GADD34: Lien moléculaire entre la détection des pathogènes et les voies intégrées de réponse au stress

GADD34: linking pathogen detection with the integrated stress response pathways

présentée et soutenue publiquement par

Nuno CLÁUDIO
Le 5 juin 2012

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Abbreviations:

APC – antigen presenting cells
ATF – activating transcription factor
bZIP – basic leucine zipper
CARD – Caspase recruitment domains
CHOP – C/EBP homologous protein
CLR – C-type lectin receptor
CRReP – constitutive repressor of eIF2α phosphorylation
DALIS – dendritic cell aggresome-like induced structures
DCs – dendritic cells
dsRNA – double-stranded RNA
ER – endoplasmic reticulum
GADD34 – growth arrest and DNA damage 34
GCN2 – general control nonrepressed 2
HCV – hepatitis C virus
IDO – indoleamine 2,3 dioxygenase
IFN – interferon
IFNAR – IFNα receptor
IFNGR – IFN-γ receptor
IPS-1 – IFNβ-promoter stimulator 1
IRF – interferon regulatory factor
ISGs – interferon-stimulated genes
ISR – integrated stress response
JAK – Janus kinase
MDA5 – melanoma differentiation-associated gene 5
MEFs – mouse embryonic fibroblast
MHC – major histocompatibility complex
mRNA – messenger RNA
mTOR – Mammalian target of rapamycin
MyD88 – myeloid differentiation factor 88
NF-κB – nuclear factor-κB
NLR – (NOD)-like receptor
NOD - Nucleotide Oligomerization Domain
PERK – double-stranded RNA-activated protein kinase-like ER kinase
PKR – Protein kinase R
poly I:C – polyinosinic:polycytidylic acid
PP1 – protein phosphatase 1
RIG-I – retinoic acid-inducible gene I
RLR – (RIG-I)-like receptor
ROS – reactive oxygen species
STATs – signal transducers and activators of transcription ()
TIR – Toll/Interleukin-1 receptor
TLR – toll-like receptor
TNF – tumor necrosis factor
TRAF – TNF receptor-associated factor
TRIF – TIR-domain-containing adapter-inducing interferon-β
uORF – upstream open reading frames
UPR – unfolded stress response
UTR – untranslated region
WARS – tryptophanyl-tRNA synthetase
Résumé

Il est maintenant bien établi que la traduction des protéines est inhibée dans les cellules exposées à différentes conditions de stress telles que l’accumulation des protéines mal repliées dans le réticulum endoplasmique (RE), la détection des virus ou encore la carence en acides aminés. Chez les mammifères l'arrêt de la synthèse des protéines peut être provoqué à travers la phosphorylation de eIF2α par quatre différentes kinases (PERK, PKR, GCN2 et HRI), qui peuvent toutes détecter des signaux de stress différents (Proud, 2005).

Mon travail de thèse a porté l’étude des conséquences qu’ont les signaux de stress différents sur la fonction des cellules dendritiques (DCs). Les DCs sont équipées avec de plusieurs récepteurs de reconnaissance des pathogènes (PRRs) et peuvent par conséquent détecter une grande diversité de produits microbiens. Après la détection des microbes, les DCs commencent un processus de maturation, caractérisé par de profonds changements fonctionnels, tels que la production de cytokines et l’augmentation de leur capacité de présentation de l’antigène (Banchereau and Steinman, 1998; Mellman and Steinman, 2001; Reis E Sousa, 2006). Les DCs jouent également un rôle important dans l’initiation de la réponse immunitaire adaptative (Banchereau and Steinman, 1998).

Les ARN de double-brins (ARNdb) sont l'une des conséquences caractéristiques de l’infection virale, qui est reconnue par les DCs principalement grâce à Toll-like receptor 3 (TLR3) (Alexopoulou et al., 2001), ou des helicas cytosoliques, telles que MDA5 (Gitlin et al., 2006) ou DDX1, DDX21, et DHX36 (Zhang et al., 2011). L’engagement de ces récepteurs conduit à la translocation nucléaire de facteurs de transcription comme IRF3 et/ou IRF7 et donc à l'induction des interférons de type I.

En tant que modèle pour l’ARNdb, l’analogue synthétique poly I: C, a été souvent utilisé (Grunberg-Manago et al., 1955; Schmidtke and Johnson, 1971). Le poly I:C et son analogue le poly-L-lysine (poly ILC) sont utilisés comme adjuvants de vaccination qui ont prouvé leur efficacité chez la souris et chez les primates non humains (Stahl-Hennig et al., 2009; Longhi et al., 2009). Chez les souris, le poly I:C est capable de générer une production systémique d’interféron (Longhi et al., 2009). Par ailleurs le poly ILC a aussi récemment été démontré d’avoir la capacité d’induire de nombreuses branches de l’immunité innée chez l’humain, en mimant les réponses observées avec un vaccin atténué du virus de la fièvre jaune (Caskey et al., 2011). Poly
I: C constitue donc un adjuvant vaccinatoire prometteur et les voies impliquées dans sa détection méritent donc d'être mieux étudiées.

La production d'interféron favorise l'induction de gènes impliqués dans le contrôle des infections virales parmi lesquels, on trouve pkr (Sadler and Williams, 2008). L'activation de PKR favorise la phosphorylation du facteur d'initiation de la traduction protéique eIF2α et par conséquent, provoque l'inhibition de la réplication virale (Donzé et al., 2004; Scheuner et al., 2006). PKR est également nécessaire pour la production d'interféron du type I par les DCs, en réponse à la stimulation ARNdb (Diebold et al., 2003; Schulz et al., 2010).

La réponse immunitaire peut générer une diversité de signaux de stress capables d'arrêter la traduction des protéines telles que la carence en acides aminés (par exemple, dans l'environnement tumoral, ou l'explosion d'oxydative après la libération d'espèces réactives de l'oxygène par les neutrophiles (Pierre, 2009). L'arrêt de traduction des protéines peut potentiellement conduire à l'apoptose, compromettant ainsi le déclenchement de la réponse immunitaire, qui serait ainsi privée de facteurs essentiels à la maturation des DCs. La stimulation des DCs avec le LPS a un effet profond sur l'intensité et la qualité de la traduction. Le contrôle de la traduction des protéines est donc étroitement coordonné avec l'état d'activation des DCs, les cellules dendritiques stimulées avec du LPS subissent ainsi une phase d’augmentation rapide de la synthèse protéique (Lelouard et al., 2007), suivie d’une fermeture progressive de celle-ci.

Avec le double objectif de mieux comprendre les voies impliquées dans la réponse à l'ARNdb et les mécanisme impliqués dans la résistance des DCs à l'arrêt traductionel, Nos résultats montrent que la traduction des protéines dans les DCs ne s'arrête pas en réponse à poly I: C (Clavarino et al., 2012b.), contrairement aux fibroblastes embryonaires de souris (MEFs) qui perdent rapidement leur capacité de traduire des protéines après lipofection du poly I:C (Clavarino et al., 2012b, 2012a). A la suite d’une analyse du transcriptome réalisée dans les cellules dendritiques dérivées de la moelle osseuse (Inaba et al., 1992) stimulées par le poly I:C (Clavarino et al., 2012b). Il a également été possible de démontrer que les DCs montent une réponse de stress intégré spécifique au cours de laquelle le facteur de transcription ATF4 et GADD34, un cofacteur de la phosphatase PP1, sont exprimés.

La phosphorylation d’eIF2α dans les DCs s’effectue de manière non-conventionnelle. Les cellules dendritiques immatures ont un niveau élevé de phosphorylation de eIF2α qui diminue après l'activation avec du poly I:C. Ceci a
également été observé chez les DCs humaines avec activation par le LPS (Ceppi et al., 2009). Le niveau basal de phosphorylation du facteur eIF2α dans les cellules dendritiques contraste donc avec celle des MEFs, qui est inexistante à l'état stationnaire. Cette observation est très importante et pourrait, à l'avenir, aider à mieux comprendre la régulation de la traduction des protéines en réponse au microbes et son importance pour les cellules dendritiques (Lelouard et al., 2007; Ceppi et al., 2009). La spécificité de la phosphorylation de eIF2α a été confirmé dans des coupes histologiques de la rate de souris, où ce haut niveau de phosphorylation a été détecté de manière évidente seulement dans les DCs et pas dans les lymphocytes T ou d'autres cellules immunitaires.

Le travail présenté ici montre que la phosphorylation d’eIF2α dans les cellules dendritiques est inversement corrélée à l'expression de GADD34 (Clavarino et al., 2012b). Le complexe PP1/GADD34 s’est donc avéré nécessaire pour contrebalancer la phosphorylation de eIF2α. Ainsi l'inhibition pharmacologique de ce complexe avec salubrinal et le guanabenz, ou l’inactivation génétique de GADD34 dans les DCs conduisent à une augmentation dramatique de la phosphorylation d’eIF2α. Nous avons aussi montré que PKR est la kinase principale provoquant la phosphorylation d’eIF2α au cours de la stimulation par le poly I:C. Toutefois, dans les DCs immatures d'autres kinases, non encore identifiées, sont responsables du haut niveau basal de phosphorylation eIF2α (Clavarino et al., 2012b).

L’expression d’ATF4 et de ses cibles (Harding et al., 2000a) nécessite la phosphorylation de eIF2α. Cependant, l’expression de GADD34 n'est pas affectée dans les cellules dendritiques déficientes en PKR, contrairement à la situation dans les fibroblastes dans lesquels, l'expression de GADD34 était totalement dépendante de l'activité de cette kinase (Clavarino et al., 2012a). Une caractérisation plus détaillée de la signalisation responsable de l'expression de GADD34 dans les DCs a pu mettre en évidence un rôle central pour l’adaptateur TRIF, en réponse à l’ARNdb.

Les MEFs et les DCs augmentent l’expression de GADD34 après une stimulation avec le poly I:C. Les cellules dendritiques sont capables de conserver intacte la traduction des protéines, et même de l'augmenter, alors que les MEFs subissent un arrêt rapide de la synthèse protétique (Clavarino et al., 2012a, 2012b). Initialement, nous avons émis l'hypothèse que l'induction de GADD34 pourrait expliquer le phénotype de résistance à l’arrêt traductionnel observé dans les DCs. Toutefois, lorsque la traduction des protéines a été suivie dans les cellules dendritiques déficientes en GADD34
(GADD34\textsuperscript{ΔC/ΔC}), aucune diminution significative de celle-ci n'a pu être observée. Néanmoins, GADD34 est absolument essentiel pour assurer la récupération au stress du RE induit dans les DCs par la thapsigargine, de manière semblable à ce qui a déjà été décrit dans des fibroblastes (Novoa et al., 2003).

C'est seulement lors de l’examen de la relation entre GADD34 et la production de cytokines que nous avons pu démontrer un rôle le plus notoire de GADD34. En effet, dans les cellules dendritiques GADD34\textsuperscript{ΔC/ΔC}, l’expression des ARN messagers de IFNβ et IL-6 sont diminués, entraînant une baisse des niveaux de production de ces cytokines (Clavarino et al., 2012b). De manière intéressante et encore plus drastique, les fibroblastes GADD34\textsuperscript{ΔC/ΔC} ne sont pas capables de sécréter l’IFNβ ou l’IL-6 dans les surnageants de culture cellulaire en réponse au poly I\textsubscript{C} (Clavarino et al., 2012a). Cependant, aucune diminution n'a été observée au niveau de la transcription des ARN messagers codant pour ces cytokines. GADD34 a donc un rôle important dans la production de cytokines et selon le type de cellule, l’impact de GADD34 sur les cytokines est exercée à différentes étapes: soit la régulation de la traduction dans les fibroblastes et ou celle de la transcription dans les cellules dendritiques. L'importance de GADD34 a été confirmée in vivo par l'injection du poly I\textsubscript{C}, chez des animaux étant inactivés pour GADD34 et ou des niveaux inférieurs de production l'IFNβ ont été mesuré dans le sérum par rapport aux animaux contrôles (Clavarino et al., 2012b).

L'importance de GADD34 a été aussi mise en évidence dans le contexte de l'infection virale. Les injections intradermiques de virus du Chikungunya (CHIKV) chez des souris déficientes en GADD34 ont révélé une capacité moindre de ces animaux à produire de l’IFN de type I. De plus, les souris nouveau-nés GADD34\textsuperscript{ΔC/ΔC} qui produisent sensiblement moins IFNβ dans le sérum et les articulations, présentent des titres de CHIKV anormalement élevés dans les muscles, les articulations et surtout le cœur. Les souriceaux GADD34\textsuperscript{ΔC/ΔC} finissent par mourir d’une myocardite aigue, liée à une nécrose massive des cardiomyocytes et une infiltration inflammatoire de monocytes et de macrophages (Clavarino et al., 2012a).

La dernière partie de mon travail de thèse est consacrée aux réponses à la carence en acides aminés. Cette carence provoque une activation de la kinase GCN2 et un arrêt de la traduction. GCN2 est impliquée dans la détection des niveaux d'acides aminés par le cerveau (Maurin et al., 2005; Hao et al., 2005), et a été aussi impliquée dans la détection du tryptophane pendant la régulation immunitaire liée à l’expression d’IDO (Munn et al., 2005; Fallarino et al., 2006). Compte tenu de la relation entre
l'appauvrissement en acides aminés et la réponse au stress intégré, j'ai étudié la conséquence de l'appauvrissement en tryptophane dans le cadre de l'activation des cellules dendritiques avec du LPS et du poly I: C. Dans des conditions de déplétion du tryptophane, les cellules dendritiques sont capables de maintenir la synthèse des protéines et la présentation des antigènes. Pourtant, de nombreux détails restent à élucider concernant le mécanisme qui permet aux cellules dendritiques de résister à ces carences.
INTRODUCTION

Along history infectious diseases have been a major cause of morbidity and mortality. Nevertheless organisms have evolved mechanisms of defense. For instance, jawed vertebrates have evolved two lines of defense, one innate and another adaptive. Innate immunity bases on the recognition of pathogens by pattern-recognition receptors (PRRs). PRRs are germ line encoded and have broad specificities for conserved and invariant features of microorganisms. The second system of defense, the adaptive, is mediated by two types of antigen receptor: T-cell receptors and B-cell receptors. Antigen receptors assemble during lymphocyte development through a series of carefully orchestrated DNA breakage and rejoining events, the somatic recombination. The capacity to rearrange genes of the immunoglobulin family allows the recognition of a great diversity of pathogen-specific motives.

1.1) Dendritic cells

Dendritic cells (DCs) are important antigen presenting cells, responsible for the initiation and modulation of the immune responses (Banchereau and Steinman, 1998). DCs were first visualized as Langerhans cells (LCs) in the skin in 1868, but their characterization only began in the early 1970s by Steinman and Cohn (Steinman and Cohn, 1973).

DCs receive their name from their cell shape and motility, as these cells can extend long dendrites in many directions from the cell body. These structures can bend, retract and re-extend (Winzler et al., 1997). The shape and motility of DCs fit their functions, which are to capture and present antigens. DCs are efficient stimulators of B and T lymphocytes (Banchereau and Steinman, 1998). Their antigen sampling and migratory capacities allow naive T cells to come into contact with peripheral antigens they would otherwise not have encountered. Inside lymph nodes DCs occupy areas where naive T cells are activated (von Andrian and Mempel, 2003). Besides priming the adaptive immune responses DCs are also responsible for induction of self-tolerance, deleting self-reactive T lymphocytes and/or expanding regulatory T cells (Steinman et al., 2003; Yamazaki et al., 2003).
Nearly all tissues contain DCs. These cells differentiate from circulating bone marrow–derived precursors and complete their differentiation after leaving the blood stream and taking residence in peripheral tissues (Steinman, 2011).

DCs can generally exist in two functional states, denominated “immature” and “mature” (Steinman, 1991). Immature DCs express lower amounts of major histocompatibility complex (MHC) and adhesion molecules, are not very motile and do not conjugate with T cells nearly as efficiently as mature DCs (Benvenuti et al., 2004). In their immature state DCs reside in peripheral tissues (Banchereau and Steinman, 1998). Upon detection of microbial products, danger signals (Matzinger, 1994) or inflammatory mediators, DCs go through a maturation process and migrate to the secondary lymphoid organs (Reis E Sousa, 2006). Mature DCs increase the expression of chemokine receptor 7 (CCR7), allowing migration to the draining lymphoid tissues in which they interact with naive T cells (Kapsenberg, 2003). Maturation is also characterized by production of pro-inflammatory cytokines and increased expression of surface markers such as MHC molecules, CD40, co-stimulators (CD80 and CD86) and increase of adhesion molecules, globally enabling enhanced adhesion and signaling with T cells. Phenotypic maturation based only on high levels of expression of CD40, CD80, CD86 and MHC molecules does not necessarily translate into T-cell immunity (Reis E Sousa, 2006). It can in fact induce tolerance, i.e. unresponsiveness to antigens. The Mellman’s lab has shown that disruption of the E-cadherin adhesion between DCs induces typical features of maturation, including the up-regulation of co-stimulatory molecules (CD80 and CD86), MHC class II, and chemokine receptors. Yet these DCs fail to release inflammatory cytokines, and consequently, T cells with a regulatory as opposed to an effector phenotype, are generated in vivo (Jiang et al., 2007).

DCs act as a switch between pathogen recognition and the adaptive T cell response. Depending on microbial and tissue-derived factors, DCs can stimulate the activation and expansion of a variety of T cells. Therefore selectively polarizing T helper 1 (Th1) versus T helper 2 (Th2) responses, or inducing T cell tolerance, the outcome depends on differential production of cytokines or alterations in the expression of surface molecules (Lanzavecchia and Sallusto, 2001). Production of cytokines IL-12, IL-23 and type I IFN by DCs polarizes T cells towards Th1 differentiation (Kadowaki et al., 2000; Trinchieri, 2003). Th1 response is generally associated with response to intracellular microbes, such as viruses and certain bacteria (Pulendran et al., 2001). Th2 cell polarization occurs in the presence of factors such as monocytic chemotactic protein.
1, OX40 ligand or the regulatory T cell-polarizing factors IL-10 and transforming growth factor β (TGF-β) (Kapsenberg, 2003). Th2 polarization is induced by extracellular pathogens such as helminthes (Pulendran et al., 2001). In terms of cytokine output, Th1 cells produce mainly IFNγ, while Th2 cells characterize for producing IL-4, IL-5, and IL-13 (Lanzavecchia and Sallusto, 2001).

The complexity of DCs is much greater than depicted here. DCs are a heterogeneous group composed of several phenotypically distinct subsets located in distinct microenvironments (Banchereau et al., 2000; Pulendran et al., 2001; Shortman and Liu, 2002). This raises the question if each subset has distinct functions. In fact there is evidence for functional specialization but also hints for functional plasticity of a given DC subset. DC subsets reveal intrinsic differences in their ability to: 1) regulate the quality of the Th response (Th1, Th2, or cytotoxic lymphocytes); 2) produce antiviral type I IFNs; and 3) cross-present exogenous antigens to CD8+ T cells (Pulendran, 2005). Furthermore, in terms of complexity the nature of the microbial stimulus also exerts great influence on the outcome of the immune response (Kapsenberg, 2003).

### 1.2) Antigen presentation

The capacity to uptake and process antigens by immature DCs is transiently increased by maturation signals. Microbial detection leads to an initial up-regulation of antigen sampling followed by a rapid shutdown of this function and transition to the mature immunostimulatory state (Pierre et al., 1997; Inaba et al., 2000; Trombetta et al., 2003; West et al., 2004; Faure-André et al., 2008). Internalized antigens are processed into peptides loaded onto MHC class I or MHC class II molecules, and presented at the cell surface to CD8+ and CD4+ T cells, respectively (Trombetta and Mellman, 2005).

The presented peptides originate from different sources. An approximation says that intracellular antigens are presented on MHC class I molecules and exogenous for MHC class II molecules but in reality the systems are much less strict (Trombetta and Mellman, 2005).
1.2.1) Cross presentation

To enlarge the spectrum of presented peptides/T cell responses, exogenous peptides can also be presented on MHC class I molecules to CD8+ T cells by a process termed “cross-presentation”. Cross-presentation occurs with greater efficiency in DCs. In order for cross-presentation to take place antigens need to egress from the endocytic compartment to the cytosol. The exit can be achieved by way of specialized channels or by intersection between the endocytic or phagocytic pathways with the ER (Trombetta and Mellman, 2005). To offer one more exception to the general “rule”, endogenous proteins can also be presented by MHC class II molecules when these proteins are degraded by autophagy, a process of recycling of cellular products with a growing panel of associated functions (Münz, 2010; Crotzer and Blum, 2010).

1.2.2) MHC class I presentation

Endogenous proteins destined for presentation on MHC class I molecules are ubiquitinated in the cytosol and directed to the proteasome where the process of fragmentation begins (Rock et al., 1994). The proteins used for presentation have their origin on proteins which are at the end of their functional lives. Defective ribosomal products (DRiPs) are also a source of proteasomal substrates, 30% to 70% of all proteins made are immediately degraded after synthesis before forming functional proteins (Schubert et al., 2000; Reits et al., 2000). Proteosome-processed peptides are further trimmed by aminopeptidases either in the cytoplasm or the ER, and transported to the ER lumen, where the loading of the MHC molecules occurs, by transporter for antigen processing (TAP). Successfully loaded MHC class I are sent to the cell surface through the standard secretory pathway (Yewdell et al., 2003; Trombetta and Mellman, 2005). Conversely, peptides and MHC class I molecules that fail to associate in the ER are returned to the cytosol for degradation (Neefjes et al., 2011).

In DCs, DRiPs can accumulate transiently as aggregates in the cytosol during maturation, in structures known as dendritic cell aggresome-like induced structures (DALIS). DALIS are also a hallmark of DC maturation. Additionally they are believed to affect the repertoire of endogenous proteins available for MHC class I presentation (Lelouard et al., 2002; Wenger et al., 2012).
1.2.3) MHC class II presentation

MHC class II molecules are mainly expressed by professional APCs, such as DCs, macrophages and B cells. MHC II molecules assemble in the ER and associate with an invariant chain (Ii). This complex is then transported to a late endosomal compartment. Here, Ii is digested, leaving a residual class II-associated Ii peptide (CLIP) in the peptide-binding groove of the MHC class II heterodimer. CLIP is later exchanged by a protein degraded in the endosomal pathway. MHC class II molecules are then transported to the plasma membrane to present their peptide cargo to CD4+ T cells (Trombetta and Mellman, 2005).

1.3) Innate Immunity

Innate immunity recognition depends on a number of receptors widely distributed in or on a variety of cell types. These receptors, known as pattern recognition receptors (PRRs), act like a molecular switch to trigger innate immune activation and tightly regulate the subsequent adaptive immune responses to microbial infections (Medzhitov, 2007). Each receptor has a broad specificity and can potentially bind to a large number of molecules that have a common structural motif or pattern. The microbe-specific molecules are often referred to as pathogen-associated molecular patterns (PAMPs). PAMPs are often components of the cell wall, such as lipopolysaccharide, peptidoglycan, lipoteichoic acids and cell-wall, for bacteria. Another important PAMP is β-glucan which is a component of fungal cell walls (Medzhitov, 2007). PAMPs also include viral DNA, double-stranded RNA (dsRNA) and single-stranded RNA (ssRNA). Discrimination between self (host) and viral nucleic acids also poses an important challenge. These two types of nucleic acids can be distinguished on the basis of specific chemical modifications and structural features that are unique to viral RNA and DNA. The cellular compartments where viral (but not host-derived) nucleic acids are normally found also contribute to the distinction (Medzhitov, 2007; Pichlmair and Reis e Sousa, 2007).
1.3.1) Innate immunity receptors

A large variety of PRRs sensing microbial infections has been identified and characterized. In common they share the feature of being germline-encoded and consisting of a recognition domain and a protein-protein-interacting region for downstream signaling (Ishii et al., 2008).

PRRs can be sorted into different classes: C-type lectin receptors (CLRs), (NOD)-like receptors (NLRs), retinoic acid-inducible gene I (RIG-I)-like receptors (RLRs) and Toll-like receptors (TLRs). TLRs can sense microbial products such as bacterial cell wall components at the plasma membrane, viral proteins at the level of the cell surface or microbial nucleic acids in endosomes and/or phagosomes (Kawai and Akira, 2011). RLRs and NLRs reside in the cytosol serving as sensors for intracellular microbial invasion. Curiously, ligands for RLRs and NLRs are often shared with TLRs (Ishii et al., 2008). CLRs comprise a heterogeneous group of transmembrane proteins. Many of them are expressed in myeloid cells and signal in response to pathogen or self-ligands to initiate or regulate cell activation (Osorio and Reis E Sousa, 2011).

1.3.2) Toll-like receptors

Toll-like receptors are the best characterized class of microbial receptors. TLRs are evolutionarily conserved from the worm Caenorhabditis elegans to mammals (Akira and Takeda, 2004). The discovery of the TLR family began with the discovery of Toll, initially identified as a gene product essential for the development of embryonic dorsoventral polarity in Drosophila (Hashimoto et al., 1988). Later, it was shown to play also a critical role in the antifungal response of flies (Lemaitre et al., 1996).

To date, 10 and 12 functional TLRs have been identified in human and mouse, respectively (Kawai and Akira, 2011). TLRs are type I integral membrane glycoproteins characterized by the extracellular domains containing varying numbers of leucine-rich-repeat (LRR) motifs. For downstream signaling, TLRs have a cytoplasmic signaling domain homologous to that of the interleukin 1 receptor (IL-1R), termed the Toll/IL-1R homology (TIR) domain (Bowie and O’Neill, 2000). TLRs are able to recognize viral nucleic acids and several bacterial products, including lipopolysaccharide and
lipoteichoic acids (Akira et al., 2006). These microbial receptors are expressed on various immune cells, including macrophages, dendritic cells (DCs), B cells, specific types of T cells, and even on non-immune cells such as fibroblasts and epithelial cells (Akira et al., 2006). Some TLRs are expressed at the surface of the cell as is the case for TLRs 1, 2, 4, 5, and 6, while other are found almost exclusively in the endosomal compartment: TLRs 3, 7, 8, and 9 (Akira et al., 2006).

TLRs are known to elicit inflammatory and antimicrobial responses after engagement with microbial ligands. Binding of TLR ligand recruits adaptor proteins, such as myeloid differentiation factor 88 (MyD88), or TIR-domain-containing adapter-inducing interferon-β (TRIF) to the cytoplasmic portion of the TLRs through interaction of their TIR domains. This association ultimately triggers downstream signaling cascades that culminate in production of pro-inflammatory cytokines and chemokines (Medzhitov, 2007).

1.3.3) Recognition of viruses in the cytoplasm

Viruses are non-living organisms and hence their replication is dependent on host cells. In order to do so viruses have develop numerous strategies for interacting with the host’s machineries. In turn, the host immune system has evolved to detect and interfere with such infections.

