LPS/IFNγ for the indicated time periods and the level of IRF8 protein was measured by western blotting (**D**). A representative blot and the mean ratios of IRF8 to β-actin from three independent experiments are shown. EAT-2 expression was determined in monocytes and in moDCs on day 5 of in vitro differentiation treated or not with LPS/IFNγ (**E**). A representative blot of three independent experiments is shown, with β-actin as loading control. *p < 0.05, ns: not significant (unpaired t test)*

4.2.5. SLAMF5 and IRF8 silencing in monocytes results in development of moDCs with overlapping changes in phenotype and cytokine secretion

To strengthen our assumption that the impact of SLAMF5 silencing on moDC functions is the consequence of IRF8 degradation, hereinafter, we investigated whether loss of IRF8 replicates the changes in moDC phenotype and cytokine production, which can be provoked by SLAMF5 silencing.

A previous study by Granato et al. reported that interference with the autophagic process in monocytes resulted in reduced expression of CD1a on moDCs [147]. Thus, we measured the induction of CD1a expression on moDCs differentiated in the absence of SLAMF5. As depicted in **Figure 20A,** silencing SLAMF5 in monocytes significantly decreased the percentage of $CD1a^+$ moDCs, consistent with the state of reduced autophagy. In accordance, IRF8-silenced cells had a phenotype similar to that of SLAMF5 knockdown cells (**Figure 20B**).

One of the major roles of autophagy in cDCs is to set a limit to the production of pro-inflammatory cytokines. In line with this, when autophagy is defective the inflammatory process is not properly controlled [29-31]. Therefore, we investigated whether SLAMF5 has an impact on this process. As expected, defective autophagy in SLAMF5-silenced moDCs coincides with enhanced secretion of IL-1β and IL-23. Interestingly, however, SLAMF5-depleted moDCs secreted less IL-12 compared with controls (**Figure 20C**). Production of these cytokines was similar in IRF8-silenced moDCs as well. Importantly, these experiments also confirmed that LPS/IFNγ-induced IL-12 secretion is fully IRF8 dependent (**Figure 20D**). The finding that depletion of IRF8 results in similar phenotypic and functional changes to those seen in SLAMF5 knockdown moDCs, further supports that IRF8 is part of the SLAMF5 autophagy regulatory pathway.

