PRODUCING, SENSING AND RESPONDING TO CELLULAR STRESS IN IMMUNITY

EDITED BY: Heitor A. Paula-Neto, Renata Meirelles Pereira and Leticia A. Carneiro

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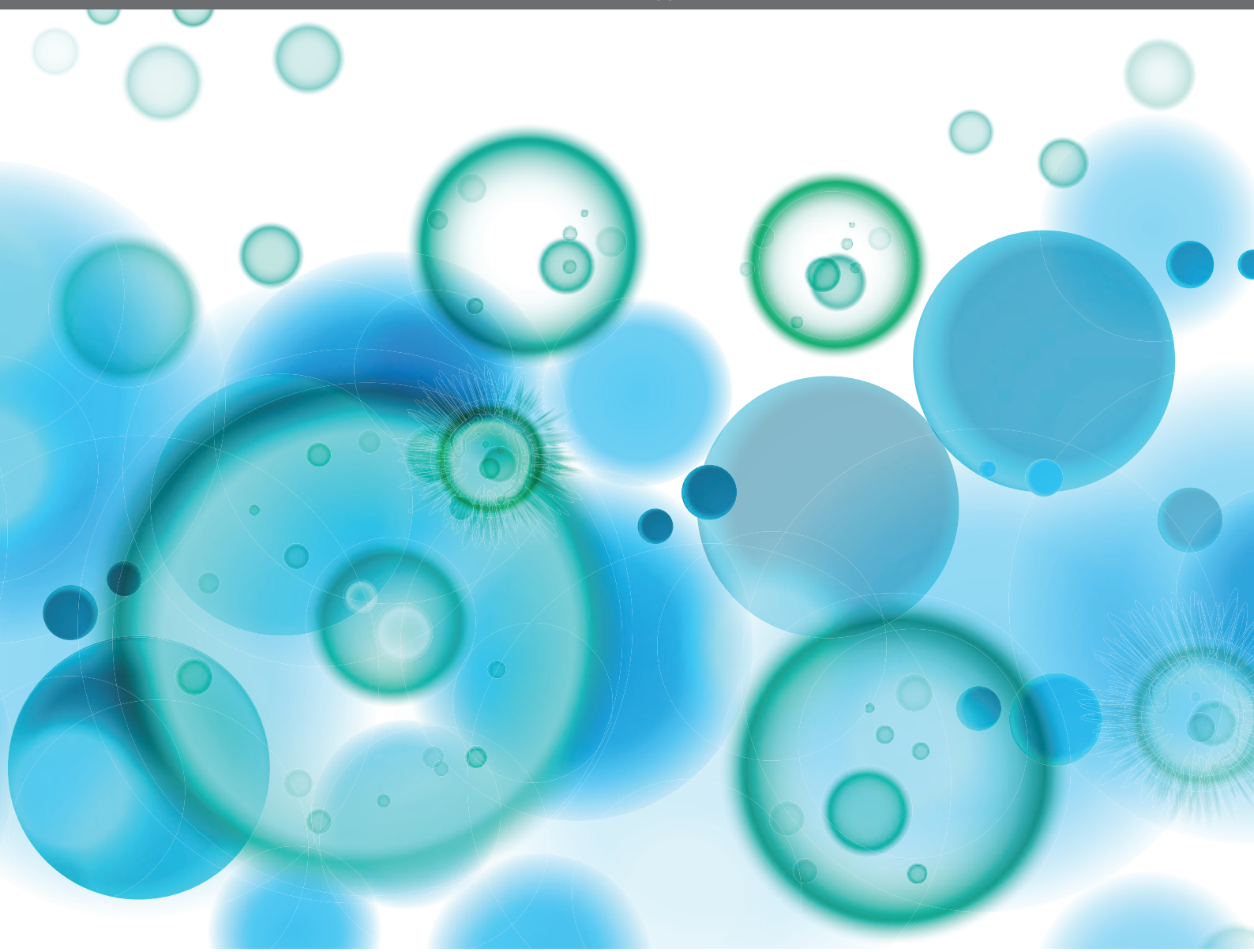

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PRODUCING, SENSING AND RESPONDING TO CELLULAR STRESS IN IMMUNITY

Topic Editors:
Heitor A. Paula-Neto, Universidade Federal do Rio de Janeiro, Brazil
Renata Meirelles Pereira, Universidade Federal do Rio de Janeiro, Brazil
Leticia A. Carneiro, Universidade Federal do Rio de Janeiro, Brazil

Cellular stress, being considered as any disturbance in cellular physiology, is a fundamental aspect of tissue and body capacity to adapt to the ever changing environment. It also surges as a consequence of tissue injury or invasion of the body by pathogens. Since the immune system was developed to sense and respond to these deleterious processes, it is reasonable to consider that immune cells are capable of sensing and responding to signs of cellular stress. Moreover, cells of the immune system undergo cellular stress during an immune response. This Research Topic presents a series of articles focusing on how cellular stress influences the outcome of immune responses, covering not only how cellular stress can be a fundamental process during immune cell activation and function, but also how cells of the immune system are capable of sensing and being influenced by factors produced by stressed cells.

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Table of Contents

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EDITORIAL
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Editorial: Producing, Sensing and Responding to Cellular Stress in Immunity

Heitor A. Paula-Neto[1]*, *Renata M. Pereira*[2]* and *Leticia A. M. Carneiro*[2]*

[1] Faculdade de Farmácia, Universidade Federal do Rio de Janeiro, Rio de Janeiro, Brazil, [2] Instituto de Microbiologia Paulo de Góes, Universidade Federal do Rio de Janeiro, Rio de Janeiro, Brazil

Keywords: stress response, homeostasis, immune response, host-pathogen, innate immunity, UPR, autphagy, integrated stress response

Editorial on the Research Topic

Producing, Sensing and Responding to Cellular Stress in Immunity

Stress, meaning any disturbance of the internal environment of a cell, can result not only from external stimuli but also from physiological processes such as the intrinsic free radical production by the metabolic functioning of mitochondria. Stressors can threaten the cell and therefore mechanisms were selected throughout evolution to cope with and adapt to cell stress. Since the immune system is, ultimately, a system to sense and respond to stress posed by tissue damage, cell injury, and/or pathogens, it is reasonable to assume that all those cell-autonomous pathways involved in stress response also play a key role in immunity. This *Frontiers in Immunology* Research Topic focuses on different stress responses and their role in host–pathogen interaction and immunity.

A clear example of intrinsic stress is given by the fundamental role of reactive oxygen species (ROS) in T-cell activation. Gnanaprakasam et al. discuss how T-cells use their antioxidant machinery to fine-tune ROS activity so that it is sufficient to activate and polarize T-cells, but well-controlled to not result in cell damage. Other non-infectious process that impacts immunity is cell death. Controlled forms of cell death are an ancestral mechanism involved in key aspects of the physiology of multicellular organisms, including the elimination of unwanted, damaged, or infected cells. Amarante-Mendes et al. provide an overview on the three major types of molecularly controlled forms of cells death—apoptosis, necroptosis, and pyroptosis—that participate in host defense through the elimination of infected cells. Furthermore, the authors discuss how these events are both regulated by signals derived from PRRs as well as a source of danger-associated molecular patterns (DAMPS) that trigger immune responses through PRRs.

The concept of DAMPs is also key to understand how self-molecules can alert the immune system that homeostasis has been compromised. Among well-known DAMPs are the chromatin-associated protein HMGB1, extracellular purine metabolites, and S100 proteins. S100 proteins are a family of cytosolic proteins with a plethora of functions in cellular homeostasis that, when released from the cell as a result of tissue damage or cellular stress, can serve as DAMPs. Xia et al. explore this aspect of S100 proteins and how it interferes with different steps of inflammatory responses including their functions as DAMPs, on macrophage migration and on tissue repair.

Apart from its role in physiological processes, such as the removal of dead cells, the immune system is well-recognized for its function in host defense, interaction with microbes, and immune surveillance. During an infection, it is critical for the host to properly assess the potential threat posed by a given pathogen. In this sense, stress response pathways can be instrumental in providing the cell with the ability to sense alterations on homeostasis and tissue damage caused during

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Francesca Granucci, University of Milano-Bicocca, Italy

***Correspondence:**
Heitor A. Paula-Neto
heitorapneto@gmail.com
Renata M. Pereira
renata.pereira@micro.ufrj.br
Leticia A. M. Carneiro
leticiac@micro.ufrj.br

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infections. Rodrigues et al. discuss how the highly conserved integrated stress response (ISR) can shape the host response to bacterial pathogens. By sensing alterations to cellular homeostasis, rather than the bacteria itself, the ISR initiates a cellular program that includes transcription of key genes, profound alterations in translation of new proteins, and cell-autonomous antimicrobial mechanisms, such as autophagy. Smith also discussed how cellular stress induced by invading pathogens (virus and bacteria) is sensed, focusing on the impacts in protein folding induced by infection. Unfolded protein response (UPR) contributes to host defense through cytokine induction. The downside of the enhancement of host response is that UPR response has been increasingly recognized in a variety of autoimmune and inflammatory diseases.

Although the host cell is partly prepared to induce pathways that intervene in infections by sensing changes in homeostasis, pathogens like *Leishmania* parasites can adapt to these pathways and even benefit from them. Vivarini et al. demonstrated that *Leishmania amazonensis* induces the activation of the transcriptional factor Nrf2 (Nuclear factor erythroid 2-related factor 2), a master regulator of phase II defense gene expression that protect cells from oxidative stress. The authors show that Nrf2 knockdown promotes oxidative stress and impairs parasite survival in macrophages. Using the combination of *in vitro*, *ex vivo*, and *in silico* approach, the group shows Nrf2/PKR cross-talk and reveals a central role of Nrf2 in human cutaneous leishmaniasis. Nrf2 activation by *L. amazonensis* also required PI3K/Akt signaling and autophagy mechanisms. Autophagy is also the focus of a review by Siqueira et al. This process, known as a cellular mechanism to recycle organelles or digest intracellular contents in times of energy shortage, also plays a key role in immunity against intracellular pathogens.

Besides its interaction with pathogenic microorganisms, the immune system is also involved in the interaction and control of commensal microbiota, which, in turn, play a major role in instructing the immune system and maintaining homeostasis. Dysbiosis, which is a disruption of the normal microbiota–host relationship, has been associated with a myriad of human diseases including metabolic disorders, autoimmunity, and cancer. Espinoza and Minamo compile evidence suggesting that dysbiosis triggers DNA damage response, either by producing genotoxins or by promoting chronic inflammation, leading to overexpression of NKG2D-L in stressed cells. Consequently, these cells are tagged to be eliminated by Natural Killer (NK) cells and various subsets of T cells, which could be linked to autoimmunity and carcinogenesis.

On the other hand, the ability of immune cells, in particular NK cells, to detect cells displaying signs of stress is crucial for tumor immunosurveillance. This has been used to design new therapies that not only have antiproliferative and cytotoxic effects but also boost antitumor immunity by rendering tumor cells better targets for NK cells. Zingoni et al. discuss how cellular stress pathways induced in various tumors by different chemotherapeutic regimens can stimulate NK cells' effector function and provide new therapeutic approaches.

This collection of review and original papers is an invitation for the reader to appreciate the view of the immune system as a platform designed for the sensing, detection, and response to stress (in a broad definition), that uses all the stress-coping machinery selected by evolution to ensure an appropriate interaction with environmental challenges and host survival.

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All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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Natural Killer Cell Response to Chemotherapy-Stressed Cancer Cells: Role in Tumor Immunosurveillance

Alessandra Zingoni[1], Cinzia Fionda[1], Cristiana Borrelli[1,2], Marco Cippitelli[1], Angela Santoni[1,3] and Alessandra Soriani[1]**

[1] Department of Molecular Medicine, Sapienza University of Rome, Laboratory Affiliated to Istituto Pasteur Italia – Fondazione Cenci Bolognetti, Rome, Italy, [2] Center for Life Nano Science@Sapienza, Istituto Italiano di Tecnologia, Rome, Italy, [3] Neuromed I.R.C.C.S. – Istituto Neurologico Mediterraneo, Pozzilli, Italy

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Edited by:
Leticia A. Carneiro, Federal University of Rio de Janeiro, Brazil

Reviewed by:
Angelo A. Manfredi, Vita-Salute San Raffaele University, Italy
Ofer Mandelboim, Hebrew University of Jerusalem, Israel
Martin Hernán Bonamino, Instituto Nacional do Câncer, Brazil

***Correspondence:**
Alessandra Zingoni
alessandra.zingoni@uniroma1.it;
Alessandra Soriani
alessandra.soriani@uniroma1.it

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Natural killer (NK) cells are innate cytotoxic lymphoid cells that actively prevent neoplastic development, growth, and metastatic dissemination in a process called cancer immunosurveillance. An equilibrium between immune control and tumor growth is maintained as long as cancer cells evade immunosurveillance. Therapies designed to kill cancer cells and to simultaneously sustain host antitumor immunity are an appealing strategy to control tumor growth. Several chemotherapeutic agents, depending on which drugs and doses are used, give rise to DNA damage and cancer cell death by means of apoptosis, immunogenic cell death, or other forms of non-apoptotic death (i.e., mitotic catastrophe, senescence, and autophagy). However, it is becoming increasingly clear that they can trigger additional stress responses. Indeed, relevant immunostimulating effects of different therapeutic programs include also the activation of pathways able to promote their recognition by immune effector cells. Among stress-inducible immunostimulating proteins, changes in the expression levels of NK cell-activating and inhibitory ligands, as well as of death receptors on tumor cells, play a critical role in their detection and elimination by innate immune effectors, including NK cells. Here, we will review recent advances in chemotherapy-mediated cellular stress pathways able to stimulate NK cell effector functions. In particular, we will address how these cytotoxic lymphocytes sense and respond to different types of drug-induced stresses contributing to anticancer activity.

Keywords: natural killer cells, immunochemotherapy, cancer, stress, natural killer cell activating ligands, damage-associated molecular patterns, death receptors, PDL-1

INTRODUCTION

Natural killer (NK) cells represent a crucial component of antitumor innate immune response displaying cytotoxic functions and secreting several cytokines/chemokines (1, 2).

Natural killer cell cytotoxic activity regulation depends on an integrated interplay between inhibitory receptors and numerous activating receptors acting in concert to efficiently eliminate tumor cells.

Relevant activating receptors for tumor cell recognition are NKG2D that recognizes MICA/B and ULBPs proteins, orthologs of the mouse RAE1 molecules, DNAM-1 that binds two ligands named poliovirus receptor (PVR/CD155) and Nectin-2 (CD112), and the receptors NKp30, NKp44, and

NKp46 belonging to the natural cytotoxicity receptors and shown to interact with a broad spectrum of ligands (3).

Natural killer cells also express inhibitory receptors for molecules of the major histocompatibility complex (MHC) class I, which are Ly49 receptors in mice, *k*iller cell *i*mmunoglobulin-like *r*eceptors (KIRs) that bind to HLA-A, -B, and -C molecules in humans, and the CD94-NKG2A heterodimer in both species (4). In addition, NK cells express two inhibitory receptors for PVR, called TACTILE (CD96) and TIGIT, that counterbalance the DNAM-1-mediated activation of NK cells (5).

The activation of NK cells leads to the release of cytotoxic granules containing perforin and various granzymes and to cytokine production, most prominently interferon-γ (IFN-γ) (6–8). In addition, the expression at the cell surface of death-inducing ligands belonging to the tumor necrosis factor (TNF) family, such as Fas ligand (FasL) and TNF-related apoptosis-inducing ligand (TRAIL), also drives the activation of the caspase enzymatic cascade through the binding to the death receptors (DRs), namely, Fas, DR4 (TRAIL-RI), and DR5 (TRAIL-RII), on target cells (9, 10).

More recently, immunological checkpoint molecules commonly associated with T cells, such as CTLA-4 and PD-1, have been described on NK cells as negative regulators of their immune function (11–13).

Conventional chemotherapies were initially designed to produce antiproliferative or cytotoxic effects on dividing tumor cells. However, as result of numerous demonstrations indicating that an endogenous antitumor immunity is essential for complete remission during tumor therapy (14–16) several antineoplastic drugs, even at low doses, have been reconsidered also as potential immunomodulatory agents (17).

In this context, it has becoming always more evident that dying or stressed cells release or expose stress molecules, called damage-associated molecular patterns (DAMPs) that can alert the immune system (18). Moreover, many chemotherapy-mediated stress pathways modulate the expression of NK cell activating and inhibitory ligands, rendering tumor cells more immunogenic.

In this review, we will summarize the effects of different chemotherapeutic agents on the activity of NK cells, emphasizing the immunomodulatory effects of both conventional and low concentrations of drugs at the interface between stressed or dying cancer cells and the immune system, in the attempt of exploiting them for therapeutic purposes.

REGULATION OF NK CELL-ACTIVATING AND -INHIBITORY LIGAND EXPRESSION BY CHEMOTHERAPEUTIC DRUGS

A number of evidence indicate that chemotherapy-induced sensitization of tumor cells to immune effectors plays an important role in anticancer therapy. Indeed, different types of drug-induced stresses can modulate the expression of NK cell-activating/or -inhibitory ligands on cancer cells thus affecting their recognition and elimination by NK cells (**Table 1**). Besides genotoxic drugs or radiotherapy, many other pharmacological compounds already approved for the treatment of different malignancies or entered in clinical trials have been described to increase NK cell-activating ligand expression (19–27). Moreover, most of these drugs are also able to downregulate NK cell-inhibitory ligand expression, so that different and multiple mechanisms concur to make tumor cells more susceptible to NK cell-mediated lysis (28–32).

In the case of genotoxic drugs or DNA replication inhibitors, the mechanisms regulating the NKp30 ligand B7-H6 expression on human cancer cells remain largely unknown (23), while much evidence indicate a major role for the DNA damage response (DDR) pathway in the upregulation of the stimulatory ligands for the NKG2D and DNAM-1 immunoreceptors. In addition, ionizing radiations represent classical stimuli to induce NKG2D ligand upregulation, through the induction of the DDR (33). The activation of the kinases ATM/ATR and the production of reactive oxygen species converge on the E2F1 factor able to activate MICA, MICB, and PVR transcription on multiple myeloma (MM) cells by doxorubicin and melphalan (34). On the other hand, a different pathway governing NKG2DLs expression by chemicals known to induce genotoxic stress has been characterized in murine lymphoma cells: DDR drives to the presence of cytosolic DNA and to STING/TBK1-dependent activation of the transcription factor IRF3, responsible for the upregulation of RAE1 expression (35). Interestingly, in murine leukemia cells, concomitantly to NKG2D ligand upregulation, DDR-activating therapeutic agents cause a loss of the inhibitory NK cell ligand Clr-b, thus enhancing the cytotoxicity mediated by $NKRP1B^+$ NK cells (36).

Non-lethal heat shock mimicking hyperthermia therapy can promote NKG2DL expression both in human and murine cancer cells but with different mechanisms. MICA and MICB upregulation occurs at the transcriptional level *via* HSF1 activation (37) and, with a similar mechanism, MICA and MICB expression on MM cells is enhanced by HSP90 chaperone inhibitors that activate this transcription factor (21). In a different way, increased surface expression of the mouse NKG2D ligand Mult1 depends on the inhibition of protein ubiquitination and lysosomal degradation (38).

Treatment of different tumor cell types with epigenetic drugs, like histone deacetylase inhibitors (HDACi) and DNA-methyltransferase inhibitors (DNMTi) (25–27, 39–43), leads to the upregulation of NKG2DLs and PVR surface levels, although it downregulates B7-H6 expression (44). For DNMTi the molecular mechanisms underlying NKG2DLs upregulation are still unclear, while different pathways cooperate in the regulation of these molecules in response to HDACi, and this might depend on the type of tumor and the dose of the drug used. In particular, valproic acid (VPA) has been reported to upregulate MICA/B with a mechanism dependent on PI3K/Akt pathway in pancreatic cancer cells (40), while the involvement of ERK in MICA/B and ULBP2 upregulation in response to VPA has been shown in MM cells (45). Moreover, Yang and colleagues proposed that the capability of the HDACi suberoylanilide-hydroxamic acid (SAHA) to increase MICA expression in hepatoma cancer cells is dependent on miR-17-92 cluster (46).

In MM cells, the bromodomain and extra terminal domain inhibitors (BETi) and immunomodulatory drugs (IMiDs) can block the repressive activity of the transcription factors IRF4 and IKZF1/3 on MICA and PVR promoters (19, 47). In addition,

TABLE 1 | Chemotherapy-induced pathways and molecular targets able to modulate natural killer (NK) cell activating ligands and PDL-1 on cancer cells.

Class of chemotherapeutic agent	Pathway/molecular target	Ligand	NK cell cytotoxicity	Cancer cell type	Reference
PROTEASOME INHIBITOR					
Bortezomib	DNA damage response (DDR)	MICA	nd	Multiple myeloma (MM)	(24)
Low doses: 0.75–10 nM	nd	MICA/B, PVR, Nec-2	+	MM	(52)
	nd	MICA/B ULBP1–3, PVR, Nec-2	nd	MM	(22)
	nd	MICA/B	+	Hepatocellular carcinoma	(114)
HISTONE DEACETYLASE INHIBITORS					
Low dose: valproic acid (1 mM)	nd	MICA/B	+	Hepatocellular carcinoma	(41)
	ERK	MICA/B, ULBP2	+	MM	(45)
	PI3K/Akt	MICA/B	+	Pancreatic cancer	(40)
Trichostatin A	HDAC1/MICA promoter	MICA/B	+	Leukemia	(42)
Suberoylanilide-hydroxamic acid	miR-17-92	MICA	+	Hepatocellular carcinoma	(46)
GENOTOXIC AGENTS					
Low doses: doxorubicin (0.05–3.5 μM); melphalan (1.5–22 μM)	Reactive oxygen species-dependent DDR	MICA/B, ULBP1–3, PVR, Nec-2	+	MM	(22, 34)
Cisplatin	nd	B7-H6	+	Tumor cell lines	(23)
Ara-C, aphidicolin	STING/TBK/IRF3	RAE1	nd	B cell lymphoma	(35)
GSK INHIBITORS					
Low doses: LiCl (10 mM), BIO (1.5 μM), SB21 (5 μM)	STAT3 inhibition	MICA	+	MM	(20)
BET INHIBITORS					
Low dose: JQ1 (0.5 μM)	IRF4	MICA	+	MM	(19)
	BRD4	PDL-1	nd	Lymphoma	(28)
HSP90 inhibitors					
Low doses: radicicol (2 μM), 17-AAG (1 μM)	HSR	MICA/B	+	MM	(21)
MICROTUBULE ASSEMBLY INHIBITORS					
Low dose: vincristine (0.05 μg/ml)	p38 MAPK	PVR, MICA, ULBP1	+	MM	(50)
Cytochalasin D Nocodazole Docetaxel	DDR Endoplasmic reticulum stress response	MICA, ULBP1–3, PVR, Nec-2, B7-H6	+	Tumor cell lines	(51)
IMMUNOMODULATORY DRUGS					
Low dose: lenalidomide (10 μM)	IKZF1/3, IRF4	MICA, PVR	+	MM	(47)

Effects on an increased NK cell recognition and killing of drug-treated tumor cells are also reported (+). Low doses of drugs that do not affect cell vitality are indicated.
nd, not done.

both these therapeutic agents can downregulate the expression of PD-L1 on cancer cells (28, 29, 31, 32). Indeed, BETi interrupt the activity of the epigenetic reader protein BRD4 on PD-L1 promoter region, by significantly reducing both the constitutive and IFN-γ inducible expression of this ligand. In this regard, the downstream mediators of IFN-γ signaling, JAK kinases, can be pharmacologically blocked to negatively regulate PD-L1 expression in cancer cells (48). Furthermore, drugs disrupting RAF/MEK/ERK signaling pathway, such as Sorafenib and the TLR3 agonists poly-IC, can synergistically reduce the percentage of tumor cells expressing PD-L1 and enhance NK and T cell activation in a mouse model of hepatocarcinoma (49).

Regarding drugs that disrupt the microtubule assembly, sub-lethal doses of Vincristine can activate p38 MAPK and regulate NKG2DL expression both at transcriptional and post-transcriptional level in MM cells (50). Moreover, Cytochalasin D, nocodazole, and docetaxel can enhance NKG2D, DNAM-1, and NKp30 ligands on tumor cell surface, with MICA upregulation being dependent on both DNA damage and endoplasmic reticulum (ER) stress response (51).

Different studies have been done by using proteasome inhibitors in MM cells. In this regard, low doses of bortezomib can induce the upregulation of both NKG2D and DNAM-1 ligands (22, 52, 53), and in accordance with these data, Jinushi

and colleagues reported a DDR-ATM-dependent upregulation of MICA surface levels (24). On the other hand, no significant change in NKG2DL expression was observed upon bortezomib treatment by Shi and colleagues (30). Interestingly, the latter study described the capability of bortezomib to downregulate HLA class I surface expression by sensitizing MM cells to NK cell–mediated lysis (30).

Chemotherapeutic agents can also contribute to the post-translational regulation of NK activating ligand expression by promoting the release of soluble NKG2DLs through the modulation of the expression and activity of metalloproteinases (MMP) and ADAM enzymes on cancer cells (54). Although an increased stimulation of the shedding process in response to genotoxic agents has been reported (55), some studies using different drugs describe an inhibitory effect. Indeed, gemcitabine treatment impaired ULBP2 shedding through downregulation of ADAM10 in pancreatic cancer (56). Likewise, the hypomethylating agents, azacitidine and decitabine, reduced MICA, MICB, and ULBP2 release in AML by increasing TIMP3 expression, a potent inhibitor of MMP family (57).

Thus, antitumor therapeutics can work also as activators of different "stress pathways" that enhance tumor sensitivity to NK cell cytolysis by modulating the expression of the activating and inhibitory ligands on tumor cells.

MODULATION OF DRs BY CANCER THERAPEUTIC AGENTS

Many cancer therapeutic drugs can induce DR expression and redistribution (58) (**Table 2**). Several studies described a role for different types of HDACi in the upregulation of TRAIL receptors on various malignant tumor cells (59–63). In this context, SAHA and trichostatin A (TSA) were shown to increase cell-surface expression of DR4 and DR5 in human MM cell lines (64). A study from Insinga et al. showed that different DR and their ligands (i.e., TRAIL, DR5, FasL, and Fas) are upregulated by HDACi on leukemic cells, but not in the normal counterpart of hematopoietic progenitors, promoting tumor apoptosis through the activation of the DR pathway (65).

A number of studies showed that bortezomib upregulated surface expression of TRAIL receptors on a variety of human tumor cell lines, enhancing their susceptibility to NK cell lysis with a mechanism mainly dependent on TRAIL (66). In another model, a bortezomib-treated murine renal carcinoma cell line is more susceptible to both NK-cell perforin/granzyme and recombinant TRAIL-mediated apoptosis, resulting in enhanced caspase-8 activity (67). Indeed, in human non-small cell lung cancer cells this drug has been shown to trigger TRAIL-induced apoptosis *via* DR5 upregulation (68). Several pieces of evidence reported that another proteasome inhibitor, namely, MG132, increases DR5 expression cooperating in establishing apoptosis in several cancer cells (69–71).

DR4 and DR5 were demonstrated to be DNA damaging-inducible and p53-regulated genes (72–76). Accordingly, many DNA damaging chemotherapeutic agents can regulate DR expression, rendering cancer cells more sensitive to DR-elicited apoptosis (74, 75, 77–81).

Altogether, these results suggest that the extrinsic apoptotic pathway has an important role in chemotherapy-induced apoptosis through the promotion of DRs-mediated recognition by cytotoxic lymphocytes. In addition, chemotherapies can promote

TABLE 2 | Chemotherapy-induced pathways and molecular targets able to modulate death receptors (DRs) on cancer cells.

Class of chemotherapeutic agent	Pathway/molecular target	DR	Cancer cell type	Reference
PROTEASOME INHIBITORS				
Low doses: bortezomib (5–20 nM)	DNA damage response	DR5	Tumor cell lines, renal carcinoma	(66, 67)
MG132	CHOP	DR5	Prostate cancer	(71)
HISTONE DEACETYLASE INHIBITORS				
Sodium butyrate	Sp1	DR5 (caspase-3 activation)	Colorectal carcinoma	(59)
Trichostatin A (TSA), suberoylanilide-hydroxamic acid (SAHA) Sodium butyrate	p53-independent mechanism	DR5 (caspase member activation)	Tumor cell lines	(60)
Low doses: SAHA (500 nM), TSA (50 nM)	p21, p27, E2F	DR4, DR5 (increase of proapototic Bcl-2 family members)	Multiple myeloma	(64)
VPA	nd	DR5, FAS	Leukemia	(65)
GENOTOXIC AGENTS				
Cisplatin, mitomycin, doxorubicin, methotrexate, etoposide	p53-dependent mechanism	FAS, DR5, DR4	Tumor cell lines	(72–74, 77)
Etoposide	NF-κB	DR5	Tumor cell lines	(76)
Doxorubicin, Ara-C, etoposide	p53-independent mechanism	DR5	Leukemia cell lines	(81)

Low doses of drugs that do not affect cell vitality are indicated.
nd, not done.

the cell death by regulating the balance between pro- and antiapoptotic proteins toward apoptosis. Many evidence show that drugs may control the cell intrinsic apoptosis by altering Bax and Bcl-2 expression in different tumor cells (82–86).

CHEMOTHERAPY-INDUCED DAMPs ALERTING NK CELLS

Many anticancer chemotherapies increase the immunogenic potential of cancer cells mainly through the establishment of immunogenic cell death, or other forms of non-apoptotic death, including autophagy, and the release of the so-called DAMPs, such as high-mobility group box 1 proteins (HMGB1), ATP, heat shock proteins (HSPs), and the ER chaperone calreticulin (87).

Damage-associated molecular patterns are intracellularly sequestered in normal physiological conditions, but they can be actively secreted or aberrantly exposed on the cell surface under conditions of cellular stress.

Engagement of various target receptors present on immune cells by DAMPs leads to the elicitation of a potent antitumor immunity. Mostly, DAMPs have been proposed to activate local APCs, thus promoting the adaptive immune system. For example, both HSP70 and HMGB1 boost dendritic cell (DC) maturation through toll-like receptor 4, favoring the induction of antigen-specific T cell-mediated antitumor immune responses (88, 89). Less is known about DAMP contribution to NK cell stimulation; thus, we will focus the attention on HMGB1 and HSPs, due to their ability to exert different effects on NK cell-mediated functions.

High-mobility group box 1 protein is an endogenous nuclear factor released both by activated immune cells or injured non-immune cells, and in the extracellular milieu acts as a DAMP alerting the immune system to danger and triggering immune response activation through the interaction either with multiple TLRs and the receptor for advanced glycation end products (RAGE), expressed on a variety of cells (90). In this regard, the chemotherapeutic agent cyclophosphamide has been recently shown to facilitate NK cell activation through a process involving HMGB1 release in a glioma mouse model (91). Accordingly, it was demonstrated that in HMGB1-deficient tumors, different innate immune cells, including NK cells, have impaired ability to reach the tumor tissue in response to DNA alkylating agent treatments (92). In addition, HMGB1 can be released by NK cells and can stimulate NK cell chemotaxis through RAGE, thus further amplifying their response to tumors (93) and can also play an important role in the cross-talk between NK and DC, by promoting DC maturation (94, 95). Interestingly, HGMB1 can induce autophagy (96), which may control the regulation of the innate and adaptive immune responses contributing to enhance antigen processing and presentation (97).

Heat shock proteins are localized in most intracellular compartments where they act as molecular chaperone by supporting protein folding and transport across membranes. Several studies demonstrated an unusual HSP70 cell membrane localization on transformed tumor cells (98–100). As already mentioned, stressful conditions can cause HSPs mobilization to the plasma membrane, or their release from cells, thus acting as potent danger signals. In this respect, therapeutic treatments including radio and chemotherapy have been shown to produce an augmentation of HSP70 cell-surface expression on tumor cells (101, 102). Several studies have shown that membrane-bound HSP70 directly promotes NK cell mediated cytotoxicity *in vitro* (103, 104) and *in vivo* (105) thus, there is an increasing interest in the therapeutic potential of targeting HSP70. Interestingly, Elsner and colleagues have shown a synergistic potentiating effect of two stress-inducible immunological danger signals HSP70 and NKG2D ligands on cytotoxicity of human (106) and mouse NK cells (107), suggesting that the drug-mediated upregulation of activating ligands and HSP70 on the cancer cell surface might be an encouraging strategy aimed at promoting the antitumor NK cell responses. Moreover, several pieces of evidence demonstrate that extracellular-located HSPs can be associated to extracellular vesicles (108–112), and a number of chemotherapeutic agents, including etoposide (109), melphalan (110), cisplatin, and 5-fluorouracil (112), have been shown to stimulate an enhanced secretion of exosomes from different types of cancer cells. Notably, colon carcinoma-derived HSP70 associated to exosomes can stimulate NK cell migration and cytotoxic activity (108). In addition, we have recently demonstrated that HSP70 on the surface of MM-derived exosomes triggers NK cell-mediated IFN-γ production through a mechanism dependent on TLR2 (110).

DIRECT EFFECTS OF CHEMOTHERAPY ON NK CELL-MEDIATED FUNCTIONS

Alterations of NK cell activities upon administration of chemotherapeutic drugs can be different in terms of cytotoxicity and immunoregulatory activity; indeed, standard chemotherapeutic protocols used in the treatment of cancer patients mainly suppress NK cell-mediated killing against cancer cells and their cytokine production. However, several studies aimed at analyzing the NK cell behavior in patients undergoing cytotoxic chemotherapy have demonstrated different and variable effects depending on both the type and the dose of the drug used.

In this regard, by producing IFN-γ, NK cells induce $CD8^+$ T cells to become CTLs, and also help to differentiate $CD4^+$ T cells toward a Th1 response. Moreover, NK cell-derived cytokines might also regulate antitumor antibody production by B cells. Thus, therapeutic strategies able to preserve NK functions in cancer patients are of pivotal importance, particularly those eligible for monoclonal antibody-based treatments. In this context, metronomic low cyclophosphamide (CTX) regimen was shown to potently stimulate NK functions in terms of cytokine production and antitumor immunity (18). A number of drugs, including bortezomib, genotoxic agents, and epigenetic drugs, exert immunosuppressive effects at high concentrations, whereas at sub-lethal doses, they can render tumor cells more immunogenic without affecting the immune cell activity (113). As an example, low doses of bortezomib capable of stimulating NK cell activating ligand expression on MM (22, 52), do not alter NK cell degranulation against sensitive targets (52). In another study, low concentrations of bortezomib reduced IFN-γ production without affecting NK cell cytotoxicity (114). Moreover, a combination of bortezomib with exogenous cytokine treatment enhanced the cytotoxic effects of NK cells against cancer cells in

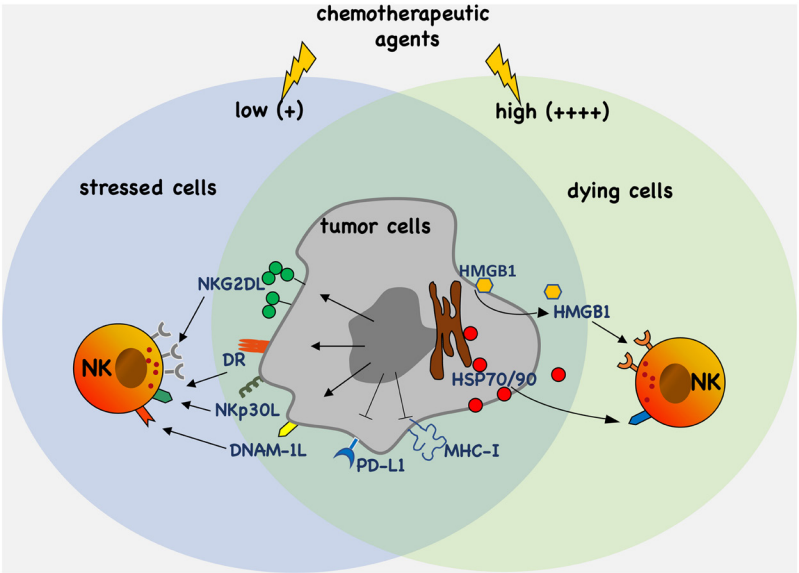

FIGURE 1 | Antitumor efficacy of chemotherapy. Chemotherapeutic agents activate molecular pathways eliciting upregulation and/or the release of stress molecules that promote tumor cell recognition and elimination by natural killer (NK) cells. Moreover, chemotherapy can also downregulate the expression of ligands such as PD-L1 and major histocompatibility complex (MHC)-I of inhibitory receptors.

two different models (115, 116). The treatment of NK cells with sub-lethal doses of doxorubicin, able to upregulate NKG2D and DNAM-1 ligands on MM cells, does not change the capacity of NK cell to degranulate in response to target cells, as well as the ability to produce IFN-γ (34). Although the wide range of HDACi, structurally different from each other, can have both stimulatory and inhibitory effects on immune cell function, the most of them (i.e., romidepsin, vorinostat, TSA, and VPA) have been shown to suppress NK cell activity at therapeutically relevant concentrations (117–119). However, some reports describe a beneficial effect on NK cells as for the narrow-spectrum HDACi entinostat that can increase NKG2D expression on NK cells without affecting their cytotoxic activity (120). Furthermore, a recent study demonstrates that the HDACi panobinostat has the capability to potentiate the antitumor effects of trastuzumab by stimulating the antibody-dependent cell-mediated cytotoxicity (ADCC) mediated by NK cells (121). Regarding the DNTMi decitabine and 5-azacytidine, treatment of NK cells leads to increased reactivity toward different tumor cells (122, 123), while another study describes that 5-azacytidine exposure compromises their activity in AML and MDS patients (124).

Immunomodulatory drugs (lenalidomide, pomalidomide, and thalidomide) exert strong immunomodulatory effects involving both innate and adaptive immunity. In particular, these compounds activate both NK and T cells by inducing their proliferation, cytokine production, and cytotoxic activity (125) and promising clinical trials have been reported their use for the treatment of hematological malignancies, such as myeloma, lymphoma, and leukemia, as well as of solid tumors (126–128). Interestingly, Lagrue and colleagues demonstrated that lenalidomide enhances NK cell response (IFN-γ production and cytotoxicity) by augmenting actin remodeling, thus rendering them able to respond to lower densities of activating ligands on tumor cells (126). Furthermore, lenalidomide has synergistic effects on NK cell functions when used in combination with monoclonal antibodies able to promote ADCC that are already approved in therapeutic protocols, such as rituximab or elotuzumab (129, 130); indeed, novel strategies in the treatment of MM combines the use of lenalidomide and the anti-inhibitory KIR antibody (IPH2101) (131, 132).

CONCLUSION

The modulation of the expression and/or the release of stress molecules has emerged as a new paradigm of the therapeutic possibilities associated with the use of chemotherapy (**Figure 1**). In this context, the characterization of novel drugs and regulatory pathways activated by cellular stress modifiers able to affect tumor growth and, at the same time, to improve the activities mediated by cytotoxic lymphocytes such as NK cells, will importantly contribute to the developing field of chemo-immunotherapy.

AUTHOR CONTRIBUTIONS

AZ, CF, CB, MC, ASantoni, and ASoriani contributed equally to writing and critically revised the paper.

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REVIEW
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S100 Proteins As an Important Regulator of Macrophage Inflammation

Chang Xia[1,2], Zachary Braunstein[3], Amelia C. Toomey[4], Jixin Zhong[2] and Xiaoquan Rao[2]**

[1] College of Health Science and Nursing, Wuhan Polytechnic University, Wuhan, China, [2] Cardiovascular Research Institute, Case Western Reserve University, Cleveland, OH, United States, [3] Boonshoft School of Medicine, Wright State University, Dayton, OH, United States, [4] Department of Health Sciences, University of Missouri, Columbia, MO, United States

The S100 proteins, a family of calcium-binding cytosolic proteins, have a broad range of intracellular and extracellular functions through regulating calcium balance, cell apoptosis, migration, proliferation, differentiation, energy metabolism, and inflammation. The intracellular functions of S100 proteins involve interaction with intracellular receptors, membrane protein recruitment/transportation, transcriptional regulation and integrating with enzymes or nucleic acids, and DNA repair. The S100 proteins could also be released from the cytoplasm, induced by tissue/cell damage and cellular stress. The extracellular S100 proteins, serving as a danger signal, are crucial in regulating immune homeostasis, post-traumatic injury, and inflammation. Extracellular S100 proteins are also considered biomarkers for some specific diseases. In this review, we will discuss the multi-functional roles of S100 proteins, especially their potential roles associated with cell migration, differentiation, tissue repair, and inflammation.

Keywords: S100 proteins, inflammation, tissue repair, biomarkers, inflammatory disease, macrophages

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****Correspondence:***
Jixin Zhong
jixin.zhong@case.edu;
Xiaoquan Rao
jannet18@163.com

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INTRODUCTION

The S100 proteins, belonging to a calcium-binding cytosolic protein family, are composed of 25 known members (1–4). They have a broad range of intracellular and extracellular functions encompassing regulation cell apoptosis, proliferation, differentiation, migration, energy metabolism, calcium balance, protein phosphorylation, and inflammation (5–8).

Based on their functional roles, s100 proteins are categorized into three main subgroups: S100 proteins that only exert intracellular functions, S100 proteins that have both intracellular and extracellular roles, and S100 proteins that mainly possess extracellular effects (7). The S100 proteins within the first subgroup only exert intracellular functions. For example, S100A1 is predominantly expressed in striated muscle (especially cardiac muscle) (9) and only exert intracellular regulatory effects such as regulating SR Ca^{2+} recycle and enhancing the gain of the calcium-induced calcium release (CICR) cascade (10–12). In addition to intracellular roles, some S100 proteins are released into the extracellular environment and may exert extracellular functions. S100B in this subgroup was known to directly interact with nuclear Dbf2-related protein kinase (NDR kinase) and block the recruitment of its substrates to NDR kinase (13). Furthermore, extracellular S100B could also activate extracellular signal-regulated protein kinase (ERK) and NFκB in chondrocytes by binding to its cell surface receptor, receptor for advanced glycation end products (RAGE) (14). The third subgroup of S100 proteins such as S100A15 mainly exerts extracellular regulatory functions. These

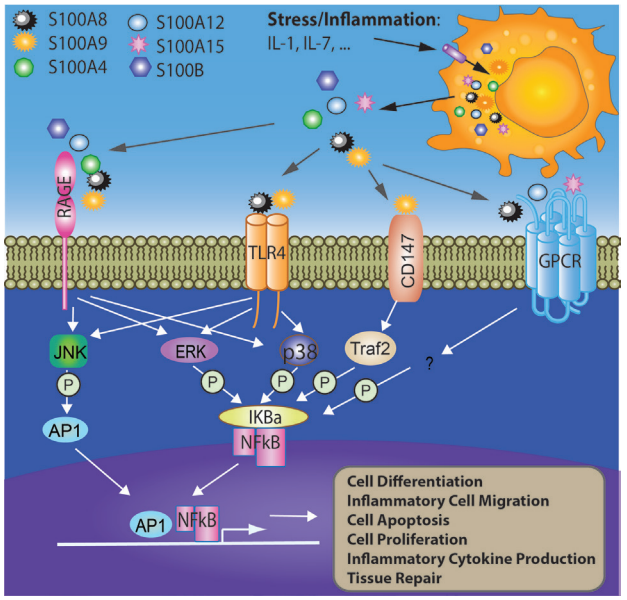

FIGURE 1 | Involvement of S100 proteins in stress and inflammation-mediated responses. Cell stress or inflammation induce the release of S100 proteins to acellular compartment where they bind cell surface receptors such as RAGE, TLR4, CD147, and GPCR. The interactions between S100 proteins and their receptors activate intracellular signaling pathways such as AP1 and NFκB, which further initiates multiple cellular processes such as cell differentiation, migration, apoptosis, proliferation, and inflammation. AP1, activator protein 1; ERK, extracellular signal-regulated protein kinase; GPCR, G-protein-coupled receptor; IL-1, interleukin 1; IL-7, interleukin 7; IκBα, nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor alpha; JNK, c-Jun N-terminal kinase; P38, p38 mitogen-activated protein kinase; RAGE, receptor for advanced glycation end products; TLR4, toll-like receptor 4; Traf2, TNF receptor-associated factor 2.

of S100 proteins are considered as potential therapeutic targets for various human disorders, including arthritis, cancer, and Alzheimer's disease (15, 16).

S100 proteins are involved in multiple intracellular functions which include: interacting with intracellular receptors or molecule subunits (17), membrane protein recruitment and transportation, transcriptional regulation (18, 19), regulating enzymes, nucleic acids, and DNA repair (20, 21) (**Figure 1**). There are two critical steps for S100 protein activation: Ca^{2+} binding (22) and homo- or hetero-dimer formation (23). Each S100 protein forming the dimer participates in ion (Ca^{2+}, Zn^{2+}, or Cu^{2+}) binding. Ca^{2+} also contributes to the formation of S100 protein oligomers, especially calprotectin (S100A8/A9 tetramer) (22, 24, 25).

When released to the extracellular space, S100 proteins have crucial activities in the regulation of immune homeostasis, post-traumatic injury, and inflammation. S100 proteins trigger inflammation through interacting with receptors RAGE and TLR4 (26). Increasing evidence has demonstrated that calprotectin (S100A8/A9) is an endogenous agonist of TLR4 (26). Binding to TLR4 initiates a signaling cascade and regulates inflammation, cell proliferation, differentiation, and tumor development in an NF-κB-dependent manner (8, 26–28). Apart from TLR4, RAGE has also been suggested to bind S100 proteins such as S100A7, S100A12, S100A8/A9, and S100B (27, 29–31). By interacting with RAGE, S100 proteins activate NF-κB, inducing the production of pro-inflammatory cytokines leading to the migration of neutrophils, monocytes, and macrophages (30, 31). In addition to the NF-κB pathway, MAP kinase-mediated signaling is also induced by S100 proteins such as S100P (32, 33). Interestingly, S100A6 activates RAGE and promotes apoptosis, while S100B inactivates RAGE by interacting with the basic fibroblast growth factor and its receptor (14, 34). Extracellular S100 proteins may regulate the apoptosis, proliferation, differentiation, and migration of a number of cell types including monocytes, macrophages, neutrophils, lymphocytes, myoblast, epithelial cells, endothelial cells, smooth muscle cells, neurons, and fibroblasts. In this review, we aim to summarize the immune regulatory role of S100 proteins and their potential involvement in inflammatory regulation, tissue repair, and tumorigenesis.

S100 GENES AND MOLECULAR STRUCTURE

Each S100 family protein is encoded by a separate gene. Most S100 genes are located within the chromosome 1q21 with a few exceptions. For example, S100A11P is located within chromosome 7q22-q3, S100B in located within chromosome 21q22, S100P is located in chromosome 4p16, S100G is located in chromosome Xp22, and S100Z is located with chromosome 5q13 (5). The sequence homology among S100 proteins varies from 22 to 57%, which is mainly due to the variance at the hinge region and C-terminus, the regions associated with their function (35).

S100 proteins are small proteins with a molecular weight of 10–12 kDa. Each S100 protein consists of two EF-hand helix–loop–helix structural motifs, which are arranged in a back-to-back manner and linked with a flexible hinge (23). The activity of the proteins is regulated by metal ions (such as calcium, zinc, and copper), which modulates the folding and oligomerization of the protein (36, 37).

EXPRESSION PATTERN AND REGULATION

Epigenetic mechanisms play a key role in the regulation of S100 protein expression. S100A3, S100A10, S10011, and S100P could be detected in various medulloblastoma cell lines treated with DNA de-methylation (38). It is reported that DNA hypomethylation could induce S100A6 overexpression in gastric cancer. Lower levels of CpG methylation in the first intron and second exon regions of the S100A6 gene, accompanied by higher levels of acetylated histone H3 binding to the promoter, have been reported in the gastric cancer tissues (39). Lower methylation in the proximal promoter region of the S100P gene was also found in prostate cancer cell lines (40). The expression of S100 proteins may also be regulated by micro RNAs, although further studies are needed to provide direct evidence. NFAT5, a transcription factor that initiates S100A4 expression (41), is regulated by miR-568 (42).

The expression of S100 proteins is strictly regulated to maintain immune homeostasis (7, 43). S100A8 and S100A9 are predominately expressed in monocytes, neutrophils, and dendritic cells (44, 45). However, they are also expressed in various other types of cells upon activation, such as fibroblasts (46), mature macrophages (47), vascular endothelial cells (48–50), and keratinocytes (51). In neutrophils, 45% of the cytosolic proteins are constituted with S100A8 and S100A9, whereas the proportion is only 1% in monocytes (52). The expression levels in different monocyte subsets also vary. The level of S100A8 mRNA is higher in classical $CD14^+/CD16^-$ human monocytes when compared to non-classical $CD14^+CD16^+$ monocytes (47).

Increasing evidence indicates that the expression of most S100 proteins is different between physiological and pathological conditions. The expression of S100A8 and S100A9 could be upregulated by a number of conditions such as oxidative stress, specific cytokines, and growth factors in many types of cells (53). S100A12 is mainly expressed in neutrophils, monocytes, and early macrophages (53, 54), but it can also be detected in endothelial cells, keratinocytes, epithelial cells, and pro-inflammatory macrophages under inflammatory conditions (51, 55–58). In human epidermal keratinocytes, interleukin (IL)-1α induces a significant increase of S100A9 expression by the p38 MAPK pathway (59). The expression of S100A5 is upregulated in bladder cancers (60). Pro-inflammatory cytokines could increase S100A7 expression in human breast cancer (61). IL-17, IL-22, and bacterial products (e.g., flagellin) can enhance S100A7 expression in keratinocytes (62). IL-6 and IL-8 released from myofibroblasts could also trigger the upregulation of S100A8/A9 in tumor-infiltrated myeloid cells (63). S100A9 was significantly higher in the peripheral blood in patients with implant-associated osteomyelitis. S100A9 expressing cells were also increased in tissue biopsies from patients with implant infections, compared with the non-infected individuals (64).

S100 PROTEINS FUNCTION AS DAMAGE-ASSOCIATED MOLECULAR PATTERN (DAMP) MOLECULES

In addition to serving as calcium-binding proteins, S100 proteins were later discovered as DAMP molecules (26, 65, 66). DAMPs were considered as a series of intracellular molecules linked with cell death and tissue damage through inducing a rapid inflammatory response or production biologically active molecules (67, 68). DAMPs are biomolecules that are released from damaged or stressed cells and could act as endogenous danger signal to activate inflammatory response (69). S100 proteins could be released from the cells after cell damage/stress or activation of phagocytes such as neutrophils and macrophages. The extracellular S100 proteins then become danger signals and activate immune cells and endothelial cells by binding to the pattern recognition receptors such as TLRs and RAGE.

They play an important role in modulating inflammatory responses (70). Once released from the cell, calprotectins function as an endogenous agonist to bind TLR4 (S100A8/A9 and S100A12) (26) and RAGE (S100A8/A9 and S100A7) [(6, 31, 71) #3535]. In the site of inflammation, calprotectin acts as a chemotactic factor by inducing neutrophils adhesion (72). Furthermore, S100A8/A9 induces apoptosis and autophagy in various cell types such as lymphocytes, macrophages, endothelial cells, and tumor cells (73). It has been shown that reactive oxygen species (ROS) is the critical factor in S100A8/A9-induced cell death and involves BNIP3. The increase of ROS production in mitochondria subsequently causes mitochondrial damage and lysosomal activation (73).

S100 PROTEINS IN MACROPHAGES MIGRATION, INVASION, AND DIFFERENTIATION

It is widely accepted that macrophages contribute to immune defense, immune regulation, and tissue repair. Based on their cytokine production and activation conditions, macrophages are categorized into two populations: pro-inflammatory M1 (classically activated macrophage) and anti-inflammatory M2 (alternatively activated macrophage). Calprotectin could induce pro-inflammatory cytokine production in monocytes and macrophages through NF-κB and p38 MAPK pathways (74). An increasing number of findings demonstrate that S100 proteins contribute to the adhesion and migration of leukocytes. For example, the release of S100A8/A9 has been suggested to facilitate monocyte and neutrophil transmigration (75, 76). The S100A8/A9 heterodimer enhances the expression of β2 integrin CD11b and the ability of adhesion in phagocytes (72, 77). Moreover, the response of $S100A9^{-/-}$ monocytes to chemotaxis was reduced when compared with wild-type cells. For example, IL 8-induced CD11b upregulation was abolished in $S100A9^{-/-}$ monocytes and neutrophils (78). S100A4 has also been shown to interact with cytoskeletal proteins to promote cell migration and deletion of *s100a4*, which leads to the deficiency of macrophage migration and chemotactic reactions (79–81). S100A12 induced the production of pro-inflammatory cytokine IL-6 and IL-8 in both a dose-dependent and time-dependent manner. This was critical to regulate the recruitment of monocytes and TNF-α release (82).

The intimate relationship between macrophages and cancer cells plays a crucial role in tumor growth and metastasis. Tumor associated macrophages influence tumor growth by modulating local inflammation, inhibiting antitumor immunity, and stimulating angiogenesis (83–85). It is commonly accepted that macrophages contribute to tumor growth and invasion. They are recruited to the site of tumors *via* chemoattractants such as CCL3-8, vascular endothelial growth factor (VEGF), and macrophage inflammatory protein-1 alpha (MIP-1α) (86). The monocytes or macrophages tend to differentiate into the M2 macrophage phenotype rather than the tumoricidal M1 phenotype, producing pro-tumor cytokines, such as macrophage colony-stimulating factor, IL-10, IL-4, and IL-13 (83, 87, 88). S100A10 was shown to mediate the migration of macrophages to the tumor site. Tumor growth was reduced in S100A10-null mice, compared with wild-type mice, and was accompanied by less macrophages within the tumor. There were many macrophages throughout the tumor in wild-type mice, where macrophages were observed

only around the absolute tumor tissue border in S100A10-null mice (89). Intraperitoneal injection of wild-type macrophages restored macrophage density within the tumor, but injection of S100A10-deficient macrophages did not. Interestingly, intratumoral injection of macrophages of either genotype could rescue tumor growth, suggesting that S100A10$^{-/-}$ macrophages still have the ability to stimulate tumor growth but lack the ability to invade into the tumor (89). Another study showed that S100A10 deficiency decreased plasmin generation and matrix metalloproteinase 9 activation in macrophages, both of which are associated with macrophage invasion and migration (90).

Downregulation of S100A8 and S100A9 is associated with the differentiation of myeloid cells toward dendritic cells and macrophages (91, 92). S100A8 and S100A9 are co-expressed in fetal myeloid progenitors, with its expression level associated with the development of the myeloid lineage (93). They are highly expressed in monocytes and neutrophils. However, the expressions of S100A8 and S100A9 are lost when monocytes terminally differentiate into tissue macrophages (93). Recent data have shown that S100A8 can be induced by oxidative stress in macrophages in an IL-10-dependent manner (51). Interestingly, S100A8/A9 has also been shown to control the cell cycle (94). S100A9 inhibited myeloid cells differentiation through generation of ROS (92). S100A9 is able to induce the differentiation of monocytes toward the osteoclast type in *in vitro* culture experiments and S100A9 derived from neutrophils and S100A9-induced osteoclast generation were considered as important reasons for bone degradation in infectious osteomyelitis (95). S100A8 and S100A9 have also been shown to mediate the arresting effect of TNF-α on the differentiation of immature myeloid-derived suppressor cells into dendritic cells and macrophages in a RAGE-dependent manner (96). Consistent with this finding, IL-6 and IL-8 released from myofibroblasts in tumor microenvironment upregulate S100A8/S100A9 in myeloid cells and induce the differentiation of myeloid cells into S100A8/S100A9-expressing myeloid-derived suppressor cells and M2 macrophages (63).

ROLE OF S100 PROTEINS IN TISSUE REPAIR

Damage-associated molecular pattern molecules play a critical role in tissue repair. S100A7, S100A8/A9, S100A12, and S100A15, well-documented DAMPs, have been shown to participate inflammatory tissue damage and tissue repair. The link between S100A12 and the severity of coronary and carotid atherosclerosis has been evidenced by multiple human studies (97–99). S100A7 is highly expressed in the skin, and the expression is increased in inflamed skin, which has been shown to be induced by pro-inflammatory cytokines (IL-17 and IL-22) and bacterial products such as flagellin (62), that the increase of S100A7 has been associated with multiple inflammatory skin diseases, such as psoriasis and atopic dermatitis (62, 100). Similarly, the expression of S100A15 was amplified in the epidermis of psoriatic lesions and acted as chemoattractants for immune cells (101). S100A8/A9 exerts anti-inflammatory function in healthy state, while oxidative stress-associated pathological conditions activate their pro-inflammatory functions (102). Increased plasma S100A8/A9 levels have been associated with atherogenesis, plaque vulnerability, myocardial infarction (MI), cardiovascular death, and heart failure. In a mouse model of angiotensin-induced cardiac damage, it was shown that S100A8/A9 released by granulocytes upregulated pro-inflammatory gene expression and induced the release of cytokines and chemokines in a RAGE-dependent manner. This process promoted myocardial tissue inflammation and fibrotic scar formation (103, 104). In a mouse model of collagenase-induced arthritis, the expression of S100A8 and S100A9 in synovial was upregulated in wild-type mice. In addition, *S100a9*$^{-/-}$ mice were protected from collagenase-induced synovitis, cartilage degradation, and osteophyte formation (105, 106). S100A9 antibodies could block the accumulation of fibroblasts and decrease fibrosis in local inflammatory microenvironment (104). In contrast, S100A1 or S100A4, released following MI, has a beneficial effect following heart injury by promoting muscle tissue repair and maintaining contractility (107, 108).