Many viruses carry out their entire infectious cycle in the cytosol. Accordingly, cells are equipped with cytoplasmic receptors, recognizing viral components able to induce signaling cascades that culminate with interferon genes induction (Pichlmair and Reis e Sousa, 2007). Double-stranded RNA (dsRNA) is the major viral signature. Long dsRNA molecules are absent from uninfected cells but can be formed during the course of an infection with an RNA virus. dsRNA is generated as an intermediate product of replication in an infected cell. Bidirectional transcription from dsDNA viral genomes also leads to abundant levels of dsRNA in the cytoplasm (Beutler et al., 2007; Ishii et al., 2008; Pichlmair and Reis e Sousa, 2007). Other viral RNA motives can be recognized besides dsRNA. RNAs from some viruses are 5’-triphosphorylated and uncapped, whereas host mRNA is capped to prevent recognition by the innate immune system (Rose, 1975; Faust et al., 1975; Moss et al., 1978).
Detection of viruses or viral intermediate products triggers an antiviral response where infected and other immune cells to produce antiviral effector molecules, including proinflammatory cytokines and type I IFNs (Pichlmair and Reis e Sousa, 2007).

Viral RNA in the cytosol is detected by RNA helicases of the family retinoic acid-inducible gene I (RIG-I)-like receptors (RLRs). RLRs comprises a family of receptors in which we can find the melanoma differentiation-associated gene 5 (MDA5), LGP2 (laboratory of genetics and physiology-2) along with the founding member retinoic-acid-inducible gene I (RIG-I) (Beutler et al., 2007; Pichlmair and Reis e Sousa, 2007; Ishii et al., 2008). The members of this family have C-terminal DEXD/H-box RNA-helicase domain, and caspase-recruitment domains (CARDs), except LGP2 (Kang et al., 2002; Yoneyama et al., 2005). RLRs interact with dsRNAs through the helicase domain, and the CARDs are responsible for triggering downstream signaling cascades. Due to the absence of CARD in LGP2, this helicase has been suggested to act as a negative regulator of RIG-I and MDA5 signaling (Rothenfusser et al., 2005; Yoneyama et al., 2005; Pippig et al., 2009).

RIG-I and MDA5 recognize different types of viral RNA. RIG-I binds and mediates innate immune activation through the 5’-triphosphate of ssRNAs and in vitro transcribed dsRNAs. While MDA5 preferentially recognizes longer dsRNAs, including the synthetic mimic poly I:C (polynosinic:polycytidylic acid) (Diebold et al., 2003; Gitlin et al., 2006; Hornung et al., 2006; Pichlmair et al., 2006; Kato et al., 2006). The different affinity of RIG-I and MDA5 for viral motives determines distinctive recognition of RNA viruses. RIG-I is essential for production of interferon in response to RNA viruses including paramyxoviruses, influenza virus and Japanese encephalitis virus (Kato et al., 2006). In contrast, MDA5 is critical for picornavirus detection. Moreover, studies with both RIG-I and MDA5-deficient mice showed that these animals are highly susceptible to infection with the respective RNA viruses (Kato et al., 2006; Gitlin et al., 2006).
1.3.4) IPS-1 signaling

Figure 1: Schematic representation of the IPS1 signaling (adapted from Kawai and Akira, 2006; and Beutler et al., 2007).

The binding of ligands recognized by MDA5 or RIG-I trigger a signaling cascade that culminates with production of interferon. It all starts with the recruitment of the adaptor protein IFN-β-promoter stimulator 1 (IPS-1) by the CARD domain of RIG-I or MDA5. IPS-1 also contains a N-terminal CARD which forms homotypic interactions with the CARDs of RIG-I and MDA5 (Xu et al., 2005; Seth et al., 2005; Kawai et al., 2005). The adaptor IPS-1 is attached to the outer mitochondrial membrane (Seth et al., 2005), which is key for cytokines production as IPS-1-deficient mice are defective in producing type I IFNs and pro-inflammatory cytokines in response to RNA viruses recognized by either RIG-I or MDA5 (Sun et al., 2006; Kumar et al., 2006). Downstream of IPS-1, TRAF3 (TNF receptor-associated factor 3) has been shown to be important for the production of IFNs. The C-terminal TRAF domain of TRAF3 associates with a TRAF-binding domain present in IPS-1. The signal is then relayed to the kinases TBK1 and IKKi which phosphorylate the transcription factors: interferon-regulatory factor 3 (IRF3) and/or IRF7, whose activity is essential for type I interferon genes expression (Kawai and Akira, 2006; Pichlmair and Reis e Sousa, 2007; Ishii et al., 2008).
1.3.5) Virus Sensing in Endosomes

Most viruses make use of the endocytic pathway, either using acidic endosomes to infect cells or because they bud into those compartments after the completion of their replication cycle (Brandenburg and Zhuang, 2007). Accordingly, a subset of TLRs is dedicated to surveying endosomes for viral presence. Their expression can be increased by IFNα and β, which can also up-regulate the RLRs (Pichlmair and Reis e Sousa, 2007). The endosomal subset of TLRs includes TLR3, TLR7, TLR8 and TLR9, which have as common ground the fact of being activated by nucleic acid. TLR9 can be activated by ssDNA oligonucleotides containing unmethylated CpG motifs (Latz et al., 2004). While the remaining are dedicated to RNA detection, TLR3 recognizes dsRNA, whereas TLR7 and TLR8 recognize genomic ssRNA (Ishii et al., 2008). TLR7 and 9 are highly expressed by DCs, in humans they are mainly expressed in the plasmacytoid DC subset but can also be expressed by other hematopoietic cells, such as B cells (Iwasaki and Medzhitov, 2004).

TLR3 has a wider distribution range including non-hematopoietic cells, where they can also be expressed at the plasma membrane (Matsumoto et al., 2002). However, it shows preferential expression in non-plasmacytoid (conventional) DCs (cDCs) (Iwasaki and Medzhitov, 2004). TLR3 recognizes dsRNA and its synthetic analogue poly I:C, such recognition activates the IFNβ promoter (Alexopoulou et al., 2001). Additionally, poly I:C-induced IL-12 production in vivo also depends on the presence of TLR3 (Alexopoulou et al., 2001). TLR3 has also been also implicated in the recognition by DCs of phagocytosed virus-infected cells containing dsRNA (Schulz et al., 2005). Noteworthy, the multitude of virus sensing pathways ends up creating redundancy; as a matter of fact, TLR3-deficient mice do not show increased susceptibility to infection with vesicular stomatitis virus (VSV), Lymphocytic choriomeningitis virus (LCMV), reovirus or Murine cytomegalovirus (MCMV) (Edelmann et al., 2004; Tabeta et al., 2004; Johansson et al., 2007). This redundancy has also been observed in humans, a young male patient carrying an autosomal recessive form of complete TLR3 deficiency, developed herpes-simplex-virus-1-induced encephalitis in childhood. But remained normally resistant to other infections, as various TLR3-deficient leukocytes from the patient, responded normally to synthetic dsRNA (poly I:C) and HSV-1, with the induction of antiviral IFN production (Guo et al., 2011).
1.3.6) TLR3 downstream signaling

The engagement of TLRs by PAMPs triggers the activation of signaling cascades involving binding of different adaptor molecules to the cytoplasmic tail of the TLRs. The usage of different adaptor molecules determines the diversity of host defense responses.

Following recognition of poly I:C by TLR3, a signaling cascade is initiated through the adaptor protein TRIF (TIR-domain-containing adapter-inducing interferon-β) (Yamamoto et al., 2003). To allow downstream signaling, a variety of molecules associate with TRIF including tumor-necrosis factor (TNF)-receptor-associated factor 3 (TRAF3), TRAF6 and receptor-interacting protein 1 (RIP1). TRAF6 and RIP1 activate the transcription factor, nuclear factor-κB (NFκB) (Meylan et al., 2004; Sato et al., 2003). TRAF3 participates in the induction of production type I IFNs (Hacker et al., 2006; Oganesyan et al., 2006), by recruiting and activating two IKK (inhibitor of NF-κB (IκB) kinase)-related kinases, TBK1 (TANK-binding kinase 1) and IKKi (inducible IKK). TBK1 and IKKi are also activated downstream of RLRs, indicating that the signaling pathways that are triggered by TLR stimulation and RLRs converge at the level of TBK1 and IKKi. Phosphorylation of IRF3 and/or IRF7 by these kinases induces the formation of homodimers. These dimers translocate into the nucleus and there play a
fundamental role in the induction of type I IFNs and other IFN-inducible genes, by binding to the IFN-stimulated response elements (ISREs) (Honda and Taniguchi, 2006).

1.3.7) Interferon

IFN constitutes the main weapon to fight viral infection. Induction of IFN production is triggered by pattern recognition receptors. Multiple cytokines are induced by virus infection, including interleukin 6 (IL-6), IL-12, and tumor necrosis factor (TNF), but the first line of antiviral defense is the production of IFN (Stetson and Medzhitov, 2006). This antiviral cytokine has been discovered in 50’s by Isaacs and Lindenmann. These authors found that influenza virus-infected chick cells produced a secreted factor that mediated the transfer of a virus-resistant state against different types of viruses (Isaacs and Lindenmann, 1957). The importance of IFN is further evidenced by its clinical uses, IFNα2 is used therapeutically to treat various forms of cancer such as leukemia and T cell lymphoma, and also hepatitis B and C (Platanias, 2005).

To date, three classes of IFN have been characterized: types I, II, III. The different classes are organized according to the receptor complex they signal through. The type II IFN has one only member, IFNγ, which binds the IFNγ receptor (IFNGR) complex, and mediates broad immune responses to pathogens other than viruses (Sadler and Williams, 2008). The discovery of type III IFN is more recent and little is known about them, nevertheless it has been reported that they are also involved in response to viruses. Type III IFN have additionally been proposed to be the ancestral type I IFNs (Levraud et al., 2007).

Type I IFNs are produced in response to viral infections and have as most relevant members IFNα, which includes 13 genes, and IFNβ, but also include IFNκ, IFNε, IFNo, IFNτ and IFNδ. The type I class of IFNs signals through IFNAR complex (Samuel, 2001; Sadler and Williams, 2008). The IFN response is fundamental to fight viral infections as IFNAR-deficient mice have increased susceptibility to a number of viruses. However these mice maintain resistance to other microbial pathogens (O’Connell et al., 2004). IFN production results in autocrine and/or paracrine IFN-mediated signaling, conferring protection against viruses on the infected cell and neighboring cells.
Binding of IFN I to its receptor induces a signaling cascade leading to the phosphorylation of members of the Janus kinase (JAK) family, and signal transducers and activators of transcription (STATs). This signaling results in amplification of IFN response as it stimulates its own synthesis. Activated STATs dimerize and translocate to the nucleus to induce the expression of more than 300 IFN-stimulated genes (ISGs) (Der et al., 1998; Stark, 2007). The IFN regulatory factor (IRF) family of transcriptional regulators, like the STATs, are important regulatory factors in the IFN response (Samuel, 2001). This family of transcription factors includes nine known members: IRF1 to 9. All share homology in their N-terminal, which is precisely the DNA binding domain. The IRFs and STATs can function together to establish the signal transduction and gene regulation events required for the antiviral response (Samuel, 2001). IRF3 and IRF7 have an important role in type I IFN production. These transcription factors can form homo or heterodimers. Mice lacking IRF3 are more susceptible to viral infections, and IFNα/β gene expression reduced in embryonic fibroblasts upon infection. The profile of IFNα mRNA subspecies is also altered in such circumstance. Moreover when both IRF3 and 7 are defective, cells completely fail completely to induce IFN genes in response to different viruses (Sato et al., 2000).

1.3.8) Interferon-induced genes

Among the plethora of targets induced by the IFN response, many encode pattern-recognition receptors and associated signaling modulators. Transcription factors are also up-regulated, being important to form an amplification loop resulting in increased IFN response which helps contain viral spread. But is also a group of ISGs encoding proteins which have a direct antiviral activity (Sadler and Williams, 2008). IFN-induced proteins with potential for direct antiviral activity include 2’-5’ oligoadenylate synthetases (OAS), ribonuclease L (RNaseL), GTPase Mx1 (myxovirus resistance 1), and also protein kinase R (PKR). These effector proteins are known to suppress various steps of viral replication. Mice with mutations or deficiencies in key steps of the pathways that are triggered by these proteins have increased susceptibility to viral infection. Other proteins with reported antiviral effect have been reported,
however proper confirmation with knockout mouse models is needed (Sadler and Williams, 2008; Samuel, 2001).

1.3.9) Protein kinase R (PKR)

PKR is a cytosolic sensor of dsRNA constitutively expressed in all tissues and up-regulated by type I (Clemens et al., 1993) and type III IFNs (Ank et al., 2006). In IFN-treated cells PKR is found predominantly in the cytoplasm and associated with ribosomes (Samuel, 2001).

PKR is peculiar compared with other PRRs for being a serine/threonine kinase. At steady state, PKR is an inactive monomer and becomes a homodimer upon dsRNA binding. dsRNA binds to the N-terminal regulatory region and induces a conformational change which frees the C-terminal kinase domain. Kinase activation is associated with the formation of a stable PKR/dsRNA complex that requires 30 to 50 bp of duplex RNA and is optimal with about 80 bp (Manche et al., 1992). The RNA-dependent dimerization of PKR involves autophosphorylation which can occur by either intra or intermolecular mechanisms. Multiples sites for autophosphorylation exist within PKR, predominantly serine residues but also threonine residues (Samuel, 2001; Williams, 2001; Sadler and Williams, 2008).

When activated PKR phosphorylates the α subunit of the eukaryotic protein synthesis initiation factor 2 (eIF2α) (Williams, 2001). PKR constitutes therefore an important link between pathogen detection and control of translation. Alternative PKR targets have been suggested such as the transcription factor inhibitor IκB (Kumar et al., 1994) and B56α, the regulatory subunit of protein phosphatase 2A (PP2A) (Williams, 2001).

PKR plays an important role in fighting viral infection since several viruses have mechanisms to subvert its functions PKR (Langland et al., 2006). PKR is able to recognize both synthetic (poly I:C) and natural dsRNA (e.g. reovirus genome dsRNA) with no sequence specificity known for the interactions to take place (Samuel, 2001). Certain highly structured single-stranded viral RNA species such as HIV TAR RNA, reovirus s1 mRNA, and hepatitis delta virus RNA can also activate PKR (Circle et al., 1997).
PKR has been implicated in type I IFN production by fibroblasts in response to exogenously added dsRNA (Visvanathan and Goodbourn, 1989; Yang et al., 1995). Additionally, experiments using PKR-deficient mouse embryonic fibroblasts show that PKR is involved in protection against infection with several RNA viruses, including HCV, Sindbis virus, encephalomyocarditis virus, and foot-and-mouth disease virus, as well as some DNA viruses like HSV.1 (Sadler and Williams, 2008). In vivo, PKR-impaired mice show impaired antiviral responses and increased susceptibility to viruses, such as vesicular stomatitis virus (VSV), influenza virus and bunyawera virus (Balachandran et al., 2000; Stojdl et al., 2000; Bergmann et al., 2000).

### 1.4) Integrated stress response

![Diagram](image)

Figure 3: phosphorylation of eIF2α leads to proteins translation arrest. The eIF2α kinases (PKR, PERK, HRI and GCN2) are activated by different stress signals and phosphorylate eIF2α which leads to protein translation arrest and synthesis of stress-response molecules.

As it has been mentioned, PKR can phosphorylate the α subunit of the eukaryotic protein synthesis initiation factor 2 (eIF2α) at serine 51, consequently leading to protein translation shutdown. This constitutes a mechanism of protein translation control which is conserved throughout eukaryotes, from budding yeast to higher mammals (Proud, 2005). Modulation of eIF2α activity and hence control of protein translation occurs in response to a wide range of stress conditions (e.g. ultraviolet irradiation, heat shock, nutrient deprivation, oxidative stress, endoplasmatic reticulum stress). Mammalian cells possess four different eIF2α kinases which are associated with recognition of different kinds of stress (Holcik and Sonenberg, 2005;
Proud, 2005; Wek et al., 2006): Heme-regulated inhibitor (HRI), GCN2 (general control non-derepressible 2), PKR and PERK (PKR-like ER kinase). HRI is activated under conditions of low haem, treatment with arsenite, osmotic or heat shock (Han et al., 2001). GCN2 is activated in response to amino-acid starvation and UV irradiation (Berlanga et al., 1999; Deng et al., 2002), GCN2 has also been reported to have an antiviral effect against RNA viruses (Berlanga et al., 2006; Won et al., 2012). PKR is activated by double-stranded RNA (Williams, 2001), whereas PERK is activated in response to ER stress (Harding et al., 2000b). These kinases share homology in their kinase catalytic domains, but their effector domains are distinct and are subject to different regulatory mechanisms (Proud, 2005; Holcik and Sonenberg, 2005).

Since all the kinases phosphorylate a common substrate, their activation leads to similar effects within cells, hence it has been termed integrated stress response (ISR) (Harding et al., 2003). The control of proteins translation through eIF2α phosphorylation is an important adaptation protecting cells against potentially toxic malfolded or modified proteins that may accumulate under stress conditions (Brostrom and Brostrom, 1998). Globally eIF2α phosphorylation induces a set of transcriptional regulators which directly or indirectly regulate the expression of a large number of genes involved in metabolism and amino acid transport; the redox status of the cell; signaling and transcription; and apoptosis (Harding et al., 2003; Wek et al., 2006).

1.4.1) Protein translation initiation

![Schematic representation of protein translation initiation](adapted from Proud, 2005)

**Figure 4:** Schematic representation of protein translation initiation (adapted from Proud, 2005)
Here the process of protein translation initiation will be briefly described in order to give a better understanding of the eIF2α-phosphorylation implications. Protein translation is divided into three phases: initiation, elongation and termination. Although all three phases are subject to regulatory mechanisms, under most circumstances it is initiation that is regulated, the rate-limiting step (Holcik and Sonenberg, 2005).

The α subunit of the eukaryotic protein synthesis initiation factor 2 (eIF2α) is part of the heterotrimeric complex eIF2 which binds guanine nucleotides and in its GTP-bound form also interacts with methionyl-initiator tRNA (Met-tRNAi). The eIF2-GTP-Met-tRNAi complex associates with the 40S ribosomal subunit forming the 43S pre-initiation complex. This multimeric complex scans the mRNA in search of the start codon (AUG) (Holcik and Sonenberg, 2005). When this point is achieved, the 60S subunit of the ribosome joins in to initiate translation. Following recognition of the start codon by the anticodon of the tRNA, GTP is hydrolyzed to GDP and Pi – a process mediated by eIF5, a GTPase-activator protein (Das et al., 2001; Paulin et al., 2001). Subsequently, the resulting eIF2-GDP complex leaves the ribosome. Protein translation is a complex process mediated by many factors, eIF5 interacts with other factors, such as eIF4G and eIF1, which plays an important role in start-site selection (Asano et al., 2000, 2001). eIF5 also interacts with the multimeric protein eIF3 which recruits the 40S subunit to the mRNA (Bandyopadhyay and Maitra, 1999).

In order for eIF2 to return to its GTP-bound state, eIF2 must undergo nucleotide exchange – a function executed by eIF2B which promotes release of GDP from eIF2 (Williams et al., 2001). GTP exchange can be inhibited by phospho-eIF2α which is a potent competitive inhibitor of eIF2B (Rowlands et al., 1988). Phosphorylation of eIF2α thus inhibits recycling of eIF2 to its active GTP-bound form, reducing the levels of eIF2–GTP. Lower levels of active eIF2 result in lower levels of translation initiation (Proud, 2005; Holcik and Sonenberg, 2005).

1.4.2) Phospho-eIF2α-induced translation

Exposure of cells to stress elicits adaptive responses that involve the coordinated expression of stress-response genes which affect cell survival, apoptosis, cell-cycle progression and differentiation (Pearce and Humphrey, 2001). Regardless of the type of
stimuli activation of the eIF2α kinases, eIF2α phosphorylation changes the translational pattern of the host cell and a common set of target genes are activated (Wek et al., 2006; Proud, 2005; Holcik and Sonenberg, 2005).

The first and best studied example of selective translation by the phosphorylation of eIF2α is given by the yeast transcription activator GCN4 (Dever et al., 1992). GCN4 enhances the expression of genes for proteins involved in amino acid biosynthesis. In response to amino acid starvation, translation of GCN4 mRNA is enhanced leading to activation of amino acid biosynthesis allowing cells to synthetize their own amino acids (Dever et al., 1992; Hinnebusch, 1984). The 5’UTR of GCN4 contains four upstream open reading frames (uORFs), each endowed with its own start and stop codons (Mueller and Hinnebusch, 1986). In resume, uORFs are short reading frames located in the 5’UTR of some mRNAs, which can either code for short peptides or be non-coding. These frames have an important role in the translational control of GCN4 mRNA. When the levels of phospho-eIF2α are low, ribosome initiates at uORF1 and then reinitiates at the next uORFs but after termination is unable to properly reinitiate so translation ends before reaching the main start codon. Thus GCN4 is not made. Contrariwise, at high levels of phospho-eIF2α (low levels of amino acids), it takes longer for the ribosomes that have already translated uORF1 to acquire active eIF2 (GTP-bound). As a consequence some 40S subunits scan through the uORFs that follow and end up reaching the main GCN4 start codon when they acquire active eIF2. Therefore the frequency of initiation of translation of the Gcn4 polypeptide is enhanced (Holcik and Sonenberg, 2005; Proud, 2005; Kozak, 2002). In mammals the activating transcription factor 4 (ATF4) has a key function in the expression of response target genes during the ISR (Lu et al., 2004). The relevance of ATF4 will be mentioned throughout the next sections.
1.5) The unfolded stress response

The majority of secreted and transmembrane proteins folds and matures in the lumen of the endoplasmic reticulum (ER). Depending on different internal and external cues, such as developmental programs, environmental conditions and the physiological state of the cell, cells adapt the flux of proteins entering the ER as well as the rate of protein folding. This process of re-adjustment is known as the unfolded protein response (UPR) and is necessary to ensure the quality of cell-surface and secreted proteins (Ron and Walter, 2007). The UPR controls the levels of molecular chaperones and enzymes involved in protein folding in the endoplasmic reticulum. When ER stress is sensed, i.e. the consequence of a mismatch between the load of unfolded/misfolded proteins, and the capacity of the cellular machinery that copes with it; a transcription program encompassing genes encoding molecular chaperones and folding enzymes localized in the ER is initiated (Schröder and Kaufman, 2005).

Without the UPR, both yeast cells and mammalian cells would be unable to survive under ER stress conditions arriving from increased protein synthesis, protein misfolding rates that exceed the capacity of available chaperones, alterations in calcium stores in the ER lumen, oxidative stress and disturbances to the redox balance in the ER lumen (Ron and Walter, 2007).

In multicellular eukaryotes, three major signaling branches sense and respond to ER stress. The main receptors of the UPR are: the transmembrane ER-resident proteins:
IRE1 (inositol requiring enzyme 1); PERK [double-stranded RNA-activated protein kinase (PKR)–like ER kinase]; and ATF6 (activating transcription factor 6) (Walter and Ron, 2011). When these ER stress sensors are activated, a signaling cascade is triggered aiming to restore homeostasis. All these three branches of the UPR are based on an integral membrane protein that senses the protein folding status in the ER lumen and transmits this information across the ER membrane to the cytoplasm. Where the downstream mechanisms of the different branches of the UPR interact with the transcriptional or translational machineries (Schröder and Kaufman, 2005).

The three branches of the UPR function in parallel and have distinct signal transduction mechanisms (Walter and Ron, 2011). Activation of each branch leads to the production of basic leucine zipper transcription (bZIP) factors, which may work alone or together to activate UPR target genes (Wek et al., 2006; Ron and Walter, 2007).

1.5.1) IRE1 (inositol requiring enzyme 1)

IRE1 works both as a kinase and as a highly specific endoribonuclease. IRE1 is the most evolutionary conserved component of the UPR, being the only one present in lower eukaryotes (Mori, 2009). In mammals, this ER stress sensor is a type 1 ER-resident transmembrane protein with a luminal domain and a cytoplasmic portion that contains both a serine/threonine kinase domain and an endoribonuclease C-terminal domain (Wang et al., 1998a). The endoribonuclease function is activated by conformational changes following oligomerization in the ER membrane and autophosphorylation. The activation of IRE1 can be accomplished by direct binding of unfolded proteins (Kimata et al., 2007) or alternatively by release of the abundant ER chaperone immunoglobulin-binding protein (BiP), which binds IRE1 and under unstressed conditions locks it into an inactivated state (Bertolotti et al., 2000).

In metazoans IRE1 cleaves the mRNA encoding for XBP1 (X-box binding protein 1), excising an intron. XBP1 a UPR-specific transcription factor. The exons are then ligated, giving rise to a spliced mRNA that is translated to the active form, XBP1s. The protein originated by the spliced mRNA is more stable (Calfon et al., 2002), whereas the unspliced form represses such gene expression (Tirosh et al., 2006;
Yoshida et al., 2006). The mature form induces the expression of a large number of genes involved in many aspects of the UPR, such as protein synthesis (but not suppression of translation initiation), folding and secretion. Its targets include genes of proteins involved in the ER-associated degradation pathway, ER-located chaperones and isomerases which promote disulphite bond formation (Yoshida et al., 2001; Lee et al., 2003). Moreover, XBP1 has been shown to increase ER biogenesis (Sriburi et al., 2004) and lipid biosynthesis (Lee et al., 2008).

Mouse embryos that lack either IRE1 or XBP-1 perish at a similar early stage of gestation, suggesting that XBP1 mediates critical functions of IRE1 (Reimold et al., 2000; Urano et al., 2000).

Under certain conditions, IRE1 can also degrade and thus block the translation of a wide variety of mRNA species (Hollien and Weissman, 2006).

1.5.2) ATF6 (Activating transcription factor 6)

ATF6 is a transmembrane metazoan-specific ER stress transducer. In unstressed conditions ATF6 associated to the ER chaperone BiP, when ER stress is detected BiP dissociates from ATF6 dictating translocation to the Golgi (Shen et al., 2002). Translocation of ATF6 to the Golgi can also be induced by underglycosylation (Hong et al., 2004). In the Golgi apparatus the constitutively-expressed version with 90 kDa is cleaved into a 50 kDa protein (ATF6f, for fragment) by resident S1P (site 1 protease) and S2P (site 2 protease) (Chen et al., 2002; Haze et al., 1999). After processing, the subcellular localization changes as ATF6f translocates into the nucleus (Haze et al., 1999; Chen et al., 2002). In the nucleus ATF6f, which contains a bZIP domain, activates the transcription of ER chaperone genes such as BiP, a major target protein of the mammalian UPR (Haze et al., 1999) and XBP1 (Wang et al., 2000).

