



DOTTORATO DI RICERCA IN
MICROBIOLOGIA MEDICA ED IMMUNOLOGIA
XIX CICLO

**Toll-like Receptor-mediated induction of type
I Interferons promotes functional
modifications and changing in the gene
expression profile along Dendritic Cell
maturation.**

Martina Severa

Tutor:
Dr. E.M. Coccia

Coordinators:
Prof. E. Garaci
Prof. C.F. Perno

Tesi per il conseguimento del titolo
Anno Accademico 2005-2006

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SUMMARY

Toll-like Receptors (TLR) interact with a growing list of pathogen-derived products and these interactions drive the activation of innate and adaptive immune responses. Dendritic cells (DC) play a key role in these events expressing a heterogeneous repertoire of TLR. Immediately after contacting the pathogens, DC undergo a process termed maturation modifying their phenotypical features and producing cytokines that regulate the immune responses. Among the released cytokines, type I Interferons (IFN) play a crucial immunoregulatory role for their ability to modulate several DC functions in an autocrine and paracrine fashion.

The main objective of this thesis has been to characterize the specific expression profile of the different type I IFN subtypes induced in DC following virus infection or TLR-triggering and to dissect the type I IFN-related functional modifications and changes in the transcriptome along DC maturation.

- We found that infection of DC with Influenza A virus (Flu) or Sendai Virus (SV) or stimulation with TLR3 and TLR4 agonists cause a selective release of the type I IFN subtypes. In particular, while all type I IFN were induced by virus infection, TLR3 and TLR4 triggering promotes mainly IFN- β expression suggesting a crucial role of this cytokine in the maturation process of DC.

- Since LPS induced IFN- β as a “signature cytokine” we characterized the IFN- β -related gene expression profile specifically induced in DC along LPS-mediated maturation. Interestingly, a specific set of genes, involved in anti-viral and anti-bacterial immune responses, was found to be IFN-regulated.

- We first characterized the IFN-dependent regulation of Viperin (*virus inhibitory protein, endoplasmic reticulum-associated, interferon- γ inducible*), a novel antiviral gene whose function is still unclear. TLR3 and TLR4 ligation highly induced Viperin *via* the TRIF/TBK1/type I IFN receptor (IFN α/β R) axis, while SV-induced Viperin gene expression was mediated independently of TLR signaling by the RNA helicase Retinoic acid-inducible gene (RIG-I) and the downstream adapter, *mitochondrial anti-viral signaling* (MAVS). We identified as the key regulator of

Viperin gene expression the IFN-stimulated gene factor (ISGF)-3 complex, whose action is counter-regulated by the positive regulatory domain I-binding factor 1 (PRDI-BF1, also called BLIMP1), a transcriptional repressor that blocks Viperin expression competing with the ISGF3 binding to the IFN stimulatory and regulatory element (ISRE) sites present within the Viperin promoter.

- Also the TLR7 gene expression was induced during LPS-induced DC maturation in a type I IFN-dependent manner. We characterized the mechanisms responsible for the activation of TLR7 gene. The induction of TLR7 in maturing DC was mainly a consequence of the transcriptional activity of IRF-1, whose binding site was located within TLR7 promoter. Moreover, we also demonstrated that “priming” with exogenous IFN- β of immature DC, that usually express TLR8 but not TLR7, induced a functionally active TLR7. In fact, treatment with the TLR7 specific synthetic ligand 3M-001 up-regulated the expression of costimulatory molecules together with the production of pro-inflammatory as well as regulatory cytokines.

These data suggest that TLR4-mediated type I IFN release activates specific transcription programs in DC amplifying the expression of pathogen sensors to correctly and combinatorially respond to a bacterium- as well as virus-host interaction.