Binding of S100B to RAGE and the subsequent increase of angiogenic factor VEGF have been shown to be essential in the development of macular degeneration (109). In addition, S100B activates the Ras-MEK-ERK1/2-NF-κB pathway in neural cells and leads to the activation of small GTPases, Rac1/Cdc 42, and neurite growth (110). In vascular smooth muscle cells, S100B induces the upregulation of ROS and recruits JAK2 and STAT3, which results in the proliferation of vascular smooth muscle cells (111). Similarly, S100B also increased cellular proliferation though activating the Phosphatidylinositol-4,5-bisphosphate 3-kinase-AKT pathway in a RAGE-dependent manner (14). On the other hand, S100B could induce apoptosis by increasing production of ROS and the release of cytochrome-c from mitochondria (110). High levels of S100B are released from injured cardiomyocytes following MI and could promote cell apoptosis through RAGE. Also, S100B released from injured skeletal muscle tissue could stimulate myoblast proliferation but inhibit myoblast differentiation by activating bFGF/FGFR1 signaling (112, 113). However, the regeneration effects of S100B on the injured myoblasts are strongly dependent on cell density, because it triggers RAGE, but not bFGF/FGFR1 signaling, at an early stage of low-density myoblast differentiation (114).

THE ROLE OF S100 PROTEINS IN INFLAMMATORY DISEASES

S100 proteins, particularly calgranulins, play a significant role in mediating innate and acquired immune responses, which contribute to the development of chronic inflammatory diseases.

Calgranulins are associated with joint inflammation in patients with rheumatoid arthritis (RA) (115). The level of S100A8/A9 in the serum and synovial fluid was significantly increased in RA (116, 117). Recent findings showed that S100A8/A9 was upregulated in early but not late phase osteoarthritis (OA) (118). S100A8/A9 plasma levels were increased at baseline in human OA participants. Meanwhile, osteophyte size was drastically reduced in S100A9$^{-/-}$ mice-induced OA (106). It has also been confirmed that S100A8/A9 contributes to cartilage degradation

and development of inflammatory arthritis in an antigen-induced arthritis model (119). Similar to S100A8/A9, human S100A7 and S100A15 were first confirmed as over-expressed in psoriatic plaques (120). Increasing evidence supports an association of S100A7 with several inflammatory skin diseases, including psoriasis and atopic dermatitis (62, 100). Evidence strongly indicates that S100A8/A9 levels are higher in hypercalprotectinemia, an extremely rare inflammatory disorder (121–123). Although the mechanism is still unclear, it is possible that the releasing of extracellular S100A8/A9 is dysregulated, which accounts for the abnormal increase of calprotectin and subsequent hyperactive inflammatory reaction. It is suggested that S100 proteins are involved in interacting with both the immune system and the pathogen. S100A12 plays a key role in fighting infections. For example, it has been shown that S100A12 plays a critical role in anti-parasite responses (124). In addition to directly killing *Mycobacterium tuberculosis* and *Mycobacterium leprae*, S10012 is also required for TLR2/1L- and IFN-γ-induced antimicrobial activity against *Mycobacterium* (125). Haley et al. also showed that S100A12 can help to repress the biogenesis and activity of *H. pylori* cag type IV secretion system by binding nutrient zinc, which results in suppressed bacterial growth and viability (126).

S100A8, S100A9, and S100A12 are abundantly expressed by neutrophils. Evidence indicates that these three members of S100 proteins are released by neutrophils, inducing MUC5AC production in airway epithelial cells through activating TLR4 and RAGE signaling pathway. This reveals the relationship between chronic neutrophilic inflammation and obstructive airway diseases such as severe asthma, COPD, and cystic fibrosis (127). In correlation with their role in the development of chronic inflammation, S100A8/A9 also participates in the hyperglycemia-induced increase of myelopoiesis occurring in a RAGE-dependent manner in diabetic mice (128). Interestingly, the amount of circulating monocytes and neutrophils were decreased when antidiabetic treatment normalized the glycemic index of $Ldlr^{-/-}$ atherosclerotic mice, which might explain the increased severity of atherosclerosis found in patients with diabetes (128). In accordance with these findings, increased serum concentrations of S100A8/A9 were detected in obese individuals (129). Furthermore, the expression of the macrophage marker CD68 was increased in the visceral adipose tissue (130). Some research of dipeptidyl peptidase-4 inhibitors for the treatment of type 2 diabetes mellitus indicates that vildagliptin could increase the mRNA expression levels of S100A9 and TNF-α in human hepatocytes. In addition, it may induce the release of S100A8/A9 complex from HL-60 cells *via* TNF-α-independent manner, which might be a contributing factor of vildagliptin-associated liver dysfunction (131).

S100 PROTEINS AS BIOMARKERS IN SPECIFIC DISEASES

Extracellular S100 proteins are involved in the activation of G protein-coupled receptors, heparan sulfate proteoglycans or N-Glycans, and scavenger receptors in autocrine and paracrine manners (132, 133). Since S100A proteins can be detected in body fluids, such as urine, cerebrospinal fluid, serum, sputum, and feces, extracellular S100 proteins are considered as biomarkers associated with certain diseases (134–137).

It has been suggested that S100A12, S100A8/A9, and S100B are linked to specific diseases and conditions such as autoinflammatory diseases, stroke, and trauma (138). The level of S100A12 in the blood is increased in the patients with diabetes, which is correlated with a higher risk of cardiovascular disease development (139). Bogdanova et al. detected the serum concentration of S100A12 and other acute-phase inflammatory markers in thirty-five patients with periodic disease (PD) (140). The level of S100A12 in PD was significantly higher compared to other familial periodic fevers. S100A12 was more sensitive to assess the subclinical activity of autoinflammatory diseases, when compared to other inflammatory biomarkers such as neutrophil counts, fibrinogen, C-reactive protein (CRP), and erythrocyte sedimentation rate (140). Similarly, the serum concentrations of S100A12, as a novel biomarker, were shown to be upregulated in patients with Familial Mediterranean fever in comparison to controls (141).

The plasma concentrations of S100A9 were significantly higher in patients with implant-associated infectious osteomyelitis when compared to patients with sterile inflammation or healthy individuals. In addition, S100A9 was associated with osteoclast generation and bone degradation. Therefore, it could serve as a novel diagnostic marker to aid in the differential diagnosis (95). Similarly, serum levels of S100A8 and S100A9 were dramatically increased in $IL\text{-}1Ra^{-/-}$ mice and contributed to bone erosion, cartilage damage, and synovial inflammation. Thus, they can be considered as a systemic or local biomarker to evaluate the extent of inflammation and inflammatory joint destruction in seronegative arthritis (142). It was shown that the expression of S100A8/A9 was high in human atherosclerotic lesions and the blood levels were also increased in the patients with coronary artery diseases (CAD), which implied S100A8/A9 might act as a biomarker for cardiovascular events (143). Recent research has shown similar findings that serum S100A8/A9 levels were elevated in 178 CAD patients with unstable angina pectoris or acute myocardial infarction, and the level of S100A8/A9 was significantly positively linked with CRP ($P < 0.01$) (144). These clinical data suggest that S100A8/A9 may become a novel biomarker for CAD (139).

In addition, more studies explored the value S100A8/A9 as a predictive biomarker for autoimmune diseases. In RA, S100A8/A9 was suggested as a potential biomarker in predicting clinical response to monitor treatment (145, 146). Some clinical investigations have indicated that S100A8/A9 levels might be a more sensitive predictor for monitoring synovial inflammation in RA patients when compared with other markers such as CRP levels (147).

The study by Shakeri et al. suggested that S100 B protein could be used as a posttraumatic biomarker for predicting brain death in severely injured patients with exclusive head trauma during the first 6 h after trauma, but found no relationship between S100 B levels and death (148). Pelinka et al. confirmed that *in vitro* S100 B concentrations increased significantly in rats with femoral fractures but not head injury (149). Interestingly, adverse results indicated that there was no difference in S100 B concentrations

between patients with and without head injury (150). S100B has also been considered as a prognostic marker of the acute phase of neurologic damage (151), predicting the outcome of traumatic brain injury and large volume cerebral infarction (152, 153). The level of serum S100B in ischemic stroke implied a worse outcome secondary to the stroke (154, 155). This research demonstrates that S100 B is correlated to trauma and a worse long-term outcome. S100B has recently been confirmed to be associated with some genetic disorders and was found to be over-expressed in patients with Down syndrome (156, 157). There was also study showing that S100B may be one of the best biomarkers of melanoma (158).

S100 PROTEINS AS THERAPEUTIC TARGETS IN DISEASE

Although direct clinical evidence is limited, increasing studies indicate that S100 proteins may also serve as a therapeutic target for certain disease conditions. As mentioned above, S100 proteins are involved in a number of diseases including inflammatory disease. It has been reported that multiple anti-allergic drugs such as amlexanox, cromolyn, and tranilast are able to bind S100A12 and S100A13, and block downstream RAGE signaling (159). Therefore, these drugs may serve as a therapeutic approach to target S100 proteins. Multiple S100 proteins such as S100A4 (160) and S100B (161) have been shown to participate in the neoplastic disorders by binding to P53 and suppressing its phosphorylation (162). Therefore, efforts are being made to restore P53 function by targeting S100 proteins (163). In an *in vitro* study, Most et al. demonstrated that extracellular S100A1 is endocytosed by the neonatal ventricular cardiomyocytes protects cardiomyocytes from 2-deoxyglucose and oxidative stress-induced apoptosis *via* activation of ERK (164). Adeno-associated virus-mediated S100A1 gene transfer in failing cardiomyocytes was also shown to be able to restore the contractile function, suggesting a potential implication of AAV-mediated S100A1 gene therapy in heart failure (165, 166). Despite the promising potentials, the feasibility and safety of these approaches and issues such as how to control and keep expression levels in the therapeutic window need to be further investigated (166).

CONCLUSION

Evidence strongly supports that S100 proteins, as a remarkable multifunctional proteins family, are involved in the regulation of several important biological processes such as the inflammatory response, protecting the intra- and extracellular environments during infection, cell proliferation and differentiation, tumor growth and metastasis, cell apoptosis, energy, and glutathione metabolism.

However, the activities of all members S100 proteins depend on the cell-specific expression patterns and binding targets even the local microenvironment. Extracellular effects of S100 proteins interact with receptors including TLR-4, RAGE, and heparan sulfate proteoglycans during infection and inflammation which associated with the pathogenesis of inflammatory such as autoimmune disease, infectious diseases, allergy, tumorigenesis and metastasis, and anti-microbial disease. Extracellular S100 proteins can also contribute to the regulation of tissue development and regeneration or repair, which is essential for elucidating their role in the pathological procession of tissue damage, cell apoptosis, or tissue repair.

Although growing evidence has begun to show the regulation of S100 proteins in detail which improves our understanding of how immune homeostasis is maintained during the development of S100 protein-associated disease, there are certain gaps in our understanding of the role of S100 proteins in pathophysiology. Among 25 known members of S100 family, only limited number of S100 proteins such as S100A8 and S100A9 have been well documented and the functional roles of other members are underappreciated. In addition, further studies are required to fully reveal the underlying mechanisms by which S100 proteins participate in a variety of disease conditions. For instance, a role of S100P has been reported in leukemia (167), while the exact function of S100P in leukemia and the signal pathways involved in this process are not completely understood. Also, the direct clinical evidence of the therapeutic potential of S100 proteins is limited at current stage. Therefore, future directions in this area could focus on the development of therapeutic approaches targeting S100 proteins, verification of the therapeutic potential of S100 proteins in both preclinical and clinical settings, and elucidation of the underlying mechanisms.

AUTHOR CONTRIBUTIONS

CX and JZ reviewed the literature and wrote the first draft. ZB, AT, JZ, and XR reviewed the literature and finalized the manuscript.

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Sensing Bacterial-Induced DNA Damaging Effects *via* Natural Killer Group 2 Member D Immune Receptor: From Dysbiosis to Autoimmunity and Carcinogenesis

J. Luis Espinoza[1]* *and Mika Minami*[2]

[1] Department of Hematology and Rheumatology, Faculty of Medicine, Kindai University, Osakasayama, Japan, [2] Faculty of Medicine, Kindai University, Higashi-osaka, Japan

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***Correspondence:**
J. Luis Espinoza
luis@med.kindai.ac.jp

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The human genome is constantly exposed to exogenous and endogenous DNA damaging factors that frequently cause DNA damages. Unless repaired, damaged DNA can result in deleterious mutations capable of causing malignant transformation. Accordingly, cells have developed an advanced and effective surveillance system, the DNA damage response (DDR) pathway, which maintains genetic integrity. In addition to well-defined outcomes, such as cell cycle arrest, apoptosis, and senescence, another consequence of DDR activation is the induction of natural killer group 2 member D ligands (NKG2D-Ls) on the surface of stressed cells. Consequently, NKG2D-Ls-expressing cells are recognized and eliminated by NKG2D receptor-expressing immune cells, including NK cells, and various subsets of T-cells. Recent pieces of evidence indicate that commensal microbial imbalance (known as dysbiosis) can trigger DDR activation in host cells, which may result in sustained inflammatory responses. Therefore, dysbiosis can be seen as an important source of DNA damage agents that may be partially responsible for the overexpression of NKG2D-Ls on intestinal epithelial cells that is frequently observed in patients with inflammatory bowel disease and other disorders associated with altered human microbiota, including the development of colorectal cancer. In this article, we discuss recent evidence that appears to link an altered human microbiota with autoimmunity and carcinogenesis *via* the activation of DDR signals and the induction of NKG2D-Ls in stressed cells.

Keywords: natural killer group 2 member D ligands, microbiota, dysbiosis, bacterial genotoxin, immunosurveillance, inflammatory bowel disease

INTRODUCTION

The DNA damage response (DDR) is a highly efficient network of cellular pathways that play a crucial role in maintaining DNA integrity (1, 2). This surveillance system is responsible for monitoring, detecting and repairing DNA lesions, in order to prevent the generation of potentially deleterious mutations, which otherwise may result in the irreversible damage of DNA molecules, leading to cancer and other alterations in cell behavior (3, 4). The accumulation of un-repaired DNA damages in non-replicating cells, such as most of the cells in the brains or muscles of adults, is believed

to contribute to the aging process in humans (5, 6). In highly replicating cells, such as hematopoietic stem cells and epithelial cells, DNA mutations that result from unrepaired DNA damages play a crucial role in malignant transformation and cancer progression (5, 7, 8). Endogenous agents capable of harming DNA, such as reactive oxygen species (ROS), lipid peroxidation products, and reactive nitrogen species (RNS) are naturally released during cell metabolic activities or hydrolytic processes (1, 9). In addition, DDR activation can be triggered by thousands of exogenous agents, including ionizing radiation, chemotherapy, virus infections, and chronic inflammation (10–13).

DNA damage response activation is controlled by three protein kinases: ataxia telangiectasia mutated (ATM), DNA-dependent protein kinase (DNA-PK), and ATM- and Rad3-related (ATR) (7, 14). Both ATM and DNA-PK are recruited by DNA double strand breaks (DSB), however, whereas DNA-PK coordinates DSB repair *via* non-homologous coupling, ATM promotes homologous recombination and cell cycle arrest at various checkpoints (14). ATR is activated in response to persistent single-stranded DNA and acts at the S-phase checkpoint (14). Upon DNA damage recognition, these kinases activate various downstream mediators including p53, CHK1, CHK2, BRCA, and H2AX, which (depending on the extent of DNA damage) may lead to cell-cycle arrest, DNA repair, senescence, or apoptosis (14–16). A key mediator of ATM signal is the checkpoint kinase Chk2, which induces G1/S checkpoint *via* Cdk2 inactivation or can block cell cycle at G2/M by preventing cyclinB1/Cdk1 complex formation (17). On the other hand, Chk1, triggered by ATR signal, activates Cdc25A phosphatase and Treslin, which induce G2 and S phase arrest (7).

Another consequence of DDR and ATM/ATR activation is the induction of cell stress molecules that are proteins expressed on the surface of damaged cells (18, 19). These stress ligands, which are usually absent in normal cells, are specifically recognized by either, the natural killer group 2 member D (NKG2D) and the DNAX accessory molecule immunoreceptors (18–20).

Natural killer group 2 member D, also known as Klrk1, is a C-type lectin-like type II transmembrane protein constitutively expressed by NK cells, activated macrophages and various T-cell subsets, such as NKT cells, $CD8^+$ αβ, $CD4^{+\alpha\beta}$, and γδ T lymphocytes (21–23). Upon engagement of specific NKG2D ligands (NKG2D-Ls), NKG2D receptor activates downstream signaling pathways resulting in effector immune responses like cytokine releases and cellular cytotoxicity (22, 24).

Recent evidence has linked various bacterial pathogens with DDR activation caused by either the direct effect of microbe produced genotoxins (25–28) or indirectly by ROS or RNS that result from the prolonged or excessive activation of host immune cells in response to certain microbes or their metabolic end-products (29, 30). This bacterial-induced DDR is not limited to highly pathogenic bacteria, since genotoxic damage induced by certain members of the commensal bacteria community (termed the "microbiota") have been also documented (31, 32). Notably, increased expression of NKG2D-Ls on the surface of intestinal epithelial cells and its recognition by NKG2D receptor-expressing immune cells is believed to contribute to the pathogenesis of inflammatory bowel diseases (IBDs), such as ulcerative colitis and Crohn's disease (33–35) and dysregulated gut microbiota has been etiologically linked to IBD and colorectal cancer (CRC) (27, 36, 37). In this article, we discuss recent pieces of evidence that appear to link alterations in gut microbiota with activation of the DDR. The potential effects that perturbations in this network have on the development of autoimmunity and cancer immunosurveillance are also discussed.

NKG2D-Ls EXPRESSION

In humans, multiple families of structurally unrelated NKG2D-Ls have been identified, including the MHC class I chain-related molecules (MICA and MICB), and the UL-16 binding proteins (ULBP1, -2, -3, -4, -5, and -6) (24, 38–40). NKG2D-Ls are absent or poorly expressed on the surfaces of normal cells but they are induced under certain pathological conditions like heat shock, virus infection, oxidative stress, and malignant transformation (39, 41). The elimination of NKG2D-Ls expressing cells by NKG2D receptor-expressing immune cells is one of the underlying grounds of the concept of cancer immunosurveillance (42–44). NKG2D-Ls upregulation has been described in various human cancers, including carcinomas of the breast (45), lung, colon (46) and prostate cancer (47), as well as in melanomas (48), gliomas (49), leukemias (18), and cervix cancer (50). The expression of these molecules is tightly regulated by mechanisms that control gene transcription, mRNA stability, protein translation, and stabilization (20, 39). Intriguingly, NKG2D-Ls expression has also been documented in certain normal cells. For example, in primary bronchial epithelial cells, MICA and ULBP1-4 are detectable mainly at intracellular level, but become detectable on the cell surface when the cells are exposed to oxidative stress (51). NKG2D-Ls (mainly ULBP1) have also been detected in peripheral blood cells (52) and these proteins are particularly upregulated in activated T cells and B cells (20, 53, 54). In addition, normal gut epithelium constitutively expresses MICA, although most cells appear to express these proteins intracellularly (55). On the other hand, the aberrant expression of NKG2D-Ls has been documented in certain autoimmune diseases, especially in the damaged tissues of patients with inflammatory bowel disease (IBD) that includes Crohn's disease (35, 56) and ulcerative colitis (33). In these disorders, NKG2D-Ls expression correlates with increased number of infiltrating $NKG2D^+$ lymphocytes in the damaged tissues (33, 35). Consistent with these observations, a randomized controlled clinical trial recently showed that a single dose of an anti-NKG2D blocking monoclonal antibody, significantly reduced disease activity in patients with active Crohn's disease (57). Despite the relatively small size of this study (78 patients) and the fact that patients with UC were not included, these encouraging data support the involvement of NKG2D/NKG2D-Ls axis in the pathogenesis or clinical course of IBD.

In patients with active Celiac disease, MICA is strongly expressed on the surface of intestinal epithelial cells and it is further upregulated by wheat gliadin, which triggers the activation of intraepithelial $NKG2D^+$ lymphocytes, leading to epithelial damage and villous atrophy (55). Notably, the probiotics *Lactobacillus fermentum* and *Bifidobacterium lactis* were found to directly

inhibit the toxic effects of gliadin in intestinal cells (58) and a gluten-free diet strongly downregulated NKG2D-Ls in intestinal epithelial cells and concomitantly decreased NKG2D receptor expression on infiltrating NK cells (59).

DYSBIOSIS AND NKG2D-Ls EXPRESSION

The community of commensal microorganisms living within the human intestines, known as gut microbiota, plays critical roles in maintaining immune tolerance and epithelial integrity (60–62).

Significant upregulation of NKG2D-Ls was observed in the intestinal mucosa of germ-free mice lacking commensal microbiota, as well as in commensal-depleted animals (ampicillin-treated mice), and low ligands expression level was restored when ampicillin treatment was stopped. Strikingly, the same study found low levels of NKG2D-Ls in animals treated with vancomycin, which was attributed to the selective propagation of the vancomycin-resistant bacterium *Akkermansia muciniphila* in mice intestines (31), indicating that NKG2D-Ls expression, at least in intestinal tissues, is largely influenced by the gut microbiota composition. Interestingly, *A. muciniphila* has been linked with anti-inflammatory protective properties against IBD (63).

The loss of microbial balance and the overgrowth of pathogenic bacteria (known as dysbiosis) is often associated with the development of autoimmune disorders and the development of CRC (62, 64, 65). Strikingly, direct microbe-induced NKG2D-Ls upregulation has been documented in human intestinal epithelial cells exposed to *Escherichia coli* strains, where the interaction between bacterial adhesin AfaE and its cellular receptor CD55 results in MICA expression (66).

Another study showed that *Pseudomonas aeruginosa* infection increased NKG2D-Ls (Rae1) in mouse airway epithelial cells *in vivo* and upregulated ULBP2 in human airway epithelial cells *in vitro*, although the mechanism of ligand induction by this pathogen is unknown (67).

Propionibacterium acnes was recently linked with Corpus-dominant lymphocytic gastritis (CDLG), a *Helicobacter pylori* negative entity and typically characterized by extensive infiltration of $CD8^+$ T-cells in the stomach epithelium. Interestingly, *P. acnes* infection correlated with increased levels of IL-15 and the upregulation of NKG2D-Ls in the inflamed gastric epithelium. Although the mechanisms leading to NKG2D-L upregulation in this entity remains unclear, a microbe-derived stimuli, probably live *P. acnes* or microbial-derived short-chain fatty acids were proposed as triggering factors (68). Notably, CDLG frequently coexists with autoimmune disorders with altered microbiota including Celiac disease (69) and Crohn's disease (70, 71). Moreover, propionic acid, derived from the fermentation of plant-derived dietary fiber mainly under the presence *Propionibacterium,* upregulated MICA/B in human cells including, activated T lymphocytes and different cancer lines (72). *Mycobacterium tuberculosis (M. tuberculosis)*-infected dendritic and airway epithelial cells also upregulate MICA expression *in vitro* and *in vivo,* and ligand recognition by Vγ2Vδ2 T cells expressing NKG2D receptor induces a potent inflammatory reaction (73).

Of note, albeit the above studies indicate that NKG2D-Ls upregulation is frequently observed in host cells exposed to various bacteria or their products, the molecular mechanisms of this phenomenon have not been elucidated. In addition, the exact mechanism that determines the fate of host cells exposed to dysbiosis (cell cycle arrest, apoptosis, malignant transformation or NKG2D-Ls upregulation) is currently unknown. Current data suggest that the extent of DNA damage and the resultant cellular responses determine cell fate under these stress conditions, hence in the context of dysbiosis, it is conceivable that cell fate may be dependent on the specific bacterium or group of bacteria dysregulated in the host.

As mentioned above, commensal bacteria play critical roles maintaining gut homeostasis, and this particular feature can be exploited for therapeutic purposes. The oral administration of commensal lactic acid bacteria effectively protected mice from dextran sulfate sodium-induced experimental colitis, which was attributed to the enhanced interferon-β production triggered by double-stranded RNA derived from commensal lactic acid bacteria (74). Although this study did not explore NKG2D-Ls expression on intestinal cells, it is worth mentioning that type I interferons have been shown to downregulate NKG2D-Ls expression impairing NK cells-dependent killing of target cells (38).

BACTERIAL GENOTOXINS AND DDR ACTIVATION

Various intestinal bacteria are known to release genotoxins (bacterial products capable of targeting host DNA), which together with the induction of sustained inflammation, promotes genomic instability and ultimately autoimmunity or cancer (**Table 1**). The first characterized bacterial genotoxin was cytolethal distending toxins (CDT), which is produced by several Gram-negative bacteria, including *E. coli*, *Campylobacter* sp., *helicobacter* sp., *Shigella dysenteriae*, *and Haemophilus ducreyi.* CDT induces DNA DSB in exposed host cells that may lead to transient cell cycle arrest or malignant transformation (32, 36, 75). Mouse liver cells exposed to CDT producing helicobacter develop dysplasia (76) and fibroblasts or intestinal epithelial cells chronically exposed to large concentrations of CDT, in the absence of immune cell clearance, show genomic instability, fail to activate DDR, and eventually become prone to malignant transformation (32).

Another bacterial-derived genotoxin is colibactin produced by *E. coli* strains of the B2 phylogroup harboring the polyketide synthetase island (*pks*), which is also found in other *Enterobacteriaceae* members such as *Proteus mirabilis* and *Klebsiella pneumoniae* (84–86). Infection with *E. coli* harboring this genomic cluster generates DSB leading to DDR activation, cell cycle arrest and genomic instability (36, 85). Notably, *E. coli* harboring *pks* are frequently detectable in patients with IBD, as well as in patients with CRC, suggesting that *pks* is directly related to disease pathogenesis (26, 92).

Escherichia coli uropathogenic-specific protein (Usp) is another bacterial toxin that induces genotoxic stress and activates

TABLE 1 | Bacterial pathogens or their products that activate DNA damage response (DDR) and may induce NKG2D ligands (NKG2D-Ls) expression in host cells.

Bacterial product	Bacterial pathogen	Target cells	Type of DNA damage	NKG2D-Ls induction	Reference
Cytotoxin					
AfaE-III adhesin subunit	*Escherichia coli*	Enterocyte-like Caco-2 cells	Unknown	MICA	(66)
Unknown ExoU? ExoA?	*Pseudomonas aeruginosa*	Airway epithelial cells Alveolar macrophages	Double stranded breaks (DSBs) Caused by reactive oxygen species (ROS) released from infected host cells	MICA ULBP2	(67, 77)
Unknown Bacterial metabolic products (propionic acid, acetate, lactate)?	*Propionibacterium acnes* *Propionibacterium* sp.	Gastric epithelial cells Activated T cells Jurkat cells	Unknown	MICA MICB ULBP2 MICA MICB	(68, 72)
Unknown	*Mycobacterium tuberculosis*	Dendritic cells Airway epithelial cells Macrophages	DSB? Endogenous ROS DDR/ataxia telangiectasia mutated (ATM)- and Rad3-related activation due to persistent activation of toll-like receptor (TLR) signal	MICA Unknown	(73) (78)
TLR ligands [LPS, Poly (IC), Zimosan]	Gram (–) bacteria *E. coli* *Listeria monocytogenes*	Macrophages	Endogenous ROS release Persistent activation of TLR signaling	MICA ULBP2	(79, 80)
CagA, VacA, γGT, urease, NapA	*Helicobacter pylori*	Gastric epithelial cells	DSB Caused by ROS released from infected host cells	NKG2D-Ls downregulation	(68)
Streptococcus pyruvate oxidase	*Streptococcus pneumoniae*	Airway epithelial cells	DSB is caused by: 1- Endogenous ROS release 2- Bacterial-secreted hydrogen peroxide	Unknown	(81, 82)
Unknown	*Salmonella typhimurium*	Murine intestinal epithelial cells	Unknown	ULBP-like transcript-1 (MULT1)	(83)
Genotoxin					
Cytolethal distending toxins	*Campylobacter jejuni*, *Haemophilus ducreyi*, *Actinobacillus actinomycetemcomitans*, *Shigella dysenteriae*, *Helicobacter cinaedi*, *Helicobacter hepaticus*, *Salmonella* sp.	Intestinal epithelial cells	Single-stranded breaks DSBs DDR activation	Unknown	(32, 36, 75)
Colibactin	*E. coli*, *Klebsiella pneumoniae*, *Enterobacter aerogenes*, *Citrobacter koseri*	Intestinal epithelial cells	Interstrand crosslink, DSBs	Unknown	(84–86)
Uropathogenic-specific protein	*E. coli*	HEK293 cells HUVE cells	DNA fragmentation		(28, 87–89)
Cyclo phenylalanine-proline	*Lactobacillus reuteri*, *Streptomyces* sp. AMLK-335, *Vibrio vulnificus*, *V. cholera*, *P. aeruginosa*, and *P. putida*	INT-407, U2OS, Huh7 cells	– ROS induction – DSB – DDR activation (ATM and downstream target CHK2)	Unknown	(90)
Pneumolysin	*S. pneumoniae*	Alveolar epithelial cells	DSB DDR activation ATM activation	Unknown	(91)

DDR in exposed cells. This genotoxin is produced by *E. coli* strains associated with pyelonephritis, prostatitis, and bacteremia (87, 88). Purified Usp cleaves linearized naked DNA *in vitro* and causes DNA fragmentation in mammalian cells (28).

Interestingly, compared with normal intestinal samples where toxin-producing bacteria constitute a minority of the commensal microbiota, human CRC tissues contain a high expression of these microorganisms (93).

Cyclo phenylalanine-proline (cFP) is other genotoxin produced by various bacteria such as *Lactobacillus reuteri*, *Streptomyces* sp. AMLK-335, *Vibrio vulnificus*, *V. cholera*, *P. aeruginosa*, and *P. putida*. Mammalian cells like INT-407, U2OS, and Huh7 cells exposed to cFP develop DSB and eventually activate ATM-CHK2 (90).

Pneumolysin, a toxin produced by *Streptococcus pneumoniae* and a key virulence factor against host cells, induces DSBs and ATM-mediated H2AX phosphorylation in epithelial alveolar cells. Consequently pneumolysin-exposed cells undergo cell cycle arrest and apoptosis, although the induction of NKG2D-Ls was not investigated in this study (91). Interestingly, pyruvate oxidase, another cytotoxin released by *S. pneumoniae*, induces DSBs and contributes to pneumolysin release (81).

Bacteria may also trigger DDR activation by inducing the expression of enzymes that enhance ROS in host cells, which lead to DNA damage or the induction of chronic inflammation (36, 94, 95). Bacterial-induced DNA damage can be further amplified by ROS released from immune cells at sites of chronic inflammation since inflammatory especially macrophages and neutrophils constitute a constant source of ROS, RNS, and cytokines that can develop in response to dysbiosis (94). Granuloma formation associated with *M. tuberculosis* infection was recently linked to the persistent inflammatory signals mediated by toll-like receptor (TLR) signals, which promotes macrophage polyploidy by regulating DDR signals *via* ATR activation (78). Although this study did not assess the expression of NKG2D-Ls in polyploidy macrophages, previous studies have shown NKG2D-Ls upregulation in macrophages exposed to bacterial-derived products *via* TLR signal activation (79, 80).

In addition, convincing evidence have established a link between infections with certain bacteria such as *E. coli, Bacteroides fragilis*, and *Fusobacterium nucleatum* with the development of CRC (26), indicating that dysbiosis may either cause carcinogenesis or autoimmunity. In some circumstances, bacterial can interrupt the DDR activation in host cells, thus allowing the cell cycle to progress in cells with unrepaired or in repaired with errors DNA resulting in mutations of critical genes associated with malignant transformation (26, 32). It is conceivable that the chronic exposure to genotoxin-secreting bacteria in host cells with fully functional DDR may result in NKG2D-Ls overexpression, *via* ATM activation, which ultimately increases the risk of autoimmunity. Conversely, in host cells with failed DDR, DSB may result in the survival of cells with unrepaired

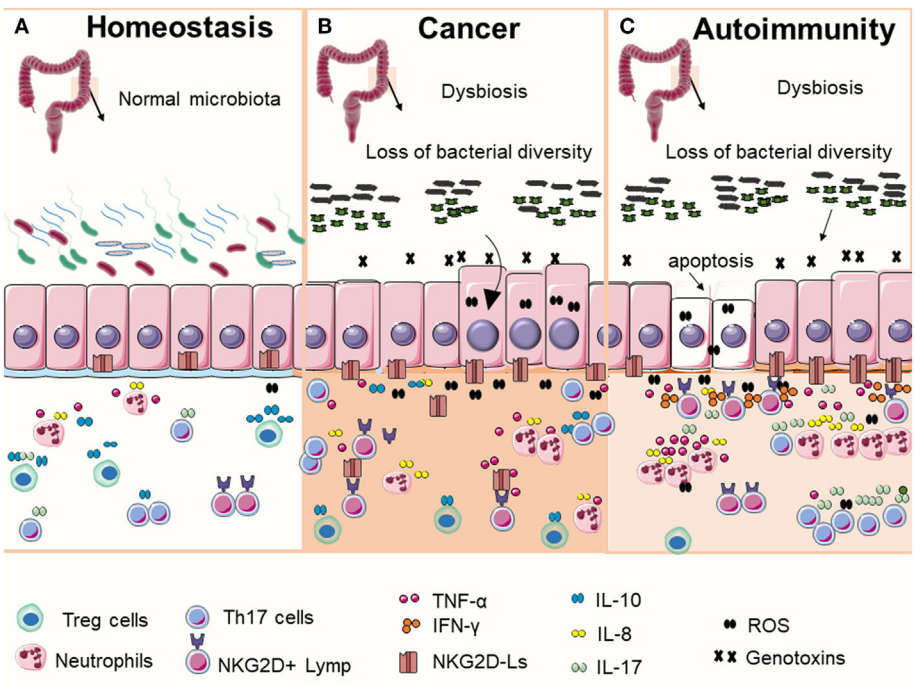

FIGURE 1 | Commensal bacteria play an important role in maintaining gut homeostasis **(A)**. In normal condition, intestinal epithelial cells express low levels of NKG2D ligands (NKG2D-Ls) (mostly intracellular) and beneficial bacterial contribute to immune education and help to maintain immune tolerance by promoting the induction and accumulation of regulatory T cells (Treg cells). In the context of microbial imbalance (dysbiosis), pathogenic bacteria may release genotoxins that generate DNA damage in host cells. DNA damage response (DDR) is then activated, which may lead to cell cycle arrest, apoptosis, or NKG2D-Ls induction in exposed cells. These events, together with the sustained immune activation in response to dysbiosis may eventually contribute to the development of autoimmune disorders or malignant transformation. Although the precise mechanisms that determine why cells take one of these two contrasting cell fates are unclear, current data appear to suggest that **(B)** bacteria-induced DNA damage in cells with failed DDR (caused by mutations or by inhibitory factors secreted by bacteria) may result in the survival and proliferation of cells with unrepaired DNA, increasing the risk of malignant transformation. Transformed cells may release or shade NKG2D-Ls that impairs NKG2D receptor-mediated functions leading to failed immune surveillance and tumor growth. **(C)** Alternatively, the chronic exposure to genotoxin-secreting bacterial in host cells with fully functional DDR may result in NKG2D-Ls overexpression *via* ataxia telangiectasia mutated activation leading to increased risk of autoimmunity.

DNA and elevated risk of malignant transformation (**Figure 1**). This assumption is supported by the observations that epithelial cells exposed to *Chlamydia trachomatis*, a bacteria associated with cervical and ovarian cancer development, undergo DNA damaged but fail to activate DDR, due to bacterial-induced impaired DNA repair. Consequently, infected cells continue to proliferate in an environment favorable for malignant transformation (96).Thus, the NKG2D/NKG2D-Ls axis maintains a delicate immune equilibrium, which is crucial for cancer immunosurveillance but that, under certain conditions, it can eventually promote autoimmunity (97).

Despite the above studies have consistently shown that bacterial genotoxin can activate DDR, unfortunately, none of them explored the potential NKG2D-Ls upregulation in host cells exposed to those bacterial products; therefore, further studied are needed to elucidate the immunological impact of genotoxins.

CONCLUDING REMARKS

Cells are continuously exposed to hostile environmental stressors, including extremes of temperature, toxins, and oxygen or nutrient deprivation. As a result, cells have evolved a wide range of molecular changes and stress responses to minimize damage which, depending on the severity and duration of stress confronted, can range from the activation of survival-promoting pathways to eliciting cell senescence or programmed cell death (98). During the last few years, remarkable progress has been made in our understanding of the molecular mechanisms of DDR activation and its role in various cellular processes like aging and cancer development, and it has become apparent that the immune system constitutes an important component of the cellular response to DNA damage stressors (99). In this regard, the recognition of NKG2D-Ls overexpressed on the surface of host cells as a consequence of DDR activation constitutes one of the mechanisms by which the immune cells expressing the NKG2D receptor can sense DNA damage in host cells (100). Importantly, dysbiosis has been causally liked with DDR activation, *via* either the release of genotoxin or by promoting chronic inflammation (92). Several questions about the causal link of dysbiosis with NKG2D-Ls induction *via* DDR remain unanswered. For example, the precise mechanisms by which DDR induced by bacterial genotoxins or dysbiosis may result in malignant transformation or autoimmunity have not been fully elucidated. In addition, the specific role of dysbiosis in the upregulation of NKG2D-Ls in the intestinal epithelial cells of patients with IBD needs to be clarified. The polymorphism rs1049174 in the NKG2D gene (generating HNK and LNK genotypes) influences NKG2D receptor expression on immune cells and is implicated in individual susceptibility to certain cancers. The HNK genotype is associated with greater NK cells cytotoxic activity and lower prevalence of epithelial cells-derived malignancies, in comparison with the low cytotoxic genotype LNK (23, 50, 101, 102). It is currently unknown if HNK and LNK genotypes can affect NKG2D receptor-mediated immune responses in the context of dysbiosis and if they are implicated in the development of autoimmune disorders like IBD. Future studies on this regard are warranted as genetic variants of human genes involved in immunity and gut architecture are associated with an altered composition of the gut microbiome (103).

Compelling evidence indicate that dysbiosis is implicated in the pathogenesis of several human diseases, ranging from metabolic disorders, autoimmunity, and cancer (64, 104, 105) and various studies have shown that manipulating human microbiota, for example by using probiotics or fecal transplantation, has promising therapeutic potential (65, 106, 107). Further research is needed to uncover the specific microbes within a dysbiotic microbiota that are directly implicated in disease etiology. Designing optimal interventions aimed to remove pathogenic microorganisms or for replacing them with beneficial ones will have enormous therapeutic potential.

AUTHOR CONTRIBUTIONS

The authors contributed extensively to the work presented in this paper. JE: conceived and designed the study; created and drew figures and wrote the manuscript. MM: wrote the manuscript, searched and collected bibliography.

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REVIEW
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Regulation of Cytokine Production by the Unfolded Protein Response; Implications for Infection and Autoimmunity

Judith A. Smith[1,2]*

[1] Department of Pediatrics, University of Wisconsin-Madison School of Medicine and Public Health, Madison, WI, United States, [2] Department of Medical Microbiology and Immunology, University of Wisconsin-Madison School of Medicine and Public Health, Madison, WI, United States

Protein folding in the endoplasmic reticulum (ER) is an essential cell function. To safeguard this process in the face of environmental threats and internal stressors, cells mount an evolutionarily conserved response known as the unfolded protein response (UPR). Invading pathogens induce cellular stress that impacts protein folding, thus the UPR is well situated to sense danger and contribute to immune responses. Cytokines (inflammatory cytokines and interferons) critically mediate host defense against pathogens, but when aberrantly produced, may also drive pathologic inflammation. The UPR influences cytokine production on multiple levels, from stimulation of pattern recognition receptors, to modulation of inflammatory signaling pathways, and the regulation of cytokine transcription factors. This review will focus on the mechanisms underlying cytokine regulation by the UPR, and the repercussions of this relationship for infection and autoimmune/autoinflammatory diseases. Interrogation of viral and bacterial infections has revealed increasing numbers of examples where pathogens induce or modulate the UPR and implicated UPR-modulated cytokines in host response. The flip side of this coin, the UPR/ER stress responses have been increasingly recognized in a variety of autoimmune and inflammatory diseases. Examples include monogenic disorders of ER function, diseases linked to misfolding protein (HLA-B27 and spondyloarthritis), diseases directly implicating UPR and autophagy genes (inflammatory bowel disease), and autoimmune diseases targeting highly secretory cells (e.g., diabetes). Given the burgeoning interest in pharmacologically targeting the UPR, greater discernment is needed regarding how the UPR regulates cytokine production during specific infections and autoimmune processes, and the relative place of this interaction in pathogenesis.

Keywords: unfolded protein response, endoplasmic reticulum stress, infection, virus, bacteria, autoimmunity, cytokine regulation, autoinflammatory disease

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***Correspondence:**
Judith A. Smith
jsmith27@wisc.edu

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INTRODUCTION: IMMUNE SENSING OF DANGER AND ENDOPLASMIC RETICULUM (ER) STRESS

How does the immune system sense pathogenic threats and respond appropriately? Cells in the immune system "see" the environment in little snippets: adaptive immune cells such as T cells bear surface receptors triggered by major histocompatibility complexes (MHC) loaded with peptides 8–20 amino acids in length (1). Even within these short stretches, the T cell receptor may

physically interact with only five amino acids (2, 3). Antibodies, constituting the B cell receptors, also recognize similarly small molecules, averaging 18–19 contact residues (up to 5 contiguous) (4). Innate immune cells, the first responders on the scene of infection, including neutrophils, macrophages, and dendritic cells, express pathogen-sensing receptors on their surfaces, inside their endosomes and cytosol collectively referred to as pattern recognition receptors (PRRs). These PRRs recognize conserved molecular arrays on pathogens, or pathogen-associated molecular patterns (PAMPs) such as bacterial lipopolysaccharide (LPS), flagellin and lipoproteins, β-glucans and mannans on yeast, and nucleic acids from viruses. The nucleic acid sensors detect types of nucleic acids that are not normally produced (e.g., dsRNA) or located in unusual settings, such as dsDNA in the cytosol or single stranded RNA in endosomes. Classes of PRRs include the toll-like receptors (TLRs), C-type lectin receptors, nucleotide-binding domain and leucine-rich repeat containing receptors (NLRs), retinoic acid inducible gene I (RIG-I) family helicases, and other cytosolic nucleic acid sensors (5–8).

When the outside world is observed in small pieces, the specificity of immune receptors becomes problematic. For instance, pathogens may express peptides with identical or functionally analogous amino acids stretches as endogenous peptides, a phenomenon referred to as "molecular mimicry" (2, 9). The classic example is the antibody cross-reactivity between streptococcal *N*-acetyl-beta-D-glucosamine and the cardiac myosin protein (10). This specificity issue, complicating the discrimination of self and infectious non-self, led to the "danger theory" put forth by Polly Matzinger in 1994, and then later refined over the years, that the immune system responds to challenges in accordance with contextual clues from damaged tissues (11–13). These damage-associated signals have been termed "danger-associated molecule patterns" (DAMPs). Different types of DAMPs have been reviewed recently in Ref. (14). When tissue is damaged, and cells destroyed by necrosis rather than apoptosis, specific molecules are released into the surrounding milieu. Examples of released products include dramatic increases in extracellular ATP, extracellular nucleic acids such as double-stranded DNA, mitochondrial DNA, chaperones such as high-mobility group box 1 (HMGB1) and heat shock proteins, interleukins IL-1α and IL-33, and uric acid (15, 16). Even in the absence of actual cellular destruction, infection or stress-triggered calcium signaling, and the generation of reactive oxygen species (ROS) may be considered DAMPs. During infections, the generation of multiple DAMPs provides the context to signal significant organismal insult.

The "danger" hypothesis was initially conceived to address issues with adaptive immune (T and B cell) self-non-self-discrimination. However, this same conceptual requirement for damage that provides context for dendritic cell activation and T cell stimulation may also help with several other specificity issues in innate immunity. Consider the microbiome: humans are widely covered on external and internal surfaces with trillions of microbes that constitute our natural microflora. Microbial-associated molecular patterns (MAMPs) also stimulate PRRs. For instance, theoretically, the same TLR4 that recognizes LPS on an invading pathogen could also be triggered by gut gram-negative bacteria. However, in the absence of tissue damage or stress, the healthy steady-state microbiome does not normally trigger inflammatory responses.

Many damage-generated endogenous products, such as extracellular matrix proteins, also stimulate PRRs (17). Indeed, the same PRRs poised to recognize PAMPs/MAMPs do "double duty" and respond to DAMPs, a testament to natural efficiency and repurposing (14). Alternatively, it has been suggested that pathogens have evolved to take advantage of PRRs evolutionarily aimed at wound repair (12). As an example of endogenous product recognition, the nucleic acids released by dying cells that are taken up into endosomes stimulate endosomal TLRs. The same non-specificity inherent in TLR4 that enables recognition of a broad variety of LPS structures may also allow TLR4 to respond to endogenous products such as fibrinogen or HMGB1 (18). The dual recognition of endogenous products and pathogens by the same receptors again poses a problem of specificity, as non-infectious damage (that releases DAMPs) may not merit an anti-pathogen response. How does the immune system determine whether to mount a wound healing response or an inflammatory response? Is there a titration by numbers or types of DAMPs (and PAMPs) and does this discrimination occasionally fail? Even in the absence of pathogens, "sterile" damage may liberate significant endogenous ligands for PRRs. One example of an over-exuberant inflammatory response in the face of sterile damage is the post-traumatic inflammatory response syndrome that occurs in the absence of inciting infection (16). Aberrant recognition of endogenous products may also drive non-resolving wound responses that lead to fibrosis (19).

Vance et al. have proposed that pathogenic organisms provide extra contextual clues that alert the immune system (20). The immune system recognizes certain bacterial products produced only by living (rather than dead), invasive pathogenic bacteria, so-called "vita PAMPs": for instance, live bacteria produce cyclic-di-nucleotides second messengers that activate the host cytosolic stimulator of interferon gene (STING) (8, 21). Access to the cytosol may be the factor that provides the key information. As an example, the lysteriolysin O that enables *Listeria* release into the cytosol is required for immunogenicity (20). Other bacterial pathogens contain secretion systems that provide a conduit between vacuoles and host cytosol. Release of products *via* this route (e.g., flagellin) may then trigger cytosolic inflammasome sensors (22, 23). Disruption of the cytoskeleton may also be directly sensed by the host cell. The mechanisms by which this occurs remain unclear, but may involve co-localization of PRRs (NOD proteins and inflammasome components) with the actin cytoskeleton (24, 25).

Disruption of fundamental cellular processes such as protein production, may also contribute to immune calibration, titrating up the threat level either appropriately, as in the case of infection, or inappropriately in autoimmunity. All cells must make protein to survive. Secreted and transmembrane proteins are manufactured in the ER. Amazingly, the ER accomplishes protein folding in a very crowded environment, estimated at 100 mg/ml, a concentration that could theoretically promote aggregation (26). The ER is also the site of sterol and phospholipid synthesis and the major cellular store for calcium. Indeed, many of the protein

folding chaperones, including the carbohydrate-binding calnexin and calreticulin, immunoglobulin heavy chain binding protein (BiP/Grp78), and protein disulfide isomerases require high concentrations of calcium for their function (27). The formation of intermolecular and intramolecular disulfide bonds during protein folding generates ROS. Thus, to maintain redox equilibrium, the ER contains buffering anti-oxidant enzymes. Related to the exigencies of the folding process, a broad variety of environmental stressors may adversely impact protein folding, such as decreased glucose or amino acids, hypoxia, decreases in ER calcium, excessive reactive oxygen radicals, increased demands in protein production, as well as the presence of misfolding proteins. To safeguard protein production and ensure quality control, ER-stress triggers the activation of several biochemical pathways collectively referred to as the unfolded protein response (UPR). The UPR restores proteostasis equilibrium by increasing capacity (ER size and chaperone production) as well as decreasing protein client load, through translational inhibition and the process of ER-associated peptide degradation (ERAD). If ER stress becomes irremediable (excessively severe or prolonged), the UPR initiates apoptosis. One could envision how infections result in the multiple ER stresses listed above: viral infections dramatically increase protein production; bacteria consume nutrient resources and stimulate oxygen radical production. Because of the universal need for protein production, and the sensitivity to a wide variety of environmental or internal stressors, the UPR is well poised to sense pathogenic danger and transduce the stress signal into a heightened immune response (**Figure 1**).

The UPR plays a physiologic role by enabling the function of highly secretory cells such as hepatocytes, plasma cells, and acinar or islet pancreatic cells. For example, mice deficient in key UPR components die early of pancreatic insufficiency and diabetes (28, 29). The UPR also supports the development of specific cells in the immune system. Even before B cells ramp up antibody production to become plasma cells, the UPR is engaged by the plasma cell differentiation program (30–32). Similarly, optimal development and survival of dendritic cells, and differentiation of eosinophils requires the UPR (33, 34). Not only does the UPR support the development of specific immune cells, but it also globally shapes the immune responses in many cell types (32, 35).

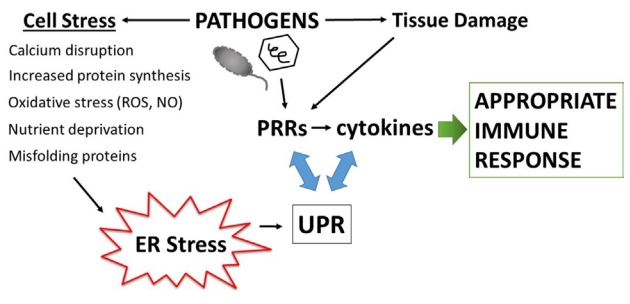

FIGURE 1 | Amplification of pathogen immune responses *via* endoplasmic reticulum (ER) stress and the unfolded protein response (UPR). Pathogens cause tissue damage, intracellular host stress, and stimulate pattern recognition receptors (PRRs) which then induce cytokines. Multiple pathogen-triggered cellular insults cause stress in the ER that impacts protein folding and thus induces the UPR. The UPR, PRR activation, and cytokine production intersects on multiple levels (see main text and **Figure 3**), with interactions going in both directions (blue two-sided arrows). This amplification mechanism generates an immune response commensurate with the degree of pathogenic threat.

Over the past 10–15 years, it has become apparent that one way in which the UPR tunes immune responses is through the modulation of cytokine production (35). Cells of the immune system communicate *via* cytokines, which are soluble secreted proteins encompassing the families of interleukins, interferons (IFNs), and tumor necrosis factor (TNF) family members among other mediators (36). Both the magnitude and types of cytokines produced program the immune response to respond appropriately to different types of threats. For instance, type I IFN-α/β induce hundreds of target genes aimed at containing and eliminating viral invasion. Cytokines such as TNF-α, IFN-γ, and IL-6 promote inflammatory innate responses that enhance antibacterial activities. IL-4, IL-5, and IL-13 heighten anti-parasitic immunity. On the other hand, IL-10 and transforming growth factor β (TGF-β) limit immune destructiveness and collateral damage to the host by toning down innate and adaptive immune responses. Following the sensing of danger signals *via* PRRs or adaptive immune leukocyte receptors, inflammatory pathways are set in motion that culminate in the activation of cytokine-regulatory transcription factors. Intriguingly, UPR pathways interweave through all levels of cytokine regulation: the UPR impacts the PRRs that sense pathogenic molecules, downstream inflammatory signaling pathways, and ultimately, the activation of cytokine-regulatory transcription factors, such as nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), activator protein 1 (AP-1), and the interferon regulatory factors (IRFs). This interaction between the UPR and inflammation is a "two-way street"; in certain tissues (e.g., the liver), inflammatory cytokines induce ER stress, setting up the potential for a positive feedback loop (37). The modulation of cytokine production by intracellular stress during infection has implications for how the immune system detects and responds appropriately to pathogens. The drawback to anti-pathogen cytokine augmentation is the potential for inappropriate boosting of immune responses resulting in autoimmunity. Below, the variety of mechanisms linking the UPR with cytokine production and the implications of this interaction for infection and autoimmunity will be addressed.

THE UPR

The metazoan UPR comprises three primary signaling pathways stemming from the activation of ER-stress sensors inositol requiring enzyme 1 (IRE1), activating transcription factor 6 (ATF6), and protein kinase R (PKR) like endoplasmic reticulum kinase (PERK) (27, 38). These three sensors reside in the membrane of the ER, poised to respond to stressors that increase the abundance of unfolded proteins. In their inactive state, the stress sensors associate with the folding chaperone BiP. When ER luminal load of unfolded proteins increases, BiP releases the sensors to preferentially bind hydrophobic patches on misfolded protein, thus resulting in the activation of the three pathways. In addition to this BiP "titration" model, alternative mechanisms

of activation have been described: crystallographic resolution of the yeast IRE1 revealed an ER luminal structure that forms an MHC-like peptide-binding groove upon dimerization, thus potentially allowing direct sensing of unfolded peptides (39). The mammalian pocket is too narrow to accommodate peptides, but may undergo a conformational change upon activation by peptide binding (40, 41). Although the PERK luminal domain has high-structural homology with IRE1, direct peptide binding has not been described for PERK. On the other hand, significant alterations in lipid content of the ER (e.g., increased acyl chain saturation) may also directly activate IRE1 and PERK, independently of their ER luminal domains (42).

Inositol requiring enzyme 1 is the most evolutionarily conserved ER stress sensor, and the only UPR pathway present in single cell organisms such as yeast. In mammals, the IRE1α (*ERN1*) isoform is ubiquitously expressed, whereas IRE1β (*ERN2*) is restricted to mucosal epithelial surfaces such as the lung and gut (43, 44). The cytosolic portion of IRE1 contains two functional domains: a kinase domain and an endonuclease domain. Upon sensing unfolded protein, IRE1 dimerizes and auto-trans phosphorylates, a prerequisite for activation. Intriguingly, the IRE1 endonuclease has only one specific mRNA target, known as Hac-1 in yeast and XBP1 in higher eukaryotes. IRE1 cleaves a 26 base pair loop out of the XBP1 mRNA, causing a frame shift mutation that removes a premature stop codon. The "unspliced" XBP1 mRNA encodes a shorter unstable protein with DNA binding domain only, but the longer "spliced" XBP1 mRNA encodes the full length transcription factor with DNA binding and transcriptional transactivating domains (45). XBP1 increases the production of folding chaperones (e.g., ERdj4), components involved in ERAD and increases phospholipid synthesis and ER size (31, 46, 47). By increasing ER capacity and decreasing ER client load, XBP1 is considered a largely "adaptive" pro-life response (48, 49). In addition to splicing XBP1, upon prolonged or severe stress, IRE1 may non-specifically degrade mRNAs in proximity to the ER in a process termed regulated IRE1-dependent decay (RIDD) of mRNA (50, 51). This non-specific endonuclease process is thought to decrease ER protein client load, as many of the degraded mRNAs encode proteins in the secretory pathway. XBP1 splicing and the RIDD functions of IRE1 may be experimentally dissociated, but the precise mechanisms governing the switch between these activities remain elusive (52). Degree of IRE1 oligomerization may regulate RNase substrate preference (53). The IRE1 kinase domain associates with other molecules in a multi-molecular complex referred to as the "UPRosome" (45, 54). Through association with TNF-receptor-associated factor 2 (TRAF2) and apoptosis signal-regulating kinase 1 (ASK1), IRE1 phosphorylates c-Jun N-terminal kinase (JNK), thus linking ER stress with autophagy, apoptosis, and inflammatory signaling (described more below) (55). Intriguingly, IRE1 also associates with the pro-apoptotic B cell lymphoma 2 (Bcl2)-family members Bcl2-antagonist/killer 1 (Bak) and Bcl2-associated X protein (Bax), which, through unknown mechanisms, enhance IRE1 kinase activity (56).

Protein kinase R like endoplasmic reticulum kinase oligomerizes and trans phosphorylates early during the UPR. PERK phosphorylates eukaryotic initiation factor 2α (eIF2α) on serine 51, thus inhibiting the guanine nucleotide-exchange activity of eIF2B required for recycling eIF2α to its GTP-bound form (57). By this mechanism, PERK inhibits ribosomal function and globally diminishes protein translation of capped mRNAs. This decrease in protein production is essential for stress adaptation, in that interference with eIF2α phosphorylation leads to proteotoxicity during ER stress (58). Certain mRNAs with inhibitory upstream short open reading frames such as the mRNA encoding the transcription factor ATF4 are preferentially translated when eIF2α is phosphorylated (59, 60). ATF4 stimulates the production of a pro-apoptotic transcription factor C/EBP homologous protein (CHOP). Together CHOP and ATF4 achieve most of the transcriptional program stemming from PERK activation, which includes the induction of proteins involved in amino acid transport, autophagy, folding chaperones, and redox regulatory proteins in addition to pro-apoptotic molecules (61, 62). ATF4 also initiates relief of the translational blockade through induction of growth arrest and DNA damage-inducible 34 (GADD34). GADD34 forms a complex with, and activates protein phosphatase 1, which dephosphorylates eIF2α (63). Thus, the global translational decrease is transient. Other molecules also impact eIF2α phosphorylation status: as an example of cross-talk between UPR pathways, XBP1 regulates $p58^{IPK}$ which binds and inhibits PERK, thus promoting eIF2α dephosphorylation (46, 64, 65). During the non-stressed state, constitutive repressor of eIF2α phosphorylation maintains eIF2α dephosphorylation (66). Interestingly, other molecules with PERK homology, such as PKR, general control non-derepressible 2 (GCN2), and Heme-regulated eIF2α kinase also phosphorylate eIF2α, in response to dsRNA, amino acid deprivation, and heme deficiency, respectively, thus broadening the scope of stressors utilizing this response pathway. For this reason, the eIF2α pathway has also been referred to as the "Integrated Stress Response" pathway (61, 67). In addition to eIF2α, PERK also phosphorylates nuclear factor erythroid 2 (Nrf2), freeing it from the Kelch-like ECH associated protein 1 inhibitory protein. PERK thus enables Nrf2 nuclear translocation and an increase in anti-oxidant protein production (68).