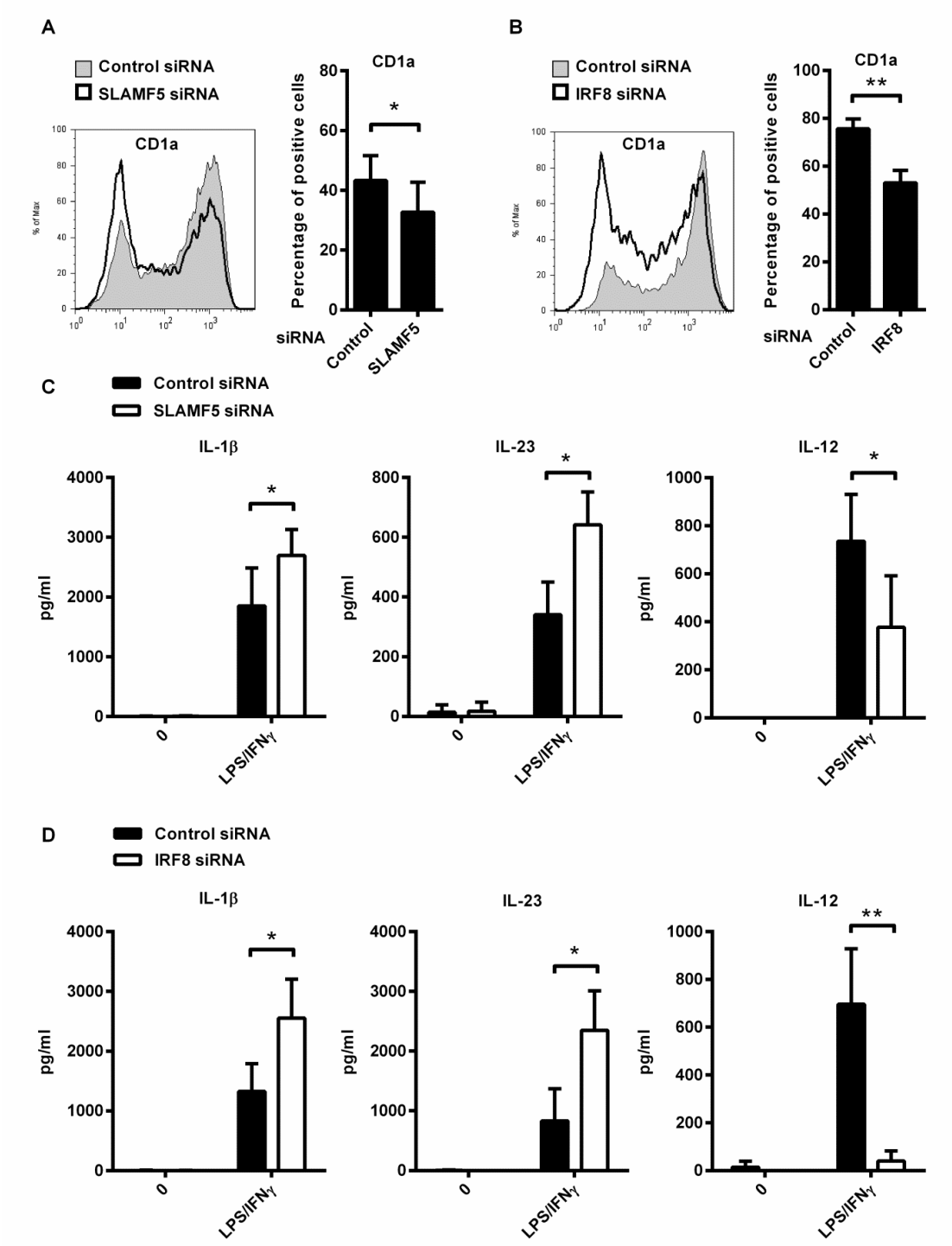


Figure 20. SLAMF5 and IRF8 silencing induced similar changes in CD1a expression and cytokine production in moDCs.

*Flow cytometric analysis of CD1a expression in SLAMF5-(**A**) or IRF8-(**B**) silenced moDCs over control cells. Representative histograms show protein expression in control (thin line with gray shading) and knockdown cells (bolded black line). Bars show the percentage of CD1a$^+$ cells. SLAMF5 (**C**) or IRF8 (**D**) knockdown moDCS were left untreated or stimulated with LPS/IFNγ and their cytokine productions were compared to cells transfected with control oligonucleotides. Cytokine concentrations in supernatants were determined at 8 hours (IL-1β and IL-23) or 12 hours (IL-12) by ELISA. Data are presented as means ± SD of at least three independent experiments. *p < 0.05, **p < 0.01 (unpaired t test)*

In summary, our work reveals a novel link between the SLAMF- and IRF8-regulated pathways and establishes SLAMF5 as a cell surface-expressed regulator of moDC autophagy that fine-tunes cytokine production in human moDCs (**Figure 21**).

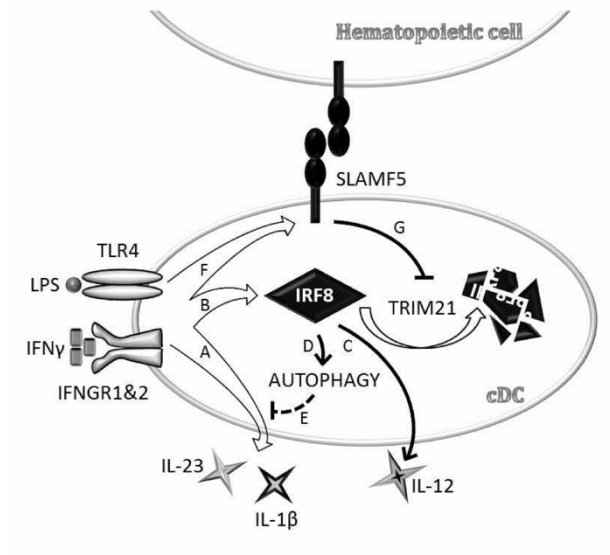


Figure 21. A model for SLAMF5-mediated regulation of autophagy and cytokine production in cDCs.