ABBREVIATIONS

α -IFNs Abs: Antibodies neutralizing type I Interferons

BM: bone marrow

CARD: caspase recruitment domain

CMV: cytomegalovirus

DC: Dendritic Cells

ds: double stranded

Flu: Influenza Virus

HAU: hemagglutination units

HCV: Hepatitis C virus

HSV: Herpes simplex Virus

IFN: Interferons

IKK: inhibitory protein κ B kinase

IL: interleukin

IRAK: IL-1R-associated kinase

IRF: IFN-regulatory factors

ISG: IFN-stimulated gene

ISGF3: IFN-stimulated gene factor 3

ISRE: IFN-stimulatory and regulatory element

JAK: Janus kinase

KO: knock out

LPS: lipopolysaccharide

LRR: leucine-rich repeats

Mal: MyD88 adapter-like

MAVS: mitochondrial anti-viral signaling

Mda-5: Melanoma differentiation associated antigen -5

Mef: murine embryonic fibroblast

MFI: median fluorescent intensity

MHC: major histocompatibility complex

MyD88: myeloid differentiation factor 88
Poly (I:C): polyinosinic-polycytidylic acid
PRDI: positive regulatory domain I
PRDI-BF1/BLIMP1: PRDI-binding factor 1/ B-lymphocyte induced maturation protein 1
PRR: pattern recognition receptor
RIG-I: Retinoic acid inducible gene I
RIP: Receptor Interacting Protein
ss: single stranded
STAT: signal transducer and activator of transcription
TBK1: TANK-binding kinase 1
TIR: Toll/IL-1 Resistance
TLR: Toll-like receptor
TRAM: TRIF-related adapter molecule
TRAF: TNF receptor-associated factor
TRIF: TIR-domain containing adapter inducing interferon- β
TSS: Transcription Starting Site
SOCS: suppressor of cytokine signaling
STAT: signal transducer and activator of transcription
SV: Sendai virus
TFBS: transcription factor binding site
TNF: tumor necrosis factor
Viperin: virus inhibitory protein, endoplasmic reticulum-associated, interferon-g inducible
VRE: virus-inducible enhancer sequence

INTRODUCTION

Dendritic Cells.

The immune system is a highly effective and dynamic organization specialized to protect a host from dangerous pathogens. Indeed, it distinguishes not only self from foreign structures but also harmful foreign antigens from innocuous antigens to prevent nonessential and self-destructive immune responses. A highly balanced immune system for the generation of antigen-specific effector cell responses as well as antigen-specific tolerance induction is mandatory for maintaining tolerance to self antigens as well as a protective immunity to pathogens.

Dendritic cells (DC) are professional antigen presenting cells that are a central component of the immune system for their extraordinary capacity to stimulate naïve T cells and initiate primary immune responses. They are continuously produced from hematopoietic stem cells in the bone marrow and, in their immature state, are strategically positioned at potential pathogen entry sites as the epidermidis, mucosal epithelia and the interstitial connective tissue of non-lymphoid organs (Banchereau and Steinman, 1998; Cella et al., 1997; Moser, 2001; Reis e Sousa et al., 1999). Immature DC including epidermal Langerhan's cells, splenic marginal zone DC and interstitial DC within non-lymphoid tissues, continuously sample self-antigen to maintain T cell self-tolerance (Banchereau and Steinman, 1998).

Immature DC play a crucial role in the surveillance of peripheral tissues by migrating through all the tissues and actively taking up foreign antigens. At this stage, DC display several features that allow them to capture antigens. First, they can take up particles and microbes by phagocytosis (Inaba et al., 1993; Reis e Sousa et al., 1993). Second, they can form large pinocytic vesicles in which extracellular fluid and solutes are sampled, a process called macropinocytosis (Sallusto et al., 1995). And third, they express receptors that mediate adsorptive endocytosis, including C-type lectin, like the mannose receptor, DEC-205 and DC-

SIGN, which recognize carbohydrate structures on pathogens (Figdor et al., 2002; Jiang et al., 1995). Once in contact with antigen, immature DC utilize several pathways to facilitate uptake. These include receptor-mediated endocytosis through C-type lectins and FcγII/IIIIR (Sallusto et al., 1995). Another class of receptors expressed on immature DC are the pattern recognition receptors (PRR), which specifically recognize pathogen-related molecules and are heterogeneously expressed by the different populations of immature DC. Toll-like Receptors (TLR; see next paragraph) are the best characterized class of PRR in the mammalian species. Studies on different DC subsets revealed distinct TLR expression patterns. Freshly isolated human plasmacytoid DC (pDC) express TLR7 and TLR9, whereas human myeloid CD11c⁺ DC (mDC, also called conventional DC) express TLR1, 2, 3, 4, 5, 6 and 8 (Hornung et al., 2002; Jarrossay et al., 2001; Kadowaki et al., 2001b). In some studies, TLR7 expression was detected on both pDC and conventional DC, even if at very low level (Ito et al., 2002; Krug et al., 2001), whereas others found TLR7 exclusively expressed in pDC (Jarrossay et al., 2001; Kadowaki et al., 2001b).

Once DC have captured an antigen, their characteristics rapidly change together with the repertoire of expression of surface molecules. This process is termed *maturation*. Maturation is a terminal differentiation process that transforms DC from cells specialized for antigen capture into cells specialized for T-cell stimulation. The events, that take place during the maturation process, are essential for understanding the control of immunity and tolerance (Banchereau and Steinman, 1998). Maturing DC rapidly lose endocytotic activity and increase the synthesis and transport of major histocompatibility complex (MHC) class I and II molecules leading to a rapid accumulation of MHC molecules on the cell surface (Cella et al., 1999; Inaba et al., 1998). This immense redistribution is accompanied by a strong up-regulation of chemokine receptors of the CCRX family, several costimulatory molecules including CD80, CD86 and CD40 (Banchereau and Steinman, 1998) and also express several adhesion molecules including ICAM-1 (CD54), ICAM-3 (CD50), LFA3 (CD58). These properties are thought to enable