In the third major UPR pathway, release of BiP from ATF6 uncovers a Golgi localization signal, enabling translocation of ATF6 from ER to Golgi (69). In the Golgi, Site-1 and Site-2 proteases cleave ATF6, liberating the active transcription factor. ATF6 also has two isoforms, ATF6α, and ATF6β. Most of the UPR-related activity is dependent upon ATF6α, but there is some redundancy required for development as deletion of both in mice is embryonic lethal (70). ATF6 binds ER stress element sites by itself or as a heterodimer with XBP1; thus, there is some overlap in function. ATF6 also upregulates *XBP1* message, another instance of UPR pathway cross-talk (71). Certain UPR target gene chaperones, such as glucose-regulated protein 94 and BiP itself are primarily ATF6-dependent (70). Besides ATF6, in specific cell types, ER stress regulates Site-1 cleavage of other basic leucine zipper transcription factor proteins [e.g., Cyclic AMP-responsive element-binding protein H (CREBH), old astrocyte specifically induced substance, CREB4] (72). In the liver, CREBH participates in inflammatory responses by activating the production of C-reactive protein and serum amyloid P components of the

acute phase response (37). For a basic summary of the three UPR pathways, see **Figure 2**.

MECHANISMS OF CYTOKINE REGULATION BY ER STRESS

Activation of the UPR is sufficient to induce low levels of inflammatory cytokine production, even in the absence of ostensible infectious stimuli or PRR ligation. In one of the earliest studies to note this phenomenon, a 2005 study by Li et al., free cholesterol loading of macrophages induced ER stress-dependent mitogen-activated protein (MAP) kinase signaling and NF-κB activation, resulting in production of IL-6 and TNF-α (73). Subsequently, studies using classic pharmacologic UPR inducers such as tunicamycin and thapsigargin have also noted low level "sterile" inflammatory cytokine production (74, 75). ER stress induces inflammatory cytokines by modulating inflammatory signaling cascades, activating "canonical" cytokine-regulatory transcription factors, as well as *via* the actions of the UPR-activated transcription factors themselves.

All three UPR pathways impact the activation of NF-κB. In the quiescent state, NF-κB family members (p50, p52, p65, RelB, and c-Rel) reside in the cytoplasm, bound to inhibitory factor κB (IκB). Upon immune signaling, IκB kinase (IKK) phosphorylates IκB, targeting it for ubiquitination and proteolytic destruction. The degradation of IκB permits NF-κB to translocate into the nucleus where it induces inflammatory cytokines such as IL-6 and TNF-α (76). IRE1 increases basal IKK activity *via* TRAF2, promoting NF-κB translocation (77–79). IRE1 may also promote NF-κB activation indirectly *via* regulation of glycogen synthase kinase 3 (80, 81). The IκBα protein has a shorter half-life compared with NF-κB, thus the PERK-dependent global translational shutdown preferentially affects IκB expression levels over NF-κB, leaving NF-κB free to translocate (82). Downstream of PERK, CHOP also enhances NF-κB signaling *via* transcriptional repression of the negative regulator peroxisome proliferator-activated receptor (83). ATF6 impacts NF-κB activation through a pathway involving mammalian target of rapamycin signaling and protein kinase B (Akt) dephosphorylation (84, 85). Finally, the calcium dysregulation and ROS generated during ER stress may contribute to NF-κB activation, either by enhancing induction of UPR pathways or other mechanisms (86). NF-κB regulates cytokine production in conjunction with other transcription factors such as the AP-1 heterodimer of Fos and Jun family transcription factors. MAP kinases [e.g., p38, extracellular regulated kinase (ERK) and JNK] regulate the activation of AP-1 factors (87). ER stress intersects with MAP kinase signaling in multiple ways [reviewed in Ref. (88)]: IRE1 promotes the activation of AP-1 family members *via* ASK-1 mediated JNK and p38 phosphorylation

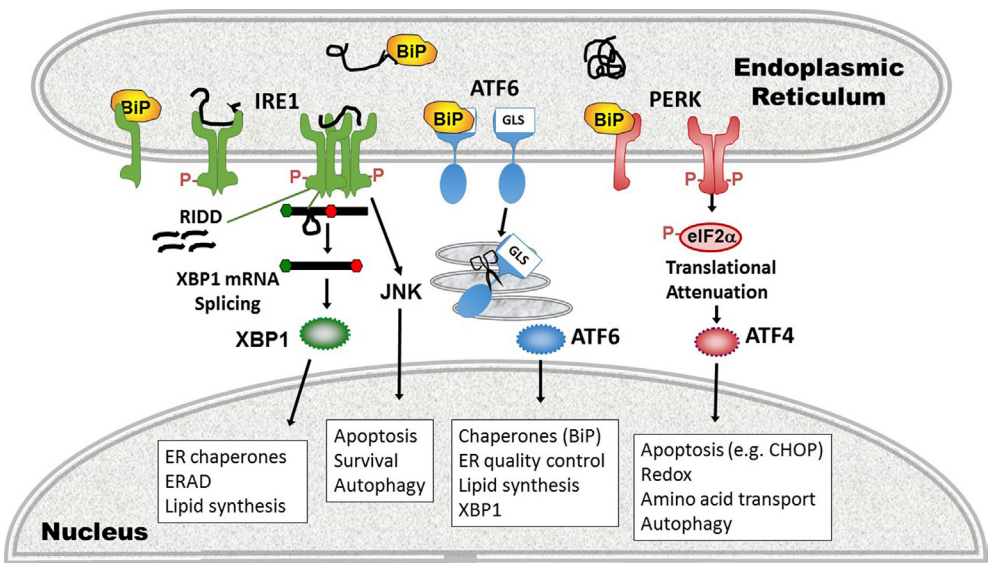

FIGURE 2 | Three pathways of the unfolded protein response (UPR). (1) inositol requiring enzyme 1 (IRE1) pathway (left, green), a dual endonuclease and kinase, binds the chaperone binding protein (BiP) in its monomeric state. On sensing unfolded/misfolded protein IRE1 oligomerizes and auto-trans phosphorylates (red Ps). Activation of the endonuclease specifically splices 26 nucleotides out of the XBP1 mRNA, causing a frameshift mutation that removes a premature stop codon, thus enabling translation of the full length transcription factor. With increased stress, the non-specific endonuclease function cleaves endoplasmic reticulum (ER)-associated mRNAs in a process called regulated IRE1-dependent decay (RIDD). The IRE1 kinase domain associates with other signaling partners that phosphorylate Jun N-terminal kinase (JNK). ERAD, ER-associated degradation. (2) Activating transcription factor 6 (ATF6) pathway (middle, blue): ATF6 release of BiP uncovers a Golgi localization signal (GLS) enabling translocation to the Golgi. There it is cleaved by Site-1 and Site-2 proteases (scissors), liberating the ATF6 transcription factor. (3) Protein kinase R like endoplasmic reticulum kinase (PERK) pathway (right, pink): in the presence of phosphorylated protein, PERK also oligomerizes and transphosphorylates, activating its kinase activity. PERK in turn phosphorylates eIF2α, resulting in transient global translational inhibition apart from a few specific mRNAs such as ATF4. ATF4 promotes transcription of the apoptosis-inducing transcription factor C/EBP homologous protein (CHOP). Cellular processes altered by the UPR pathways and key gene targets that are UPR components are in boxes.

(55, 89). ERK phosphorylation during ER stress is also partially IRE1-dependent (90). In bronchial epithelial cells, PERK and ATF6 promote ERK and p38 signaling, and in cholesterol loaded macrophages, CHOP was required for ERK activation (73, 91). P38 positively feeds back on the UPR, phosphorylating CHOP and ATF6, and thus increasing their activities (92–94).

In addition to the classic pro-inflamamtory cytokines, the UPR regulates type I IFN. IFN-β is one of the earliest IFNs produced in response to viral infection and PRR engagement, and by binding the type I IFN receptor (IFNAR) and upregulating IRF7, promotes the production of multiple IFN-α species and induction of the full anti-viral interferon program (95). In the *ifnb1* promoter "enhanceosome" region, NF-κB, AP-1, and IRF3 bind cooperatively to initiate transcription (96–98). Like NF-κB, unactuated IRF3 remains cytoplasmic. Upon phosphorylation on multiple serines and threonines, IRF3 dimerizes and translocates into the nucleus where it binds its gene targets (99). IRF3 phosphorylation also enables association with the transcriptional co-activator CREB-binding protein (CBP/p300) (100). ER stress induces IRF3 phosphorylation and nuclear translocation, although the precise mechanisms are not yet clear and may depend upon the type of stress. Calcium disruption (as through the SERCA pump inhibitor thapsigargin) and oxygen glucose deprivation activate IRF3 through a STING-dependent mechanism, whereas agents that disrupt N-linked glycosylation (e.g., tunicamycin) appear to utilize a STING-independent, but Site-1/Site-2 protease (ATF6?)-dependent pathway (101).

In addition to the activation of canonical inflammatory transcription factors and IRFs, the classic UPR transcription factors which orchestrate the UPR bind directly to genetic cytokine-regulatory elements. Through chromatin precipitation analyses, XBP1 was detected at the promoters of the IL-6, and TNF-α encoding genes, a *tnf* enhancer, as well as an enhancer element downstream of the *ifnb1* gene (74, 102). In response to short chain fatty acids, ATF4 (downstream of PERK and the integrated stress response) binds the cAMP response element in the *Il6* promoter (103). CHOP binds the IL-23p19 (*Il23A*) promoter in dendritic cells in response to LPS, ER stress, and most fully to the combination of LPS and ER stress (104). On the other hand, certain UPR-regulated transcription factors such as ATF3 have anti-inflammatory effects, and may play a role in regulating pathogen responses, ischemic preconditioning, and cancer (105–109).

More recently, evidence has suggested that beyond directly regulating transcription factors or cytokine promoters, ER stress also impacts the activation of upstream PRRs. For example, ER stress activates the inflammasome, thus promoting IL-1β production and potentially programmed cell death. IRE1 activation, possibly *via* RIDD, inhibits a micro-RNA, miR-17, that downregulates the production of thioredoxin-interacting protein (TXNIP) (110, 111). Thus, ER stress rapidly increases TXNIP expression (111, 112). PERK also increases *TXNIP* expression *via* the induction of transcription factors carbohydrate-responsive element-binding protein and ATF5 (112). TXNIP associates with and activates the NLRP3 inflammasome at the mitochondria. NLRP3 in turn, in a caspase-2 and BH3 domain interacting agonist (Bid)-dependent mechanism, causes mitochondrial damage, cytochrome C release, and production of oxygen radicals that further stimulates inflammasome production of IL-1β (113). The IRE1-RIDD function has also been implicated in the generation of small RNAs that trigger RIG-I-dependent NF-κB activation (114). UPR-dependent mitochondrial damage and mitochondrial DNA release may also play a role in the activation of another cytosolic sensor STING: mitochondrial DNA triggers the molecule cGAS, which in turn generates a cGAMP ligand that stimulates STING (115). As noted, certain types of ER stress mobilize STING translocation and STING-dependent IFN production (101). However, the link between *ER stress*-dependent mitochondrial damage and STING activation remains speculative. ER stress is well poised to initiate mitochondrial ROS-dependent events that activate and amplify innate immune signaling: protein folding is an oxidative process (116). The UPR and ROS trigger calcium release from the ER through activation of the inositol-1,4,5-triphosphate (IP3) receptor and ryanodine receptor ER channels. ER and mitochondria are spatially juxtaposed at the mitochondria-associated ER membranes, where ER IP3R channels are linked *via* chaperones to mitochondrial voltage-dependent anion channels (117). Increased cytosolic calcium thus triggers ROS release from mitochondria, which induces increased levels of ER stress, resulting in a relentless feed-forward loop (116). Finally, the UPR also interacts with the cytosolic peptidoglycan receptors NOD1 and NOD2 to induce production of the pro-inflammatory cytokine IL-6. Activation of this NOD1/2-dependent pathway by thapsigargin or infection with *Brucella abortus* was suppressed by the general UPR inhibitor tauroursodeoxycholic acid (TUDCA) and the IRE1 kinase inhibitor KIRA6 (118). The proposed mechanism involves IRE1 kinase activation and recruitment of NOD-interacting proteins TRAF2 and receptor-interacting serine/threonine-*protein* kinase 2 (119). For a summary highlighting mechanisms at the intersection of UPR and cytokine induction, see **Figure 3**.

Sterile ER stress results in relatively low levels of cytokine production, particularly compared with PRR stimulation (74, 120). In the case of IFN-β, this is perhaps surprising, as ER stress activates the critical enhanceosome components NF-κB, AP-1, and IRF3. One possible explanation is that another signal is required (e.g., PRR ligation) for full phosphorylation of IRF3 at multiple sites (101). Alternatively, although multiple UPR pathways activate NF-κB, it may still be at a low level compared with that induced by PRR ligation. Another possibility, extending to other cytokines, is that PRR ligation may be required to generate certain transcriptional co-factors or epigenetic modifiers.

In contrast to situations involving either sterile ER stress or isolated PRR stimulation, subsequent PRR ligation of cells undergoing ER stress has profound consequences for inflammation. Specifically, induction of ER stress has the capacity to render cells exquisitely sensitive to PRR stimulation, resulting in dramatically synergistic production of certain cytokines. This synergism has been demonstrated using pharmacologic UPR inducers, XBP1 overexpression, and misfolding proteins (74, 104, 120–122). Prominently increased cytokines include IL-6, TNF-α, IL-23, and IFN-β. In the cases of IL-6, TNF-α, and IFN-β, synergy appears to be XBP1 dependent but for IL-23, it is CHOP-dependent,

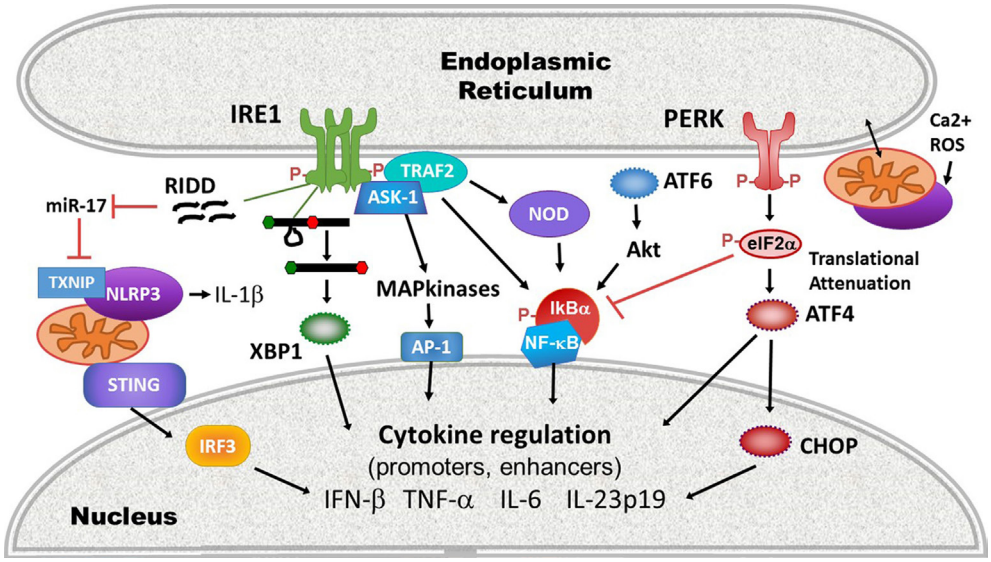

FIGURE 3 | Intersections of endoplasmic reticulum (ER) stress/unfolded protein response (UPR) and immune signaling. ER stress and the UPR impact innate immune signaling and cytokine production on many levels between pathogen-sensing pattern recognition receptors (PRRs) and ultimate cytokine production: (1) PRR activation: ER stress and the UPR activate multiple PRRs (purple) including stimulator of interferon gene (STING), NLRP3, and other inflammasomes *via* thioredoxin-interacting protein (TXNIP) upregulation and reactive oxygen species (ROS), and the NOD1/2 receptors. Much of this interaction occurs at the mitochondrial-ER interface, where released calcium (Ca2+) and ROS feed into PRR activation. (2) The UPR enhances inflammatory signaling pathways leading to mitogen-activated protein kinase activation [IRE1 shown here, but protein kinase R-like endoplasmic reticulum kinase (PERK) and activating transcription factor 6 (ATF6) also impact ERK and p38 activation], and inhibitory factor κB (IκB) phosphorylation and degradation. (3) Transcription factors: the UPR activates canonical pro-inflammatory and IFN-regulatory transcription factors activator protein 1 (AP-1), nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), and IRF3. Core UPR generated transcription factors such as XBP1 and C/EBP homologous protein (CHOP) also directly stimulate cytokine production by binding cytokine promoter and enhancer elements.

consistent with their detected binding to specific cytokine gene regulatory elements (74, 102, 104). ER stress may also enable cells to produce IL-1β in response to TLR4 ligation in a TRIF (TIR domain containing adaptor protein inducing interferon beta)-dependent and caspase 8-dependent, but XBP1 and CHOP independent manner (123). Synergy is not the invariable outcome of PRR stimulation of stressed cells but may depend upon the context. In ischemic preconditioning, which induces ER stress, inflammatory cytokine production is blunted, possibly *via* ATF3 induction or decreased NF-κB activity (106, 124).

Direct ligation of PRRs on the other hand, in the absence of a specific ER stressor, appears to partially activate UPR signaling pathways and selectively suppress others. Woo et al. reported that TLR3 or TLR4 stimulation suppressed subsequent ER stress-induced ATF4 and CHOP activation (but not upstream PERK or eIF2α phosphorylation) in a TRIF-dependent manner (125). LPS suppression of CHOP limited apoptosis (126). Stimulation of TLR2 and TLR4 activates IRE1 sufficiently to induce XBP1 mRNA splicing and binding of XBP1 to cytokine promoters. Interestingly, in this setting the nominal XBP1 UPR targets genes (e.g., *ERdj4*) were not transcribed. TLR signaling did not trigger the other two UPR pathways, as assessed by PERK phosphorylation and ATF6 cleavage, and inhibited tunicamycin-dependent upregulation of CHOP and the ATF6 target BiP. Canonical TLR signaling pathways and ROS appear to be involved in TLR-induced XBP1 splicing, as NOX2, TRAF6, and TLR adaptors myeloid differentiation primary response 88 (MyD88) and/or TRIF were all required (74). As another example of partial UPR activation and modification, viral infections that release dsRNA stimulate PKR, eIF2α phosphorylation, and GADD34 induction, in a TRIF-dependent manner. Interestingly, in the setting of virus/dsRNA, GADD34 relieves the translational inhibition of IFN-β and IL-6, but not global translation (127, 128). The basis of this specificity, or the resistance of global translational reversal remains unclear. Likewise, it is not yet understood why TLR4 induced XBP1 would promote the production of cytokines, but not its nominal chaperone targets. This phenomenon of partial UPR signaling and modulation in response to PRR ligation has been termed the "microbial stress response" pathway (129). As a net result, PRR adaptation of the UPR machinery potentially boosts cytokine production while avoiding the apoptotic sequelae of a fully engaged UPR.

One other mode of UPR-cytokine cross-talk occurs between cells rather than within individual cells. Surface translocation of calreticulin in cancer cells due to ER stress enhances immunogenicity and phagocytic uptake by dendritic cells—an immunostimulatory "eat me" signal (130). In a subsequent study, thapsigargin but not tunicamycin treatment of fibroblasts increased surface calreticulin expression and phagocytic uptake by co-incubated dendritic cells, suggesting the type of ER stress may be important. Interestingly, incubation of these thapsigargin-treated fibroblasts with bone marrow cells augmented LPS-induced production of pro-inflammatory cytokines such as IL-1β, IL-12, IL-23, and TNF-α (75).

IMPLICATIONS FOR VIRAL INFECTIONS

Intracellular infections provide a stage where ER stress interacts with signals from multiple PAMPs and DAMPs. The impact of the UPR on host-pathogen interaction has been increasingly recognized in viral, bacterial, and parasitic infections. UPR in the setting of parasitic infections has been reviewed recently and will not be discussed below (131).

The dramatic synergy observed by multiple groups between UPR and PRR signaling in the induction of type I IFN has particularly compelling implications for viral infection where the IFN response forms the capstone of host resistance. Viruses notoriously sabotage IFN production in a variety of ways. Several viruses interfere with the signaling leading to IRF3 activation or association with CBP/p300 (132–134). For instance, Dengue virus infection cleaves STING and also targets its upstream ligand-generator cGAS (135, 136). Other viruses target the type I IFN receptor IFNAR for proteolytic degradation (137). Paramyxovirus V proteins target STAT1 and STAT2 for proteolytic degradation (138). Therefore, one could speculate, that given all the viral obstacles to mounting an effective IFN response, even a partial UPR with XBP1 splicing or GADD34 induction to promote IFN-β transcription and translation might improve the odds.

Viruses induce ER stress through multiple mechanisms: during viral infection, cells dramatically increase protein production to manufacture new progeny virus. Some viruses reorganize the ER to develop replication platforms (e.g., Hepatitis C virus, coronavirus), and disrupt ER-Golgi trafficking (e.g., Picornavirus) (139–142). Viral infection also generate ROS. Beyond the host's direct response to ER stress, the catalog of viral proteins that induce or manipulate UPR pathways has grown exponentially. One could envisage how the UPR could be both helpful and harmful to viral infection, even aside from any effects on the anti-viral IFN program. On the one hand, adaptive pathways within the UPR could enable host cells to survive the inordinate stress of significantly increased viral protein production by significantly increasing ER capacity. However, both translational inhibition and ERAD could diminish viral protein production. Premature UPR-related apoptosis could also limit viral replication and spread.

In recent reviews, 35 animal viruses and several plant viruses have been reported to provoke ER stress and/or UPR induction (143, 144). Viruses vary greatly in their capacity to both induce and inhibit individual UPR pathways. Multiple RNA viruses (e.g., Dengue virus, Hepatitis C, Coxsackie B3, and SARS coronavirus) and DNA viruses (Ebstein Barr virus, Hepatitis B) induce all three UPR signaling axes (65, 114, 143, 145–150). Several viruses have been reported to induce two UPR axes, for instance IRE1 and PERK (Sindbis) or IRE1 and ATF6 (Influenza A, Chikungunya), whereas some may induce only one arm [e.g., ATF6, lymphocytic choriomeningitis virus (LCMV)] (151–153). Different aspects of the UPR may also prevail at specific times during the viral life cycle (145). The basis for this selective activation is not well understood but may depend upon specific viral factors and intracellular lifestyle.

Viruses have co-evolved multiple mechanisms to manipulate specific UPR pathways to avoid some of the potentially detrimental effects of UPR induction. For instance, several viruses encode GADD34 homologs: the Herpes Simplex Virus 1 product $\gamma_1$34.5 forms a complex with protein phosphatase 1, which dephosphorylates eIF2α, thus limiting translational inhibition (154). Further, $\gamma_1$34.5 contributes to viral resistance to IFN-α/β (155). The African swine fever virus DP71L functions similarly, inhibiting induction of ATF4 and CHOP (156). Japanese encephalitis virus induces RIDD to enhance replication, but intriguingly appears resistant to the RNAse activity (157). Herpes Simplex Virus UL41 protein suppresses XBP1 mRNA induction and splicing, possibly to decrease ERAD (158). There are also examples of viruses (e.g., Hepatitis C) that are permissive for XBP1 splicing, but prevent induction of XBP1's nominal UPR gene targets, which would include ERAD proteins such as ER degradation-enhancing α-mannosidase-like protein EDEM (159). This separation of XBP1 splicing and UPR target induction is reminiscent of the TLR-induced XBP1 disjunction. In these cases, it would be interesting to determine if the "blocked" XBP1s could still synergize in promoting IFN or pro-inflammatory cytokine production.

Modulation of the UPR appears to have varying effects, depending upon the virus and the type of UPR inhibition used. Viruses often induce pathways that enhance their replication. For instance, LCMV induces ATF6 activation, and cells defective in Site-2 protease produce lower titers of infectious virus (153). Likewise, blocking IRE1 with pharmacologic agents inhibits Influenza A replication (160). There are also multiple examples of viruses where the UPR appears to limit replication, suggesting a contribution to host defense. For instance, PERK is required for control of Dengue replication and pharmacologic eIF2α phosphorylators exhibit anti-viral activity (145, 161). Similarly, West Nile virus replicates at much greater titers in the absence of pro-apoptotic CHOP (162). Together, these studies support a general, but not universal concept that the IRE1 and ATF6 pathways are more likely to benefit virus, but the PERK pathway supports host defense. As an example where integrated stress response benefits virus, HIV induced ATF4 directly promotes HIV transcription through its long terminal repeat (163).

Although the UPR limits some viral infections, direct evidence for the role of the UPR in promoting type I IFN or other inflammatory cytokines during viral infection has been limited. It can also be difficult to tease apart cytokine vs. other effects of UPR modulation. For instance, in a Dengue model, induction of the UPR with a BiP inhibitor increased activation of IRF3 and NF-κB. However, it was not clear if these transcription factor effects contributed to anti-Dengue activity (164). There is some evidence viruses induce collateral damaging inflammation *via* UPR activation. For instance, the Hepatitis B protein HBx induced inflammatory cyclooxygenase 2 *via* an eIF2α-ATF4 pathway (114). Dengue virus-induced PERK/Nrf2 activation enhanced TNF-α production *via* increases in c-type lectin domain family 5, member A (CLELC5A), thus exacerbating pathology in a mouse model (165). Regarding IFN, in dendritic cells, XBP1 overexpression enhanced IFN-β production and markedly suppressed Vesicular stomatitis virus replication (122). In murine embryonic fibroblasts, GADD34

was required for dsRNA induced IFN-β and IL-6 production and resistance to Chikungunya virus. *In vivo*, IFN-dependence upon GADD34 appeared age-specific: adult mice were resistant to Chikungunya. However GADD34−/− neonates produced greatly diminished IFN-β in response to infection and rapidly succumbed (127). These two studies support a role for the UPR or microbial stress response pathways in supporting IFN and anti-viral immunity. However, viruses can also manipulate the UPR to limit IFN production. For instance, vesicular stomatitis virus and hepatitis C virus target IFNAR for proteolytic degradation *via* a PERK-dependent pathway, and this pathway appeared to enhance viral infection (137). Hepatitis C activation of UPR-autophagy pathways, including induction of CHOP and autophagy protein 5, also limited IFN-β production (166). Overall, the precise role of UPR pathways in supporting or limiting IFN or other cytokine production during viral infection, and the ultimate effect on pathogenesis remain important areas for further investigation.

IMPLICATIONS FOR BACTERIAL INFECTIONS

The study of the UPR in bacterial infections is much younger and less well developed than for viral infection, but the complexity of bacterial lifestyles promises many interesting variations on the interactions between host UPR and immunity. The list of bacteria inducing UPR pathways through their intracellular lifecycles or elaboration of bacterial products is steadily growing. Regarding bacterial products, Subtilase toxin, produced by Shiga endotoxic *Escherichia coli*, cleaves BiP, thus initiating all three arms of the UPR (167, 168). Interestingly, this UPR activation may either promote apoptosis, or dampen NF-κB responses and endotoxic pathology at subcytotoxic doses (169, 170). Listeriolysin O, produced by *Listeria monocytogenes*, also induces all three axes of the UPR (171). The current mechanism is unknown, but may involve depletion of intracellular calcium stores (172). Cholera toxin selectively binds IRE1, activating its RIDD activity (173). *Brucella abortus* secretes a factor VceC *via* its type IV secretion system that binds BiP and selectively induced IRE1activation (174). Interestingly, when ectopically expressed, several other *Brucella* type IV secretion system substrates also appear to accumulate in the ER, inhibit protein secretion and induce varying amounts of ER stress (175).

Beyond secretion of ER/UPR modifying factors, several pathogens form intimate spatial relationships with the ER during their intracellular lifecycle. For instance, *Legionella* and *Brucella* traffic in the endosomal pathway, preventing full phagosome-lysosome fusion, and establish replicative vacuoles within ER-derived compartments (176, 177). *Chlamydia* containing inclusion compartments also contact the ER (178). Intriguingly, reports of the interactions of these three ER-localized pathogens with ER stress responses have varied. One group reported that persistent (non-productive) *Chlamydia* infection induced transient BiP upregulation and eIF2α phosphorylation but not ATF6 cleavage or XBP1 splicing (179). However, in another study, *Chlamydia* stimulated "robust" IRE1 activation and XBP1 splicing, and induced CHOP in a GCN2-dependent manner (180). *Legionella* actively inhibited XBP1 splicing *via* bacterial translation elongation inhibitors (181). *Brucella* infection induces pronounced activation of UPR pathways. Within 24–48 h of infection, *Brucella* causes massive restructuring of the ER marked by condensation, fragmentation, and vacuolization (176, 182). This restructuring is mediated, at least in part *via* a microtubule stabilizing factor produced by *Brucella*, TcpB, which also has UPR-inducing properties (182, 183). Although the UPR induced by *B. melitensis* involves all three axes, with prominent CHOP induction, the *B. abortus* triggered UPR appears more targeted in scope (174, 182). Interestingly, the UPR appears to benefit *B. melitensis* replication in that targeting IRE1 with RNAi in a *Drosophila* S2 cell line or in IRE1−/− fibroblasts, or treatment of macrophages with the general UPR inhibitor TUDCA all diminished replication (182, 184). The UPR may help the host cell to survive the tremendous structural insult to its protein producing factory through its adaptive pro-survival ER stress coping mechanisms. The UPR also induces autophagy through multiple pathways, thus providing increased nutrients to "feed" the bacteria (185). Autophagy may also promote cell–cell spread (186). In contrast to *B. melitensis*, *B. abortus* replication was not affected by TUDCA (118). The basis for this species difference in UPR induction and consequence is not clear.

Several lines of evidence support a role for the UPR in innate immune sensing of bacterial infection and control of infection or collateral inflammation. The cytokine response to *Chlamydia* involves multiple ER stress pathways: CHOP critically contributed to *Chlamydia*-induced IL-23 production (104). Chlamydia also induced PKR-dependent IFN-β through a mechanism requiring TLR4 and IRE1 RNase activity. Interestingly, this TLR4 activity may limit CHOP induction, stressing the importance of the multiple innate immune and ER stress inputs that impact cytokine production during infection (180). XBP1 deficiency significantly decreased TLR2-dependent TNF-α and IL-6 responses to *Francisella in vitro*. Furthermore, XBP1 conditional knockout mice infected with *F. tularensis* exhibited greater organ disease burden (74). UPR augmentation of cytokine production may be particularly important in *Brucella* infection because of the unusually low endotoxicity of its LPS, as well as the sabotage of TLR signaling by TIR-domain analog-containing bacterial factors (e.g., TcpB) (187, 188). In *B. abortus* infected macrophages, VceC and IRE1 was required for optimal IL-6 responses *in vitro* (174). In a subsequent study, this same group implicated the NOD1/NOD2 PRRs downstream of ER stress in *Brucella*-stimulated IL-6 production (118). *In vivo*, the VceC mutant stimulated much less splenic IL-6 production, despite similar bacterial burden. Furthermore, in an inflammatory abortion model, the VceC mutant, TUDCA treatment, or NOD1/2 deficiency all decreased placentitis, placenta IL-6 expression, and increased mouse pup survival (118). Thus, ultimately, the net benefit of UPR-supported inflammatory responses during infection may represent a balance between augmented host sensing of infection, containment, and collateral inflammatory damage.

IMPLICATIONS FOR AUTOIMMUNITY AND AUTOINFLAMMATORY DISEASES

The UPR potentially enhances host responses to invading pathogens by boosting PRR signals. However, the down side to immune augmentation is the capacity to cause pathologic cytokine production, even in the absence of infection. Aberrant cytokine production plays a critical role in fomenting inflammatory disease, as attested to by the tremendous clinical utility of cytokine blocking antibody therapies. Cytokine targeting therapy has been remarkably effective in both autoimmune disease [e.g., rheumatoid arthritis (RA)], where "self" autoantigens play key roles in disease pathogenesis, as well as autoinflammatory diseases, which are driven primarily by abnormalities in cytokine production [e.g., TNF-receptor-associated periodic fever syndrome (TRAPs) and cryopyrinopathies] (189). Some of the diseases discussed below [inflammatory bowel disease (IBD), spondyloarthritis (SpA)], although not a result of a monogenic cytokine dysregulation, also have prominent autoinflammatory features. For instance, in mouse models, exogenous expression of IL-23 (generated by genetic minicircle infusion) reproduces many of the clinical features of SpA, including sacroiliitis, enthesitis, and inflammatory skin disease (190). General overexpression of human TNF in mice phenocopies RA, whereas a stabilized TNF-α in mice (TNFΔARE) produces aggressive widespread (polyarticular) joint disease and Crohn's like IBD, with arthritis occurring independently of T or B cells (191–193). In humans, genome wide association studies in polygenic autoimmune and autoinflammatory disorders have identified numerous associations with polymorphisms in cytokine or cytokine-regulatory genes (194–196). Thus, given the centrality of cytokine production in these inflammatory diseases, as indicated by clinical data, mouse models, and genetic studies, ER stress could theoretically have a major impact on disease pathogenesis. Indeed, the UPR has been implicated in an increasingly greater number of inflammatory diseases. A few themes will be highlighted below, including linkage of UPR components to polygenic autoimmune diseases, diseases of altered ER function, misfolding protein diseases, and autoimmunity in highly secretory cells.

Inflammatory bowel disease results from the aberrant, overexuberant response to endogenous gut flora (197). Further, the association with *NOD2*, the first major gene linked to IBD, implicates innate immunity in the abnormal gut inflammation (198, 199). IBD is also one of the first polygenic disease to be genetically linked to UPR components (200). Specifically, a hypomorphic allele of *XBP1* increases risk of developing IBD. XBP1$^{\Delta IEX}$ mice, lacking XP1 in intestinal epithelial cells, develop spontaneous mild enteritis and are more susceptible to Dextran sodium sulfate-induced colitis (an experimental IBD model) (200). Autophagy or the process of "self-eating" interacts with the UPR on multiple levels, in that the UPR induces autophagic pathways and autophagy may limit the UPR (185). Interestingly, in the case of IBD, *ATG16L1*, encoding a core autophagy effector, also associates with IBD in human genetic screens, and ATG16L1$^{\Delta IEX}$ mice develop spontaneous Crohn's like ileitis (201–203). ATG16L1$^{\Delta IEC}$ and XBP1$^{\Delta IEC}$ double knockout mice develop very severe colitis, suggesting a functional synergy between defective autophagy and UPR in predisposing to colitis (202). Part of the role of the UPR in colitis appears to be in support of gut-protective secretion: XBP1 supports Paneth cell development and function (200). However, there is also a more direct inflammatory consequence of XBP1 deletion. Through an unclear mechanism, XBP1 deficiency results in hyperactivation of IRE1. ATG16L1 deficiency in gut intraepithelial cells independently results in increased IRE1, related to defective IRE1 clearance by autophagy (203). Increased IRE1 kinase activity induces augmented NF-κB activation and thus pro-inflammatory cytokine production. Indeed, gut deficiency of IRE1 or TNFR1 relieves the XBP1$^{\Delta IEC}$ inflammatory phenotype (202). Mucin production maintains the barrier between gut flora and epithelial cells; "Winnie" and "Eyeore" mice expressing misfolding Mucin2 have a deficiency in mucin production, exhibit increased gut permeability and strong UPR induction, and develop gut inflammation (innate immune and Th17) (204, 205). Two other ER genes have also been linked to IBD in humans, anterior gradient 2 (*AGR2*), encoding a protein disulfide isomerase, and Orosomucoid-like 3 (*ORMDL3*), which regulates ER calcium and induces UPR pathways (206–209). Agr2−/− mice develop severe spontaneous ileocolitis associated with defective mucin folding and ER stress (210). At this time, it is not clear how ORMDL3 regulates gut inflammation. Together these studies suggest that the UPR-autophagy interaction regulates the extent of inflammatory responses to gut flora and that defects in this axis predispose to IBD.

More recently, protein mishandling/altered trafficking at the ER has been identified as a monogenic cause of an autoimmune syndrome. Patients with mutations in *COPA* develop inflammatory interstitial lung disease with pulmonary hemorrhages, arthritis, autoantibody production, and renal disease (211, 212). *COPA* encodes a component of the COP I complex responsible for Golgi-ER retrograde transit and the syndromic mutations in this gene appear to disturb protein cargo recognition. *COPA* mutant cells display signs of ER stress with increased *BiP*, *ATF4*, and *CHOP* expression, although the precise mechanism linking this defect in retrograde transit with ER stress are not yet clear. The ER stress correlates with increased expression of IL-1β, IL-6, and IL-23 in immortalized B cells from these subjects, previously noted ER stress augmented cytokines (74, 104, 123). Perhaps as a result of increases in these specific cytokines, patients also exhibit an expansion of T helper 17 CD4+ T cells, a cell type implicated in autoimmunity (213). Interestingly, a number of these patients also have evidence for a type I IFN-regulated gene signature in their peripheral blood (214).

TNFR1-associated periodic fever syndrome is an autosomal dominant monogenic autoinflammatory disease that manifests with episodes of prolonged high fever, rash, abdominal pain, periorbital edema, and myalgia (189). Defective surface shedding of TNF receptors (and thus prolonged TNF signaling) was initially postulated as a pathogenic mechanism; however, several studies have shown that TNFR1-associated mutants form oligomers and aggregates in the ER, resulting in ER retention (215). Interestingly, these mutations were also associated with defective autophagy, and increasing autophagy with geldanamycin decreased IL-β production (216). Patient peripheral blood mononuclear cells (PBMC) expressed elevated levels of phosphor PERK and

spliced XBP1 mRNA, but not increases in other UPR-associated transcripts. Their monocytes had increased ROS as well (217). Transfection of cells with mutant TNFR1 did not induce BiP or CHOP expression, suggesting the UPR is not a direct contributor (215). However, cells from patients expressing mutant TNFR1 displayed increased mitochondrial ROS production, which promotes inflammatory cytokine production (218). Thus, ER stress may link misfolding TNFR1 to inflammation *via* ROS.

Spondyloarthritis encompasses a group of genetically and pathologically related inflammatory diseases which manifest with axial (spinal) arthritis, enthesitis, uveitis, gut inflammation, and psoriasis (219). SpA is highly linked to an MHC protein HLA-B27 that misfolds during biogenesis: in patients with the prototypic SpA, ankylosing spondylitis, 80–90% of subjects are HLA-B27 positive vs 6% of the United States population (220, 221). Although ankylosing spondylitis is a polygenic disease, the presence of HLA-B27 accounts for the preponderance (67%) of the currently identified heritability, conferring an odds ratio of >50 (222, 223). This misfolding propensity and prolonged association with BiP in the ER results from specific amino acids in its peptide-binding B pocket and unpaired cysteines (224–226). The subtypes of B27 with differential disease association also exhibit variance in biochemical features including thermos-stability, folding rates, and intracellular aggregation (227–229).

Transgenic HLA-B27 expression alone is sufficient to drive an inflammatory disease analogous to SpA in susceptible rat strains, although disease requires very high-transgene numbers (230, 231). Interestingly, disease does not occur in germ-free rats, but requires microbiota (232). Although there are many reasons why this might be the case, in light of the current discussion, one could speculate that microbiota may also be required to provide PRR signals that synergize with ER stress. Interestingly, CD8+ T cells are dispensable for disease development in rats, suggesting another property of HLA-B27 besides its antigen-presenting capacity may be important in driving disease (233). Bone marrow-derived macrophages from HLA-B27, but not HLA-B7 transgenic rats showed evidence for a UPR gene signature, particularly when class I MHC was acutely upregulated by cytokines such as TNF-α and/or IFN (234). These ER stressed macrophages responded to TLR agonists with greatly increased type I IFN *in vitro* (120). Interestingly, the bone marrow macrophages from the diseased B27 transgenic animals displaying a UPR gene signature also exhibited a very prominent IFN signature (234). However, the role of IFN, if any, in SpA has not been established. The inflamed colons in diseased animals exhibited upregulation of UPR target genes, along with increased IL-23, IL-17, IFN-γ expression, and expansion of Th17 cells (121). In an effort to more directly address the role of the UPR in these rats, one study interbred HLA-B27 transgenic rats with human beta-2 microglobulin overexpressing rats to stabilize and aid in HLA-B27 folding. This breeding did indeed reduce misfolding in Con-A stimulated splenocytes, although macrophages and tissue UPR were not assessed (235, 236). Surprisingly, these animals developed more severe arthritis, without changes to their colitis. This study suggests the role of HLA-B27-linked UPR may be discordant in the joints and the gut during SpA and raises further questions regarding HLA-B27 misfolding, UPR, and disease pathogenesis.

Although HLA-B27 can induce a UPR, it is not clear this property is the culprit in human subjects expressing at most two copies of the MHC molecule. HLA-B27 also forms surface dimers that can stimulate IL-17 producing cells, providing an alternative mechanism (237). Studies examining UPR in human subjects have yielded inconsistent results: increased BiP has been observed in knee fluid macrophages from ankylosing spondylitis patients (238). PBMC monocytes have been reported to express higher levels of UPR target genes, although other groups have reported a lack of UPR in PBMC and synovium (239, 240). Blood-derived macrophages from ankylosing spondylitis patients produce increased IL-23 in response to LPS without increased UPR target gene expression (241). Misfolded HLA-B27 has been detected in gut biopsies from SpA patients, but associated with activation of autophagy rather than UPR (242). Also, not all SpA (or even ankylosing spondylitis) patients are HLA-B27 positive. Interestingly, in a mouse model with altered autoreactive T cell repertoire, curdlan or zymosan treatment induces an SpA-like disease with enteritis, sacroiliitis, enthesitis, and psoriatic skin inflammation (243, 244). This disease model is also cytokine (IL-23 in particular) and gut microbiome-dependent (245). Interestingly, the inflamed colons from these animals showed evidence of UPR target gene induction (243). Thus, misfolding HLA is not an absolute prerequisite for UPR induction in SpA pathogenesis. These observations also raise the possibility that the UPR may be an integral part of the developing inflammatory process and not just the inciting event.

Myositis is another rheumatologic entity linking aberrant MHC, a type I IFN signature and the UPR. This group of diseases includes dermatomyositis, inclusion body myositis and dermatomyositis. Muscle biopsies from these patients exhibit either CD4+ or CD8+ T cell infiltrate, along with macrophages, and dendritic cells, implicating adaptive and innate immunity (246). Both peripheral blood (dermatomyostis and polymyositis) and muscle biopsies (dermatomyositis) showed evidence for a type I IFN signature and the blood signature correlated with disease activity (247–249). Muscle biopsies from autoimmune myositis patients and inclusion body myositis patients also showed evidence for UPR activation, supported by increased expression of BiP, PERK, GADD 153, ATF3, and chaperones such as grp94, calnexin, calreticulin, and ERp72 (250, 251). Myocytes do not typically express abundant MHC class I, but class I molecules are highly expressed in muscle from these patients, in conjunction with elevated ER stress markers and NF-κB activation (250, 252). Although the link between aberrant MHC expression and ER stress driven inflammation in human cells is mainly correlative, in mice, transgenic overexpression of H-2Kb in skeletal muscle drives an inflammatory myositis phenotype associated with autoantibodies and ER stress (253, 254). Myositis was particularly severe in young mice compared with adults (254).

Besides myositis, an increasing number of rheumatologic conditions appear to be associated with a type I IFN gene signature. This list prominently includes systemic lupus erythematosus (SLE), Sjogren's disease and systemic sclerosis (255). Moreover, in SLE, the gene signature also correlates with disease activity (256). Outside of plasma B cell development, current evidence for UPR activation in SLE is meager: lupus PBMC showed increased

XBP1s but decreased expression of IRE1, PERK, and CHOP (257). T lymphocytes from SLE patients may be more susceptible to ER stress-induced apoptosis, related to defective BiP and autophagy (258). On the other hand, anti-double-stranded DNA antibodies, which are characteristic of lupus, induced both ER stress and cytokine production from human kidney mesangial cells (259). In systemic sclerosis, PBMC from patients showed upregulation of BiP, ATF4, ATF6, XBP1s, along with increased DNAJB1 and IFN-related genes. Furthermore, ER stress markers correlated with disease severity (the presence of pulmonary arterial hypertension) and IL-6 levels (260). Systemic sclerosis involves overproduction of pro-fibrotic cytokines, such as TGF-β, aberrant tissue deposition of collagen, and differentiation of fibroblasts and epithelial cells into myofibroblasts (261). TGF-β increased ER stress in lung fibroblasts, as evident by BiP, ATF6, and XBP1s induction, and also increased expression of α-smooth muscle actin and collagen. Indeed, ER stress may mediate the induction of the myofibroblast proteins, as the chemical chaperone 4-PBA inhibited TGF-β induced α-smooth muscle actin and collagen induction (262). The IRE1α endonuclease pathway also regulated TGF-β driven myofibroblast differentiation in human cells (263).

Finally, autoimmunity frequently targets physiologically highly secretory cells. Autoimmune thyroid diseases are the most prevalent autoimmune conditions and thyrocytes pump out abundant thyroglobulin (264). Melanocytes mount a UPR to cope with melanin production and become targets in vitiligo (265). In the pancreas, β-cells are insulin-producing factories that increase production up to 25-fold in response to glucose (266). In vitiligo and diabetes, CD8+ T cells appear to kill their cellular targets very specifically, without damage to neighboring tissue (265). However, although the autoimmune destruction is carried out by adaptive immune cells, pro-inflammatory cytokine production plays a critical inciting role. The T cell recruiting IFN-regulated chemokine CXCL10 is critical for the development and maintenance of vitiligo (267). In diabetes, IL-1β and IFN-γ induce β-cell apoptosis by stimulating reactive oxygen and nitrogen species (268). Beta-cell death generates autoantigen. Beta-cells also secrete chemokines CXCL10 and CXCL9 that recruit T lymphocytes to the islets (269).

Endoplasmic reticulum stress and the UPR interweave through diabetes pathogenesis on multiple levels. The UPR is absolutely required for basal pancreatic function; PERK−/− mice die early from diabetes and exocrine pancreas failure (28). IRE1/XBP1s activity was also required for glucose-stimulated increases in insulin production and protection from oxidative stress (270). Islets from both diabetes-prone non-obese diabetic (NOD) mice and early human diabetes patients exhibited signs of chronic ER stress with increased CHOP expression and decreased pro-adaptive XBP1 and ATF6 (271, 272). Furthermore, treatment of the NOD mice with TUDCA restored UPR function and markedly protected NOD mice from the development of diabetes (271). Pro-inflammatory cytokines, particularly TNF-α, IFN-γ, and IL-1β induced ER stress (particularly CHOP upregulation) in β-cells (273, 274). TUDCA also protected islet cells from cytokine-induced JNK activation and apoptosis (274). The pro-insulin molecule is prone to misfolding, and human mutations that increase misfolding cause infantile diabetes (275, 276). In the Akita mouse model of diabetes, a mutation in the *Ins2* gene that prevents proper proinsulin folding (C96Y) leads to early onset diabetes associated with ER stress. CHOP deficiency delayed diabetes onset in this model by 8–10 weeks (277). In β-cells, activation of IRE1 promoted increased TXNIP expression *via* miR-17 degradation. TXNIP induction also depended upon PERK. ER stress-induced IL-1β and TXNIP-dependent apoptosis in islets.

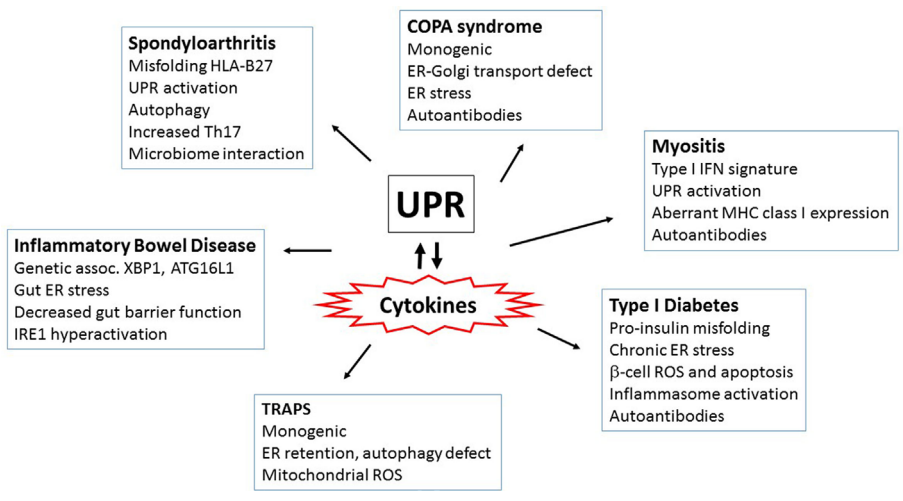

FIGURE 4 | Autoimmune and autoinflammatory diseases involving the unfolded protein response (UPR) and endoplasmic reticulum (ER) stress. Aberrant or excess cytokine production plays a key role in driving autoimmune and autoinflammatory disorders. Interestingly, ER stress and/or the UPR has been increasingly implicated in these same diseases. Thus, the multiple mechanisms by which the UPR interacts with cytokines (both cytokines inducing ER stress and UPR regulating cytokine production) have repercussions for the pathogenesis of inflammatory diseases. Several of the diseases highlighted in this review and the prominent features surrounding ER stress and cytokine induction are in boxes. More autoinflammatory disorders are to the left and autoimmune on the right.

In THP-1 monocytes, induction of IL-1β depended upon TXNIP and NLRP3 (111). Other studies have also linked NLRP3 and islet IL-1β in type 2 diabetes (278). In the Akita model, deletion of TXNIP protected against β-cell apoptosis and ameliorates diabetes severity (111). Interestingly, NLRP3 deficiency did not prevent diabetes in Akita mice, suggesting other inflammasomes or TXNIP activities may play a role (279). IRE1α has also been linked to the development of diabetes in the NOD mice: targeting the ABL kinases that hyperactivate IRE1 (and thus decreasing IRE1 activity) reversed diabetes in NOD mice (280). These studies provide tantalizing clues that link diabetes and IRE1 activation; however, the connection between ER stress and early cytokine production and apoptosis in these autoimmune conditions remains an open area of investigation. For a summary of the autoimmune and autoinflammatory disorders highlighted above, see **Figure 4**.

CONCLUSION AND PERSPECTIVES

In summary, the ER plays an indispensable role in cell function and is sensitive to many types of stress; the ER is thus perhaps uniquely poised to transmute significant threats to cell function into amplified immune responses. Because of this role in sensing threats that perturb proteostasis, ER stress has been referred to as a "dyshomeostatic DAMP" (14). From an evolutionary perspective, it may not be mere serendipity that UPR molecules exhibit homology with ancient cytosolic anti-viral proteins, PERK with PKR and IRE1 with RNaseL (281, 282). Numerous pathways interweave the UPR and inflammation, making the ER an effective nidus for promoting sterile inflammation or dramatically amplifying PRR responses. Specifically, the UPR regulates cytokine production through a variety of mechanisms extending from PRR sensing to inflammatory signaling and cytokine transcription factor activation. During infection, the UPR may enable cells to titer the degree of threat, providing greater cytokine responses for threats that impact cell function vs. those that merely stimulate PRRs. The UPR may also enable infected cells to sense invasion by pathogens that otherwise sabotage PRR signaling. Perhaps one of the costs of this inflammatory amplification is the potential for inappropriate activation in the absence of pathogens. The UPR has been increasingly implicated in the pathogenesis of a number of autoimmune and autoinflammatory conditions where cytokines play a central role. However, at this point, much of this is guilt by associations. Although the pieces are there (evidence for UPR, aberrant cytokine production), the exact causative relationships await further definition.

The material presented above raise a number of questions, ranging from mechanistic to teleological. Several questions surround the regulation of the different modes of IRE1 (kinase, RIDD, and XBP1 splicing) activation. Is degree of oligomerization critical or association with co-factors? Does XBP1 directly or indirectly limit kinase activity? Is this occurring *via* ERAD of IRE1? During TLR4 ligation how does XBP1 promote cytokine production but not its other UPR gene targets? Is this also related to co-factor or heterotypic binding? During viral infections, how does GADD34 promote IFN production and not translation of other targets? For that matter, how does Japanese encephalitis virus trigger RIDD but specifically avoid degradation? The relationship between the proposed microbial stress response and UPR also requires further clarification. Drawbacks to the TLR-mediated suppression of ATF6 and PERK include inhibiting cytokine promotion by these pathways (e.g., NF-κB activation) or adaptive pathways that enable cells to survive stress or commit apoptosis when infected. Infections may induce both ER stress and stimulate multiple PRRs. Perhaps the relative balance of PRR stimulation vs degree of ER stress sways the cell toward either UPR or microbial stress response.

Although the UPR *can* regulate cytokines, how much of a role *does* the UPR actually play in cytokine induction during infection and autoimmunity? Moving from the relatively clean results obtained with selective pharmacologic UPR agonists or PRR agonists to the "messy" reality of an intracellular infection or autoimmune disease has been challenging, related to the tremendous increase in complexity. Beyond cytokine regulation, the UPR heavily influences autophagy, nutrient mobilization, and cell death. These other effects of the UPR make it difficult to assign particular responsibility to its effects on cytokines. For instance, it is challenging to tease apart the direct effect of the UPR on viral replication vs. augmented IFN production. In autoimmunity, the UPR may critically regulate autoantigen generation (and presentation) or the basal function of immune type cells. This may be a deus ex machina concept, but perhaps the sheer number of intersections between cytokine regulation and the UPR and the magnitude of effect (e.g., log-fold for IFN) provide support for their significance in disease pathogenesis.

The availability of small molecule inhibitors or agonists for different UPR pathways has grown exponentially, driven by the interest in developing novel therapeutic approaches to cancer and autoimmunity. As an example of repurposed cancer drugs, proteosome inhibitors, which affect proteostasis (and thus ER function) and cytokine production, have demonstrated efficacy in murine lupus models (283, 284). UPR modulating agents may also be useful for intractable infectious diseases or vaccine development. Some of these UPR drugs have already moved to clinical trials. For instance, Celgosivir, which inhibits N-linked glycosylation, is undergoing a phase II trial for Dengue (285). Better understanding of the role of the UPR in specific settings will be critical for the judicious trial of these new therapies; given the complexity of UPR-immune interactions, carefully conceived pre-clinical studies may be necessary to gage the net effect of individual UPR modulating agents on specific infectious or inflammatory conditions. It will be important not to generalize, as the role of the UPR is likely to be highly context specific, even between species of pathogen. An example described above, *B. melitensis* and *B. abortus* have been reported to induce different degrees of UPR activation and blockade with TUDCA appears to have different effects on replication (118, 182). Also, it will be important to balance the anti-pathogenic effects of UPR modulation against the potential of disturbing physiologic UPR responses. Given the exciting clinical potential for UPR modulation, clarification of these issues has become a compelling mandate.

AUTHOR CONTRIBUTIONS

The author confirms being the sole contributor of this work and approved it for publication.

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REVIEW
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Metabolic Reprogramming in Modulating T Cell Reactive Oxygen Species Generation and Antioxidant Capacity

*Josephin N. Rashida Gnanaprakasam, Ruohan Wu and Ruoning Wang**

Center for Childhood Cancer & Blood Diseases, Hematology/Oncology & BMT, The Research Institute at Nationwide Children's Hospital, Ohio State University, Columbus, OH, United States

A robust adaptive immune response requires a phase of proliferative burst which is followed by the polarization of T cells into relevant functional subsets. Both processes are associated with dramatically increased bioenergetics demands, biosynthetic demands, and redox demands. T cells meet these demands by rewiring their central metabolic pathways that generate energy and biosynthetic precursors by catabolizing and oxidizing nutrients into carbon dioxide. Simultaneously, oxidative metabolism also produces reactive oxygen species (ROS), which are tightly controlled by antioxidants and plays important role in regulating T cell functions. In this review, we discuss how metabolic rewiring during T cell activation influence ROS production and antioxidant capacity.

Keywords: reactive oxygen species, oxidative stress, metabolism, T cell, antioxidant

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****Correspondence:***
Ruoning Wang
ruoning.wang@nationwidechildrens.org

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INTRODUCTION

T cells are central orchestrators of antigen-specific adaptive immunity and tolerance. Upon stimulation of antigen receptors, T cells rapidly transit from naïve to an active state followed by massive clonal expansion. Depending on the nature of pathogens and the surrounding cytokine milieu, proliferating T cells can differentiate into diverse phenotypic and functional subsets to elicit a robust immune response. After the clearance of pathogens, the majority of effector T cells die through apoptosis and the remaining memory T (T_{mem}) cells are responsible for immunity upon re-exposure to the same pathogen. Accumulating evidence suggests that a coordinated rewiring of cellular metabolism is required for T cell activation and differentiation by fulfilling the bioenergetic, biosynthetic, and redox demands (1–9). Importantly, different phenotypic and functional T cell subsets are characterized by distinct metabolic programs (**Table 1**), which are largely controlled by immune modulatory signaling cascades (10–17). Naïve T (T_{nai}) cells, T_{mem} cells, and immune-suppressive regulatory T (T_{reg}) cells predominantly rely on fatty acid oxidation (FAO) and oxidative phosphorylation (OXPHOS) to meet their relatively low-energy needs (14, 15, 18, 19). However, persistent aerobic glycolysis, the pentose phosphate pathway (PPP), and glutaminolysis are required to drive cell growth, clonal expansion, and effector functions in both $CD4^+$ subsets and $CD8^+$ effector T (T_{eff}) cells (**Table 1**) (10, 15, 16, 18, 20–31).

