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Review

Mapping the crossroads of immune activation and cellular stress response pathways

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The innate immune cell network detects specific microbes and damages to cell integrity in order to coordinate and polarize the immune response against invading pathogens. In recent years, a cross-talk between microbial-sensing pathways and endoplasmic reticulum (ER) homeostasis has been discovered and have attracted the attention of many researchers from the inflammation field. Abnormal accumulation of proteins in the ER can be seen as a sign of cellular malfunction and triggers a collection of conserved emergency rescue pathways. These signalling cascades, which increase ER homeostasis and favour cell survival, are collectively known as the unfolded protein response (UPR). The induction or activation by microbial stimuli of several molecules linked to the ER stress response pathway have led to the conclusion that microbe sensing by immunocytes is generally associated with an UPR, which serves as a signal amplification cascade favouring inflammatory cytokines production. Induction of the UPR alone was shown to promote inflammation in different cellular and pathological models. Here we discuss how the innate immune and ER-signalling pathways intersect. Moreover, we propose that the induction of UPR-related molecules by microbial products does not necessarily reflect ER stress, but instead is an integral part of a specific transcription programme controlled by innate immunity receptors.

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Introduction

Cells are constantly subjected to diverse stresses such as nutrient deprivation, radiation, oxidative stress and also infection by microbial pathogens that can lead to damage and cell death. Cells have therefore evolved different mechanisms to cope with these exogenous stresses. As protein synthesis is a fundamental cell function, the control of mRNA translation plays a central role in most stress responses. mRNA translation can be divided into three phases: initiation, elongation and termination. Although all phases are subject to regulatory mechanisms, initiation is regarded as the rate-limiting step (Holcik and Sonenberg, 2005). Much of this control involves different post-translational modifications of initiation factors. Among them, phosphorylation of the α subunit of the eukaryotic protein synthesis initiation factor 2 (eIF2α) provided one of the first examples of the control of eukaryotic protein synthesis by protein phosphorylation (Proud, 2005). This mechanism of protein translation control is triggered by diverse stresses and is conserved from budding yeast to higher mammals (Proud, 2005).

Phosphorylation of eIF2α at serine 51 by eIF2α kinases abolishes the formation of the translation initiation ternary complex (eIF2α/GTP/methionyl tRNA) by inhibiting the GTP exchange factor eIF2B (Holcik and Sonenberg, 2005), leading to translation initiation suppression and promotion of a specific transcriptional response (Harding *et al*, 2003). Mice bearing a homozygous mutation (S51A) at serine 51 residue die within the first day after birth from severe hypoglycemia, resulting from low plasma insulin levels (Scheuner *et al*, 2001). These results suggest that aberrant eIF2α phosphorylation, resulting from malfunction or misregulation of eIF2α kinases and phosphatases, could play a role in different cellular pathologies.

In recent years, the regulation of eIF2α phosphorylation has been implicated in biological processes as diverse as synaptic plasticity, inflammation and metabolic diseases (Deng *et al*, 2004; Nakamura *et al*, 2010; Tabas and Ron, 2011; Trinh *et al*, 2012). Most of the available biochemical and genomic data about eIF2α biology were obtained during the study of the unfolded protein response (UPR) in the endoplasmic reticulum (ER) (Ron and Walter, 2007). The ER is an essential cellular compartment for the synthesis and folding of secreted and transmembrane proteins. Only correctly folded proteins are exported to the Golgi apparatus (Schröder and Kaufman, 2005; Ron and Walter, 2007; Yoshida, 2007). This organelle is also responsible for intracellular calcium homeostasis and lipid biosynthesis, which are required for cell survival and normal cellular functions. Certain environmental conditions induce the accumulation of misfolded/unfolded proteins leading to ER

stress. In cells of higher eukaryotes, three major signalling cascades, commonly known as the UPR, connect ER stress detection with the regulation of the transcriptional and translational machineries (Schröder and Kaufman, 2005; Ron and Walter, 2007) (Figure 1). (1) IRE1 (inositol-requiring enzyme 1) cleaves the mRNA encoding for the transcription factor X-box-binding protein-1 (XBP1) (Yoshida *et al*, 2001; Ron and Walter, 2007). XBP1 activates the expression of a large number of genes regulating ER homeostasis and involved in protein folding, disulphide bond formation, lipid biosynthesis or ER-associated degradation (ERAD) such *Bip* or *ERdj4* (Yoshida *et al*, 2001; Lee *et al*, 2003, 2008). (2) Upon ER stress, activating transcription factor 6 (ATF6) is transported to the Golgi and is processed into an active transcription factor (Haze *et al*, 1999; Chen *et al*, 2002; Shen *et al*, 2002). After nuclear translocation, ATF6 induces the transcription of ER chaperone genes, such as *Bip*, again and several major targets of the mammalian UPR (Haze *et al*, 1999), including *Xbp1* (Wang *et al*, 2000). (3) PERK (protein kinase RNA (PKR)-like ER kinase) is a kinase that phosphorylates eIF2α inhibiting the flux of neo-synthetized proteins and activating the expression of the transcription factor ATF4, and its downstream targets, including the pro-apoptotic transcription factor C/EBP homologous protein (CHOP/GADD153) and, the growth arrest and DNA-damage-inducible protein 34 (GADD34, also known as PPP1R15a or Myd116), a phosphatase 1 cofactor that functions as a negative-feedback regulator of eIF2α phosphorylation.

Innate sensing is the first line of defense against pathogens and is necessary for efficient activation of adaptive immunity. Microbes detection is mediated by pattern recognition receptors (PRRs), which detect conserved structures of pathogens called pathogen-associated molecular patterns (PAMPs), such as lipopolysaccharides (LPS) or nucleic acids. Toll-like receptors (TLRs) are the most characterized PRRs and their triggering results in the induction of multiple signalling cascades that lead to the expression of genes involved in shaping specific immune responses against infectious pathogens (Kawai and Akira, 2010). Similarities have recently been noted in signalling pathways stemming from innate immune and ER-stress-signalling pathways (Zhang and Kaufman, 2008). Both IRE1 and TLRs can trigger antimicrobial response and engage molecular adaptors to trigger inflammatory responses through NF-kB or mitogen-activated protein kinase activation (Urano *et al*, 2000; Martinon *et al*, 2010). Moreover, results from experiments investigating the molecular connections