mature DC to attract and cluster with naïve T lymphocytes as has been observed *in vivo* (Ingulli et al., 1997). The distinctive functional properties of DC are strictly dependent on the maturing and modulating stimuli produced in the local environment (Steinman et al., 2003). For instance, induction of the differentiation of subtype 1 helper T-cells (Th1) versus Th2 is mediated by 2 groups of signals. Microbial pathogen–derived signals induce several proinflammatory cytokines, including type I Interferons (IFN), tumor necrosis factor (TNF)- α , Interleukin (IL)-12 and IL-1 that promote Th1 responses (Kalinski et al., 1999; Proietto et al., 2004). In contrast, pathogens such as fungi, schistosomes, and cholera toxin, as well as anti-inflammatory cytokines such as IL-10 and transforming growth factor- β , provoke DC to induce Th2 responses (Bozza et al., 2002; Gagliardi et al., 2000; Kalinski et al., 1999; MacDonald et al., 2002). For an overview on the events occurring along DC maturation see *Figure A*.

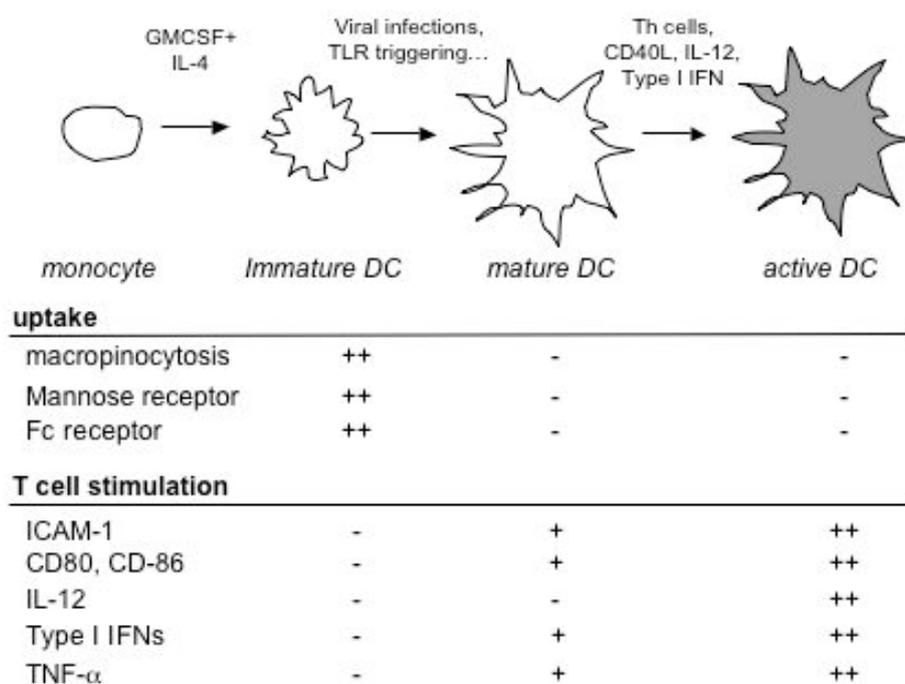


Figure A. From monocyte progenitors to mature DC.

Immature DC play a crucial role in the surveillance of peripheral tissues and actively taking up foreign antigens. Once DC have captured an antigen they undergo a process termed maturation where their features rapidly change together with the expression pattern of surface molecules and the release of regulatory cytokines.

The released cytokines can act in autocrine and paracrine fashion on DC modulating many aspects of the immune response. It has been shown that type I IFN may induce the expression of certain chemokines genes, such as CXCL-9, CXCL-10, and CXCL-11 (Lande et al., 2003a; Matikainen et al., 2000; Moser and Loetscher, 2001) involved in recruitment and selective homing into inflamed tissues of activated/effector immune cells, such as T and Natural Killer cells. Functional maturation culminates with DC residing in T cell-rich areas of lymphoid tissues presenting peptide antigens acquired in the periphery in the context of MHC to passing T cells. Mature DC are terminally differentiated and typically short lived. Presumably the DC that enter lymph nodes die, unless they encounter cognate T cells. Indeed, the life of mature DC, expressing high levels of CD40, can be prolonged by interaction with CD40 ligand localized on the surface on T cells (Caux et al., 1994).

TLR family members

The strategy of pathogen identification is based on pattern recognition, the detection of a limited set of conserved molecular patterns that are unique in the microbial world (Janeway and Medzhitov, 1998). Pathogen patterns (the so-called pathogen-associated molecular patterns or PAMPS) are detected by PRR. Many types of PRR are expressed on DC, including C-type lectins, mannose receptors, and TLR, thus enabling the identification of various types of microbial antigens (Geijtenbeek et al., 2004; Iwasaki and Medzhitov, 2004).