These metabolic programs actively support ATP production by providing mitochondrial OXPHOS substrates, support biomass accumulation by generating metabolic precursors for the biosynthesis of protein, lipids, and nucleic acids, and maintain redox balance through generation and elimination of reactive oxygen species (ROS).

TABLE 1 | The metabolic profiles of T cell subsets.

T cell type	Naïve	Active	Differentiated	Memory T cell (T_{mem})
Metabolic profile	FAO OXPHOS	Aerobic glycolysis PPP Glutaminolysis	Th1: aerobic glycolysis/some OXPHOS Th2: aerobic glycolysis Th9: aerobic glycolysis Th17: aerobic glycolysis, glutaminolysis Tfh: aerobic glycolysis, OXPHOS T_{reg}: FAO, OXPHOS CTL: aerobic glycolysis CAT: oxidation, phosphorylation	FAO OXPHOS

FAO, fatty acid oxidation; OXPHOS, oxidative phosphorylation; PPP, pentose phosphate pathway; Th1, T helper 1 cell; Th2, T helper 2 cell; Th9, T helper 9 cell; Th17, T helper 17 cell; Tfh, follicular helper T cell; T_{reg}, regulatory T cell; CTL, cytotoxic T lymphocyte; CAT, chronically activated T cell; T_{mem}, memory T.

MITOCHONDRIAL OXPHOS AND NADPH OXIDASES (NOXs) IN GENERATING ROS IN T CELLS

The mitochondria are the central metabolic hub and powerhouse of all eukaryotic cells. The oxidation of acetyl-CoA to carbon dioxide (CO_2) by the tricarboxylic acid (TCA) cycle is the central metabolic process for fueling ATP production. While glycolysis and FAO primarily provide the OXPHOX substrate, acetyl-CoA, for mitochondria in T_{nai} cells, T_{mem} cells, and T_{reg} cells (14, 15, 18, 19), heightened mitochondrial biogenesis during T cell activation leads to higher numbers of mitochondria and likely the enhanced mitochondrial dependent metabolic flux in T_{eff} cells compared with T_{nai} cells (23, 32, 33). In particular, a surplus of 3-, 4-, and 5-carbon metabolites (anaplerotic substrates) including pyruvate, malate, and α-ketoglutarate (α-KG) feed into the TCA cycle during the catabolism of glutamine and other amino acids (5, 13, 15, 34). The electron transport chain (ETC) constantly transfers electrons from NADH and FADH2 to oxygen while allowing protons (H^+) to pass through the inner mitochondrial membrane to form an electrochemical proton gradient that drives ATP synthesis. However, both protons and electrons can leak from the ETC due to the uncoupling of ATP synthase from the proton gradient and a premature exit of electron before reaching cytochrome *c* oxidase, respectively. Electron leak largely occurs at the sites of complex I (NADH–ubiquinone oxidoreductase) and complex III (ubiquinone–cytochrome *c* oxidoreductase) in the ETC and results in the partial reduction of oxygen, generating superoxide $\left(O_2^{-\bullet}\right)$. Subsequently, mitochondrial dismutase acts to convert superoxide to hydrogen peroxide (H_2O_2), which is free to diffuse into cytosol and act as a redox signaling molecule to elicit different cellular responses (35–37). Thus, increased ROS production in T cells can occur as a result of metabolic reprogramming during T cell activation. Besides mitochondria, cytoplasmic ROS is generated by NOXs, which is also an important source of ROS in T cell. NOX family proteins are transmembrane proteins that transport the electrons from nicotinamide adenine dinucleotide (phosphate), NAD(P)H, to oxygen and generate superoxide anion as the intermediate product of oxidase and subsequently H_2O_2, as the product of dismutation of the superoxide. There are different isoforms of the NOX enzyme including NOX1, NOX2, NOX3, NOX4, NOX5, dual oxidase 1, and DUOX2, and the expression of these subunits varies among different tissues. NOX-2 is an important source of ROS in T cells (38, 39). The ROS production by NOX is regulated at various levels including the assembly of functional NOX complex, the availability of prosthetic group, flavin adenine dinucleotide, the intracellular concentration of calcium, cell surface receptor signals mediated by G protein-coupled receptors, complement, T cell receptor (TCR), and CD28 (35–37, 40, 41).

ROS SIGNALING IN REGULATING T CELL ACTIVATION AND DIFFERENTIATION

T cell activation requires ligation of TCR and the major histocompatibility complex molecules. This interaction will initiate the signaling cascade and activation of transcriptional factors such as nuclear factor of activated T cells (NFAT), activator protein 1 (AP-1), and nuclear factor of kappa light chain enhancer in B cells (42). It has been reported that TCR ligation increases the production of ROS from OXPHOS and cytoplasmic ROS from NADPH oxidases (NOXs), a family of plasma membrane-associated oxidases (36, 40, 41). ROS-mediated signaling events are required for driving T cell activation, proliferation, and differentiation (**Figure 1**) (36, 41). T cells with reduced production of mitochondrial ROS display impaired production of interleukin-2 and antigen-specific proliferation, indicating an essential signaling role for mitochondrial ROS in driving optimal TCR signaling. The proximal TCR signaling machinery, including zeta chain-associated protein kinase 70, linker of activated T cell, SH2 domain-containing leukocyte protein, phospholipase Cγ1, and protein kinase Cθ, is involved in driving ROS production upon T cell activation (36, 41, 43). Conversely, physiologically relevant levels of ROS facilitate the activation of oxidation-dependent transcription factors, such as NF-κB and AP-1, which are required for driving essential signaling events to support T cell-mediated immune responses (44–46). However, excessive ROS production following ablation of *de novo* glutathione (GSH) synthesis suppresses the activity of mammalian target of rapamycin and the expression of transcription factors NFAT and c-MYC, the latter of which control metabolic reprogramming following T cell activation (15, 47, 48). Thus, T cells fail to meet their increased energy and biosynthetic needs and display compromised proliferation (48). In addition, uncontrolled ROS production is involved in the activation-induced T-cell death by affecting expression of apoptosis related genes including Bcl-2

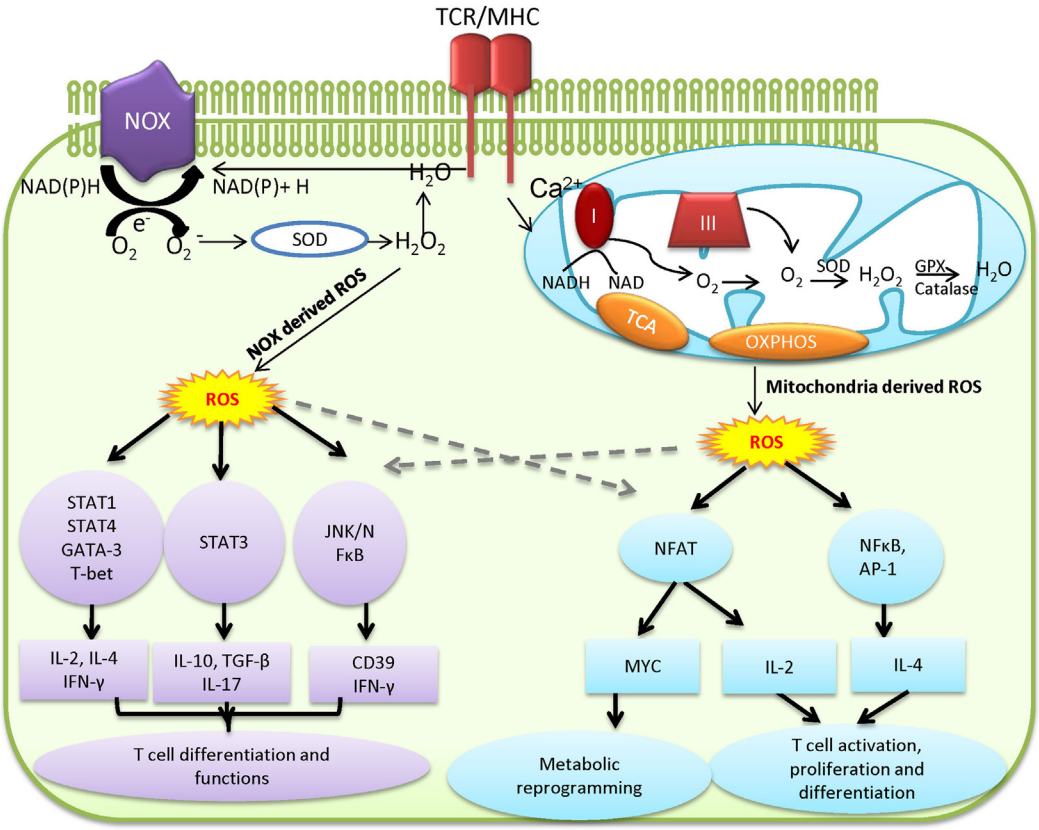

FIGURE 1 | Mitochondria and NADPH oxidases (NOX)-derived reactive oxygen species (ROS) regulates T cell activation, differentiation, and metabolism. Mitochondria and NOX are the two major sources of ROS. The stimulation of T cell receptor (TCR) initiates signaling and metabolic events that drive ROS production in cytoplasm through NOX-dependent reaction and ROS production in mitochondria *via* mitochondria electron transport chain (ETC). Excess ROS causes damage and cell death. However, physiologically relevant levels of ROS mediate essential redox signaling through nodulation of a wide spectrum of redox-sensitive transcription factors to drive T cell activation and function.

and FasL and mitochondrial membrane potential (43, 49–52). NOX-derived ROS modulates the function of GATA-binding protein 3, signal transducer and activator of transcription, and T-box transcription factor to collectively control T cell activation and differentiation. T cells from NOX-deficient animals showed a skewed Th17 phenotype, whereas NOX-intact cells exhibited a preferred Th1 response (39, 53–55). In CD8 T cells, NOX-derived ROS is involved in regulating the production of IFN-γ and CD39 expression through c-Jun N-terminal kinase and NFκB signaling (40, 56). Importantly, the impact of ROS on T cell activation can be extended to the later T cell differentiation stages. Fine tuning of ROS is required for polarizing T cell in part by engaging lineage-specific transcription factors and modulating cytokine profiles, and consequently directs T cell-mediated inflammatory responses (39, 40, 53–55, 57–61).

METABOLIC PATHWAYS IN MODULATING ANTIOXIDANT CAPACITIES

Excessive ROS production causes collateral damage to macromolecules, cellular organelles, and eventually necrosis, which can lead to uncontrolled hyper-inflammation and tissue damage. Thus, a fine-tuned balance between ROS production and antioxidant capacity ensures appropriate levels of intracellular ROS (**Figure 2**) (44, 55, 62). GSH, a tripeptide of glutamine, cysteine, and glycine, is the most abundant antioxidant capable of providing reducing equivalents and also serves as a versatile nucleophilic cofactor in a wide spectrum of metabolic reactions in aerobic organisms (63, 64). Thioredoxin (TXN) is a class of small redox proteins that are involved in modulating cell surface receptors and confers tolerance to oxidative stress in T cells (65–69). A reciprocal redox reaction can be coupled between these two antioxidant systems to act as a backup for each other under certain conditions (70–77). Supporting these findings, the inhibition of thioredoxin reductase (TXNRD) conferred an increased susceptibility of cancer cells to GSH depletion (78–80). Glutathione-disulfide reductase (GSR) regenerates GSH from its oxidized form, glutathione disulfide (GSSG), whereas TXNRD is responsible for the regeneration of TXN once it has been oxidized. Importantly, both GSR and TXNRD require NADPH as a reducing agent. Upon antigen stimulation, both PPP and glutaminolysis are significantly upregulated and further enhance T cell antioxidant capacities by generating NADPH through

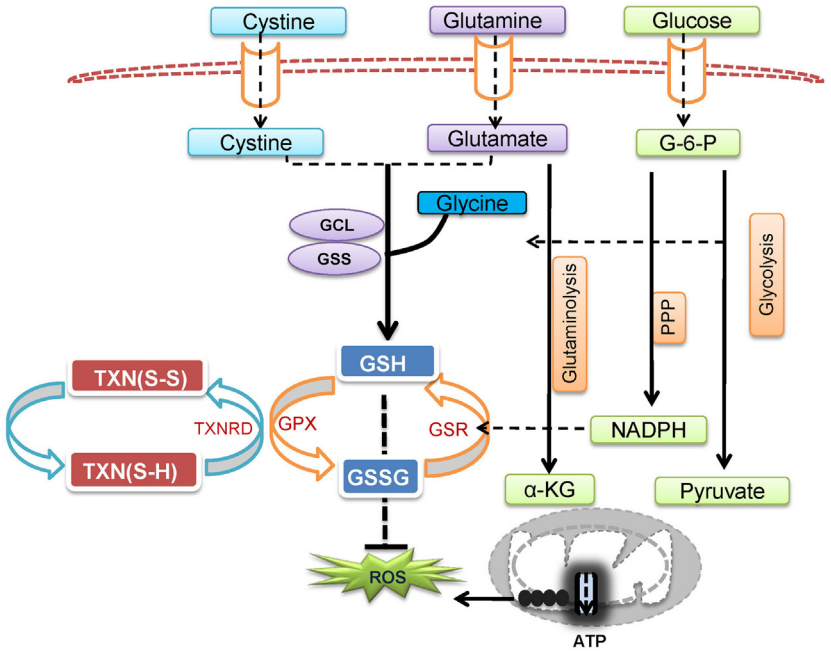

FIGURE 2 | T cell metabolic programs that link to reactive oxygen species (ROS) production and the *de novo* synthesis of GSH. Pyruvate that is derived from glucose *via* glycolysis is shuttled to the mitochondria and drives the tricarboxylic acid (TCA) cycle and fuels oxidative phosphorylation (OXPHOS). Glucose-derived glucose-6-phosphate feeds into the pentose phosphate pathway (PPP) and produces NADPH in the cytoplasm. In addition, glutamate feeds the TCA cycle through α-ketoglutarate (α-KG) to fuel OXPHOS and generate ROS. Excessive ROS production is regulated by glutathione (GSH), a tripeptide of glutamine, cysteine, and glycine, which is synthesized *de novo* by glutamate-cysteine ligase (GCL) and glutathione synthetase (GSS). In addition, NADPH, glutathione-disulfide reductase (GSR), and glutathione peroxidase (GPX) are involved in regenerating GSH from glutathione disulfide (GSSG), whereas thioredoxin reductase (TXNRD) is responsible for the regeneration of thioredoxin (TXN) to control oxidative stress in T cell.

metabolic reactions that are controlled by glucose-6-phosphate dehydrogenase, phosphoglycerate dehydrogenase, malic enzyme 1, and isocitrate dehydrogenase 1. The intracellular GSH concentrations are normally in a range of three orders of magnitude higher than extracellular GSH. Even though some cells are able to recycle extracellular GSH, it may only play a minor role in maintaining intracellular GSH pool (63, 64, 81–86). By contrast, both the regeneration of GSH from GSSG (recycling pathway) and *de novo* synthesis of GSH, by glutamate-cysteine ligase (GCL) and glutathione synthase (GS), are required to maintain intracellular GSH levels (64, 87). The ligation of glutamate and cysteine to form dipeptide ϒ-glutamylcysteine (ϒ-GC) is the first and also the rate-limiting step of GSH *de novo* synthesis, which is controlled by ATP-dependent ligase GCL, a heterodimer of a catalytic subunit (GCLC) and modifier subunit (GCLM). Subsequently, GSH is formed by GS-mediated ligation of ϒ-GC and glycine (88, 89). Thus, the supply of intracellular cysteine, glycine, and glutamate must fulfill the need of *de novo* synthesis of GSH during T cell activation. Supporting this idea, the metabolic processes that are involved in providing three amino acids are tightly regulated upon T cell activation (13, 15, 90–92). Upon T cell activation, heightened glycolysis, PPP, and glutaminolysis intersect with the *de novo* synthesis of GSH through promoting cysteine uptake and providing glycine, glutamine, and NADPH (93–95). As such, the genetic abrogation of *de novo* synthesis of GSH, the glucose, or glutamine starvation significantly dampens T cell activation (10, 13, 15, 20, 48).

GLUTAMINE CATABOLISM IN COORDINATING THE PRODUCTION OF ROS AND GSH

Glutamine has been known as a key nutrient, which supports a diverse range of cellular functions (93–102). Glutamine provides high proportions of the energy from OXPHOS, provides precursors for various biosynthetic pathways, as a key nitrogen and carbon donor, and also is catabolized to various intermediate metabolites that have signaling roles in modulating cellular processes. In specialized cells, such as the cells of the nervous system, glutamine catabolism intersects with signaling networks to support the production of central neurotransmitters including glutamate, GABA, and aspartate (103–106). To meet bioenergetic and biosynthetic demand during T cell growth and proliferation, glutaminolysis replenishes the anapleurotic substrate α-KG that fuels OXPHOS *via* the TCA cycle and also provides sources of nitrogen and carbon to support the biosynthesis of nonessential amino acids, lipids, nucleotides, and polyamines (13, 15, 102, 107). Similar to cancer cells, *de novo* synthesis of GSH in T cells, which relies on glutamine to provide precursors, plays

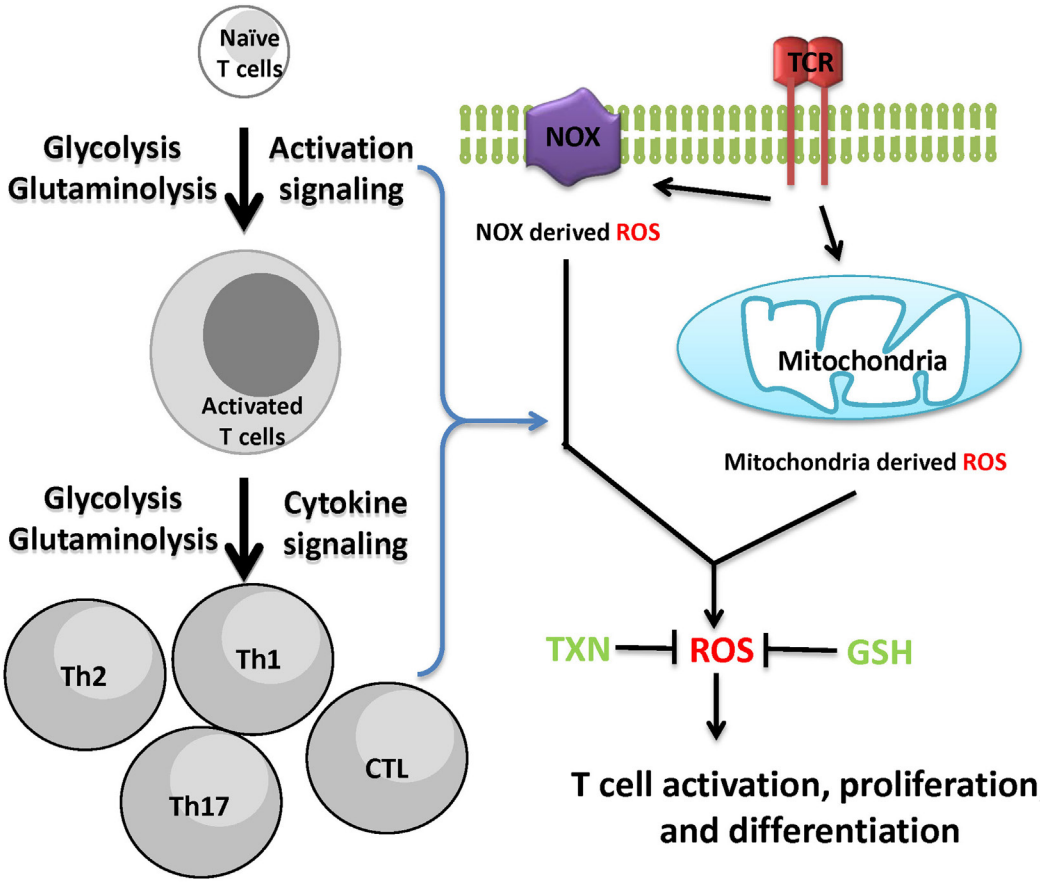

FIGURE 3 | Cellular redox homeostasis is essential for mounting an effective T cell-mediated immune response. In addition to generate ATP and provide biosynthetic precursors, T cell activation-induced metabolic reprogramming actively regulates redox homeostasis. The coordination of *de novo* synthesis of glutathione (GSH) and the production of reactive oxygen species (ROS) ensures T cell redox balance and a fine-tuned T cell response.

an essential role in suppressing oxidative stress. Accordingly, glutaminolysis is a branched pathway that consists of several paths, enabling energy production through oxidation and biomolecule production, including GSH through biosynthesis (93–95). While the ATP generating capacity of glutaminolysis is considered to be redundant with glucose oxidation and/or FAO, the oxidation of glutamine is indispensable for driving T cell proliferation and differentiation (13, 15, 102). However, enhanced glutamine oxidation in the mitochondria also increases the production of its by-product, mitochondrial ROS, the main source of cellular ROS in T cells (35, 37). Therefore, glutamate represents a key branch point in glutaminolysis that can be committed toward mitochondrial oxidation to produce ATP and ROS, or toward *de novo* synthesis of GSH to modulate redox balance and suppress oxidative stress. In addition, the high rate of glutaminolysis ensures that the capacity to supply glutamate, the most abundant intracellular metabolite in cells, exceeds the demand for glutamate from each of the downstream metabolic branches. The branched pathways in glutaminolysis enable the production of counteracting metabolites, i.e., ROS and GSH, from a common metabolic precursor, and permit a fine-tuned coordination between the metabolic flux shunted toward GSH synthesis and the metabolic flux shunted toward OXPHOS. Consistent with this idea, the overall high consumption rate of glutamine in proliferative cells is suggested to provide a sensitive and precise regulation on intermediate metabolites that can be committed toward several metabolic branches, hence permitting rapid responses to meet the demands for energy production or antioxidant production (99, 108). In addition to increasing antioxidant capacity, T cells may adapt by shifting glucose catabolism from OXPHOS toward aerobic glycolysis, which could provide biosynthetic precursors and rapidly produce ATP by the substrate level of phosphorylation.

CONCLUSION AND PERSPECTIVE

Reactive oxygen species is not only a by-product of cellular metabolic programs but also a key signaling molecule involved in directing T cell activation and differentiation. However, uncontrolled ROS production causes collateral damage to biomolecules and cellular organelles. Under pathophysiological conditions, ROS generation from mitochondria can contribute to the initiation and progression of inflammatory and autoimmune diseases. However, oxidative stress caused by elevated ROS

may also render key immune effector cells vulnerable to agents that can either modulate stress response or modulate metabolic pathways for ROS and GSH production (**Figure 3**). Redox signaling is essential to regulate T cell metabolism. Technological advancement in genetic models and metabolomics will allow us to understand the key metabolic processes that dictate T cell fate through modulation ROS and GSH production. Thus, further research is expected to illustrate the complex interplay between cellular metabolism and redox signaling in T cells, thereby offering novel therapies for treating inflammatory and autoimmune diseases.

AUTHOR CONTRIBUTIONS

JG and RHW wrote the manuscript. RW wrote and finalized the manuscript.

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REVIEW
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Autophagy and Its Interaction With Intracellular Bacterial Pathogens

*Mariana da Silva Siqueira, Renato de Moraes Ribeiro and Leonardo H. Travassos**

Laboratory of Immunoreceptors and Signaling, Immunobiology Program, Institute of Biophysics Carlos Chagas Filho, Federal University of Rio de Janeiro, Rio de Janeiro, Brazil

Cellular responses to stress can be defined by the overwhelming number of changes that cells go through upon contact with and stressful conditions such as infection and modifications in nutritional status. One of the main cellular responses to stress is autophagy. Much progress has been made in the understanding of the mechanisms involved in the induction of autophagy during infection by intracellular bacteria. This review aims to discuss recent findings on the role of autophagy as a cellular response to intracellular bacterial pathogens such as, *Streptococcus pyogenes*, *Mycobacterium tuberculosis*, *Shigella flexneri*, *Salmonella typhimurium*, *Listeria monocytogenes*, *and Legionella pneumophila*, how the autophagic machinery senses these bacteria directly or indirectly (through the detection of bacteria-induced nutritional stress), and how some of these bacterial pathogens manage to escape from autophagy.

Keywords: autophagy, infection, *Mycobacterium tuberculosis*, *Shigella flexneri*, *Listeria monocytogenes*, *Salmonella typhimurium*, *Streptococcus pyogenes*, *Legionella pneumophila*

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***Correspondence:**
Leonardo H. Travassos
leo.travassos@biof.ufrj.br

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INTRODUCTION

Autophagy is a homeostatic and highly conserved survival mechanism in which portions of the cytoplasm such as long-lived proteins and damaged organelles are sequestered in double-membrane vesicles (called autophagosomes). Then, autophagosomes fuse with lysosomes, leading to the degradation of the sequestered content and recycling of functional blocks for anabolic processes, especially during nutrient shortages (1). Indeed, for many years, autophagy was mainly considered as a breakdown process to degrade macromolecules to generate energy during nutrient deprivation. To date, three types of autophagy have been described, chaperone-mediated autophagy, microautophagy, and macroautophagy (1). Here, we discuss the interaction of the best-characterized type of autophagy (macroautophagy, hereafter autophagy), with intracellular bacterial pathogens, a process designated xenophagy.

The first report demonstrating induction of autophagy by bacteria was published in 1984. In this study, Rikihisa described the presence of vesicles containing glycogen granules and rickettsiae in Guinea pig polymorphonuclear (PMNs) cells infected with the bacteria (2). Despite this initial study, it was only after the studies from Nakagawa et al. and Gutierrez et al. that autophagy was regarded as an important cell autonomous arm of the innate immune system against intracellular bacteria. In their seminal and independent studies, Nakagawa et al. and Gutierrez et al. demonstrated a crucial role for autophagy in the sequestration and degradation of group A *Streptococcus* (GAS) and *Mycobacterium bovis* BCG, respectively (3, 4). Since then, an amazing number of elegant studies have demonstrated a key role of autophagy in the control of infection by different bacterial pathogens and also how some of these most well-succeeded pathogens circumvent or even use autophagy to establish replicative niches inside different cell types (5–7).

The Autophagosome Formation Core Machinery

Possibly one of the most exciting areas in the field of autophagy, the mechanisms involved in the formation of autophagosomes, the hallmark of this process, have been the focus of many research groups. Morphologically, autophagy begins with the formation of a cup-shaped double-membrane structure that surrounds the cargo. Upon its complete closure, the phagophore is now called an autophagosome, a transient organelle that delivers its content for degradation in lysosomes (8). After extensive work from several groups, the proteins that participate in autophagosome biogenesis can be categorized into complexes that take place in different steps of the autophagosome formation (1). Below, we will summarize the different steps of the autophagic process and the major protein groups that take part in each step of the whole process and discuss critical findings linking these proteins with bacterial-induced autophagy. For extensive literature on autophagosome formation machinery, please refer to Suzuki et al. (9) and Yin et al. (10).

Signal Induction

The ULK Complex and Autophagy Induction

The uncoordinated-51-like kinase (ULK1) complex comprising ULK1, ATG13, FAK family kinase-interacting protein of 200 kDa (FIP200), and ATG101 is responsible for sensing changes in nutrient status within the cell. Its activation is instrumental in the initiation of autophagy. This complex works downstream mammalian target of rapamycin complex 1 (mTORC1) and under, nutrient-rich conditions is phosphorylated by mTOR, which inhibits ULK1 recruitment to the phagophore assembly site (PAS).

Under nutrient starvation, however, mTORC1 is inactivated, and ULK1 is released, allowing FIP200 phosphorylation and translocation of the complex to PAS for the recruitment of ATG proteins, required for autophagosome formation (11). Interestingly, components of the ULK complex have also been shown to target bacterial vacuoles during infection with intracellular bacteria (12). This is the case of FIP200 during infection with *Salmonella typhimurium*. Experiments performed by Kageyama et al. suggest that this protein is recruited to the vicinity of vacuoles containing *S. typhimurium.* See below for more detailed information regarding autophagy induced by this pathogen.

Nucleation

Class III Phosphatidylinositol 3-Kinase (PtdIns3K) Complex and Trafficking of Atg9 for Autophagosome Nucleation

The class III PtdIns3K complex consisting of Beclin 1, ATG14L, phosphoinositide 3-kinase regulatory subunit 4 (PIK3R4) are recruited to PAS to initiate phagophore membrane nucleation through the activation of PtdIns3-kinase class III (PtdIns3KC3). As a result, PtdIns3P is generated at this site, and the PtdIns3P-binding protein WD-repeat domain phosphoinositide-interacting 1 (WIPI1) and 2 (WIPI2) are recruited to the PAS, allowing ATG proteins to be recruited later on (13). Mammalian Atg9 (mAtg9) is another protein required for the assembly of phagophore, although its role is still not completely understood. It has been demonstrated that mAtg9 is not necessary for LC3 recruitment to phagophore, but essential for its generation following infection with *Salmonella typhimurium* (12).

Expansion

Ubiquitin-Like Conjugation Systems and Autophagosome Expansion

Pivotal for the formation of autophagosomes are two ubiquitin-like conjugation systems: Atg8/LC3 and Atg12. The Atg8/LC3 system modifies the core autophagy protein microtubule-associated 1 light chain 3 (LC3). LC3 has a diffuse cytosolic distribution pattern and is cleaved at its C-terminus by the cysteine protease Atg4 to form LC3-I, which has a C-terminal glycine residue. Upon autophagy induction, LC3-I is sequentially modified by the E1-like enzyme Atg7 and the E2-like enzyme Atg3 to form LC3-II after the conjugation of LC3-I to phosphatidylethanolamine (PE). This lipidated form of LC3 is attached to both outer and inner phagophore membrane being eventually removed from the autophagosomal membrane by Atg4 before the fusion with late endosomes/lysosomes (1, 14). In the Atg12 conjugation system, Atg5 and Atg12 proteins form a complex through the covalent binding of Atg12 to the C-terminus of Atg5 in a reaction involving Atg7 and Atg10. Then, the scaffold protein Atg16L1 is conjugated to Atg5 *via* its N-terminus, forming the 800 kDa Atg12–Atg5–Atg16L1 complex. It has been proposed that the Atg16L1 complex works as an E3-like enzyme to target LC3-I to its membrane site of lipid conjugation (15). Data from the literature suggest that these two systems work coordinately as in Atg3-deficient cells, where no LC3-II is found, Atg12–Atg5 conjugation is dramatically reduced (16).

Alternative (non-canonical) forms of autophagy have been identified and reported to target invading bacteria (17–19). In this review, however, we will focus on xenophagy and its implication in intracellular bacterial infections.

Cargo Selection During Infection With Bacterial Pathogens

Invasion of host cytosol by bacteria imposes a significant challenge to homeostasis and triggers several cellular and immune responses such as proinflammatory cascades and cell-autonomous in an attempt to control of bacterial replication, such as xenophagy.

In addition to the steps discussed above, autophagy has an additional and essential step that is cargo selection. One of the central questions regarding xenophagy relates to its specificity and how autophagy machinery specifically recognizes bacteria. This is of major importance as xenophagy, which eventually aims to reduce not only bacterial load but also prevent cellular stress resulting, for instance, from bacteria-induced amino acid starvation (see later in this review). To explain the central mechanisms involved in the selection of intracellular bacteria by the autophagy machinery, we will focus on bacterial models that helped us shape the field.

Mycobacterium tuberculosis

Mycobacterium tuberculosis is the causative agent of tuberculosis (TB), possibly one of the oldest human pathogens and still

among the top 10 causes of death worldwide (20). *M. tuberculosis* is a non-motile and facultative intracellular pathogen of macrophages. In this regard, the infection of alveolar macrophages is a crucial requisite toward the establishment of a successful replicative niche. Experiments using mice depleted for resident alveolar macrophages have shown that these animals become protected from *M. tuberculosis* (21). One of the main features of TB pathogenesis is the ability of *M. tuberculosis* to survive within alveolar macrophages through the interference with phagolysosome biogenesis (3, 22).

In the last decade, autophagy emerged as an essential protective strategy employed by the host to restrict the spread of *M. tuberculosis*. The first piece of evidence on the role of autophagy in the control of *Mycobacterium* was provided by the cornerstone study of Gutierrez et al. (3). The authors demonstrated that upon the induction of autophagy by starvation or rapamycin *M. tuberculosis* variant *bovis* BCG colocalized to LC3$^+$ compartments in RAW 264.7 macrophages. Moreover, BCG phagosomes were shown to be positive for markers of acidification such as cathepsin D and Lamp-1, suggesting that xenophagy induction was able to override the blockade in phagosome maturation by BCG, with a clear impact on bacterial killing (**Figure 1**). Interferon-γ (IFN-γ) is essential for resistance to infection, by interfering with the transcription of more than 2,000 genes (23). In a more physiological context, Gutierrez et al. demonstrated that IFN-γ, a potent activator of macrophages, was able to mimic the effects of rapamycin or starvation on the induction of autophagy, through the immunity-related p47 guanosine triphosphatases (IRG) Irgm1 (LRG-47) (3) (**Figure 1**). These results put autophagy on the center stage of the immune mechanisms involved in the

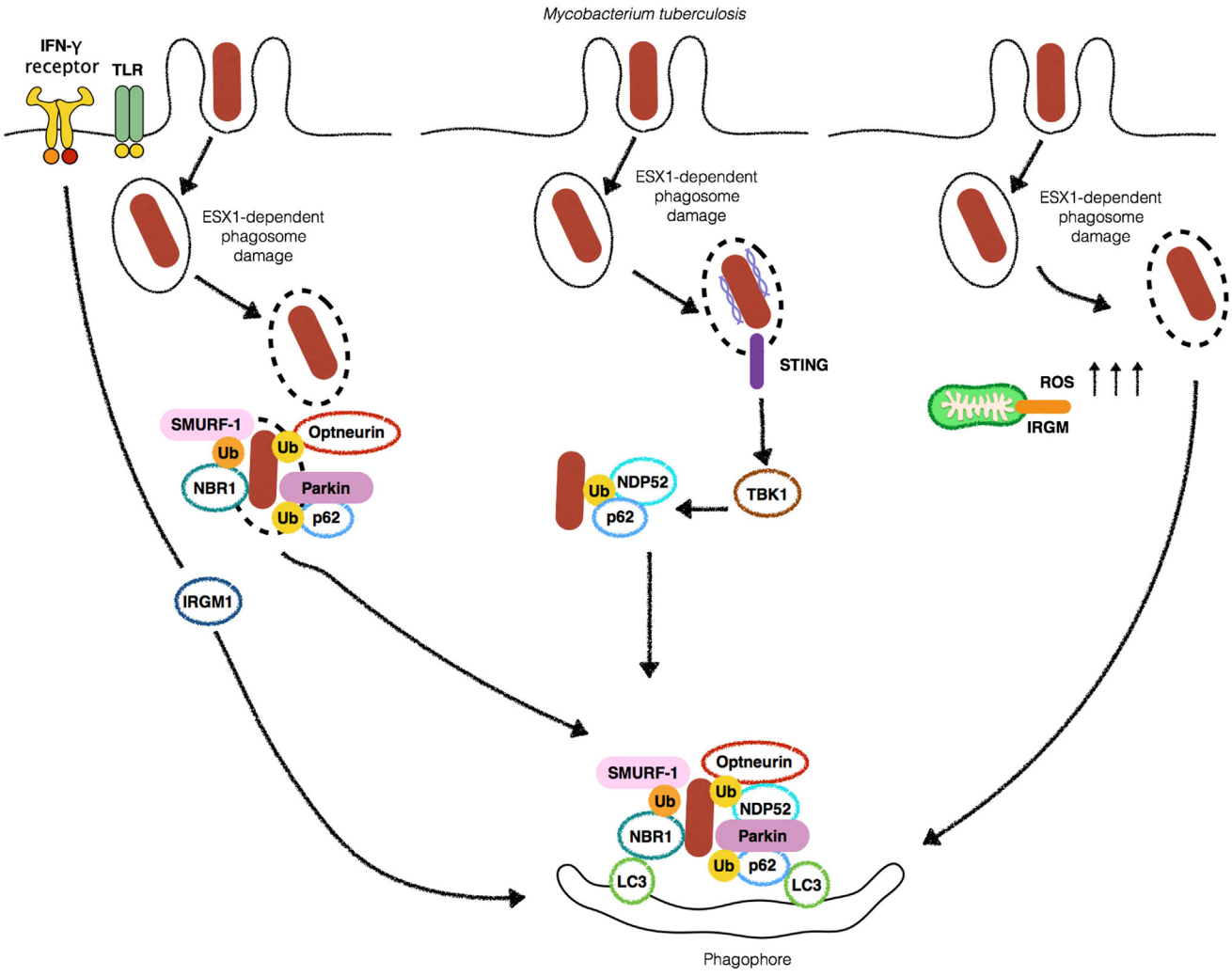

FIGURE 1 | Autophagy targets *Mycobacterium tuberculosis* (Mtb) by different mechanisms. Stimulation with IFN-γ or Toll-like receptors (TLRs) ligands leads to an increase in the localization of (Mtb) into autophagosomes (left). 6-kDa early secretory antigenic target (ESAT-6) secretion system 1 (ESX-1)-induced phagosomal damage induces the exposure of Mtb to cytosolic autophagy adaptors such as Optneurin, p62, NDP52 and NBR1 which bind to ubiquitin associated with Mtb as a consequence of the E3-ligases SMURF-1 and Parkin, culminating with targeting of the bacteria for autophagic degradation (center). Extracellular bacterial DNA from Mtb is detected by STING to activate TBK1 and lead to Mtb ubiquitination and recruitment of p62 and NDP52 (middle-left). IRGM-induced increase in ROS provokes autophagic targeting of Mtb (right).

protection against *M. tuberculosis* infection. After their initial discoveries, in a subsequent study, the same group demonstrated that both Irgm1 and its human ortholog IRGM are necessary for the induction of autophagy, generating large autolysosomes that contributed with *M. tuberculosis* intracellular growth restriction upon macrophage activation by IFN-γ (24). The mechanism behind IRGM restriction of *M. tuberculosis* seems to rely on its interaction with cardiolipin in mitochondria to generate ROS and mitochondrial fission, both necessary for *M. tuberculosis* killing (25). The role of IFN-γ in autophagy also involves the participation of interferon-induced guanylate-binding (GBP), which are also upregulated in the presence of the cytokine. It has been demonstrated that GBPs promote oxidative killing and the delivery of antimicrobial peptides to autophagolysosomes, contributing to *M. tuberculosis* intracellular replication control (26) (**Figure 1**). Altogether, these studies demonstrated an essential *in vitro* role for xenophagy and its induction by IFN-γ in the control of *M. tuberculosis* intracellular replication.

Although the link between deficiencies in ATG proteins and defective xenophagy has been widely reported upon infection with *M. tuberculosis*, ATG5 have also been described to play a critical autophagy-independent role in an *in vivo* TB mouse model. Kimmey et al. have demonstrated that the deletion of *Atg14L*, *Atg12*, *Atg16L1*, *Atg7*, and *Atg3* in the myeloid compartment did not affect the outcome of *M. tuberculosis* infection, suggesting that the loss of autophagy is not implicated with the progression of the disease. In sharp contrast, the authors reported that the loss of ATG5 in PMN but not in alveolar macrophages led to exacerbated imunopathology, sensitizing mice to *M. tuberculosis*. Together, these findings suggest that ATG5 has unique autophagy-independent features that are not shared with other ATG proteins, pointing for a reinterpretation of the role of ATG5 in the control of *M. tuberculosis* infection *in vivo* (27).

One primary open question that remained to be answered was how eukaryotic cells sense *M. tuberculosis* infection to induce autophagy. Toll-like receptors (TLRs) detect a myriad of extracellular and endolysosome located microbial products. It has been reported that Poly (I:C), LPS, and ssRNA, ligands for TLR3, TLR4, and TLR7, respectively, induce autophagosome formation through MyD88-dependent pathways. Interestingly, activation of TLR7 by its ligand increased the ability of macrophages to kill BCG (28) (**Figure 1**). However, it was not clear how TLRs would be able to detect BCG to induce autophagy in the absence of exogenous stimulation with their cognate ligands. The first molecular evidence of the detection of *M. tuberculosis*-derived microbial-associated molecular pattern (MAMP) triggering autophagy demonstrated that stimulation of interferon genes (STING), an important adaptor of TANK-binding kinase (TBK1) in the interferon stimulatory DNA pathway, senses the presence of cytosolic DNA to trigger the ubiquitination of *M. tuberculosis* after phagosome damage. Upon sensing of extracellular DNA from *M. tuberculosis* by STING, *M. tuberculosis* is ubiquitinated, leading to the recruitment by the autophagic adaptors p62/SQSTM1 (hereafter p62), a multi-domain protein that functions as an autophagic adaptor. p62 possesses an LC3-interacting protein region (LIR) and a C-terminal ubiquitin-associated (UBA) domain that binds ubiquitinated substrates and an LIR. Together with p62 and nuclear dot protein 52 kDa (NDP52) work to link ubiquitinated substrates to LC3 recruitment, ensuring the efficient delivery of *M. tuberculosis* to autophagosomes (29) (**Figure 1**). Although the sequestration of *Mycobacteria* by xenophagy has been demonstrated to be mostly dependent on ATG proteins, one report has been shown that sequestration of ubiquitinated mycobacteria can occur in ATG5-independent manner. The authors found that following 6-kDa early secretory antigenic target (ESAT-6) secretion system 1 (ESX-1)-mediated phagosome escape, ubiquitinated bacteria were resequestered by structures that resembled autophagosomes and localized to Lamp-1$^+$ compartments. Notably, ubiquitinated *M. marinum* were never decorated with LC3 and ATG5 deficiency and did not affect bacterial counts. It remains to be elucidated if the finding that *M. marinum* did not localize to LC3$^+$ compartments represents a potential specific mechanism of escape from autophagy (30).

Upon phagosome damage mediated by ESX-1, *M. tuberculosis* is ubiquitinated, in an essential step required for the recruitment of the autophagic adaptors p62 and NDP52 and LC3. Although it has not been determined, which bacterial or host proteins (or both) are ubiquitinated during xenophagy, much progress has been made in the identification of host proteins that mediate ubiquitination involved in xenophagy. Several ubiquitin-ligases have been described as participants of bacterial ubiquitination. Parkin has a well-established role in mitophagy where it promotes the ubiquitination of mitochondrial surface proteins prior to the recruitment of p62 in order to direct malfunctioning mitochondria for autophagic degradation. In 2013, Parkin was also reported to be crucial in the conjugation of K63-ubiquitin chains to *M. tuberculosis* inside macrophages. In line with this finding, *Park2*$^{-/-}$ displayed increased *M. tuberculosis* replication in an *in vivo* TB model (31) (**Figure 1**). Of note, Parkin has also been demonstrated to participate in ubiquitination of other mycobacterial species such as *M. leprae* (32). Similarly, SMAD-specific E3 ubiquitin-ligase protein 1 (Smurf1) has been demonstrated to mediate K48- but not K63-ubiquitination and the recruitment of the autophagy adaptor NBR1 during *M. tuberculosis* infection to control its replication in human macrophages and to associate with bacteria present in the lung of patients with pulmonary TB (33). In their study, Franco et al. reported that Smurf1- but not Parkin-dependent ubiquitination is necessary for the recruitment of proteasome and NBR1 for the vicinity of *M. tuberculosis*. In contrast, K63 ubiquitination by Parkin but not Smurf1 is required for the recruitment of p62 to the bacterial surface (**Figure 1**). It remains to be elucidated why host cells employ different ubiquitin-ligases with apparent redundant roles for targeting *M. tuberculosis* for xenophagy. One possibility is that the apparent redundancy of Smurf-1 and Parkin is a countermeasure resulting from the ability of *M. tuberculosis* to escape from autophagy. Also, the different ubiquitin moieties added to *M. tuberculosis* surface could help in the recruitment of various adaptors. Indeed, Smurf1-mediated ubiquitination recruits the adaptor NBR1, which is not recruited by Parkin-mediated activity.

Several recent studies have reported that *M. tuberculosis* uses sophisticated mechanisms to escape xenophagy and replicate inside host cells. In addition to the induction of miR33 and

miR33* expression to manipulate cellular metabolism and energy levels (34) and miRNA125a to inhibit UVRAG expression (35) (discussed later in this review), *M. tuberculosis* also induces the expression of other microRNAs (miRNAs) to circumvent xenophagy by interfering with different aspects of cellular physiology. This is the case of miR30A that has its expression increased during infection with *M. tuberculosis* to decrease Beclin 1 expression levels, leading to inhibition autophagosome elongation to promote intracellular survival of *M. tuberculosis* (36). Similarly, miR144* inhibits antimicrobial responses against *M. tuberculosis* in monocytes by targeting the expression of DNA damage-regulated autophagy modulator 2, allowing *M. tuberculosis* replication (37). In contrast, miR155 has been demonstrated to play a pro-autophagic role during *M. tuberculosis* infection. Wang et al. reported that miR155 targets Ras homolog enriched in brain (Rheb), a negative regulator of autophagy to accelerate the process of xenophagy. Inhibition of autophagy by *M. tuberculosis* seems to aim not only xenophagy but other essential steps of the immune response as well. It has been recently demonstrated that the bacterial factor PE_PGRS47 inhibits autophagy through an unknown mechanism to block MHC II antigen presentation and dampen adaptative immune responses against *M. tuberculosis* (38). Altogether, these studies provide compelling evidence that despite the crucial role of xenophagy as an antimycobacterial mechanism, *M. tuberculosis* has developed means to escape autophagy and replicate within macrophages.

Streptococcus pyogenes

Streptococcus pyogenes is the causative agent of a variety of infections, ranging from such as pharyngitis and skin infections to life-threatening necrotizin fasciitis and bacteremia (39). In 2004, Nakagawa et al. provide one of the first definitive evidence of the role of autophagy as a cell-autonomous antimicrobial mechanism. In this study, HeLa cells were shown to specifically target cytosolic GAS. This process was dependent on the toxin streptolysin O (SLO), a cholesterol-dependent pore-forming cytolysin (40). Nakagawa et al. demonstrated that the majority of the cytosolic population of GAS colocalized to LC3+ compartments, in contrast to SLO-deficient mutants in which no colocalization with LC3 was found (4) (**Figure 2**). As demonstrated for other intracellular bacteria, the adaptors p62, NDP52, and NBR1 are essential for recognition of ubiquitin decorated GAS and recruitment of LC3 before autophagic degradation (41, 42). Evasion of xenophagy by GAS has been reported, and GAS has been shown to evade ubiquitin recognition by the abovementioned autophagic adaptors. Barnett et al. have found that the globally disseminated serotype M1T1 (strain 5448) clone of GAS can avoid xenophagy to replicate in the cytosol. This is achieved by the expression of SpeB, a cysteine protease that degrades p62, NDP52, and NBR1. M1T1 Δ*speB* mutants fail to evade recognition by these proteins and are efficiently degraded through xenophagy (41) (**Figure 2**). These findings reveal a new mechanism by which GAS evades elimination by xenophagy. Notably, data from the literature demonstrate that xenophagy efficiently eliminates other GAS serotypes such as M6, M49, and M89. GAS is a successful human bacterial pathogen that causes a vast array of diseases and the work of Barnett et al. uncovers autophagy evasion as a determinant feature for the dissemination of GAS. The mechanisms employed by autophagy to target intracellular GAS also include the engagement of the CD46 pathogen receptor (43). CD46 is a glycoprotein expressed by all nucleated human cells that physically binds several pathogens such as adenoviruses B and D, human herpesvirus 6, *Neisseria*, and GAS (44) (**Figure 2**). Although several innate immune receptors such as TLRs have been described to trigger xenophagy upon infection or ligand stimulation, how these receptors are connected to the selective targeting of intracellular bacteria to lysosomes is still unclear. The findings from Joubert et al. provide an important piece of data to this open question. One possibility that needs to be experimentally tested is that CD46 might be concomitantly activated together with TLRs to promote xenophagy. Another known host factor that has been reported to participate in GAS targeting for xenophagy is 8-nitroguanosine 3',5'-cyclic monophosphate (8-nitro-cGMP), a downstream mediator of nitric oxide that has been shown to promote protein *S*-guanylation on bacterial surface, which are then K63 ubiquitinated prior to the recruitment of LC3 (45). Although these findings shed light into a new xenophagy targeting mechanism during infection with GAS, some open questions remain, such as (i) is this mechanism specific for GAS? and (ii) which autophagy adaptors and ubiquitin-ligases

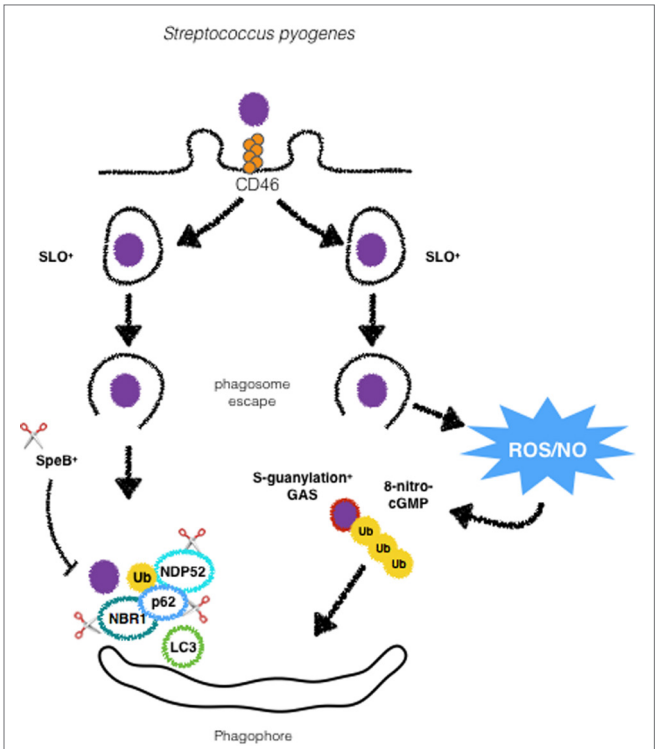

FIGURE 2 | GAS is targeted by xenophagy by different mechanisms. Following activation of CD46, GAS is directed to autophagosomes. Streptolysin O promotes escape from phagosomes and ubiquitination and recognition by autophagic adaptors p62, NDP52 and NBR1. SpeB producing strains are able to degrade such adaptors to escape from xenophagy. GAS can also undergo modifications by ROS/NO-induced 8-nitro-cGMP via S-guanylation of its surface proteins followed by ubiquitination and targeting to autophagosomes.

participate in this process. One interesting question regarding the induction of xenophagy by GAS is the role of endothelial cells in this process. Despite different reports showing that xenophagy plays an important role in the clearance of intracellular GAS, in endothelial cells, the results are contrasting. While, Cutting et al. demonstrated the ability of endothelial cells to upregulate xenophagy in order to control GAS infection, a recent study from Lu et al. reports that endothelial cells fail to target GAS for degradation due to an intrinsic defect in the ubiquitination of intracellular bacteria (46, 47). Even though much progress has been done in the understanding of the mechanisms of GAS-induced autophagy, further studies are required in order to clarify whether endothelial cells are in fact defective in xenophagy, if this defect is specific for infection with GAS or if GAS can halt xenophagy in these cells and not in epithelial cells.

Shigella flexneri

Shigella spp. are Gram-negative and highly invasive enteropathogens and a significant cause of disease, especially in children under the age of 5 years, causing approximately one million deaths worldwide (48). A few minutes after its invasion of epithelial cells and macrophages, *S. flexneri* is able to lyse the phagocytic vacuole and access the cytosolic compartment where it replicates (49). As countermeasures, host cells trigger autophagy to restrict *S. flexneri* intracellular growth and cell-to-cell spreading. The first evidence of an interaction between *Shigella* and autophagy was provided by a study dating from 2005. In this study, it was demonstrated that wild-type *S. flexneri* can escape from autophagic targeting by employing IcsB, one of the effectors of its type 3 secretion system (T3SS). Ogawa et al. observed that deletion mutants for IcsB, which is secreted by cytosolic bacteria and localizes to the bacterial surface were more efficiently targeted by autophagosomes. These results suggest that *S. flexneri* is able to escape from xenophagy. According to this study, the escape mechanism employed by *S. flexneri* involves IcsA/VirG, a 52 kDa protein that requires the bacterial chaperone IpgA for its stability, activates the complex-related proteins (Arp) 2/3 complex through the recruitment and activation of N-WASP, to induce actin polymerization and bacterial motility within the cell (50–54). Mechanistically, the study of Ogawa et al. demonstrated that, in Δ*icsB* mutants, IcsA/VirG triggers autophagy by binding to ATG5. According to the authors, IcsB inhibits IcsA/VirG affinity for ATG5. Thus, in wild-type *S. flexneri,* IcsB reduces IcsA/VirG affinity for ATG5 to initiate xenophagy (52) (**Figure 3**). More recently, a study added more complexity to the role of IcsB as a factor contributing to *S. flexneri* escape from autophagy. Baxt and Goldberg reported that IcsB also contributes to *S. flexneri* escape from xenophagy by recruiting transducer of CDC42-dependent actin assembly 1 (Toca-1) to prevent the recruitment of the adaptor NDP52 and LC3 (55).

The ubiquitination of *S. flexneri* has been reported to be essential for the recruitment of the adaptors p62 and NDP52 (56). However, in contrast to *S. typhimurium* and *M. tuberculosis* for which several ubiquitin-ligases that ubiquitinate bacterial surface have been described, the mechanism used to host cells to tag *S. flexneri* with ubiquitin is not clear. LUBAC is an ubiquitin-ligase that mediates the formation of M1-linked ubiquitin chains that culminate with xenophagy and bacterial degradation of *S. typhimurium* (see below). In contrast, LUBAC was reported to play no major role in the trafficking of *S. flexneri* to autophagosomes

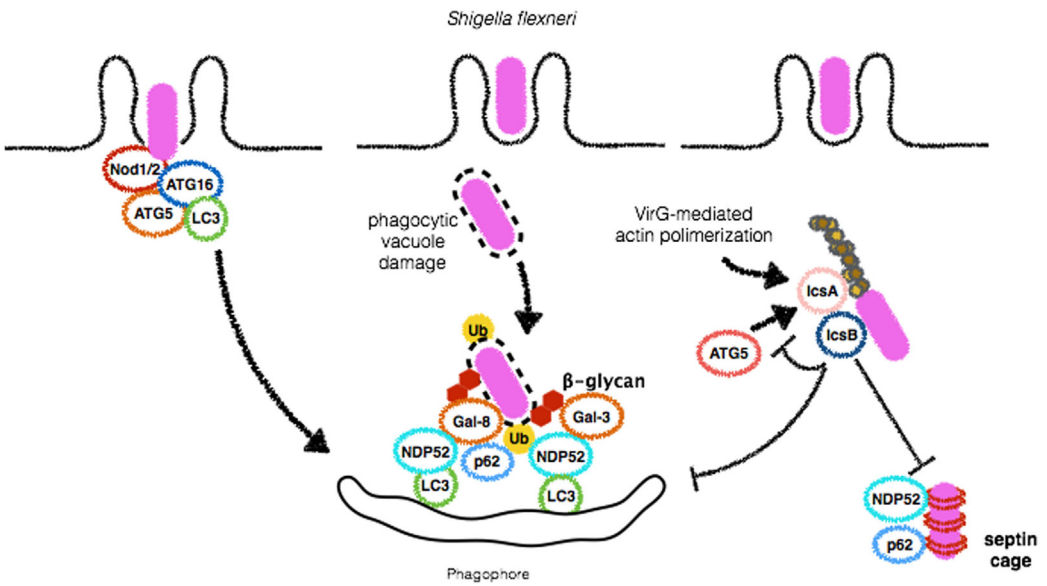

FIGURE 3 | *Shigella flexneri* employs different mechanisms to escape from autophagy. During bacterial entry into host cells, Nod1 and Nod2 recruit ATG16L1 to initiate autophagosome formation in order to restrict *S. flexneri* replication (left). Vacuole damage leads to β-glycan exposure and recognition by Galectins 3 and 8 and recruitment of NDP52, followed by bacterial ubiquitination and binding to p62 and NDP52, culminating to *S. flexneri* targeting for autophagic degradation (center). IcsB plays a central role in disguising autophagic machinery. This protein competes with IcsA/VirG for binding to ATG5, preventing p62, and NDP52 binding, septin caging and autophagosome formation (right). Septin caging and further recruitment of autophagic adaptors are blocked by IcsB expression.

(57). According to this study, *S. flexneri* escapes from LUBAC-dependent ubiquitination by secreting the effector E3-ligase H1.4 to antagonize the activity of LUBAC (57). Despite the lack of substantial data on the how *S. flexneri* is ubiquitinated, different adaptors that bind ubiquitin are independently recruited to the bacterial surface. For instance, p62 and NDP52 have been demonstrated to be recruited to *S. flexneri* surface and to regulate xenophagy mediated by each other. In agreement with the notion of its anti-autophagic role, IcsB also contributed to *S. flexneri* escape from autophagy by hiding IcsA/VirG from ubiquitin coating (56) (**Figure 3**). The reason why *S. flexneri* recruits different adaptors *S. flexneri* is not clear. However, it is possible that p62 and NDP52 may recognize different ubiquitin linkages as a result of the activity of different ubiquitin-ligases. Another hypothesis is that their LIR domains could be able to recruit different LC3 homologs and different adaptors that could contribute to membrane recruitment from various sources for the formation of autophagosome around bacteria. These hypotheses still lack experimental confirmation.