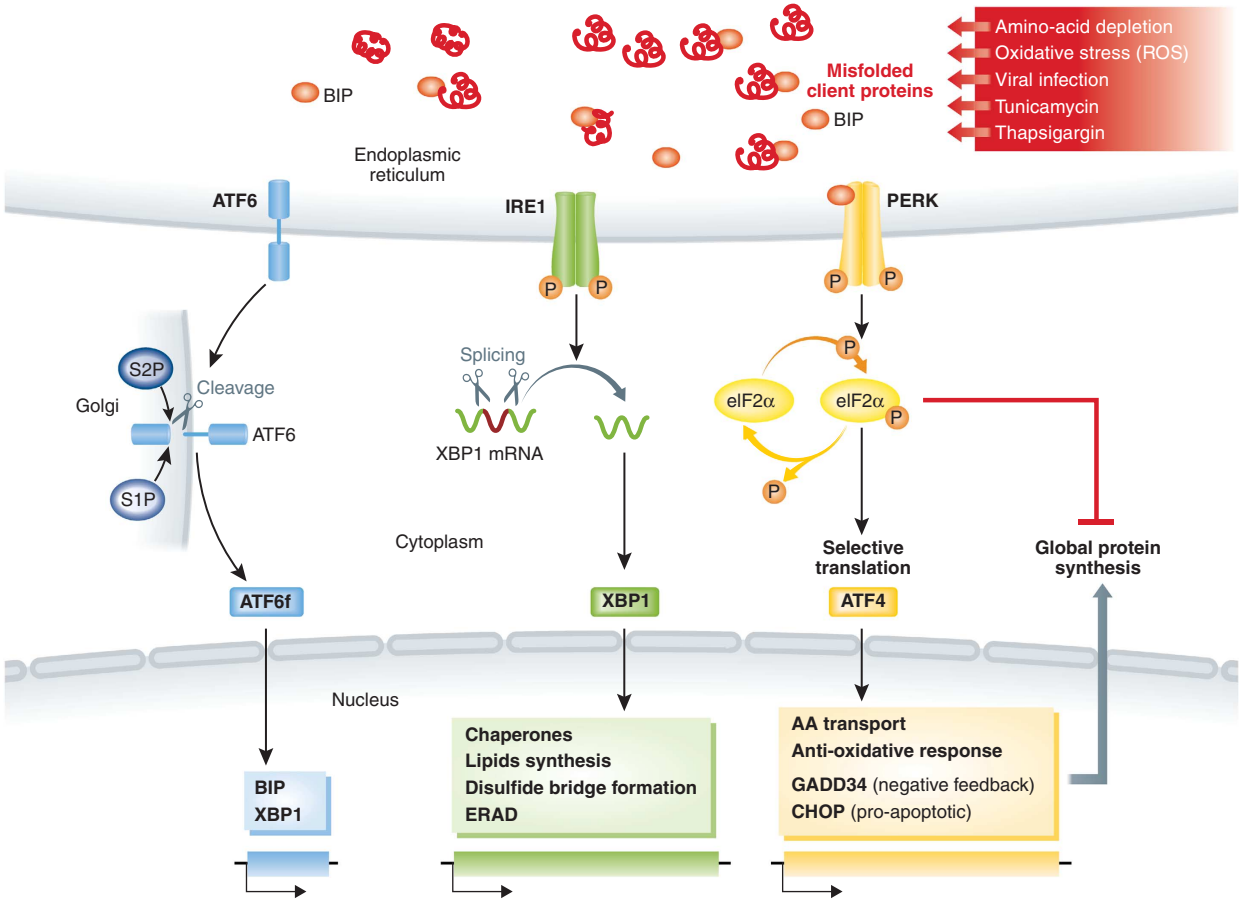

Figure 1 Schematic description of the unfolded-protein response (UPR). Misfolded protein accumulation in the ER activates three distinct sensors: ATF6, inositol-requiring transmembrane kinase/endonuclease 1 (IRE1) and pancreatic ER kinase (PERK). ER stress can be induced by stressors, such as ROS, leading to IRE1-dependent XBP1 mRNA splicing and translation. XBP1 nuclear translocation drives the transcriptional activation of multiple genes involved in ER and molecular chaperones homeostasy. Other UPR transcription factors, such as ATF4, and CHOP are induced upon eIF2α phosphorylation by PERK, which also inhibits translation initiation. The phosphatase 1 cofactor, GADD34, functions in a negative-feedback loop driven by ATF4, which dephosphorylates eIF2α and restores protein synthesis upon stress relief.

between microbe-sensing and protein synthesis regulation, and in particular the role of GADD34 in this process (Clavarino *et al*, 2012a, b), indicate that although several key genes including *ATF4*, *CHOP* and *GADD34* are upregulated both by microbial detection and ER stress, these gene expression programmes are part of distinct transcriptional responses. We propose that this stress response, which is embedded within the larger innate defense gene expression signature driven by microbe recognition, should be considered as microbe specific and not merely a reflection of accentuated ER stress (Leber *et al*, 2008; Seimon *et al*, 2010; Hetz, 2012) (Table I).

The negative-feedback-signalling loop of the UPR

Mammalians possess four different eIF2α kinases (Holcik and Sonenberg, 2005; Proud, 2005; Wek *et al*, 2006): PKR, PERK, GCN2 (general control non-derepressible-2) and HRI (haem-regulated inhibitor). All of them act via phosphorylation of serine 51 of eIF2α, thereby limiting global protein synthesis (Figure 2). Each kinase is associated with the response to different kinds of stress. HRI is activated under conditions of low haem, as well as by oxidative, osmotic or heat shock (Han *et al*, 2001). PERK is mostly activated in response to ER stress, through the quenching of the ER-resident HSP70