1.5.3) PERK (PKR-like ER kinase)

The third branch of the UPR is governed by PERK, an ER-resident transmembrane kinase. Like IRE1, PERK is an ER-localized type I transmembrane
protein with lumenal stress-sensing domains. The lumenal domains of IRE1 and PERK present a small degree of homology conserved throughout all eukaryotes. Despite only weak sequence similarity in their lumenal domains it has been shown that these domains of IRE1 and PERK are functionally interchangeable in mediating an ER stress response (Bertolotti et al., 2000).

As it has been mentioned earlier in the text, PERK is also part of the integrated stress response, a signaling pathway that senses different stress signals and represses translation by phosphorylating eIF2α. The cytoplasmic projection of PERK contains a protein kinase domain (Harding et al., 1999). Similarly to IRE1 the lumenal of PERK is bound to the chaperone BiP in absence of stress. Under conditions that perturb protein folding in the ER BiP binding is loss and correlates with the formation of high molecular mass complexes of activated PERK (Bertolotti et al., 2000). When activated by ER stress PERK also phosphorylates itself and the translation initiation factor eIF2α, inhibiting global protein translation (Harding et al., 1999, 2003).

Lower levels of translation initiation mean a reduced flux of newly synthesized proteins entering the ER lumen. Although global protein translation is inhibited, PERK-mediated eIF2α phosphorylation activates translation of mRNAs encoding several short upstream open reading frames (uORFs) (Kozak, 2002; Lu et al., 2004). One example is the bZIP (basic leucine zipper) activating transcription factor 4 (ATF4) (Lu et al., 2004). Similarly to what has been described for yeast GCN4, conditions that limit eIF2 activity lead ribosomes to skip the inhibitory uORFs allowing translation of ATF4. ATF4 has two uORFs, the 5’-proximal uORF1 encodes a peptide only three amino acid residues in length, and uORF2 is 59 residues long and overlaps the ATF4 coding region (Schröder and Kaufman, 2005; Wek et al., 2006).

1.5.4) GADD34

In order to allow recovery from ER stress and an efficient response to prolonged ER stress, translational inhibition by PERK is transient (Schröder and Kaufman, 2005; Ron and Walter, 2007). Sustained eIF2α phosphorylation results lethal to cells (Srivastava et al., 1998). Cells must therefore tightly regulate the level of phosphorylated eIF2α in order to survive. The regulatory subunits GADD34 (growth
arrest and DNA-damage inducible protein-34) and CReP (constitutive repressor of eIF2a phosphorylation) when bound to protein phosphatase 1 (PP1) through their homologous C-terminal domains enable dephosphorylation of eIF2α by PP1 (Novoa et al., 2001, 2003; Jousse et al., 2003). CReP is constitutively expressed and contributes to baseline eIF2α dephosphorylation (Jousse et al., 2003). GADD34, also known as MyD116, is a member of a family of GADD genes that are induced by DNA damage, growth factor deprivation, and other forms of cell stress (Zhan et al., 1994; Fornace et al., 1989).

Tunicamycin-induced ER stress and amino acids depletion promotes the expression of GADD34 (Novoa et al., 2001). GADD34 expression is part of the alternative transcription program initiated by eIF2α phosphorylation (Brush et al., 2003; Ma and Hendershot, 2003; Novoa et al., 2003). The expression of GADD34 is dependent on ATF4, an important transcription factor translated during the eIF2α-mediated translational block (Ma and Hendershot, 2003). ATF4 binds to a conserved ATF site in the promoter region of GADD34 in a stress-dependent manner, inducing its transcription. ATF4-deficient cells besides being unable to up-regulate GADD34 in response to ER stress and amino acid deprivation, also revealed prolonged eIF2α phosphorylation, delayed protein synthesis recovery, and diminished translational up-regulation of BiP (an important cytoprotective induced during the UPR) during ER stress (Ma and Hendershot, 2003).

The PP1/GADD34 assembly functions as a negative feedback loop that attenuates signaling in the integrated stress response, promoting recovery from translational inhibition in the unfolded protein response (Novoa et al., 2003).

An apparent paradox of the UPR is the simultaneous activation of both adaptive responses to stress and pro-apoptotic pathways. eIF2α phosphorylation is normally transient and protein translation returns to its baseline state after ER stress has abated. If homeostasis of the ER cannot be re-established, apoptotic pathways are activated to destroy the damaged cell in order to ultimately protect the organism (Nakagawa et al., 2000). The best characterized of these pro-apoptotic pathways is production of the transcription factor C/EBP homologous protein (CHOP also known as GADD153). CHOP is a downstream target of ATF4 (Harding et al., 2000a) and activates the promoter of target genes that are believed to play a role in programmed cell death and tissue regeneration (Zinszner et al., 1998; Wang et al., 1998b). It has also been proposed that CHOP leads to transcriptional repression of the anti-apoptotic BCL-2 protein (Marciniak et al., 2004), and to up-regulation of the death receptor family member DR5.
GADD34 has been found to attenuate CHOP activation by both protein malfolding in the endoplasmic reticulum, and amino acid deprivation (Novoa et al., 2001).

GADD34 and CReP have also been associated to apoptotic signaling downstream of ER stress. Rescuing protein translation in a scenario of ER stress may increase misfolded proteins, which can therefore compromise cell functions. GADD34-deficient cells display persistent eIF2α phosphorylation and fewer misfolded protein aggregates in the ER lumen (Novoa et al., 2003; Marciniak et al., 2004).

Different viruses have been reported to have GADD34-like genes, it is the case of herpes simplex virus (HSV). Its ICP34.5 represents an essential virulence factor of this virus (He et al., 1997). The C-terminus of ICP34.5 contains a region of 84 amino acids that confers PP1 binding and is highly conserved in mammalian GADD34. In infected cells, ICP34.5 associates with PP1 constituting an active eIF2α-dephosphorylation complex, counteracting the action of PKR. This allows the virus to override the host cell-mediated shutoff of protein synthesis and the ensuing programmed cell death (He et al., 1997).

The developed by me and here presented gives a new dimension to GADD34, the connection of the stress-response pathways with the immune response.

![Diagram](image.png)

**Figure 6:** Negative feedback loop of the integrated stress response. Phosphorylation of eIF2α as a consequence of ER stress, or amino acids depletion leads to protein translation arrest and translation of the transcription factor ATF4. ATF4 induces both CHOP and GADD34. GADD34 conjugates with protein phosphatase 1 (PP1) and alleviates the level of eIF2α phosphorylation. Under “normal” conditions the constitutively expressed co-factor, CReP, can associate with PP1 and dephosphorylate eIF2α.
1.6) Amino acid response

In mammals, nutrients may be provided on an irregular basis which obliges cells to adapt their metabolism accordingly. In healthy adult humans, nine amino acids are considered essential (valine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, histidine and tryptophan), this means they cannot be synthesized de novo and have to be supplied by the diet (Jousse et al., 2004). It is therefore important that cells regulate the amino acid metabolism, especially considering that mammals don’t have the capacity to store amino acids. When imbalances occur in consequence of physiological or pathological conditions, a set of genes is regulated enabling cells to become accustomed to low amino acid availability.

Limiting the extracellular supply of amino acids increases uncharged tRNA which bind and activate GCN2 (Berlanga et al., 1999; Sood et al., 2000). Once activated, GCN2 kinase phosphorylates eIF2α leading to a decrease in global protein synthesis (Scorsone et al., 1987). Despite protein translation shutdown, specific genes are up-regulated, a typical feature of the ISR. From the up-regulated genes, a large number encodes plasma membrane amino acid transporters, but also genes encoding transcription factors, ribosomal proteins or proteins involved in the signal transduction processes (Kilberg et al., 2005; Chaveroux et al., 2010). In mammals, increase of eIF2α phosphorylation induced by amino acid starvation increases the expression of the master regulator ATF4 (Harding et al., 2000a) – ATF4 has also been shown to be indispensable for amino acid regulation of most of the genes studied (Kilberg et al., 2005; Chaveroux et al., 2010). CHOP, a downstream target of ATF4 is highly expressed during amino acids starvation (Bruhat et al., 1997; Averous et al., 2004). Another important factor for the induction of CHOP, when there is a limitation of amino acids is ATF2 (Chaveroux et al., 2009). ATF2 is involved in histone modification in order to enhance CHOP and ATF3 transcription in response to amino acid starvation (Bruhat et al., 2007). ATF3 in association with other factors acts as a negative feedback regulator of ATF4 regulated transcription (Chaveroux et al., 2010).

In the promoter regions of amino acid-regulated genes an amino acid response element (AARE) can be found. These regulatory elements are associated with binding of transcription factors and regulatory proteins, mainly related to the C/EBP and ATF/CREB families, which help modulate the rate of transcription in the response to
amino acids starvation (Kilberg et al., 2005). In resume, response to amino acids depletion activates a highly coordinated program of interaction between a defined set of bZIP transcription factors and amino acid response element leading to transcriptional activation of CHOP and other AARE regulated genes (Chaveroux et al., 2010).

The GCN2-ATF4 axis is a major signaling pathway for gene regulation in response to amino acid starvation, however is not the only one (Deval et al., 2009).

1.6.1) Mammalian target of rapamycin (mTOR)

Cells possess another amino acid-sensitive regulatory pathway, mTORC1, which is inhibited by amino acid starvation (Hara et al., 1998; Wang et al., 1998c). The signaling pathway that is centered on the mammalian kinase target of rapamycin (mTOR) is able to sense energy, nutrients and stress as well as growth factors. mTOR serves as an interface between nutrient availability and cell growth and proliferation. This signaling pathway is implicated in disease states where growth is deregulated and homeostasis is compromised, namely cancer, metabolic diseases and ageing. TOR belongs to the phosphoinositide 3-kinase (PI3K)-related protein kinases family, which comprises large proteins that enable adaptation to metabolic, environmental and genetic stresses (Zoncu et al., 2011b).

mTOR is the catalytic subunit of two distinct complexes called mTOR complex 1 (mTORC1) and mTORC2. Unique accessory proteins distinguish these complexes, determining the binding of different substrates and regulators.

Amino acid sufficiency activates the mTOR kinase cascade that ultimately results in phosphorylation of S6 kinase 1 (S6K1) and eIF4E binding protein 1 (4E-BP1) – S6K1 and 4E-BP1 associate with mRNAs and regulate protein translation initiation and progression, thus controlling the global rate of protein synthesis (Ma and Blenis, 2009).

In vertebrates, amino acids may be sensed intracellularly rather than at the plasma membrane (Christie et al., 2002); accordingly, a system of amino acid transporters plays an important part in mTORC1 signaling. This system imports leucine, which is a key amino acid for mTORC1 activation, into the cell (Nicklin et al., 2009). Very recently D. Sabatini’s lab has proposed a model in which amino acids must
accumulate in the lysosomal lumen to initiate signaling (Zoncu et al., 2011a). Amino acids have been described to activate the Rag(A-D) guanosine triphosphatases (GTPases) (Sancak et al., 2008; Kim et al., 2008). These GTPases form heterodimers in which the two members of the heterodimer have opposite nucleotide binding states. In the absence of amino acids, Rag GTPases are found in an inactive conformation. Amino acids cause Rag GTPases to switch into an activated conformation by inversion of the nucleotide loading states. The interaction of the active Rag heterodimer with mTORC1 causes it to cluster onto the surface of late endosomes and lysosomes – the site of mTORC1 activation (Sancak et al., 2008) where the Rag GTPases are also located. The anchoring of the Rag GTPases to the membrane the late endosomal structures is ensured by a scaffolding complex named Regulator (Sancak et al., 2010). The key factor in this signaling model is the vacuolar H\(^+\)–adenosine triphosphatase ATPase (v-ATPase), which engages in extensive amino acid–sensitive interactions with the Regulator. ATP hydrolysis and associated rotation of the v-ATPase is necessary to relay an amino acid signal from the lysosomal lumen to the Rag GTPases and activate mTORC1 (Zoncu et al., 2011a).

1.6.2) Role of GCN2 pathway in the immune system

The GCN2 pathway has also an important role in the regulation of immune processes. Particularly indoleamine 2,3 dioxygenase (IDO), IDO is a tryptophan-degrading enzyme whose expression induces local depletion of tryptophan (Mellor and Munn, 2004; Puccetti and Grohmann, 2007). IDO’s immunoregulatory activity is involved in a variety of pathophysiological conditions including pregnancy, infection, allergy, autoimmunity, chronic inflammation, transplantation and mechanisms for the escape of tumors from the immune system (Mellor & Munn, 2004; Puccetti & Grohmann, 2007).

IDO is basally expressed in tissues such as the gut, lungs, epididymis, brain, and thymus. But its highest expression is found in in professional antigen-presenting cells (APCs) such as DCs (Grohmann et al., 2003; Mellor and Munn, 2004). Inflammation induces counter-regulatory mechanisms which are key to limit potentially dangerous immune responses and reduce collateral tissue damage. IDO is one of several of these
mechanisms. Pro-inflammatory mediators, such as IFNγ induce the expression of IDO in several tissues. Although IFNγ is more effective in inducing IDO, the mouse and human IDO gene promoters contain multiple sequence elements that confer responsiveness to type I interferon (IFNa/β) (Hassanain et al., 1993; Baban et al., 2005; Mellor et al., 2005). Ligation of soluble cytotoxic T-lymphocyte antigen 4 (CTLA4)–immunoglobulin fusion protein was also found to induce IDO expression, through the ligation of cell-surface CD80 and CD86 molecules (Grohmann et al., 2002). IDO activity suppresses adaptive immunity, DCs expressing the tryptophan-catabolizing enzyme are capable of inhibiting T cell proliferation in vitro (Hwu et al., 2000; Munn et al., 1999) and reduce T cell immune responses in vivo (Munn et al., 1998; Mellor et al., 2002; Grohmann et al., 2001).

IDO-expressing plasmacytoid DCs activate the GCN2 kinase pathway in responding T cells, leading to cell cycle arrest, anergy, as well as differentiation in regulatory T cells (Tregs) (Munn et al., 2005). GCN2 acts as a molecular sensor in T cells, allowing them to detect and respond to conditions created by IDO. GCN2-disrupted T cells are not susceptible to IDO-mediated suppression of proliferation in vitro and in vivo (Munn et al., 2005).

Besides depleting local concentration of tryptophan, IDO generates immunosuppressive metabolites of tryptophan called kynurenines. Certain T cells are sensitive to the toxic effects of tryptophan metabolites that can be produced by cells expressing IDO (Fallarino et al., 2002).
RESULTS
Global summary of the results

It now is well established that protein translation is inhibited in cells exposed to different stress conditions such as protein malfolding in the ER, detection of virus or amino acids depletion. In mammals protein synthesis arrest can be mediated through phosphorylation of eIF2α by four different kinases (PERK, PKR, GCN2 and HRI), each one sensing different stress signals (Proud, 2005).

The work I developed was focused in investigating the consequences that different stress signals have on dendritic cells’ function. DCs are equipped with several PRRs and can consequently detect a great diversity of conserved microbial products. Upon microbial sensing DCs begin a maturation process characterized by dramatic functional changes, such as cytokine production and up-regulation of antigen presentation capacity (Banchereau and Steinman, 1998; Mellman and Steinman, 2001; Reis E Sousa, 2006). DCs play also an important role initiating the adaptive immune response (Banchereau and Steinman, 1998).

Double-stranded RNA is one of different typical features of viral replication/viral infection which can be recognized by DCs mainly through: Toll-like receptor 3 (TLR3) (Alexopoulou et al., 2001), or cytosolic RNA DExD/H-box helicases, such as melanoma-associated gene-5 (MDA5) (Gitlin et al., 2006) or the DDX1, DDX21, and DHX36 (Zhang et al., 2011). Engagement with these receptors leads to nuclear translocation of IRF3 and/or IRF7 and induction of type I IFN production.

As a model for dsRNA, poly I:C, a synthetic dsRNA mimic, was used (Grunberg-Manago et al., 1955; Schmidtke and Johnson, 1971). Poly I:C and its poly-L-lysine (poly ICLC) analogue are used as vaccine adjuvants which have proven to be effective in mice and in nonhuman primates (Stahl-Hennig et al., 2009; Longhi et al., 2009). In mice poly I:C is capable of generating a systemic type I IFN response (Longhi et al., 2009), moreover poly ICLC has recently been shown to have the capacity to induce many branches of innate immunity in humans mimicking the responses observed with a live attenuated vaccine of yellow fever virus (Caskey et al., 2011). Poly I:C constitutes a promising vaccine adjuvant and therefore pathways involved on its detection/response deserve to be better studied.
Type I interferon production promotes the up-regulation of a series of genes involved in viral infection control among which we find PKR (Sadler and Williams, 2008). The activation of PKR promotes phosphorylation of eIF2α causing protein translation shutdown and inhibition of viral replication (Donzé et al., 2004; Scheuner et al., 2006). PKR is also necessary for normal type I IFN secretion by DCs in response to dsRNA stimulation (Diebold et al., 2003; Schulz et al., 2010).

The immune response can pose a diversity of stress signals capable of arresting protein translation such as amino acid starvation (e.g. in the tumoral environment, or oxidative bursts after release of reactive oxygen species (ROS) by neutrophils) (Pierre, 2009). Protein translation shutdown may potentially lead to apoptosis, thus compromising the onset of the immune response, which would be deprived of essential factors synthesized in the course of DC maturation. DC stimulation with LPS has a profound effect on the intensity and quality of translation. Translation control is tightly coordinated with the state of DC activation, LPS-stimulated DCs undergo a phase of rapid up-regulation of protein synthesis (Lelouard et al., 2007).

With the dual objective of better understanding the pathways involved in response to dsRNA and the mechanism underlying DC resistance to protein translation shutdown, a transcriptomic analysis was performed in bone marrow-derived DCs (Inaba et al., 1992) stimulated with poly I:C (Clavarino et al., 2012b). The results evidence that protein translation in DCs does not stop in response to poly I:C (Clavarino et al., 2012b). Thus differing from MEFs which lose protein translation after poly I:C lipofection (Clavarino et al., 2012b, 2012a). Our work has also been able to show that DCs mount a specific integrated stress response during which the ISR transcription factor ATF4, and the growth arrest and DNA damage-inducible protein 34 (GADD34/Ppp1r15a), a phosphatase 1 (PP1) cofactor, are expressed.

DCs display an unconventional pattern of eIF2α phosphorylation. Immature DCs (iDCs) possess a high level of phosphorylation that decreases following activation with poly I:C. Such pattern has also been observed in human DCs with LPS activation (Ceppi et al., 2009). The basal level of eIF2α phosphorylation of DCs contrasts with that of murine embryonic fibroblasts which is inexistent at steady state. This represents a very important observation which may in the future help disclose more about the translational regulation of DCs and function (Lelouard et al., 2007; Ceppi et al., 2009). The pattern of eIF2α phosphorylation (P-eIF2α) was confirmed in histological sections
of mouse spleen, where in immunohistochemistry high P-eIF2α was mostly detected in resting CD11c⁺ DCs and not in neighboring CD3⁺ T cells.

The presented work demonstrates that the pattern of P-eIF2α in DCs is inversely correlated with the expression of the PP1 co-factor GADD34 (Clavarino et al., 2012b). The complex PP1/GADD34 has been proven to be necessary to counterbalance eIF2α phosphorylation, either pharmacological inhibition of this complex with salubrinal and guanabenz, or GADD34-inactivated DCs (GADD34ΔC/ΔC) lead to a dramatic increase of phosphorylated eIF2α. It is shown that PKR is the main kinase mediating eIF2α phosphorylating during poly I:C stimulation. However in iDCs other kinases are responsible for the high basal level of eIF2α phosphorylation (Clavarino et al., 2012b), such kinases remain still to be identified. Expression of ATF4 and its downstream targets (Harding et al., 2000) requires eIF2α phosphorylation, however GADD34 expression was not affected in PKR-deficient DCs. Contrary to what happens in DCs, in MEFs the expression of GADD34 was totally dependent on the kinase activity of PKR (Clavarino et al., 2012a). Further characterization of the signaling responsible for GADD34 expression in DCs evidenced a central role for the adaptor TRIF, in response to dsRNA.

Both MEFs and DCs were found to up-regulate GADD34 after stimulation with poly I:C, DCs are able to conserve protein translation intact, and even up-regulate it while MEFs register a dramatic shutdown of protein synthesis (Clavarino et al., 2012a, 2012b). This might be explained by the increasing eIF2α phosphorylation observed in MEFs, a decrease was only observed at late time points. Initially it was hypothesized that GADD34 could account for the protein translation-resistant phenotype of DCs. Yet, when protein translation was monitored in GADD34-deficient DCs, no significant decrease could be observed after poly I:C delivery. Still, GADD34 is absolutely essential to recover from thapsigargin-induced ER stress in iDCs, similar to what has previously been described in MEFs by the Ron’s lab (Novoa et al., 2003).

It was only when we questioned if GADD34 would be involved in cytokine production that we found the most relevant phenotype of GADD34 deficiency until now. In GADD34-deficient DCs IFNβ and IL-6 mRNA are diminished which results in decreased levels of these cytokines in the supernatants (Clavarino et al., 2012b). In fibroblasts a similar phenotype was found, after poly I:C delivery, GADD34ΔC/ΔC MEFs presented no IFNβ or IL-6 in the cell culture supernatants (Clavarino et al., 2012a). Nevertheless no decrease was observed at the level of the transcripts of these cytokines.
GADD34 has hence an important role in cytokine production and according to the cell type, the regulation of GADD34 on cytokines is exercised at different steps: translational regulation in MEFs and transcriptional in DCs. The importance of GADD34 was confirmed in vivo, by injecting mice with poly I:C it was shown that GADD34-deficient animals had lower levels of IFNβ in the serum (Clavarino et al., 2012b).

The relevance of the PP1 co-factor was further evidenced in the context of viral infection. Intradermal injections of CHIKV to WT (FVB) and GADD34\(^{AC/AC}\) mice revealed less capacity of the later to produce type I IFN. GADD34\(^{AC/AC}\) pups displayed significantly less IFNβ in the serum and joints, while more elevated CHIKV titers were found in muscle, joints and heart, which are known to be primarily targeted by the virus. GADD34\(^{AC/AC}\) neonates ended up dying few days after infection with Chikungunya virus. The premature death of GADD34-deficient pups is associated with necrotic myocarditis, as these animal showed signs of severe cardiomyocytes necrosis with inflammatory infiltrates by monocytes/macrophages (Clavarino et al., 2012a).

The last part of the presented work is dedicated to responses to amino acids depletion. Depletion of amino acids from the cells medium causes activation of GCN2 and eIF2α-mediated protein translation stop. GCN2 is involved in nutritional sensing of amino acid levels by the brain (Maurin et al., 2005; Hao et al., 2005), and has been implicated in the sensing of tryptophan levels during IDO-mediated immune regulation (Munn et al., 2005; Fallarino et al., 2006). Given the relationship between depletion of amino acids and the integrated stress response, I investigated the consequence of tryptophan depletion in the context of dendritic cells activation with LPS and poly I:C. Under tryptophan starvation conditions DCs are able to maintain protein synthesis and antigen presentation. Yet many details remain to unravel concerning the mechanism that permits DCs to resist to amino acids depletion.
Published papers
I. **Ppp1r15a/GADD34** regulates cytokines production in polyinosinic:polycytidylic acid stimulated dendritic cells.

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Ppp1r15a/GADD34 regulates cytokines production in polyinosinic:polycytidylic acid stimulated dendritic cells

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Abstract

In response to inflammatory stimulation, dendritic cells (DCs) have a remarkable pattern of differentiation that exhibits specific mechanisms to control the immune response. Here we show that in response to polyriboinosinic:polyribocytidylic acid (pI:C), DCs mount a specific integrated stress response during which the transcription factor ATF4 and the growth arrest and DNA damage-inducible protein 34 (GADD34/Ppp1r15a), a phosphatase 1 (PP1) cofactor, are expressed. In agreement with increased GADD34 levels, an extensive dephosphorylation of the translation initiation factor eIF2α was observed during DC activation. Unexpectedly, although DCs display an unusual resistance to protein synthesis inhibition induced in response to cytosolic dsRNA, GADD34 expression did not have a major impact on protein synthesis. GADD34, however, was shown to be required for normal cytokine production both in vitro and in vivo. These observations have important implications in linking further pathogen detection with the integrated stress response pathways.

Keywords:

type-I interferon | Toll-like receptor | protein kinase RNA activated |
puromycin | unfolded protein response
Introduction

Dendritic cells (DCs) are regulators of the immune response, which, upon stimulation by conserved microbial products (PAMPs), are most efficient at inducing differentiation of naïve T cells through massive cytokine production and optimization of their antigen presentation capacity (1). Double-stranded RNAs (dsRNA), a hallmark of virus replication, is recognized by Toll-like receptor 3 (TLR3) (2) or cytosolic RNA DExD/H-box helicases, such as melanoma-associated gene-5 (MDA5) (3) or the DDX1, DDX21, and DHX36 (4). Detection of pI:C, a dsRNA mimic, promotes the nuclear translocation of IRF-3 and IRF-7 and antiviral type-I IFN production (5). IFN triggers, among many other antiviral genes, the dsRNA-dependent protein kinase (PKR) (6, 7). PKR activation by dsRNA promotes translation initiation factor 2 alpha (eIF2α) phosphorylation leading to protein synthesis shutoff and inhibition of viral replication (8). PKR is also necessary for normal type-I IFN secretion by DCs in response to dsRNA stimulation (9, 10).

Different stress signals trigger eIF2α phosphorylation, thus attenuating mRNA translation and promoting a specific transcriptional response known as the integrated stress response (ISR) (11). To date four eIF2α kinases have been identified, PKR, PKR-like ER kinase (PERK) (12), general control nonderepressible-2 (GCN2) (13), and heme-regulated inhibitor (HRI) (14). PERK, which is activated by an excess of unfolded proteins in the ER lumen (12), is necessary to mount part of a particular ISR, known as the unfolded protein response (UPR). The UPR encompasses a group of signals that cope with perturbations in ER homeostasis. In addition to PERK, the UPR is initiated by additional ER sensors and associated transcription factors such as IRE1, XBP1 and ATF6 (15, 16). In addition to inhibiting global protein synthesis, eIF2α phosphorylation by PERK allows the specific translation of the transcription factor
ATF4 (14). This factor promotes cell adaptation to stress by heightening ER functions through specific mRNA transcription. Immune responses and immune cell development can be profoundly affected by abnormalities in the UPR (17, 18) and TLR-dependent activation of XBP1, via the production of reactive oxygen species (ROS), is required for normal production of pro-inflammatory cytokines in macrophages (19).