Autophagy, that is normally decreased transiently upon microbial sensing by cDC, is later on restored via the induction of IRF8 to prevent overproduction of IL-1β and IL-23. Concomitantly, IRF8 also induces the secretion of IL-12. We found that SLAMF5 is upregulated upon LPS/IFNγ treatment and inhibits TRIM21-mediated proteasomal degradation of IRF8. Thereby, SLAMF5 enhances autophagy and fine-tunes cytokine production in human cDCs.

5. DISCUSSION

The immune system is in a constant race with pathogens that have developed a plethora of suppressor mechanisms to evade immunosurveillance. In some cases, however, it is not obvious how pathogens may benefit from inducing a strong inflammatory response. One must remember that most effector functions delivered by the immune system (release of proteases, ROS) are not specific and cause significant tissue damage in the host. Moreover, exuberant immune reactions induce recruitment and activation of suppressor cells and mechanisms. While tissue destruction may pave the way for pathogen invasion, secondary activation of suppressor cells and mechanisms may extend their existence in the host. Thus, delicate regulatory mechanisms are required to deliver efficient antimicrobial responses and, at the same time, prevent exuberant innate immune response that may cause excessive tissue damage, chronic inflammation and even lead to development of autoimmune diseases. DCs are primary orchestrators of a proper powerful, yet controlled immune response, whereby pathogens are neutralized while the damage to the host is minimal. Several molecular cues guide DC functions obtained through multiple cross-talk mechanisms between DCs and other cells of their local microenvironment and also via intrinsic cellular mechanisms such as signalling from their own mitochondria. In our work, we first examined how pDC functionality is affected by mitochondrially produced ROS. In parallel, we investigated the regulatory role of cell-cell communication by studying the modulatory effects of the cell surface receptor SLAMF5 on the autophagic process of cDC and exploring the mechanism behind it.

The dynamic balance between the production and elimination of ROS is disturbed when the host becomes infected with a virus [148]. Increased ROS might modulate infected cell responses, immune defences, viral replication and contribute to the pathogenesis of infections [149]. Our group has previously shown that TLR7-induced cellular responses of primary human pDCs are highly sensitive to oxidative stress [51]. Thus, in inflamed peripheral tissues where pDCs are exposed to ROS, TLR-mediated pathways are presumably suppressed. Research described in this dissertation extended our current understanding of pDC functions significantly by showing that similar to exogenous H_2O_2, mtROS restrain endosomal TLR-induced production of IFNα, irrespective of the activation state of pDCs. Strikingly, we found that mtROS amplify RIG-I signalling both in GEN2.2 cells and primary human pDCs. Although a booster effect for mtROS on RIG-I-induced type I IFN production has been described in MEFs, our research team was the first to show up-regulation of RIG-I signalling and type I IFN production in response to mtROS in pDCs, the chief regulators of the antiviral response. Based on these observations, we propose that during the early phase of viral infection, systemic type I IFN response of pDCs is driven by TLR-mediated signals, while their late, local antiviral response - when pDCs gain access to peripheral tissues - might be mediated primarily by the cytosolic receptor, RIG-I. These findings, however, raise the question of how this context-