Table I Characteristics of the UPR and the MSR

UPR	MSR
Induced in response to unfolded protein accumulation in the ER. UPR counteracts the harmful effect of unfolded proteins and promotes ER homeostasis (Schröder and Kaufman, 2005; Ron and Walter, 2007).	Activated in response to PAMPs. MSR counteracts the physiological consequences of infection or microbial detection, while promoting immune defenses (Woo *et al*, 2009; Goodall *et al*, 2010; Martinon *et al*, 2010; Clavarino *et al*, 2012a, b)
UPR comprises three different signal cascades, including the PERK/ATF4, ATF6 and IRE1/XBP1 pathways (Schröder and Kaufman, 2005; Ron and Walter, 2007).	MSR comprises at least the TRIF/ATF4 or PKR/ATF4 pathways, mostly without CHOP protein expression and can display some IRE1 activation in specific cells types (Clavarino *et al*, 2012a, b)
Upregulation of *Xbp1, Bip, Ero1, ERdj4* and $p58^{IPK}$, as well as other ER chaperones, and induction of protein degradation pathways. CHOP transcription and synthesis are strongly induced (Lee *et al*, 2003; Schröder and Kaufman, 2005; Ron and Walter, 2007). *Atf3* and *Gadd34* are strongly induced and ATF4 is synthetized.	No upregulation of *Bip, Ero1, ERdj4* and $p58^{IPK}$. Cell-type-dependent limited upregulation of *Xbp1* (Martinon *et al*, 2010). CHOP induction is limited both transcriptionaly and translationaly. *Atf3* and *Gadd34* are strongly induced and ATF4 is synthetized.
Translation is temporarily arrested, but is reinitiated upon eIF2α dephosphorylation by GADD34 (Schröder and Kaufman, 2005; Ron and Walter, 2007).	Protein translation activity is induced and can be regulated independently of eIF2α dephosphorylation (Clavarino *et al*, 2012a, b).
Potential role in sterile inflammatory cytokine transcription, but no demonstrated action on type-I IFN expression (Deng *et al*, 2004; Hsu *et al*, 2004).	The ATF4/GADD34 axis regulates cytokine expression both transcriptionally and translationally in a PKR- or TRIF-dependent manner. Correct type-I IFN expression requires GADD34 and XBP1 (Clavarino *et al*, 2012a, b; Martinon *et al*, 2010).
Can be ROS dependent (Hetz, 2012).	Can be ROS independent (Li *et al*, 2010; Martinon *et al*, 2010).

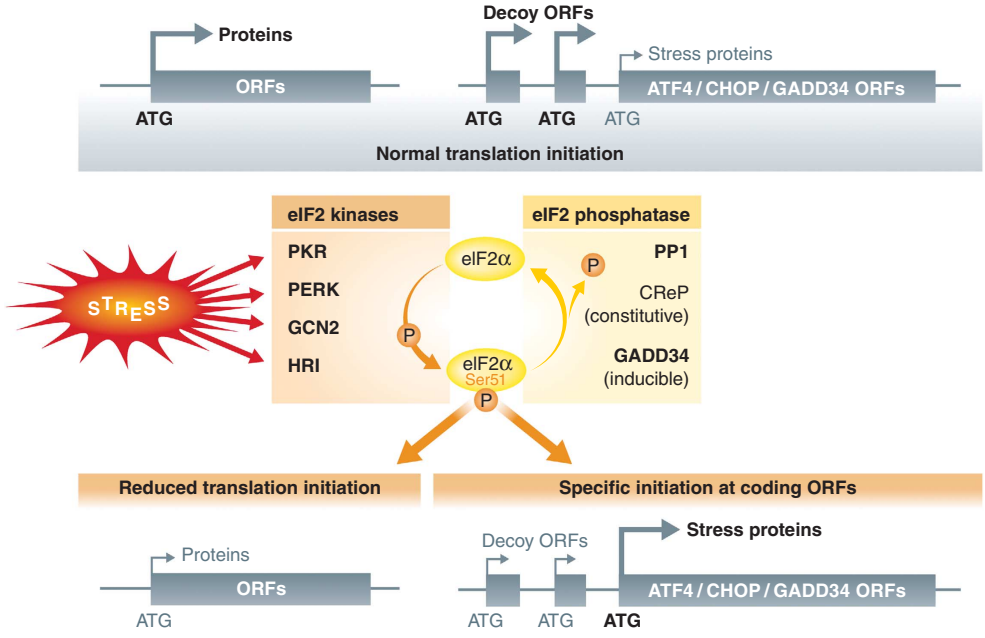

Figure 2 Schematic description of eIF2a phosphorylation pathway. Upon stress sensing, four known different eIF2α kinases, PKR, PERK, GCN2 and HRI, act via phosphorylation of serine 51 of eIF2α to limit global protein synthesis. GADD34 (PPP1R15a) and CReP (PPP1R15b) are regulatory subunits of PP1 that promote eIF2α dephosphorylation and counteract eIF2α kinases activity. Under normal conditions, ATF4, GADD34 and CHOP mRNA translation is repressed by competition for translation initiation of several short open reading frames (decoy ORFs) located upstream and frame shifted from the true translation initiation site. Upon phosphorylation of eIF2α, translation can now initiate at the AUG of the downstream coding regions allowing synthesis of these molecules during stress-induced protein synthesis inhibition.

chaperone BiP by an excess of misfolded client proteins (Figure 1) (Harding *et al*, 2000b). GCN2 is activated in response to amino-acid starvation and UV irradiation (Berlanga *et al*, 1999; Deng *et al*, 2002). GCN2, which mostly senses unloaded tRNAs, has also been reported to play a role in defense against RNA and DNA viruses *in vitro* and *in vivo* (Berlanga *et al*, 2006; Won *et al*, 2012). Similarly, the type-I interferon (IFN)-inducible PKR exerts an antiviral activity through its activation by double-stranded RNA (Williams, 2001; Dabo and Meurs, 2012), but is also regulated by cellular cofactors such as p58IPK, ribosomal protein L18, the TAR RNA-binding protein (TRBP) and the PKR activator (PACT) (Daher *et al*, 2009).

Thus, viral infection or accumulation of misfolded proteins can result in a sustained eIF2α phosphorylation by PKR, GCN2 or PERK, which can become lethal if prolonged (Srivastava *et al*, 1998). Cells must therefore tightly regulate the level of phosphorylated eIF2α in order to survive and carry on with their function (Tabas and Ron, 2011). GADD34 (PPP1R15a) and the constitutive repressor of eIF2α phosphorylation CReP (PPP1R15b) are regulatory subunits of protein phosphatase 1 (PP1) that promote the dephosphorylation of eIF2α (Connor *et al*, 2001; Novoa *et al*, 2001, 2003; Jousse *et al*, 2004). CReP contributes to a basal level of eIF2α dephosphorylation (Jousse *et al*, 2004), while GADD34 negatively controls eIF2α phosphorylation during the UPR and other stress, including viral infection (Clavarino *et al*, 2012a). GADD34 expression is mostly dependent on ATF4 (Brush *et al*, 2003; Ma and Hendershot, 2003; Novoa *et al*, 2003), which binds to a conserved consensus site in the promoter region of the *GADD34* gene and induces its transcription (Ma and Hendershot, 2003). *GADD34* transcription is also known to involve other transcription factors, such as the pro-apoptotic CHOP, ATF3 (Jiang *et al*, 2004; Marciniak *et al*, 2004) and potentially ATF6, whose proteolytic activation contributes to the UPR. GADD34 expression and its eIF2α phosphatase activity are therefore critical to determine cellular fate following various forms of stress (Harding *et al*, 2003) and to define a biochemical response commensurate to stress intensity and duration.