Shortly after *S. flexneri* entry in epithelial cells, the phagocytic vacuole is ruptured, membrane remnants expose host sugars in the cytosol, and galectin 3 promotes ubiquitination and recruitment of p62 to support xenophagy (58). In contrast to other reports from the literature, the authors did not observe increased recruitment of p62 in Δ*icsB*.

Interestingly, members of the NF-κB pathway such as TRAF6 and NEMO and the peptidoglycan receptor Nod1 were reported to localize to these membrane remnants (58). Similarly, NLRP3, NLRC4, ASC, and Caspase-1 were also found associated with Shigella vacuolar membrane remnants. The physiological meaning of these findings is still to be defined. One possibility is that these membrane portions might be used for the activation of inflammatory cascades and that this process is likely to be regulated by autophagy. Another possibility is that by recruiting these proteins to its vicinity, *S. flexneri* modulates NF-κB activation and inflammation to favor its replication and spread.

Septins are conserved GTP-binding proteins that play critical roles in cell division, cytoskeletal dynamics, and membrane remodeling (59). These proteins have been shown to form cages around *S. flexneri* actively polymerizing actin. Interestingly, colocalization of septins, p62, and LC3 on *S. flexneri* bacterial surface has been demonstrated and depletion of septins markedly reduced xenophagy of *S. flexneri*, suggesting an intimate relationship between these two processes (60). More recently, the precise mechanisms involved in *S. flexneri*-cage assembly were revealed. Sirianni et al. have found that mitochondrial proteins associate with *S. flexneri*-septin cages and that mitochondria promote the formation of septin cage assembly around *S. flexneri* for antibacterial xenophagy (61). *S. flexneri* has been demonstrated to induce mitochondrial damage and in the study by Siriani et al., this aspect was linked to dampening of septin cages and escape (61, 62). Of note, IcsB contributes to masking *S. flexneri* from septin caging (60). These results demonstrate that IcsB dampens xenophagy by at least three different mechanisms: competing with IcsA/VirG for binding to ATG5, by avoiding septin caging and targeting to autophagosomes, and by recruiting Toca-1 to inhibit the recruitment of NDP52 and LC3.

In addition to direct interaction of its virulence factors and autophagy proteins, pattern-recognition receptors also seem to participate in the interplay between *S. flexneri* and autophagic pathways. It has been demonstrated that the infection of macrophages by *S. flexneri* induces a robust activation of Caspase-1 that leads to inflammasome activation and cell death by pyroptosis in an NLRC4-dependent but ASC-independent mechanism (63). Interestingly, both Caspase-1 and NLRC4 were shown to negatively regulate autophagosome formation in macrophages infected with *S. flexneri* as demonstrated by studies in which bone marrow-derived macrophages (BMDMs) from knockout mice for the genes encoding these proteins were shown to induce the formation of GFP-LC3 positive membranes around bacteria in contrast to wild-type BMDMs (63). In contrast to previous studies, IcsA/VirG was not implicated in autophagy induction (52, 63), which can be explained by the different cell types used in these studies. In contrast to negative regulation of autophagy by NLRC4, NLRC1 (Nod1), and NLRC2 (Nod2), the founding members of the NLR family have been linked to autophagy induction. Nod1 and Nod2 are sensors of intracellular peptidoglycan that upon engagement lead to the activation of NF-κB activation through the recruitment of the adaptor protein RIP2 (64). Both Nod1 and Nod2 have been shown to recruit ATG16L1 at early stages of infection by *S. flexneri* to initiate autophagosome formation. As a result, Nod1- and Nod2-deficient MEFs display decreased numbers of GFP-LC3 positive bacteria, and interestingly, these findings did not rely on recruitment of RIP2 or NF-κB activation. Notably, in this study, the most common Nod2 mutation associated with Crohn disease (CD) resulted in impaired recruitment of ATG16L1 to the bacterial entry site and much less xenophagy, underscoring the notion that dysregulation of bacterial autophagy is likely to play an important role in the pathogenesis of CD (49) (**Figure 3**). It remains to be clarified if and in which conditions Nod1/2-dependent pro-autophagic signals would prevail over NLRC4-dependent anti-autophagy ones and *vice versa*.

Salmonella typhimurium

Salmonella typhimurium is a pathogenic Gram-negative bacterium found in the intestinal lumen and a major cause of gastroenteritis in humans and other mammals (65). This pathogen uses two T3SS, encoded by *Salmonella* pathogenicity island 1 and 2 (SPI2) to enter non-phagocytic cells and establish a replicative niche within vacuoles termed *Salmonella*-containing vacuole (SCV). In 2006, it was first reported that a fraction of the bacterial population within the SCV previously demonstrated to form pores in this compartment was able to reach the cytosol being immediately targeted by LC3 and ATG proteins. In this study, the authors showed that *Atg5*-deficient MEFs infected with *S. typhimurium* had decreased fusion of $LC3^+$ bacteria colocalized with Lamp1, suggesting diminished bacterial degradation in lysosomes. Indeed, these cells harbored increased bacterial numbers, confirming the role of autophagy in the control of *S. typhimurium* infection (66) (**Figure 4**). Importantly, xenophagy has been reported to be essential in the control of *S. typhimurium* in other models such as *Caenorhabditis elegans* and *Dictyostelium discoideum*, suggesting that the role of xenophagy as an

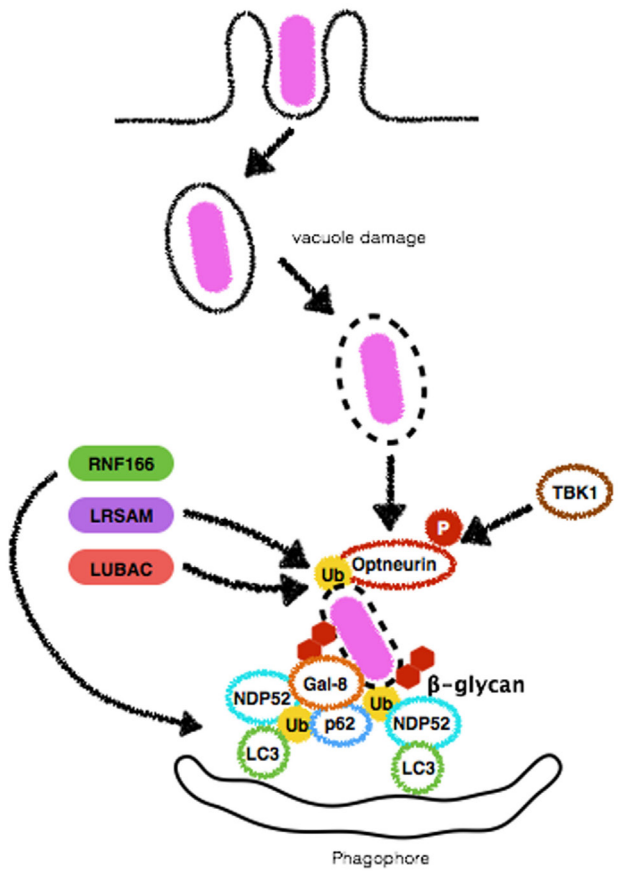

FIGURE 4 | Mechanisms of autophagy induction by *Salmonella Typhimurium*. Upon entry in epithelial cells, *S. Typhimurium* resides in a specialized compartment, the Salmonella-containing vacuole (SCV). A fraction of the bacterial population damages and/or escapes from SCV and initiates to replicate in the cytosol. Either still within the SCV or free in the cytosolic compartment, *S. Typhimurium* triggers autophagy by several means. β-glycan present in vacuole remnants is recognized by Galectin-8 (Gal-8) and targets bacteria to autophagosomes. *S. Typhimurium* can also be ubiquitinated by the E3-ligases LRSAM or LUBAC, allowing its recognition by autophagic adaptors Optneurin, p62 or NDP52. RNF166, another E3-ligase, ubiquitinates p62 to increase the ability of this protein to bind bacteria-associated ubiquitin. Diacylglycerol (DAG) recognition and autophagy induction upon *S. Typhimurium* infection are not depicted here.

anti-*S. typhimurium* mechanism has been conserved throughout evolution (67).

Although *S. typhimurium* targeting by autophagy was demonstrated, the means by which autophagosome formation machinery recognizes cytosolic *Salmonella* is not completely clear and has been the subject of many studies. Cytosolic, but not SCV residing bacteria, have been shown to be decorated with ubiquitin early during infection (66, 68). Interestingly, Ub^+ bacteria colocalize with LC3, suggesting that the autophagic machinery can detect ubiquitinated substrates. Indeed, p62 has been shown to play a crucial role in the recognition, targeting to lysosomes and restriction of cytosolic ubiquitinated *S. typhimurium* (69). Other ubiquitin-binding proteins have also been reported to participate in *Salmonella*-induced autophagy. Similarly to p62, Optineurin harbors LIR and UBA domains and was shown to be necessary for the control of *S. typhimurium*. Interestingly, this mechanism requires Optineurin to be phosphorylated by TBK1 on serine-177 in order to enhance ubiquitin- and LC3-binding affinity to promote bacterial clearance (70). One aspect of *S. typhimurium* recognition by autophagy machinery that remained elusive was which bacterial substrates are ubiquitinated prior to detection by the adaptors p62, NDP52, and Optineurin. A recent study from Fiskin et al. in which ubiquitination site profiling was performed during infection with *S. typhimurium* revealed that outer membrane proteins are targets for ubiquitination (71).

Several ubiquitin-ligases have been reported as necessary for ubiquitination of *S. typhimurium*. Leucine-rich repeat and sterile α-motif-containing 1 (LRSAM1) was shown to play an essential role in the autophagic degradation of *S. typhimurium*. This E3-ligase was found to localize to cytosolic *Salmonella* upon infection of epithelial cells to ensure proper ubiquitination and autophagic control of bacterial replication. In line with these findings, a cohort study reported that lymphoblasts from patients with Charcot-Marie-Tooth disease, which harbor a frameshift mutation that truncates the RING domain of LRSAM1, present limited antibacterial activity as compared to cells from control individuals (72, 73). Another E3-ligase demonstrated to be involved in autophagic targeting of *S. typhimurium* is RNF166. This gene was identified in a screening for human E3-ligases as necessary for the recruitment of p62, NDP52, and LC3 for the bacterial surface in order to limit *S. typhimurium* replication. A unique feature of RNF166 is that, rather than tagging bacteria, it drives K29- and K33-linked ubiquitination of p62 at K91 and K189. According to the authors, this step is essential for p62-dependent bacterial targeting for autophagosomes (74) (**Figure 4**). More recently, the role of LUBAC, another E3-ligase, has been described. LUBAC generates linear (M1-linked) polyubiquitin patches on the surface of *S. typhimurium* to recruit the adaptors Optineurin, NDP52 and p62 and direct bacteria for autophagic degradation. Indeed, MEFs from *cpdm*$^{-/-}$ mice, which harbor a spontaneous mutation in LUBAC or MEFs silenced for the protein, display an increased time-dependent replication of *S. typhimurium* in comparison to wild-type or control-silenced cells, respectively (57). In addition to the recruitment of autophagy adaptors, LUBAC was reported to be crucial in triggering pro-inflammatory roles during infection with *S. typhimurium* (**Figure 4**). LUBAC-dependent generation of M1-linked polyubiquitin chains on the surface of the bacteria also recruits NEMO to this site (57, 75). These findings are of particular interest as it suggests that bacterial surface can provide mechanical support for the assembly of signaling platforms such as NF-κB activation, a major transcription factor that controls the production of inflammatory mediators such as cytokines and chemokines. Given that LRSAM1 was found to be only partially responsible for *S. typhimurium* ubiquitination, which RNF166 ubiquitinates p62 rather than bacteria and that LUBAC required an upstream E3-ligase, Polajnar et al. hypothesized that other ubiquitin ligases were involved in the ubiquitination of *S. typhimurium* and identified Ring-between-Ring E3 ligase

ARIH1 (also known as HHARI) as an important protein ubiquitin-ligase for targeting this pathogen to autophagosomes (76). Notably, this study demonstrated that depletion of LRSAM1 and ARIH1 led to an enhancement in LUBAC-dependent ubiquitination and NF-κB activation, culminating with increased bacterial replication, in contrast to previous findings, reporting that NF-κB activation led to bacterial growth restriction (57, 75, 76). Together, these data indicate that recruitment of different ubiquitin-ligases (with different ubiquitin linkage abilities) to the bacterial surface may endow cells with several layers of protection against the replication of cytosolic *S. typhimurium*.

In addition to bacterial ubiquitination, lipid second messengers have also been reported to be required for efficient targeting of *S. typhimurium*. Shahnazari et al. demonstrated that diacylglycerol (DAG) is produced during infection with *S. typhimurium* in a phospholipase D- and phosphatidic acid phosphatase-dependent manner. DAG localization in bacteria-containing phagosomes seemed to be a requisite for autophagy and may occur in parallel to independent p62 and NDP52 recruitment, once again suggesting several layers of proteins involved in bacterial targeting (77).

The detection of damage in the SCV has been demonstrated to be an important step in the targeting of *S. typhimurium* for autophagic degradation (66). Galectin-8 is a β-galactoside-binding lectin that has been reported to monitor endosomal and lysosomal integrity and detects bacterial invasion by binding host glycans exposed on damaged SCVs. Recently, it has been demonstrated that among galectins 1–4, 7–10, and 12–14, only Galectin-8 colocalized to *S. typhimurium* during infection of HeLa cells. Interestingly, NDP52 was recruited to cytosolic exposed *S. typhimurium*, directly binding to Galectin-8 to restrict bacterial replication. These and previous findings lead to a model in which, upon SCV damage, host sugar molecules such as β-galactoside, usually confined to the lumen of endosomes are exposed in the cytosol and sensed by Galectin-8 that in turn recruits NDP52 and LC3 to SCV to initiate lysosomal degradation of *S. typhimurium* (42, 78). Despite their role in mediating *S. typhimurium*-induced autophagic degradation, p62 and NDP52 show independent targeting activity. In a study in which HeLa cells were silenced for p62 or NDP52, there was no interference in the number of $NDP52^+$ or $p62^+$ bacteria, respectively (**Figure 4**). Interestingly, it was demonstrated that these adaptors recognize ubiquitin deposited in distinct microdomains at the bacterial surface that could result from the activity from different ubiquitin-ligases (79). Future studies must provide explanations if and why cells preferably decide toward the employment of one or the other ubiquitin-ligase and autophagy adaptors.

Listeria monocytogenes

Listeria monocytogenes is a Gram-positive bacterial pathogen that causes listeriosis, a self-limiting disease in healthy individuals that become severe in immunocompromised or elderly individuals and pregnant women (80). One of the main features of *L. monocytogenes* is its ability to replicate within several cell types during infection, including macrophages, a cell type usually able to kill the majority of intracellular bacteria (81).

Before its replication in the cytosol, *L. monocytogenes* must escape from the phagosome. This is achieved through the expression of several virulence factors rapidly upon entry. Possibly, the main bacterial factor associated with phagosome escape, listeriolysin O (LLO), is a cholesterol-dependent, pore-forming cytolysin that form pores in the phagosomal membrane immediately after bacteria uptake (82–85). In experiments with fluorescently labeled molecules of increasing sizes, it has been demonstrated that the pores grow in size until large enough to allow bacterial escape (86). In addition to LLO pore-forming activity, two C-type phospholipases, phosphatidylinositol-specific (PI-PLC, plcB), and a broad-range phosphatidylcholine (PC-PLC, plcA) also contribute to *L. monocytogenes* escape from phagosome, likely digesting its membrane (86).

In order to successfully replicate in the cytosol, *L. monocytogenes* needs to circumvent several layers of host defense. Autophagy has been reported to contribute to the control of infection, although several studies show that the bacteria are able to escape from autophagic degradation (87, 88). Infection of RAW 264.7 macrophages of wild-type *L. monocytogenes* showed that ~40% of the intracellular bacterial population was targeted by LC3 by 1 h postinfection (p.i) in an LLO-dependent manner (**Figure 5**). However, at 8 h p.i, only 10% of the bacterial population was LC3-positive. These results suggest that *L. monocytogenes* was able to escape from autophagic degradation. Indeed, after initial targeting by LC3, replication rates robustly increased, in line with the drop in bacterial colocalization with LC3 observed at later stages of infection (89).

ActA, a key virulence factor of *L. monocytogenes* involved in intracellular motility, has also been implicated in autophagy evasion. *In vitro* studies demonstrate contradictory results with Δ*actA* mutants in different genetic backgrounds and cell types. While EGDe Δ*actA* mutants infecting Hela cells show time-dependent increase in the colocalization with LC3, 10403S Δ*actA* mutants in the 10403S background infecting macrophages loses its staining for LC3 at later time points during infection (88, 90). It remains to be elucidated whether the differences observed for both genetic backgrounds are related or not to the different cell types used. Despite this controversy, it is important to note that Δ*actA* mutants in both backgrounds display comparable replication *in vitro* (88–90). Importantly, ActA-dependent escape of autophagy does not rely on its ability to mediate bacterial motility. Using a series of *ActA* truncated mutants, Yoshikawa et al. demonstrated that as long as the capacity of ActA to recruit actin-related proteins (Arp) 2/3 complex, vasodilator-stimulated phosphoprotein or actin, is retained, bacteria are able to disguise autophagic recognition (88) (**Figure 5**).

The ubiquitination of *L. monocytogenes* and the involvement of autophagy adaptors such as p62 and NDP52 have been reported in the targeting *L. monocytogenes* to autophagosomes. In HeLa cells, p62 and NDP52 were shown to be recruited independently during the infection with the Δ*actA* EGDe (56). Recently, these strains were compared in regards to LC3, p62, and Ub during infection of macrophages. Although Δ*actA* mutants in EGDe and 10403S genetic backgrounds were reported to be sharply different regarding colocalization with LC3, p62, and Ub recruitment and replication were identical for both strains suggesting that Δ*actA* can block xenophagy downstream of ubiquitination and LC3 recruitment (90). The ubiquitin-ligases Parkin and SMURF1 were

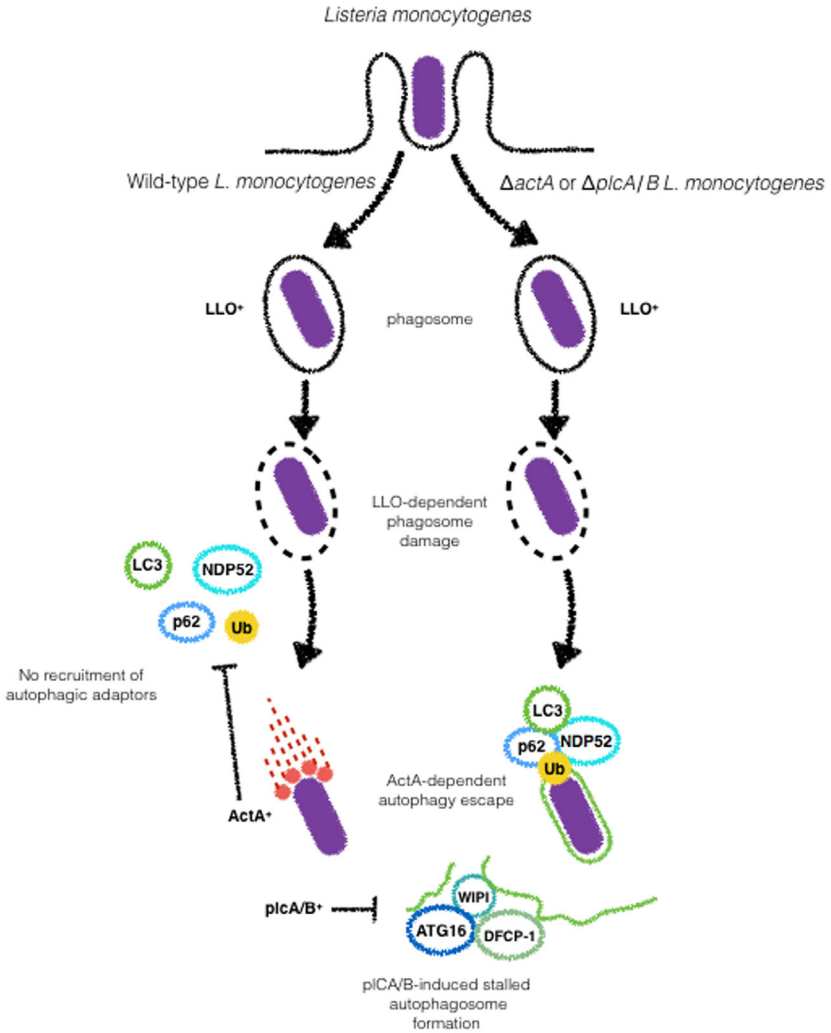

FIGURE 5 | ActA and phospholipases (plc) A and B are major *Listeria monocytogenes* virulence factors involved in the escape from autophagy. Upon Listeriolysin O-dependent escape from phagosome wild-type *L. monocytogenes* escapes from autophagic targeting due to the expression of ActA, that blocks the recruitment of p62 and NDP52 to the bacterial surface. The expression of plcA/B stalls the formation of autophagosomal membranes. Galectin recognition of damaged vacuoles and autophagy induction are not shown here.

demonstrated to play a role in the ubiquitination of *L. monocytogenes*. *Park2*$^{-/-}$ mice infected with *L. monocytogenes* showed up to 20-fold higher bacterial load relative to wild-type animals (31). *Smurf1*$^{-/-}$ macrophages infected with Δ*actA L. monocytogenes* do not show recruitment of K48-ubiquitin to the bacterial surface while K63-linked ubiquitination was not affected. In line with this finding, *Smurf1*$^{-/-}$ mice infected with *L. monocytogenes* were shown to harbor significantly higher bacterial burdens in comparison to wild-type (33). As ubiquitination of cytosolic bacteria has been known to be essential for autophagic degradation, it is assumed that the higher bacterial burden in *Smurf1*$^{-/-}$ mice is a consequence of dampened antibacterial autophagy. More recently, NEDD4 (neuronal precursor cell expressed, developmentally downregulated 4), another ubiquitin-ligase has been implicated autophagic degradation of *L. monocytogenes* (**Figure 5**). However, in contrast to Parkin and Smurf1, NEDD4 does not recruit ubiquitin to the bacterial surface but enhances the mediated K6- and K27-linkage ubiquitination of BECN1, leading to higher stability of BECN1 and increased autophagy (91).

Listeria monocytogenes has been reported to induce amino acid starvation and activation of the general control nonderepressible 2 (GCN2)-eIF2α pathway upstream mTOR. GCN2 is one of four "stress kinases" that block translation by phosphorylating eIF2α. GCN2 is thought to bind uncharged tRNAs to "sense" amino acids availability (92). Upon detection of a decrease in the amino acid pool, mTOR activity is reduced leading to autophagy activation to normalize this condition. Unlike what is observed during the infection of epithelial cells with *S. flexneri*, in *L. monocytogenes-infected* cells, autophagy is kept repressed, suggesting that *L. monocytogenes* possesses other virulence weapons to block autophagy (93–95). In addition to Δ*actA*-mediated escape from autophagy, *L. monocytogenes* employs its two C-type

phospholipases to disrupt the autophagosome elongation step in order to inhibit autophagy-dependent degradation. In an *in vitro* study, it was observed that *L. monocytogenes* deleted for plcA and plcB were more strongly targeted to autophagosomes than wild-type bacteria at later time points of infection. In parallel, wild-type bacteria induced the accumulation of granules positive for LC3, ATG16L1, and as well as WIPI-2, a phosphatidylinositol 3-phosphate-binding protein that is present on maturing phagophores, suggesting blockade of pre-autophagosome structures. Interestingly, the authors demonstrate that in plcA/plcB *L. monocytogenes* mutants, the accumulation of such structures was not observed (93, 95). These results, together with the previous findings of Mitchell et al. point toward the combined effects of ActA and *L. monocytogenes* phospholipases in the escape from autophagy (87) (**Figure 4**).

The detection of MAMPs has also been described as an autophagy trigger during the infection of *L. monocytogenes*. In 2008, a study using *Drosophila melanogaster* as a model for *L. monocytogenes* infection reported that peptidoglycan-recognition protein (PGRP-LE) mediated autophagy-dependent control of bacterial replication *in vitro* and *in vivo* (96). Interestingly, the intracellular peptidoglycan receptor Nod1 has also been linked to xenophagy of *L. monocytogenes in vitro*. MEFs from Nod1-deficient mice were demonstrated to be defective in targeting *L. monocytogenes* to autophagosomes, indicating an important role for peptidoglycan recognition in the induction of autophagy during infection with this bacterium in mammals as well (49).

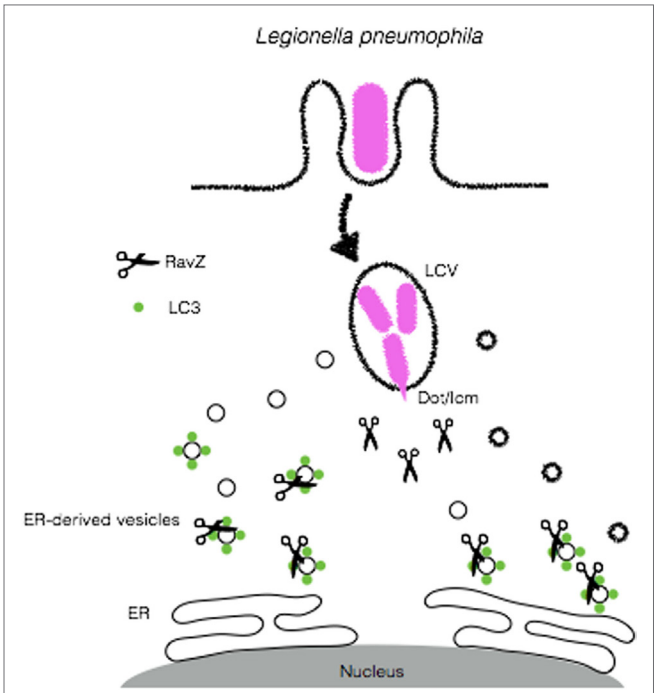

FIGURE 6 | *Legionella pneumophila* disrupts autophagy to create a replicative niche. *L. pneumophila* secretes RavZ through its Dot/Icm apparatus in order to deconjugate LC3 from ER-derived vesicles and block autophagy in order to escape from autophagic degradation.

Legionella pneumophila

The Gram-negative bacterium *L. pneumophila* was first identified as the causative agent of an epidemic of pneumonia at an American Legion convention in Philadelphia, PA, USA in 1976 (97). This disease is characterized by the inhalation of aerosols containing high numbers of *L. pneumophila* (98). Although usually found in freshwater protozoa and amebae, *L. pneumophila* can accidentally replicate in alveolar macrophages in human lung, especially in immune-compromised patients (99, 100). In order to replicate within its eukaryotic host, *L. pneumophila* employs strategies that involve blocking the fusion of phagosomes with lysosomes after phagocytic ingestion of the bacteria and the generation of endoplasmic reticulum (ER)-like compartment that affords its replication (7, 101). The *L. pneumophila*-containing vacuoles (LCVs) present features that are shared by autophagosomes, including its close association with ER membrane (100, 102). This led to the speculation that the formation of biogenesis could involve the autophagy machinery (103, 104). Initial studies that focused on the characterization of the LCV reported that this compartment did not fuse with acidic vesicles since proteins that localize to endolysosomes, such as Lamp-1 and Rab7 were absent in LCV membrane and that the ability of *L. pneumophila* to evade phagosomal maturation was dependent on its viability (98, 105). Further studies using avirulent strains of *L. pneumophila* identified the intracellular multiplication (icm) and defect in organelle trafficking (dot) loci as the genetic loci determinants required for intracellular multiplication and evasion phagosome–lysosome fusion (106, 107). The emergence of autophagy as an antimicrobial effector led to the examination of the role of this process in the pathogenesis of *L. pneumophila* infection. Since LC3 is a major marker for autophagosome membranes, several cell biology approaches aimed to analyze the recruitment of $LC3^+$ compartments to LCVs (100). Interestingly, following infection of macrophages with *L. pneumophila,* the formation of autophagosomes was blunted. In line with the role of Dot/Icm in the virulence of this bacterium, infection of macrophages with an isogenic Dot/Icm-deficient dotA mutant was unable to induce defects in autophagy induction (108). To identify the bacterial factors involved in autophagy inhibition, Choy et al. conducted a genetic screen that mapped the defect in autophagy to a chromosomal region encoding for 10 effectors. Analysis of the effects of the individual effectors revealed the protein RavZ as necessary and sufficient for blocking autophagy (108). *In vitro* analysis demonstrated that RavZ, which displays cysteine-protease activity, acts to deconjugate LC3 from autophagosomes and block its reconjugation (108). Furthermore, recent reports demonstrate that RavZ might participate not only in the deconjugation of LC3 but also in other steps that interfere with xenophagy. Kubori et al. have found in co-infection experiments with *L. pneumophila* and *S. typhimurium* that the recruitment of ubiquitin, p62, and NDP52 to the surface of *S. typhimurium* was dampened, suggesting a deubiquitinase-like enzymatic activity for RavZ (109). The resolution of the crystal structure of RavZ yielded new clues to its mechanisms. According to this study, by targeting autophagosomes through PIP3- and curvature-sensing motifs, RavZ limits its activity only to LC3 that is bound to autophagosomes (110) (**Figure 6**). Other RavZ-independent mechanisms for *L. pneumophila* evasion

of autophagy have been described as well. Phylogenetic analyses suggested a high degree of similarity between one *L. pneumophila* and the eukaryotic sphingosine-1 phosphate lyase (SPL) (111). The *L. pneumophila* SPL homolog (LpSlp) has similar enzymatic activities to the eukaryotic SPL, which finely regulates intracellular levels of sphingosine-1-phosphate (S1P) (112), which have been shown to stimulate autophagy (111, 113). Infection of macrophages with wild-type *L. pneumophila* but not the LpSpl-deficient mutant leads to a depletion in S1P levels and inhibition of autophagy, indicating that *L. pneumophila* uses molecular mimicry to block autophagy and replicate within macrophages (111).

The Role AMP-Dependent Protein Kinase (AMPK) Activation and Bacterial-Induced Amino Acid Starvation in Bacterial Xenophagy

Living organisms obtain energy from the catabolism of nutrients whose molecular blocks are then converted into ATP and NADPH. The fact that cells are continually synthesizing ATP keeps its level close to maximal, with only small variations (114). However, under nutrient stress, when ATP levels drop, adenylate kinase shifts to an ATP synthesis mode to restore its levels. In turn, AMP levels increase significantly and, physiologically, changes in AMP concentrations are much higher than those observed to ATP (115), which makes the AMP/ATP ratio the most reliable marker of the cellular energetic status (114). Under such conditions, AMPK detects tiny changes in AMP levels and represents the principal cellular metabolism regulator (114). One of the main direct consequences of AMPK engagement is the activation of ULK1, suppressing mTORC1 inhibitory activity to allow the formation of autophagosomes (116).

In addition to its crucial role as a metabolic sensor, AMPK has also been widely reported to be involved in the activation of autophagy by bacteria. In a bacterial peritonitis-induced sepsis model, the use of the AMPK activator aminoimidazole carboxamide ribonucleotide (AICAR) increased bacterial killing, suggesting the implication of AMPK in the enhancement of the activity of phagocytic cells. Indeed, the use of these activators led to increased chemotaxis, phagocytosis, and bacterial killing of neutrophils infected with *Escherichia coli* (117). Evidence from the literature demonstrates that activation of AMPK by treatment with AICAR can also increase targeting of *M. tuberculosis* to LC3-positive compartments. Of note, when key autophagic proteins such as ATG7 were silenced, this effect was not observed, suggesting AICAR promotes the targeting of *M. tuberculosis* to autophagosomes. Moreover, AICAR-induced xenophagy was shown to contribute to bacterial killing *in vitro*, in a mechanism involving mTOR inhibition and increased mitochondrial biogenesis and ATP generation, likely as a result of energy drop during *M. tuberculosis* infection. Since it has been previously demonstrated that peroxisome proliferator-activated receptor-gamma, coactivator 1α (PPARGC1A) is important for the regulation of mitochondrial gene expression and glucose metabolism, it was speculated that PPARGC1A was involved in AICAR-induced xenophagy of *M. tuberculosis*. When PPARGC1A expression was silenced in macrophages infected with *M. tuberculosis*, the robust increase in mitochondrial biogenesis, ATP generation, and decreased *M. tuberculosis* replication induced by AICAR treatment were not observed (118, 119). The findings of this study support those from Gutierrez et al. (3) demonstrating that induction of autophagy through rapamycin enhances antimicrobial defenses against *M. tuberculosis*. Although AMPK activation was found to be involved in the efficient xenophagy-dependent control of *M. tuberculosis*, this pathogen developed sophisticated mechanisms to manipulate AMPK activity in order to favor its replication. In another recent evidence, miRNAs emerged as important "fine-tuners" of gene expression in response to pathophysiological stimuli. These RNAs bind to the 3'-untranslated region of specific mRNAs to reduce protein expression by blocking mRNA translation or inducing its degradation (120). Accumulating evidence shows that many miRNAs regulate the complex interplay between mycobacterial survival strategies and host innate immune and metabolic pathways (121). One of these miRNAs, miR33 has been shown to the regulation of fatty acid metabolism and insulin signaling (122). *M. tuberculosis* seems to use the expression of miRNAs to subvert autophagy to create a favorable replicative niche. *M. tuberculosis* infection of macrophages induces the expression of miR-33 and its passenger strand miR-33* to dampen mitochondrial fatty acid oxidation and lipophagy (autophagy of lipid droplets) to increase cellular lipid content, which is essential for the bacilli as a nutrient source during infection (34). According to this study, autophagy inhibition was achieved by inhibition of AMPK, which controls transcription factor EB and Forkhead box transcription factor class O (FOXO3), transcriptional regulators of autophagy and lysosomal biogenesis gene programs, respectively (123, 124). Altogether, these studies demonstrate that AMPK is activated during infection with intracellular bacteria.

The mechanisms by which intracellular bacteria initiate xenophagy are not completely elucidated, but compelling evidence from the literature suggests that these pathogens trigger energy imbalance and cellular nutritional stress that result in the activation of cellular responses culminating in the upregulation of autophagic activity (93). It has been reported that the infection of epithelial cells with *S. flexneri* infection induces a general and persistent loss of amino acids, leading to amino acid starvation-induced stress (94). In contrast, *S. typhimurium* induces only a rapid and transient depletion of amino acid pools. Of note, during *S. flexneri*- and *S. typhimurium*-induced amino acid depletion, robust relocalization of mTORC1 is observed. While in *S. flexneri*-infected cells, S6K1 and 4EBP1, two major targets of mTOR, are downregulated, and mTORC1 dispersed in the cytosol throughout infection, during the infection with *S. typhimurium*, this is observed only in early timepoints, suggesting that this bacterial pathogen developed means to manipulate mTOR signaling to favor its intracellular survival (94) (**Figure 7**). Indeed, in a recent study, Ganesan et al. demonstrated that despite sustained low levels of ATP in macrophages infected by *S. typhimurium*, AMPK was only transiently activated at early timepoints and then returned to basal levels (125). AMPK activation is known to be regulated by a cytosolic complex consisting of liver kinase B1 (LKB1) and Sirtuin-1 (Sirt1), where Sirt1 is necessary for deacetylation and activation of LKB1 (126). Interestingly, the study by Ganesan et al. reports that *S. typhimurium* induces the lysosomal degradation of AMPK, LKB1, and Sirt1 to reactivate mTORC1 activity in order to inhibit autophagosome formation

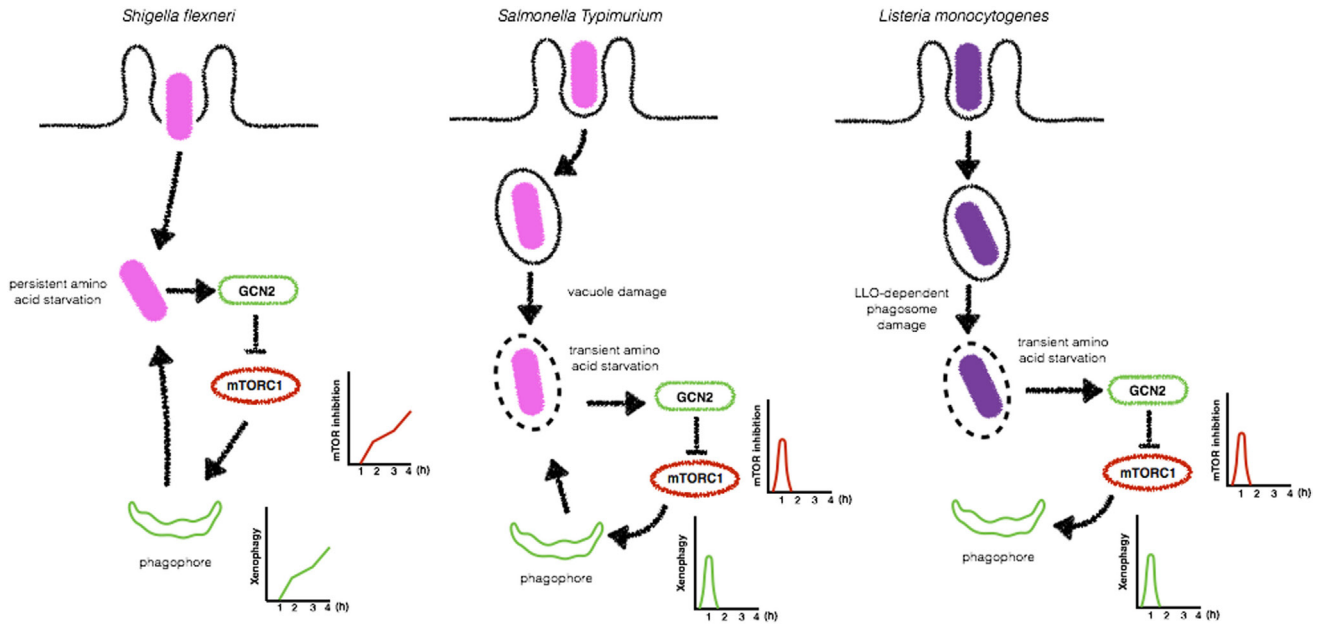

FIGURE 7 | Bacteria-induced amino acid starvation stress triggers xenophagy. *Shigella flexneri* induces a persistent amino acid starvation that leads to GCN2 activation and inhibition of mTORC1 to allow the formation of bacteria targeted autophagosomes (left). In contrast, *Salmonella Typhimurium* (center) and *Listeria monocytogenes* (right) trigger only a transient amino acid starvation and inhibition of mTORC1, allowing its reactivation at later time-points to block the formation of autophagosomes.

and escape from xenophagy. Notably, this reactivation was shown to be dependent on SsrB, a regulator of pathogenicity island 2 (SPI2) encoded virulence factors (127), and SsaV a component of the SPI2 type III secretion apparatus (128). *S. typhimurium* mutants lacking SsrB and SsaV failed to induce lysosomal degradation of the AMPK/LKB1/Sirt1 circuit and are efficiently targeted to autophagosomes (125). Together, the studies from Tattoli et al. and Ganesan et al. demonstrate that nutritional cellular stress induced by bacterial infection triggers xenophagy to control bacterial replication (in the case of *S. flexneri* infection) and that *S. typhimurium* developed mechanisms to escape from autophagy by reactivating mTORC1 activity. Together, the studies from Tattoli et al. and Ganesan et al. demonstrate that nutritional cellular stress induced by bacterial infection triggers xenophagy to control bacterial replication (in the case of *S. flexneri* infection) and that *S. typhimurium* developed mechanisms to escape from autophagy by reactivating mTORC1 activity.

Listeria monocytogenes has also been reported to induce amino acid starvation-induced cellular stress and activation of the GCN2-eIF2α pathway upstream mTOR. Upon de detection of a decrease in the amino acid pool, mTOR activity is reduced leading to autophagy activation in order to normalize this condition. Unlike what is observed during the infection of epithelial cells with *S. flexneri*, in *L. monocytogenes* cells, autophagy is kept repressed, suggesting that *L. monocytogenes* possesses other virulence weapons to block autophagy (93–95) (**Figure 7**). Finally, AMPK has also been implicated in the enhancement of xenophagy during the infection with *E. coli*. According to this study, *E. coli* infection leads to an increase in intracellular calcium levels, which activates Ca(2+)/calmodulin-dependent protein kinase kinase β (CaMKKβ) to promote AMPK activation. AMPK was undoubtfully implicated in CaMKKβ-mediated xenophagy when macrophages were silenced for AMPK and control of *E. coli* replication was dampened (129).

CONCLUDING REMARKS

Xenophagy has been widely reported to target bacteria for autophagic degradation, with clear impact on intracellular bacterial handling. Even with major advances in our understanding of the mechanisms involved in cargo selection, many questions remain unanswered. For example, why so many different mechanisms to target bacterial pathogens exposed to the cytosol? Still, why different autophagic adaptors and ubiquitin-ligases with apparent redundant functions? Although no evidence in this direction has been reported, we cannot exclude that different types of autophagosomes exist. Thus, p62, NDP52, NBR1, and Optineurin would function as sorters for different autophagosomes. It is possible that the different ubiquitin-ligases work in this direction as well by adding different ubiquitin linkages to the bacterial surface. Regarding bacteria-induced nutritional stress and autophagy induction, it is still to be elucidated whether amino acid starvation is induced upon infection with bacterial pathogens other than *Shigella*, *Salmonella*, and *Listeria*. Also, why bacteria induce amino acid starvation that leads to autophagy to subsequently inhibit it?

In *in vitro* studies, it is clear that only a fraction of the intracellular bacterial population is targeted to autophagosomes, with modest impact in bacterial replication control following autophagy ablation. This is in sharp contrast to *in vivo* studies, which demonstrate much more pronounced differences in

bacterial replication in the absence of autophagy. How would these differences be explained?

Future goals in the field must address these open questions to provide a full understanding of the role of autophagy in bacterial infections.

AUTHOR CONTRIBUTIONS

LT, MS, and RM wrote the paper. LT also revised the final document.

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REVIEW
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Integrated Stress Responses to Bacterial Pathogenesis Patterns

*Larissa O. C. P. Rodrigues, Rodrigo S. F. Graça and Leticia A. M. Carneiro**

Laboratório de Inflamação e Imunidade, Instituto de Microbiologia Paulo de Góes, Universidade Federal do Rio de Janeiro, Rio de Janeiro, Brazil

Activation of an appropriate innate immune response to bacterial infection is critical to limit microbial spread and generate cytokines and chemokines to instruct appropriate adaptive immune responses. Recognition of bacteria or bacterial products by pattern recognition molecules is crucial to initiate this response. However, it is increasingly clear that the context in which this recognition occurs can dictate the quality of the response and determine the outcome of an infection. The cross talk established between host and pathogen results in profound alterations on cellular homeostasis triggering specific cellular stress responses. In particular, the highly conserved integrated stress response (ISR) has been shown to shape the host response to bacterial pathogens by sensing cellular insults resulting from infection and modulating transcription of key genes, translation of new proteins and cell autonomous antimicrobial mechanisms such as autophagy. Here, we review the growing body of evidence demonstrating a role for the ISR as an integral part of the innate immune response to bacterial pathogens.

Keywords: eukaryotic translation initiation factor 2 alpha, cellular stress, bacterial pathogens, heme-regulated eIF2α kinase, general control non-derepressible 2, PKR-like ER kinase, PKR

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Barbara M. Bröker, University of Greifswald, Germany

***Correspondence:**
Leticia A. M. Carneiro
leticiac@micro.ufrj.br

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INTRODUCTION

Microbial sensing by pattern recognition molecules (PRMs) triggers a robust innate immune response with the production of cytokines, chemokines, and antimicrobial factors (1–4). In the last decade, the concept that, in addition to microbial-associated molecular patterns recognition by PRMs, the host response can be tuned by the recognition of alterations in homeostasis induced by pathogens during progression of disease has been established (5–7). Such alterations on cell homeostasis allow the host to differentiate pathogenic organisms from those that do not represent a threat and, thus, adequate the immune responses to deal with the attack being mounted accordingly. It is interesting that despite the multitude of virulence mechanisms among bacterial species, most of them converge to few common "patterns of pathogenesis" that include membrane damage, access to the cytosol, disruption of cytoskeleton, and protein aggregation among others (5–10). In a sense, these patterns of pathogenesis would align with the concept of danger-associated molecular patterns (DAMPs), which are host molecules whose presence indicate that there has been tissue damage such as, for example, extracellular ATP or the chromatin-associated protein high-mobility group box 1 (11–13). But they are not exactly the same as DAMPs as these are molecules that are released as a result of cellular death and that are recognized by receptors on other cells whereas patterns of pathogenesis induce alterations of cell homeostasis during infection and provides the infected cell with information to mount a more refined response and to adapt and, in many cases recover from the insult.

In this context, the cellular mechanisms to sense and respond to stress can be regarded as an integral part of the innate immune response. The integrated stress response (ISR), a common adaptive pathway that eukaryotic cells activate in response to diverse stress stimuli is one such mechanism. The core event in this pathway is the phosphorylation of eukaryotic translation

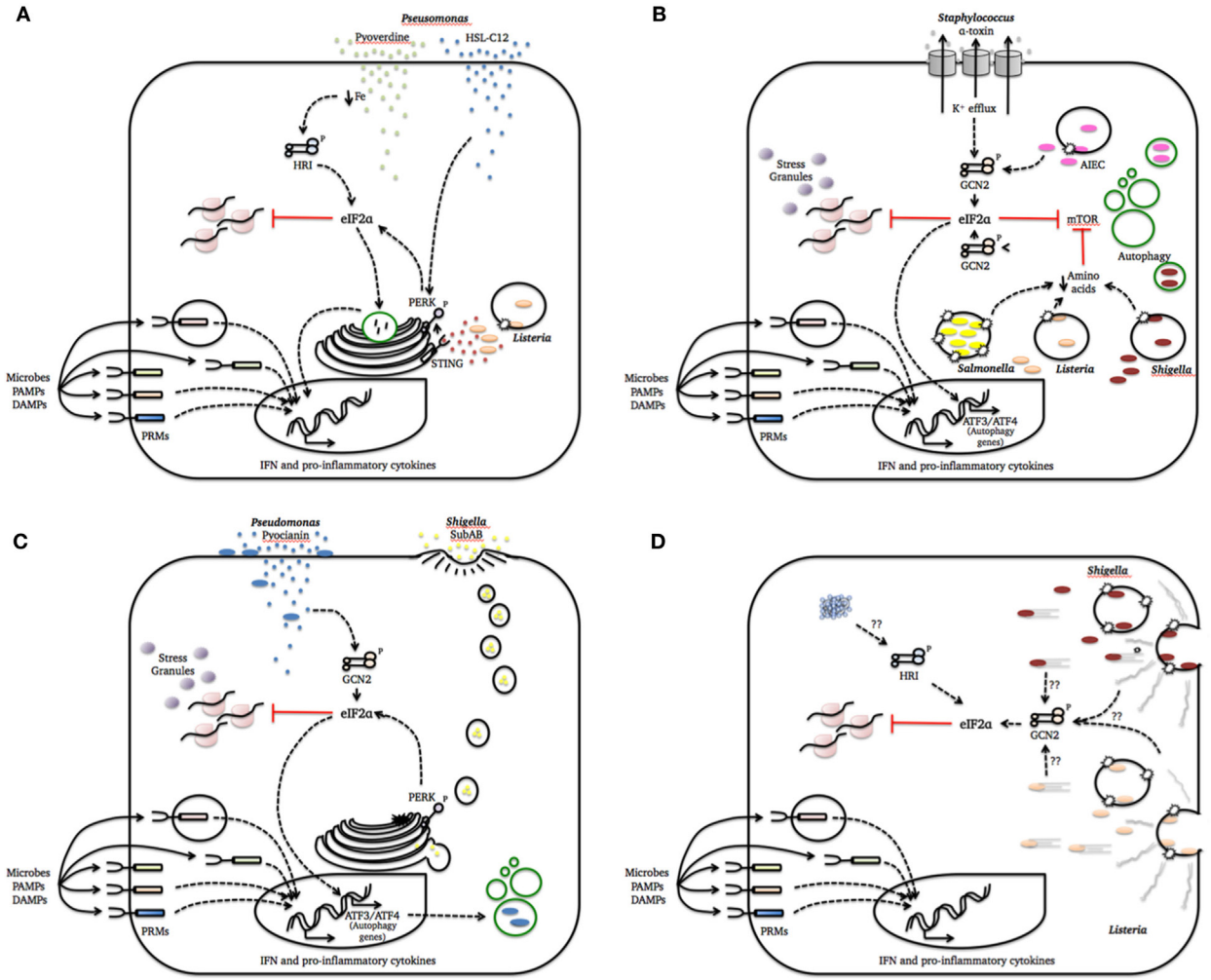

FIGURE 1 | Integrated stress response (ISR) activation by bacterial pathogenesis patterns. This figure summarizes how cellular damage induced by different bacterial species is sensed by one or more eukaryotic translation initiation factor 2 alpha (eIF2α) kinases to activate defense mechanisms and homeostatic programs. We intentionally included a simplified representation of pattern recognition molecules (PRMs) recognition of microbes, PAMPs, and danger-associated molecular patterns (DAMPs) in all cartoons to strengthen the notion that these system act together to refine the cell response to the infection. **(A)** Pathogenesis pattern: bacterial growth; **(B)** pathogenesis pattern: membrane damage; **(C)** pathogenesis pattern: access to cytosol; **(D)** pathogenesis pattern: cytoskeleton disruption and protein aggregation.

initiation factor 2 alpha (eIF2α) by one or more of four members of the eIF2α kinase family (6). The phosphorylation of eIF2α results in a marked decrease in global protein synthesis accompanied by the induction of selected genes, including the transcription factor ATF4, both of which are important to promote cellular recovery (6, 7, 14). This type of response to stress mediated by the eIF2α kinases, parallels those mediated by the mTOR pathway or by autophagy in the sense that are highly conserved signaling modules that regulate essential metabolic circuits, both in homeostatic and stress conditions, from yeast to mammals (6, 8, 15). In the context of an infection, the power of this type of "sensing system" relies on the fact that it does not recognizes pathogens *per se* but rather utilizes an ancient system that detects cellular stress/damage to sense insults that are caused by pathogenic bacteria regardless of its specific virulence factors.

In the present review, we focus on the emerging role of the ISR on host response to bacterial pathogens, which only recently began to be appreciated, in contrast to its well-established role in response to viruses. As obligate intracellular pathogens that highjack the host cell machinery to produce its own proteins, the link between viruses and the ISR is more obvious and more generally accepted. The impact of the ISR on viral infections has been extensively reviewed elsewhere (16–21). Here, we discuss recent data that implicate the ISR as an important component of cell autonomous anti-bacterial responses. As an emerging topic, there are still many gaps in our understanding of the mechanisms underlying this process but we believe that our current knowledge already provides a conceptual framework to work with. As much as we tried to bring together evidence of a role for ISR in different bacterial infections, this is by no means an exhaustive review.

eIF2α AND eIF2α-KINASES

Regulation of translation may be useful to coordinate several innate immune functions such as microbial sensing, microbial replication control, and induction of inflammatory cytokines. Translation shut down can help cells to cope with stress conditions and prevent further damage until the insult is gone. However, this happens in a context where cells still need to communicate that they are under attack in order to prevent infection spread and initiate adequate immune responses. Among metabolic sensors, eIF2α kinases have major roles in adjusting the protein synthesis machinery to enhance translation of mRNAs that are relevant to deal with the source of stress, including those induced by PRM activation, while shutting down the translation of unrelated proteins (6, 7, 21). This ability to screen and modulate host protein synthesis can affect the quality of the innate immune responses both at the transcriptional and translational levels. In addition, the gene expression program induced during ISR adjusts the stress response according to cellular context, nature, and intensity of stress stimuli (6, 7). Finally, although ISR is primarily a homeostatic-preserving program by which cells adapt to survive, severe and/or long-lasting stress can tip the balance toward cell death signaling by regulating the cell autonomous processes of autophagy and apoptosis (6, 7, 15).

The eIF2α kinases act as early responders to alterations in cellular homeostasis which is mainly due to the fact that these proteins are at the same time the sensors of stress and the kinases that phosphorylate eIF2α (6, 10, 20, 22). Each kinase dimerizes and autophosphorilates for full activation in response to distinct environmental and physiological types of stress. Double-stranded RNA (dsRNA)-dependent protein kinase (PKR) is activated mainly by dsRNA during viral infection but also by oxidative and ER stress, growth factor deprivation, cytokines, bacterial infections, and ribotoxic stress (23–27). Interestingly, caspase activity in the early stages of apoptosis was also shown to activate PKR, indicating a role for protein synthesis inhibition in apoptosis (28). PKR-like ER kinase (PERK) is activated by accumulation of unfolded proteins in the ER or perturbations in calcium homeostasis, cellular energy, or redox status (29–31). It has also been reported to respond to ATP depletion and subsequent sarcoplasmic/ER Ca^{2+}-ATPase pump inhibition in the context of glucose deprivation in neuronal cells and in pancreatic β cells (32, 33). Heme-regulated eIF2α kinase (HRI) is a sensor for low levels of intracellular heme as well as arsenite-induced oxidative stress, heat shock, nitric oxide, 26S proteasome inhibition, and osmotic stress (34–37). This array of types of stress activate HRI independently of heme but require the presence of heat shock proteins HSP90 and HSP70 (37). General control non-derepressible 2 (GCN2) is highly conserved from yeasts to humans and is activated in response to amino acid deprivation when it binds to deacylated transfer RNAs (tRNAs) *via* histidyl-tRNA synthetase-related domain (38, 39). As one can appreciate, some types of stress can potentially activate more than one of these four kinases. Most likely, the eIF2α kinases act cooperatively to specifically tune cellular responses stress. Of note, all of these kinases have been reported to have roles independent of eIF2α phosphorylation but here we will focus on the ISR, which signals through eIF2α phosphorylation.

The common signaling hub for all the stress stimuli that activate ISR is phosphorylation of the subunit α of eIF2 on serine 51 (6, 10, 20, 22). eIF2 is constituted by three subunits (α, β, and γ). When bound to GTP and Met-$tRNA_i^{Met}$ (initiator methionyl-tRNA), eIF2 form a ternary complex that delivers the initiator tRNA to the 40S ribosomal subunit. eIF2 is released from the ribosome bound to a GDP and to be ready for another round of translation initiation, the eIF2 complex must be recycled back to its active GTP-bound form. The guanine nucleotide exchange factor eIF2B exchanges GDP for GTP on the γ subunit and maintains the levels of the ternary complex available for new rounds of translation. Under a variety of stress conditions, however, phosphorylation of the α subunit of eIF2 at Ser51 blocks general translation initiation, as it converts eIF2 to a competitive inhibitor of eIF2B by blocking the GDP–GTP exchange reaction and reducing the dissociation rate of eIF2 from eIF2B (6, 40, 41). Phosphorylation of eIF2α leads to a global arrest in translation but it does not affect all mRNA transcripts alike. A subset of mRNAs that contain upstream open reading frames and often encode proteins that are important for stress recovery and re-establishment of homeostasis have selective increased translation (6).

One of the genes that are upregulated following eIF2α phosphorylation is the transcription factor ATF4. Studies using ATF4-deficient mice have shown it has critical roles in the regulation of normal metabolic as well as redox processes such as regulation of obesity, glucose homeostasis, energy expenditure, and neural plasticity (42–44). Under stress conditions, increased ATF4 expression represents a signature of the ISR and is mainly due to translational control, as *Atf4* is one of those mRNAs that have its translation augmented upon eIF2α phosphorylation in contrast with the general translational arrest observed for most transcripts (6, 45). As a transcription factor, ATF4 can activate several transcriptional programs that will ultimately determine the cell fate—from cell death to re-establishment of homeostasis. The ability of ATF4 to interact with multiple other transcription factors allows it to generate distinct tailored responses to different types of cellular stress. Thus, despite ATF4 being a master common regulator of ISR, its target genes will be highly dependent on stress intensity and cellular context (45–47). For example, when acting in combination with ATF3, ATF4 is a part of a program that aims to re-establish cellular homeostasis and promote survival (48). Conversely, when interacting with CHOP, ATF4 promotes cell death following ER stress (49). In addition to the interacting partners that cooperate with ATF4 to promote transcription of target genes, another set of interacting partners prevent ATF4 transcriptional activity as is the case for PHD3 during hypoxia and TRIB3 during amino acid starvation and ER (50–52).

ISR AND BACTERIAL INFECTIONS

Eukaryotes have evolved in a context of constant interactions with prokaryotes and it is clear that the latter have contributed to shape those organisms throughout evolution. A human being harbors

more than 1,000 bacterial species as part of their microbiota and interacts with another incalculable number of bacterial species during its lifetime (53). The vast majority of these interactions does not result in disease and, in many cases, they are actually beneficial to the hosts. However, despite representing less than 1% of the total number of estimated bacterial species in our planet, pathogenic bacteria still cause millions of deaths every year.

In general, those bacteria that are considered as pathogenic are the ones endowed with certain attributes that allow them to (1) colonize the host; (2) find a nutritionally compatible niche in the host body; (3) avoid, subvert, or circumvent the host innate and adaptive immune responses; (4) replicate, using host resources; and (5) exit and spread to a new host (54). However, even though some bacteria display very well-defined virulence attributes, the pathogenic potential of a given bacterium can only really be observed upon interaction with its host. The final outcome of an infection is never the result of bacterial virulence alone but rather a cross talk between the host and the pathogen. This complicates the definition of "true pathogen" as the same bacterial pathogen can have different impact in different individuals. Thus, for the host, it is critical to be able to assess the potential threat that a given pathogen represents in order to establish an appropriate response.