The discovery of the TLR represents a key milestone in understanding how cells recognize and react to invading pathogens (Janeway and Medzhitov, 2002). At present, 13 TLR have been identified: TLR1-9 are common to mouse and human, while TLR10 is unique to humans and TLR11-13 are unique to the mouse (Tabeta et al., 2004; Takeda et al., 2003; Zhang et al., 2004).

Several lines of evidence indicate that the TLR involved in the recognition of molecular structures unique to bacteria and fungi (TLR1, TLR2, TLR4, TLR5,

TLR6) are localized to the plasma membrane and can be recruited to the phagosome, whereas the TLR, that detect viral and bacterial nucleic acids (TLR3, 7, 8 and 9), are localized in the endosomal compartment (*Figure B*).

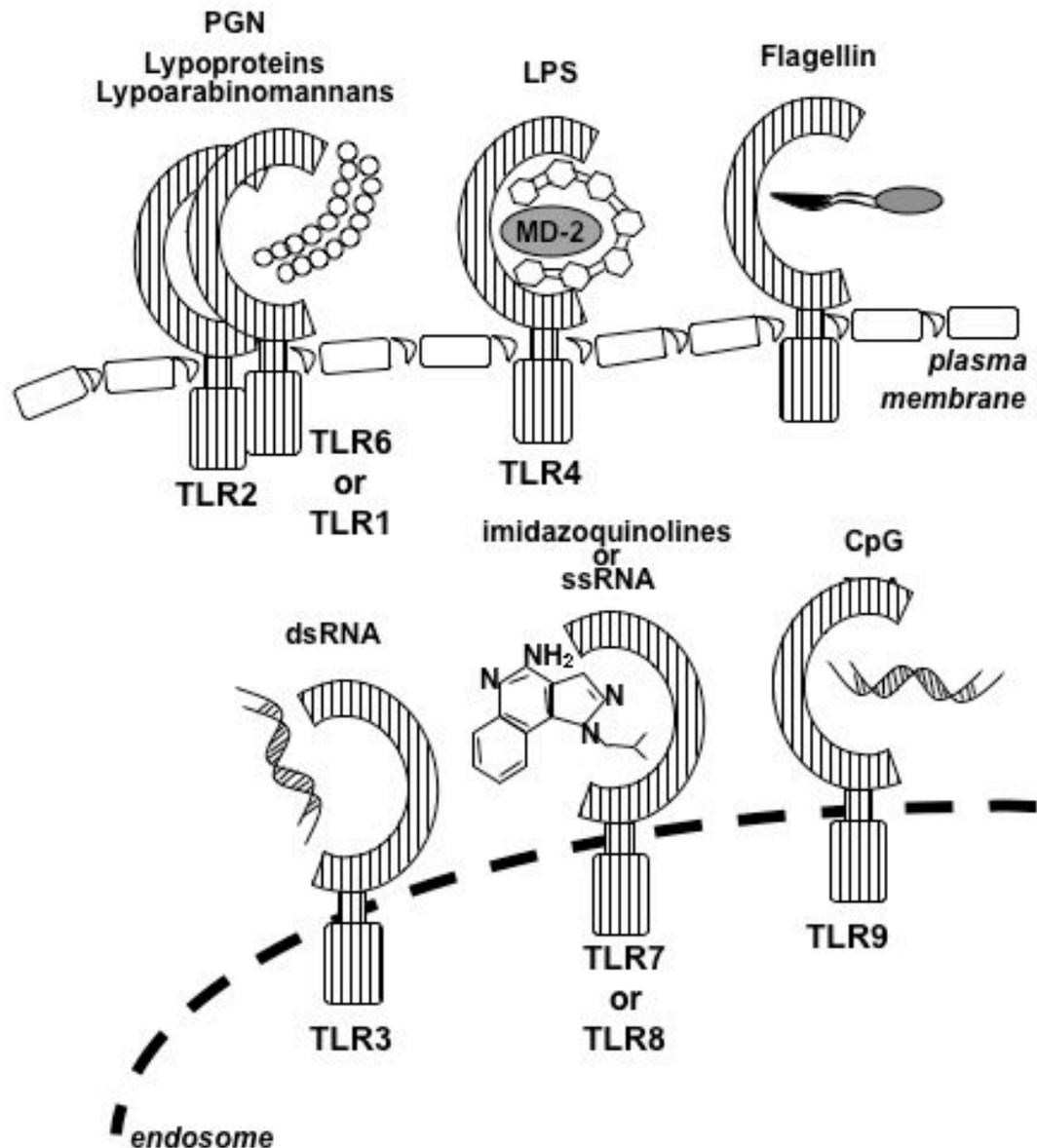


Figure B. TLR family members.