During stress, eIF2α phosphorylation and its inhibitory impact on translation initiation are essential to promote ATF4 synthesis (Figure 2). Under normal conditions, ATF4 mRNA translation is repressed by competition for translation initiation of several short open reading frames (uORFs) located upstream and frame shifted from the true translation initiation site. These uORFs are translated by ribosomes, which generally initiate translation on the first available AUG codon placed in the right neighbouring nucleotide sequence context. As a consequence, the last downstream ORF that encodes ATF4 is only translated at low levels, if at all. During stress conditions, phosphorylation of eIF2α and the accompanying reduction in the levels of eIF2α-GTP increase the time required for the scanning ribosomes to become competent to initiate translation. This delay allows the ribosomes to scan through the uORFs and initiate at the AUG of the downstream *ATF4*-coding region allowing full translation of this transcription factor (Holcik and Sonenberg, 2005). This in turn promotes the transcription of the downstream target genes *CHOP* and *GADD34* (Lee *et al*, 2009; Palam *et al*, 2011) that share a similar upstream uORF-competition translation mechanism (Lee *et al*, 2009; Palam *et al*, 2011) (Figure 2).

Importantly, most of our current knowledge on ATF4 and GADD34 was obtained by investigating their role during artificial induction of the UPR. Recent work from our laboratory, however, indicates that ATF4 and GADD34 induction are also important components of antimicrobial responses, although the modalities of their expression upon infection are clearly distinct from the 'classical' UPR.

Phosphorylation of eIF2α and viral detection

In addition to ER stress, virus infection and dsRNA also induce eIF2α phosphorylation via PKR to inhibit cellular protein synthesis and viral replication (Dabo and Meurs, 2012). During their replication, RNA and DNA viruses generate RNA intermediates that elicit antiviral responses mostly through type-I IFN production (Kawai and Akira, 2006; Pichlmair and Reis e Sousa, 2007). In addition to PKR, several families of proteins are known to sense dsRNA and trigger IFN release, including endocytic TLR3 (Alexopoulou *et al*, 2001) and several cytosolic DEAD-box RNA helicases, such as MDA5 (Yoneyama and Fujita, 2007; Loo and Gale, 2011) (Figure 3). Type-1 IFN binding to cell surface receptors leads to activation of the Janus tyrosine kinase pathway, which induces the expression of a wide spectrum of IFN-stimulated genes, including PKR itself, which participates in the cellular defense against viral infection (Williams, 1999). PKR mediates phosphorylation of eIF2α, leading to inhibition of translation and triggering of apoptosis (Williams, 1999, 2001; Dabo and Meurs, 2012). Initial analysis revealed that the $PKR^{-/-}$ mice (129terSv × C57/BL6) are healthy and presented normal antiviral responses after intravascular inoculation of EMCV or Vaccinia virus (Yang *et al*, 1995; Abraham *et al*, 1999). However, differences in genetic backgrounds that may compensate for the PKR deficiency were later observed, and PKR-deficient mice in the 129terSv × BALB/c background died due to intranasal infection with Vesicular Stomatitis Virus (VSV). The $PKR^{-/-}$ mice also showed increased susceptibility to influenza virus infection (Balachandran *et al*, 2000).

Independently of PKR detection, some viruses use the ER as a site of replication, which can lead to the activation of ER stress and PERK (Cheng *et al*, 2005). The GCN2 and PERK eIF2α-kinases can thus phosphorylate eIF2α upon viral detection (Won *et al*, 2012), and like PKR, limit infection by preventing viral replication and inducing apoptosis in contaminated cells. These three eIF2α kinases can therefore contribute to a powerful antiviral pathway (Langland *et al*, 2006; Domingo-Gil *et al*, 2011). Consequently, many viruses have evolved strategies to ensure completion of their infection cycle and efficient spreading, by escaping host translation shut-down, for example through antagonizing PKR (Schneider and Mohr, 2003). Some viruses have thus acquired factors homologous to GADD34 containing PP1-activating motives to promote eIF2α dephosphorylation (Cruz *et al*, 2011). The protein ICP34.5 of herpes simplex virus 1 (HSV-1) enables HSV-1 to escape the inhibitory effect of both PKR and PERK activation (He *et al*, 1997, 1998; Cheng *et al*, 2005), via its C-terminal domain, which mimics the C-terminal region of GADD34. Functional

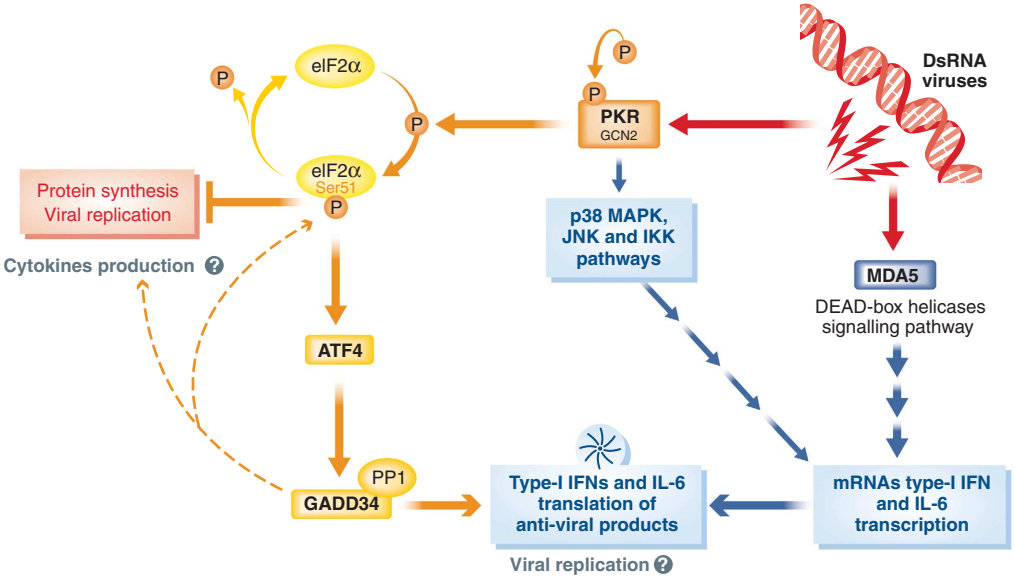

Figure 3 Schematic description of dsRNA and viral sensing in the cytosol. During their replication, viruses generate RNA intermediates, which are sensed by several cytosolic DEAD-box RNA helicases, such as MDA5, which signal to promote type-I IFN production, through a cascade of adaptors leading to IRF3 phosphorylation and nuclear translocation. Concomitantly upon dsRNA sensing, PKR or GCN2 autophosphorylate and mediates phosphorylation of eIF2α, leading to inhibition of translation, while activating other signalling pathways promoting cytokines expression (e.g., p38 and JNK). The large production of inflammatory cytokines and antiviral factors, despite this rapid and efficient concomitant shut down of cellular protein synthesis, implies the existence of specific regulatory mechanisms allowing the translation of host antiviral mRNAs during eIF2α phosphorylation. ATF4 and GADD34 are necessary to allow cytokine translation.