dependent regulation of type I IFNs by mtROS is to the advantage of the host? The most appealing answer is that such control mechanism may exist to restrict production of excessive amounts of type I IFNs, which could be detrimental for the host. Multiple studies indicate that chronic activation of pDCs resulting in maintained high level secretion of type I IFNs could lead to autoimmunity. In this regard, in SLE, DNA- and RNA-protein complexes released by damaged cells form immune complexes with autoantibodies that could induce type I IFN production in pDCs via endosomal TLRs [150, 151]. It was previously reported that IFNα production of pDCs, triggered by endogenous IFN inducers such as RNA-containing immune complexes, is potently inhibited by $CD14^+$ monocytes from healthy individuals. Importantly, the use of ROS scavangers revealed that the inhibitory activity of monocytes was due to ROS production. Although this report concludes that monocyte-derived ROS affect IFNα production of pDCs mostly indirectly, through inhibition its stimulation by NK cells, our results suggest that ROS also have a direct inhibitory effect on TLR9-induced type I IFN production of pDCs [152]. Taking into consideration those previous observations and considering our present findings, we propose that in inflamed tissues, elevated level of ROS contributes to diminish the amplitude of type I IFN signalling via TLRs, likely to avoid destruction of healthy tissues.

It is not surprising that several viruses developed strategies to increase mtROS, thus might usurp the above regulatory mechanism to escape TLR-induced antiviral responses unleashed by pDCs [38-40]. Perhaps the most significant discovery of the above described findings is the identification of the RIG-I-dependent "salvage pathway" for type I IFN production by which under virally-induced oxidative stress the RIG-I pathway could compensate for the lack of a functional TLR pathway and support immunity to viruses. Therefore, the TLR-induced RIG-I signalling pathway in pDCs might be one of the crucial mechanisms to circumvent virus escape from the innate immune response.

The key transcription factor of type I IFN genes during RIG-I activation is IRF3 [50]. We found its increased phosphorylation upon AMA treatment of RIG-I-stimulated GEN2.2 cells; therefore, we propose that mtROS promote the above described RIG-I-mediated type I IFN production through enhancing activation of IRF3 in human pDCs. NOX-derived ROS-mediated up-regulation of MAVS was reported to be one of the mechanisms that lead to IRF3 activation and subsequent type I IFN production upon RIG-I activation [139]. Moreover, Buskiewicz et al showed that oxidative stress can lead to MAVS oligomerization and subsequent activation independently of RIG-I helicases [138]. In consistent with these reported findings, we could detect an mtROS-dependent but RIG-I-independent up-regulation of MAVS. Based on this, we speculate that mtROS might boost the RIG-I-mediated type I IFN production by influencing MAVS activity, however, this assumption requires further investigation.

Another proposed mechanism for mtROS-mediated increase of the RIG-I signalling pathway is associated with Akt. A common immune escape strategy employed by viruses to promote viral replication is up-regulation of the PI3K/Akt pathway to suppress apoptotic events in host immune cells [153, 154]. Conversely, Yeon et al. suggested that Akt is important in RIG-I mediated antiviral

signalling pathways in murine macrophages. They found Akt associated with MAVS upon RIG-I stimulation to promote IRF3 activation and type I IFN production [140]. Besides, a recent study demonstrated ROS-dependent phosphorylation of Akt in response to the release of mtROS into the cytoplasm in human amniotic cells [155]. In line with these reports, we found that in GEN2.2 cells Akt phosphorylation is induced upon activation of RIG-I that could be further enhanced by AMA treatment. This result indicated that the mtROS-induced IRF3 activation and type I IFN production might be mediated at least in part through Akt activation in pDCs.

Throughout evolution, a wide array of tactical solutions has emerged by which viruses could evade host innate defences that continuously forced the host's immune system to evolve mechanisms that overcome viral infections. It is tempting to speculate that the crosstalk of TLR and RIG-I signalling cascades in pDCs and their opposing regulation by mtROS are the consequence of another battle of the ancient war between virus and host.

Another mechanism to ensure protective antimicrobial responses while reducing the severity of inflammation and tissue damage is via the strict regulation of autophagy in cDCs. This requires communication between sensors of the immune environment and the regulatory pathways of autophagy [28]. Our experiments identified SLAMF5 as a novel cell surface regulator of cDC autophagy. We presented evidence that inhibition of SLAMF5 expression by specific siRNA inhibited, while cross-linking of SLAMF5 with an agonistic antibody increased the autophagic flux of human moDCs both under steady state conditions and following activation with LPS/IFNγ. As neither the mTOR-inhibitor rapamycin nor the late phase inhibitor BafA1 could restore the level of autophagy in SLAMF5-silenced moDCs, we propose that SLAMF5 influences the early steps of autophagy, independently of mTOR.