Different pathogen-associated molecular patterns selectively stimulate different members of the TLR family localized in different cellular compartments. TLR involved in the recognition of molecular structures unique to bacteria and fungi (TLR1, TLR2, TLR4, TLR5, TLR6) are localized to the plasma membrane, whereas the TLR that detect viral and bacterial nucleic acids (TLR3, 7, 8 and 9) are localized in the endosomal compartment.

The first evidence of TLR in the recognition of pathogens was reported from studies with mice carrying a point-mutated or disrupted *Tlr4* gene (Hoshino et al., 1999; Poltorak et al., 1998). These mice are unresponsive to bacterial lipopolysaccharide (LPS), an integral component of the outer membranes of Gram-negative bacteria that can cause endotoxin shock. Subsequently, the generation of knockout mice for each TLR gene has revealed respective pathogens that can be recognized by each TLR. TLR2 is involved in the responses to a variety of bacterial components that include peptidoglycan, lipoproteins/lipopeptides, glycosyl-phosphatidylinositol anchors from *Trypanosoma cruzi*, and zymosan (Takeuchi and Akira, 2002; Takeuchi et al., 1999; Takeuchi et al., 2000). However, recognition of these TLR2 ligands requires another TLR family member. The mycoplasmal diacylated lipopeptide Pam2csk4 or MALP-2 is recognized by a heterodimer of TLR2 and TLR6, whereas the bacterial triacylated lipopeptide Pam3csk4 is recognized by a heterodimer of TLR2 and TLR1 (Takeuchi et al., 2001; Takeuchi et al., 2002). Flagellin, a 55 kDa monomer obtained from bacterial flagellum, the polymeric rod-like appendage extending from the outer membrane of Gram-negative bacteria, is also a potent pro-inflammatory inducer, which is recognized by TLR5 (Hayashi et al., 2001).

Moreover, a number of viral products are sensed by the cells of the innate immune system and, among them, double-stranded (ds) RNA is a common signature of viral replication and is generated in infected cells by most (if not all) viruses. In 2001 it was described for the first time that TLR3 mediates responses to Polyinosinic-polycytidylic acid [poly (I:C)], a synthetic analogue of dsRNA. Indeed TLR3 knockout mice were resistant to poly (I:C)-induced shock compared to wild-type mice (Alexopoulou et al., 2001). TLR3 has been implicated in the immune response to several viruses. TLR3 controls inflammatory cytokine and chemokine production in Respiratory Syncytial Virus (RSV)-infected cells (Rudd et al., 2005). RSV-induced CXCL-10 and CCL-5 production, but not CXCL-8 production or viral replication, were shown to be impaired in the absence of TLR3. Hoebe *et al.* reported that mice homozygous for the *Lps2* mutation, a distal frameshift error in

TRIF, are hypersusceptible to mouse cytomegalovirus (CMV) (Hoebe et al., 2003), and a role for TLR3 in the response to mouse CMV was confirmed using TLR3 knockout mice (Tabeta et al., 2004). TLR7 and TLR8 have also been shown to recognize viral nucleic acids. Firstly, TLR7 and 8 were shown to trigger IFN production in response to the imidazoquinolines, imiquimod and resiquimod (or R-848). These are low molecular weight immune response modifiers with potent anti-viral and anti-tumor properties that are used clinically in the treatment of external genital warts caused by human papilloma virus infection (Hemmi et al., 2002). Furthermore, TLR7 and 8 sense guanosine- and uridine-rich single-stranded (ss) RNA oligonucleotides derived from human immunodeficiency virus-1 (HIV-1) and synthetic ssRNA (polyU) or ssRNA derived from wild-type Influenza virus (Diebold et al., 2004b; Heil et al., 2004).

TLR9 recognizes unmethylated 2'-deoxyribo(cytidine-phosphate-guanosine) (CpG) DNA motifs commonly present in bacterial and viral genomes that have immunostimulatory activities (Hemmi et al., 2000). The CpG motifs are also found in abundance in some viral genomes, such as the dsRNA virus, Herpes simplex Virus (HSV). The pDC, which express TLR9, respond to HSV-1 by secreting high levels of type I IFN, releasing IL-12 and up-regulating co-stimulatory molecules (Dalod et al., 2002) and the pDC responsiveness to HSV-1 *in vitro* is indeed TLR9-dependent (Krug et al., 2004). It is highly likely that other large DNA viruses whose genomes are rich in CpG motifs are also recognized by TLR9. Indeed, only very recently, Basner-Tschakarjan *et al.* reported that the dsDNA virus, Adenovirus efficiently activates pDC in a TLR9-dependent manner, resulting in maturation and IFN- α production (Basner-Tschakarjan et al., 2006). Furthermore, it has recently been shown that TLR11, which is abundant in the kidney and bladder, senses uropathogenic bacteria (Zhang et al., 2004).