homology was established by domain-swapping experiments demonstrating that the infectivity of an ICP34.5-deleted mutant of HSV-1 could be rescued with the PP1-interacting domain of GADD34 (Chou and Roizman, 1990, 1994; Zhan *et al*, 1994). The current list of identified GADD34 viral homologues include DP71L from African swine fever virus (ASFV) (Zsak *et al*, 1996), Gene 7 of the transmissible gastroenteritis virus (TGEV) (Malathi *et al*, 2007; Cruz *et al*, 2011) and by the human papillomavirus (HPV) type 18 E6 oncoprotein (Kazemi *et al*, 2004).

Unresolved issues concerning innate immunity and eIF2α phosphorylation

Although the antiviral role of mammalian eIF2α kinases is well established, several ambiguities have to be resolved to fully understand the integration of antiviral host cell responses within the more complex systemic immune responses. First, the large production of inflammatory cytokines and antiviral factors, including type-I IFN, despite a rapid and efficient shut down of cellular protein synthesis in infected cells, implies the existence of specific regulatory mechanisms allowing the translation of host antiviral mRNAs during PKR-dependent eIF2α phosphorylation. Conversely, if one applies to viral infection, the biochemical model built using the observations drawn from PERK activation and ATF4 production during the UPR, phosphorylation of eIF2α in response to direct dsRNA-dependent activation of PKR, should lead to a rapid ATF4 and GADD34 induction in infected cells (Figure 3). GADD34 will, in turn, promote eIF2α dephosphorylation, which in the particular context of viral infection should antagonize PKR and promote viral replication, in a similar fashion to what is observed for ICP34.5 and other GADD34 viral homologues. Thus, based on the UPR model, eIF2α phosphorylation-mediated GADD34 induction in infected cells would be counter productive for the host. Dendritic cells (DCs) and macrophages, which are key for the initiation of the immune response, are equipped with a broad array of these microbial PRRs (Kawai and Akira, 2011) and are able to detect and control a large variety of pathogens. In these cells, bacterial LPS detection by TLR4 (Hsu *et al*, 2004; Nakamura *et al*, 2010) and exposure to inflammasome agonists induce PKR autophosphorylation (Figure 4) and activation (Lu *et al*, 2012). PKR deficiency in these stimulated cells significantly inhibits the expression of cytokines, like type-I IFN (Diebold *et al*, 2003), and prevents the secretion of IL-1β, IL-18 and HMGB1 (Lu *et al*, 2012). However, despite PKR activation, protein synthesis is enhanced and not inhibited in TLR-stimulated cells (Hsu *et al*, 2004; Lelouard *et al*, 2007; Clavarino *et al*, 2012b), indicating that differently from the UPR, microbial activation of eIF2α kinases is not always associated with translational arrest and increased phosphorylation of eIF2α (Goldfinger *et al*, 2011). These observations can be also extended to plasma cells stimulated with LPS, although in these cells, a precise molecular dissection of the pathways involved is made more complex by the exhibition of a chronic ER stress due to their massive immunoglobulin secretory activity (Cenci *et al*, 2006; Goldfinger *et al*, 2011). Some clues to these yet unresolved issues could be given by the surprising observation that upon dsRNA sensing and viral infection, GADD34 is necessary to ensure efficient inflammatory cytokines production both at the transcriptional and translational level (Clavarino *et al*, 2012a, b), thereby unravelling novel and important features of this molecule during infection and the initiation of innate immunity.