During a bacterial infection, a multitude of signals exchanged by the two organisms establishes a cross talk that will ultimately determine the outcome of the infectious process. Many known bacterial virulence factors are only synthesized when bacteria go through major changes in metabolism in order to adapt to the dynamic conditions of the host environment (55). While doing that, bacterial pathogens may have profound effects on host cell homeostasis that, in turn, trigger cellular stress responses. Bellow, we will discuss how the ISR can be triggered by cellular alterations caused by bacterial infections and the impact of this response on host–pathogen interactions. The data discussed in the next sections are summarized in **Figure 1**.

BACTERIAL GROWTH

The ability to survive and grow inside the host upon infection is one of the most common pathogenesis patterns as it represents the ability of a given pathogen to scape the host response and establish a replicative niche. For the host, being able to differentiate growing and dying bacteria, especially in the context of an acute infection, is key to mount a proper response. Molecules whose presence could indicate bacterial growth include peptidoglycan fragments released during bacterial cell division, quorum-sensing inducers that are produced once the bacterial population reaches a certain density and bacterial pyrophosphates such as HMB-PP (5, 56–58). Alternatively, instead of direct detection of a molecule, bacterial growth sensing could be achieved by sensing of altered local levels of cellular nutrients such amino acids or oxygen (59–61).

Recently, the definition of PAMPs has been updated to allow the classification of those produced specifically by living microorganisms, the so-called *Vita*-PAMPs, and those that represent the degradation products of dead microorganisms, named PAPMs-*postmortem* (PAMPs-PM), as two different categories that have different biological activities (10, 62). Moretti et al. (10) has recently identified cyclic-di-adenosine monophosphate (c-di-AMP), a second messenger that is produced by live Gram-positive bacteria, as a *Vita*-PAMP. The authors show that phagocytes are able to discriminate live and dead *Listeria innocua* by sensing this *Vita*-PAMP through the innate immune sensor stimulator of interferon genes resulting in ER stress, PERK and eIF2α phosphorylation. Subsequently, an autophagic response ensued to sequester stressed ER membranes and prevent stress-induced cell death while also inducing an IFN-dependent response. Importantly, this response was blunted in phagocytes lacking PERK. Finally, following *L. monocytogenes* infection, mice engineered to have PERK-deficient macrophages presented lower systemic levels of IFN-I and higher bacterial burden on both liver and spleen when compared with WT controls. In this model, at a cellular level, there was no difference between the response induced by live *L. innocua*, a non-pathogenic bacteria, and live *L. monocytogenes*, but there was differences when these were compared to dead bacteria. The response to any infection is multilayered and dependent on the interaction of multiple sensing systems—each one of these systems provides the cells with different information that when combined determine the cells response and, ultimately, its fate. In this case, the ISR provided the cells with the ability to distinguish live from dead bacteria, which is crucial to mount appropriate response even though it was not able to differentiate between a pathogenic from a non-pathogenic species. Of note, when the authors tested their hypothesis *in vivo,* they used only *L monocytogenes*, most likely because *L. innocua* would have been readily cleared given its lack of virulence and would have not generated any of the responses observed against *L. monocytogenes*. This, once again, illustrates how important the context is: in the natural course of a real infection, *L. innocua* would probably have never caused the systemic infection that *L. monocytogenes* does and would have not reached circulating phagocytic cells or the liver or the spleen. However, when given directly to these cells *in vitro*, it induced the same response that the bacterial species that would have encountered these cells during infection. It would also be interesting to investigate if this response is restricted to phagocytic cells or can occur in other cell types.

A contrasting study showed that PERK activation and IFN-I production by myeloid cells during infection with *L. monocytogens* or treatment with the pore-forming toxin LLO is actually detrimental to the host. In this model, the PERK pathway is amplified by IFN-I resulting in the activation of another eIF2α-kinase, PKR. This, in turn, served as an amplification loop for PERK-signaling leading to excessive ER stress and cell death. Consistent with this, mice deficient on CHOP, a pro-apoptotic factor that is downstream of PERK, are more resistant to *L. monocytogenes* infection than WT controls (63). This model could provide a partial explanation for why mice lacking IFN-I receptor have been consistently reported to be more resistant to *L. monocytogenes* than WT mice (64–66).

The opportunistic Gram-negative *Pseudomonas aeruginosa* causes both acute and chronic infections, especially in the respiratory tract (31, 67). Its ability to scape or subvert the host immune response constitutes its main virulence attribute. *P. aeruginosa* is able to form biofilms, a complex biological system

that protects the bacteria from host immune defense mechanisms and promotes persistent infection. This bacterium coordinates the production of biofilms and other virulence factors using quorum sensing, a cell-to-cell communication system that allow bacteria to perceive their population density by producing and sensing diffusible signal molecules. One of the quorum-sensing auto inducers produced by *P. aeruginosa* to regulate gene expression and communicate is *N*-(30oxododecanoyl)-homoserin lactone (HSL-C12) (31, 67). HSL-C12 is a lipid-like diffusible molecule that has multiple effects on mammalian cells including apoptosis and release of Ca^{2+} from the ER stores. By perturbing ER homeostasis, HSL-C12 induces the activation of PERK and eIF2α phosphorylation resulting in protein synthesis inhibition (31). If in the one hand this inhibition results in increased NF-κB activation and transcription of pro-inflammatory genes because IκB re-synthesis is blocked, on the other hand it prevents the translation into proteins of the transcribed genes resulting in an overall downregulation of the host response and, thus, can be considered a pathogen scape mechanism. This would be one instance where the pathogen evolved to manipulate and take advantage of a cell host sensing system.

As mentioned above, nutrient availability is a critical limitation for invading microorganisms. Iron is a nutrient indispensable for growth of almost living organisms and is unlikely to be readily available for invading microorganisms resulting in fierce competition between host and pathogens (59). Like many other bacteria, *P. aeruginosa* has developed several mechanisms to acquire iron during infection. In a recent study, it was demonstrated that the iron-chelating siderophore pyoverdine produced by *P. aeruginosa* limits the concentration of iron in the cell medium resulting in the activation of HRI, eIF2α phosphorylation, and induction of *Gadd34* transcription in human bronchial epithelial cells. This response had cytoprotective effect and was turned off when the medium was supplemented with iron (61).

These few examples demonstrate that host cells can detect growth of bacteria by sensing molecules that accumulate as the number of bacteria increase including those that bacteria use to communicate with each other, such as quorum-sensing auto inducers and second messengers as well as molecules that bacteria use to acquire nutrients.

MEMBRANE INTEGRITY

The detection of this type of stress is highly conserved. Damage of the plasma membrane is an archaic threat that needs to be faced with efficient cell autonomous defense mechanisms (5). Recently, a pivotal role for GCN2 in the response to membrane damage has been uncovered in different models. For example, it has been demonstrated that membrane permeabilization by the detergent digitonin induces a robust response characterized by GCN2 phosphorilation and ATF3 expression (68). In *Drosophila*, the damage caused by *Pseudomonas entomophila* in gut cells induces a starvation-like state, resulting in GCN2 and eIF2α phosphorylation and concomitant inhibition of the mTOR pathway by the AMP-activated kinase (AMPK). In this model, these two stress response pathways together shut down translation of new proteins and trigger innate immune responses (69).

In mammalian cells, disturbance of membrane integrity caused by bacterial pathogens can also trigger stress responses (8, 15, 68). Pore-forming toxins represent an important class of bacterial exoproducts that can induce membrane damage leading to stress responses (70). In human epithelial cells, the α-toxin produced by *Staphylococcus aureus* induces the formation of pores on cellular membranes resulting in potassium efflux, failure of nutrient transport and loss of ATP which, in turn, activates both GCN2 and the energy sensor AMPK, with subsequent eIF2α phosphorylation and mTORC1 deactivation (similar to what was reported in *Drosophila*) (15, 71). Low intracellular concentrations of potassium is known to trigger several responses in infected or stressed cells including the activation of inflammasomes and caspases (15, 71), as well as activation of multiple kinases such as p38 and CREB, in addition to the aforementioned AMPK and GCN2 (71–73). Activation of GCN2 induced by potassium efflux caused by membrane perforation indicates that cells may exploit the dependence of nutrient transport across the plasma membrane on physiological ion gradients to indirectly sense perturbations on ion concentration. Both removal of the pore from the plasma membrane by dynamin-dependent endocytosis and the metabolic reprogramming activated by the ISR are essential for cellular recovery as cells that are not able to activate this program are more susceptible to α-toxin (74, 75).

Invasive bacteria such as *Salmonella* Tiphymurium, *Shigella flexneri*, and *Listeria monocytogenes* also cause membrane damage during their internalization process. Similar to what was described above, all three bacteria trigger an acute intracellular amino acid starvation program that induces stress responses dependent on GCN2 and eIF2α phosphorylation at the same time as it disarms mTOR signaling unleashing an autophagic response (8, 68). However, the response that ensues is different for these three bacteria. (i) During infection with *S. flexneri*, a Gram-negative bacterium that escapes to and replicates in the host cell cytoplasm, amino acid starvation persists up to 4 h after infection allowing not only the induction of autophagy but also GCN2- and eIF2α-dependent formation of stress granules in the cytosol as well as reprogramming of the transcriptional response orchestrated by ATF3 (8, 76). (ii) *L. monocytogenes*, a Gram-positive bacterium that similar to *S. flexneri* escapes to and replicates in the cytosol, also triggers a state of amino acid starvation characterized by activation of GCN2, eIF2α phosphorylation, and transcriptional upregulation of ATF3. In this case, however, this response is very transient peaking at 1 h and is completely normalized after 4 h post-infection. The kinetics of this response parallels the kinetics of the pore-forming toxin LLO-dependent scape from the internalization vacuole and coincides with the maximal targeting of *L. monocytogenes* to autophagosomes (68). (iii) *Salmonella*, in contrast to the bacteria described above, remains in vesicles known as *Salmonella*-containing vacuoles (SCV) after its internalization. The damages to the SCV membranes trigger the same GCN2-dependent early amino acid starvation program described above. However, following *Salmonella* infection membrane integrity and cytosolic amino acid concentration are readily normalized allowing mTOR to be reactivated at the surface of the SCV and

promoting bacterial scape from autophagy (8). Thus, these three model invasive bacteria all induce GCN2-dependent ISR during their entry processes but each one of them deal with it in different ways once again highlighting that bacteria have also evolved to counteract ISR-mediated responses.

Adherent-invasive *Escherichia coli* (AIEC), which is abnormally abundant in the intestinal mucosa of Crohn's disease patients, also induces phosphorylation of GCN2 with subsequent eIF2α phosphorylation and increased ATF4 levels. Upon activation of this pathway, ATF4 binds to promoters of multiple autophagy-related genes including *MAP1LC3B*, *Becn1*, *SQSTM1*, *ATG3*, and *ATG7*. This is necessary to initiate autophagy and restrict bacterial growth as depleting cells from GCN2 resulted in impaired autophagy, increased bacterial replication, and elevated pro-inflammatory cytokine production both *in vitro* and *in vivo*. The authors go on to show that the GCN2–eIF2α–ATF4 pathway is activated in ileal biopsies from patients with noninflamed Crohn's disease but not on those with inflamed Crohn's disease, indicating that failure to activate this stress response could be one of the mechanisms contributing to active disease (77).

Thus, it appears that a nutrient sensor, GCN2, may also function as a sentinel of membrane integrity and that the responses it triggers are essential to prevent abyssal ATP loss and irreversible damage. In addition, in the case of invasive pathogens, this response might affect their ability to replicate within the host cell due to increase in autophagic activity as a consequence of amino acid starvation as well as production of inflammatory factors induced by the stress transcription factors ATF3 and ATF4.

ACCESS TO CYTOSOL

Many pathogens are able to deliver molecules directly into the cytosol of host cell. This may be achieved by AB-toxins when the B subunit binds to specific receptors on the surface of the cells and translocates the active subunit A into the cell (78), by pore-forming toxins such as listeriolysin O (mentioned above) and streptolysin O (70), or secretion systems such as the type III secretion systems of *Yersinia* and *Salmonella* (79), the type IV secretion system of *Legionella*, *Coxiella*, and *Brucella* (80), and the type VI secretion system of *Pseudomonas* and *Vibrio* (81, 82).

Shiga-toxigenic *E. coli* produces Shiga toxin (Stx) 1 and 2 that cause hemorrhagic colitis and hemolytic uremic syndrome. A newly described toxin, namely subtilase cytotoxin (SubAB), was shown to bind to and be internalized by target cells through clathrin-, lipid rafts-, and actin-dependent pathways. Once it reaches the ER, SubAB cleaves the chaperone Bip/Grp78 initiating an ER-stress induced ISR resulting in cytotoxicity. This response also included the formation of stress granules induced not only by PERK but also as a result of PKR activation (14).

Yang et al. (83) show that *P. aeruginosa* infection induces a strong activation of the GCN2–eIF2α–ATF4 pathway that is largely dependent on production of pyocianin during initial infection and that ultimately results in bacterial clearance through autophagy. Pyocianin is a cell permeable toxin considered to be a major virulence factor for *P aeruginosa*. *In vivo*, in rats, infection with a mutant bacterial strain that does not produce pyocianin and, thus, does not activate of the GCN2–eIF2α–ATF4 pathway results in higher number of colony-forming units in the lungs, more extensive alveolar wall thickening and higher mortality when compared to infection with the WT strain. Although indirect, these data suggest a role for the ISR in preventing prolonged infection and immunopathology. Interestingly, reduction of pyocianin production by *P. aeruginosa* in chronic airways infections has been associated with better host adaptation and worse outcomes in cystic fibrosis patients (84).

ACTIN CYTOSKELETON DISRUPTION

Another common feature employed by various highly divergent pathogenic bacterial species is the disruption of the host cell cytoskeleton. Invasive bacteria such as *S. flexneri*, *L. monocytogenes*, *Mycobacterium marinum*, and Rickettsial species exploit the actin-based motility to move inside the cell and from one cell to the other without never being exposed to immune defenses outside the cells (5, 85). Other bacterial pathogens, such as *E. coli* and *Citrobacter freundii,* produce hallmark attaching and effacing lesions that are characterized by localized destruction of the brush border villi of enterocytes, intimate attachment of bacteria to the residual apical membrane and formation of a dense plaque of actin cytoskeletal filaments beneath adherent bacteria that is essential for their pathogenesis (86, 87). Finally, some pathogens manipulate host actin cytoskeleton to either induce their own uptake or to avoid phagocytosis (88–90).

Polysomes, mRNAs, elongations factors, and aminoacyl-tRNA synthetases are found associated with actin filaments indicating that the cytoskeleton might actually act as a platform to facilitate the assembly of components involved translation (91–93). GCN2 has been recently implicated as a sensor of F-actin depolymerization. Disruption of the actin cytoskeleton by drugs such as latruculin-B and cytochalasin-D induces GCN2 activation followed by eIF2α phosphorylation, attenuation of global translation, and augmented ATF4 and CHOP expression (94). In nutrient-replete cells, GCN2 is kept in a latent state by the interaction with other proteins such as the eukaryotic elongation factor 1A (eEF1A) that delivers aminoacyl-tRNAs to ribosomes during the elongation step of protein synthesis (95, 96). During starvation periods, however, uncharged tRNA displaces eEF1A from GCN2 allowing its autophosphorilation and eIF2α phosphorylation (97, 98). Another binding partner of eEF1A is F-actin. In yeasts, the same mutations that affect binding of eEF1A to aminoacyl-tRNAs also result in actin binding and buding defects that lead to GCN2-dependent eIF2α phosphorylation (99, 100). Thus, it has been proposed that upon F-actin disruption eEF1A is displaced from GCN2 and bound to F-actin leaving GCN2 free to initiate the ISR (94). In addition, F-actin disruption also leads to deacylated tRNA accumulation, which in turn might also contribute to the activation of GCN2 resulting in global protein synthesis arrest and reduction of amino acylated tRNA levels (94).

As mentioned above, two invasive pathogens—*L. monocytogenes* and *S. flexneri*—that exploit the actin cytoskeleton of the cell to move around the cell and infect neighboring cells were shown to induce a GCN2-dependent starvation program as a consequence of membrane damage. It is possible that disruption

of F-actin could impede the proper function of amino acid transporters on the plasma membrane triggering this response. In summary, infection with *L. monocytogenes* and *S. flexneri* could potentially activate GCN2 in multiple ways: when bacteria escape from the vacuole into the cytosol causing membrane damage (as it has been experimentally demonstrated) or by disrupting the actin cytoskeleton.

PROTEIN AGGREGATION

Heme-regulated eIF2α kinase is able to sense and respond to a variety of types of cellular stress including heme deprivation, oxidative stress, heat shock, and proteasome inhibition, all of which are known to result in accumulation of misfolded protein aggregates in the cytosol (35, 36, 101, 102). As it has been previously shown that infection with bacterial pathogens trigger the formation of large PRM oligomeric complexes in the cytosol, one may speculate that this is the common feature among all these types of stresses that is actually sensed by HRI (103–106). This could serve as a sensing system to monitor misfolding of large protein complexes and formation of toxic aggregates in the cytosol and trigger damage control mechanisms such as ISR and autophagy.

CONCLUDING REMARKS

Even at a single cell level, the response to an infection is multilayered and involves sensing, effector, and homeostatic mechanisms. Each one of these elements has, in itself, multiple layers of complexity and, together, they generate a full-blown response. Sensing of microbes or their products by PRMs is pivotal and activates robust inflammatory responses. Since the discovery of PRMs, there has been much debate on how the cells can tailor the response to specific pathogens using a limited number of receptors that recognize structures that are present in many different microorganisms, including non-pathogenic. This can be achieved by different means including the combinatorial effect of several PRMs (107), the compartmentalization of PRMs that only allows recognition of certain PAMPs when presented in specific compartments of the cell (108) and the sensing of *vita*-PAMPs versus PAMPs-PM (62). The recognition of pathogenesis patterns by the ISR represents another layer in the host response. Sensing alterations on homeostasis and cell damaged caused by infection can instruct the host to generate a more refined and specific response while triggering protective gene expression programs that enable cells to recover from the initial stress and re-establish homeostasis. Given its origins early on evolution, stress responses may actually represent an ancient innate defense mechanism against invading pathogens.

In this review, we discussed evidence showing that the ISR can have an important role in shaping the autonomous cell response to bacteria with varying levels of virulence. In this context, the ISR acts in concert with other sensing systems to adequate the response to the threat. Thus, the ISR during bacterial infection cannot be analyzed isolated from the context. This generates a complexity that represents a challenge for dissecting the precise role and the relevance of each component in the final response. While there are still many gaps to be filled before we have a more comprehensive overview, the picture that emerges is that the ISR can influence the quality of the response initiated by innate immune recognition.

For the most part, the studies discussed in this review show that several bacteria are able to activate or manipulate the ISR during infection, through different eIF2α kinases and signaling pathways, resulting in specific transcriptional programs. However, in many cases, it is yet to be defined how this affects the outcome of the infection. In some cases, it is clear that it can affect the ensuing immune response. For example, ISR activation in phagocytic cells infected by *Listeria* was shown to be critical for IFN-I production and bacterial clearance. On the other hand, eIF2α phosphorylation induced by the HSL-C12 from *P. aeruginosa* results in downregulation of translation of pro-inflammatory cytokines such as IL-6 and KC. We believe that understanding how the ISR can affect qualitatively the response to a given pathogen is a major avenue for future work. In this sense, as we progress, it would be important to determine how the ISR impact on how cells communicate infection to the neighboring cells as well as to immune cells and how this can qualitatively affect the immune response, including in subsequent exposures to the same pathogen. Finally, it would be interesting to see if the homeostatic adaptations during infection can lead to persistent alterations in the infected cell rendering it more resistant to following infections.

The interplay between ISR and autophagy is also a common theme in most of the studies mentioned here. Interestingly, two studies discussed above showed that in the absence of the GCN2–eIF2α–ATF4-autophagy pathway, opportunistic bacteria such as AIEC and *P. aeruginosa* establish persistent infection that perpetuate inflammation contributing to worsen the pathology in Crohn's disease and cystic fibrosis, respectively. It will interesting to investigate how ISR could affect the development of chronic complex diseases that are known to have a microbial component to it such as the two mentioned above.

As we mentioned above, co-evolution of pathogens and their hosts have shaped (and continue to do so) their interactions. In this constant arms race, both sides try to adapt in order to survive. Thus, it should come as no surprise that some bacteria might be able to scape or even take advantage of the ISR to manipulate the host cell response. Indeed, being able to subvert host responses is part of the very definition of what a pathogen is. In this case, failure to activate the ISR properly could lead the host to underestimate the infectious threat. As it is well documented for several viruses, we expect that as our knowledge increases, we will uncover many bacterial strategies to tamper with the ISR.

Because of the significant overlap between the eIF2α kinases in addition to the complexity of many host–pathogen interactions, at this point, it is difficult to clear define the role of each of the ISR sensors in response to bacterial pathogens and most likely a combination of them are responsible for an appropriate response. Future work will help us understand how these pathways are activated and manipulated by bacterial pathogens and how can we use this knowledge to develop new treatments to prevent or cure infection. For example, if we are able to safely increase the signals generated by cells of the innate immunity by manipulating

the ISR, we might be able to improve the adaptive immunity generated by vaccines.

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REVIEW
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Pattern Recognition Receptors and the Host Cell Death Molecular Machinery

Gustavo P. Amarante-Mendes[1,2], Sandy Adjemian[3,4], Laura Migliari Branco[5,6], Larissa C. Zanetti[7], Ricardo Weinlich[7]* and Karina R. Bortoluci[5,6]**

[1] Departamento de Imunologia, Instituto de Ciências Biomédicas, Universidade de São Paulo, São Paulo, Brazil, [2] Instituto de Investigação em Imunologia, Instituto Nacional de Ciência e Tecnologia (INCT), São Paulo, Brazil, [3] Molecular Signaling and Cell Death Unit, Inflammation Research Center, VIB, Ghent, Belgium, [4] Department of Biomedical Molecular Biology, Ghent University, Ghent, Belgium, [5] Departamento de Ciências Biológicas, Universidade Federal de São Paulo, Diadema, Brazil, [6] Centro de Terapia Celular e Molecular (CTC-Mol), Universidade Federal de São Paulo, São Paulo, Brazil, [7] Instituto Israelita de Ensino e Pesquisa, Hospital Israelita Albert Einstein, São Paulo, Brazil

Pattern Recognition Receptors (PRRs) are proteins capable of recognizing molecules frequently found in pathogens (the so-called Pathogen-Associated Molecular Patterns—PAMPs), or molecules released by damaged cells (the Damage-Associated Molecular Patterns—DAMPs). They emerged phylogenetically prior to the appearance of the adaptive immunity and, therefore, are considered part of the innate immune system. Signals derived from the engagement of PRRs on the immune cells activate microbicidal and pro-inflammatory responses required to eliminate or, at least, to contain infectious agents. Molecularly controlled forms of cell death are also part of a very ancestral mechanism involved in key aspects of the physiology of multicellular organism, including the elimination of unwanted, damaged or infected cells. Interestingly, each form of cell death has its particular effect on inflammation and on the development of innate and adaptive immune responses. In this review article, we discuss some aspects of the molecular interplay between the cell death machinery and signals initiated by the activation of PRRs by PAMPs and DAMPs.

Keywords: PRR, pathogen recognition receptor, apoptosis, necroptosis, pyroptosis, inflammation

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****Correspondence:***
Gustavo P. Amarante-Mendes
gpam@usp.br
Ricardo Weinlich
ricardo.weinlich@einstein.br
Karina R. Bortoluci
kbortoluci@gmail.com

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INTRODUCTION

In 1989, Charles Janeway Jr. proposed the existence of a collection of receptors expressed by innate immune cells responsible for detecting conserved products of microbial origin (1). After 25 years of intense research, fierce debates, and a Nobel Prize granted on this subject, it is unquestionable that Janeway's ingenious idea has revolutionized our understanding of the immune system. Indeed, his seminal article is considered as one of the pillars of immunology (2).

The so-called Pattern Recognition Receptors (PRRs) are proteins capable of recognizing molecules frequently associated with pathogens (aka Pathogen-Associated Molecular Patterns—PAMPs). A more comprehensive description of PRRs and their signaling transduction pathways can be found elsewhere (3). Briefly, PRRs can be found associated to subcellular compartments, such as the cellular and endosomal membranes, the cytosol, as well as extracellularly, in secreted forms present in the bloodstream and interstitial fluids (3). There are four major sub-families of PRRs—the Toll-like receptors (TLRs), the nucleotide-binding oligomerization domain (NOD)- Leucin Rich Repeats (LRR)-containing receptors (NLR),

the retinoic acid-inducible gene 1 (RIG-1) -like receptors (RLR; aka RIG-1-like helicases—RLH), and the C-type lectin receptors (CLRs) (4). As predicted by Janeway, the engagement of PRRs on the innate immune cells induces co-stimulatory signals for the adaptive immune cells (particularly T lymphocytes) (5). In addition, they activate microbicidal and pro-inflammatory responses required to eliminate (or at least to contain) infectious agents, including the induction of infected cell death (6), as discussed below.

Another ingenious idea came from Polly Matzinger (7), who proposed that the immune system is less concerned with the origin of the antigens (self vs. non-self) than with the context of their encounter with our body (tissue damage vs. tissue homeostasis). In her "Danger Theory," Matzinger suggested that during tissue stress or damage, endogenous molecules are released or activated and initiate or propagate the inflammatory response, which, among other things, empower antigen-presenting cells to activate the adaptive immune response. Today, these molecules are collectively known as DAMPs (Damage-Associated Molecular Patterns). Importantly, soon enough it became clear that similarly to PAMPs, DAMPs could also engage PRRs.

These two theories together put forward the idea that our body is equipped to distinguish "healthy," homeostatic tissue turnover or encounters with foreign "friendly" microorganisms, from potential "danger" that may come from pathogens and/or tissue damage.

CELL DEATH PROGRAMS

Molecularly controlled forms of cell death are part of a very ancestral mechanism involved in key aspects of the physiology of multicellular organism, including the elimination of unwanted, damaged or infected cells. Importantly to our discussion, cell death can have a direct or an indirect impact upon the course of infection, as the elimination of infected cells may eradicate or at least restrain the growth of a given pathogen. Moreover, the recognition of dying cells or their by-products modulates both inflammatory and immune responses. In the following sections, we will briefly describe the mechanisms that govern the three major types of molecularly controlled forms of cell death, namely apoptosis, necroptosis and pyroptosis, that participate in host defense through elimination of infected cells, and how they are regulated by signals derived from PRRs. For information regarding other cell death modes please refer to the work published by the Nomenclature on Cell Death Committee 2018 (8)

Apoptosis

Apoptosis was the first type of programmed cell death to be described, initially based on morphological features that distinguished it from necrosis, an uncontrolled, accidental form of cell death observed upon extreme physicochemical insults (9). In this regard, apoptosis is characterized by chromatin condensation, nuclear fragmentation, cell shrinkage with formation of cellular membrane blebs, and, finally, cellular disintegration into fragments known as apoptotic bodies (10). Importantly, during apoptosis, the plasma membrane integrity is preserved, avoiding the release of intracellular contents to the extracellular milieu. This feature contributes to the concept that apoptosis is an (relatively) inflammatory-silent form of cell death. Indeed, recognition and elimination of apoptotic cells during physiological circumstances, such as tissue/organ sculpture during development and tissue homeostasis, occurs without the cardinal signs of inflammation. In addition, it is well established that recognition of apoptotic cells by macrophages, in particular, results in the production of anti-inflammatory molecules, such as TGF-β and PGE_2 (11). On the other hand, it is also known that apoptotic cells release a series of so-called "find-me" signals, such as extracellular ATP and lysophosphatidylcholine (LPC), capable of recruiting phagocytes to the site of apoptotic corpses, characterizing, therefore, at least one aspect of an inflammatory reaction (12, 13). Besides, more recently, it was shown that apoptosis initiated via the FAS/CD95 death receptor is associated with the release of chemokines and other immunologically active proteins that coordinates the migration of phagocytes and proper removal of apoptotic cells (14). Taken together, it is reasonable to say that although not completely "silent," apoptosis is a form of cell death that does not trigger an overt inflammatory response.

From the molecular point of view, much of our knowledge about the regulation of apoptosis came from works with the nematode *Caenorhabditis elegans*. In a series of elegant studies, Bob Horvitz and colleagues identified four crucial genes (*Ced-3, Ced-4, Ced-9, and Egl-1*) responsible for the control of developmental cell death in *C. elegans* (15), which granted him the Nobel Prize in Physiology or Medicine in 2002, together with John Sulston and Sidney Brenner, "*for their discoveries concerning genetic regulation of organ development and programmed cell death.*" Soon after Horvitz discoveries, it became clear that cell death in *C. elegans* and apoptosis in mammals shared a very similar, phylogenetically conserved mechanism. Apoptosis is executed by certain members of a family of cysteine aspartate-specific proteases called caspases (16–18). Importantly, not all caspases induces apoptosis. Caspases-1, -4, -5, -11, -12, -13, and-14 are inflammatory caspases not related to the initiation or execution of the apoptotic program. Caspases are produced as an inactive pro-form (zymogen) that can be activated either through proteolytic processing by upstream caspases (in the case of caspases-3, -6, and-7) or via dimerization in the context of multimolecular platforms, such as the apoptosome (caspase-9), the DISC (death-inducing signaling complex) (caspases-8 and-10), the PIDDosome (caspase-2), and the inflammasome (caspase-1 and-11) (16). Executioner or effector caspases, such as caspase-3, -6, and-7 (and CED-3 in *C. elegans*), are responsible for the induction of the morphological as well as the biochemical features associated with apoptosis, including oligonucleosomal DNA fragmentation and externalization of phosphatidylserine (PS) residues from the inner to the outer leaflet of the plasma membrane (19). Interestingly, in mammals, although the inhibition of effector caspases prevents apoptosis, it does not preclude cell death, which proceeds with different morphological and biochemical characteristics (20). Because of this, it has been proposed that apoptosis in mammals may not be actually a cell death mechanism, but perhaps a termination step of a cell-death

program aimed to properly dispose damaged or unwanted cells without initiating inflammatory responses (18).

There are two signaling pathways of apoptosis (**Figure 1**). The intrinsic pathway deals with signals derived from intracellular stress, such as DNA damage, oxidative stress, dysregulation of Ca^{2+} homeostasis, interference with the cytoskeleton structure, endoplasmic reticulum stress, etc. Its first layer of regulation comprises the differential expression/activation of BCL-2 family members, responsible for controlling the mitochondria outer membrane permeabilization (MOMP) (21). When the pro-apoptotic stress is too strong for a given cell, MOMP allows the selective release of certain mitochondrial proteins, such as SMAC (second mitochondria-derived activator of caspases)/Diablo (direct IAP binding protein with low pI), HtrA2 (high temperature requirement protein A2)/Omi, and cytochrome c to the cytosol. Cytochrome c associates with APAF-1 (apoptosis-activating factor-1), the mammalian CED-4 homolog, and pro-caspase-9, thereby assembling the apoptosome and enabling caspase-9 to activate the downstream effector caspases. SMAC/Diablo and HtrA2/Omi facilitate apoptosis by preventing the inhibitory action of the inhibitors of apoptosis proteins (IAPs) on the effector caspases. The extrinsic pathway, in comparison, is initiated by the interaction of trimeric, extracellular ligands (TNF-α, CD95L, and TRAIL) to their cognate receptors (TNFR1, CD95 and TRAILRI, or TRAILRII, respectively) present on the plasma membrane (10, 22, 23). The stimulation of these so-called death receptors (DRs) leads to the recruitment of adaptor molecules, such as TRADD (Tumor necrosis factor receptor type 1-associated death domain protein) and/or FADD (Fas-associated protein with death domain), and the pro-caspase-8, giving rise to the conventional DISC. Next, caspase-8 directly activates the effector caspases or amplifies the cell death signal by engaging BID (BH3 interacting-domain death agonist), a pro-apoptotic member of the BCL-2 (B-cell lymphoma 2) family, leading to MOMP, cytochrome c release and assembly of the apoptosome (**Figure 1**). It is important to mention that the activation of caspase-8 in the context of DISC can be regulated by c-FLIP (cellular FLICE-like inhibitory protein), a catalytically-dead caspase-8 homolog (24).

In some instances, apoptosis can also be triggered by TLR stimulation, as a defense mechanism against infection. TLR2 was the first PRR to be associated with induction of apoptosis, by virtue of its ability to recruit FADD via MyD88 (Myeloid differentiation primary response 88), and the consequent activation of caspase-8 (25). Likewise, bacterial lipoproteins were reported to trigger apoptosis through this TLR2 pathway (26, 27) and *Mycobacterium tuberculosis* was also shown to induce TLR-2/caspase-8-dependent apoptosis in macrophages (28). Interestingly, TLR3-induced apoptosis is mediated via TRIF (TIR-domain-containing adapter-inducing interferon-β), which interacts with RIPK1 (Receptor Interacting Serine/Threonine Kinase 1) through its RHIM (RIP homotypic interaction motif) domain (please refer to necroptosis section for further information on these protein-protein interactions). FADD is then recruited, and activates caspase-8 leading to apoptosis (25, 29). In human keratinocytes, poly I:C-induced apoptosis required the stimulation of TLR3 and its adaptor TRIF, thus inducing caspase-8 activation (30); the same molecules were shown to induce apoptosis in human breast cancer cells (31). Not surprisingly, TLR4 can induce apoptosis either via MyD88 or TRIF, and depending on the cell type or conditions engage the extrinsic or intrinsic pathways. For instance, *Yersinia* was shown to induce TLR4-mediated apoptosis of macrophages through TRIF (32, 33). TRIF-mediated apoptosis seems to be executed through the extrinsic pathway, with no evidence of the involvement of the mitochondrial pathway (34). Interestingly, UV irradiation was shown to induce apoptosis in murine macrophages through TLR4 and MyD88 (35). Despite these observations and a number of other examples that we have not presented here, it is important to emphasize that PRR-induced apoptosis is a relatively minor event compared to all other triggers of apoptosis and that PRR activation leads preferentially to other forms of regulated cell death, as we will discuss below.

Necroptosis

Evidence of a molecularly controlled necrotic cell death was first provided by studies showing that Tumor Necrosis Factor Receptor 1 (TNFR1) and CD95 ligation were capable of inducing necrosis, particularly when caspase activity was inhibited (36, 37). This idea was further supported by a study that demonstrated that the cowpox virus could induce necrosis in porcine kidney cells when it harbored the caspase inhibitor CrmA (cytokine response modifier A) (38). This cell death mode was named "Necroptosis," as it reflects the existence of a molecular pathway (like apoptosis) but with a necrotic phenotype.

The first molecule to be identified in the necroptotic pathway was RIPK1 as its kinase activity inhibitor, necrostatin-1 (Nec-1), was shown to suppress cell death triggered by caspase inhibition during TNFR1/Fas stimulation (39). RIPK1 has been previously involved in apoptotic and survival pathways, functioning as a scaffold protein to the assembly of the respective signaling platforms (40). Contrastingly, the RIPK1 kinase activity is indispensible for death receptor-triggered necroptosis, as its auto-phosphorylation induces a conformational change that allows RIPK1 to recruit, via their respective RHIM domains, the next member of this pathway, namely RIPK3 (41–43). Once recruited, RIPK3 gets activated by auto-phosphorylation and forms an amyloid-like structure, which promotes the recruitment and activation of Mixed Lineage Kinase Domain-Like (MLKL) (42, 44–47). RIPK3-phosphorylated MLKL oligomerizes and translocates to the plasma membrane, where it interacts with phosphatidylinositides and induces plasma membrane disruption [(48–51); **Figure 2**]. Distinct effector mechanisms were raised to account for the MLKL-driven permeabilization of the plasma membrane, either directly by pore or cation channel formation, or indirectly, by activation of TRPM or other ion channels (48–52). It is still unclear, however, which of these mechanisms are physiologically relevant. Nonetheless, in all cases, MLKL induces a loss of osmolality control, which causes cell swelling and membrane rupture. Recently, ESCRT-III machinery was suggested to counter these effects by shedding out the MLKL-damaged plasma membrane regions (53).

Necroptosis can be initiated by a variety of signals. The first to be described and most thoroughly studied was TNFR1

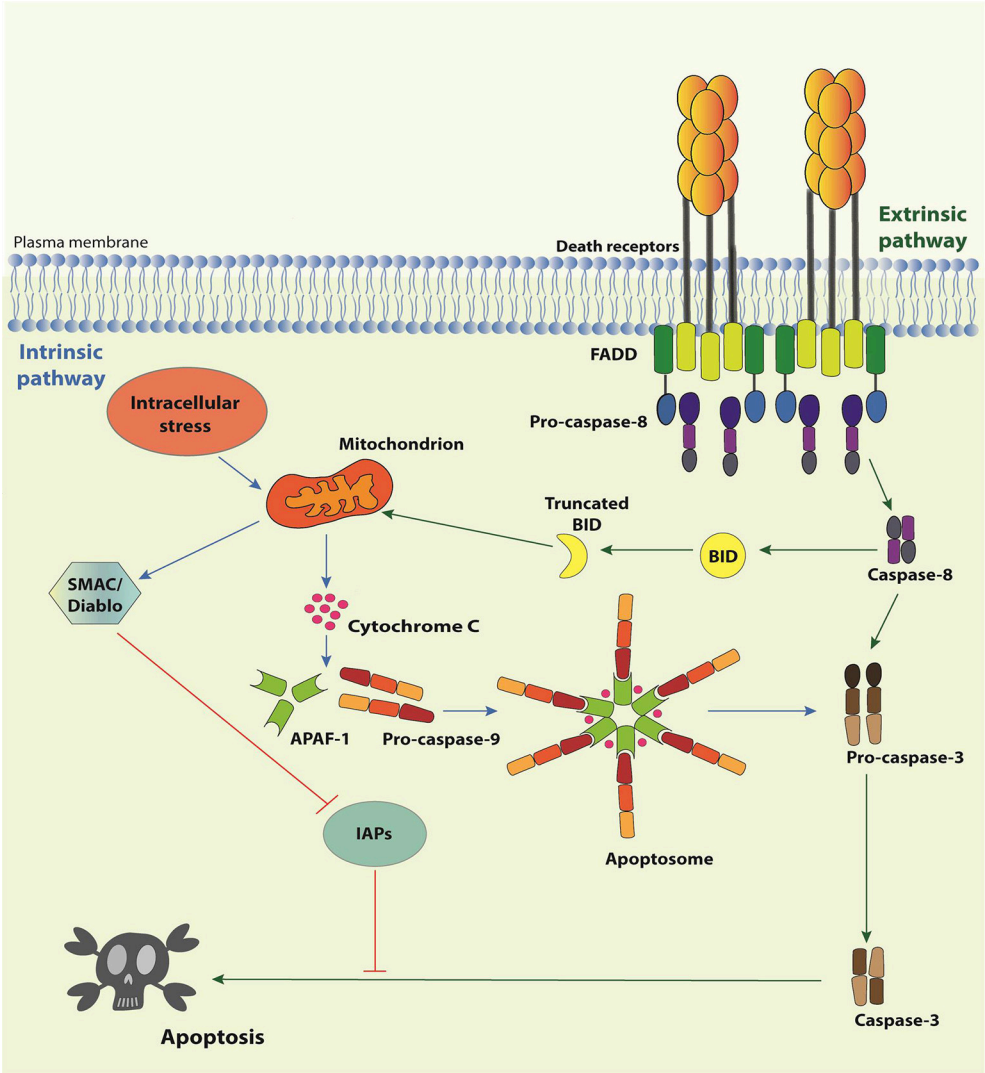

FIGURE 1 | Apoptosis pathways. The Intrinsic Pathway of apoptosis is activated when intracellular "stresses," such as DNA or cytoskeleton damage or absence of growth/survival factors, are "perceived" by BH3-only members of the Bcl-2 family. These molecules become activated and migrate to the mitochondria where they facilitate or actively induce the release of apoptogenic factors, such as cytochrome c and SMAC/Diablo, to the cytosol. Cytochrome c associates with APAF-1 and pro-caspase-9 to form the apoptosome, resulting in the activation of caspase-9, which activates the effector caspases-3, -6, and -7, responsible for the biochemical and morphological modifications associated to apoptosis. SMAC/Diablo participates by preventing inhibition of caspases by IAPs. The extrinsic pathway of apoptosis initiates by the engagement of Death Receptor by their cognate Death Receptor Ligands causing the formation of the Death-inducing signaling complex (DISC). DISC is formed by the intracellular portion of the Death Receptors, the adaptor proteins TRADD and/or FADD and the pro-caspase-8 (or pro-caspase-10). Activated caspase-8 may directly activate the effector caspases or process the BH3-only protein Bid. Truncated Bid migrates to mitochondria and activates the extrinsic pathway of apoptosis.

ligation (36). Upon its ligation, TNFR1 typically assembles a multimolecular complex (Complex I) composed by TRADD, RIPK1, TRAF2, TRAF5, cIAP1, cIAP2, and LUBAC (linear ubiquitin chain assembly complex), which is involved in NF-κB activation, pro-inflammatory cytokines synthesis and cell survival (54). Sustained TNFRI ligation leads to CYLD-mediated deubiquitination of this complex, which disassembles, allowing the formation of a secondary complex (Complex II) in the cytosol, constituted by TRADD, FADD, RIPK1, caspase-8, and occasionally c-FLIP (54). As pointed out above, when c-FLIP levels are low, caspase-8 forms active homodimers and triggers downstream events that culminate in apoptosis. However, in the absence of FADD, c-FLIP or a functional caspase-8, TNFRI signaling results in the recruitment of TRADD and RIPK1, forming a platform called complex IIb or necrosome, wherein RIPK3 and MLKL are activated to execute necroptosis (55). Although slightly differing on how RIPK1 is brought to the complex, this molecule has also a central role in Fas and TRAILR-induced necroptosis, as RIPK1 is, in all these cases, mandatory to

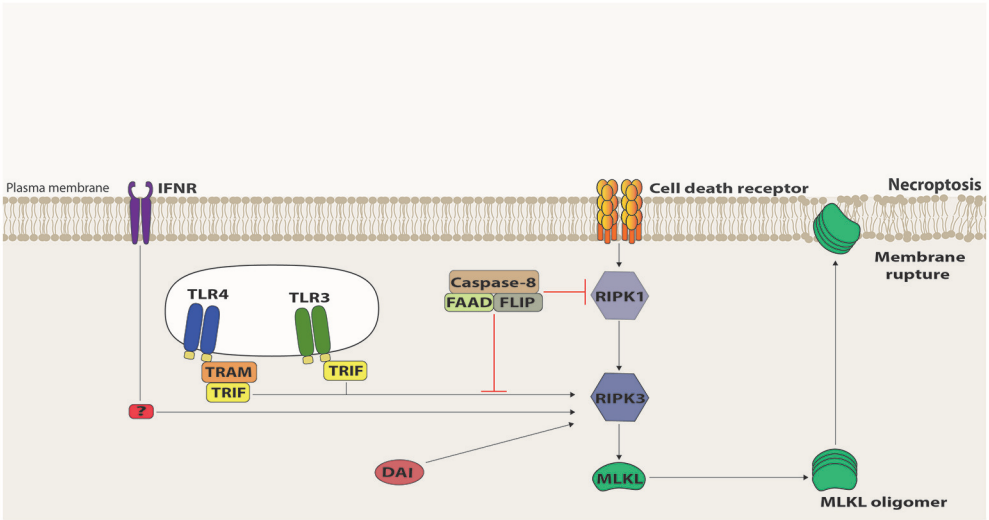

FIGURE 2 | Necroptosis signaling. Death Receptor (DR)-induced necroptosis requires RIPK1 kinase activity to recruit RIPK3 that, in turn, recruits and activates MLKL via phosphorylation of its pseudokinase domain. Once phosphorylated, MLKL oligomerizes and migrates to the plasma membrane, where it interacts with phosphatidylinositol phosphates and induces membrane destabilization and rupture. Necroptosis signaling mediated by TRIF, IFNR, and DAI can directly activate RIPK3 and, in this case, RIPK1 acts as a negative regulator, mostly by recruiting to the signaling platform the suppressive complex containing Caspase-8, FADD and c-FLIP.

recruit RIPK3 via their RHIM homotypic domain interactions (54).

Necroptosis can also be triggered by PRRs, such as TLR3 and TLR4, intracellular sensing proteins, such as DAI, RIG-I and MDA-5 as well as interferon signaling [(56–59); **Figure 2**]. Intriguingly, however, RIPK1 is dispensable for or even inhibitory of the necrosome formation during TLR3-, TLR4-, DAI-, and interferon-mediated necroptosis (57, 60). In these cases, RIPK3 is directly recruited to the signaling platforms, and the presence of RIPK1 slows down or halts the RIPK3-mediated activation of MLKL (60, 61). The ability of RIPK1 to recruit FADD, and consequently, caspase-8 and FLIP accounts, at least in part, for its inhibitory property. Therefore, from the molecular point of view, necroptosis ought to be defined as a RIPK3-dependent form of cell death.

Many other stimuli have been described as capable to induce necroptosis, ranging from UV irradiation, chemotherapeutic drugs (such as cisplatin, etoposide, and staurosporine), natural compounds (such as shikonin and its analogs), to DNA damage, hypoxia, ischemia/reperfusion and oxidative stress (62). The signaling pathways that lead to necroptosis in each of these cases are still to be fully elucidated. Further studies are required to evaluate whether they are dependent on RIPK1 and also whether they directly signal to a RIPK3-activating platform or indirectly, via up regulation of a classic necroptotic inducer, such as TNF or FasL. For example, UV irradiation was reported to induce necroptosis via TNF upregulation but also via spontaneous aggregation of RIPK1 and RIPK3, independently of any death receptor ligation (29, 63). Particularly puzzling is the fact that shikonin, a naphthoquinone compound obtained from a plant extract, can induce necroptosis even in the absence of FADD/caspase-8/FLIP inhibition, which is thought to be mandatory for this type of cell death (64). Thus, either this compound can itself somehow block their activity, or it shall be instrumental to decipher alternative ways in which MLKL is activated and necroptosis is executed.

Nonetheless, despite the different mechanisms that initiate necroptosis, in all cases cells undergo rapid MLKL-mediated plasma membrane permeabilization with consequent release of intracellular contents, including many DAMPs, such as lysosomal proteases, DNA, mtDNA, ATP, and HMGB1 [(55); **Table 1**]. Therefore, similarly to pyroptosis (see below), necroptosis is considered a pro-inflammatory form of cell death. Even so, it is still to be determined whether the pro-inflammatory properties of necroptotic cells are the result of the intracellular content leakage or, rather, they can actively produce and/or modify specific DAMPs. Evidence for the latter comes from ESCRT-III-deficient cells that undergo necroptosis much faster, which limits the amount of inflammatory cytokines and chemokines produced and hinders antigen cross-presentation (53). Moreover, both RIPK3 and MLKL have been associated with inflammasome and NF-κB activation, supporting the notion that the pro-inflammatory potential of necroptotic cells goes beyond the passive release of their intracellular content (110–112).

Necroptotic cells not only induce a potent inflammatory response but they are also highly immunogenic, which may be instrumental against infection and during anti-tumoral responses. For example, mice injected with necroptotic cells present a higher $CD8^+$ T cell cross-priming and increased tumor immunity when compared with animals injected with apoptotic cells (113, 114). Likewise, RIPK3 deficiency in mice inhibits immune cell infiltration and attenuates organ injury during sepsis

TABLE 1 | DAMPs released by cell death and its role in the immune system.

DAMPs	Immunogenic function	Receptors	Related cell death	References
Adenosine triphosphate (ATP)	DC and Mφ activation Inflammasome activation	P2Y2,6,12, P2X1,3,7 NLRP3	Apoptosis Pyroptosis Necroptosis NCD	(65–69)
Annexin A1 (ANXA1)	"Eat me" signal Immunogenicity	FPR1	Apoptosis	(70)
ASC specks	Lysosomal damage IL-1β activation	unknown	Pyroptosis	(71)
Calreticulin	"Eat me signal" Immunogenicity	CD91	Apoptosis	(72, 73)
Cyclophilin A	Cytokine induction	CD147	Necroptosis NCD	(74, 75)
Defensin α	Antimicrobial Anti-inflammatory	CCR2, CCR6, TLR4	Apoptosis NCD	(76)
Heat shock proteins (HSPs)	Monocytes and neutrophils attraction DC maturation	CD91, TLR2, TLR4, SREC1 and FEEL1	Necroptosis NCD	(77–79)
HMGB1	DCs and Mφ activation Cytokine activation	CXCR4, RAGE, TLR2,4,9	Apoptosis Necroptosis Pyroptosis	(69, 80–84)
HMGN1	Leukocyte recruitment DC maturation	TLR4	Necroptosis NCD	(85–87)
IL-1α	DC and Mφ activation Cytokine induction	IL-1R	Necroptosis Pyroptosis NCD	(88–90)
IL-33	Cytokine induction DC activation	ST2	Necroptosis NCD	(91, 92)
IL-6	Immune responses T cell differentiation	IL6R and GP130	Necroptosis NCD	(61, 93)
Lysophosphatidylcholine (LPC)	Monocyte and Mφ recruitment DC maturation "Eat me" signal	G2A	Apoptosis	(94, 95)
Mitochondrial DNA (mtDNA)	Mφ activation PMNs activation NLRP3 activation	TLR9	Necroptosis Pyroptosis	(96–99)
N-formyl peptides (NFP)	PMNs activation Monocyte activation	FPR1	NCD	(97, 100)
Nucleic acids (dsDNA/dsRNA)	DC activation Inflammasome activation Cytokine induction	TLR3, TLR7/8, TLR9, AIM2	Apoptosis Necroptosis Pyroptosis NCD	(68, 79, 101, 102)
Peroxiredoxin 1 (Prx1)	Cytokine induction CD maturation	TLR4	NCD	(103)
S100	Leukocyte recruitment Cytokine induction	RAGE, TLR4	Necroptosis NCD	(79, 104, 105)
SAP130	Mφ activation Neutrophil recruitment Cytokine induction	Mincle	Necroptosis NCD	(106, 107)
Uric acid	DC activation Inflammasome activation	P2X7, NLRP3	NCD	(108, 109)

NCD stands for Necrotic Cell Death, which means the referred papers only characterized the cell death by its necrotic morphology. AIM2, absent in melanoma 2; ASC, Apoptosis-associated speck-like protein containing a CARD; CCR, CC chemokine receptor; CD, cluster of differentiation; CXCR, CXC chemokine receptor; DC, dendritic cells; FEEL-1, fasciclin EGF-like laminin-type EGF-like and link domain-containing scavenger receptor-1; FPR-1, formyl peptides receptor-1; G2A, G2 accumulation; GP130, Glycoprotein 130; HMGB1, high-mobility group box 1 protein; HMGN1, high-mobility group nucleosome-binding domain 1 protein; IL, interleukin; LRR and PYD domains-containing protein 3; Mincle, Macrophage inducible Ca^{2+}-dependent lectin receptor; Mφ, macrophage; NLRP3, NACHT LRR and PYD domains-containing protein 3; P2XR, P2X receptor; P2YR, P2Y receptor; PMNs, polymorphonuclear leukocytes; RAGE, receptor for advanced glycation end-products; SAP130, Sin3A Associated Protein 130; SREC-I, Scavenger receptor expressed by endothelial cells; ST2, Interleukin 1 receptor-like 1; TLR, Toll-like receptor.

(115). Therefore, given that necroptosis is highly immunogenic, disruption in the necroptotic pathway would be expected in some pathophysiological conditions. Indeed, it was reported that most of the *in vitro* transformed cells as well as human tumor samples have low or no expression of RIPK3 (116), and a cohort of chronic lymphocytic leukemia patients present down regulation of CYLD (117). Furthermore, patients with lower expression of RIPK3 or MLKL have worse prognosis for breast cancer or ovarian cancer, respectively (116, 118), suggesting that resistance to necroptosis is positively selected during tumor growth and/or development. This may be associated with an increased ability to evade immune attack, either by prolonging the lifespan of the transformed cells, by decreasing the availability of DAMPs, or by avoiding the activation of antigen-presenting cells during the immune responses. Therefore, induction of necroptosis in tumors may change its immunogenicity and promote a better immune response against it. This is particularly exciting, as we are currently witnessing novel and promising approaches in tumor treatment that are based on stimulation of the immune system. On the other hand, it is possible that the inflammation generated by necroptosis may promote tumor development by stimulating angiogenesis and metastasis (119). Therefore, thorough investigation of the benefits and pitfalls of inducing inflammatory cell death for each cancer type will be required in order to determine whether inducing necroptosis is indeed a good option in the specific cancer treatment.

Besides its impact on tumorigenesis and tumor progression, deficient necroptotic signaling can be detrimental during viral infection. Mice lacking RIPK3 are highly sensitive to vaccinia virus due to widespread infection (120). Likewise, RIPK3-deficient mice are more susceptible to Influenza A virus (IAV) than the wild-type animals (121). Remarkably, seasonal IAV, but not the 1918 and 2009 pandemic IAV strains, induces RIPK3-mediated immunogenic death of dendritic cells (122). The pandemic strains' ability to suppress necroptosis was mapped to the hemagglutinin (HA) genomic segment (122), indicating that either the pandemic strains' HA do not induce necroptosis or it may directly interfere with the necroptotic signaling pathway.

Keeping with the notion that suppressing necroptosis is advantageous to the infectious agent, there is accumulating evidence that viruses can encode molecules that are able to directly interfere with the necroptotic signaling. vIRA, a molecule expressed by MCMV that contains a RHIM-like domain blocks RIPK3 recruitment to RIPK1 and to DAI (57). MCMV expressing vIRA mutated in its RHIM domain produces an attenuated viremia in wild-type mice, which is reverted in RIPK3-deficient animals (57). Likewise, HSV-1 and HSV-2 express ICP-6 and ICP-10, respectively, which are able to suppress necroptosis in human cells through a similar RHIM-dependent mechanism (123, 124). Curiously, in mice, ICP-6 was shown to promote necroptosis through direct aggregation with RIPK3, restricting virus propagation (124, 125). A different mode of action was reported for the IE1-regulated gene product expressed by HCMV, which suppresses necroptosis downstream of RIPK3 activation and MLKL recruitment (126).

Bacteria can also induce necroptosis, at least *in vitro*. It is less clear, though, whether necroptosis plays a central role in bacterial infections *in vivo*. Loss of RIPK3 in combination with deletion or inhibition of caspase-8 or FADD renders mice susceptible to a number of pathogens, including *Yersinia* and *Citrobacter* (127, 128). However, the relative contribution of necroptosis and caspase-8-mediated apoptosis in these models were not yet tested, as caspase-8- or FADD–deficient animals are not viable (129–131).

Necroptosis, though, may not always be protective against infection. Macrophage death by necroptosis correlates with increased susceptibility to *Salmonella* infection (132). Also, HIV-specific $CD8^+$ T cell response, which is a key indicator of infection control, is impaired due to increased necroptosis levels in this cell population (133). Taken together, necroptosis seems to be detrimental when it eliminates the population that is central for the immune control of the infection. In the other cases, necroptosis limits infection, mostly likely by destroying the pathogen's replicative niche through a cell death mode that generates a pro-inflammatory and immunogenic environment. However, it is important to note that, as mentioned above, RIPK3 and MLKL were shown to participate of additional signaling platforms, including inflammasome activation, NF-κB signaling and even apoptosis induction (134). Therefore, in the light of these novel RIPK3 and MLKL roles, it is essential to reevaluate the relative contribution of necroptosis to the phenotypes observed. A good illustration comes from the fact that while RIPK3-deficient mice are more susceptible to IAV, MLKL-deficient animals are not, indicating that necroptosis is not the sole RIPK3-mediated mechanism important in IAV control (121). In fact, it was shown that IAV also triggers RIPK3-mediated apoptosis, via recruitment of RIPK1, FADD and caspase-8. This was further supported by the fact that MLKL-caspase-8 double deficient mice present similar levels of susceptibility to IAV infection observed with the RIPK3-deficient animals (121). Another example is that RIPK3-deficient mice are less susceptible to *Staphylococcus aureus* lung damage and present reduced bacterial loads and inflammation, while MLKL-deficient animals present an opposite outcome, suggesting that these molecules have independent, non-necroptotic roles (135).

Pyroptosis

Pyroptosis is a necrotic form of regulated cell death distinct from necroptosis, mainly due to the requirement of inflammatory caspase-1 and/or caspase-11 (murine caspase-11 corresponds to caspases-4 and -5 in humans) [(136); **Figure 3**]. It is the result of pore formation in the plasma membrane that increases osmotic pressure ensuing in osmotic lysis and, consequently, the release of the intracellular content, including pro-inflammatory cytokines and DAMPs (137). Although distinct from the typical oligonucleosomal fragmentation observed during apoptosis, DNA fragmentation is also a hallmark of pyroptosis, which seems to occur independently of the caspase-activated DNase (CAD) (138).