We show here that activated DCs resist the translation arrest, normally elicited by the PKR-dependent eIF2α phosphorylation in response to cytosolic dsRNA delivery. Concomitantly, pI:C detection promotes the expression of the inducible phosphatase 1 (PP1)-cofactor, GADD34 (20). GADD34 efficiently dephosphorylates eIF2α in activated DCs, but has little impact on the specific resistance of DCs to dsRNA-triggered translation arrest. Alternatively, GADD34 is required for optimal transcription and production of the cytokines IFN-β and interleukin-6 (IL-6), suggesting that induction of the ATF4-dependent branch of the UPR in DCs is a critical signaling module of the innate response to dsRNA.
Results

DC Stimulation by pI:C Induces the ATF4 Transcription Factor.

To identify signaling pathways involved in dsRNA response, a genomewide expression analysis was performed in pI:C-stimulated mouse bone marrow-derived DCs (bmDCs) using Affymetrix Mouse Genome 430 2.0 arrays. We found that at least nine transcripts, typically expressed in different cells exposed to tunicamycin (11, 21, 22), were also induced in DCs responding to pI:C (Table S1).

Up-regulation of these transcripts was confirmed by quantitative RT-PCR (qPCR) (Fig. 1 and Fig. S1). Among them, the key UPR transcription factors, ATF4 and CCAAT/enhancer binding protein homologous protein (CHOP) mRNAs were increased by two- and eightfold, respectively (Fig. 1 A and C). ATF4 mRNA is normally poorly translated in unstressed cells, but upon eIF2α phosphorylation, a rapid synthesis of the ATF4 protein has been observed (14). In DCs, ATF4 protein levels were increased upon pI:C stimulation and detected in the nuclear extracts after 8 h of stimulation, similarly to control DCs, in which a bona fide UPR was induced with thapsigargin (Fig. 1B and Fig. S2).

Nuclear translocation of ATF4 normally induces CHOP expression, which can trigger apoptosis, and enhance the transcription of several ATF4 target genes such as GADD34 and ERO1-α (22). As expected from the array analysis, increased CHOP mRNA levels were detected (Fig. 1C), however, we failed to visualize protein expression in LPS and pI:C-stimulated DCs extracts. In contrast, CHOP was detected in control tunicamycin-treated DC as well as in LPS-stimulated mouse leukaemic macrophages (RAW 264.7 cell line) (Fig. 1D). We tested different doses and modes of pI:C delivery to DCs and in agreement with the lack of CHOP expression, we found no major apoptotic death induction in our experimental system (Fig. S3A). Thus, pI:C-
activated DCs display an ATF4 transcriptional signature during which, CHOP expression seems to be specifically down-modulated at the translational level, which presumably contributes to the prevention of apoptosis in activated DCs (23).

**GADD34 Is Up-Regulated in Activated DCs.**

GADD34 acts together with PP1 to dephosphorylate eIF2α and relieves translation repression during ER stress (22, 24, 25). In response to soluble pI:C, GADD34 mRNA transcription was enhanced at least 14-fold (Fig. 1E), contrasting with the modest 1.5-fold up-regulation of PP1 expression (Fig. 1G) and 2-fold up-regulation of the “constitutive” protein phosphatase 1 regulatory subunit 15B (CReP) mRNA (Fig. 1H) (26). Matching the transcriptional analysis, and irrespectively of the mode of pI:C delivery (soluble or lipofected), GADD34 protein levels were induced and continuously increased during activation (Fig. 1F). Addition of proteasome inhibitor MG132 during the last 2 h of pI:C treatment facilitated the detection of short-lived GADD34 by preventing its degradation (Fig. 1F) (27) and confirmed GADD34 expression during DC maturation.

**eIF2α Is Dephosphorylated During DC Activation.**

Protein synthesis was monitored using puromycin labeling followed by immunoblot (Fig. 2A) (28). As previously shown for LPS-activated DCs (29), translation was enhanced in the first hours of pI:C stimulation followed by a reduction at 16 h. The levels of phosphorylated eIF2α (P-eIF2α) were gradually lost during pI:C stimulation (Fig. 2B), independently of the mode of dsRNA delivery (Fig. S3B). Levels of P-eIF2α were surprisingly high in nonstimulated DCs compared with mouse embryonic fibroblasts (MEFs), and no up-regulation of P-eIF2α could be visualized.
We further investigated whether these high levels of P-eIF2α were physiologically relevant by analyzing purified mouse spleen CD11c+ DCs. Explanted CD11c+ DCs displayed even higher P-eIF2α levels than nonstimulated bmDCs. This observation was further confirmed by immunohistochemistry of spleen sections, in which high P-eIF2α was mostly detected in resting CD11c+ DCs and not in neighboring CD3+ T cells in situ (Fig. S4). Spleen DCs and nonstimulated bmDCs, therefore, display naturally high levels of P-eIF2α, which might explain their low level of translation in vitro and in vivo and might be important for exerting their sentinel function (29).

Because P-eIF2α intensity was inversely correlated with GADD34 expression in activated cells, we tested the function of PP1-GADD34 and -CReP complexes by inhibiting their activity with the pharmacological inhibitors salubrinal and guanabenz (30, 31). In the presence of both drugs, P-eIF2α levels were enhanced by pl:C treatment (Fig. 2D). The same result was found when using DCs inactivated for GADD34 (GADD34ΔC/ΔC) (25), in which P-eIF2α was considerably augmented upon activation compared with WT cells (Fig. 2E and quantification in Fig. S3C). Thus, GADD34 and PP1 dephosphorylate eIF2α and counteract efficiently the activation of eIF2α kinases in pI:C-stimulated DCs.

**PKR Is Up-Regulated and Phosphorylates eIF2α in pI:C-Activated DCs.**

PKR acts as a signal transducer in the proinflammatory response to different PAMPs through TLRsignaling and direct activation by cytosolic dsRNA. PKR activation results in eIF2α phosphorylation and protein synthesis arrest (7, 32). As expected, PKR, being type I IFN-inducible, was strongly up-regulated upon pl:C stimulation (Figs. 2E and 3 A and B). In nonactivated PKR−/− DCs, P-eIF2α levels
were close to normal, indicating that eIF2α kinases other than PKR are responsible for the high degree of eIF2α phosphorylation in nonstimulated cells (Fig. 3A). Importantly, eIF2α phosphorylation was nearly abolished in activated PKR−/− DCs, indicating that PKR is mostly responsible for this activity after pI:C stimulation (Fig. 3A). Cytosolic dsRNA delivery by lipofection also decreased P-eIF2α levels in maturing cells, being more evident in PKR−/− cells (Fig. 3B). Independently of the mode of pI:C delivery, PKR activity is therefore efficiently counteracted by GADD34 induction. This observation contrasts with previous work on MEFs exposed to cytosolic pI:C or Sindbis virus (33). Moreover, although PKR was shown to promote apoptosis in LPS-stimulated macrophages (34), we found that procaspase 3 cleavage was reduced in response to pI:C, again coinciding with eIF2α dephosphorylation and contrasting with the UPR-inducing drug, thapsigargin, which enhanced both caspase-3 cleavage and eIF2α phosphorylation in DCs (Fig. S3B).

GADD34 expression was not affected by PKR deletion (Fig. 3C), correlating with the absence of abundant P-eIF2α in PKR−/−-activated cells (Fig. 3A). Recently, PKR and its NOX2-dependent activation have been implicated in amplifying part of the UPR and linking it to inflammation (19, 35, 36). To gain further insights into the signaling pathways controlling GADD34 expression during DC activation by pI:C, we monitored GADD34 expression in DCs derived from different knockout mice. Conversely to what was observed in NOX2−/− macrophages (35), NOX2 deletion did not interfere with GADD34 induction upon DC activation (Fig. 3D). However, we found that toll/interleukin 1 receptor domain-containing adapter inducing IFN-β (TRIF) was absolutely required to induce GADD34 expression in response to soluble and lipofected pI:C. GADD34 remained undetectable in TRIF−/− DCs (Fig. 3E), even using longer kinetics of activation (Fig. S5A), whereas its expression was found normal in
activated MDA5−/− cells (Fig. 3F). Thus, the cytosolic helicase MDA5 is dispensable for GADD34-inducible expression by dsRNA even upon lipofection delivery. When we investigated ATF4 expression, we also found that its synthesis was attenuated upon TRIF inactivation, albeit not completely abolished (Fig. S2). This result further suggests that at least two signaling pathways are working in parallel upon pI:C detection and that GADD34 and ATF4 expression are mostly TRIF dependent in pI:C-activated DCs.

**Cytosolic Delivery of pI:C Does Not Inhibit Protein Synthesis in DCs.**

As cytosolic accumulation of dsRNA normally induces a PKR-dependent translational arrest (32), protein synthesis was monitored in DCs and fibroblasts exposed to soluble or lipofected pI:C. pI:C lipofection of MEFs efficiently caused a PKR-dependent translation arrest within 8 h (Fig. 4A). The translation arrest observed in WT MEFs suggests that soluble dsRNA can efficiently access the cytosol of these cells and interact with PKR. In the case of DCs, and as anticipated from the low levels of P-eIF2α induced by pI:C lipofection (Fig. 3B and Fig. S3B), translation was not inhibited even after 8 h of exposure to equivalent levels of cytosolic pI:C (Fig. 4A and Fig. S5B). We also observed by confocal microscopy the efficient access of soluble pI:C in the DC cytosol (Fig. S5C), likely to induce concomitantly the different dsRNA-sensing pathways (e.g., TLR3 and MDA5), as suggested by the levels of IFN-β production observed in TRIF-inactivated cells upon soluble pI:C stimulation in vitro (Fig. S5D) (4) and in vivo (37). On the basis of these observations and for greater consistency in our experiments, we decided to use only lipofected pI:C, which is more likely to be relevant physiologically (38).

When GADD34ΔC/ΔC DCs were exposed to cytosolic pI:C, their translation levels remained similar to those of WT cells (Fig. 4B). GADD34ΔC/ΔC DCs were
clearly activated as shown by the pattern of S6 ribosomal protein phosphorylation (P-S6) normally associated with TRIF-dependent mTOR activation and protein synthesis enhancement (29). Activated DCs are therefore able to resist PKR-dependent translational arrest and rely on the induction of GADD34 to shift the biochemical equilibrium toward eIF2α dephosphorylation. GADD34, however, is not important to establish or maintain DC resistance to dsRNA-induced translation arrest (Fig. 4B). In contrast, GADD34 was absolutely required for eIF2α dephosphorylation and translation recovery upon thapsigargin treatment (Fig. 4C). Thus, GADD34 seems to play a specific role in the innate response toward dsRNA independently of its previously characterized function in the UPR.

**Cytokine Production Is Affected by GADD34 Inactivation.**

GADD34 impact on translational initiation being limited, we examined the phenotype of activated GADD34ΔC/ΔC DCs. Lipofected pI:C-driven maturation of GADD34ΔC/ΔC CD11c+ cells was found normal, judging by the increase of surface MHC II and CD86 levels (Fig. S6). In contrast with these results, a reduction in IFN-β and IL-6 levels was detected in the cell culture supernatants of GADD34ΔC/ΔC DCs (Fig. 5A). To define whether GADD34 deficiency impacted cytokine production at the transcriptional or translational level, we measured the mRNA expression of IFN-β and IL-6 transcripts and observed that, in the absence of functional GADD34, the levels of these transcripts were reduced by half after 8 h of pI:C stimulation (Fig. 5B). GADD34 activity, therefore, enhances the transcription of different cytokines downstream of the TRIF adapter.
**GADD34 Regulates Levels of IFN-β in Vivo.**

To evaluate the consequences of GADD34 deletion at the whole animal level, we injected i.v. the Friend leukemia virus B sensitive mouse strain (FVB) mice with pI:C complexed with the liposomal transfection reagent DOTAP. We could show that under these conditions, GADD34 is induced rapidly in CD11c+ splenocytes (Fig. S7). We next analyzed IFN-β blood levels at 3 and 6 h postinjection. In FVB WT animals, production of IFN-β peaked at 3 h postinjection, whereas after 6 h the levels returned to basal levels (Fig. 5C). As predicted from the in vitro data and compared with WT mice, a marked reduction of IFN-β was observed in GADD34ΔC/ΔC mice 3 h postinjection (Fig. 5C). We tested next the relevance of GADD34 during viral infection by monitoring type-I IFN production in Chikungunya virus (CHIKV)-infected 12-d-old mice. We turned to this small RNA enveloped virus, because it is known to be a strong inducer of type-I IFN in vivo (39), a response key for mouse neonates to control the infection (40). At 72 h postinoculation, GADD34ΔC/ΔC pups displayed significantly less IFN-β in the serum and in the joints than their WT littermates, whereas as expected, virus titers were increased in GADD34ΔC/ΔC organs (Fig. 5D). GADD34 is therefore an immunologically relevant dsRNA-inducible factor that participates in the optimization of cytokine production in response to pI:C and viral infection.

**Discussion**

DCs treated with pI:C display an ATF4 expression signature sharing common features with an ISR, including GADD34 induction. Interestingly, GADD34 induction has also been singled out in a transcriptome analysis of *Listeria monocytogenes*-infected macrophages (41) and during corona virus infection of fibroblasts (42), suggesting its association with pathogen detection. In vitro, the penetration of dsRNA in all cellular
compartments can be directly detected through TLR3, DExD/H-box helicases, and PKR (43), which can also be rapidly activated by TLR ligation to promote p38 and NF-κB signaling (32). This eIF2α kinase is necessary to achieve functional DC maturation (9); however, its activation should normally lead to translation inhibition through eIF2α phosphorylation. Surprisingly, and in contrast to fibroblasts, DCs are not affected by dsRNA-induced translation inhibition. This specificity could allow DCs to prioritize the signal transduction pathways governing their innate immunity function over the pathways normally protecting cellular integrity from viral infection. When fully activated, these pathways would lead to protein translational arrest and/or apoptosis, through CHOP production and caspase cleavage, thus impairing DC function at a crucial time of the immune response (29). The lack of translation inhibition together with the absence of CHOP production, could allow DCs to carry their function even when infected with viruses as already evoked for human monocyte-derived DCs submitted to influenza virus infection (44).

GADD34 expression has primarily been shown to operate as a negative feedback loop during the UPR and allows for translation recovery through eIF2α dephosphorylation. We have shown that in DCs, GADD34 is required to prevent thapsigargin-induced protein synthesis arrest but GADD34 inactivation does not impact significantly on protein synthesis in response to dsRNA delivery. Activated DCs are therefore atypical cells in which eIF2α phosphorylation levels do not fully correlate with protein synthesis intensity and confirms that specific translation regulation pathways operate in during microbe detection, as observed during the proteasome-dependent cleavage of eIF4GI and DAP5 translation factors at late stages of their activation (29).
DCs have been reported to express high levels of XBP1, a transcription factor essential for ER homeostasis during UPR (15, 45, 46) and necessary for normal DC development (17, 47). In macrophages, TLR4 and TLR2 stimulation activates the ER-stress sensor kinase IRE1α and its downstream target, XBP1, without inducing other UPR branches (19). Our observations suggest that in DCs, the ATF4-dependent pathway is activated upon dsRNA detection. Interestingly nonactivated DCs possess abnormally high levels of P-eIF2α, which could, like XBP1 transcription, reflect a specific activation of the UPR during their differentiation and explain their low mRNA translation activity (29). GADD34 induction by pI:C in DCs requires the adapter TRIF and, contrary to XBP1, is not linked to NOX2 activity. The fact that pI:C enters efficiently the cell cytosol and the recent discovery of the TRIF-dependent DExD/H-box helicases DDX1, DDX21, and DHX36 (4), suggests that according to the mode of dsRNA delivery, TRIF could integrate different signals initiated by TLR3 and/or these helicases. The total absence of GADD34 induction by cytosolic pI:C in TRIF−/− DCs suggests that this pathway, leading to GADD34 expression, works in parallel with the induction of the MDA-5 sensing pathway to produce cytokines such as IFN-β efficiently.

Our observations also imply the existence of alternative signaling pathways, which allow ATF4 and GADD34 translation in the absence of increased P-eIF2α levels (48). Potentially, the high levels of P-eIF2α present in iDCs are sufficient to promote their synthesis during the early stages of DC activation. CHOP mRNA transcription was also found to be up-regulated in DCs, but no translation product could be detected, suggesting that CHOP synthesis is also tightly regulated in this cell type, as anticipated from the potent protective effect mediated by TLR agonists injection in mice subjected to systemic ER stress (49).
dsRNA is probably an extreme example of microbial-associated stressor because it can also induce PKR through direct recognition in the cytosol. Interestingly, although GADD34 controls eIF2α dephosphorylation, its inactivation does not overly impact translational control. However, like for XBP1 deficiency, we could show that IFN-β and IL-6 transcriptions were decreased in GADD34ΔC/ΔC DCs in vitro and in deficient mouse in vivo. In absence of an obvious effect of GADD34 deletion on protein synthesis, this observation suggests that GADD34/PP1 is required to dephosphorylate/activate a key factor in the signal transduction pathway downstream of TRIF. Recently, PP1-mediated deactivation of IkB kinase (IKK) in response to TNF-α, has been linked to GADD34 recruitment by the CUE domain-containing 2 protein (CUEDC2) (50). CUEDC2 silencing in macrophages led to increased transcription of inflammatory cytokine IL-6, by decreasing putative GADD34-dependent dephosphorylation of IKK. We could not recapitulate this finding in DCs, because IL-6 mRNA was less induced in activated GADD34ΔC/ΔC cells. A search for additional GADD34/PP1 targets will allow better understanding of the regulation pathways controlled by this specific pathogen-induced stress response. Activation of ATF4 and GADD34 should therefore be considered as an integral part of the innate immunity signaling cascades downstream of dsRNA sensors.

**Experimental Procedures**

Mouse bmDCs were treated with pI:C for different time periods. An Affymetrix Mouse Genome MOE 430 2.0 gene microarray chip was used to identify genes involved in dsRNA response of pI:C-activated DCs. Potential targets were confirmed by qPCR. Protein extracts of DCs from WT or different gene-targeted mice were analyzed by immunoblot for the presence of GADD34, P-eIF2α, or to study protein translation
through puromycin incorporation (28). In vivo injections of pI:C were performed as shown before (3). Cytokine levels were measured by ELISA. Detailed experimental procedures can be found in SI Experimental Procedures.

ACKNOWLEDGMENTS.

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References


Figure 1. ATF4 and GADD34 are induced during DCs activation with pI:C.

Experiments were performed in BMDCs stimulated with 10 μg/ml of pI:C or, when indicated, in RAW macrophages with 100 ng of LPS. mRNA levels of ATF4 (A), CHOP (C), GADD34 (E), PP1 (G) and CReP (H) were measured by qPCR. (B) ATF4 protein levels were detected by immunoblot in nuclear extracts. HDAC-1 is shown as loading control. Protein levels of CHOP (D) and GADD34 (F) were detected by immunoblot in total cell lysates. pI:C was added as soluble or lipofected (pI:C-lip). When indicated, MG132 was used to inhibit proteasomal degradation of GADD34 and was added for 2 h before harvesting the cells. ER stress-inducing drugs thapsigargin (th) or tunicamycin (tun) serves as positive control treatments for the expression of ATF4, CHOP and GADD34. Data represented in A, C, E, G and H are mean ±SD of three independent experiments.
Figure 2. eIF2α dephosphorylation is controlled by GADD34 during pI:C-induced DC maturation.

(A) Protein synthesis was quantified in protein extracts of pI:C-activated DCs using puromycin labelling followed by immunoblot. Cells not treated with puromycin (co) or cycloheximide (chx) were used as controls. (B) Cell extracts were blotted for P-eIF2α and total eIF2α, P-eIF2α decreases with time after pI:C stimulation. (C) Steady state levels of P-eIF2α non-stimulated bmDC (iDC), MEF and CD11c+ splenocytes. (D, E) Immature and pI:C-stimulated DCs were treated with GADD34 inhibitors guanabenz and salubrinal. Both drugs were added at the same time as pI:C, and for a period of 8h to non-stimulated cells. Protein extracts were blotted for P-eIF2α and eIF2α. (E) P-eIF2α levels of WT and GADD34\textsubscript{∆C/∆C} DCs stimulated with lipofected (lipo) or soluble pI:C (sol). Non-stimulated cells were treated with lipofectamine only. PKR blot is shown as control of DC activation. Thapsigargin (th) was used as positive control for P-eIF2α.
Figure 3. PKR phosphorylates eIF2α in pI:C-activated DCs.

WT and PKR−/− DCs were stimulated with pI:C alone (sol) or in combination with lipofectamine (lip) for the indicated time points (A, C) or for 8h (B). Protein extracts were blotted for PKR, P-eIF2α (A, B), and GADD34 (C). Sodium arsenite (as) was used as a positive control for P-eIF2α induction. (D) GADD34 immunoblot was performed on lysate of NOX2−/− bmDCs activated with either pI:C or LPS for 8 and 24h. (E) WT and TRIF−/− bmDCs were stimulated with pI:C as indicated and tested for the presence of GADD34 in protein extracts. (F) WT and MDA5−/− bmDCs were stimulated with lipofected pI:C and immunoblotted for GADD34. Treatment with MG132 (2h) was used to facilitate GADD34 visualization.
Figure 4. DCs are protected from translation inhibition induced by cytosolic pI:C detection. (A) Protein synthesis was monitored by immune-detection of puromycin incorporation in WT and PKR⁻/⁻ MEFs and DCs treated with soluble pI:C (sol) or lipofected (lip) for 8h. Controls: no puromycin (co), chx and lipofectamine-treated (mock) cells. (B) WT and GADD34ΔC/ΔC DCs were lipofected with pI:C for the indicated time and translation measured by anti-puromycin immunoblot. (C) bmDCs from WT and GADD34ΔC/ΔC mice were challenged with thapsigargin for the indicated time. Proteins translation, P-eIF2α and total eIF2α were detected by immunoblotting.
Figure 5. GADD34 promotes normal secretion of IFN-β and IL-6 in response to pI:C. (A) Culture supernatants of WT and GADD34^{ΔC/ΔC} bmDCs in which pI:C was delivered in the cytoplasm by lipofection were analysed by ELISA for IFN-β and IL-6. (B) IFN-β and IL-6 mRNA expression levels were measured by qPCR. One of four independent experiments with similar results is shown for each panel. (C, D) GADD34 is relevant for normal IFN-β protein levels *in vivo*. IFN-β levels were measured in the serum of mice that were i.v.-injected with 20 µg/ml of pI:C for 3h or 6h. pI:C was delivered conjugated with DOTAP, controls were performed injecting PBS with DOTAP. (D) IFN-β dosage in tissues and serum of mice inoculated with 10^{6} PFU of CHIKV via the ID route. 15 days old mice were analyzed 72h after inoculation. Each data point represents the arithmetic mean +/- SD for at least 4 mice. Average virus titers in the different organs are indicated.
Supplementary figures

Figure S1. Induction of UPR genes upon pI:C stimulation of DCs. mRNA levels of genes coding for Wars, Sec23b, Got1, Ero1l, Atf3 and Ndrg1 were monitored in BM-DCs at different time points of pI:C stimulation. Data represented are mean ± SD of three independent experiments. Wars: tryptophanyl tRNA synthetase; Sec23b: Sec23 protein transport B; Got1: glutamate oxaloacetate transaminase 1; Ero1l: Endoplasmic oxidoreducin-1-like protein; Atf3: activating transcription factor 3; Ndrg1: N-myc downstream regulated gene 1.
Figure S2. ATF4 induction is reduced in TRIF −/− DCs upon pI:C treatment.
ATF4 blots of nuclear extracts from dendritic cells treated for 8 and 20h with soluble or lipofected pI:C. Thapsigargin is used as control. Loading control is provided by HDAC1.
Figure S3. pI:C activation decreases apoptosis in DCs.

(A) DCs were treated with different concentrations of pI:C in the soluble and lipofected form, over a period of 8 or 20h. Cells were analyzed by FACS for the apoptosis marker Annexin V. The displayed graph shows the percentage of Annexin V positive cells in the CD11c⁺ and PI negative population. (B) Dendritic cells were treated with 10µg/ml of pI:C for 8 and 20h, the dsRNA mimic was administrated in the soluble and lipofected form. Protein extracts were immunoblotted for caspase 3. The levels of P-eIF2α were also verified in the same samples. Thapsigargin and tunicamycin were used as controls. (C) Quantification of P-eIF2α/eIF2α total ratio in WT and GADD34ΔC/ΔC DCs shown in Fig. 2E. P-eIF2α levels decrease in WT but not in GADD34ΔC/ΔC activated DCs.
Figure S4. High levels of eIF2α phosphorylation in spleen CD11c⁺ dendritic cells.
Immunohistochemistry of mouse spleen centered on the T-cell zones within the white pulp. T cells expressing CD3 are marked in green, DCs expressing CD11c in blue and phosphorylated eIF2α (P-eIF2α) (A, top panels) or eIF2α (B, bottom panels) in red. Single color images are shown in right rows (original magnification x40). Boxed areas in top row are presented at higher magnification and different color combination in bottom rows. High P-eIF2α levels (red) defines a cell population within the T cell zone, which is mostly found also positive for CD11c (blue, white arrowheads). T cells (green) express low level of P-eIF2α (red) collapsed in several small cytosolic dots (green arrowheads). Level of eIF2α are relatively homogenous within the T cell zones (green arrowheads), with no strong and obvious preferential expression in CD11c⁺ DCs. Outside of T cell areas some heterogeneity of staining can be observed suggesting that eIF2α expression and phosphorylation are dynamically regulated at steady state in different lymphocytes population. Our observations confirm, however, that high eIF2α phosphorylation occurs at steady state in dendritic cells in situ.
Figure S5. pI:C entry in MEFs and DCs.

(A) BM-DCs from WT and TRIF−/− were stimulated with pI:C and tested for the presence of GADD34 in protein extracts. Treatment with MG132 (2h) was used to prevent GADD34 degradation. (B) MEFs and DCs were treated for 8h with lipofected Cy5-labeled pI:C and the number of pI:C positive cell assessed by FACS. (C) Soluble pI:C enters DC cytosol. DCs were treated for 8h with 10 µg/ml Cy5-coupled-poly I:C (blue), alone (sol) or in combination with lipofectamine (lip). Cells were then stained with the anti-LAMP2 antibodies (red) and visualised by confocal microscopy. Bars, 10 µM. Data are representative of two independent experiments with similar results. (D) Culture supernatants of WT and TRIF−/− BM-DCs in which pI:C was delivered exogenously or in the cytoplasm by lipofection were analysed by ELISA for IFN-β and IL-6. One of three independent experiments with similar results is shown.
Figure S6. CD11c\(^+\) expression and phenotypic maturation are not affected in GADD34\(^{ΔC/ΔC}\) BM-DCs.