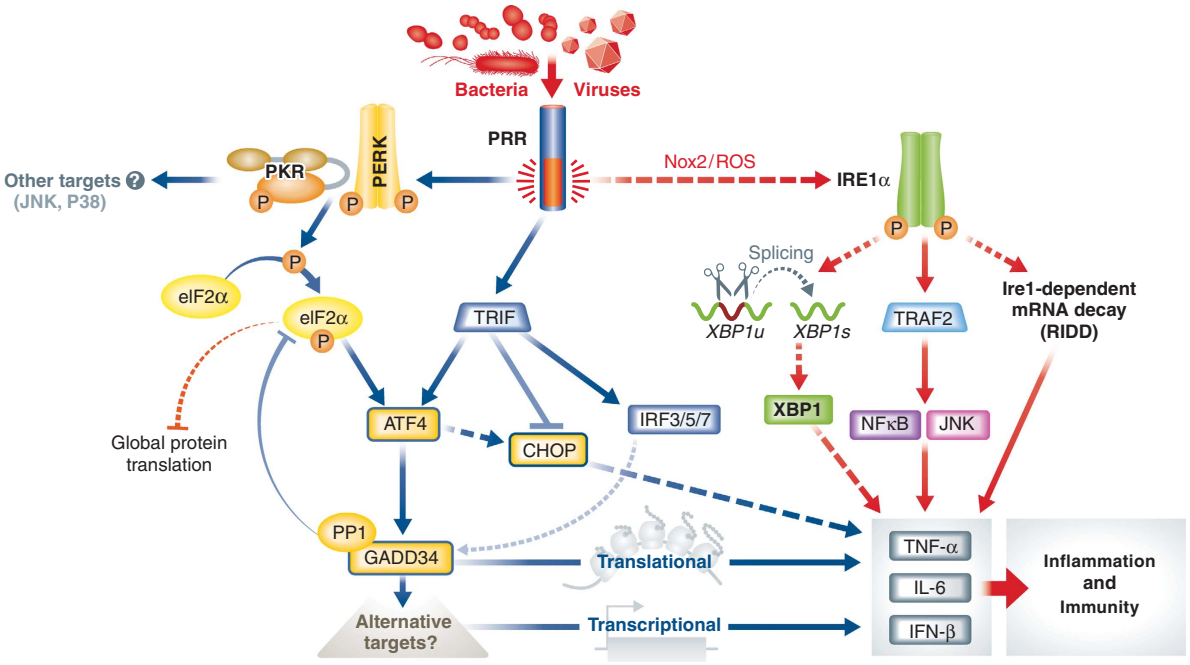

Figure 4 Schematic description of the MSR. During the MSR, microbes- and virus-associated molecular patterns (e.g., LPS) are sensed directly or indirectly by different receptors, such as Toll-like-receptors (TLRs), Rig I-like receptors (RLRs) or the dsRNA-sensing kinase (PKR), which through complex signalling cascades, involving the TRIF adaptor and different TRAF ubiquitin ligase, leads to the nuclear translocation of NFkB or IRF-3, and subsequent IFN-I and inflammatory cytokines transcription. Microbial detection leads also to GADD34 expression, which, however, in this context has little effect on controlling global translation, while participates in the regulation of cytokine production both at the translational and transcriptional level. During the MSR, XBP1 splicing levels varies greatly according to cell models and microbe stimulus used; however, a striking distinctive feature of this pathway is the translational inhibition of CHOP synthesis, together with enhanced level of eIF2α de-phosphorylation, GADD34 and expression. GADD34, ATF4 and XBP1 are likely to favour the expression of cytokines through the targeting of yet undefined partners at the translational, signal transduction and transcriptional level. Cross-talks between the UPR and MSR clearly exist, and the direct activation of the TRAF2 or RIDD pathway by IRE1 and subsequent inflammatory cytokines transcription could be an example of those commonalities.

GADD34, a novel player in the cellular antiviral response

In cells not expressing TLR3, GADD34 is strongly induced upon cytosolic delivery and detection of the synthetic dsRNA analogue polyriboinosinic:polyribocytidylic acid (poly I:C) (Figure 3). GADD34 expression obeys the UPR/PERK paradigm during which phosphorylation of eIF2α by its cognate kinase induces translation inhibition while favouring ATF4 synthesis and subsequent GADD34 expression (Clavarino *et al*, 2012a). As expected, GADD34 induction in response to cytosolic poly I:C is PKR- and ATF4-dependent, and triggers the negative control loop of eIF2α dephosphorylation, despite the continuous presence of the dsRNA stimulus and steadily increased PKR activation. Conversely, in GADD34-deficient cells, eIF2α phosphorylation is strongly increased in response to poly I:C, demonstrating the functionality of the PP1 cofactor in this system. Apparently, this biochemical cascade is closely related to what is observed during the UPR. However, conversely to the drug-induced UPR during which translation is only profoundly inhibited for few hours prior full recovery, cytosolic poly I:C induces a near to complete and irreversible protein synthesis extinction within 8 h of cytosolic delivery, despite a rapid and concomitant induction of GADD34 and eIF2α dephosphorylation (Clavarino *et al*, 2012a). Although eIF2α phosphorylation and PKR are required for the initiation of protein synthesis inhibition, this process becomes rapidly eIF2α-independent and, surprisingly, GADD34 inactivation has no impact on neither the intensity nor the speed of translation loss. This observation contrasts with the UPR, during which functional GADD34 is absolutely required to prevent total and rapid protein synthesis inhibition in response to the PERK-activating drug thapsigargin. These observations clearly show that although eIF2α phosphorylation and GADD34 expression represent common consequences of PERK and PKR activation, their impact on the cell physiology are absolutely not equivalent. The functional importance of GADD34 induction in response to dsRNA remained unclear, until it was demonstrated that GADD34-deficient cells were unable to produce type-I IFN and IL-6 proteins in response to poly I:C or Chikungunya Virus (ChikV) infection, despite close to normal mRNA induction (Clavarino *et al*, 2012a).

At the mechanistic level, these observations point directly to a role of GADD34 in controlling the translation of specific mRNAs upon PKR-dependent eIF2α phosphorylation and global translation repression, which is prolonged by yet other undefined factors than eIF2α phosphorylation itself. Interestingly, the synthesis of PKR does not seem inhibited in any of these situations, suggesting that some mRNAs are insensitive to the translation repression exerted by poly I:C detection and do not absolutely depend on GADD34 for their synthesis. However, apart from IFN-β and IL-6, the identity of the mRNAs dependent on GADD34 for their translation is still ill defined. This list could encompass messengers translated

specifically by ER-associated polysomes, such as those coding for secreted and membrane-associated proteins expressed after microbial detection. This possibility infers to the existence of a compartment-specific regulation of mRNA translation, enabling controlled synthesis of selected proteins possibly at specific cellular locations in a globally repressed environment. The existence of such segregation has been proposed for ER-associated translation during the UPR by Nicchita and collaborators (Stephens *et al*, 2005). This compartmentalization of protein synthesis might possibly be extremely important during PKR-dependent responses, and GADD34 could have a qualitative role on the selection of mRNAs being translated during viral infections. The activity of GADD34 viral homologues, such as ICP34.5, might therefore be more subtle, than merely counteracting PKR, and could influence the translation of specific messengers, leaving others untouched. Consequently, the importance of GADD34 for cytokines production makes this molecule a novel actor in the antiviral arsenal, with a role particularly obvious in neonates mice, that are exquisitely sensitive to ChikV infection and die of myocarditis in the absence of functional GADD34 (Clavarino *et al*, 2012a).