It was recently reported that the transcription of many autophagy genes is dependent on IRF8 in murine DCs. Our experiments described above show that, similar to murine cells, IRF8 controls the autophagic process of human moDCs. The dramatically decreased IRF8 protein level of SLAMF5-silenced cells together with the observation that the autophagy promoting effect of the SLAMF5-specific antibody is reversed by silencing IRF8 expression of moDCs suggests that SLAMF5 controls cDC autophagy via an IRF8-dependent mechanism. However, we cannot exclude contribution of other pathways such as regulation of the Vps34/Beclin-1 macrocomplex that was described to be regulated by other members of the SLAM family. The amount of IRF8 protein in macrophages is reportedly modified by TRIM21-mediated polyubiquitination and subsequent proteasomal degradation [128]. In line with this, we observed a TRIM21-dependent degradation of IRF8 in SLAMF5-silenced cells, suggesting that SLAMF5 sustains IRF8 signalling in cDCs via inhibiting this E3 ubiquitin ligase.

In human PBMCs a consequence of autophagic block, either by the PI3K inhibitor 3-methyladenine or by knockdown of the autophagy protein Atg7, is increased production of IL-1β in response to either LPS or Mycobacterium tuberculosis (Mtb) [30, 31]. Likewise, inhibition of autophagy in moDCs allowed excessive IL-23 secretion, whereas induction of autophagy had the

opposite effect [29]. We found that SLAMF5- and IRF8-silenced moDCs stimulated with LPS/IFNγ showed a similar increase of IL-1β and IL-23 production to that of autophagy-deficient cells. However, impaired autophagy in both SLAMF5- and IRF8-silenced cells associated with diminished production of IL-12. This seems to be in contradiction with previous studies describing increased IL-12 production in case of autophagic block. This was demonstrated by both myeloid cell-specific deletion of the autophagy protein Atg5 in mice followed by Mtb infection, and treating microglia with the PI3K inhibitor 3-methyladenine as well as silencing Atg5 or Beclin-1 by RNA interference prior to LPS stimulation [156, 157]. This discrepancy, however, can be explained by the IRF8 dependence of LPS/IFNγ-induced IL-12 production in macrophages and, as our results demonstrate, in human moDCs [158].

Beyond suppressing inflammation, autophagy partakes in direct elimination of pathogens, when they manage to invade the host cell interior. For this reason, many intracellular pathogens have evolved strategies to interfere with the autophagic microbicidal defence [124]. It was recently reported that hepatitis C virus (HCV), derived from sera of infected patients, blocked the autophagic process in differentiating monocytes, which resulted in impaired transition of $CD1a^-$ cells to $CD1a^+$ moDCs. The authors speculated that block of autophagy is part of the escape strategy by which HCV may keep the antiviral response under control. Beyond interfering with moDC development, blocking autophagy could further promote HCV-mediated immune subversion by decreasing degradation of viral proteins within the autophagosomes, generating peptides available for presentation in complex with MHC class II [147]. Importantly, we observed a similar decrease in the subset of $CD1a^+$ moDCs in the absence of either SLAMF5 or IRF8. The viability of moDCs and the yield of their differentiation however remained intact in the absence of SLAMF5 suggesting that the survival-promoting effect of SLAMF5, observed by Binsky et al in chronic lymphocytic leukemia cells, is either context-dependent, restricted to the lymphoid lineage or even specific to transformed cells [142]. The phenotypic maturation of SLAMF5-silenced moDCs was also comparable to that of control cells. These findings indicate that SLAMF5 signalling more possibly contribute to the secretion of a specific pattern of cytokines rather than to an overall downregulation of LPS/IFNγ response. The SLAMF5-mediated altered cytokine profile of LPS/IFNγ-stimulated moDCs together with the intact co-stimulatory receptor expression should allow cDCs to modulate the polarization of naïve T cells while supporting their proliferation. The cytokines IL-1β and IL-23, which are suppressed by SLAMF5, are important for the differentiation and expansion of Th17 cells from naïve $CD4^+$ T cells, while IL-12, that is increased by SLAMF5, would support Th1 cell differentiation [29, 159]. However, SLAMF5-mediated modulation of T cell polarization may be more complex. Besides Th1 cells, differentiation of Tfh-like IL-21 producing $CD4^+$ T cells also depends on IL-12 [160]. Though the exact effects of cDC-derived SLAMF5 signals on T cell responses requires further clarification, our findings suggest that in human cDCs, SLAMF5 supports antimicrobial responses against intracellular pathogens by both enhancing autophagy and by regulation of T cell differentiation.