(A) Percentage of CD11c\(^+\) cells was assessed in 8h pI:C-stimulated and non-stimulated WT and GADD34\(^{ΔC/ΔC}\) BM-DCs. (B) Surface expression of CD86 and MHC class II (IA/IE) was quantified in the CD11c\(^+\) population. One of three independent experiments with similar results is shown.
Figure S7. *In vivo* expression of GADD34.

WT FVB mice were injected with 50µg of pIC complexed with DOTAP. Control mice were injected with PBS and DOTAP. The spleens of the animals were collected 4h after injection and CD11c+ splenocytes purified by magnetic separation. Proteins extracts from the CD11c+ cells were immunoblotted for GADD34 and eIF2α.
## Supplementary table

### Table S1. Induction of UPR transcripts in poly I:C activated DCs

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* fold induction confirmed by qPCR

## Experimental material and procedures

### Mice
Male C57BL/6 6-week-old mice were purchased from Charles River Laboratories. TRIF−/− (C57BL/6) mice were a gift from L. Alexopoulou (CIML, Marseille). NOX2−/− (gp91phox−/−) (C57BL/6) mice were donated by S. Meresse and A. Savina (Institut Curie, Paris). MDA5−/− and wild-type mouse bone marrow (from B6/129SvJ mice) were a gift from M. Colonna (Department of Pathology and Immunology, Washington University). PKR−/− and wild-type mouse bone marrow and MEFs SV40 transformed (from C57BL/6 mice) were a gift from C. Reis e Sousa (Cancer Research UK, London). GADD34Δ/Δ mice (FVB background) were originally obtained from L. Wrabetz (San Raffaele Institute, Milan) and maintained in the animal facility of CIML under specific pathogen-free conditions; age and sex-matched FVB wild-type mice were purchased from Charles River Laboratories. The principles of good laboratory animal care were followed all along the experimental process.

**Cell culture**

Bone marrow-derived DCs were obtained and cultured as described previously (1). Wild-type and PKR−/− MEFs were cultured in DMEM (GIBCO), 10% FCS (HyClone, PERBIO), 100 units/ml penicillin and 100 µg/ml streptomycin (GIBCO). RAW macrophages were cultured in DMEM, 10% FCS, 100 units/ml penicillin, 100 µg/ml streptomycin and 1mM sodium pyruvate. All cells were cultured at 37°C and 5% CO₂.

**Reagents**

DCs, RAW cells and MEFs were treated for the indicated time with 10 µg/ml poly I : C (InvivoGen), alone or in combination with lipofectamine 2000 (Invitrogen). DCs and RAW macrophages were treated for the indicated time with 100 ng/ml LPS (SIGMA). Tunicamycin, thapsigargin (added for 4h) and sodium arsenite (30’ treatment) were
purchased from Sigma and were used at 2 µg/ml, 500 nM and 0.5 mM, respectively. MG132 (Biomol International) was used at 2 µM and added 2h before harvesting. Salubrinal (Calbiochem) was used at 75 µM. Guanabenz (gift from David Ron, Cambridge, UK) was used at 50µM.

**Affymetrix microarray hybridization and data mining**

Total RNA was extracted from bmDCs at different times after poly I:C stimulation using the RNeasy miniprep kit (Qiagen). For each condition 100 ng of total RNA were employed to synthesize double-stranded cDNA using two successive reverse-transcription reactions according to standard Affymetrix protocols (GeneChip Two-Cycle Target Labelling, Affymetrix). Linear amplification with T7-RNA polymerase and biotin labelling were performed by in vitro transcription by standard Affymetrix procedures. The resulting biotin-labeled cRNA was fragmented and hybridized to the Affymetrix Mouse Genome MOE 430 2.0 oligonucleotide 39,000-gene microarray chip for 16 h at 45ºC. Following hybridization, the probe array was washed and stained on a fluidics station and immediately scanned on a Affymetrix GCS 3000 GeneArray Scanner. The data generated from the scan were then analyzed using the MicroArray Suite software (MAS 5.0, Affymetrix) and normalized using the GC-RMA algorithm. Bioinformatic analysis was performed using the GeneSpring GX 9.0 software (Agilent).

**Quantitative PCR**

Total RNA was isolated from DCs using the RNeasy miniprep kit (Qiagen) combined with a DNA digestion step (RNase-free DNase set, Qiagen). cDNA was synthesized from 1µg of total RNA using the Superscript II reverse transcriptase (Invitrogen) and random hexamer primers. Quantitative PCR amplification was carried out using
complete SYBR Green PCR master mix (Applied Biosystems) on a 7500 Fast Real-PCR system (Applied Biosystems) thermal cycler. cDNA concentration in each sample were normalized by using HPRT. The primers used for gene amplification (designed with Primer3 software) were the following: ATF4 (S 5'-GGACAGATTGGATGTTGGAGA-3', AS 5'-AGAGGGGCAAAAAGATCACAT-3'); CHOP (S 5'-ATGAAGGAGAAGGAGCAGGAG-3', AS 5'-CACTTCCGGAGAGACACAGACAG-3'); GADD34 (S 5'-GACCCCTCCAACCTCTCTTC-3', AS 5'-CTTCCTCAGCCCTCAGCATC-3'); PP1 (S 5'-GCCTCAACCGATGATATGT-3', AS 5'-CTTCCTGTGAAAGTGCCTGAT-3'); CReP (S 5'-GGAAATCCACCACCTTCGTAT-3', AS 5'-GCTGGGTTATCTGTGCAATGT-3'); Wars (S 5'-GGAACGTGTTGGAATTCAGAT-3', AS 5'-TCTTTGGAAAGAGTTGCTGA-3'); Sec23b (S 5'-GGTGTTGGAATTCAGAT-3', AS 5'-GCAGTTCAGATTAGCAACATC-3'); Got1 (S 5'-ACATCCGGCCCTATTTGCTAC-3', AS 5'-CATGGAGAACCATGAGCTGA-3'); Ero1l (S 5'-TGATGGGATTCTGACTGAAGG-3', AS 5'-TTCCCAGTGAAAGCTGAAAA-3'); ATF3 (S 5'-TTCCCATCCAGAATAAACAC-3', AS 5'-GCCTCAGACTTGGTGACTGAC-3'); Ndg1 (S 5'-GAGAGCATCAGTGCTTCTC-3', AS 5'-TCTCTGGCATGACGTCCATCGA-3'); HPRT (S 5'-CAGGGCACTTTGTGGAAT-3', AS 5'-TTGCGCTCATCTTGGGCTT-3'); IFN-β (S 5'-CCCTATGGAGATGACGGAGA-3', AS 5'-AACCAGGTGGAGAATTTG-3'); IL-6 (S 5'-CATGTTCAGATGACTGCA-3', AS 5'-TCCAGTATTGGTAATCCATC-3'). Data were analyzed using the 7500 Fast System Applied Biosystems software.
Translation intensity measurement

Puromycin labelling for measuring the intensity of translation was performed as previously described (2). For immunoblots, 10 µg/ml puromycin (Sigma, min 98% TLC, cell culture tested, P8833, diluted in PBS) was added to the culture medium 10 min before harvesting at 37°C and 5% CO₂. Where indicated, 25 µM cycloheximide (Sigma) was added 5 min before puromycin. Cells were then harvested in cold 1% FCS in PBS, centrifuged at 4°C and washed with cold PBS prior to cell lysis and immunoblotting with the 12D10 antibody.

Immunoblotting

Cells were lysed in 1% Triton X-100, 50 mM Hepes, 10 mM NaCl, 2.5 mM MgCl₂, 2 mM EDTA, 10% glycerol, supplemented with Complete Mini Protease Inhibitor Cocktail Tablets (Roche). Protein quantification was performed using the BCA Protein Assay (Pierce). 20-25 µg of Triton X-100-soluble material were loaded on 2-12% gradient or non-gradient 8 or 10% SDS-PAGE before immunoblotting and chemiluminescence detection (SuperSignal West Pico Chemiluminescent Substrate, Pierce). Nuclear extraction was performed using the Nuclear Complex Co-IP kit (Active Motif). Rabbit polyclonal antibodies against ATF4 (CREB-2, C-20), eIF2α (FL-315) and GADD34 C-terminal domain (C-19), as well as mouse monoclonal antibodies against PKR (B-10) and CHOP (GADD153, B-3) were purchased from Santa Cruz Biotechnology. Rabbit polyclonal antibody against P-eIF2α (Ser 51) was from BioSource. Rabbit polyclonal antibodies against P-S6 ribosomal protein and S6 were from Cell Signaling Technology. Mouse monoclonal antibodies against β-actin and histone H1 were from Sigma and Upstate respectively. Mouse monoclonal 12D10
antibody against puromycin has been previously described (2). Secondary antibodies were purchased from Jackson ImmunoResearch Laboratories.

**Confocal microscopy**

DCs were harvested and let adhere on 1% Alcian Blue-treated coverslips for 10 min at 37°C, fixed with 3% paraformaldehyde in PBS for 10 min at room temperature, permeabilized with 0.5% saponin in PBS/5% FCS/100 mM glycine for 15 min at room temperature and stained 1h with a rat monoclonal antibody against LAMP2 (from I. Mellman, Genentech, USA). Anti rat-Alexa 568 secondary antibody (30 min staining) was obtained from Molecular Probes (Invitrogen). pI :C was coupled with Cy5 using the Label IT Tracker Cy5 labeling kit (Mirus Bio Corporation). Coverslips were mounted on a slide and images taken with a laser-scanning confocal microscope (LSM 510; Carl Zeiss MicroImaging) using a 63x objective and accompanying imaging software.

**ELISA**

IFN-β and IL-6 quantification in cell culture supernatant was performed using the Mouse IFN-β ELISA kit (PBL InterferonSource) and Mouse Interleukin-6 ELISA kit (eBioscience) according to manufacturer instructions.

**Flow cytometry analysis**

Cells were stained with specific antibodies for cell surface markers: CD86-PE-Cy7, IA/IE-PE and CD11c-FITC (BD Pharmingen) (30min in ice, in 1% FCS in PBS). After washing, cells were fixed in 2% paraformaldehyde in PBS. Events were collected on a
**FACSCanto II** (Becton Dickinson), data were acquired using the Diva software (BD Biosciences) and analysed with FlowJo software.

**in vivo poly I:C injections**

Age and sex-matched FVB wild-type and GADD34\(^{\Delta C/\Delta C}\) were injected with 20 µg of poly I:C i.v. in combination with DOTAP (Roche). Controls were injected with PBS with DOTAP. The serum samples were taken 3 and 6 h after injection. Interferon-β was measured by ELISA.

**Immunohistochemistry**

Spleens were snap-frozen in Tissue Tek (Sakura Finetek, The Netherlands). Frozen sections (8 µm) were fixed with acetone and then stained with the following antibodies: anti-CD3ε-FITC (145-2C11) (BD pharmingen), anti-CD11c (N418) (Biolegend), anti-eIF2α (FL-315) (Santa cruz) and anti-P-eIF2α (S52) (Invitrogen). Alexa Fluor 647 goat anti-hamster (Invitrogen) and Alexa Fluor 555 donkey anti-rabbit IgG (Invitrogen) were the secondary antibodies used. Technical details have been described previously (3-4). Images were collected using a Zeiss LSM 510 confocal microscope (Welwyn Garden City, Hertfordshire, UK). Image processing was performed with Zeiss LSM software.

**CHIKV infection in mice**

GADD34\(^{\Delta C/\Delta C}\) and control FVB 12 days mice were anesthetized and inoculated via the intra-dermal route with 10\(^6\) PFU of CHIKV-21 isolate. Interferon titers in tissues and serum were determined as described (5). Viral titers in tissues and serum were determined as described (5), and expressed as tissue cytopathic infectious dose 50 (TCID50)/g or TCID50/ml, respectively.
Supplementary references:


II. Induction of GADD34 is necessary for dsRNA-dependent Interferon-β production and participates in the control of Chikungunya virus infection

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Induction of GADD34 is necessary for dsRNA-dependent Interferon-β production and participates in the control of Chikungunya virus infection

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Abstract

Nucleic acid sensing by cells is a key feature of antiviral responses, which generally result in type-I Interferon production and tissue protection. However, detection of double-stranded RNAs in virus-infected cells promotes two concomitant and apparently conflicting events. The dsRNA-dependent protein kinase (PKR) phosphorylates translation initiation factor 2-alpha (eIF2α) and inhibits protein synthesis, whereas cytosolic DExD/H box RNA helicases induce expression of type I-IFN and other cytokines. We demonstrate that the phosphatase-1 cofactor, growth arrest and DNA damage-inducible protein 34 (GADD34/ Ppp1r15a), an important component of the unfolded protein response (UPR), is absolutely required for type I-IFN and IL-6 production by mouse embryonic fibroblasts (MEFs) in response to dsRNA. GADD34 expression in MEFs is dependent on PKR activation, linking cytosolic microbial sensing with the ATF4 branch of the UPR. The importance of this link for anti-viral immunity is underlined by the extreme susceptibility of GADD34-deficient fibroblasts and neonate mice to Chikungunya virus infection.
Author Summary

Nucleic acids detection by multiple molecular sensors results in type-I interferon production, which protects cells and tissues from viral infections. At the intracellular level, the detection of double-stranded RNA by one of these sensors, the dsRNA-dependent protein kinase also leads to the profound inhibition of protein synthesis. We describe here that the inducible phosphatase 1 co-factor Ppp1r15a/GADD34, a well known player in the endoplasmic reticulum unfolded protein response (UPR), is activated during double-stranded RNA detection and is absolutely necessary to allow cytokine production in cells exposed to poly I:C or Chikungunya virus. Our data shows that the cellular response to nucleic acids can reveal unanticipated connections between innate immunity and fundamental stress pathways, such as the ATF4 branch of the UPR.

Keywords: innate immunity/PKR/RLR/TLR/poly I:C /puromycin/
Introduction

During their replication in host cells, RNA and DNA viruses generate RNA intermediates, which elicit antiviral responses mostly through type-I interferon (IFN) production [1,2]. Several families of proteins are known to sense double-stranded RNA (dsRNA), including endocytic Toll-like receptor 3 (TLR3) [3], the dsRNA-dependent protein kinase (PKR) [4] and the interferon-inducible 2′-5′-oligoadenylates and endoribonuclease L system (OAS/2-5A/RNase L) [5]. Viral dsRNA and the synthetic dsRNA analog polyriboinosinic-polyribocytidylic acid (poly I:C) are also detected by different cytosolic DExD/H box RNA helicases such as the melanoma differentiation-associated gene 5 (MDA5), DDX1, DDX21, and DHX36, which, once activated, trigger indirectly the phosphorylation and the nuclear translocation of transcription factors such as IRF-3 and IRF-7, resulting predominantly in abundant type-I IFN and pro-inflammatory cytokines production by the infected cells [1,6,7].

Alphaviruses such as Chikungunya virus (CHIKV) are small enveloped viruses with a message-sense RNA genome, which are known to be strong inducers of type-I IFN in vivo [8,9], a key response for the host to control the infection [10,11,12]. In vitro, however, response to RNA viruses is heterogeneous, since Sindbis virus (SINV), do not elicit detectable IFN-α/β production in infected of murine embryonic fibroblasts (MEFs) [13]. The specific points of blockage of type-I IFN production during infection are still not well delineated, but SINV and other alphaviruses could antagonize IFN production by shut-off of host macromolecular synthesis in infected cells [14,15,16]. Recently, human fibroblasts infection by CHIKV was shown to trigger abundant IFN-α/β mRNA transcription, while preventing mRNA translation and secretion of these antiviral cytokines [13,15]. Contrasting with these reports, other
groups using different CHIKV strains have observed abundant type-I IFNs release in the
culture supernatants of CHIKV-infected human monocytes [17], human lung cells (MRC-5),
human foreskin fibroblasts and MEFs [10]. Type-I IFN stimulation of non-hematopoietic cells
has also been shown to be essential to clear infection upon CHIKV inoculation in mouse, but
CHIKV was found to be a poor inducer of IFN secretion by human plasmacytoid dendritic
cells [10]. Thus, great disparities regarding alphavirus-triggered IFN responses exist between
viral strains and the nature of host cells or animal models.

Once bound to their receptor on the cell surface (IFNAR), type-I IFNs activate the Janus
tyrosine kinase pathway, which induces the expression of a wide spectrum of cellular genes
including Pkr [18]. These different genes participate in the cellular defense against the viral
infection. PKR is a serine–threonine kinase that binds dsRNA in its N-terminal regulatory
region and induces phosphorylation of translation initiation factor 2-alpha (eIF2α) on serine
51 [19,20], leading to protein synthesis shut-off and apoptosis. PKR has been also been
shown to participate in several important signaling cascades, including the p38 and JNK
pathways [21], as well as type-I IFN production [22,23]. Inhibition of translation, IFN
responses and triggering of apoptosis combine to make PKR a powerful antiviral molecule,
and many viruses have evolved strategies to antagonize it [24,25]. Interestingly, several
positive RNA-strand viruses (eg. Togaviridae or Picornaviridae) have been shown to activate
PKR, resulting in phosphorylation of eIF2α and host translation arrest [26], while viral mRNA
could initiate translation in an eIF2-independent manner by means of a dedicated RNA
structure, that stalls the scanning 40S ribosome on the initiation codon [25].

Despite the existence of these viral PKR-evading strategies, the importance of PKR for type-I
IFN production has been strongly debated over the years and even considered dispensable since the discovery of the innate immunity function of the DExD/H box RNA helicases [27,28]. However, several PKR-deficient cell types have reduced type-I IFN production in response to poly I:C [23,29,30], while PKR was demonstrated to be required for IFN-α/β production in response to a subset of RNA viruses including Theiler’s murine encephalomyelitis, West Nile (WNV) and Semliki Forest virus (SFV), but not influenza, Newcastle disease, nor Sendai virus [31,32,33,34]. These studies raise therefore the possibility that some but not all viruses induce IFN-α/β in a PKR-dependent and cell specific manner. Infection of PKR or RNAse L deficient mice demonstrated that these enzymes were not absolutely necessary for type I IFN-mediated protection from alphaviruses such as SFV or WNV, but still contributed to levels of serum IFN and clearance of infectious virus from the central nervous system [25,35]. Similarly, deficient mice for both PKR and RNAse L showed no increase in morbidity following SINV infection, although, like during WNV infection, increased viral loads in draining lymph nodes were observed [35,36]. These observations support a non-redundant and cell specific role for these enzymes in the amplification of type-I IFN responses to viral infection more than in their initiation [31,32,35]. Nevertheless, the exacerbated phenotypes observed upon alphavirus infection of mice deficient for type-I IFN receptor (IFNAR), underlines the limits of the individual contributions of PKR and RNAse L to the anti-viral resistance of adult animals [10,35,36].

In addition to dsRNA detection, different stress signals trigger eIF2α phosphorylation, thus attenuating mRNA translation and activating gene expression programs known globally as the integrated stress response (ISR) [37]. To date, four kinases have been identified to mediate eIF2α phosphorylation: PKR, PERK (protein kinase RNA (PKR)-like ER kinase) [38],
GCN2 (general control non-derepressible-2) [39,40] and HRI (heme-regulated inhibitor) [41,42]. ER stress–mediated eIF2α phosphorylation is carried out by PERK, which is activated by an excess of unfolded proteins accumulating in the ER lumen [38]. Activated PERK phosphorylates eIF2α, attenuating protein synthesis and triggering the translation of specific molecules such as the transcription factor ATF4, which is necessary to mount part of a particular ISR, known as the unfolded protein response (UPR) [43,44]. Interestingly DNA viruses, such as HSV, that use the ER as a part of its replication cycle, have been reported to interfere with the ER stress response through different mechanisms, such as the dephosphorylation of eIF2α by the viral phosphatase 1 activator, ICP34.5 [45,46].

We show here, using SUnSET, a non-radioactive method to monitor protein synthesis [47], that independently of any active viral replication, cytosolic poly I:C detection in mouse embryonic fibroblasts (MEFs) promotes a PKR-dependent mRNA translation arrest and an ISR-like response. During the course of this response, ATF4 and its downstream target, the phosphatase-1 (PP1) cofactor, growth arrest and DNA damage-inducible protein 34 (GADD34, also known as MyD116 and Ppp1r15a) [48], are strongly up-regulated. Importantly, although the translation of most mRNAs is strongly inhibited by poly I:C, that of IFN-β and Interleukin-6 (IL-6) is considerably increased under these conditions. We further demonstrate that PKR-dependent expression of GADD34 is critically required for the normal translation of IFN-β and IL-6 mRNAs. We prove the relevance of these observations for antiviral responses using CHIKV as a model: we show that GADD34-deficient MEFs are unable to produce IFN-β during infection and become permissive to CHIKV. We further show that CHIKV induces 100% lethality in 12-day-old GADD34-deficient mice, whereas WT controls do not succumb to infection. Our observations demonstrate that induction of
GADD34 is part of the anti-viral response and imply the existence of distinct and segregated groups of mRNA, which require GADD34 for their efficient translation upon dsRNA-induced eIF2α phosphorylation.

**Results**

**Poly I:C induces translational arrest and IFN-β production**

We monitored protein synthesis in MEFs and NIH-3T3 cells after poly I:C stimulation, using puromycin labeling followed by immunodetection with the anti-puromycin mAb 12D10 [47]. Poly I:C delivery to MEFs and NIH-3T3, rapidly and durably inhibited protein synthesis, concomitant with increased eIF2α phosphorylation (P-eIF2α) (Fig. 1A and Fig. S1A). In MEFs, a strong eIF2α phosphorylation was observed after 4h of poly I:C treatment, followed by a steady dephosphorylation at later times (Fig. 1A). Protein synthesis arrest was confirmed in individual cells by concomitant imaging of poly I:C delivery, mRNA translation and P-eIF2α (Fig. 1B and Fig. S1B), as well as with a wide range of dsRNA concentrations (Fig S1C). Poly I:C-induced eIF2α phosphorylation and subsequent translation arrest were not observed in PKR-deficient MEFs (Fig. 1C and 1D), while eIF2α phosphorylation induced by the UPR-inducing drug thapsigargin (th) (an inhibitor of SERCA ATPases) or arsenite (as) were unchanged in PKR -/- cells (Fig. 1C). PKR is therefore necessary to induce protein synthesis inhibition in response to cytosolic poly I:C.

When levels of IFN-β were quantified in culture supernatants and compared to total protein synthesis intensity, we found that most of the cytokine production occurred after 4 to 8h of pIC delivery (Fig. 1E, WT, and S1D), a time at which mRNA translation was already considerably decreased (Fig. 1A and S1E). We measured the amount of cytokine produced in
NIH-3T3 cells at a time (7h) at which translation was already strongly inhibited (Fig. 1G and 1F). To prove that IFN-β production truly occurred during this poly I:C-induced translation arrest, cells exposed for 7h to poly I:C were washed and old culture supernatants replaced with fresh media for 1h (with or without CHX), prior translation monitoring (Fig. 1F, right) and IFN-β dosage (Fig. 1G, right). We observed that close to 30% of the total IFN-β produced over 8h of poly I:C stimulation is achieved during this 1h period, despite a close to undetectable protein synthesis in the dsRNA-treated cells (Fig. 1F). The neosynthetic nature of this IFN was further demonstrated by the absence of the cytokine in CHX-treated cell supernatants. IFN-β production in response to poly I:C is therefore likely to be specifically regulated and occurs to a large extent independently of the globally repressed translational context. As previously observed in MEFs, IFN-β production in response to poly I:C was independent of PKR (Fig. 1E) [31]. This suggests that although its production occurs during cap-mediated translation inhibition, it does not directly depend on a specialized open reading frame organization, as described for the translation of the mRNAs coding for the UPR transcription factor ATF4 or the SV 26S mRNA upon eIF2α phosphorylation [26,49]. This hypothesis is also supported by the ability of MEFs expressing the non-phosphorylatable eIF2α Ser51 to Ala mutant (eIF2α A/A), to produce normal levels of IFN-β in response to poly I:C (Fig. 1E), while global translation was not inhibited by poly I:C in these cells (Fig. S2).

**GADD34 expression is induced by cytosolic poly I:C detection**

We went on to investigate the molecular mechanisms promoting this paradoxical IFN-β synthesis in an otherwise translationally repressed environment. Induction of eIF2α phosphorylation by PERK during ER stress promotes rapid ATF4 synthesis and nuclear translocation, followed by the transcription of many downstream target genes important for
the UPR [50]. Similarly, in presence of PKR, nuclear ATF4 levels were found to be up-regulated in MEFs responding to cytosolic poly I:C, albeit less importantly than upon a \textit{bona fide} UPR induced by thapsigargin (Fig. 2A).

One of the key molecules involved in the control of eIF2\(\alpha\) phosphorylation is the protein phosphatase 1 co-factor GADD34, which relieves translation repression during ER stress by promoting eIF2\(\alpha\) dephosphorylation [50,51],[52]. GADD34 is a direct downstream transcription target of ATF4 [53]. Expression of GADD34 was quantified by qPCR and immunoblot in WT and PKR\(^{-/-}\) MEFs (Fig. 2B). In WT cells GADD34 mRNA expression was clearly up-regulated (20 fold) in response to poly I:C, while GADD34 protein induction was equivalent in poly I:C- and thapsigargin-treated cells. GADD34 mRNA transcription and translation were not observed in PKR\(^{-/-}\) cells responding to poly I:C, but occurred normally upon thapsigargin treatment, paragoning eIF2\(\alpha\) phosphorylation (Fig. 2B, right).

We next investigated the importance of ATF4 for GADD34 transcription by monitoring the levels of GADD34 mRNA in ATF4-deficient cells. ATF4\(^{-/-}\) MEFs displayed higher basal levels of GADD34 mRNA than WT cells. However, in absence of ATF4, MEFs were unable to efficiently induce GADD34 mRNA transcription in response to any of the stimuli tested (Fig. S3). GADD34 mRNA expression was induced only 2 fold in ATF4\(^{-/-}\) MEFs exposed to poly I:C, suggesting that its transcription is mostly dependent on ATF4 in this context. We further investigated P-eIF2\(\alpha\) requirement for GADD34 expression and found that eIF2\(\alpha\) A/A expressing MEFs were incapable of up-regulating GADD34 in response to poly I:C (Fig. 2C). Phosphorylation of eIF2\(\alpha\) by PKR in response to cytosolic poly I:C induces therefore a specific
integrated stress response (ISR), that allows ATF4 translation, its nuclear translocation and subsequent GADD34 mRNA transcription.