TLR-dependent intracellular pathways

TLR are transmembrane proteins and their extracellular domains contain a repetitive structure rich in leucine residues, the leucine-rich repeats (LRR), that are involved in ligand recognition. The intracellular region includes a common structure to all TLR and IL-1 receptor family members, and is referred to as the Toll/IL-1 Resistance (TIR) domain, which is essential for signal transduction. Every TLR triggers a specific cellular activation program via the recruitment of different combinations of specific adaptor molecules to its TIR domain. These adaptors include myeloid differentiation factor 88 (MyD88) (Muzio et al., 1997), MyD88 adapter-like (Mal) (Fitzgerald et al., 2001) (also called TIRAP (Hornig et al., 2001), TIR-domain containing adapter inducing interferon- β (TRIF) (Yamamoto et al., 2002) (Hoebe et al., 2003) (also called TICAM1 (Oshiumi et al., 2003a) and TRIF-related adapter molecule (TRAM) (Fitzgerald et al., 2003b) (also called TICAM2 (Oshiumi et al., 2003b). Only recently, another TIR-domain containing adapter has been described, SARM (SAM- and ARM-containing protein), which contains sterile alpha (SAM) and HEAT/Armadillo (ARM) motifs, as well as a TIR domain (Liberati et al., 2004). SARM has recently been shown to act as a negative regulator of TLR signaling (Carty et al., 2006).

The recruitment of these TIR-domain containing adapters to the TIR domain of activated TLR leads to the activation of several transcription factors, including NF- κ B, ATF2/c-Jun, and the IFN-regulatory factors (IRF), with the subsequent induction of type I IFN and IFN-dependent responses (*Figure C*).

Among the five different adapter molecules containing the TIR domain, MyD88 was the first identified and shown to be critical for TLR and IL1R family signaling (Kawai et al., 1999). MyD88 can associate with all TLR (Medzhitov et al., 1998) with the exception of TLR3 (Oshiumi et al., 2003a; Yamamoto et al., 2003). MyD88 has an amino terminal death domain and a carboxy-terminal TIR domain. The TIR domain is involved in the interaction with TLR and other adapters (see

below) while the death domain associates with members of the IL-1R-associated

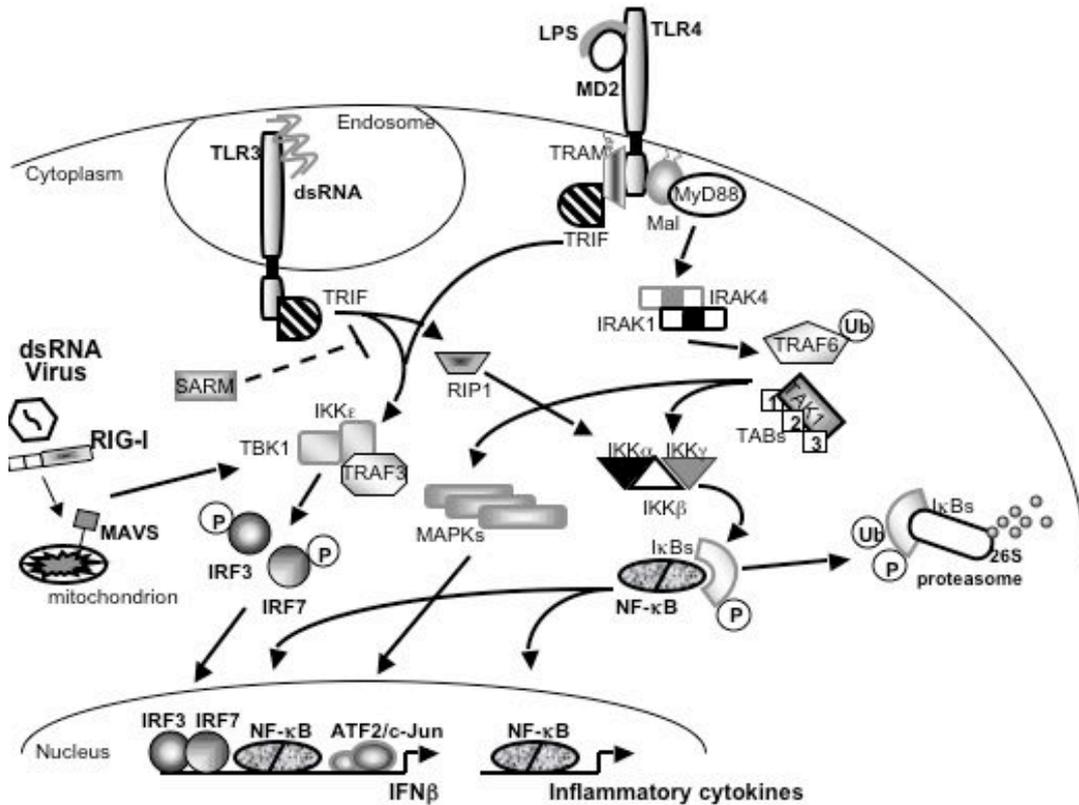


Figure C. TLR-dependent and -independent signaling pathways.