Specificity of the innate immunity-sensing pathways

Independently of direct cellular infection by pathogens, activation of innate cells by microbial products is a key event in the initiation of a productive immune response. Interestingly, TLR stimulations by LPS or other microbial agonists, such as soluble poly I:C, result in the activation of multiple signalling cascades resulting in inflammatory cytokines and type-I IFN production. In macrophages, PKR has been shown to be activated downstream of TLR4 and TLR3 in a TIR-domain-containing adaptor-inducing IFN-β (TRIF)-dependent manner (Hsu *et al*, 2004) and to participate to the induction of type-1 IFN in macrophages and DCs (Diebold *et al*, 2003; Hsu *et al*, 2004). Interestingly, prior to PKR activation, non-activated DCs display extremely high level of eIF2α phosphorylation both *in vitro* and *in vivo* (Clavarino *et al*, 2012b). This unconventional eIF2α phosphorylation pattern may reflect special needs of DCs for translational regulation to fully exert their function (Lelouard *et al*, 2007; Ceppi *et al*, 2009). PKR activation in response to TLR triggering was found not to increase further the levels of phosphorylated eIF2α (P-eIF2α), which are even decreased rapidly upon microbial products detection (Hsu *et al*, 2004; Lelouard *et al*, 2007; Ceppi *et al*, 2009; Goldfinger *et al*, 2011; Clavarino *et al*, 2012b). In DCs, eIF2α dephosphorylation was shown to be mediated by GADD34 induction, which occurs together with ATF4 synthesis during TLR-dependent LPS or poly I:C detection (Clavarino *et al*, 2012b). This type of transcriptional response has been also observed in macrophages infected with *L. monocytogenes* or *Mycobacterium tuberculosis* during which transcription of *Chop*, *Gadd34* and *activating transcription factor 3* (*Atf3*) are also strongly induced (Leber *et al*, 2008; Seimon *et al*, 2010). Importantly ATF3, in addition to PAMPs detection, is induced by many forms of stress, including tunicamycin and tapsigargin treatments (Mungrue *et al*, 2009). $Atf3^{-/-}$ animals exhibit no obvious developmental phenotypes, only when the mice are challenged with LPS do they display significantly elevated IL-6 and IL-12 serum levels compared to wild-type controls, suggesting that ATF3 negatively regulates pro-inflammatory cytokine production (Thompson *et al*, 2009).

Gadd34 together with *Atf3* and *Atf4* should therefore be considered as TLR-induced genes, and the signal transduction pathways leading to their expression seem different from what is classically observed during the UPR. Interestingly, recent work on the signalling cascades induced in DCs by West Nile virus infection suggests that GADD34 represents a key signature gene associated to the triggering of RLRs and of mitochondria-associated adapter molecule (MAVS, also called IPS-1, VISA or CARDIF), and could depend on IRF5 translocation for its expression (Lazear *et al*, 2013). This view is confirmed by the direct comparison of the mRNA transcription signatures obtained from cells exposed to tunicamycin treatment or poly I:C exposure, which are clearly different, and contain only few co-regulated genes, including *ATF3, ATF4 and GADD34* (Clavarino *et al*, 2012b) (Table I). Expression of ATF4 and several of its downstream targets, including GADD34, normally requires increased eIF2α phosphorylation to allow their translation at the right initiation codon (Harding *et al*, 2000a) (Figure 2). At an early stage of DC activation, the level of eIF2α phosphorylation could be high enough to permit the synthesis of ATF4 and GADD34 immediately upon transcriptional induction. It is, however, striking that ATF4 is not synthetized in non-stimulated cells, which display massive eIF2α phosphorylation, and that it solely accumulates in activated DCs, mostly concomitant with GADD34-dependent eIF2α dephosphorylation (Clavarino *et al*, 2012b). Moreover, GADD34 and ATF4 expressions are occurring normally in PKR-deficient DCs, which display, upon activation, much lower levels of eIF2α phosphorylation than their normal counterparts. Thus, ATF4 and GADD34 expression are part of a specific response to pathogens and their function seems to have other purposes than solely participating in ER homeostasis and global translation regulation during stress (Figure 4). In fact, it has been recently proposed that IFN regulatory factor (IRF) 7, a master regulator of type-I IFN gene expression, upregulates ATF4 activity and expression, whereas ATF4 in return inhibits IRF7 activation, suggesting a cross-regulation between the IFN response and a participation of ATF4 in a negative-feedback loop of the IFN antiviral response (Liang *et al*, 2011). Interestingly, TLR stimulation seems to induce cellular resistance to eIF2α phosphorylation-dependent inhibition of translation, and this independently of the presence of functional GADD34 explaining how TLR-activated GADD34-deficient DCs are able to synthesize cytokines.

In GADD34-deficient DCs, the transcription levels of IFN-β and IL-6 in response to lipofected dsRNA were found significantly decreased, indicating that during microbial induction, GADD34 probably impacts other signalling cascades important for the transcriptional regulation of cytokines. In the absence of inflammatory stimuli, NF-κB remains in an inactive state via its binding to inhibitor of NF-κB (IκB), which is constitutively expressed. Amplification of NF-κB signalling and inflammatory cytokines production through a P–eIF2α-mediated attenuation of translation has been proposed on the basis that the half-life of IκB is much shorter than that of NF-κB (Schröder and Kaufman, 2005). Translation attenuation should increase the ratio of free

NF-κB to IκB, thereby reducing NF-κB quenching by neo-synthesized IκB and prolonging NF-Kb-dependent transcription events in response to ER stress. This phenomenon is, however, clearly not at work during DC responses to lipofected dsRNA, since protein synthesis is not attenuated and GADD34 inactivation was found to decrease cytokines expression. Thus, contrary to a *bona fide* UPR, GADD34 activity on eIF2α phosphorylation and protein synthesis might become secondary to its other regulatory functions during TLR stimulation.

ER stress Response versus Microbial Stress Response

Interestingly, although functional ATF4 and GADD34 are clearly detected in activated DCs, microbial stimulation alleviates the synthesis of the pro-apoptotic transcription factor CHOP (Marciniak *et al*, 2004; Nakayama *et al*, 2010), despite an upregulation of its transcripts. Transient CHOP expression has been suggested to be beneficial during ER stress, possibly by avoiding the action of pro-apoptotic regulator Bax (Sok *et al*, 1999). However, when the stress is permanent, expression of CHOP is prolonged and cell death induced (Boyce and Yuan, 2006; Rutkowski *et al*, 2006). This observation was confirmed by work in macrophages demonstrating that CHOP expression in response to UPR-inducing agent is inhibited by TLR stimulation (Figure 4) (Woo *et al*, 2009; Nakayama *et al*, 2010; Woo *et al*, 2012). This inhibition is potentially favouring cell survival during microbial detection, since LPS-induced apoptosis is suppressed in different CHOP-deficient cell types (Endo *et al*, 2005, 2006). However, these experiments (Woo *et al*, 2009, 2012) were carried out in the presence of UPR-inducing drugs. Loss of CHOP expression in response to TLR activation was attributed to a deficit in ATF4 synthesis and nuclear translocation, therefore limiting the level of ATF4-dependent CHOP mRNA transcription in stressed macrophages (Woo *et al*, 2009, 2012). Contrasting with these studies, experiments performed in LPS-activated human monocyte-derived DCs have demonstrated that CHOP participates in the enhanced production of IL-23 p19 (Goodall *et al*, 2010), suggesting that CHOP expression is not always inhibited by LPS sensing and associated with increased apoptosis. In activated bone-marrow-derived DCs, ATF4 is synthetized and translocated, while GADD34 and CHOP mRNAs are induced, suggesting that, in these cells, the control of a potentially detrimental CHOP synthesis is occuring at the translational level. This extinction of CHOP synthesis, while maintaining the translation of ATF4 and GADD34 active, again, clearly singularizes the microbial-induced stress genes transcriptional response from a classical ER stress response.