**GADD34 expression is required for global translation recovery in response to thapsigargin but not to poly I:C.**

We next evaluated the relevance of GADD34 induction, by treating WT and GADD34$^{ΔC/ΔC}$ fibroblasts with poly I:C or with drugs known to induce ER stress, such as thapsigargin and the N-glycosylation inhibitor tunicamycin [52]. As expected, in WT cells eIF2$α$ phosphorylation was rapidly increased in response to all ISR-inducing stimuli and decreased concomitantly with the expression of GADD34 over time (Fig. 3A and S4) [52]. Consequently eIF2$α$ phosphorylation was greatly increased in GADD34$^{ΔC/ΔC}$ MEFs in all the conditions tested (Fig. 3A and S4A).

In thapsigargin-treated cells, protein synthesis was reduced in the first hour of treatment and rapidly recovered (Fig. 3B) [54]. Poly I:C, however, nearly completely inhibited translation despite active eIF2$α$ dephosphorylation. This was particularly obvious when poly I:C was co-administrated together with thapsigargin. Indeed, poly I:C dominated the response by preventing the translation recovery normally observed after few hours of drug treatment (Fig. 3B). Surprisingly, in absence of functional GADD34, although eIF2$α$ phosphorylation induction by poly I:C was augmented dramatically, no further decrease in protein synthesis was observed upon treatment of GADD34$^{ΔC/ΔC}$ cells with the dsRNA mimic (Fig. 3A and 3C). The functionality of GADD34 in translation restoration was, however, fully demonstrated, when the same cells were treated with thapsigargin, and protein synthesis was completely inhibited by this treatment [52] (Fig. 3C). Thus, cytosolic dsRNA delivery
induces a type of protein synthesis inhibition, which requires eIF2α phosphorylation for its initiation, but conversely cannot be reverted by GADD34 induction and subsequent GADD34-dependent eIF2α dephosphorylation.

The potential contribution of the OAS/2-5A/RNAse L system to this P-eIF2α-independent inhibitory process was evaluated by investigating RNA integrity in MEFs exposed to poly I:C. We used capillary electrophoresis to establish precise RNA integrity numbers (RIN) computed from different electrophoretic traces (pre-, 5S-, fast-, inter-, precursor-, post-region, 18S, 28S, marker) and quantify the degradation level of mRNA and rRNA potentially resulting from the activation of this well characterized anti-viral pathway. No major RNA degradation could be observed upon poly I:C delivery (Fig. S5), suggesting that global RNA degradation does not contribute extensively to the long term translation inhibition observed upon poly I:C delivery in our experimental system.

GADD34 is required for cytokine production induced by poly I:C

We have observed that GADD34 expression counterbalances PKR activation by promoting eIF2α dephosphorylation, however it has little impact on reversing the global translation inhibition initiated by poly I:C. We next monitored the production of specific proteins and cytokines in WT and GADD34ΔC/ΔC MEFs (Fig. 4). Cystatin C, a cysteine protease inhibitor was chosen as a model protein, since its secretion ensures a relative short intracellular residency time so that its intracellular levels directly reflect its synthesis rate [55]. This is confirmed by the N-glycosylated- and total Cystatin C accumulation in cells treated with brefeldin A (Fig. 4A, left panel). Cystatin C levels were found to follow a similar trend to that observed with total translation, being strongly reduced upon poly I:C exposure and not profoundly
influenced by GADD34 inactivation (Fig. 4A, right panel). Thapsigargin treatment induced a brief drop in cystatin C levels, prior to some levels of GADD34-dependent recovery. 6 hours of tunicamycin treatment affected more cystatin C accumulation than anticipated (Fig. 4A, right panel), probably due to interference with the N-glycosylation and associated folding of this di-sulfide bridge containing protein [55], thereby promoting its degradation by endoplasmic reticulum-associated protein degradation (ERAD) [56].

We next turned towards PKR, which displayed a pattern of expression completely different from cystatin C (Fig. 4B). As expected from its IFN-inducible transcription, levels of PKR were increased in poly I:C-treated MEFs (Fig. 4B), despite the strong global translation inhibition observed in these cells (Fig. 3). GADD34 inactivation appeared to influence the accumulation of PKR, since the cytoplasmic dsRNA sensor levels were not up-regulated and even decreased in poly I:C-treated GADD34ΔC/ΔC MEFs (Fig. 4B). Control treatment with tunicamycin and thapsigargin did not alter significantly PKR levels (Fig. 4B), suggesting that ER stress did not influence the kinase expression. The absence of PKR up-regulation in the poly I:C-treated GADD34ΔC/ΔC MEFs led us to investigate the capacity of these cells to produce anti-viral and inflammatory cytokines, which normally drive PKR expression through an autocrine loop. We ruled out any interference from the UPR in triggering IFN-β production in our experimental system, since, as anticipated from PKR expression, tunicamycin and thapsigargin treatments were not sufficient to promote cytokine production in MEFs (Fig. S6) [43,44].

We therefore investigated IFN-β and IL-6 production in response to dsRNA in WT, GADD34ΔC/ΔC and CReP−/− MEFs. CReP−/− MEFs were used as a control, since CReP (Ppp1r15b)
is a non-inducible co-factor of PP1 and displays some functional redundancy with GADD34 [57]. Although basal levels of elf2α phosphorylation were higher in CReP /−, PKR expression and translation inhibition upon poly I:C delivery were equivalent in WT and CReP /− MEFs (Fig. S7A and S7B). Quantification of IFN-β and IL-6 levels in culture supernatants indicated that, although abundant and comparable amounts of these cytokines were secreted by WT and CReP /− cells, they were both absent in poly I:C-treated GADD34ΔC/ΔC MEFs (Fig. 4C and S7C).

Quantitative PCR analysis revealed that, IFN-β, IL-6 and PKR transcripts were potently induced in poly I:C treated GADD34ΔC/ΔC MEFs (Fig. 4D), thus excluding any major transcriptional alterations in these cells, as confirmed by the normal levels of cystatin C mRNA, which remained constant in all conditions studied. Moreover, using confocal immunofluorescence microscopy, we could not detect intracellular IFN-β in poly I:C-stimulated GADD34ΔC/ΔC MEFs, in contrast to WT cells, which abundantly expressed the cytokine, despite the global translation arrest (Fig. S8). Thus, we could attribute the deficit in cytokine secretion of the GADD34ΔC/ΔC MEFs to a profound inability of these cells to synthesize cytokines, rather than to a defect in transcription or general protein secretion. GADD34 induction by poly I:C is therefore absolutely necessary to maintain the synthesis of specific cytokines and probably several other proteins in an otherwise translationally repressed context. Importantly, GADD34 exerts its rescuing activity only on a selected group of mRNAs including those coding for IFN-β and IL-6, but not on all ER-translocated proteins, since cystatin C synthesis was strongly inhibited by poly I:C in all conditions tested.
Interestingly, in GADD34ΔC/ΔC MEFs, PKR mRNA strongly accumulated in response to poly I:C (Fig. 4D), despite the absence of detectable IFN-β production and PKR protein increase (Fig. 4B). This continuous accumulation of PKR mRNA in response to poly I:C suggests the existence of alternative molecular mechanisms, capable of promoting PKR mRNA transcription and stabilization independently of autocrine IFN-β detection. Nevertheless in these conditions PKR expression, like IFN-β, was found to be dependent on the presence of GADD34 for its synthesis (Fig. 4B).

Recent results indicate that PKR participates to the production of IFN-α/β proteins in response to a subset of RNA viruses including encephalomyocarditis, Theiler's murine encephalomyelitis, and Semliki Forest virus [31]. Even though IFN-α/β mRNA induction is normal in PKR-deficient cells, a high proportion of mRNA transcripts lack their poly(A) tail [31]. As GADD34 induction by poly I:C was completely PKR-dependent, we wondered whether the phenotypes observed in PKR−/− cells and GADD34ΔC/ΔC MEFs could be related. Oligo-dT purified mRNA extracted from cells exposed to poly I:C were therefore analyzed by qPCR. PolyA+ mRNAs coding for IFN-β and IL-6 were equivalently purified and amplified from WT and GADD34ΔC/ΔC MEFs (Fig. S9). This confirms that albeit the phenotypes of PKR−/− and GADD34ΔC/ΔC cells might be linked, mRNA instability is not the primary cause of the cytokine production defect observed in GADD34ΔC/ΔC. Taken together these observations suggest the existence of a specific mRNAs pool, encompassing cardinal immune effectors such as IFN-β, IL-6, and PKR, which are specifically translated in response to dsRNA sensing and increased levels of P-eIF2α. This mRNAs pool requires GADD34 for their translation during the global protein synthesis shut-down triggered by dsRNA detection.
**GADD34 rescues cytokine production in GADD34\(^{ΔC/ΔC}\) MEFs.**

We verified that GADD34 inactivation, and no other deficiency, was truly responsible for the loss of cytokine production by complementing GADD34\(^{ΔC/ΔC}\) MEFs with GADD34 cDNA prior to poly I:C delivery. IFN-β secretion was partially restored in transfected GADD34\(^{ΔC/ΔC}\) cells while eIF2α was efficiently dephosphorylated in both WT and GADD34\(^{ΔC/ΔC}\) transfected MEFs (Fig. 4E). To further demonstrate that the phosphatase activity of GADD34 controls cytokine production upon dsRNA detection, we treated WT MEFs with guanabenz, a small molecule, which selectively impairs GADD34-dependent eIF2α dephosphorylation [58]. Upon treatment with this compound, a dose dependent inhibition of IFN-β secretion was observed in poly I:C-treated MEFs, confirming the importance of GADD34 in this process (Fig. S10).

**GADD34 is necessary for IFN production and to control Chikungunya virus infection**

Fibroblasts of both human and mouse origin constitute a major target cell of Chikungunya virus (CHIKV) during the acute phase of infection [59]. In adult mice with a totally abrogated type-I IFN signaling, CHIKV-associated disease is particularly severe and correlates with higher viral loads. Importantly, mice with one copy of the IFN-α/β receptor (IFNAR) gene develop a mild disease, strengthening the implication of type-I IFN signaling in the control of CHIKV replication [59]. Recently, human fibroblasts infection by CHIKV was shown to induce IFN-α/β mRNA transcription, while preventing mRNA translation and secretion of these antiviral cytokines. CHIKV was found to trigger eIF2α phosphorylation through PKR activation, however this response is not required for the block of host protein synthesis [15].

We tested the importance of PKR during CHIKV infection by infecting WT and PKR\(^{-/}\) MEFs with CHIKV-GFP, at a multiplicity of infection (MOI) of 10 and 50. Productive infection was...
estimated by GFP expression (Fig. 5A, left panel), while culture supernatants were monitored for the presence of IFN-β (5A, right panel). PKR was found to be necessary to control CHIKV infection in vitro, since at least 60% of PKR-inactivated cells were infected after 24 of viral exposure, compared to only 15% in the control fibroblasts population. WT MEFs produced efficiently IFN-β, while the hypersensitivity to infection of the PKR−/− MEFs was correlated to a reduced type-I IFN production capacity after infection. Thus, during CHIKV infection, PKR is required for normal IFN production by MEFs. We also monitored protein synthesis in infected WT and PKR−/− fibroblasts using puromycin labeling followed by immunofluorescence confocal microscopy (Fig. 5B). CHIKV-GFP positive PKR−/− MEFs were found to incorporate efficiently puromycin, while in their infected WT counterpart protein synthesis was efficiently inhibited. Thus CHIKV, in this experimental model, induces a PKR-dependent protein synthesis inhibition and is therefore particularly relevant to further confirm our observations on the role of GADD34 in controlling type-I IFN production during response to viral RNAs.

GADD34ΔC/ΔC MEFs were exposed to CHIKV-GFP (MOI of 10 or 50) for 24 and 48 h. Productive infection was estimated by GFP expression and virus titration (Fig. 6A), and culture supernatants monitored for the presence of type-I IFN (Fig. 6B, left). Only minimal CHIKV infection (15%) could be observed at maximum MOI in WT MEFs (Fig. 6A, left), while robust IFN-β amounts were already produced at the lowest MOI (Fig. 6B). Contrasting with WT cells and regardless of the MOI used, a higher level of viral replication was observed in GADD34ΔC/ΔC MEFs (Fig. 6A). The GADD34-inactivated cells were clearly more sensitive to CHIKV, displaying a 50% infection rate after 24h of infection (MOI 50) and a log more of virus titer in culture supernatants (Fig. 6A, right). Correlated with their susceptibility to CHIKV
infection, IFN-β production was nearly undetectable in GADD34ΔC/ΔC MEFs (Fig. 6B). Such observation confirms the incapacity of GADD34-deficient cells to produce cytokines in response to cytosolic dsRNA, a deficiency likely to facilitate viral replication. This interpretation is further supported by the abrogation of viral replication in both WT and GADD34ΔC/ΔC MEFs briefly treated with IFN-β (Fig. 6C). Thus, GADD34 inactivation does not favor viral replication per se, but is critical for type-I IFN production. Interestingly, infection levels were found to be higher in PKR -/- than in GADD34ΔC/ΔC MEFs, although this difference could be attributed to clonal MEFs variation, it more likely suggests that PKR-dependent translation arrest could be key in preventing early viral replication in this system. In addition, the relatively lower permissivity of GADD34ΔC/ΔC MEFs to infection at high MOI could indicate the existence of GADD34-dependent defense mechanisms, which could be independent from IFN production and eIF2-α dephosphorylation. To strengthen and generalize these observations, we treated a different strain of WT MEFs with guanabenz and examined the consequences for CHIKV infection. Biochemically, GADD34 expression was induced upon CHIKV infection, and guanabenz treatment resulted in a clear increase in eIF2α phosphorylation, demonstrating the importance of GADD34 in limiting this process during infection (Fig. 6D, right). As observed with GADD34ΔC/ΔC cells, pharmacological and RNAi inhibition of GADD34 was found to increase significantly the sensitivity of MEFs to infection, while reducing their IFN-β production (Fig. 6D and S10). Thus, induction of GADD34 and its phosphatase activity during CHIKV infection, in vitro, participates to normal type-I IFN production and control of viral dissemination.

Several components of the innate immune response have been shown to impact on the resistance of adult mice and to restrict efficiently CHIKV infection and its consequences in
We decided to investigate the importance of GADD34 upon intradermal injections of CHIKV to WT (FVB) and GADD34ΔC/ΔC mice. Neither strain of adult mice was affected by intradermal injections of CHIKV, with little statistically significant differences in the virus titers found in the different organs. Thus, GADD34 deficiency does not annihilate all the sources of type-I IFN in the infected adult animals, a situation exemplified by the capacity of GADD34ΔC/ΔC bone-marrow derived dendritic cells to produce reduced, but measurable IFN-β in response to poly I:C [60]. This also infers that the light impact of GADD34 inactivation on mouse development [61] does not render these animals more sensitive to CHIKV infection.

As in Humans, CHIKV pathogenicity is strongly age-dependent in mice, and in less than 12 day-old mouse neonates, CHIKV induces a severe disease accompanied with a high mortality rate [59]. GADD34 function was therefore evaluated in this more sensitive context by injecting intradermally CHIKV to FVB (WT) and GADD34ΔC/ΔC neonatal mice. As previously observed for C57/BL6 mice [59], when CHIKV was inoculated to FVB neonates, a rate of 50% of mortality was observed 3 days after the infection of 9-day-old mice, while 12-day-old pups were found essentially resistant to the virus lethal effect (Fig. 7A). Strongly contrasting with these results, all CHIKV infected GADD34ΔC/ΔC neonates died within 3-5 days post inoculation whatever their age (Fig. 7A). When infection was monitored 5 days post-inoculation of 12-day-old mice at, GADD34ΔC/ΔC pups displayed considerably more elevated CHIKV titers (10-100 folds) in most organs tested, including liver, muscle, spleen and joints, the later being primarily targeted by the virus (Fig. 7B, left). As expected, and in full agreement with the in vitro data, infected GADD34ΔC/ΔC tissues showed a considerably reduced IFN-β production (40-50%) compared to control tissues (Figure 7B, right), while serum levels were reduced by 20% (not shown). Although Infectious virus was poorly
detected in the heart of WT animals, elevated titers of virus were observed in the heart of GADD34-deficient pups, matching the limited production of IFN in this organ. We further investigated the possible pathological consequences of cardiac tissue infection by carrying-out comparative histopathology. Hearts of infected GADD34-deficient animals displayed severe cardiomyocytes necrosis with inflammatory infiltrates by monocytes/macrophages and very important calcium deposition (Fig. 8), all being indicative signs of grave necrotic myocarditis. As a consequence, the left ventricles were strongly dilated, being probably the cause of acute cardiac failures and of the important death rate observed in GADD34ΔC/ΔC infected pups. Histology of infected FVB mice hearts was, however, normal with only few inflammatory cells (mainly lymphocytes) observed in the close vicinity of capillaries.

GADD34 expression is therefore necessary to allow normal type-I interferon production during viral infection and to promote the survival of young infected animals. We could circumvent the age-related acquisition of viral resistance in GADD34ΔC/ΔC mice to 17 days, since mice inoculated at that age survived CHIKV inoculation. In these animals, 3 days post-infection, enhanced viral replication was observed in the spleen and muscles, matching the relatively low level of type-IFN production in these tissues (Figure 7C). Functional GADD34 is therefore required to mount a normal innate response against the virus, but in older mice type-I IFN production by non-infected innate cells is probably capable to gradually overcome GADD34-deficiency and limit viral proliferation in vital organs, such as the heart.

Discussion

Translation inhibition occurs in response to stress, when other cellular activities have to be reassigned or suspended momentarily. We demonstrate here that the activation of PKR by
cytosolic dsRNA results in a stress response, leading to ATF4 and GADD34 induction. GADD34 expression has been observed during the infection of cells by different types of viruses [62] or intracellular bacteria such as *Listeria monocytogenes* [63]. Our observations demonstrate that GADD34 expression is a direct consequence of PKR activation and dsRNA sensing. Interestingly, although GADD34 induction by poly I:C promotes eIF2α dephosphorylation, this is not sufficient to prevent global protein synthesis arrest. The uncoupling of efficient eIF2α dephosphorylation from global translation recovery in response to cytosolic poly I:C implies therefore the existence of additional mechanisms inhibiting global translation. The 2-5A/RNAse L pathway does not seem to be sufficiently active in our experimental setting to explain this prolonged protein synthesis inhibition. The cleavage or the inactivation of other translation factors could work in concert with eIF2α to block or affect the efficiency of other individual steps of mRNA translation [64]. For instance, the phosphorylation of translation elongation factor 2 (eEF-2) is also controlled by eIF2α phosphorylation. Thus, Thr56 phosphorylation of eEF-2, which is known to inhibit its translational function by reducing its affinity for ribosomes, could contribute directly to the protein synthesis inhibition induced by PKR activation [65]. Independently of general protein synthesis inhibition, eIF2α dephosphorylation is necessary for the production of specific proteins upon dsRNA-induced translation inhibition. As demonstrated for ATF4, translation of a given mRNA during stress could rely on the structure and organization of its coding sequence, as well as the presence of multiple alternative initiation codons [49]. Surprisingly, functional GADD34 expression was found necessary for the translation of IL-6, IFN-β, and PKR. This observation points to the existence of a distinct group of mRNAs efficiently translated upon dsRNA detection and dependent on GADD34 activity.
GADD34 is extremely short lived and has been shown to accumulate on the ER, when over-expressed [51]. GADD34 could mediate its activity at the ER level and influence differently eIF2α sub-cellular distribution according to the type, localization, and level of activity displayed by the different eIF2α kinases. The strong eIF2α phosphorylation mediated by PKR in response to poly I:C or viral infection and leading to the initiation of translation inhibition, could be circumvented through GADD34 activity solely at the ER level, thereby allowing local cytokine production in absence of other functional protein synthesis. This selectivity for translation of several specific mRNAs among other ER-secreted molecules suggests further that GADD34 dependent mRNAs might display specific features allowing their efficient identification by GADD34 and associated molecules, as well as allowing their translation in presence of minimal levels of active guanine nucleotide exchange factor eIF2B.

GADD34 and PKR are necessary to produce anti-viral cytokines during CHIKV infection, and probably other types of infection. PKR, ATF4 and GADD34 should therefore be considered as an essential module of the innate anti-viral response machinery. The importance of PKR in anti-viral type-I IFN responses has been the object of contradictory reports [30,31,66,67]. Our observations, however, suggest that PKR function should be re-evaluated by integrating the impact of viral detection on cellular translation. In eIF2A/A and PKR/− cells, cytokine transcription is induced normally following poly I:C detection by DExD/H box RNA helicases, while as expected in these cells, no eIF2α phosphorylation and subsequent host translation inhibition are observed. This lack of translation arrest in the absence of potent eIF2α phosphorylation allows for normal cytokine production during dsRNA detection, with no requirement for an operational GADD34 feedback loop. The importance of PKR and GADD34 for IFN-β and other cytokines production could therefore be directly linked to the efficiency
of the cellular translation inhibition induced by RNA viruses, as exemplified here with CHIKV, which in MEFs strongly activates PKR and subsequent protein synthesis inhibition.

GADD34ΔC/ΔC neonates are extremely sensitive to CHIKV infection and display signs of acute myocarditis and ventricles dilatation probably causing recurrent cardiac failures. CHIKV cardiac tropism is not normally observed in WT mouse and inability of heart tissues to produce sufficient type-I IFN in GADD34ΔC/ΔC could allow abnormally high viral replication, myocarditis and dilated cardiomyopathy. Interestingly many cases of myopericarditis induced by CHIKV and leading to dilated cardiomyopathies in infected patients have been reported since the 1970s after the different western Indian Ocean islands and Indian subcontinent disease outbreaks[73, 74]. These particular symptoms and complications might therefore be the consequences of great variation in the tissue-specific type-I IFN levels induced in CHIKV-infected patients, who might display particular polymorphisms in their innate viral sensing pathways increasing their peculiar susceptibility to viral dissemination in the heart.

Importantly, our data reveal a link between pathogen-associated molecular patterns (PAMPs) and the UPR through the activation of the eIF2-α/ATF4 branch [68]. Similarly, several laboratories have reported that TLR stimulation activates the XBP-1 branch of the UPR and that XBP-1 production was needed to promote a sustained production of inflammatory mediators, including IL-6 [69,70]. Here, we identify GADD34 as a novel functional link between ISR and PAMPs detection in MEFs, required for the production of cytokines including type-I IFN. It will now be important to explore the therapeutic potential of targeting GADD34 to reduce cytokines overproduction during inflammatory conditions.
Materials and methods

Ethics Statement

This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals the French Ministry of Agriculture and of the European Union. The protocol was approved by the Committee on the Ethics of Animal Experiments of the Institut Pasteur and Région PACA (Autorisation # 13.116 issued by DDSV/Préfecture des Bouches du Rhône, Marseille, France) and were performed in compliance with the NIH Animal Welfare Insurance #A5476-01 issued on 02/07/2007. All experiments were performed under isoflurane anesthesia (Forene, Abbott Laboratories Ltd, United-Kingdom), and all efforts were made to minimize suffering. Animals were housed in the Institut Pasteur and CIML animal facilities accredited by the French Ministry of Agriculture to perform experiments on live mice.

Cells

Matched wild-type (129 SvEv), and PKR\(^{-/-}\) MEFs (Yang et al., 1995) were a gift from Caetano Reis e Sousa (Cancer Research UK, London); primary eIF2\(\alpha\) S/S and eIF2\(\alpha\) A/A MEFs were a gift from Randal J. Kaufman (Department of Biological Chemistry, University of Michigan Medical Center, USA); Matched wild-type (129 SvEv), ATF4\(^{-/-}\), GADD34\(^{\Delta C/\Delta C}\) and CReP\(^{-/-}\) MEFs were a gift from David Ron (Skirball Institute of Biomolecular Medicine, New York). All MEFs were cultured in DMEM, 10% FCS (HyClone, Perbio), 100 units/ml penicillin, 100 \(\mu\)g/ml streptomycin, 2mM glutamine, 1 x MEM non-essential amino acids and 50 \(\mu\)M 2-mercaptoethanol. NIH3T3 cells were cultured in RPMI 1640 (Gibco) supplemented with 10%
FCS (HyClone, PERBIO), 100 units/ml penicillin and 100 µg/ml streptomycin. All cells were cultured at 37°C and 5% CO₂. MEFs and NIH3T3 were treated for the indicated time with 10 µg/ml poly I:C (InvivoGen) in combination with lipofectamine 2000 (Invitrogen). Thapsigargin, tunicamycin, sodium arsenite, and guanabenz (all from SIGMA) were used at 200 nM, 2µg/ml, 0.5 mM, and 10µM respectively. The plasmid GADD34 (FLAG epitope tagged at N-terminus, CMV2-based mammalian expression) was a kind gift from David Ron (Institute of Metabolic Sciences, University of Cambridge, UK).

Translation intensity measurement
Puromycin labelling for measuring the intensity of translation was performed as previously described [47]. For immunoblots, 10 µg/ml puromycin (Sigma, min 98% TLC, cell culture tested, P8833, diluted in PBS) was added in the culture medium and the cells were incubated for 10 min at 37°C and 5% CO₂. Where indicated, 25 µM cycloheximide (Sigma) was added 5 min before puromycin. Cells were then harvested, centrifuged at 4°C and washed with cold PBS prior to cell lysis and immunoblotting with the 12D10 antibody.

Immunoblotting
Cells were lysed in 1% Triton X-100, 50 mM Hepes, 10 mM NaCl, 2.5 mM MgCl₂, 2 mM EDTA, 10% glycerol, supplemented with Complete Mini Protease Inhibitor Cocktail Tablets (Roche). Protein quantification was performed using the BCA Protein Assay (Pierce). 25-50 µg of Triton X-100-soluble material was loaded on 2%-12% gradient or 8% SDS-PAGE before immunoblotting and chemiluminescence detection (SuperSignal West Pico Chemiluminescent Substrate, Pierce). Nuclear extraction was performed using the Nuclear Complex Co-IP kit (Active Motif). Rabbit polyclonal antibodies recognizing ATF4 (CREB-2, C-
20), GADD34 (C-19), Lamin A (H-102) and eIF2-α (FL-315) were from Santa Cruz Biotechnology, as well as mouse monoclonal anti-PKR (B-10). GADD34/PPP1R15A (Catalog No. 10449-1-AP) rabbit polyclonal antibody was purchased from PROTEINTECH. Rabbit polyclonal anti-eIF2α[pS52] and Cystatin C were from Invitrogen and Upstate Biotechnology, respectively. Mouse monoclonal antibodies for β-actin and HDAC1 (10E2) were purchased from Sigma and Cell Signaling Technologies. Secondary antibodies were from Jackson ImmunoResearch Laboratories.