Stimulation of TLR or RNA helicases (see next paragraph) by specific ligands triggers different intracellular signaling pathways leading to the activation of NF- κ B, ATF-2/c-Jun and IRFs. These transcription factors control the transcription of inflammatory cytokines and type I IFN.

kinase (IRAK) family (Martin and Wesche, 2002). IRAK-1 is recruited to MyD88 via DD-DD interactions within a complex with another protein termed Toll-interacting protein (Tollip) (Burns et al., 2000). This IRAK1-MyD88 association triggers hyper-phosphorylation of IRAK1 by itself as well as phosphorylation by the related kinase, IRAK-4 (Cao et al., 1996; Li et al., 2002). These events lead to the dissociation of IRAK1 from MyD88 and Tollip and its interaction with the downstream adaptor TNF receptor-associated factor 6 (TRAF-6) (Burns et al., 2000). TRAF-6, a RING domain ubiquitin ligase activates the TAK1 kinase through K63-linked polyubiquitination [reviewed in (Chen, 2005)]. TAK1 in turn activates the inhibitory protein κ B kinase (IKK) complex, which phosphorylates I κ Bs and targets these NF- κ B inhibitors for ubiquitination and degradation by the

proteasome. NF- κ B is then released and translocates to the nucleus where it can induce several hundred target genes (Medzhitov et al., 1997; O'Neill, 2002).

The diversity of TLR signaling pathways was revealed following the analysis of the response of MyD88-deficient macrophages to Gram negative bacteria-derived LPS (Kawai et al., 1999). LPS which signals via TLR4 and MD2 can still trigger the activation of NF- κ B and MAPK in cells from MyD88 knockout mice, albeit with delayed kinetics compared with wild type cells, whereas most other TLR ligands are completely ineffective at triggering these events in the absence of MyD88. Although MyD88-deficient mice lose their ability to induce proinflammatory cytokines in response to LPS, they are still able to upregulate co-stimulatory molecules and induce type I IFN and IFN-stimulated genes (ISGs) (Kaisho et al., 2001; Kawai et al., 2001). Subsequent studies from several groups identified another adapter, TRIF, which regulates these MyD88-independent pathways (Fitzgerald et al., 2003b; Hoebe et al., 2003; Yamamoto et al., 2003). TRIF knockout mice are compromised in the induction of type I IFN and the expression of ISGs in response to LPS and the dsRNA mimetic poly(I:C), a TLR3 ligand. Both TLR4 (Navarro and David, 1999) and TLR3 (Fitzgerald et al., 2003b; Oshiumi et al., 2003a; Yamamoto et al., 2002) signaling cascades activate the nuclear translocation and DNA binding of the transcriptional regulator, IRF3, a key regulator of IFN- β and ISGs, a process mediated solely by TRIF in the case of TLR3 signaling (Fitzgerald et al., 2003b; Hoebe et al., 2003; Yamamoto et al., 2003). In the case of TLR4 signaling an additional adapter, TRAM is also required to recruit TRIF to TLR4 (Bin et al., 2003; Fitzgerald et al., 2003b; Oshiumi et al., 2003b). TRAM is modified by N-terminal myristoylation, which is important in tethering TRAM to the plasma membrane, where it co-localizes with TLR4 (Rowe et al., 2006). This function of TRAM appears to be important in recruiting TRIF to membrane localized TLR4. A fourth adapter molecule Mal also participates in TLR4 signaling. In contrast to TRIF and TRAM, however, Mal appears to be important in the recruitment of MyD88 to TLR4 to regulate inflammatory cytokine genes (Fitzgerald et al., 2001; Horng et al., 2001) (*Figure C*).

TLR3-mediated NF- κ B activation is also triggered by a TRIF-dependent mechanism. The C-terminus of TRIF associates with the serine threonine kinase Receptor Interacting Protein-1 (RIP1) through a RIP homotypic interaction motif (Meylan et al., 2004). RIP-1 deficient cells fail to activate NF- κ B in response to poly (I:C) (Meylan et al., 2004), whereas IRF3 activation remains intact (Cusson-Hernance et al., 2005). The TRIF N-terminal region has also been shown to associate with TRAF6 in overexpression systems (Sato et al., 2003). Studies using macrophages from TRAF6-deficient mice however, suggest that the exact requirement for TRAF6 in the TLR3 response to NF- κ B is still a little unclear, probably due to functional redundancy with other TRAF proteins in certain cell types (Gohda et al., 2004). TAK-1 is also involved in TLR3-mediated NF- κ B and MAPK activation (Sato et al., 2005). Recent studies have also shown that TRIF and MyD88 can bind to a second TRAF family member TRAF3, which activates the IRF factors to induce type IFN. However, TRAF3 does not appear to be required for the induction of proinflammatory cytokines (Hacker et al., 2006; Oganessian et al., 2006) (*Figure C*).