Pyroptosis is a form of cell death initiated in response to the engagement of certain members of the PRRs, which are capable of assembling complex structures called inflammasomes. These platforms are composed by a sensor protein, either from the NLR or the pyrin and HIN domain-containing protein

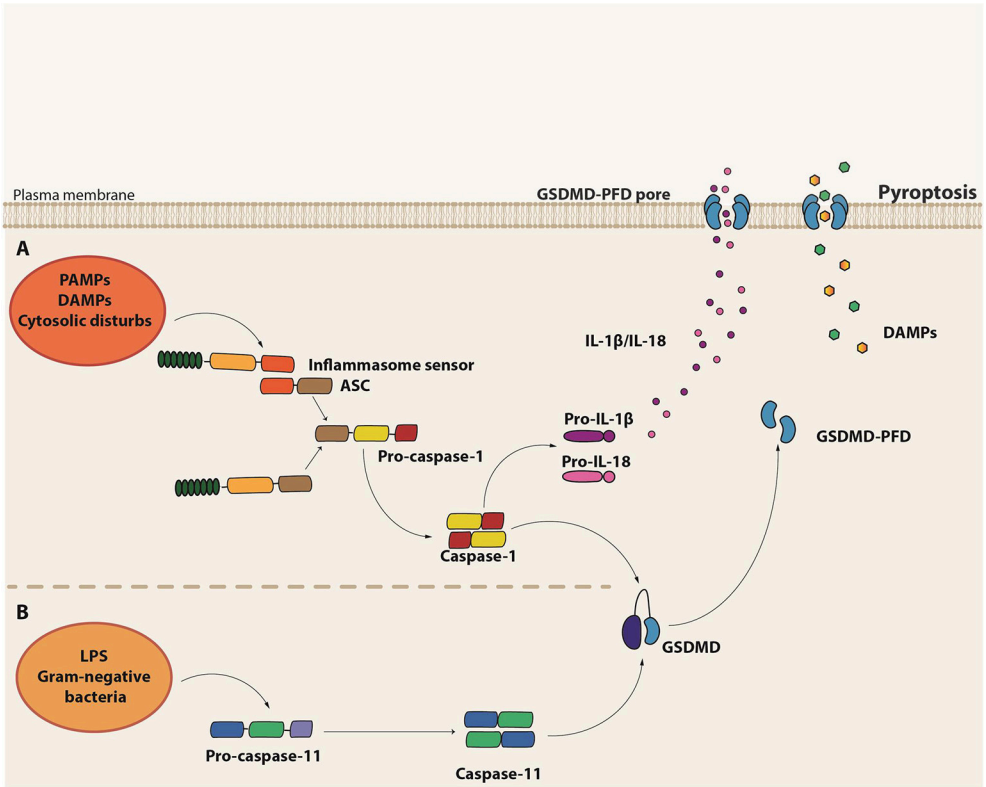

FIGURE 3 | Molecular basis of pyroptosis. **(A)** Canonical inflammasome assembly upon sensing of PAMPs, DAMPs or other cytosolic disturbs leads to the recruitment and activation of caspase-1 directly or via the recruitment of the adaptor protein ASC. Caspase-1 induces the maturation of pro-IL-1β and pro-IL-18 into their active forms as well as cleavage of Gasdermin D (GSDMD). The GSDMD pore form domain (PFD) interacts with the plasma membrane to form the GSDMD pore, leading to the release of the intracellular content, including IL-1β and IL-18. **(B)** Non-canonical inflammasome activation is initiated by the detection of cytosolic LPS from gram-negative bacteria by the pro-caspase-11 itself. Activated caspase-11 (caspase-4 or caspase-5 in humans), in turn, induces GSDMD cleavage and consequent pyroptosis.

(PYHIN) families of cytosolic PRRs, in addition to the adaptor molecule apoptosis-associated speck-like protein containing a caspase activating and recruitment domain (ASC) and pro-caspase-1. There are five major types of the so-called canonical inflammasomes—NLR pyrin domain-containing 3 (NLRP3), NLRP1, neuronal apoptosis inhibitory protein (NAIP)/NLR CARD-containing 4 (NLRC4), absent in melanoma 2 (AIM2), and PYRIN inflammasomes (139). Activation of one of these cytosolic sensors in response to PAMPs, DAMPs or cytosolic disturbances such as ionic imbalance leads to the recruitment and activation of caspase-1 either directly or through the ASC adaptor molecule [(140); **Figure 3**]. Besides the induction of pyroptosis, caspase-1 also leads to the processing and release of the pro-inflammatory cytokines IL-1β and IL-18 (139).

In contrast to the canonical inflammasomes, which require multicomplex structures, the non-canonical inflammasome seems to be composed solely by pro-caspase-11, which plays the role of the sensor as well as the executor (141, 142). During intracellular gram-negative bacteria infections, Lipid A, a component of LPS, can directly bind to the CARD domain of pro-caspase-11 (143), which gets activated and induces pyroptosis. Interestingly, the non-canonical caspase-11 inflammasome acts independently of LPS recognition by TLR4 and does not directly induce IL1-β and IL-18 maturation [(141, 142); **Figure 3**]. In monocytes, however, non-canonical inflammasome stimulation may result in minor production of IL-1β and IL-18 through the bystander induction of NLRP3 activation (144). Interestingly, LPS-induced lethal shock is driven by the activation of the non-canonical inflammasome. Since IL-1β and IL-18 release are not a major outcome of caspase-11 activation, the exacerbated inflammatory response observed in sepsis seems to be mainly driven by pyroptosis, probably due to the efflux of DAMPs, such as High Mobility Group Box 1 (HMGB1) and IL-1α [**Table 1**; (145)].

Such non-canonical inflammasome-mediated responses have drawn the attention of different research groups that became interested in unraveling the relevant as well as the pathogenic caspase-11 downstream targets. In 2015, two concurrent studies reported that Gasdermin D (GSDMD), a member of the GSDM family, was the effector component of the non-canonical inflammasome pathway (146, 147), which was later confirmed by a third study (148). Kayagaki and colleagues performed N-ethyl-N-nitrosourea mutagenesis screening for mutations

that compromised LPS-induced IL-1β release and pyroptosis (146) while Shi and colleagues employed the clustered regularly interspaced palindromic repeat (CRISPR)/Cas9 genome-editing screens in TLR4 deficient mouse bone marrow-derived macrophages for guide RNAs that protected from LPS-induced cell death (147). Both studies hit GSDMD as a substrate for caspase-11 and the effector of pyroptosis. GSDMD is composed by a C-terminal and a N-terminal domain linked by a long loop. Caspase-11 cleaves an aspartate residue within the linking loop, releasing the N-terminal fragment from the inhibitory C-terminus (146, 147). The N-terminal domain, also called Pore-Forming Domain (PFD) (149) oligomerizes and associates with lipids in the inner plasma membrane to form 10–33 nm pores leading to cell swelling and eventually to cell lysis (150–153). Importantly, it was also demonstrated that caspase-1 cleaves GSDMD at the same site as caspase-11, establishing that GSDMD is also required for the canonical inflammasome-driven pyroptosis (147).

Until the discovery of GSDMD as the pyroptosis executioner, the physiological function of GSDM proteins was largely unknown. However, recent studies described that the PFD is highly conserved among several members the GSMD family. Indeed, expression of PFD from GSDMA, GSDMA3, GSDMB, GSDMC, GSDME, or GSDMA3 in HEK293 was able to induce pore formation and a cell death phenotype similar to pyroptosis (147, 151). Moreover, GSDMA3 cleavage by caspase-3 in HEK293 and macrophages results in a secondary necrotic cell death after apoptosis (154). This necrotic cell death might contribute to hearing loss in GSDMA3 spontaneous mutations that are associated with deafness (155). Thus, given the cytotoxic activity of different GSDM PFD, some authors have proposed a redefinition of pyroptosis as a GSDM-mediated cell death (146). However, it is controversial how other GSDM members are activated and whether these proteins participate in cell death pathways. Also, GSDMD seems not to be required for pyroptosis during prolonged inflammasome activation in response to the classical agonists, ATP, and flagellin (146). Moreover, in the absence of caspase-1 protease activity, caspase-8 accounts for GSDMD-independent cell death in response to inflammasome agonists (156–158). Since some of these processes share features of pyroptosis, it is hard to define pyroptosis solely as being a process of cell death regulated by inflammatory caspases or mediated by GSDM proteins, since we can find exceptions to the rules that govern both concepts.

From the biological point-of-view, cell death by pyroptosis results in a fast removal of infected cell leading to the elimination of the replication niche. Conversely to the previous idea of liberation of bacteria to the extracellular milieu by pyroptotic cells (159), the current knowledge predicts that, instead, the damaged bacteria remain trapped within the pyroptotic corpses. This structure is called pore-induced trap (PIT) and it prevents bacterial dissemination (160, 161). Despite that PIT does not directly kill intracellular bacteria, pyroptosis renders them more susceptible to H_20_2, to the antimicrobial peptide polymyxin B and to the antibiotic ciprofloxacin (157). As a consequence, the recovered bacteria from PIT are less capable to infect neighbor cells.

The inflammatory milieu created by the release of the intracellular content from pyroptotic cells recruits circulating phagocytes to the infectious site. Subsequently, neutrophils efferocyte the PIT and kill the pathogen by a mechanism dependent on reactive oxygen species (ROS) (161). Extracellular bacteria can also be controlled by the action of antimicrobial peptides (160, 161) and potentially by the GSDMD N-terminal domain released during cell lysis due to its affinity to cardiolipin and phosphatidylserine expressed in some bacterial cell membranes, such as *Escherichia coli* and *Listeria monocytogenes* (152, 162). Interestingly, canonical and non-canonical inflammasomes are required for intestinal epithelial cells (IECs) responses to infections (163, 164). The activation of NLRC4 inflammasomes in IECs results in a lytic cell death prior to a non-conventional process of cell expulsion that contributes to control bacterial replication. Although caspase-1 and Gasdermin-D were required for IEC pyroptosis, both molecules were dispensable for cell expulsion, demonstrating that coordinated inflammasome responses in IECs are important to prevent bacterial translocation to deeper tissues (163, 164).

Interestingly, neutrophils seems to be more resistant to pyroptosis than macrophages in response to *Salmonella* and are able to maintain a sustained IL-1β production and secretion, which could be important to control the infection (165). However, Kambara et al. (166) recently described that a specific neutrophil elastase (ELANE) is able to cleave GSDMD independently of caspases activity, promoting a lytic cell death in these cells. Interestingly, these authors demonstrated that GSDMD-dependent neutrophil death impairs the control of extracellular bacteria *E. coli*, thus suggesting that GSDMD could exert an anti-inflammatory role depending on the infection context.

In addition to its role in the elimination of replicative niche, the pyroptosis machinery is involved in IL-1β and IL-18 release. As these cytokines lack the signal peptide, their release is considered to occur by non-conventional pathways (167). Among the different pathways that have been proposed to explain their secretion, mechanisms involving cell death are particularly subject to intense debate in the literature. Growing evidences suggest that IL-1β can be released by viable monocytes (168), dendritic cells (DCs) (169), and macrophages (170). GSDMD pore is large enough to allow IL-1β release concomitant with the influx of cationic ions (148). Notably, in viable cells, GSDMD seems to be required for IL-1β translocation to the extracellular space in response to stimuli that hyperactivate phagocytes, such as oxidized phospholipids (oxPAPC) in DCs or LPS in human monocytes (170–172). Nonetheless, it is difficult to establish whether the cells were actually viable, since cell death can precede cell lysis, thus suggesting that pyroptosis and cell lysis can be uncoupled events (173). Moreover, the assessment of cell death by the detection of lactate dehydrogenase release (LDH), used in several studies as the only viability assay, might be insufficient to discriminate viable cells from dying cells since both viable and unviable cells can release LDH to the cell culture (170, 173, 174).

Although many studies have demonstrated the requirement of canonical and non-canonical inflammasomes to host defense

against pathogens, the precise contribution of pyroptosis and other inflammasome-related mechanisms are poorly understood and arose mainly from *in vitro* assays or bacterial infection models in mice deficient for molecules that compose these platforms (159, 175). In *L. monocytogenes*, *S. typhimurium,* and *B. thailandensis in vivo* infections, the lack of caspase-1/11 was more deleterious to the host than IL-1β/IL-18 deficiency (176–179), while in mice infected with *F. novicida*, the treatment with recombinant IL-1β/IL-18 only partially recovered the resistance to infection, suggesting that cytokine secretion was not sufficient to protect the host (180). The susceptibility of GSDMD-deficient mice seems to correlate with that demonstrated by caspase-1/11-deficient mice, although the deletion of GSDMD also culminated in reduced IL-1β/IL-18 release (146–148). Even though (a) IL-1β/IL-18 secretion might occur independently of pyroptosis and (b) caspase-1 or caspase-11 deletion is more severe than IL-1β/IL-18 ablation in some bacterial infections, usually cytokine release and cell death are overlapping events necessary for optimal host defense (159). In any case, the susceptibility of GSDMD and other GSDM deficient mouse strains to infectious agents and its comparison to mice lacking caspase-1, caspase-11, IL-1β, and/or IL-18 remains to be established, especially in non-bacterial infection contexts. For example, despite clear evidences of the involvement of inflammatory caspases in the host control of some fungal infections such as *Candida albicans*, *Aspergillus fumigatus,* and *Paracoccidioides brasiliensis* (172), the requirement of GSDMD to cell death and the consequences to the host resistance against these infections is still to be elucidated.

Notwithstanding, the highly pro inflammatory outcome of pyroptosis as well as the cell loss can be prejudicial to the host during the response to pathogens. In HIV patients, the quiescent CD4 T cells depletion seems to be mainly mediated by pyroptosis (181, 182). During HIV abortive infection, the engagement of the interferon-gamma-inducible-protein 16 (IFI16) in response to cytosolic viral DNA leads to inflammasome assembly and caspase-1 mediated CD4 T cells pyroptosis in lymphoid tissues (181, 182). Interestingly, co-cultivation of lymphoid-derived cells sensitizes blood-derived CD4 T cells to HIV-induced pyroptosis (183). Moreover, pyroptotic peripheral blood $CD14^{+}CD16^{-}$ monocytes from HIV-infected patients release ASC specks, a hallmark of inflammasome activation. Therefore, besides the depletion of CD4 T cells, pyroptosis of CD4 T cells and monocytes contributes to the chronic inflammation that characterizes the disease (184).

The identification of the non-canonical inflammasome and the discovery of GSDMD as the executioner of pyroptosis have expanded our understanding of the mechanisms driving this type of cell death. However, further studies are necessary to elucidate the precise role of inflammatory and non-inflammatory caspases and the participation of members from GSDM family and/or other effector proteases in the molecular regulation of pyroptosis. In addition, the understanding of its role during infection or inflammatory processes *in vivo* will contribute to better understand the biological relevance of this regulated cell death induced in response to the PRRs activation.

PRR SENSING OF CELL DEATH AND CELL DEATH PRODUCTS

The notion that cells undergoing cell death release or expose several intracellular molecules regardless of the accidental nature or the different regulated death programs (apoptosis, necrosis or pyroptosis) is widely recognized. Although mainly non-inflammatory in the intracellular space, molecules released/exposed from damaged cells can participate in the activation of inflammation and immune responses. Indeed, a broad range of receptors, including PRR, sense these DAMPs and alert the immune system by inducing immune cell migration, increasing phagocytosis by macrophages and DCs, stimulating the production of pro-inflammatory cytokines or even contributing to the maturation of DCs, among other key functions (72, 80, 185). A number of studies have been dedicated to the characterization of putative DAMPs, and it became apparent that the type of cell death, as well as the nature of cell death stimuli, influence the quality and quantity of DAMPs release (**Table 1**).

Importantly, the stress or damage before the cellular demise itself is determinant to set in motion a sequence of events leading to an immunogenic cell death (ICD). The sensing of this stress regulates the cell death process thus initiating signaling pathways that will actively—or not—generate danger signals (186). Other DAMPs will be passively released as a result of membrane rupture during necroptosis or pyroptosis. These DAMPs define in part the immunogenicity of cell death, but are not sufficient to elicit a specific anti-tumor immune response, for instance. Indeed, they are released or exposed by the dying cells and act as adjuvant providing that antigens are exhibited conjointly (187). In contrast, a non-immunogenic cell death does not provide the required levels of DAMPs and antigens to evoke an adaptive immune response (187).

Together, these concepts redefined the widely accepted paradigm stating that apoptosis is always a silent cell death modality as opposed to necrosis, which is inflammatory and immunogenic. Therefore, a non-immunogenic apoptosis is characterized by the absence of plasma membrane leakage and the rapid phagocytosis of apoptotic bodies prevents the release of DAMPs and the consequent inflammatory reaction. Indeed the apoptotic process reduce cell immunogenicity by diverse ways including, (1) preservation of intracellular structures and plasma membrane, thereby blocking the release of DAMPS; (2) reduction of cellular volume, by condensation of the nucleus and shedding of small vesicles, which favors its rapid elimination by the surrounding tissue; (3) expression of "find-me" and "eat-me" signals, which increases the speed of cell clearance (12, 188); (4) inhibition of the production of interferons and pro-inflammatory cytokines, as the DNA is being chopped and condensed (189); (5) induction of an antigenic tolerance in the engulfing APCs (190); and (6) post-translational modifications on DAMPS and alarmins that decrease their pro-inflammatory potential (191, 192).

Interestingly, depending on the trigger, apoptosis can be immunogenic. Indeed, some chemotherapeutic agents, such as anthracyclines, as well as radiation and hypericin-based photodynamic therapy, were found to strongly prime immune

TABLE 2 | PRR agonists and consequent cell death program.

Stimuli/DAMPS	PRRs	Cell death mode	References
Pam3CSK4	TLR1	Apoptosis	(209, 210)
Pam3CysK Lipoproteins	TLR2	Necroptosis Apoptosis	(27, 211, 212)
Poly(I:C)	TLR3	Necroptosis Apoptosis	(211, 213, 214)
LPS HMGB1	TLR4	Necroptosis Apoptosis	(211–213, 215, 216)
Flagellin	TLR5	Necroptosis Pyroptosis	(211)
CpG DNA	TLR9	Necroptosis	(211)
LPS	CASPASE-11	Pyroptosis	(141–143)
Crystals/particulate-matter	NLRP3	Pyroptosis	(207, 217–220)
ATP	NLRP3	Pyroptosis	(221)
Bacterial pore-forming toxins	NLRP3	Pyroptosis	(221–224)
Bacterial RNA	NLRP3	Pyroptosis	(225)
dsRNA	NLRP3	Pyroptosis	(226)
Saturated-fatty acids	NLRP3	Pyroptosis	(227)
Flagellin T3SS/T4SS needle and inner rod proteins	NAIP/NLRC4	Pyroptosis	(228–233)
dsDNA	AIM2	Pyroptosis	(234)
Bacillus anthracis protective agent	NALP1	Pyroptosis	(235)
Muramyl dipeptide	NALP1	Pyroptosis	(236)
Toxin-modified RHO GTPase	PYRIN	Pyroptosis	(237)
ATP	P2X7	Apoptosis	(238, 239)
ssRNA shRNA	RIG-I	Necroptosis Apoptosis	(57, 240)
dsDNA Genomic RNA	DAI (DLM-1/ZBP)	Necroptosis Apoptosis	(56, 241)

AIM2, absent in melanoma 2; DAI (DLM-1/ZBP), DNA-dependent activator of IFN-regulatory factors; dsDNA, double stranded DNA; dsRNA, double stranded RNA; HMGB1, high-mobility group box 1 protein; LPS, Lipopolysaccharides; NAIP/NLRC4, NLR family CARD domain-containing protein 4; NALP1, NACHT, LRR and PYD domains-containing protein 1; P2X7, P2X purinoceptor 7; PAM3CSK4/Pam3CysK, TLR2 receptor agonist; Poly(I:C), Polyinosinic:polycytidylic acid; RIG-I, retinoic acid-inducible gene-I-like receptors; shRNA, short hairpin RNA; ssRNA, single stranded RNA; T3SS/T4SS, Type III/IV secretion system; TLR, Toll-like receptor.

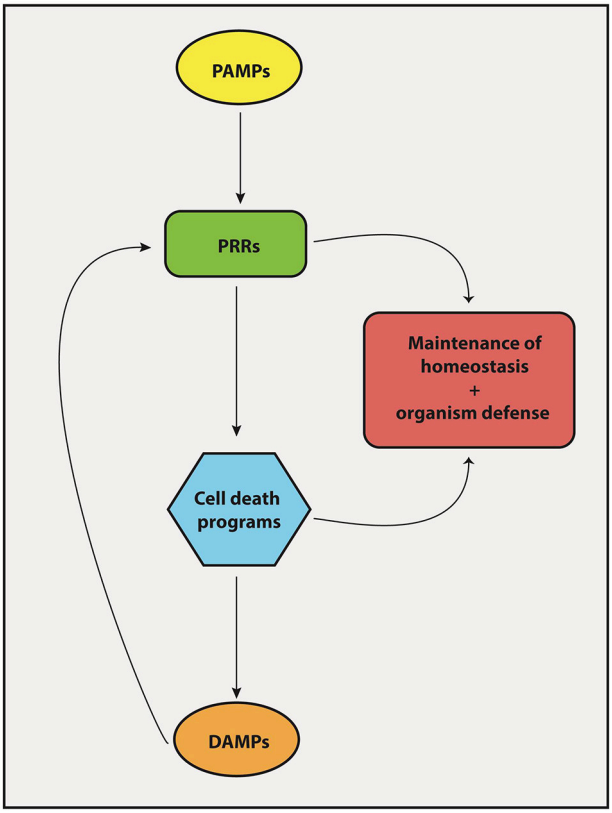

FIGURE 4 | Interplay between PRRs and cell death mechanisms. The engagement of PRRs in response to PAMPs induces the activation of different cell death machineries in order to promote tissue homeostasis and host-defense against pathogens. Importantly, cell death products known collectively as DAMPs forms a feedback loop that stimulate PRRs to induce inflammatory/immune responses.

responses through the induction of ICD (65, 185). Among these, immunogenic chemotherapies are well characterized and involve the emission of a number of danger signals. The pre-apoptotic release or exposure on the plasma membrane of ER-chaperones, such as calreticulin and Heat Shock Proteins (HSPs), constitutes an early event of ICD, which relies on the induction of an ER-stress. Calreticulin promotes the uptake of dying cells by DCs (72) and the inhibition of its exposure during anthracycline-induced apoptosis of murine tumor cell lines abolished their immunogenic potential (72). The early apoptotic secretion of ATP, which binds to P2X7 or P2Y2 purinergic receptor on DCs, stimulates the formation of the NLRP3 inflammasome, thereby inducing the release of IL-1β (66). Moreover, ATP released by dying cells undergoing ICD is responsible for the recruitment and differentiation of myeloid precursors into inflammatory DCs, mediating a specific antitumor immune response (193). Passive release of the nuclear protein HMGB1 occurs during secondary necrosis (i.e., late-stage apoptotic cells), which interacts with TLR4 on DCs, and through Myd88 signaling, enables efficient tumor antigen processing and cross-presentation (80). Additionally, anthracyclines have been shown to induce the release of RNA, thereby stimulating TLR3 as a mimic of viral infection. Activation of TLR3 is then responsible for type I IFNs production that acts in an autocrine and paracrine manner to promote the secretion of CXCL10 (194). Release of Annexin A1 has also been described after anthracyclines treatment, stimulating the Formyl Peptide Receptor 1 (FPR1), thus directing the final trajectory of DCs to dying tumor cells (195).

Besides chemotherapeutic agents, bacterial and viral infection can also trigger an immunogenic apoptosis. In this case, PAMPs, such as LPS or double-stranded RNA, expressed by the pathogen can stimulate TLR signaling and induce an immune response. Indeed, phagocytosis of infected apoptotic cells could trigger TLR

activation and lead to IL-6 and TGF-β production that induce the development of infection-specific as well as self-reactive T_H17 cells, linking infection to autoimmunity (196, 197). Finally, defects in mechanisms of apoptotic cell clearance are linked to autoimmunity disorders, including lupus and rheumatoid arthritis, likely due to the increased risk of loss of cell integrity with the consequent release of DAMPs and increased availability of circulating self-antigens (198, 199).

Accidental or programmed forms of necrosis are responsible for the release of an usually larger panel of DAMPs compared to apoptotic cells, mainly due to plasma membrane permeabilization. Also, RIPK1-mediated activation of the NF-κB pathway, through upregulation of pro-inflammatory cytokines and increased antigen presentation, was reported to play a role in the immunogenicity of necroptotic cell death mode (186). Recently, it was show that necroptosis is accompanied by the release of the classical and potent DAMPs—HSPs, ATP, and HMGB1 (200, 201). While HSPs were shown to stimulate TLR2 and 4, inducing DCs maturation, HMGB1 was reported to interact with TLR3, 4 and 9, as well as RAGE, to activate DCs and macrophages (202–204). Necrotic cells can potentially release intact mitochondria, a major source of ATP, which may activate the NLRP3 inflammasome resulting in IL-1β secretion and neutrophil recruitment (67, 96, 205). Moreover, mitochondrial DAMPs, such as formyl peptides and mitochondrial DNA, can potentially act on FPR1 and TLR9, respectively, inducing neutrophils recruitment and degranulation (97, 115). Additionally, Mincle, the C-type lectin receptor 4E was reported to interact with the necrotic DAMP SAP130 (spliceosome-associated protein 130), normally involved in spliceosomes assembly. The stimulation of this PRR was also reported to induce recruitment of neutrophils (106). Uric acid has been described as a product of accidental necrosis (108). Once released, this DAMP can precipitate and form monosodium urate (MSU) crystals able to induce the expression of costimulatory molecules by DCs, as well as activating the NLRP3 inflammasome to trigger the production of IL-1β and IL-18 (206, 207). RNA and double-stranded DNA (dsDNA) are also released during necrotic cell death, and while RNA stimulates TLR3, dsDNA stimulates TLR9 and two members of the RLR family, RIG-I and MDA5, responsible for the release of IFN-β and CXCL10 through IRF3 and NF-κb pathways. The AIM2 inflammasome can sense dsDNA released by necrotic cells, thereby inducing IL-1β secretion (204). Finally, it is important to mention that some proteins considered DAMPs also stimulate receptors that are not PRRs. For instance, IL-1α acts on IL-1R1 to trigger an inflammatory response mediated by the Myd88 pathway (208) and full-length IL-33 is another DAMP released during necrosis, with immunological property due to the absence of caspase processing (191).

CONCLUSIONS

The protective response of our body against pathogens and tumor cells depends on proper activation of both innate and adaptive immunity. Particularly, macrophages and DCs reside on the center of these two arms of immunity. They are powerful antigen-presenting cells that may elicit effector T cell responses (protection) or induce T cells to become regulatory (tolerance), depending on their activation status. They express PRRs, which are very ancient proteins that help us identify and react to pathogens and danger signals. Upon engagement, through the interaction with conserved molecular patterns frequently associated with pathogens (PAMPs), PRRs trigger a series of biochemical signaling cascades that activates pro-inflammatory programs on DCs that enable the differentiation of antigen-specific T cells into protective effector TH1, TH2, and TH17 cells. PRR engagement also triggers programs of cell death, particularly necroptosis and pyroptosis, the necrotic forms of cell death associated with a pro-inflammatory outcome (**Table 2**). These forms of cell death release larger amounts of DAMPs, which in turn, stimulate surrounding cells via PRRs, thus constituting a positive feedback loop capable of amplifying host defense mechanisms (**Figure 4**). Apoptosis, on the other hand, is a cell death program mostly related to non-inflammatory outcomes and likely to take major role in the maintenance of homeostasis by "silently" eliminating unwanted or damaged cells. However, apoptosis may also participate in elimination of infectious agents or tumor cells. Therefore, recognition of pathogen- and damage/danger-associated molecules by the same set of immune receptors (PRRs) is a powerful strategy that bridges intrinsic cell death programs and complex immune cell interactions to preserve homeostasis and at the same time protects the organism against infection and cellular transformation (**Figure 4**).

AUTHOR CONTRIBUTIONS

LM designed the figures. LZ and SA prepared Table. GA-M, RW, and KB designed the review organization. All authors contributed to the writing, reviewed, and approved the manuscript.

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Systems Approach Reveals Nuclear Factor Erythroid 2-Related Factor 2/Protein Kinase R Crosstalk in Human Cutaneous Leishmaniasis

Áislan de Carvalho Vivarini[1], Teresa Cristina Calegari-Silva[1], Alessandra Mattos Saliba[2], Viviane Sampaio Boaventura[3,4], Jaqueline França-Costa[3,4], Ricardo Khouri[3,4], Tim Dierckx[5], Karina Luiza Dias-Teixeira[1], Nicolas Fasel[6], Aldina Maria Prado Barral[3,4], Valéria Matos Borges[3,4], Johan Van Weyenbergh[5] and Ulisses Gazos Lopes[1]**

[1] Laboratory of Molecular Parasitology, Carlos Chagas Filho Biophysics Institute, Center of Health Science, Federal University of Rio de Janeiro, Rio de Janeiro, Rio de Janeiro, Brazil, [2] Department of Microbiology, Immunology and Parasitology – FCM/UERJ, State University of Rio de Janeiro, Rio de Janeiro, Rio de Janeiro, Brazil, [3] Gonçalo Moniz Institute, Oswaldo Cruz Foundation, Salvador, Bahia, Brazil, [4] Federal University of Bahia, Salvador, Bahia, Brazil, [5] Department of Microbiology and Immunology, Rega Institute for Medical Research, KU Leuven, Leuven, Belgium, [6] Faculty of Biology and Medicine, Department of Biochemistry, University of Lausanne, Lausanne, Switzerland

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Leticia A. Carneiro, Federal University of Rio de Janeiro, Brazil

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Min Zhang, Indiana University School of Medicine, United States
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***Correspondence:**
Johan Van Weyenbergh
j.vw@live.be;
Ulisses Gazos Lopes
lopesu@biof.ufrj.br

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Leishmania parasites infect macrophages, causing a wide spectrum of human diseases, from cutaneous to visceral forms. In search of novel therapeutic targets, we performed comprehensive *in vitro* and *ex vivo* mapping of the signaling pathways upstream and downstream of antioxidant transcription factor [nuclear factor erythroid 2-related factor 2 (Nrf2)] in cutaneous leishmaniasis (CL), by combining functional assays in human and murine macrophages with a systems biology analysis of *in situ* (skin biopsies) CL patient samples. First, we show the PKR pathway controls the expression and activation of Nrf2 in *Leishmania amazonensis* infection *in vitro*. Nrf2 activation also required PI3K/Akt signaling and autophagy mechanisms. Nrf2- or PKR/Akt-deficient macrophages exhibited increased levels of ROS/RNS and reduced expression of *Sod1* Nrf2-dependent gene and reduced parasite load. *L. amazonensis* counteracted the Nrf2 inhibitor Keap1 through the upregulation of p62 *via* PKR. This Nrf2/Keap1 observation was confirmed *in situ* in skin biopsies from *Leishmania*-infected patients. Next, we explored the *ex vivo* transcriptome in CL patients, as compared to healthy controls. We found the antioxidant response element/Nrf2 signaling pathway was significantly upregulated in CL, including downstream target p62. *In silico* enrichment analysis confirmed upstream signaling by interferon and PI3K/Akt, and validated our *in vitro* findings. Our integrated *in vitro, ex vivo*, and *in silico* approach establish Nrf2 as a central player in human cutaneous leishmaniasis and reveal Nrf2/PKR crosstalk and PI3K/Akt pathways as potential therapeutic targets.

Keywords: ***Leishmania*, macrophage, nuclear factor erythroid 2-related factor 2, PKR, Sod1**

INTRODUCTION

Human cutaneous leishmaniasis (CL) is spread worldwide, and the incidence is estimated to be from 0.7 to 1.2 million cases each year (1). Different clinical manifestations occur in humans due to the immune response and the infection by distinct *Leishmania* species (2). *Leishmania* parasites exhibit a plethora of adaptive mechanisms that interfere with several macrophage functions through the manipulation of host signaling pathways (3).

The imbalance between oxidative stress and cytoprotective systems of detoxification dictates the outcome of intracellular parasitic infections (4). The transcription factor [nuclear factor erythroid 2-related factor 2 (Nrf2)] is a master regulator of phase II defense gene expression that may protect cells from oxidative stress. The DNA promoter sequences of phase II defense genes share the canonical antioxidant response element (ARE), which is recognized by Nrf2 (5). Nrf2-dependent expression leads to profound effects on the suppression of the inflammatory response and immune activation through *Toll*-like receptors (6, 7).

The control of Nrf2 activation is dictated by different post-translational modifications. Multiple sites on the Nrf2 protein are phosphorylated by kinases, such as PERK, members of the MAPK family, PKCζ, and GSK3β, increasing the nuclear translocation and binding of the protein to ARE elements on the promoters of target genes (8–10).

The PI3K/Akt pathway modulates Nrf2 signaling (11) and, importantly, recent reports have demonstrated the modulation of the PI3K/Akt pathway upon *Leishmania amazonensis* infection (12). The enzyme GSK3, a target of Akt1, phosphorylates the Nh6 domain of Nrf2 and facilitates the action of ubiquitin ligase, leading to proteasomal degradation. The inhibition of GSK3 by phosphorylation allows the nuclear translocation of Nrf2 (13).

Kelch-like ECH-associated protein 1 (Keap1) is a major inhibitor of Nrf2 that constitutively induces the ubiquitination of the Nh2 domain, directing Nrf2 to proteasomal degradation. Keap1 is uncoupled from Nrf2 because of post-translational modifications due to oxidative stress, releasing Nrf2 for nuclear translocation (14).

Autophagy may modulate Nrf2 activation *via* Keap1 degradation (15). The components of the autophagy pathway are sensors of oxidative stress (16), and the increase in the expression of *p62* (*Sqstm1*), an Nrf2 target autophagy gene (17), favors the cellular capacity to process proteins destined for the autophagosome, decreasing oxidative stress (18).

The phosphorylation of p62 allows its binding to several cargo proteins, including Keap1, leading to autophagy as well as the Nrf2 stability and activation (19). The activation of Nrf2 through the p62/autophagy non-canonical pathway has also been demonstrated in macrophages treated with LPS, PolyI:C and peptidoglycan (PGN) upon the engagement of TLR4, TLR3, and TLR2, respectively (20).

Double-stranded RNA-dependent protein kinase [protein kinase R (PKR)] has in the Nh2-terminal domain two double-stranded RNA-binding motifs, and its kinase catalytic domain is located in the carboxyl-terminal (21, 22). On binding dsRNA, PKR dimerizes and undergoes autophosphorylation at multiple sites (23). Expression of catalytically defective mutant PKR (K296R) in cells inhibited the autophosphorylation and subsequent the activation of its major substrate eIF2-α (24, 25). PKR-mediated signaling may promote autophagy through eIF2-α phosphorylation (26). In several viral infections, PKR plays an essential role in the autophagy trigger (27). In STAT3$^{-/-}$ cells, PKR is able to induce autophagy through LC3-I to LC3-II conversion and the formation of vacuole compartments (28). In cells treated with type I interferon (IFN-I), both LC3 activation and p62 expression are increased (29).

In this work, we unveil the mechanisms that regulate Nrf2 gene expression in a PKR-dependent fashion. We describe for the first time the signaling pathway that coordinates Nrf2 activation during *Leishmania* infection. Finally, the induction of cytoprotective genes through the novel PKR/Nrf2 pathway may represent a prominent therapeutic mechanism for treatment and guide the development of novel targets in both infectious and inflammatory diseases.

MATERIALS AND METHODS

Reagents

Chloroquine diphosphate salt, DL-sulforaphane (SFN), phorbol-12 myristate-13 acetate (PMA), *N*-acetyl-L-cysteine (NAC), Wortmannin, and LY294002 hydrochloride were purchased from Sigma-Aldrich (St. Louis, MO, USA). AKTi (AKT inhibitor VIII, Akt1/2) was purchased from Santa Cruz Biotechnology (Dallas, TX, USA). Poly (cytidylic-inosic) acid potassium salt (PolyI:C) and the PKR inhibitor CAS 608512-97-6 were purchase from Calbiochem-Millipore (Darmstadt, Germany). Human recombinant interferon-alpha 2b was obtained from Blausiegel (Cotia, SP, Brazil).

Cell Lines and Culture

The mouse macrophage leukemia cell line RAW 264.7 (TIB-71; American Type Culture Collection (ATCC), Manassas, VA, USA), the human monocytic leukemia cell line THP-1 (ATCC:-TIB202TM) and the human embryonic kidney cell line HEK-293T (ATCC:CRL-11268) were maintained in DMEM medium with high glucose (Vitrocell Embriolife, Campinas, SP, Brazil) supplemented with 10% heat-inactivated fetal bovine serum (Sigma-Aldrich, St. Louis, MO, USA). THP-1 cells were differentiated to macrophages with 40 ng/mL of PMA for 3 days. Afterward, the cells were washed three times with PBS and incubated with fresh medium for an additional 3 days. RAW 264.7 cells expressing either empty vector (RAW-Bla cells) or a dominant-negative PKR K296R (RAW-DN-PKR cells) were donated by Dr. Aristóbolo Silva, Federal University of Minas Gerais, Brazil.

Peritoneal Macrophages

Ten-week-old male 129/SvEv PKR$^{-/-}$ (PKR-ko) and their respective wild-type littermates (WT) were used for experiments. Briefly, 4 days before peritoneal lavage, 2 mL of 3% thioglycolate were intra-peritoneally injected in each mouse. Thioglycolate-elicited peritoneal macrophages from wild-type or PKR-knockout 129Sv/Ev were obtained by injecting 8 mL of serum-free DMEM into the peritoneal cavity. After 1 h, the cells were washed once in PBS and then plated in in DMEM medium supplemented with 10% FBS on glass coverslips at 2×10^5/well in 6-well or 24-well polystyrene plates for subsequent *Leishmania* infection assays.

Cell Treatment

To induce the activation of Nrf2, 10 mM SFN were used as positive controls. For the inhibition of PKR activity, we pretreated the

cells for 1 h with 300 nM of the PKR inhibitor (PKRi). To induce PKR activation, poly(inosinic-cytidylic-) acid potassium salt (PolyI:C) at a final concentration of 25 μg/mL or recombinant IFNα-2b at 1,000 U/mL were used. PI3K/Akt inhibition was accomplished by cell treatment with 10 μM LY294002, 10 mM Wortmannin or 5 mM AKTI (AKT inhibitor VIII Akt1/2). To inhibit autophagy, we used 40 μM chloroquine. *N*-acetylcysteine (NAC) was used at a concentration of 10 mM.

Parasites, Culture Conditions, and Infection

Leishmania (Leishmania) amazonensis (WHOM/BR/75/Josefa) and *Leishmania (Viannia) braziliensis* (BA788) were used in this study. The *L. (L.) amazonensis* strains obtained from biopsies of patients with diffuse cutaneous leishmaniasis (DCL) (Ba276, Ba336, and Ba760) or localized cutaneous leishmaniasis (Ba69, Ba73, and Ba125) were also used *in vitro* assays. The promastigote forms were grown at 26°C in Schneider's Insect Medium (Sigma-Aldrich) with 10% fetal bovine serum, and metacyclic promastigotes were collected from stationary cultures and used for cell infections. Macrophages were infected with *Leishmania* promastigotes at a parasite:cell ratio of 10:1 at 37°C. Infected macrophages were counted in a Neubauer Chamber by light microscopy to assess the infection index, which was calculated by multiplying the percentage of infected macrophages by the average number of parasites per macrophage in Giemsa-stained slides.

Immunoblotting

THP-1 cells (1×10^6 cells) were washed twice with ice-cold PBS and then lysed in 100 μL of lysis buffer (50 mM Tris-HCl, pH 7.5, 5 mM EDTA, 10 mM EGTA, 50 mM NaF, 20 mM β-glycerophosphate, 250 mM NaCl, 0.1% Triton X-100, 1 μg/mL BSA, and a 1:100 dilution of protease inhibitor cocktail, Sigma-Aldrich, St. Louis, MO, USA) for total protein extraction. For nuclear protein extraction, after infection and/or treatment, the cells were washed twice with 1x PBS and then lysed with 100 μL of buffer A (HEPES 10 mM pH 7.9. 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, NP-40 0,25% (v/v); cocktail of protease inhibitors) for 10 min on ice. The lysed cells were centrifuged at 14,000 *g* for 1 min at 4°C, and the pellet was resuspended in 60 μL of buffer C (20 mM HEPES pH 7.9, 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 20% glycerol, protease inhibitor cocktail) and incubated on ice for 20 min. The lysate was centrifuged at 14,000 *g* for 5 min, and the supernatant containing nuclear proteins was collected in a new tube. The protein extracts were subjected to electrophoresis on 10% SDS-polyacrylamide gels and transferred to nitrocellulose membranes (Amersham Biosciences, Piscataway, NJ, USA). After blocking with 5% non-fat dry milk in TBS with 0.1% Tween-20 (TBS-T), the blots were incubated over-night with antibodies against PKR (12297), Nrf2 (12721), GSK3 (9369), Sqstm1/p62 (5114), LC3B (2775), phospho-GSK3β-Ser9 (9336), phospho-Akt-Ser473 (9271), phospho-eIF2α-Ser51 (9721), α-Tubulin (2144), β-Tubulin (2146), and Lamin A/C (2032) from Cell Signaling Technology; phospho-PKR Th451 (07-886) from Millipore; keap1 (150654) from Abcam; and β-actin (47778), Sod1 (8637), followed by anti-rabbit (2004) or anti-mouse (2005) horseradish peroxidase-conjugated IgG (1:4,000) from Santa Cruz Biotechnology. The membranes were then submitted to three washes with 0.1% TBS-T after each incubation, and the proteins were detected using the ECL chemiluminescent detection system (Amersham Biosciences).

Immunohistochemistry

To validate the differential expression of Nrf2 (C20—Santa Cruz Biotechnology) and keap1 (150654—Abcam) in DCL and LCL samples, immunohistochemistry was performed on formalin-fixed, paraffin-embedded (FFPE) sections. Briefly, after deparaffinization, rehydration and target retrieval (DAKO Corporation, Hamburg, Germany), slides from five DCL and five LCL cases were incubated with serum-free protein block reagent and then incubated overnight with Nrf2 or Keap1 (4and 10 mg/mL, respectively, both from Abcam, Cambridge, United Kingdom) or anti-rabbit isotype control antibodies. After the sequential application of a peroxidase-blocking reagent, DAKO EnVision + System-HRP (DAKO Corporation, Hamburg, Germany), digital images of the tissue sections were captured using a Nikon E600 light microscope and a Q-color 1 Olympus digital camera. Sections of prostate and lung adenocarcinoma were used as positive controls. Quantification of the stained areas was performed using Image Pro Plus software (Media Cybernetics).

Luciferase Assays

To investigate the promoter activity, RAW-264.7 cells (1×10^5 cells per well) was plated in 48-well polystyrene plates and transfected with 1 μg of reporter plasmids using LIPOFECTAMINE 2000 reagent (Invitrogen, Carlsbad, CA, USA). THP-1 cells (2×10^6) were transfected with 0.5 μg of luciferase reporter plasmids using Nucleofector™ Technology (Lonza, Basel, Switzerland) according to the manufacturer's instructions. The following plasmids were employed in the assays: Sod1-basal, Sod1-ΔARE, Sod1-WT, 3xARE, and Nrf2-WT. For normalization of the luciferase readout, the plasmid pRL-CMV (Promega) was used. After infection and treatment, the cells were washed with PBS, lysed according to the Dual Luciferase System protocol (Promega), and analyzed using the GloMax®-Multi detection system (Promega Corp., Madison, WI, USA).

Chromatin Immunoprecipitation Assay (ChIP)

Chromatin immunoprecipitation assay analysis was carried out according to the Simple ChIP Enzymatic Chromatin IP kit protocol (Cell Signaling). RAW 264.7 (WT-PKR and DN-PKR) cells or the human monocytic leukemia cell line THP-1 (ATCC:TIB202TM) were plated to confluence in 15 cm dishes. After infection, the cells were fixed with 1% formaldehyde for 10 min at room temperature, followed by the addition of glycine to a final concentration of 125 mM for 5 min at room temperature prior to cell lysis. One unit of micrococcal nuclease was added to the sample and incubated for 20 min at 37°C to digest DNA to the length of approximately 150–800 base-pairs. The chromatin was immunoprecipitated with 5 μg/mL anti-Nrf2 antibody (D1Z9C-XP—Cell

Signaling Technology, Danvers, MA, USA) at 4°C under rotation for 16 h. The DNA isolated from the immunoprecipitated material was amplified by real-time PCR using SybrGreen, and the DNA sequences of the primers used were Sod1-ARE.chip-F: 5′-AAGTCCGGGTCCCAGCTCAGAG-3′ and Sod1-ARE.chip-R: 5′-TTGGTGCAAGCACACCGGGAG-3′; p62-ARE.chip-F: 5′-CCCCACAGTTCCCCATTGGC-3′ and p62-ARE.chip-R: 5′-GACAGTGGGGACGCAAAGGC-3′; and Nrf2-AREL2chip-F: 5′-AAGTCCGGGTCCCAGCTCAGAG-3′ and Nrf2-AREL2 chip-R: 5′-TTGGTGCAAGCACACCGGGAG-3′. As a control, 1/50 of the digested input chromatin was similarly processed and analyzed in the absence of immunoprecipitation. To calculate the input percentage of the samples, the input was adjusted to 100% (average Ct of input − Log_2 of 50), followed by the application of the $100 \times 2^{(\text{adjusted input} - \text{average Ct(IP)})}$ formula.

Cloning and Generation of Luciferase Reporter Plasmids

Total DNA was extracted from THP-1 cells using a Wizard® Genomic DNA Purification kit (Promega) and measured using a BioPhotometer (Eppendorf). One PCR was carried out with primers spanning different regions of the *Sod1* and *Nrf2* promoters, yielding different fragment sizes, in the following conditions: 20 ng of genomic DNA and 35 cycles of 95°C for 1 min, 58°C for 1 min, and 72°C for 1 min. The DNA sequences of the primers used were Sod1.wt-F: 5′- GTCTCGA GCTGTAGGGTTGTGGCCTTGCCAAA-3′, Sod1.ΔARE-F: 5′-GTCTCGAGGCCAATTTCGCGTACTGCAACCG-3′, Sod1. basal-F: 5′-GTCTCGAGCTCGCGACCCGAGGCTG-3′ and Sod1-R: 5′-GTAGATCTCAGGAGACTACGACGCAAACCAG C-3′; and Nrf2-F: 5’ AAGTCCGGGTCCCAGCTCAGAG 3′ and Nrf2-R: 5′-TGGGGGCGGAACAAGGACCTAG-3′. A 1.8% agarose gel was run for 50 min at 100 V, and the amplicons were extracted from the gel and purified with the Zymoclean Gel DNA Recovery kit ™ (Zymo Research). The amplicons were ligated into a pJet-Blunt plasmid (Fermentas) with T4 ligase (Promega) for the first selection of positive colonies. After confirming positivity through PCR and a digestion assay, a colony was selected and grown, and a new plasmid extraction was performed. Digestion of the pJet-Blunt vector containing subcloned amplicons was performed with the Bgl-II enzyme (Promega), and the products were subjected to electrophoresis on a 2% agarose gel to extract the gel fragments. The pGL2-basic plasmid was also digested with the Bgl-II enzyme for the subsequent binding of the amplicons with T4 ligase enzyme (Promega). The cloned fragments and final vectors were then transformed into DH5α bacteria, and colonies were selected for further confirmation by sequencing. To obtain a luciferase-expressing pGL2-basic plasmid containing three copies of the sequence regulatory region ARE (3xARE), two oligos (5′-ATGCCGCTCGAGAATGACATTTCTAGAATG ACATTTCTAGAATGACATTTCTAGAGATCTCGG CCG-3′ and 3′-TACGGCGAGCTCTTACTGTAAAGATCTT ACTGTAAAGATCTTACTGTAAAGATCTCTAGAGC CGGC-5′) were designed and annealed to serve as templates for a PCR under the following conditions: 20 ng of DNA oligo and 35 cycles of 95°C for 1 min, 52°C for 1 min, and 72°C for 1 min with the primers 3xARE-F: 5′-ATGCCGCTCGAGAATG 3′, and 3xARE-R: 5′-CGGCCGAGATCTCTAGA 3′. The binding reactions and digestion with the Bgl-II enzyme followed the same protocol as described above.

Lentiviral Production and THP-1 Transduction

HEK-293T cells were used for shNrf2 lentiviral production. Initially, we co-transfected the cells with two packaging plasmids (pΔ8.9 and pVSVG) containing accessory proteins for the generation of the virus and capsid, respectively, along with the plasmid pLKO.1-shMission-Nrf2 (Sigma-Aldrich). For HEK-293T transfection, 60 μL of FuGENE HD reagent (Promega) was used in a 100 mm dish containing approximately 4×10^6 cells, along with 10 μg of target plasmid, 6 μg of pVSVG and 4 μg of pΔ8.9. After 24 h of transfection, the culture medium was changed and, over the next 2 days, the supernatants were collected at 10 mL/day. The 20 mL of supernatant was ultracentrifuged at 16,000 rpm for 90 min at 4°C, and the pellet was resuspended in 1 mL of DMEM without serum. Viral transduction in THP-1 cells was accomplished in 2×10^6 cells incubated with 1 mL of virus preparation for 48 h.

Fluorimetric Assays

The production of reactive oxygen species (ROS), nitric oxide (NO), and peroxynitrite (OONO) was performed by fluorimetry. For this, 10^5 cells were seeded in black 96-well plates and maintained for 24 h in DMEM containing 10% fetal bovine serum. The day after, the cells were washed three times with PBS, and HBSS medium without serum was added and incubated for 1 h at 37°C and 5% CO_2. The cells were incubated with different fluorescent probes for 1 h. Then, the cells were washed with PBS and treated with medium or infected with *L. amazonensis*. Fluorescence counting was monitored after incubation at 1-h intervals for up to 6 h (GloMax™). The production of ROS was detected using the probe CM-H2DCFDA (5 mM, Molecular Probes), with excitation at 495 nm and emission at 525 nm. For NO production, the DAF-FM probe (5 mM, Molecular Probes) was used, with excitation at 495 nm and emission at 515 nm. For the production of $OONO^-$, the probe HPF (5 mM, molecular probes) was used, with excitation at 490 nm and emission at 515 nm.

Patient Characteristics

Diffuse cutaneous leishmaniasis patients ($n = 4$) were recruited at our reference clinic in São Luiz, Maranhão, Brazil. DCL patients exhibited chronic progression of the disease with several remissions, multiple nodular and highly parasitized lesions throughout the skin, and a negative DTH response. LCL patients ($n = 5$), recruited at our reference clinic in Jiquiriçá, Bahia, Brazil, had a single or a few ulcerated lesions present for up to 2 months and a positive DTH response (30). The clinical and epidemiological data from patients with DCL and those with LCL are summarized in Table S3 in Supplementary Material. Skin biopsies were preserved as paraffin-embedded specimens.

Patient Recruitment and Diagnosis for Transcriptomic Analysis

This study was approved by the Ethics Committee of the Gonçalo Moniz Research Center (FioCruz-Bahia). Informed consent was obtained from all patients and healthy controls. CL patients were diagnosed according to characteristic lesion morphology, positive skin test, seropositivity toward *Leishmania* antigen and/or the presence of parasites in the lesion. LCL patients infected with *Leishmania braziliensis* ($n = 18$, 10 male, 29.6 ± 2.3 years) were recruited at diagnosis (before treatment) in two outpatient clinics (Jequié and Jiquiriçá-BA, NE Brazil) covering the same rural area.

Ethics Statement

Written informed consent was obtained from all participants or legal guardians, and all of the data analyzed were anonymized. The project was approved by the Institutional Review Board of Centro de Pesquisas Gonçalo Moniz, FIOCRUZ–BA (license number 136/2007) and complies with the guidelines of the Declaration of Helsinki.

Microarray Analysis

PBMCs from LCL patients and healthy controls were processed in parallel and immediately frozen in Trizol to preserve RNA integrity. Following Trizol extraction, total RNA was further purified using an RNeasy kit according to the manufacturer's protocol (QIAGEN, Venlo, Netherlands). Affymetrix Whole Genome microarray analysis was performed by the VIB MicroArray Facility (Leuven, Belgium) using a GeneChip® Human Gene 1.0 ST Array with the WT PLUS reagent kit (Affymetrix, Santa Clara, CA, USA) according to the manufacturer's specifications. Data preprocessing (RMA) was performed using the Bioconductor xps package. Microarray data were deposited in GEO (accession number: GEO Submission (GSE80008) (NCBI tracking system #17832057)).

nCounter Digital Transcriptomics

RNA extraction from skin biopsies was performed as above. Digital quantification of selected genes (NRF2, PKR, SOD1, SOD2, KEAP1, HMOX1) was performed by nCounter (Nanostring). Two-step normalization using internal positive and negative control RNAs, as well as PTPRC (CD45) normalization to correct for differences in tissue leukocyte infiltration, was performed as previously described (31).

Enrichment Analysis

The ingenuity pathway analysis (IPA) program was used to perform the initial pathway/function level analysis on genes determined to be differentially expressed in the microarray analysis (IPA version 9.0, Build 116623, Content version 3211, Ingenuity Systems, Red Wood City, CA, USA). Uncorrected *p*-values and absolute fold-changes were used with cut-offs of $p < 0.05$. Based on a scientific literature database, the genes were sorted into gene networks and canonical pathways, and significantly overrepresented pathways were identified. Further enrichment analysis was performed, including Gene Ontology (GO) term enrichment using the WEB-based GEne SeT AnaLysis Toolkit (WebGestalt), KEGG pathway enrichment using the pathway database from the Kyoto Encyclopedia of Genes, and Genomes and transcription factor target enrichment using data from the Broad Institute Molecular Signatures Database (MSigDB). Genesets from the GO, KEGG pathways, WikiPathways, and Pathway Commons databases, as well as transcription factors, were considered overrepresented if their corrected *p*-value was smaller than 0.05.

Statistical Analysis

The data were analyzed by one-way ANOVA for independent samples or Mann–Whitney (two-sided *t*-test) using Prism 5 software. The data represent the mean ± SD of the mean. The data are expressed as the average of three independent determinations, and significant differences were indicated by $^*p < 0.05$.

RESULTS

Leishmania Induces Nrf2 *via* PKR

The oxidative stress response plays a determinant role in the control of intracellular pathogens such as *Leishmania* (32). *L. amazonensis* dampens some macrophage functions, including the induction of oxidative stress (33, 34). Importantly, Nrf2 activation may promote infection tolerance, thus favoring pathogen survival. We sought to investigate whether *L. amazonensis* would induce Nrf2 *via* PKR. **Figure 1A** and Figure S1A in Supplementary Material shows that Nrf2 translocated to the nuclei of macrophages during the initial phase of interaction with the parasite. Importantly, Nrf2 translocation was not observed in infected pkr-ko and DN-PKR macrophages, respectively. Nrf2 levels were augmented in 6 h of infection and were induced by PKR signaling (**Figure 1B**; Figure S1B in Supplementary Material). The main target of PKR, eIf2α, is also not activated by phosphorylation in PKR-deficient cells (Figures S1C,D in Supplementary Material). PKR activation by inducers, such as PolyI:C or IFN-I added to macrophages also induced Nrf2 translocation and expression (**Figure 1C**). Next, we investigated the binding of Nrf2 to cognate *Nrf2* promoter (**Figure 1D**). Our data show that Nrf2 only occupied the ARE sequences in infected wild-type macrophages by ChIP. To address whether ARE genes are activated in *Leishmania* infection, we constructed two luciferase reporter plasmids. The 3xARE construct contains the canonical ARE promoter response element, while the other construct contains the *Nrf2* promoter (also spanning an ARE-like element). **Figure 1E** shows that the 3xARE regulatory sequence drove luciferase expression in infected wild-type macrophages, while luciferase expression was abrogated in DN-PKR cells. Importantly, the *Nrf2* promoter was also induced in infected wild-type macrophages. In summary, our results show that *L. amazonensis* induces Nrf2 in a PKR-dependent manner.