**Immunofluorescence**

MEFs and NIH3T3 were grown on coverslips overnight and stimulated for the indicated time with poly I:C complexed with Lipofectamine 2000. Cells were fixed with 3% paraformaldehyde in PBS for 10 min at room temperature, permeabilized with 0.5% saponin in 5% FCS PBS with 100 mM glycine, for 15 min at room temperature and stained for 1h with indicated primary antibodies. Anti-P-eIF2α was from BioSource; anti-dsRNA (clone K1) from English & Scientific Consulting Bt.; anti-IFN-β-FITC-conjugated from PBL Interferon Source; anti-puromycin (clone 2G11, mouse IgG1) has been previously described [47]. Alexa-conjugated secondary antibodies (30 min staining) were from Molecular Probes (Invitrogen). Coverslips were mounted on a slide and images taken with a laser-scanning confocal microscope (LSM 510; Carl Zeiss MicrolImaging) using a 63x objective and accompanying imaging software. When PKR WT and PKR−/− were infected with CHIKV, protocol was performed as follows: cells were fixed with 4% paraformaldehyde in PBS for 20 min, then permeabilized for 30 min in 0.1% Triton 100X (Sigma) and blocked in 10% of normal goat serum (Vector Laboratories). Cells were stained with a mouse monoclonal antibody directed against CHIKV capsid coupled to Alexa-488 and a mouse antibody against puromycin coupled
to Alexa-555 and a rabbit antibody anti-eIF2α[pS\textsuperscript{52}] (Invitrogen) and a Cyanin-3 secondary antibody, and finally counterstained with Hoechst (Vector Lab). Cells were observed with an AxioObserver microscope (Zeiss). Pictures and Z-stacks were obtained using the AxioVision 4.5 software.

**ELISA**

IFN-β and IL-6 quantification in culture supernatant was performed using the Mouse Interferon Beta ELISA kit (PBL InterferonSource) and Mouse Interleukin-6 ELISA kit (eBioscience) respectively, according to manufacturer instructions.

**Quantitative PCR**

Total RNA was isolated from cells using the RNeasy miniprep kit (QIAGEN) combined with a DNA digestion step (RNase-free DNase set, QIAGEN). cDNA was synthesized using the Superscript II reverse transcriptase (Invitrogen) and random hexamer primers. Quantitative PCR amplification was carried out using complete SYBR Green PCR master mix (Applied Biosystems) and 200 nM of each specific primer. 5 µl of cDNA template was added to 20 µl of PCR mix, and the amplification was tracked via SYBR Green incorporation by an Applied Biosystems thermal cycler. cDNA concentration in each sample were normalized by using HPRT. A nontemplate control was also routinely performed. The primers used for gene amplification (designed with Primer3 software) were the following: GADD34 (S 5'-GACCCCTCAACTCTCCTC-3', AS 5'-CTTCCTTCAGCCTCAGATCC-3'); HPRT (S 5'-AGGCCAGACTTTGTTGAGATT-3', AS 5'-GGCTTTGTATTTGGCTTTTCC-3'); IFN-β (S 5'-CCCTATGGAGATGACGGAGA-3', AS 5'-ACCCAGTGCTGGAGAAATTG-3'); IL-6 (S 5'-CATGGTCTCTGGGAAATCGTG-3', AS 5'-TCCAGTTTGCTTAGCATCCATC-3'); PKR (S 5'-
CGGTGCCTCTTTATTCAAA-3’, AS 5’-ACTCCGGTCACGATTGTTCT-3’); Cystatin C (S 5’-GAGTACAACAGGGGCAAC-3’, AS 5’-TCAAATTTGCTGGGACTTG-3’). ATF4 (5’-GGACAGATTGGATGGGAGA-3’, AS 5’-AGAGGGGCAAAAAGATACAT3-’)
mRNA isolation from total RNA was performed with oligoT columns (Genelute mRNA miniprep kit (Sigma). Data were analyzed using the 7500 Fast System Applied Biosystems software.

RNA integrity measurement

RNA integrity upon poly I:C stimulation was measured by capillary electrophoresis using the Agilent RNA 6000 Pico Chip kit (Agilent Technologies) in an Agilent 2100 Bioanalyser, according to manufacturer instructions.

MEFs infection with CHIKV

GADD34ΔC/ΔC and the corresponding WT control MEFs were infected at a multiplicity of infection (MOI) of 10 or 50 with CHIKV-GFP generated using a full-length infectious cDNA clone provided by S. Higgs [71]. By 24h and 48h post infection, 30 000 cells were analyzed in triplicate by FACS for expression of GFP. At the same time-points, culture supernatants were collected and IFN-β protein assessed by ELISA. In experiments with exogenous IFN-β, cells were treated with mouse IFN-β (PBL InterferonSource) for 3 h before infection with CHIKV-GFP. When guanabenz was used to specifically inhibit GADD34, MEFs cells were treated for 2h with 10µM of Guanabenz or DMSO and then infected in the same medium. Three hours post infection the inoculum was removed and fresh medium with Guanabenz or DMSO was added and maintained all along the experiment. RNAi for GADD34 was performed as described in [60].
**CHIKV infection in mice**

FVB WT mice were obtained from Charles River Laboratories (France). GADD34^{ΔC/ΔC} FVB mice were obtained from L. Wrabetz (Milan). Mice were anesthetized and inoculated via the intradermal route with $10^6$ PFU of CHIKV-21 isolate [72]. Viral titers in tissues and serum were determined as described before [59], and expressed as tissue cytopathic infectious dose 50 (TCID50)/g or TCID50/ml, respectively. Organs including heart, liver, skeletal muscles and spleen were collected for histopathological procedures. Organs were then fixed in 4% paraformaldehyde solution, paraffin-embedded, sectioned coronally in 5-10 µm thickness and stained with hematoxylin-eosin.

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Figure 1. Translation inhibition and IFN-β production are induced by poly I:C in MEFs.

A) Protein synthesis was monitored in poly I:C(stimulated MEFs using puromycin labelling followed by immunoblot with the anti-puromycin mAb 12D10. Controls are cells not treated with puromycin (No puro) and cells treated with cycloheximide (chx) 5 min prior puromycin incorporation. β-actin immunoblot is shown for equal loading control. Quantification of puromycin signal was quantified with ImageJ software and is represented above the immunoblot. Phosphorylation of eIF2α (P-eIF2α) was assessed in the same MEFs extracts.

B) Immunofluorescence staining for puromycin, P-eIF2α and dsRNA of MEFs treated with poly I:C for 4h and labeled with puromycin for 1h. Scale bar, 10 µm.

C) WT and PKR−/− MEFs were stimulated for 8h with poly I:C (pI:C), thapsigargin (th) or arsenite (as). PKR and P-eIF2α were detected by immunoblot.

D) WT and PKR−/− MEFs were stimulated for 8h with poly I:C and protein synthesis was monitored like in (A). β-actin immunoblot is shown for equal loading control.

E) IFN-β levels were measured, by ELISA, in cell culture supernatants of WT, PKR−/−, eIF2αA/A and control eIF2αS/S MEFs after 4 and 8 h of poly I:C stimulation. Data are mean ± standard deviation of 3 independent experiments.

F) Protein synthesis was measured in NIH3T3 cells by puromycin incorporation after 7h of poly I:C treatment. Where indicated, a chase of 1h with fresh media was performed prior to puromycin labeling and immunoblotting. Samples with cycloheximide (chx) and arsenite (as) added respectively 5 min and 30 min before the puromycin pulse are shown as controls.

G) IFN-β was quantified by ELISA in culture supernatants in the conditions described above after 7h of poly I:C stimulation or 7h of poly I:C stimulation followed by 1h with fresh media (chase). Data are mean ± standard deviation of 4 independent experiments.
Figure 2. PKR is required for ATF4 and GADD34 expression in response to poly I:C.

A) WT and PKR\(^{-/-}\) MEFs were stimulated for 8h with poly I:C (pI:C), or the UPR-inducing drug, thapsigargin (th) for 6h. ATF4 protein expression was detected by immunoblot on nuclear extracts. Nuclear HDAC1 immunoblot is shown for equal loading control. * indicates unspecific band.

B) GADD34 mRNA levels were quantified by qPCR after 6h of poly I:C (pI:C) treatment in WT and PKR\(^{-/-}\) MEFs. For the same cell extracts, immunoblots of GADD34 (middle panel), PKR and P-eIF2\(\alpha\) (right panel) were performed. C) The same analysis was performed in eIF2\(\alpha\) A/A and control eIF2\(\alpha\) S/S MEFs. Treatment with thapsigargin (th), for 6h was used as control to induce GADD34 and P-eIF2\(\alpha\). eIF2\(\alpha\) and \(\beta\)-actin immunoblots are shown for equal loading control. Quantitative PCR data are the mean ± standard deviation of 3 independent experiments.
Figure 3. GADD34 mediates eIF2α dephosphorylation but not global translation recovery in response to poly I:C.

A) After treatment with poly I:C, protein extracts of WT and GADD34ΔC/ΔC MEFs were immunoblotted for GADD34 and P-eIF2α.

B) Protein synthesis was analyzed in WT cells treated for 1 to 6 hours with poly I:C (pI:C) alone or together with thapsigargin (th). Controls are cells not treated with puromycin (co) and cells treated with cycloheximide (chx) 5 min before puromycin incorporation.
C) Protein synthesis was analyzed in GADD34ΔC/ΔC cells treated for 1 to 6 hours with poly I:C (pi:C) alone or together with thapsigargin (th). Tubulin or β-actin immunoblot are shown for equal loading control. In GADD34ΔC/ΔC cells translation is strongly impacted by thapsigargin, but not poly I:C.
Figure 4. GADD34 is required for cytokine production in poly I:C-stimulated MEFs.

A) Left panel, immunoblot for cystatin C after treatment or not with brefeldin A (BFA) in poly I:C-stimulated WT MEFs. Arrow indicates N-glycosylated-Cystatin C. Right panel, WT and GADD34ΔC/ΔC MEFs were treated with poly I:C (pI:C), thapsigargin (th) or tunicamycin (tun)
for the indicated times. Levels of GADD34 and Cystatin C (CysC) were examined by immunoblot. β-actin immunoblot is shown as equal loading control.

**B)** Immunoblots for GADD34 and PKR in WT and GADD34-inactivated cells treated with poly I:C for the indicated periods of time. The UPR-inducing drugs, Thapsigargin (th) and tunicamycin (tun), were used as controls to induce GADD34. Immunoblot of tubulin is shown as equal loading control.

**C)** Amount of IFN-β (left panel) and IL-6 (right panel) in cell culture supernatants of WT and GADD34ΔC/ΔC MEFs after 6h of poly I:C stimulation. Mock are samples treated with lipofectamine alone. Data are mean ± standard deviation of five (IFN-β) and three (IL-6) independent experiments.

**D)** Transcription of IFN-β, IL-6, PKR and Cystatin C was analyzed by qPCR in samples of WT and GADD34ΔC/ΔC MEFs treated with poly I:C (pI:C). Mock represent samples treated with lipofectamine alone.

**E)** WT and GADD34ΔC/ΔC MEFs were transfected overnight with an expression plasmid carrying the murine GADD34 (G34) cDNA and then treated with poly I:C for 6h. IFN-β production was quantified by ELISA, left panel, in cell culture supernatants and plotted as a ratio of IFN-β to total cell proteins to compensate for different cell mortality levels induced by the transfection. In the right panel immunoblots for GADD34 and P-eIF2α in the same experimental conditions are shown. One representative analysis of 3 independent experiments is shown.
Figure 5. PKR is required to control CHIKV infection and IFN-β production in MEFs.

A) WT and PKR⁻/⁻ MEFs were infected with CHIKV-GFP at an MOI of 10 or 50, for 24h and 48h. The amount of infected cells was determined by GFP expression, left panel. Interferon β present in the cell culture supernatants was measured by ELISA, right panel. Data represented are mean ± standard deviation from 3 experiments.

B) WT (top panel) and PKR⁻/⁻ MEFs (bottom panel) were infected for 24h with CHIKV-GFP, then labeled with puromycin for 1h prior fixation. GFP-CHIKV positive (green) were visualized by confocal microscopy after staining with specific antibodies for puromycin (cyan).
and phospho-eIF2α (red). Cell Nuclei are stained with Hoechst 33258 (blue). Infection by CHIKV inhibits protein synthesis (visualized by puromycin incorporation) in WT, but not in PKR-deficient cells (arrows). In WT MEFs, eIF2α phosphorylation levels correlate with translation inhibition, although variability is observed among different infected cells, presumably due to GADD34 activity and time of infection. Non-infected WT and PKR<sup>−/−</sup> cells serve as a reference for normal translation activity and are indicated by an arrowhead. Scale bar 10 µm.
Figure 6. CHIKV infection and IFN-β production are controlled by GADD34 in MEFs.

A) WT and GADD34ΔC/ΔC cells were infected with CHIKV-GFP for a period of 24 and 48h. The percentage of infected, GFP positive, cells and viral titers were analyzed.

B) Levels of IFN-β in cell culture supernatants of WT and GADD34ΔC/ΔC MEFs infected with CHIKV-GFP for 24h and 48h.

C) Murine IFN-β was added 3h before infection of WT and GADD34ΔC/ΔC MEFs with CHIKV-GFP (10 MOI). Productive infection was estimated by GFP expression 24h after CHIKV exposure.

D) Cells were treated with guanabenz 3h before and during infection (24h) with CHIKV-GFP, percentage of infected cells (left) and corresponding IFN-β production (middle), are shown.
Immunoblot of GADD34 and P-eIF2α are shown on the right. NI stands for non-infected. Percentage of infected cells, viral titers and IFN-β measurements data represent mean ± standard deviation of 3 experiments. p values shown in (D) were obtained applying a Student’s t test.
Figure 7

A

9-day-old mice

Survivors (%)

0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21
day post infection

WT
GADD34<sup>−/−</sup>

12-day-old mice

Survivors (%)

0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21
day post infection

WT
GADD34<sup>−/−</sup>

B

day 5 post-infection of 12-day-old mice

C

day 3 post-infection of 17-day-old mice
Figure 7. CHIKV infection in mouse neonates.

A) Kaplan–Meier plots representing the survival of FVB (WT) and GADD34ΔC/ΔC mouse neonates 9-day-old (n=11 per group) (upper panel) or 12-day-old (n=14 per group) (lower panel) after intradermal inoculation with 10⁶ PFU of CHIKV and observed for 21 days.

B) Left panel, viral titers in different tissues and serum of 12-day-old mice inoculated with 10⁶ PFU of CHIKV via the intradermal route. Mice were sacrificed 5 days after infection and the amount of infectious virus in serum and tissues quantified by TCID₅₀ (see methods) (n=5). In addition of considerably increased levels of viral replication in CHIKV target tissues, GADD34ΔC/ΔC neonates also display signs of heart infection. Right panel, Quantification of IFN-β for the same different tissues, CHIKV-infected target tissues of GADD34ΔC/ΔC mice produced less IFN-β than WT.

C) 17-day-old mice were infected with 10⁶ PFU of CHIKV via the intradermal route, and sacrificed 72h later. Quantification of viral titers and IFN-β/viral titers ratio is presented for different tissues. A broken line indicates the detection threshold. In B and C represented data are arithmetic mean ± standard deviation, n=5. In B and C p values were calculated using a Student’s t test, *p≤0.1, **p≤0.05.
Figure 8

Day 5 post-infection

WT

GADD34Δ

A

B

x10

C

D

x100

E

F

x400
Figure 8. CHIKV infection causes severe myocarditis in mouse neonates.

Histological appearance of horizontal sections of the heart through left and right ventricles of 12-day FVB (A, C and E) and GADD34ΔC/ΔC mice at D5 pi (B, D and F). Normal appearance of heart of FVB infected mice, at low magnification (A, x10) with normal cardiomyocytes (C, x100) and exceptional small foci of lymphocytes (E, x400). Numerous foci of necrosis in the heart of GADD34ΔC/ΔC infected mice, at low magnification (B, x10) and extensive through the ventricular wall (D, x100). Higher magnification shows few residual cardiomyocytes (arrow head) and inflammation mainly composed of monocytes as well as extensive deposition of calcium (F, x400). The mice were inoculated with $10^6$ PFU of CHIKV via the intradermal route.
Figure S1. Poly I:C stimulation induces protein translation inhibition and IFN-β production in NIH3T3 cells.

A) Protein synthesis was quantified in poly I:C-stimulated NIH3T3 using puromycin labeling followed by immunoblot with anti-puromycin mAb 12D10. Protein synthesis was strongly reduced upon poly I:C stimulation. Immunoblot for phosphorylated (P-eIF2α) and total eIF2α
were performed on the same NIH3T3 extract. Cycloheximide (chx) was added 5 min before puromycin incorporation. β-actin immunoblot is shown for equal loading control.

**B)** Puromycin integration was analysed by immunofluorescence in NIH3T3 cells treated for 4h with poly I:C and labeled with puromycin in the last 10 min. Fluorescence intensity profiles were generated with LSM 510 Carl Zeiss MicroImaging software. Upper profile refers to the red line in the upper cell (no poly I:C); lower profile refers to the red line in the lower cells (poly I:C-transfected). The puromycin intensity (green) decreases in the presence of poly I:C (red). Data shown in (A) and (B) are representative of three independent experiments with similar results. Scale bar, 10 µm.

**C)** Protein synthesis quantification of WT MEFs treated for 8h with different doses of poly I:C. One of two independent experiments with similar results is shown.

**D)** IFN-β was quantified in cell culture supernatants of NIH3T3 cells after treatment with poly I:C.

**E)** Protein translation was monitored in the same experimental conditions by immunoblot. One of two independent experiments with similar results is shown in (D) and (E).
Figure S2. Protein translation in cells with non-phosphorylatable eIF2α.

Protein synthesis was quantified in MEFs with non-phosphorylatable eIF2α, eIF2αA/A and the corresponding control cells, eIF2αS/S. After poly I:C or thapsigargin treatment, puromycin labeling followed by immunoblot, was performed. Puromycin labeling was quantified with ImageJ software and protein translation was depicted as percentage of steady state. Cycloheximide (chx) was added 5 min before puromycin incorporation. Tubulin immunoblot is shown for equal loading control. One of two independent experiments with similar results is shown.
Figure S3. GADD34 mRNA induction in ATF4-deficient MEFs stimulated with cytosolic poly I:C. The levels of GADD34 transcript were determined by qPCR in WT and ATF4−/− cells after 8h of poly I:C stimulation. Treatment with tunicamycin and thapsigargin were used as positive controls for GADD34 induction. Results are displayed according to both WT internal reference (left) and ATF4−/− internal reference (right).
Figure S4. GADD34 mediates eIF2α dephosphorylation in MEFs stimulated with poly I:C

**A**) Wild-type and GADD34ΔC/ΔC MEFs were treated for the indicated times with poly I:C (pI:C), tunicamycin (tun) or thapsigargin (th) and eIF2α phosphorylation was monitored by immunoblot.

**B**) GADD34 expression was analyzed by immunoblot in samples treated for 1 or 6 hours with poly I:C alone or together with tunicamycin (tun) or thapsigargin (th).

Data shown in (A) and (B) are representative of three independent experiments with similar results.
Figure S5. RNA integrity upon poly I:C exposure.

WT MEFs were treated with poly I:C for the indicated times and RNA integrity evaluated by capillary electrophoresis (Agilent RNA 6000). RNA Integrity Numbers (RIN) between 8.2 and 9.2 were obtained, indicating a high level of RNA integrity. Data shown are representative of three independent experiments with similar results.
**Figure S6.** UPR-inducing drugs do not elicit IFN-β production.

Cell culture supernatants of murine embryonic fibroblasts were tested for the presence of IFN-β, after treatment with poly I:C (8h), tunicamycin and thapsigargin (6h). The results shown are representative of 4 experiments.
Figure S7. Deletion of the constitutively-expressed PP1 co-factor, CReP, does not impact protein translation and IFN-β production in MEFs.

A) WT and CReP−/− MEFs were treated with poly I:C (pI:C) for the indicated times and the levels of P-eIF2α and PKR were analyzed by immunoblot. Although basal levels of P-eIF2α were higher in CReP−/− MEFs, increase of phosphorylation upon poly I:C exposure was similar to the WT. PKR expression upon poly I:C treatment was equivalent in CReP−/− and WT MEFs.

B) Protein synthesis was quantified using puromycin labeling followed by immunoblot with the anti-puromycin mAb 12D10. Where indicated, cells were treated with cycloheximide (chx) 5 min before puromycin incorporation. No major differences were found between WT and CReP−/− cells at the level of translation inhibition following poly I:C exposure.

C) IFN-β quantification in cell culture supernatants after 8h of poly I:C (pI:C) treatment.
Data shown in this figure are representative of two independent experiments with similar results.

**Figure S8**

**Figure S8. GADD34 is necessary for IFN-β production in response to poly I:C stimulation.** WT and GADD34ΔC/ΔC MEFs were treated with poly I:C for 8h and labeled with puromycin for the last 10 min. Immunofluorescence staining for intracellular IFN-β (green), puromycin (red) and dsRNA (poly I:C, blue) was performed and samples were imaged by confocal microscopy. Scale bar, 10 µm. Data shown are representative of three independent experiments with similar results.
Figure S9. IFN-β and IL-6 polyA+ mRNAs are induced equally in WT and GADD34ΔC/ΔC MEFs in response to dsRNA.

WT and GADD34ΔC/ΔC MEFs were treated for 6h with poly I:C, total RNA extracted and poly A+ mRNAs purified on an oligo-dT column. Quantitative PCR was performed after reverse transcription. Data shown are representative of two independent experiments with similar results.
Figure S10. Specific inhibition of GADD34 with guanabenz or by RNAi decreases IFN-β production.

A) WT MEFs were treated with different doses of guanabenz (or DMSO as control) during 2 hours before being stimulated with poly I:C for 8 hours in the presence or absence of guanabenz. IFN-β levels were monitored in cell culture supernatants after the treatments. Guanabenz decreased IFN-β levels in a dose-dependent fashion. Data shown is representative of three independent experiments with similar results. B) MEFs treated with con and GADD34 siRNAs were infected with CHIKV-GFP for a period of 24h. The percentage of infected GFP positive cells and resulting IFN-β production were analyzed.
Effect of tryptophan depletion on
dendritic cells function

Several immune regulatory mechanisms have been described having as basis the depletion of amino acids (Grohmann and Bronte, 2010). Amino acids starvation induces phosphorylation of eIF2α and translation inhibition, such response could interfere with the initiation of adaptive immunity by DCs.

A transcriptional analysis performed in our lab to identify signaling pathways involved in dsRNA response in DCs showed that the levels of tryptophanyl-tRNA synthetase (WARS) mRNA were up-regulated during this process (Clavarino et al., 2012b). Accurate protein synthesis requires aminoacyl-tRNA synthetases to couple the correct amino acid onto the cognate tRNA molecule, providing the substrates for the growing polypeptide chain: the aminoacyl-tRNAs (Hausmann and Ibba, 2008). Up-regulation of WARS could in theory endow DCs with the capacity of counteracting depletion of tryptophan. Mounting an immune response to infection is energy demanding, as protein synthesis intensifies during DC maturation also does the requirement for amino acids. The availability of tryptophan becomes further relevant in the context of expression of the immunoregulatory enzyme indoleamine 2,3-dioxygenase (IDO). IDO degrades tryptophan increasing the amount of uncharged tRNA, which in T cells result in downstream phosphorylation of eIF2α by GCN2 kinase (Munn et al., 2005).

I decided therefore to study the consequences of tryptophan depletion in bone marrow-derived dendritic cells, in an in vitro culture using RPMI without tryptophan and dialyzed fetal calf serum (FCS) with a cut-off of 10,000 MW. Firstly I began by evaluating the impact of tryptophan-depleted medium in global protein translation in MEFs and Hela cells. In Hela cells substitution of the culture media by the tryptophan-depleted lines resulted in increased eIF2α phosphorylation and decrease of protein translation (Figure 1). Likewise, in WT MEFs the intensity of protein synthesis was decreased as soon as 2h after depleting tryptophan from the cell medium. Intriguingly eIF2α phosphorylation did not increase much under tryptophan starvation conditions, being that only 8h after medium change did eIF2α phosphorylation stand out (Figure 1B). The GCN2 kinase phosphorylates eIF2α when amino acids levels are decreased or
depleted thus stopping protein translation. To test if the dampening of protein synthesis observed was due to the action of this kinase, cell culture medium was replaced by tryptophan-depleted medium in GCN2 KO MEFs and eIF2α phosphorylation and protein translation evaluated (Figure 1B). Protein translation of GCN2 KO MEFs was resistant to tryptophan removal, however a decrease was observed which may account for loss of growth factors eventually during the process of dialysis of the FCS. Strangely the levels of eIF2α phosphorylation also increased in GCN2 MEFs meaning that other kinases may be at play.

In order to differentiate in vitro, bone marrow-derived DCs (BMDCs) need the growth factor GM-CSF (Inaba et al., 1992). Since mTOR signaling can be affected by growth factor and consequently influence protein translation (Zoncu et al., 2011) medium with and without GM-CSF were compared in their capacity to influence protein translation. Cells incubated in medium with GM-CSF did indeed display slightly increased protein translation intensity compared to medium without the differentiation factor (Figure 2). BMDCs differentiate in vitro for 6 days in the presence of GM-CSF and the experiments performed at day 6 of culture. Consequently at this point DCs are fully differentiated and therefore GM-CSF becomes irrelevant for differentiation proposes.

When immature DCs (iDCs) were incubated in medium without tryptophan, for 8h, a small decrease in protein translation levels was observed (Figure 2, left). Additionally, phosphorylation of eIF2α was increased under the same conditions. Stimulation of DCs with poly I:C or LPS made protein translation resistant to tryptophan starvation as no substantial differences could be appointed between cells incubated in medium with or without tryptophan (Figure 2, right). Additionally, eIF2α phosphorylation was not increased under tryptophan-depleted conditions after DC maturation (Figure 2, right). Altogether it can be concluded that mature DCs (mDCs) are able to resist tryptophan starvation in contrast to MEFs and Hela cells.

During maturation process DCs increase protein translation, which is essential for the process. When compared iDCs and mDCs, even under tryptophan-depleted conditions an increase in the intensity of protein translation can be observed (Figure 2). Another typical feature of DC maturation is the increased surface expression of CD86 and MHC class II as can be observed in Figure 3A this feature is conserved in DCs maturing in tryptophan-depleted conditions. On inflammatory stimulation, DCs accumulate newly synthesized ubiquitinated proteins in large cytosolic structures
denominated DALIS (Lelouard et al., 2002). Here I was able to show that even under tryptophan starvation these structures are still formed (Figure 3B); hinting once more that protein translation is not majorly affected since these structures are dependent on protein synthesis (Lelouard et al., 2002). Concerning cytokines, secretion of the pro-inflammatory IL-12 was not majorly impacted during tryptophan starvation (Figure 3C). It can therefore be concluded that under conditions of tryptophan depletion DC maturation is not affected.