In contrast with the ubiquitous basal autophagy apparatus, SLAMF5 is expressed solely on hematopoietic cells. Thus, SLAMF5 is likely part of a regulatory module required for the fine-tuning of autophagy in specific immune cells. However, whether its autophagy regulatory effect varies in different immune cell types still needs to be established. Considering the protective role of autophagy during an antimicrobial immune response, enhancing autophagy in immune cells may be beneficial in treatments of infectious diseases. In support of this concept, Garcia et al. have recently reported that autophagy was induced by Mtb-derived antigens in $CD14^{+}$ monocytes obtained from patients with active tuberculosis, and its level correlated with disease severity [161]. In addition, other reports have shown that vitamin D inhibited both HIV and Mtb replication in human macrophages through an autophagy-dependent mechanism [162, 163].

It should be noted that in our experiments, SLAMF5 signalling occurs during DC-DC interaction. Whether this interaction represents the most relevant cDC-interactions needs to be investigated. Heterophil cell-cell interactions between cDCs and other cell types in peripheral lymphoid and/or non-lymphoid tissues are likely to play an important role. CDCs localize in close proximity to other immune cells, such as T cells, B cells and mast cells, which potentially influence the immune modulatory capacities of cDCs [164]. The homophilic SLAMF5 receptor is expressed by these cell types and has been shown to regulate TCR-, BCR-, and FCεRI-mediated signal-transduction [165-167]. To understand how bidirectional SLAMF5 signalization between cDCs and other immune cell types affect immune responses awaits further studies.

Altogether, our experiments identified SLAMF5 as a novel cell surface modulator of autophagy and revealed an unexpected link between the SLAMF and IRF8 signalling pathways, both implicated in multiple human pathologies. Additional work is required to establish how this novel pathway may be harnessed to modulate regulatory circuits of autophagy and inflammation to improve current therapies in various infectious and/or autoimmune diseases.

6. SUMMARY

Inflammation today is considered as an essential, integral part of the immune response. Uncontrolled, exuberant or chronic inflammation however leads to extensive tissue damage that can contribute to the development of multiple human pathologies including various autoimmune diseases and cancers. The two major subsets of dendritic cells (DC), the conventional (cDC) and the plasmacytoid DCs (pDC) play an instrumental role in setting the threshold and regulating the length and intensity of the inflammatory response. The common theme in this work was to study the role of extracellular and intrinsic signals on the antimicrobial mechanisms of DCs against intracellular pathogens. More specifically, we analysed the role of mitochondrial reactive oxidative species (mtROS) on the activity of TLR- and RIG-I-induced signalling in pDCs and studied the role of SLAMF5, a member of the Signaling Lymphocyte Activation Molecule Family (SLAMF) on the regulation of autophagy in cDCs.

Generation of mtROS, triggered by stress or metabolic changes in the cells, is one of the key regulators of virus-stimulated signalling pathways. We found that in TLR9 agonist-induced pDCs elevated level of mtROS markedly reduced the expression of type I interferon (IFN) genes via blocking phosphorylation of IRF7, the key transcription factor of type I IFNs. In contrast, mtROS enhanced the expression of type I IFN genes induced by RIG-I agonist in pDCs via increasing the expression and phosphorylation of stimulatory signalling proteins in this pathway. The identified novel mechanism allows pDCs to maintain viral sensing and activation in the inflammatory environment, where TLR9-induced signalling is inhibited by the elevated levels of ROS.