Nrf2 and PKR Signaling Control SOD1 Gene Expression

Recent reports have demonstrated that *L. amazonensis* activates the classical antiviral response mediated by PKR, leading to Sod1

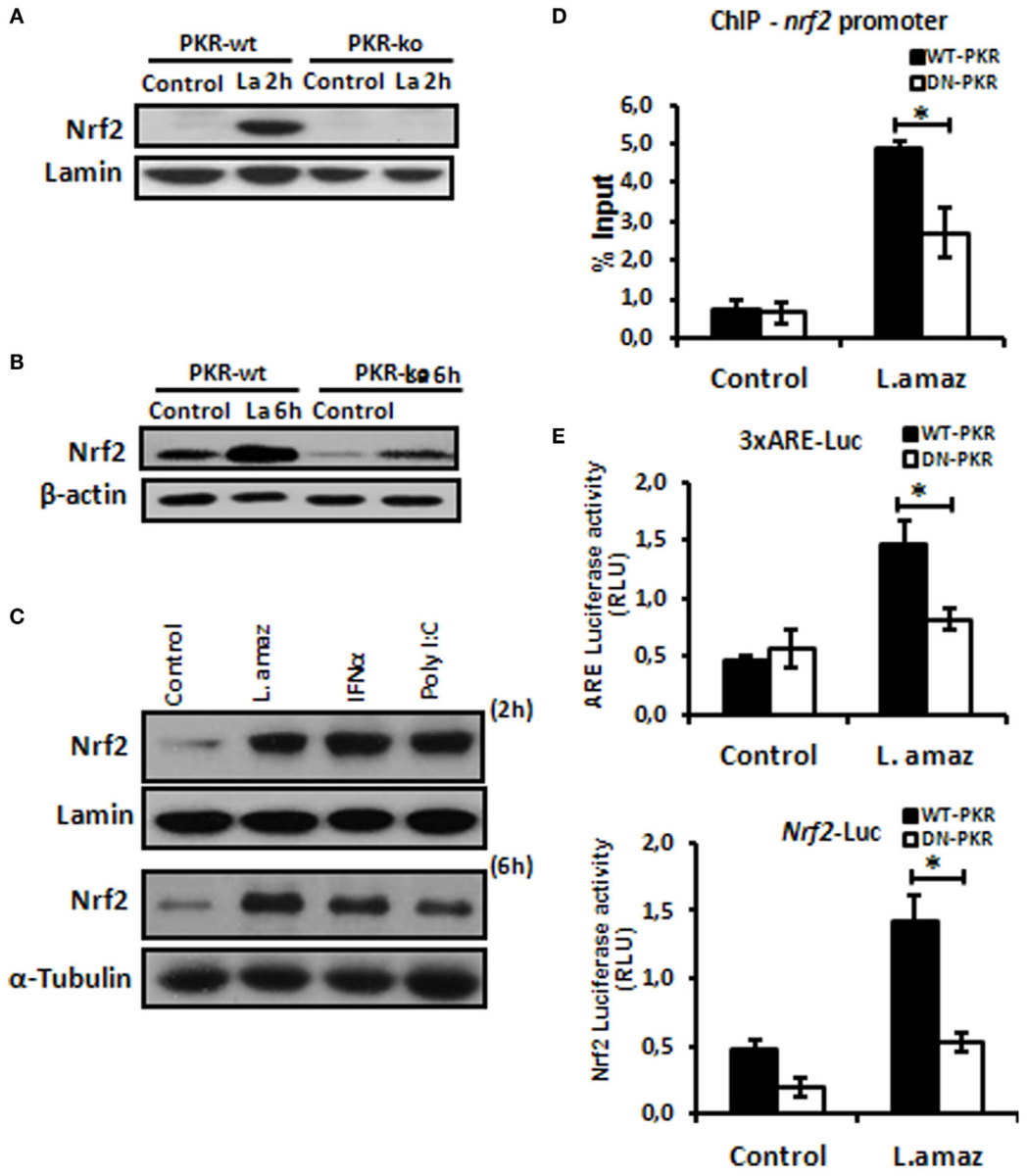

FIGURE 1 | *Leishmania amazonensis* induced nuclear factor erythroid 2-related factor 2 (Nrf2) expression and nuclear translocation in a protein kinase R (PKR)-dependent manner. Peritoneal macrophages from wild-type or PKR-ko 129/sv mice were infected with stationary promastigotes forms of *L. amazonensis* for 2 h **(A)** or 6 h **(B)**. Western-blot was carried out for nuclear or total protein extract, respectively, and then assay was performed using Nrf2 antibody. **(C)** THP-1 cells were infected with *Leishmania amazonensis* or treated with IFN-α or PolyI:C for 2 h for nuclear extract or 6 h for total protein extract, before western-blot analysis with Nrf2 antibody. **(D)** RAW-WT-PKR and RAW-DN-PKR cells were infected with stationary promastigotes forms of *L. amazonensis* for 4 h and then submitted to chromatin immunoprecipitation assay (ChIP) using Nrf2 ChIP-antibody. **(E)** RAW 264.7 cells were transiently transfected with p3xARE- or pNrf2-promoter-luciferase reporter plasmids constructs and infected with *L. amazonensis* 24 h post-transfection. Whole-cell lysates were analyzed for luciferase activity 24 h later. Results are representative of three independent experiments. $^*p < 0.05$.

expression, favoring parasite growth in infected macrophages (35, 36). To address the role of Nrf2 on Sod1 expression in infected macrophages, we cloned the *Sod1* promoter and deleted the regulatory regions in the Luciferase vector (pGL2) (**Figure 2A**). The *Sod1* promoter was induced in wild-type infected macrophages, while the deletion of the ARE sequence disrupted Luc expression. Accordingly, Sod1 expression, which is controlled by Nrf2, was only increased in wild-type infected macrophages (**Figure 2B**). Our data show that ARE element on Sod1 promoter was occupied by Nrf2 only in infected wild-type macrophages by ChIP (**Figure 2C**). We aimed to test the hypothesis that Sod1 dependence of Nrf2 activity, we developed a macrophage shNrf2 knockdown cell line. In only wild-type infected macrophages, the parasites induce Sod1 expression (**Figure 2D**). The quantification of infection index show a decrease on proliferation of *Leishmania* in Nrf2 knockdown cells

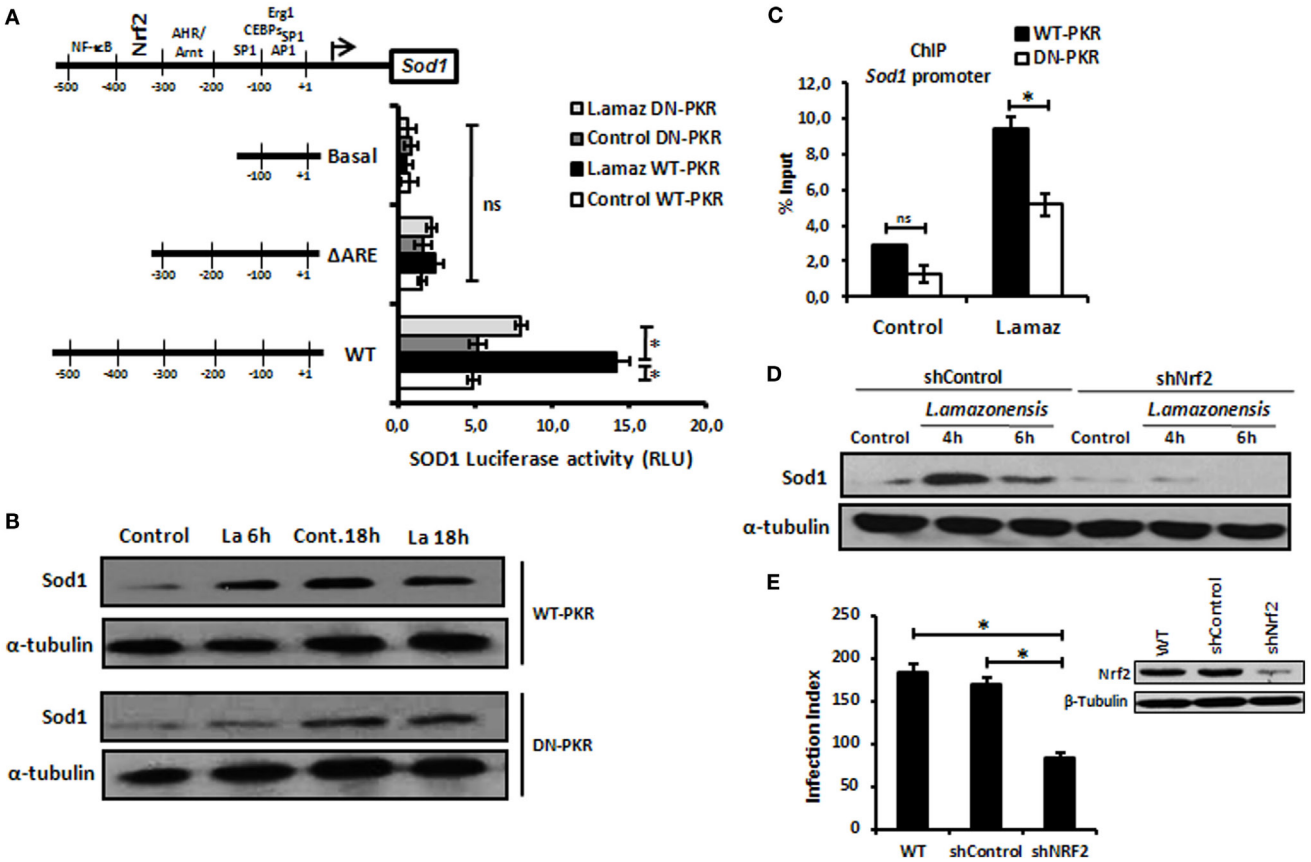

FIGURE 2 | Sod1 regulation through nuclear factor erythroid 2-related factor 2 (Nrf2) and protein kinase R (PKR) signaling. **(A)** RAW 264.7 WT or DN-PKR cells were transiently transfected with Sod1-Luc plasmids and infected with stationary promastigotes of *Leishmania amazonensis* for additional 24 h before luciferase activity assay. **(B)** The same cells were also infected with stationary promastigotes of *L. amazonensis* for 18 h before total protein extract for western-blot analyzes with Sod1 and α-tubulin antibodies. **(C)** RAW 264.7 cells were infected with stationary promastigotes forms of *L. amazonensis* for 4 h and then submitted to chromatin immunoprecipitation assay (ChIP) using Nrf2 ChIP-antibody. shNrf2 or shControl THP-1 cells were infected with stationary promastigotes of *L. amazonensis*, **(D)** Sod1 protein expression was analyzed, and **(E)** infection index was evaluated. Results are representative of three independent experiments. *$p < 0.05$.

(**Figure 2E**). These data support the link between Sod1 and two major signaling pathways represent by Nrf2 and PKR.

Akt1 Controls Nrf2 Induction in Infected Macrophages

Nrf2 activation is controlled at different levels, including indirect phosphorylation by Akt1 (11). Because *L. amazonensis* promotes Akt1 activation (12), we aimed to investigate its role in Nrf2 induction. Initially, we examined whether the induction of Akt1 by *L. amazonensis* relied on PKR expression. **Figure 3A** shows that GSK3 phosphorylation due to Akt1 depended on PKR. The phosphorylation of Akt depends on PKR during *Leishmania* infection (Figures S3A,B in Supplementary Material). Of note, Nrf2 induction required Akt signaling, as shown in infected shAkt1 macrophages (**Figures 3B,C**). In macrophages treated with pharmacological inhibitors of Akt1/2 and PI3K (**Figures 3D,E**), we also observed the same pattern of Nrf2 repression in nucleus translocation and protein expression. As predicted, ARE element, *Nrf2* Luciferase and *Nrf2* promoter occupancy in ChIP assay were induced by *L. amazonensis* infection in an Akt1-dependent manner (**Figures 3F,G**). Likewise, Sod1 expression followed the same PI3K/Akt1 dependence pattern. ChIP assays corroborated these findings, where the occupancy of ARE in the *Sod1* promoter by Nrf2 depended on Akt1 (**Figure 3H**).

Nrf2 Knockdown Promotes Oxidative Stress and Impairs Parasite Survival in Macrophages

We aimed to test the hypothesis that Nrf2 knockdown would favor oxidative stress, leading to the reduction of the parasite load in macrophages. We measured the production of OONO, NO, and ROS as components of the oxidative stress pathway in Nrf2-knockdown infected macrophages (**Figure 4A**). As expected, the production of ROS and the formation of OONO and NO were enhanced in infected Nrf2-knockdown macrophages. **Figure 4B** shows that PKR or Akt1 inhibition leads to a similar oxidative stress profile upon infection. Silencing of Nrf2 decreased the infection index, whereas the parasite load was rescued when infected Nrf2-knockdown macrophages were treated with the antioxidant NAC compound (**Figure 4C**).

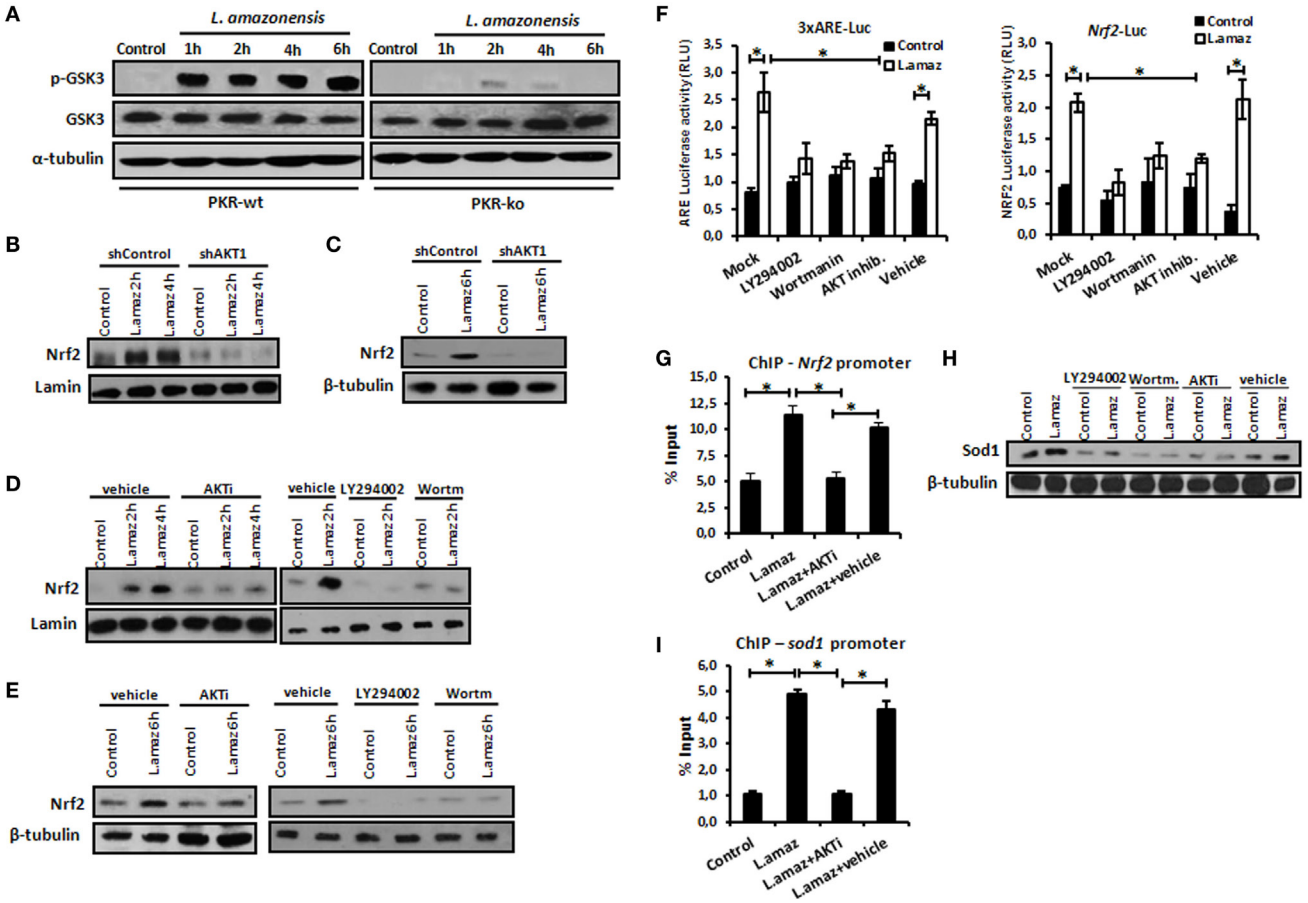

FIGURE 3 | Protein kinase R (PKR)-dependent PIK3/Akt signaling activation controls positively the nuclear factor erythroid 2-related factor 2 (Nrf2) functions in *Leishmania*-infected macrophages. **(A)** Peritoneal macrophages from wild-type or PKR-ko 129/sv mice were infected with stationary promastigotes forms of *Leishmania amazonensis* at indicated times. Western-blot was carried out for total protein extract with anti-phospho-GSK3 and anti-GSK3. THP-1 cells stably knocked-down for Akt1 expression **(B,C)** and treated with PI3K/Akt inhibitors (LY294002, Wortmannin and Akt-inhibitor-VIII) **(D,E)** were infected with stationary promastigotes forms of *Leishmania amazonensis* at indicate times. Nuclear and total protein extracts were analyzed using Nrf2 antibody. **(F)** THP-1 cells were transiently transfected with p3xARE- or pNrf2-promoter Luciferase reporter plasmids. Twenty-four hours post-transfection, cells were differentiated into macrophages with phorbol-12 myristate-13 acetate (PMA) treatment for 6 days. The cells were infected with stationary promastigotes forms of *L. amazonensis* and/or treated with PI3K/Akt inhibitors for additional 24 h. Whole-cell lysates were analyzed for luciferase activity 24 h later. THP-1 cells were infected with stationary promastigotes forms of *L. amazonensis* and/or treated with Akt-inhibitor-VIII for 4 h and then submitted for chromatin immunoprecipitation assay (ChIP) using Nrf2 ChIP-antibody and primers for *Nrf2* **(G)** and *Sod1* **(I)** promoters. **(H)** Western-blot for total protein extract analyses with Sod1 antibody was performed at same conditions of infection and treatment. Results are representative of three independent experiments. *$p < 0.05$.

Notably, the Nrf2 inducer sulforaphane augmented the infection index.

Leishmania Down-Regulates the Nrf2 Negative Regulator Keap1 and Induces Autophagy

Nuclear factor erythroid 2-related factor 2 is sequestered in the cytosol by a homodimer of Keap1, which limits its nuclear translocation. Nrf2 associated with Keap1 is directed to proteasomal degradation by Cul3-mediated poly-ubiquitination (37). However, Keap1 is degraded through p62-mediated autophagy, releasing Nrf2 into the nucleus (38). Given that *Leishmania* induces autophagy in infected macrophages (39), we sought to investigate the levels of Keap1 in *Leishmania* infection. **Figure 5A** shows the prompt decrease in Keap1 levels in infected macrophages and demonstrates that PKR inactivation prevented Keap1 degradation. Moreover, Keap1 reduction was prevented by chloroquine, an autophagy inhibitor (**Figure 5B**). Given that Nrf2 released *via* Keap1 degradation promotes the antioxidant response, we addressed whether the inhibition of autophagy would increase the oxidative stress of infected macrophages. As observed in **Figure 5C**, the levels of ROS, OONO, and NO increased in infected cells treated with chloroquine. We also confirmed that *L. amazonensis* triggers LC3-I conversion to LC3-II, a marker of autophagy (**Figure 5D**). Given that the formation of the LC3-p62-Keap1 ternary complex on the autophagosome membrane directs Keap1 to degradation

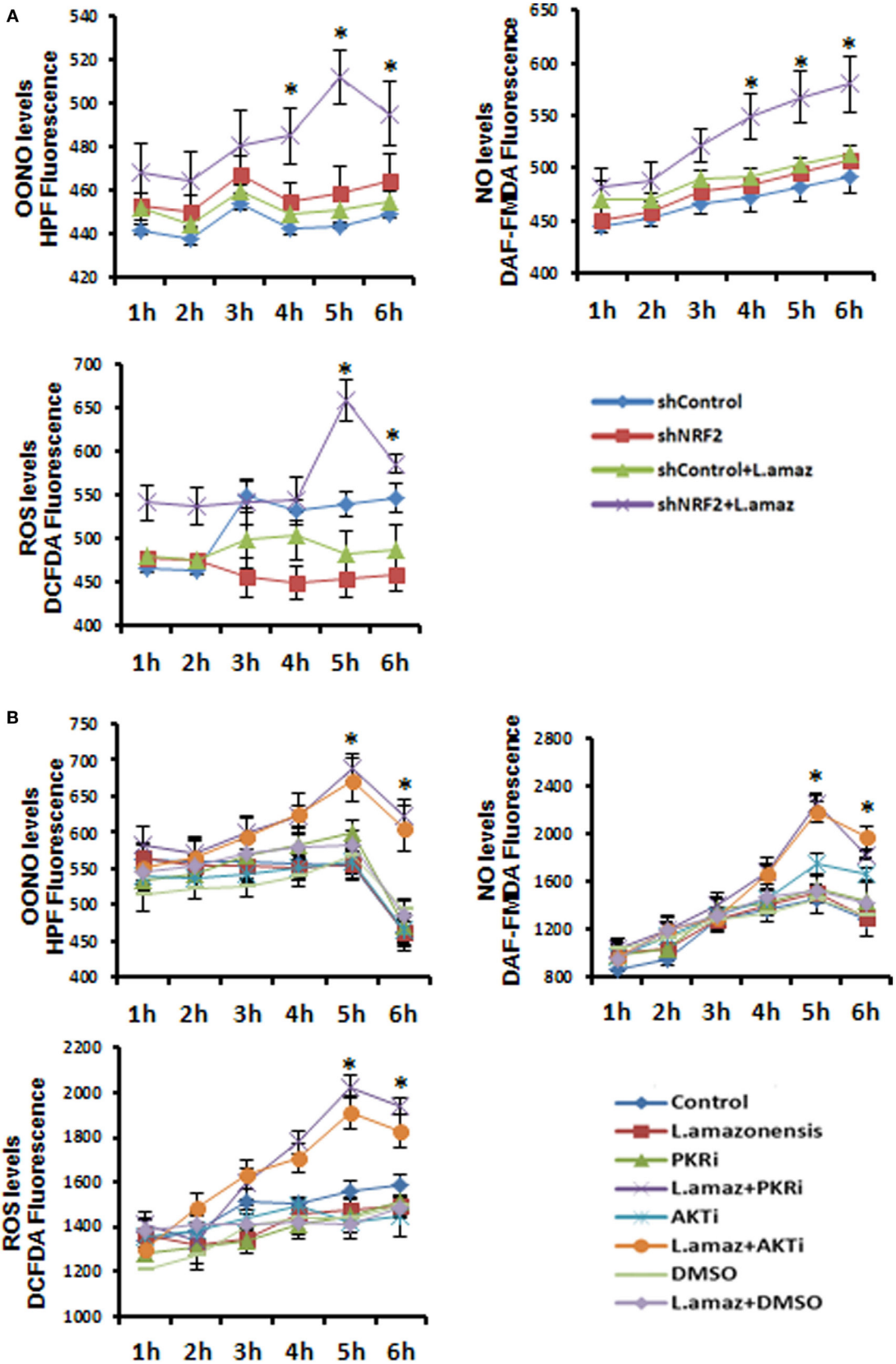

FIGURE 4 | Continued

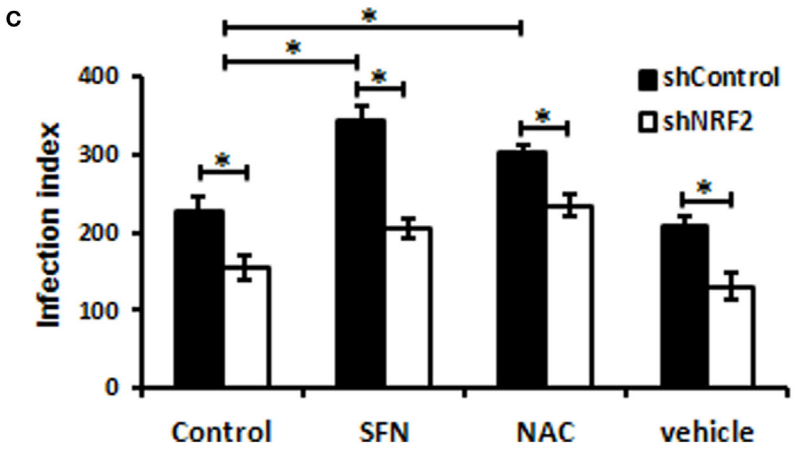

FIGURE 4 | The reactive oxygen species (ROS) were enhanced upon *Leishmania amazonensis* infection in nuclear factor erythroid 2-related factor 2 (NRF2)/protein kinase R (PKR)/Akt-deficient macrophages. **(A)** shNrf2 or shControl and **(B)** wild-type THP-1 cells treated with PKR-inhibitor or Akt-inhibitor-VIII were infected with stationary promastigotes forms of *L. amazonensis* at indicated times together with probes for quantifying peroxynitrite (OONO), nitric oxide (NO), and ROS, and then analyzed as described in material and methods. **(C)** THP-1 transiently knocked-down for Nrf2 expression or shControl cells were infected with stationary promastigotes forms of *L. amazonensis* for 24 h and treated for additional 24 h with sulforaphane (SFN) or NAC (N-acetylcysteine) before the analysis of infection index. The asterisk means the statistic significant differences between the groups. Results are representative of three independent experiments. *$p < 0.05$.

(40), we investigated the induction of p62 in the infection. We show that p62 was induced in infected macrophages, and this effect relied on PKR and Akt1 (**Figure 5E** and Figures S5A in Supplementary Material, respectively). Moreover, the ablation of Nrf2 expression prevented p62 induction due to infection (**Figure 5F**). Given that our data indicate that PKR and Akt control the induction of Nrf2, we tested the occupancy of the *p62* promoter by Nrf2 in the context of infection. Our data show that *Leishmania* promoted Nrf2 occupancy, and the inhibition of either PKR or Akt signaling prevented this effect (**Figure 5G** and Figure S5B in Supplementary Material, respectively).

Nrf2 Protein Levels Are Elevated in Human CL, and *L. braziliensis* also Induces Nrf2 *In Vitro*

We aimed to address whether other *L. amazonensis* strains isolated from patients with localized cutaneous lesions (LCL) or DCL would induce Nrf2 nuclear translocation and the activation of PKR. **Figure 6A** shows that all distinct strains of *L. amazonensis* activated PKR and Nrf2. Given that most of the cases of human CL in Brazil are caused by *L. braziliensis*, we decided to address whether this species would induce PKR and Nrf2. **Figure 6B** shows that *L. braziliensis* activated PKR and Nrf2. Nrf2 activation depended on PKR function (**Figure 6C**). Moreover, the expression of the Nrf2 target genes p62 and Sod1 was reduced in Nrf2-silenced *L. braziliensis*-infected macrophages (**Figure 6E**), and the growth of amastigotes was impaired in Nrf2-knockdown macrophages (**Figure 6D**). These results prompted us to investigate the levels of Nrf2 and the negative regulator Keap1 in clinical samples from LCL or DCL patients. **Figure 6F** shows the marked expression of Nrf2 in DCL samples compared to LCL tissues. Accordingly, Keap1 expression was enriched in LCL samples. Altogether, the data show that Nrf2 induction is triggered by distinct species and strains of *L. amazonensis*, and high levels of Nrf2 are found in patients with DCL, a severe clinical condition that presents with a high number of parasites and poor prognosis (41).

Transcriptomic Analysis Reveals a Pivotal Role of Nrf2 Signaling in CL Patient Samples

Next, we tested for transcription factor enrichment among the 413 genes composing the systemic LCL disease signature. Only five transcription factor motifs were significantly enriched among the promoters of the 413 genes of the LCL disease signature. After the E4F1 motif, the Nrf2 binding site was the second-most significantly represented, being present in 15 of the 413 genes composing the LCL disease signature (**Table 1**). Among those, p62 (Sqstm1), in bold, was confirmed, in agreement with our *in vitro* data. We herein present the first disease signature of LCL using a systems biology analysis of the PBMC transcriptome of LCL patients ($n = 18$) vs. healthy controls ($n = 12$). Using Affymetrix microarrays (HuGene 1.0), we found that Nrf2 was significantly overexpressed in patient PBMCs vs. controls (1.8-fold, uncorrected $p = 0.0002$, $p = 0.033$ using the Benjamini–Hochberg correction for genome-wide testing). The top 50 upregulated genes in patients vs. controls are shown in Table S1 in Supplementary Material. Next, we used IPA to determine which biological pathways and molecular networks were enriched among the LCL disease signature. As shown in Table S2 in Supplementary Material, three antioxidant pathways, i.e., the thioredoxin pathway, the antioxidant action of Vitamin C and the Nrf2 pathway, were significantly enriched in the LCL disease signature.

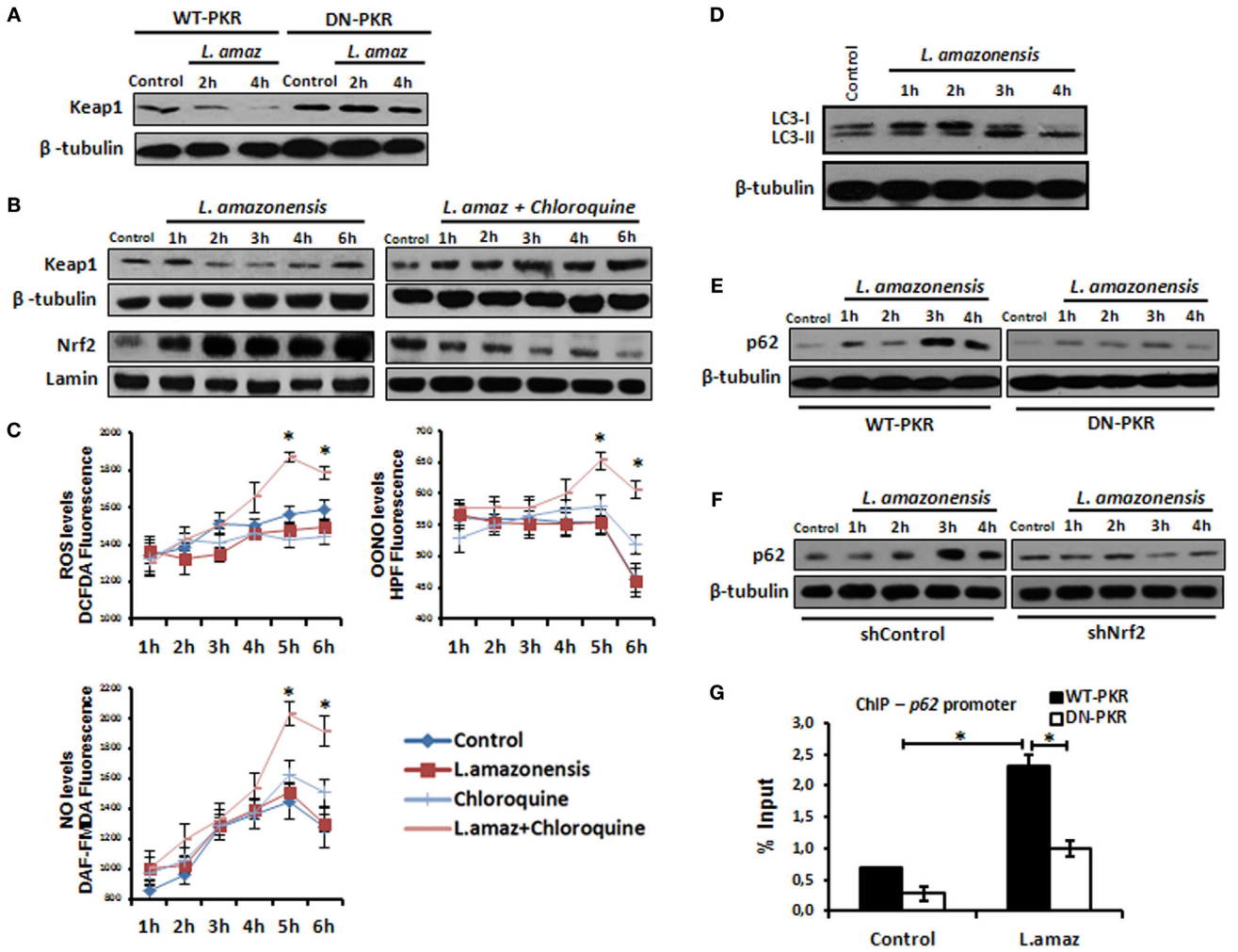

FIGURE 5 | The nuclear factor erythroid 2-related factor 2 (Nrf2)-inhibitor Kelch-like ECH-associated protein 1 (Keap1) is modulated negatively through protein kinase R (PKR) signaling and p62 autophagy-dependent manner in *Leishmania* infection. **(A)** RAW-WT-PKR and RAW-DN-PKR cells were infected with stationary promastigotes forms of *Leishmania amazonensis* for 2 or 4 h and then western-blot assay were performed with total protein extract using Keap1 antibody. **(B)** THP-1 cells were infected with stationary promastigotes forms of *L. amazonensis* at indicate times and/or treated with chloroquine and the total or nuclear protein extracts were analyzed using Keap1 and Nrf2 antibodies. **(C)** THP-1 cells treated with chloroquine were infected with stationary promastigotes forms of *L. amazonensis* at indicated times together with probes for quantifying OONO, NO, and ROS. **(D)** THP-1 cells were infected with stationary promastigotes forms of *L. amazonensis* and western-blot for LC3-I/II protein was performed. RAW-WT-PKR and RAW-DN-PKR cells **(E)**, and shNrf2 or shControl THP-1 cells **(F)** were infected with stationary promastigotes forms of *L. amazonensis* and then the total protein extract was analyzed by western-blot assay with p62 antibody. **(G)** RAW-WT-PKR and RAW-DN-PKR were infected with stationary promastigotes forms of *L. amazonensis* for 4 h and then submitted for ChIP assay using Nrf2 ChIP-antibody and primers for *p62* promoter. Results are representative of three independent experiments. $^*p < 0.05$.

Nrf2 Transcriptome-Wide Correlations Confirm the Links between IFN-I/PKR, ARE, PIK3, and Autophagy Signaling Pathways *In Situ*

Then, we performed a transcriptome-wide correlation analysis to further investigate whether the molecular links we described at the protein level *in vitro* might be confirmed at the transcriptional level *ex vivo*. The expression of a large number of genes was significantly correlated to Nrf2 transcript levels, even following stringent Benjamini–Hochberg correction for multiple testing. Among those, *PKR*, *PIK3C*, *Sod1*, and *p62* (*SQSTM1*) transcripts were positively correlated, whereas *Keap1* was negatively correlated, to *Nrf2* transcript levels, with minor differences between LCL patients and controls (**Figure 7A**), thus confirming our protein data of *Nrf2* regulation, both upstream or downstream. To validate these microarray results, we performed a targeted analysis of key genes in the Nrf2/PKR crosstalk using nCounter digital transcriptomic quantification in LCL ($n = 6$) as well as healthy skin biopsies ($n = 4$). As shown in **Figure 7B**, unsupervised hierarchical clustering of *in situ* transcriptomes revealed two major clusters, which coincided with either LCL patients or normal donors (ND). Thus, LCL skin biopsies could be discriminated from healthy skin by

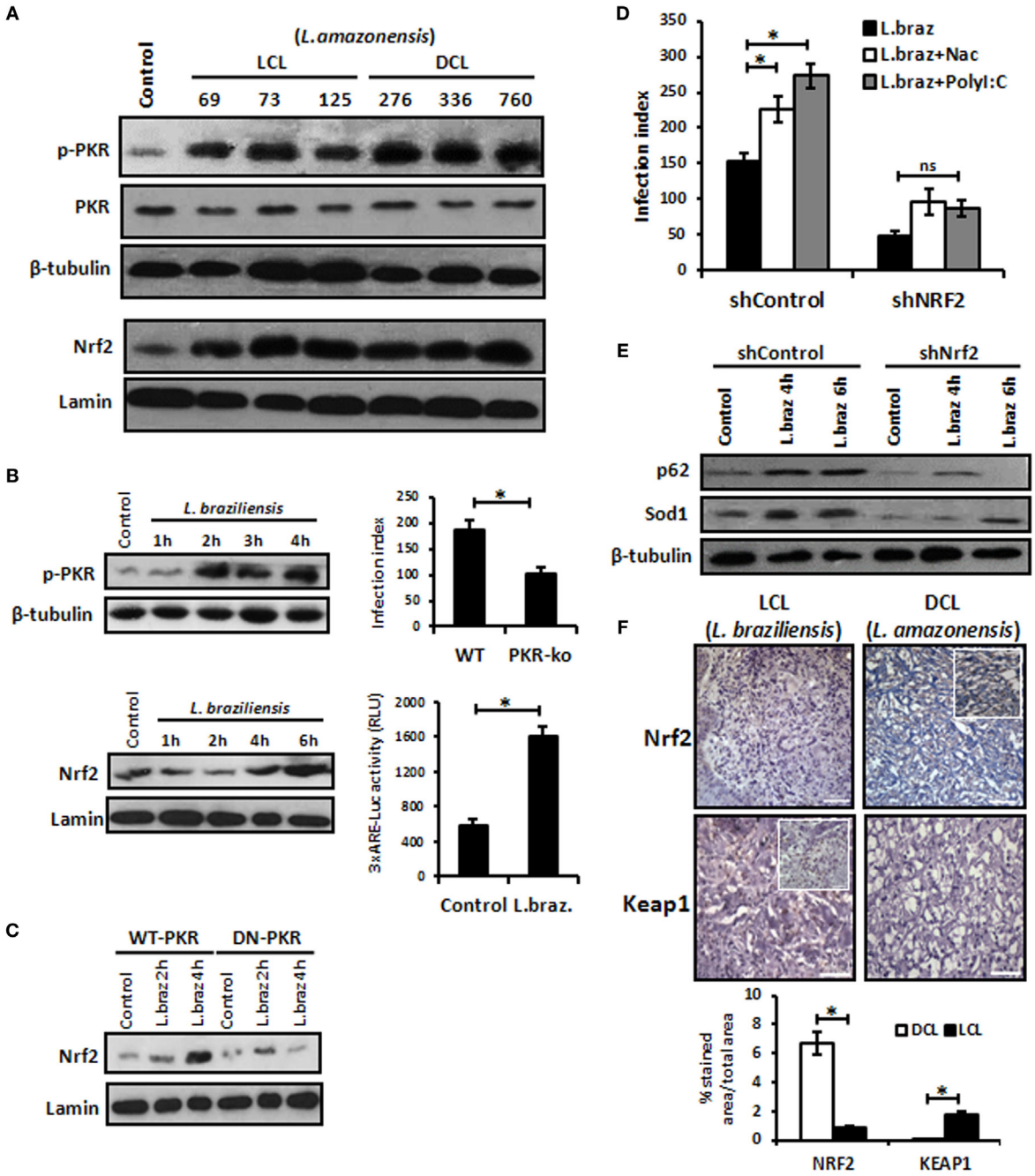

FIGURE 6 | *Leishmania braziliensis* and different strains of *Leishmania amazonensis* infections also modulated positively the protein kinase R (PKR)/nuclear factor erythroid 2-related factor 2 (Nrf2) axis pathway. **(A)** *L. amazonensis* strains from LCL or diffuse cutaneous leishmaniasis (DCL) patients were used to infect THP-1 cells. Total or nuclear protein extracts were processed and then analyzed by western-blot with phospho-PKR and Nrf2 antibodies, respectively. **(B)** THP-1 cells were infected with stationary promastigotes forms of *L. braziliensis* at indicate times and then performed for western-blot with phospho-PKR and Nrf2 antibodies, and 3xARE-promoter Luciferase assays. Peritoneal macrophages of wild-type or PKR-ko mice were infected with *L. braziliensis* and infection index assays were then analyzed. **(C)** RAW-WT-PKR and RAW-DN-PKR cells were infected with *L. braziliensis* and western-blot with anti-Nrf2 was then analyzed. **(D)** shNrf2 or shControl THP-1 cells were infected with stationary promastigotes forms of *L. amazonensis* for 24 h before treatment with NAC or polyI:C for additional 24 h. After this time, the cells were fixed and the infection index was evaluated. **(E)** THP-1 transiently knocked-down for Nrf2 expression or shControl cells were infected with stationary promastigotes forms of *L. amazonensis* for 4 or 6 h before total protein extract for western-blot analyzes with p62 and Sod1 antibodies. **(F)** Histological sections from biopsies obtained from lesions of patients with DCL ($n = 4$) or with LCL ($n = 5$) were submitted to immunohistochemical reaction with primary antibodies against Nrf2 or Keap1 as previously described. All sections were counterstained with hematoxylin. Digital images (400× magnification) were captured using a Nikon E600 microscope and an Olympus Q-Color 1 digital camera with the Image Pro Plus program. Bars represent 10 μm. Positive cell density was obtained. Graph represents the analysis of reactive positive cells for Nrf2 and Keap1 compared with isotype controls as percentage of positive stained area per total tissue area. Results are representative of three independent experiments. $^*p < 0.05$.

TABLE 1 | Gene promoters in LCL disease signature are enriched for nuclear factor erythroid 2-related factor 2 transcription factor binding sites.

Index	Gene symbol	Gene name	Entrez gene
1	TXNRD1	Thioredoxin reductase 1	7296
2	TFAP4	Transcription factor AP-4	7023
3	**SQSTM1**	**Sequestosome 1**	**8878**
4	RB1CC1	RB1-inducible coiled-coil 1	9821
5	CDH23	Cadherin-related 23	64072
6	SLC16A6	Solute carrier family 16, member 6	9120
7	KBTBD8	Kelch repeat and BTB (POZ) domain containing 8	84541
8	FBXO30	F-box protein 30	84085
9	ATP1B1	ATPase, Na+/K+ transporting, beta 1 polypeptide	481
10	PRDM1	PR domain containing 1, with ZNF domain	639
11	MAST2	Microtubule-associated serine/threonine kinase 2	23139
12	CLC	Charcot–Leyden crystal protein	1178
13	SYTL1	Synaptotagmin-like 1	84958
14	SFXN5	Sideroflexin 5	94097
15	TMEM57	Transmembrane protein 57	55219

differential expression of only six transcripts (*Nrf2, PKR, Sod1, Sod2, Keap1*, and *Hmox1*).

DISCUSSION

The oxidative burst in infected cells is a key microbicide mechanism exhibited by macrophages. However, *Leishmania* parasites present a repertoire of adaptive mechanisms to cope with the altered redox state of infected macrophages by expressing antioxidant enzymes or interfering with macrophage signaling pathways (42). A growing number of reports indicate that PKR modulates infections caused by intracellular pathogens (43). Notably, increased levels of Sod1 are expressed in macrophages infected by *L. amazonensis* due to PKR activation (35). Given that the transcription factor Nrf2 is the main regulator of Sod1 expression (44) among other genes involved in the anti-oxidative response, we studied the regulation of Nrf2 in the context of *Leishmania* infection and tested the hypothesis that PKR is actually a positive regulator of the ARE *via* Nrf2.

Our results demonstrated that Nrf2 activation depended on PKR signaling. Simple treatment with PKR inducers such as IFN-α and PolyI:C increased the expression and nuclear translocation of Nrf2, demonstrating that the mechanisms of Nrf2 activation through PKR pathway signaling are not exclusively due to *Leishmania* infection. Remarkably, PKR activation induces a significant increase in Nrf2 expression. PKR phosphorylates eIF2-α, which reduces protein synthesis while upregulating the expression of some genes such as ATF-4. The 5′untranslated region of Nrf2 mRNA presents an internal ribosomal entry site (IRES), allowing enhanced Nrf2 translation in eIF2-α-mediated protein translation (45, 46).

The control of Nrf2 activation requires different post-translational modifications as well as its repression and subsequent degradation via the proteasome (8–11, 47). The non-canonical PI3K/Akt signaling pathway has been linked to the activation of Nrf2 in a number of models. GSK3-mediated inhibitory phosphorylation induces Nrf2 by inhibiting the phosphorylation signal and sequential ubiquitination in the Neh6-Nrf2 domain, allowing its stability and activity (13). In *Leishmania* infection, PI3K and Akt inhibition reduced the expression of Nrf2 and Sod1 (**Figure 3**). Our results led us to conclude that PI3K/Akt activation as a result of *Leishmania* infection is a positive Nrf2 regulator in host cells.

The rise of ROS seems to be a key regulator of infection by intracellular pathogens (48), and the co-evolution of host cells and parasites results in a shared pattern of subversion in the production of these radicals. For example, *Trypanosoma cruzi* infection in THP-1 cells requires a level of oxidative stress for successful parasitism, given that the overexpression of Nrf2 reduces parasitism (49). Our data from *in vitro* Nrf2-knockdown macrophages revealed the spontaneous increase of oxidative stress, measured through the levels of ROS, NO, and OONO. The same change in phenotype occurred when PKR and Akt were inhibited, probably due to the reduction of Sod1 and other targets. However, the infection index increased when the cells were treated with SFN and NAC. Our data suggest that Nrf2 activation induces Sod1, thus counteracting the oxidative boost in the cell milieu in infected macrophages.

Several reports (50) have highlighted the close relationship between oxidative stress and the autophagy process. The autophagy pathway plays an important role in resistance to various infections, although it could be subverted, thus favoring some infections (51). It is conceivable that autophagy induced by *L. amazonensis* may be controlled by PKR, as revealed in other models, thus regulating Nrf2 levels. Accordingly, some studies have shown the importance of oxidative stress sensing in autophagy (15, 16) and have demonstrated that the degradation of Keap1 *via* autophagy allows cellular redox homeostasis in liver cells. Our data showed that infected macrophages display an increase in LC3-I to LC3-II conversion, thus corroborating the importance of autophagy through this marker during infection.

Kelch-like ECH-associated protein 1 is a negative regulator of Nrf2, and in the context of infection by *Leishmania*, we demonstrated that Keap1 is regulated after 18 h of infection in a PKR-independent manner (data not included). However, Keap1 stability is decreased in a PKR-dependent manner between 2 and 4 h of infection. When autophagy was inhibited by chloroquine, we noted a cytoplasmic accumulation of Nrf2 and stabilization of Keap1 levels, which was accompanied by high levels of oxidative stress.

Several studies have shown the involvement of the p62 (Sqstm1) protein as a central regulator between Keap1 and Nrf2. Oxidative stress decreases when cells overexpressing p62 bind to this inhibitory protein, leading to autophagosome formation (19, 20). Other studies have shown that Nrf2 positively regulates the expression of p62 (17). Importantly, TLR2 activation culminates in M2 polarization of macrophages (MOX

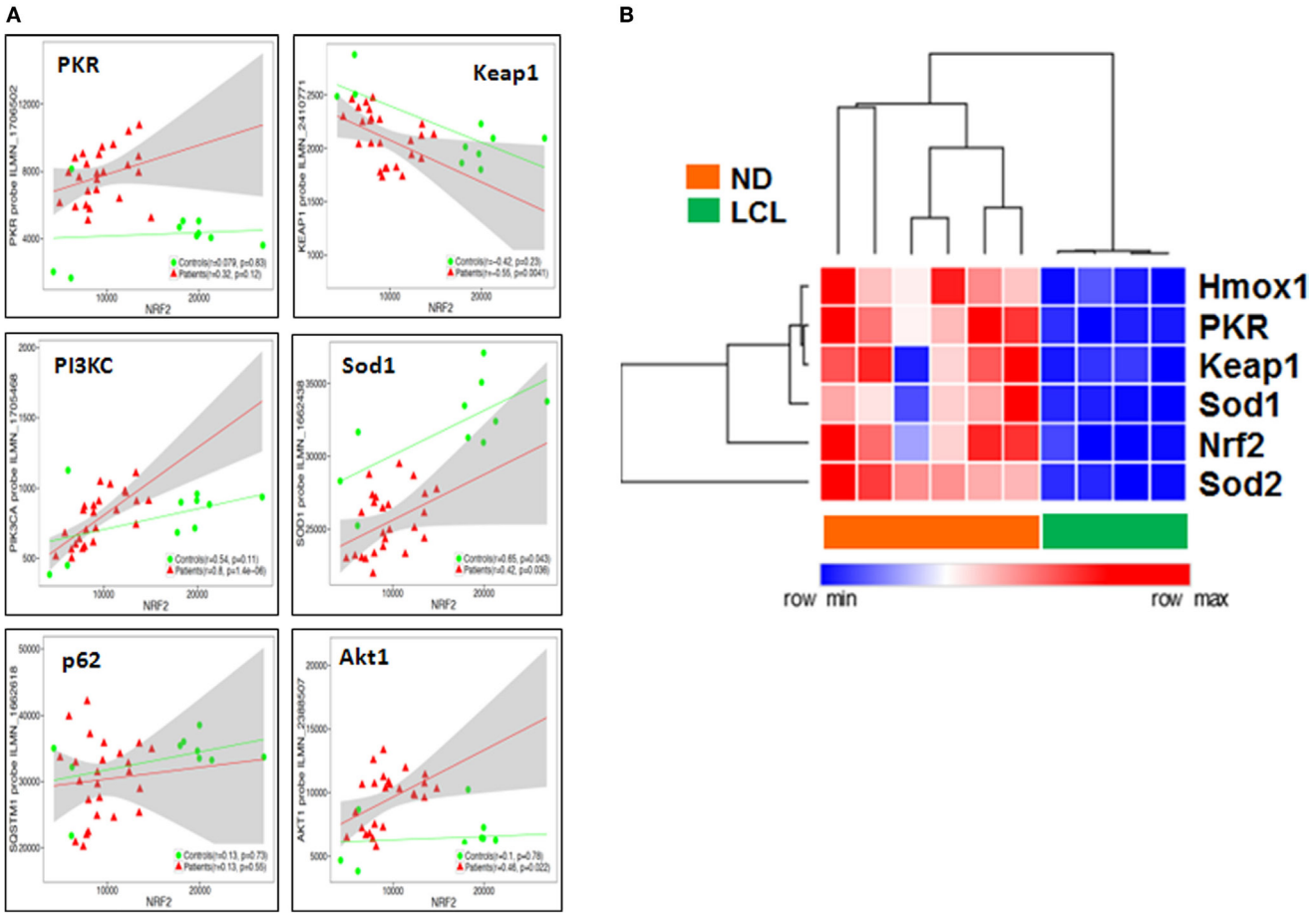

FIGURE 7 | Nuclear factor erythroid 2-related factor 2 (Nrf2) transcriptome-wide correlations confirm the links between IFN/protein kinase R (PKR), antioxidant response element (ARE), PIK3, and autophagy signaling pathways *in situ*. **(A)** Transcriptome-wide correlation analysis was applied to Nrf2 transcript levels in microarray data (Illumina) obtained from skin biopsies from LCL patients (n = 20, red) and healthy controls (n = 10, green). Spearman correlation coefficient and p-values are shown for individual transcripts, 95% confidence intervals are shown in gray for patients only. **(B)** Validation of increased PKR/Nrf2 signaling pathway members by digital mRNA quantification. Heat map and hierarchical cluster analysis (Eucledian distance) of selected genes quantified by nCounter digital transcriptomics (Nanostring) in skin biopsies from normal donors (ND, n = 4) and LCL patients (n = 6), normalized according to PTPRC (CD45) expression levels, to account for differences in tissue leukocyte infiltration.

macrophages), which leads to NF-κB-p65 degradation through p62 and lysosomes, characterized by selective autophagy (52). M2 macrophages exhibit antioxidant properties, as judged by the expression of Cox2, IL1β, HO-1, VEGF, and Nrf2 (53). Considering the cascade of signals, our data support the notion that the PKR–PI3K/Akt↔Nrf2 axis regulates p62 gene expression in *Leishmania* infection and passively triggers the autophagy pathway that culminates in Keap1 degradation, activating Nrf2 and resulting in oxidative cellular homeostasis.

Patients with localized cutaneous lesion (LCL) exhibit predominant expression of iNOS, IL-1β, IL-6, MCP-1, TNF-α, and IFN-γ, while anergic diffuse cutaneous leishmaniasis (ADCL) lesions are characterized by the presence of IL-4, IL-5, IL-10, and MIP-1α and the low expression of iNOS (54, 55). Our *in vitro* data showed that *L. braziliensis*, the prominent causative agent of LCL, also induces Nrf2 in a PKR-dependent fashion. This observation underlines the importance of this signaling pathway in other *Leishmania* species besides *L. amazonensis*. However, the immunohistochemistry analysis of LCL vs. DCL lesions revealed a strong Nrf2 reaction in the latter group, while the Keap1 signal was predominant in the former clinical samples. These results indicate that Nrf2 activation may contribute to the poor oxidative response and, consequently, the high parasite burden in DCL patients.

The data obtained in this study confirm and extend our previous finding of an IFN-I/Sod1 axis, linked to increased parasite burden (56) and therapeutic failure in both localized cutaneous leishmaniasis and DCL (31). This study now reveals that this IFN-I/Sod1 link is critically mediated by Nrf2/ARE signaling. Our genome-wide study revealed Nrf2 as a master regulator of the *in situ* (skin biopsies) transcriptome (**Figure 7**), both in health and disease, which is in agreement with its central role in proteostasis and ancient molecular networks, conserved in evolution from Drosophila to man (57). There is a negative correlation with the Keap1 transcript skin biopsies, which was corroborated by our findings at the protein level in both LCL

and DCL skin biopsies (**Figure 6F**). These results point to a possible compartmentalization of the pathogen-driven immune response between tissues in CL, where cutaneous ulcers in LCL are exposed to a complex microbiome, which strongly influences the local immune response, in addition to *Leishmania* antigens (58).

Due to its strong pleiotropic effects and its essential function in normal homeostasis, Nrf2 itself is not yet a target of choice for therapeutic intervention in LCL. However, this study reiterates our previous suggestion that downstream targets of Nrf2, such as Sod1, represent excellent therapeutic targets in LCL. Previous works from our and other groups (59–62) have shown that the Sod1 inhibitor DETC or its precursor molecule, disulfiram, are plausible therapeutic alternatives that have been used extensively in humans for decades with an excellent safety profile. In conclusion, we demonstrate for the first time the prominent role of Nrf2 and the PKR↔PI3K/Akt↔p62/autophagy axis in human and experimental leishmaniasis (**Figure 7B**). Collectively, our data propose a signaling-based scenario that may reveal a pivotal molecular basis for CL pathogenesis as well as its therapeutic potential. A schematic model based in our results is depicted in **Figure 8**.

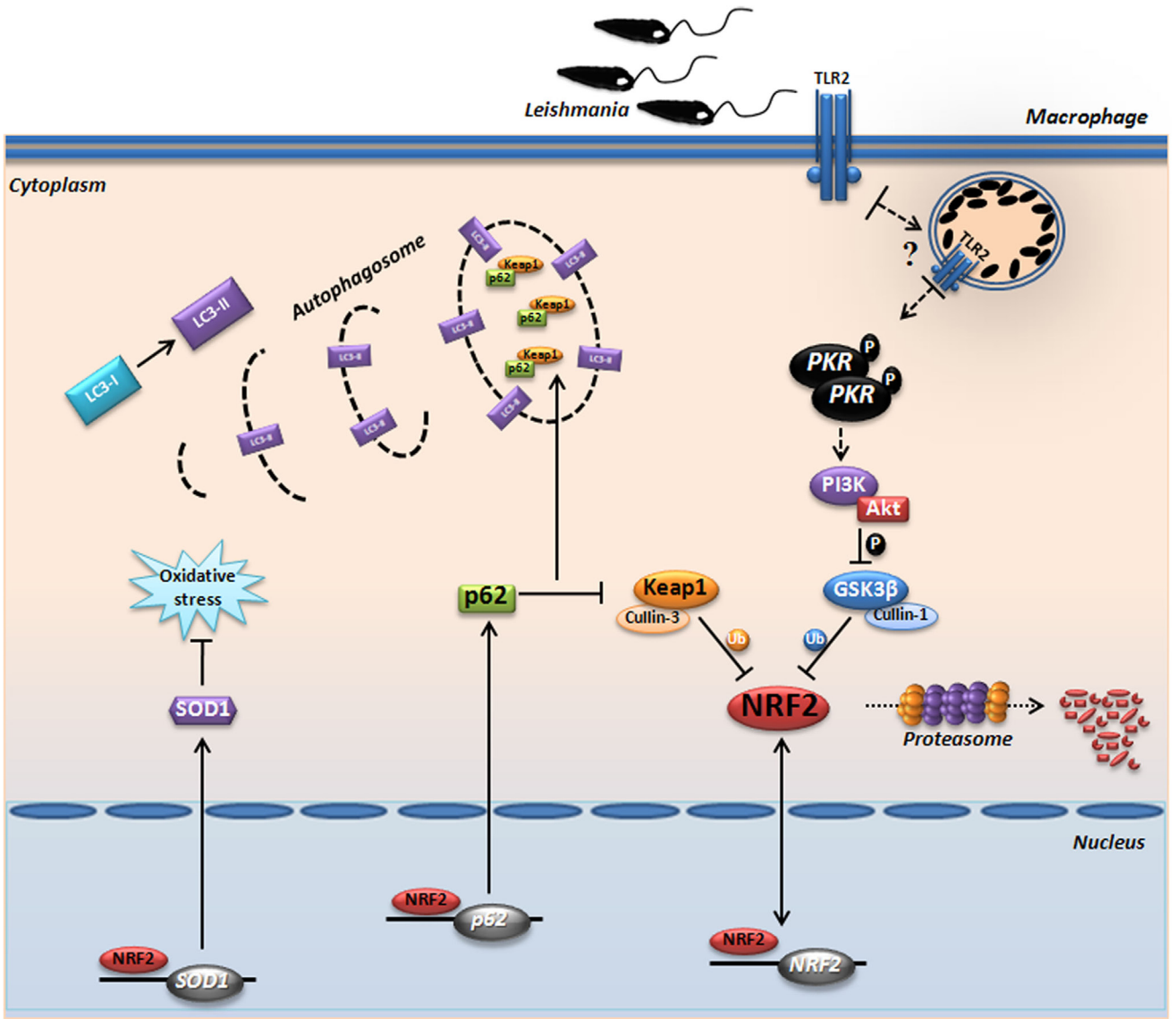

FIGURE 8 | Proposed model for the protein kinase R (PKR)-dependent nuclear factor erythroid 2-related factor 2 (Nrf2) activation in *Leishmania* infection. Internalized parasite signals through the endosomal compartment *via* TLR2 and induce activation of PKR by dimerization and subsequent autophosphorylation. Subsequently, we found that GSK3 phosphorylation is dependent of PKR signaling, allowing that not occur inhibition of Nrf2 through Neh6 inhibitory domain. This activation of Nrf2 is also dependent of Keap1 inhibition through of autophagic and PKR pathways. These mechanisms induce nuclear translocation Nrf2, increasing the gene expression of *Sod1*, *Nrf2*, and *p62*. The sequestosome-1 (p62) could be recruiting, together with processed LC3-II and Keap1 for autophagic vacuoles, allowing greater Nrf2 activation and inhibition of oxidative stress through antioxidant enzymes.

ETHICS STATEMENT

Written informed consent was obtained from all participants or legal guardians, and all of the data analyzed were anonymized. The project was approved by the Institutional Review Board of Centro de Pesquisas Gonçalo Moniz, FIOCRUZ–BA (license number 136/2007) and complies with the guidelines of the Declaration of Helsinki.

AUTHOR CONTRIBUTIONS

AV—designed and performed experiments, analyzed data, and wrote the manuscript; TC-S—performed initial experiments for the study; AS—provided reagents, supervised experiments, and critically evaluated the manuscript; VB, JF-C, RK, AB, TD, JW, and VB—carried out experiments with patient samples, analyzed the results, and provided input for experimental design and interpretation; NF—critically reviewed the manuscript and analyzed data, and UL—directed the study, analyzed the data, and wrote the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at http://journal.frontiersin.org/article/10.3389/fimmu.2017.01127/full#supplementary-material.

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