I hypothesized if the expression of the PP1 co-factor GADD34 could help DCs maintain protein translation upon TLR activation in tryptophan-depletion conditions. Although GADD34 is expressed under these conditions (Figure 4B), we have demonstrated that the PP1 co-factor has no role maintaining global protein translation in DCs stimulated with poly I:C (Clavarino et al., 2012b). To better understand the mechanisms behind DC resistance to amino acids depletion, and the involvement of GADD34, future work should focus on transcriptional analysis of WT and GADD34-deficient DCs under tryptophan-depleted conditions.

An interesting observation made in the context of GADD34 expression in DCs was the strict regulation of this protein under tryptophan starvation conditions. Tryptophan or complete amino acids depletion from the medium did not lead to GADD34 expression in iDCs (Figure 4B). While in MEFs when tryptophan is depleted GADD34 is readily expressed (Figure 4A) but unable to avoid translational arrest (Figure 2).

During the process of maturation, DCs increase the expression of nutrient transporters (Duclos et al., 2011). We have also observed that upon TLR activation DCs mount a specific integrated stress response, during which the transcription factor ATF4 is up-regulated. ATF4 is known to be involved in the expression of amino acids transporters (Harding et al., 2003). It is therefore possible that DC activation and resistance to amino acids starvation are strongly connected.

Antigen presentation is a major function of DCs. MHC class I presentation depends largely on defective peptides, meaning that it depends on protein synthesis in a great extent (Princiotta et al., 2003; Qian et al., 2006a). To determine if the MHC I/peptide complexes were affected by tryptophan depletion the generation of MHC class I/peptide complexes was globally evaluated. DCs were acid-stripped and MHC class I/peptide recovery detected with the HB-176 mAb (Qian et al., 2006b; Lelouard et al., 2007). The HB-176 mAb recognizes conformed H-2kb molecules independently of the
nature of bound peptides (Qian et al., 2006b). Tryptophan depletion reduced slightly the ratios of MHC I/peptide complexes recovery but did not inhibit the up-regulation upon DC maturation (Figure 5).

To better understand the consequences of tryptophan starvation on DC activation of T cells, DCs were externally loaded with a MHC class I peptide (SIINFEKL) and mixed with the immortalized T cell line B3Z. T cells are very susceptible to amino acid depletions (Munn et al., 2005; Cobbold et al., 2009). Accordingly, when B3Z cells were put in medium without tryptophan protein translation almost completely ceased (Figure 6). Such observation helps to understand why when B3Z and DCs are incubated overnight in medium without tryptophan the lacZ signal is abrogated (figure 6). For this reason DCs and B3Z had to be incubated in complete medium. Therefore we can conclude that: 1) DCs are unique since contrary to other cell types are able to resist tryptophan depletion, maintaining protein translation; 2) tryptophan depletion does not impact antigen presentation, which is in agreement with the MHC I/peptide complexes present at the cell surface.

The experimental setting here used has only an in vitro base. Nevertheless the results provide important information concerning DC resistance to tryptophan starvation, which can be immunologically relevant. In the future it will be important to find a more physiological model in order to better understand the significance of the results here presented.
Figure 1. Effect of tryptophan depletion on protein translation.

Hela cells (A) and MEFs (WT and GCN2 KO) (B) were incubated in tryptophan depleted medium for different periods of time. Protein synthesis was monitored using puromycin labelling followed by immunoblot with the anti-puromycin mAb 12D10 (Schmidt et al., 2009). Immunoblots for phosphorylated (P-eIF2α) and total eIF2α were performed on the same extracts. Actin was used as loading control.
Figure 2. Effect of tryptophan depletion on protein translation of dendritic cells.
DCs were harvested and washed twice in PBS before being incubated in medium with (GM or +) or without tryptophan (-). GM, stands for medium with the growth factor GM-CSF. DCs were stimulated with 10μg/ml of soluble poly I:C (pIC) or 100ng/ml of LPS, for 8 hours. Untouched represents cells that were not washed. Protein synthesis was monitored using puromycin labeling followed by immunoblot with the anti-puromycin mAb 12D10 (Schmidt et al., 2009). Immunoblot for phosphorylated (P-eIF2α) and total eIF2α were performed on the same extracts. Cells treated with cycloheximide (CHX) 5 min prior puromycin incorporation were used as control. Actin was used as loading control.
Figure 3. DC maturation under tryptophan starvation conditions.

(A) DCs were harvested and washed twice in PBS before being incubated in medium with (Trp+) or without tryptophan (Trp-). DCs were stimulated with 10µg/ml of soluble poly I:C (pIC) or 100ng/ml of LPS for 8 hours. The surface expression of CD86 and MHC class II were analyzed by FACS.

(B) Immunofluorescence staining for CD11c (green) and poly-ubiquitin (white).

(C) IL-12 levels were measured, by ELISA, in cell culture supernatants of DCs stimulated with LPS in the presence or absence of tryptophan.
Figure 4. GADD34 expression under tryptophan starvation conditions.

Immunoblotting of GADD34 was performed on cell extracts of WT MEFs (A) and DCs (B) cultured in tryptophan-depleted medium. In (B) iDCs were incubated in medium with (Trp+) or without (Trp-) for 8h. The cells were also challenged with complete depletion of amino acids (HBSS) and mechanical stress by pipetting up and down. DCs were stimulated with 10µg/ml of soluble poly I:C (pIC). The proteasome inhibitor MG132 was added in DCs 2h before harvesting in order to enhance GADD34’s signal. The UPR-inducing drug, tunicamycin (tun), was used as controls to induce GADD34. Actin was used as loading control.
Figure 5. Effect of tryptophan depletion on the generation of MHC class I/peptide.
DCs were harvested and washed twice in PBS before being incubated in medium with (trp+) or without tryptophan (trp-). After different periods of stimulation with poly I:C, cells were again harvested and then acid washed being let to recover for 2h in the respective medium. Cells were stained with CD11c and HB-176 mAb and analyzed by FACS. The rates of MHC class I/peptide recovery were calculated for the CD11c+ population.
Figure 6. Tryptophan depletion effect on MHC class I presentation.

(A) B3Z cells were incubated either in the presence or absence of tryptophan for 8h and protein synthesis monitored using puromycin labeling followed by immunoblotting.

(B) DCs were stimulated 8h with LPS in presence or absence of tryptophan, and the MHC class I peptide, SIINFEKL was externally loaded. DCs and B3Z were mixed in a 1:4 ratio O/N. B3Z expansion was measured assessing the LacZ signal.
DISCUSSION

The capacity to sense and adapt to the environmental cues is key for DC function and initiation of the adaptive immune response. At present the body of evidence linking ER-stress response pathways and immunity is growing fast. For instance inflammation is closely linked with the UPR (Zhang and Kaufman, 2008; Hotamisligil, 2010). NFκB is a key transcriptional regulator with a central role during the onset of inflammation (Rius et al., 2008). In response to ER stress, the UPR can directly promote NFκB activation through PERK by attenuating translation. Under these circumstances the ratio the of NFκB to IκB (the inhibitor of NFκB) increases given that IκB has a shorter half-life, consequently NFκB is freed and translocates into the nucleus (Deng et al., 2004). The ER stress sensor IREα is also able to activate NFκB, when activated IREα recruits TRAF2 (TNFα-receptor-associated factor 2) which then recruits IκB kinase culminating in activation and nuclear translocation of NFκB (Hu et al., 2006). The IRE1α/TRAF2 complex can also recruit the protein kinase JNK which when activated induces expression of inflammatory genes (Davis, 2000).

The work here presented shows that DC stimulation with the dsRNA mimic, poly I:C, gives rise to a transcriptional profile reminiscent of the ATF4 branch of the integrated stress response. Transcripts of ATF4 and its downstream targets, CHOP and GADD34 were found up-regulated (Clavarino et al., 2012b). Despite the increased levels of CHOP mRNA we were unable to retrieve the protein by western blotting. During physiologic ER stress, transient CHOP expression has been suggested as beneficial, possibly by inducing the carbonic anhydrase VI which may decrease the intracellular pH during ER stress, hence avoiding the action of proapoptotic regulator Bax (Sok et al., 1999). However when the stress is permanent, expression of CHOP is prolonged and cell death induced (Rutkowski et al., 2006; Boyce and Yuan, 2006). The detrimental role of CHOP has been implicated in a number of diseases, including neurodegenerative diseases, atherosclerosis, diabetes and renal disease (Tabas and Ron, 2011; Oyadomari and Mori, 2004).

Based on our results, we reckon that DCs might have a mechanism that protects them against prolonged expression of CHOP, which is supported by the fact that we did not observe increased cell death after stimulation with poly I:C (Clavarino et al., 2012b). It has been reported that poly I:C can induce apoptosis in human breast cancer
cells in a TLR3-dependent manner (Salaun et al., 2006). We are therefore in presence of evidence pointing out a differentiated regulation of DCs in response to dsRNA compared to fibroblasts. However the mechanism responsible for the down-modulation of CHOP translation in DCs remains elusive. In macrophages substantial progress has been made concerning the regulation of CHOP levels. These multifaceted cells of the immune system have the capacity to suppress the ATF4-CHOP branch avoiding apoptosis and translational suppression of critical proteins by toll-like receptor signaling (Woo et al., 2009). Treatment with a low dose of LPS, before inducing ER stress with tunicamycin, allows the reduction of the amount of CHOP in a TRIF-dependent fashion. The mechanism is independent from upstream inducers of CHOP – phosphorylation of PERK and eIF2α. Despite GADD34 expression in the settings used by the authors, eIF2α phosphorylation is not decreased (Woo et al., 2009). In this matter we can evidence a significant difference between DCs and macrophages. As revealed by our work, DCs possess a particular pattern of eIF2α phosphorylation characterized by high phosphorylation in resting conditions and dephosphorylation upon maturation (Clavarino et al., 2012b).

It has recently been proposed that the mechanism governing CHOP’s down-regulation is centered on the regulation of translation factor eIF2B phosphorylation (Woo et al., 2012). Protein phosphatase 2A (PP2A) dephosphorylates the ε subunit of eIF2B, restoring its guanine nucleotide exchange activity, which is normally inhibited by phospho-eIF2α. Decrease of phospho-eIF2B levels may counteract the translation of ATF4 and its downstream target, CHOP (Holcik and Sonenberg, 2005).

Differently from macrophages, in DCs ATF4 translation is increased after stimulation with poly I:C, translation of ATF4 is in fact attenuated in TRIF-deficient DCs (Clavarino et al., 2012b). A relevant lead coming from both our work (Clavarino et al., 2012b) and others (Woo et al., 2009, 2012) is the apparent link between the adaptor TRIF and ATF4, establishing a connection between PAMPs detection and the ATF4 branch of the integrated stress response. TRIF has been shown to be used by TLR3 and TLR4 signaling and more recently the RNA DExD/H-box helicases DDX1, DDX21, and DHX36 (Zhang et al., 2011). Elucidating the pathways linking microbial detection to the regulation of ATF4 translation and its targets represents a major interest for future research. A first approach to this question should focus on known signaling proteins downstream of TRIF. Interfering with TANK-binding kinase 1 (TBK1) a kinase that mediates TRIF signaling (Kenny and O’Neill, 2008) could help us understand better the
signaling leading to GADD34 induction. IRF5 and IRF7 are two other signal transducers downstream of TRIF during TLR3 or TLR4 signaling (Fitzgerald et al., 2003; Takaoka et al., 2005), in macrophages ablation of IRF5 or IRF7 prevents down-regulation of CHOP levels (Woo et al., 2009).

The NADPH oxidase NOX2 plays an important role in the signaling downstream of the TLR (Matsuzawa et al., 2005). In macrophages NOX2 is intimately linked to IL-6 and IFNβ production, in response to TLR2 and TLR4 activation (Martinon et al., 2010). NOX2 is required for maturation (splicing) of XBP1, yet the process is independent of ER stress as downstream targets of XBP1 normally activated in the context of ER stress are not induced (Martinon et al., 2010). Interestingly poly I:C does not activate XBP1 splicing, thus IRE-XBP1 signaling branch is not part of the response to dsRNA (Martinon et al., 2010). In our studies the NADPH oxidase didn’t prove necessary for GADD34 expression (Clavarino et al., 2012b).

In DCs, TLR activation induces protein synthesis up-regulation therefore increasing the amount of client proteins in the ER. It is important to evaluate PERK activation upon DC maturation since there is the possibility that under these conditions PERK may phosphorylate eIF2α, thus opening a window for ATF4 translation. Upon stimulation with poly I:C the phosphorylation of eIF2α is mostly due to PKR, but PKR does not seem to participate in the elevated levels of phospho-eIF2α displayed by DCs at steady state (Clavarino et al., 2012b). The kinases involved in the initial high phosphorylation of eIF2α may therefore pre-dispose cells to activate the ATF4 pathway. It is however questionable if such mechanism would also apply for cells such as MEFs, firstly because we have clearly shown that PKR is the key activator of ATF4 and GADD34 in these cells. And second, translation is shutdown after poly I:C delivery in MEFs (Clavarino et al. unpublished).

The capacity to deal with ER stress is a key process for cell homeostasis, but in recent years it has also been reported to amplify cytokine production and concomitant protection of the host against pathogens. The entry of pathogens and their intracellular actions may condition cells to activate ER stress pathways (Sansonetti, 2006). Goodall and colleagues have reported the ER stress-induced transcription factor CHOP as essential for IL-23 induction in monocyte-derived dendritic (Goodall et al., 2010). IL-23 promoter has a CHOP-binding region responsible for the enhanced production of this cytokine during ER stress condition. Similarly, thapsigargin-induce ER stress increased the production of IFN after LPS stimulation in macrophages (Smith et al., 2008; Zeng et
This synergistic phenomenon is dependent on ER stress-induced transcription factor XBP1, which binds to a downstream region of *ifnβ1* along with IRF3 and CREB binding protein, a broad-range transcriptional co-activator (Zeng et al., 2010). XBP1 has additionally been reported to be recruited to the promoters of genes encoding some inflammatory mediators, including IL-6 and TNF in macrophages (Martinon et al., 2010).

We have made important observations linking GADD34 with cytokine production namely IFNβ and IL-6. GADD34 has a dual role in this process; it can either be at the transcriptional, as shown in DCs (Clavarino et al., 2012b), or translational level in MEFs (Clavarino et al. unpublished). GADD34 is important in the context of infection with Chikungunya virus, animals with functional GADD34 have better capacity to produce IFN and thus lower viral loads associated with a better survival outcome. Globally these observations evidence a direct transcriptional regulation of cytokines by ER stress response mediators. Tackling GADD34’s role in the regulation of transcription of IFNβ and IL-6 in DCs during response to dsRNA will require a transcriptional analysis of WT and GADD34-deficient cells, in order to have a more complete map of the pathways involved. Besides its role on eIF2α dephosphorylation other functions of GADD34 are relatively unknown.

In DCs GADD34 protein expression was observed to be subjected to strict regulation, even though the mRNA levels of GADD34 are clearly augmented during poly I:C stimulation, the protein can only be properly observed inhibiting the proteasome (Clavarino et al., 2012b). Our observation is in agreement with a previous report in human cell lines, where it has been shown that GADD34 is polyubiquitinated and this modification enhanced its degradation by 26S proteasome (Brush and Shenolikar, 2008). The tight regulation of GADD34 expression could also be observed in the context of amino acids depletion, in MEFs GADD34 is readily expressed upon tryptophan removal from the culture media. Whereas in DCs GADD34 is only expressed with TLR stimulation, immature DCs do not express GADD34 in tryptophan or even when all amino acids are depleted. Thus strengthening the link between the PP1 co-factor and the immune response.

GADD34 has been described as potentially pro-apoptotic (Boyce et al., 2005; Rutkowski et al., 2006) which may help explain the necessity of tight regulation. The evidence is provided by the fact that ER stressed GADD34-deficient cells display persistent eIF2α phosphorylation (Novoa et al., 2003) and fewer misfolded protein...
aggregates in the ER lumen (Marciniak et al., 2004). Consequently, GADD34-mediated dephosphorylation of eIF2α can be seen as pro-apoptotic, however this scenario does not comply with poly I:C-stimulated DCs since apoptosis is contained (Clavarino et al., 2012b).

From our work it remains to identify the mechanism behind cytokine regulation by GADD34. Specifically the work conducted in MEFs points to the existence of a distinct group of mRNAs efficiently translated upon dsRNA detection and dependent on GADD34 activity. Studies on GADD34 have demonstrated its presence in the cytoplasm and several membrane compartments including ER, mitochondria, and Golgi bodies (Zhou et al., 2011). Subcellular location may correlate with different functions. GADD34 owns an amphipathic helix, whose hydrophobic surface drives endoplasmic reticulum localization (Zhou et al., 2011). ER binding has been reported to attenuate the proteasomal degradation of GADD34 and modifies ER morphology, suggesting that redistribution or trafficking of GADD34 may regulate the levels of GADD34 protein and potentially control protein translation (Novoa et al., 2001; Zhou et al., 2011). The ER location of GADD34 predicts that it may regulate IFNβ and IL-6 translation at this important site of protein translation. To gain better understanding on which mRNAs are regulated by GADD34 it will be necessary to isolate the ER-bound polysomes (Stephens et al., 2005) and perform transcriptional analysis in WT and GADD34∆C/∆C MEFs treated with poly I:C.

The detection of microbial products by DCs initiates a program of “maturation” characterized by changes at the biochemical and morphological levels (Reis E Sousa, 2006). One feature of this multifaceted process is the up-regulation of metabolism-related proteins. The expression of most transporters, especially those from the solute carrier family, is increased, meaning that metabolic requirements increase during maturation (Duclos et al., 2011). Recent work suggests a strong link between inflammation and metabolism (Tannahill and O’Neill, 2011). After exposure to TLR agonists, DCs undergo a metabolic transition from oxidative phosphorylation to aerobic glycolysis (Krawczyk et al., 2010). Such metabolic shift allows DCs to produce energy rapidly and in hypoxic environments such as those encountered at sites of inflammation.

Work in our lab has shown that following contact with LPS and poly I:C protein translation increases massively in DCs (Leluard et al., 2007; Ceppi et al., 2009; Clavarino et al., 2012b). A steady supply of amino acids is therefore necessary to avoid protein synthesis shutdown which could compromise antigen presentation and the onset
of adaptive immunity. Amino acids depletion can occur under inflammatory context as part of immunosuppressor mechanisms that metabolize specific amino acids (Grohmann and Bronte, 2010), and induce differentiation of tolerogenic T cells (Cobbold et al., 2009).

Up-regulation of amino acid-transporters constitutes a strategy to avoid nutritional stress, human monocyte-derived macrophages express high-affinity transporters of tryptophan under conditions of low substrate concentration (Seymour et al., 2006). Nevertheless other mechanisms to resist amino acids depletion might exist, in our work tryptophanyl-tRNA synthetase (WARS) appeared up-regulated after poly I:C stimulation of DCs. One can hypothesize that this may help DCs to maintain protein translation and its accuracy. As has been shown in the results section, DCs are able to maintain protein synthesis in conditions where tryptophan is depleted, contrary to fibroblasts and Hela cells. However, more studies are needed to understand which mechanisms help DCs resist tryptophan depletion.

Aminoacyl tRNA synthetases catalyze the ligation of amino acids to their cognate tRNAs, but some have been associated with a range of alternative functions (Park et al., 2008). The unconventional functions of WARS are linked to an alternative spliced form, induced by IFNγ, which is involved in angiogenesis (Tolstrup et al., 1995; Wakasugi et al., 2002). WARS has also been reported to be up-regulated during tunicamycin-induced ER stress (Harding et al., 2003), but no specific function has been assigned to it in this context.

The tryptophan resistant phenotype can also be associated to GADD34. Although the PP1 co-factor is not responsible for the maintenance of global protein translation in DCs after poly I:C stimulation, it was shown to regulate a specific group of mRNAs. GADD34 is key to keep eIF2α dephosphorylated being predictable that the influence of this co-factor is not only restricted to the regulation of IL-6 and IFNβ. Transcriptional analysis aiming to elucidate GADD34’s role in immunity will surely help address the players responsible for the mechanisms allowing DCs to resist different cell stress scenarios. This work can give way to important developments in DC vaccination. The pivotal role of DCs in immunity makes them an attractive target for manipulation of immune responses (Palucka et al., 2010). However there is a number of challenges, among others the incomplete understanding of what adjuvants or DC maturation signals to use and under what conditions (Delamarre and Mellman, 2011). I consider the work developed by us in DCs (Clavarino et al., 2012b) important to deepen
the understanding of DC responses to poly I:C, a potential adjuvant for human vaccination (Caskey et al., 2011). To better achieve this goal, in the future, work must be directed towards the comprehension of GADD34 function in human DC subsets.

**PERSPECTIVE**

The work here presented has important implications in linking pathogen detection with the integrated stress response pathways. GADD34, a PP1 co-factor, associated with dephosphorylation of eIF2α, and consequent derepression of protein synthesis in the course of stress responses, gains in this work a new dimension which is the involvement in cytokine production. GADD34 can be therefore grouped together with XBP1 and CHOP, constituting a group of factors with important functions in stress-response pathways such as the unfolded protein response and the integrated stress response, and also determinant for cytokine production. The participation of GADD34 in this process remains however ambiguous, depending on the cell type, the level at which GADD34 participates differs. In MEFs, the PP1 co-factor is preponderant for the translation of IFNβ and IL-6, in response poly I:C, while in DCs the impact of GADD34 inside on the transcription of those cytokines. These observations may open a large spectrum for GADD34’s functions and targets. We can speculate that GADD34 may have functions that do not involve its canonical partner PP1, interaction with other phosphatases cannot be excluded. In macrophages the phosphatase PP2A is necessary to dephosphorylate eIF2B to allow for TRIF-dependent down-regulation of the ATF4-CHOP axis (Woo et al., 2012). Even though in DCs ATF4 translation is increased, eIF2B dephosphorylation may favor local regulation of IFNβ and IL-6 translation, namely at the ER level, where GADD34 is located (Zhou et al., 2011). Alternative functions of GADD34, not related with dephosphorylation, can be predicted from the fact that GADD34 has been shown to bind the human SNF5/INI1 protein, a member of the SNF/SWI complex that can remodel chromatin and activate transcription (Adler et al., 1999; Wu et al., 2002). This can indeed corroborate a function in cytokine transcription as anticipated by our DC work. The field of action for GADD34 remains wide. In the near future we hope to unveil more about GADD34 function in response to dsRNA by means of transcription analysis comparing WT and GADD34-deficient cells.
(MEFs and DCs). This analysis will permit to gather more information about the targets and the mechanisms of action of GADD34.

As has been shown by our work (Clavarino et al., 2012b) and others (Woo et al., 2009), the adaptor TRIF assumes a key role connecting pathogen detection with ATF4. It is important to identify the downstream targets of TRIF that influences the translation of ATF4, in order to better comprehend the connection between the immune response and the ISR.

The discovery of the involvement of GADD34 in cytokine production opens the possibility of new therapies for pathologies where excessive cytokine production constitutes a burden, such as lupus or inflammatory bowel disease. Still, more information about the full spectrum of cytokines influenced by GADD34, as well as its mechanism of action need to be better understood. Recently, guanabenz an α2-adrenergic receptor agonist used in the treatment of hypertension, has been shown to specifically inhibit GADD34 (Tsaytler et al., 2011). The fact that guanabenz is a FDA-approved drug may help explore the clinical value of GADD34.

![Figure 7](image_url)

**Figure 7:** Schematic representation of the mechanisms of GADD34 induction and action in response to dsRNA.
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**GADD34: Lien moléculaire entre la détection des pathogènes et les voies intégrées de réponse au stress**

Les cellules dendritiques (DCs) sont les plus efficaces cellules présentatrices d'antigène. La détection de motifs pathogènes, tel que lipopolysaccharides bactériens et ARNs double-brins (ARNdb) viraux, par les DCs provoque leur maturation et induit de nombreux changements morphologiques et biochimiques permettant aux DCs d’acquérir leurs puissants fonctions activatrices des cellules T. Dans ce travail, les réponses des DCs à l’ARNdb ont été analysées. Nous avons montré que, en réponse à au poly I:C, un analogue synthétique des ARNdb, les DCs montent une réponse de stress intégré spécifique au cours de laquelle le facteur de transcription ATF4 et le cofacteur de la phosphatase 1, GADD34, sont exprimés. Les DCs activées par le poly I:C présentent un profil de transcrits similaire à ce qui est produit au cours d’une ‘unfolded protein response’. GADD34 est important pour contrebalancer la phosphorylation du facteur d’initiation de la synthèse protéique eIF2α par la kinase PKR au cours de l’activation des DCs. Contrairement aux fibroblastes embryonnaires murins, les DCs résistent à l’inhibition de la synthèse des protéines induite en réponse à la stimulation avec poly I:C. Néanmoins, l'expression de GADD34 n'a pas un impact majeur sur la synthèse protéique globale. Par contre, GADD34 a été démontré être absolument nécessaire à la production d’interféron du type I et d'IL-6 par les fibroblastes et les DCs en réponse à l’ARNdb. Cette observation a des implications importantes en liant la détection des pathogènes avec les voies intégrés de réponse au stress. L'importance de ce lien a été mise en évidence par l'extrême sensibilité des fibroblastes et des souris nouveau-nées déficientes en GADD34 à l'infection par le virus du Chikungunya.

**GADD34: linking pathogen detection with the integrated stress response pathways**

Dendritic cells (DCs) are the most important antigen presenting cells. In response to inflammatory stimulation, DCs display a distinct pattern of differentiation that exhibits specific mechanisms to control the immune response. In this work the responses to dsRNA were analyzed. We have shown that in response to a mimic of dsRNA, polyriboinosinic:polyribocytidylic acid (poly I:C), DCs mount a specific integrated stress response during which the transcription factor ATF4 and the growth arrest and DNA damage-inducible protein 34 (GADD34), a phosphatase 1 (PP1) cofactor, are expressed. GADD34 is important to counteract phosphorylation of eIF2α by PKR. In contrast to murine embryonic fibroblasts (MEFs), DCs resist to protein synthesis inhibition induced in response to cytosolic dsRNA. Nevertheless, GADD34 expression does not have a major impact on global protein synthesis. Importantly, GADD34 was shown to be absolutely required for type I-IFN and IL-6 production by MEFs and DCs in response to dsRNA. This observation has important implications in linking pathogen detection with the integrated stress response pathways. The importance of this link is further underlined by the extreme susceptibility of GADD34-deficient fibroblasts and neonate mice to Chikungunya virus infection.

**Discipline:** Immunologie

**Mots-clés:** cellules dendritiques, GADD34, poly I :C, eIF2α, traduction

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