SLAMF receptors are cell surface proteins, expressed on hematopoietic cells, which facilitate communication among immune cells. This work describes the identification of SLAMF5 as a new member of cell-surface expressed regulators of autophagy. We discovered that, unlike SLAMF1 and SLAMF4 that are regulators of the Vps34/Beclin-1 autophagy macrocomplex, SLAMF5 exerts its regulatory function via inhibiting proteolytic degradation of IRF8, a master regulator of autophagy by a mechanism dependent on the E3 ubiquitin ligase TRIM21. As an autophagy regulator, SLAMF5 influences the ratio of $CD1a^+$ cells in differentiating cDCs and partakes in the regulation of IL-1β, IL-23 and IL-12 production in LPS/IFNγ-activated cDCs in a manner that is consistent with its effect on IRF8 stability.

Taken together, the presented research led to identification of novel mechanisms in signalling pathways involved in fundamental cell biological functions, e.g. stress-induced production of mtROS and autophagy that should contribute to our understanding of the inflammatory process as well as the regulatory circuits governing the immune response to intracellular pathogens.

ÖSSZEFOGLALÁS

Napjainkra nyilvánvalóvá vált, hogy a gyulladás az immunválasz nélkülözhetetlen része. A szabályozatlan, túlságosan intenzív, vagy krónikus gyulladás azonban kiterjedt szövetkárosodást okoz, mely számos kórkép kialakulásához vezethet, beleértve autoimmun illetve tumoros megbetegedéseket. A dendritikus sejtek (DC) két fő alpopulációja, a konvencionális (cDC) valamint a plazmacitoid DC-k (pDC) meghatározó szerepet töltenek be a gyulladásos válasz elindításában és mértékének szabályozásában. Munkánk során átfogóan tanulmányoztuk a DC-k intracelluláris patogének elleni védelmi mechanizmusait befolyásolni képes intra- és intercelluláris kommunikációs folyamatokat. Kísérleteinkkel sikerült részletesebben megismernünk a mitokondriális eredetű reaktív oxigén gyökök (mtROS) TLR- és RIG-I receptorok jelátvitelére kifejtett hatását pDC-ben, valamint a SLAMF receptor családba tartozó SLAMF5 sejtfelszíni molekula autofágiára gyakorolt hatását cDC-ben.

A metabolikus változások vagy stressz hatására megemelkedett mtROS termelés a vírusok által stimulált jelátviteli utak egyik kulcsfontosságú szabályozója is. Eredményeink szerint emelkedett mtROS jelenlétében jelentősen csökken a pDC-k TLR9 agonisták által indukált I-es típusú interferon (IFN) termelése, döntően az útvonalban központi szereppel bíró IRF7 transzkripciós faktor foszforilációjának gátlásán keresztül. Ezzel ellentétben, a RIG-I agonista által indukált I-es típusú IFN gének kifejeződésére pozitív hatást gyakorol a fokozott mtROS termelés, mely ezen jelátviteli útvonal pozitív szabályozóinak fokozott aktivációjának következménye. A kísérleteink során azonosított mechanizmuson keresztül a pDC-k képesek a vírusok érzékelésére és I-es típusú IFN-ok termelésére a gyulladásos környezetben is, ahol a magasabb ROS koncentráció következtében a TLR9-indukált jelátvitel gátolt.