When the IRE-1α and XBP-1 pathways (Figure 1) were explored during macrophage stimulation with different microbial products, it was found that although XBP-1 mRNA splicing could be detected, the transcriptional consequences of this unusual IRE1α activation were qualitatively different from the traditional UPR response (Martinon *et al*, 2010). The NADPH oxidase NOX2 plays an important role in the signalling downstream of the TLRs, through the production of reactive oxygen species (ROS) and their damaging activities on cell structures (Matsuzawa *et al*, 2005). NOX2-dependent activation of IRE1 was shown to be required for XBP1 mRNA maturation, yet this process was found independent of ER stress as downstream targets of XBP1 normally activated in the context the UPR were not induced in response to LPS (Table I) (Martinon *et al*, 2010). Indeed, transcription of the XBP1 canonical UPR targets, such as *BiP* or *ERdj4*, was not observed. Like for *Chop* transcription, when LPS was administered together with tunicamycin, the induction of several UPR-related mRNAs was suppressed. Conversely, during a chemically induced UPR, XBP1 synthesis did not promote the transcription of inflammatory cytokines such as IL-6 or TNF-α, whereas in the context of microbial activation, XBP1 clearly enhanced the transcription of these different cytokines. Independently of XBP1 mRNA splicing, Ire1-dependent decay (RIDD), a recently described pathway specialized in the degradation of different mRNAs by IRE-1 homologous to the type-I-inducible antiviral RNAse L, could also participate in the inflammatory response through host mRNAs or viral mRNA degradation during infection (Malathi *et al*, 2007; Hollien *et al*, 2009). Interestingly, XBP-1 mRNA splicing is not very efficient in DCs activated with LPS or poly I:C (A Dalet, personal communication). The lower microbicidal activities of DCs compared to macrophages could explain this difference, as also suggested by the fact that activated *Nox2*-deficient DCs display normal GADD34 expression levels (Clavarino *et al*, 2012b). In that case, GADD34 expression was found to be TRIF-dependent, further suggesting that ATF4 and GADD34 inductions are a direct consequence of TLR triggering and not part of an indirect response to ER stress, linked to protein overload or misfolding in microbe-stimulated innate cells.

Perspective

The induction or activation of several molecules linked to the ER stress response pathway by microbial stimuli have created the impression that innate sensing is always associated with an UPR. The detection of transcriptional programme linked to the UPR in numerous human diseases, including atherosclerosis, cancer, diabetes and neurodegenerative disorders, have naturally led a wealth of data describing the importance of the UPR in the generation and maintenance of inflammation by immune cells, including macrophages and DCs. However, we would like to underline that the characterization of non-canonical roles of ATF4/GADD34 and IRE-1/XBP-1 deeply linked to the immune context suggests the existence of a specific microbial stress response (MSR) distinct from the now 'classical' UPR. Like the UPR during ER stress, the MSR could allow individual cells to cope with the considerable and deleterious impact of microbe detection on their physiology, while they are expected to participate actively in systemic response to infection. The coexistence and synergy of the two responses are obviously not excluded, as suggested by plasma cell biology or several viral infection situations, but we suspect that the MSR could be dominant in some respect, since TLR stimulation has been shown to counteract most of the conserved features of the UPR (Martinon *et al*, 2010), such as *Bip or ERdj4* transcription (Martinon *et al*, 2010), CHOP synthesis (Woo *et al*, 2009, 2012), eIF2α phosphorylation and translation inhibition (Goldfinger *et al*, 2011; Clavarino *et al*, 2012b). There is emerging evidence that immune

responses can be negatively affected by abnormalities in the UPR and that this could contribute to the development of autoimmunity and metabolic diseases (Todd *et al*, 2008; Hotamisligil, 2010). Artificial induction of the UPR, together with microbial stimulation, has become a standard to study UPR contribution to inflammation (Woo *et al*, 2009; Goodall *et al*, 2010; Hotamisligil, 2010; Woo *et al*, 2012). However, XBP-1, ATF4 and ATF3 synthesis and their nuclear translocations are directly induced by TLR triggering or type-I IFN stimulation (Litvak *et al*, 2009; Martinon *et al*, 2010; Clavarino *et al*, 2012b), with poor experimental evidences for concomitant protein misfolding and true ER stress induction, independent of XBP-1 or ATF4 expressions themselves.

GADD34 induction during TLR activation might have an additional purpose than mediating eIF2α dephosphorylation. GADD34 can form stable interactions with the Tuberous sclerosis complex (TSC1/2) and inhibits mTOR signalling (Uddin *et al*, 2011). A cross-talk between stress-inducible GADD34 and the mTOR-signalling pathway might therefore exist (Goldfinger *et al*, 2011) and play an important role in innate signalling, as well as autophagy regulation (Hyrskyluoto *et al*, 2012). In parallel, GADD34 has been reported to interact with CUE domain-containing 2 (CUEDC2) and form together a stable complex with PP1. This complex has been proposed to be part of a negative-feedback loop that specifically dephosphorylates IKKα and IKKβ after their TLR-dependent activation, thereby decreasing the levels of activated transcription factor NF-κB (Li *et al*, 2008). Thus, CUEDC2 and, by association, GADD34 were proposed to function as an anti-inflammatory complex promoting NF-κB inactivation. These observations contrast, however, with the result obtained in GADD34-inactivated DCs, in which inflammatory cytokines production is decreased suggesting that GADD34 expression can be pro-inflammatory (Clavarino *et al*, 2012b).

Thus, we would like to propose that microbial recognition induces a specific stress response (MSR) with some molecular determinants in common with the UPR, but with different levels of regulation and, more importantly, with a different functional outcome (Table I). Consequently, as transduction cascades downstream of TLRs could be seen as parallel and complementary signalling modules, the MSR should be considered as a novel signal transduction and transcription module involved in the coordination of inflammatory cytokines induction (e.g., IFN-β/IRFs and IL6/NFkB), metabolism activation (e.g., mTOR) and cellular antimicrobial pathways (e.g., PKR and eIF2α) during the detection of microbial products and pathogens.

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Conflict of interest

The authors declare that they have no conflict of interest.

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