A SLAMF receptorok hematopoetikus sejtek felszínén expresszálódnak, elősegítve az immunsejtek közötti kommunikációt. Megfigyeléseink alapján a SLAMF5 receptor azon kevés sejtfelszíni molekulák közé sorolható, melyek képesek az autofágia folyamatának szabályozására. Eredményeink azt mutatják, hogy a SLAMF1 és SLAMF4 receptoroktól eltérően, melyek a Vps34/Beclin-1 komplexen keresztül szabályozzák ezt a folyamatot, a SLAMF5 az autofágiát szabályozó gének egyik fő transzkripciós faktorán, az IRF8 fehérjén keresztül fejti ki hatását. Megállapítottuk, hogy a SLAMF5 a TRIM21 E3 ubikvitin ligáz gátlása révén képes gátolni az IRF8 proteoszomális degradációját. Kimutattuk továbbá, hogy az autofágiát szabályozó funkciójának megfelelően a SLAMF5 jelátvitele hatással van a monocita-DC differenciáció során a $CD1a^+$ sejtek arányára, valamint összhangban a receptor IRF8 stabilizáló hatásával, LPS/IFNγ aktivációt követően befolyásolja az IL-1β, IL-23 és IL-12 citokinek termelését.

Ezen kutatási eredmények új mechanizmusokat tártak fel alapvető sejt funkciókat érintő jelátviteli folyamatokban, mint például a stressz hatására termelődő mtROS vagy autofágia, melyek

reményeink szerint hozzájárulnak a gyulladás valamint az intracelluláris patogének elleni védelmi reakciókat szabályozó folyamatok jobb megértéséhez.

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Subject: PhD Publikációs Lista

Candidate: Zsófia Agod
Neptun ID: IWCMYY
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List of publications related to the dissertation

1. Agod, Z., Pázmándi, K. L., Bencze, D., Vereb, G., Bíró, T., Szabó, A., Rajnavölgyi, É., Bácsi, A., Engel, P., Lányi, Á.: Signaling Lymphocyte Activation Molecule Family 5 Enhances Autophagy and Fine-Tunes Cytokine Response in Monocyte-Derived Dendritic Cells via Stabilization of Interferon Regulatory Factor 8.
   Front. Immunol. 9 (62), 2018.
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2. Agod, Z., Fekete, T., Budai, M. M., Varga, A., Szabó, A., Moon, H., Boldogh, I., Bíró, T., Lányi, Á., Bácsi, A., Pázmándi, K. L.: Regulation of type I interferon responses by mitochondria-derived reactive oxygen species in plasmacytoid dendritic cells.
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3. Pázmándi, K. L., Agod, Z., Kumar, B. V., Szabó, A., Fekete, T., Somogyi, V., Veres, Á., Boldogh, I., Rajnavölgyi, É., Lányi, Á., Bácsi, A.: Oxidative modification enhances the immunostimulatory effects of extracellular mitochondrial DNA on plasmacytoid dendritic cells. Free Radic. Biol. Med. 77, 281-290, 2014.
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4. Szabó, A., Osman, R. M., Bacskai, I., Kumar, B. V., Agod, Z., Lányi, Á., Gogolák, P., Rajnavölgyi, É.: Temporally designed treatment of melanoma cells by ATRA and polyI:C results in enhanced chemokine and IFNb secretion controlled differently by TLR3 and MDA5. Melanoma Res. 22 (5), 351-361, 2012.
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8. KEYWORDS

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TÁRGYSZAVAK

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10. SUPPLEMETARY

SUPPLEMENTARY 1

Zsofia Agod, Tünde Fekete, Marietta M. Budai, Aliz Varga, Attila Szabo, Hyelim Moon, Istvan Boldogh, Tamas Biro, Arpad Lanyi, Attila Bacsi, Kitti Pazmandi

Regulation of type I interferon responses by mitochondria-derived reactive oxygen species in plasmacytoid dendritic cells

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SUPPLEMENTARY 2

Zsofia Agod, Kitti Pazmandi, Dora Bencze, Gyorgy Vereb, Tamas Biro, Attila Szabo, Eva Rajnavolgyi, Attila Bacsi, Pablo Engel and Arpad Lanyi

Signaling Lymphocyte Activation Molecule Family 5 Enhances Autophagy and Fine-Tunes Cytokine Response in Monocyte-Derived Dendritic Cells via Stabilization of Interferon Regulatory Factor 8

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