Recognition of virus by RNA helicases

Recently, it has been described that there are also TLR-independent mechanisms of virus sensing and the search for additional anti-viral sensors has led to the identification of a second dsRNA sensing system, localized in the cytoplasm. This second dsRNA sensing system is mediated by the DExD/H box RNA helicases, Retinoic acid inducible gene (Rig-I) (Yoneyama et al., 2004) and Melanoma differentiation associated antigen -5 (Mda-5) (Andrejeva et al., 2004; Gitlin et al., 2006; Kato et al., 2006). The RIG-I gene encodes a protein of 925 amino acids that contains an N-terminal region characterized by the presence of two caspase recruitment domains (CARD) and a C-terminal region harboring ATP-dependent RNA helicase activity. Mda-5 has a similar structure to RIG-I, containing two

CARD domains and a C-terminal RNA helicase domain (Andrejeva et al., 2004). RIG-I and Mda-5 have been shown to activate both NF- κ B and IRF3 via the N-terminal CARD domain (Yoneyama et al., 2005; Yoneyama et al., 2004). RIG-I and Mda-5 activate downstream signaling *via* CARD domains, which engage MAVS (*mitochondrial anti-viral signaling* (Seth et al., 2005), also called IPS1 (Kawai et al., 2005) or CARDif (Meylan et al., 2005) or VISA (Xu et al., 2005)) a CARD-domain containing adapter via homotypic interactions. Importantly, MAVS is localized to the outer mitochondrial membrane via a transmembrane domain at its C-terminus and it initiates the downstream signaling, culminating in the activation of NF- κ B, MAPK and IRF pathways (*Figure C*).

Recently genetic evidence has revealed that RIG-I and Mda-5 discriminate between different classes of RNA viruses (Kato et al., 2005; Kato et al., 2006). RIG-I is required for triggering anti-viral responses against Newcastle disease virus, Sendai virus (SV) and Vesicular stomatitis virus (VSV) (Kato et al., 2005), whereas Mda-5 is required for the response against encephalomyocarditis virus (Gitlin et al., 2006; Kato et al., 2006), and probably other picornaviruses that are pathogenic to humans including poliovirus, rhinovirus and Coxsackie B virus. Furthermore, RIG-I and Mda-5 discriminate between *in vitro* transcribed dsRNA and poly (I:C), respectively (Kato et al., 2006). Therefore RIG-I and Mda-5 recognize different RNA structures and are critical for the elimination of RNA viruses *in vivo*.

Type I IFN

Interferons were discovered 50 years ago by Isaacs and Lindenmann as soluble proteins released by almost all cell types upon viral infections and capable of “interfering” with virus replication (Isaacs and Lindenmann, 1957). IFN are cytokines that exert antiviral, antiproliferative and immunomodulatory effects. Because of these important properties, in the past two decades, major research

efforts have been undertaken to understand the signaling mechanisms through which these cytokines induce their effects.

The IFN family includes two main classes of related cytokines: type I and type II IFN (Pestka et al., 2004). There are many type I IFNs, all of which have considerable structural homology. These include IFN- α (which can be further subdivided into 12 different subtypes, IFN- α 1/13, - α 2, - α 4, - α 5, - α 6, - α 7, - α 8, - α 10, - α 14, - α 16, - α 17 and - α 21), IFN- β , IFN- δ , IFN- ϵ , IFN- κ , IFN- τ and IFN- ω and IFN- ζ (or Limitin) (Pestka, 1997). IFN- α s, IFN- β , IFN- ϵ , IFN- κ and IFN- ω exist in humans, whereas IFN- ζ has been described only for mouse and IFN- δ and IFN- τ only for pigs and cattle, respectively, and do not have human homologues. The genes that encode type I IFN are clustered on chromosome 9 in humans and on chromosome 4 in mice (Chen et al., 2004). All type I IFNs bind a common cell-surface receptor, which is known as the type I IFN receptor or IFNAR (see below). Conversely, there is only one type II IFN, IFN- γ . The gene that encodes this cytokine is located on chromosome 12 in humans and chromosome 10 in mice, and the protein does not have marked structural homology with type I IFN (Parmar and Platanius, 2003). IFN- γ binds a different cell-surface receptor, which is known as the type II IFN receptor or IFNGR (Bach et al., 1997; Pestka et al., 1997). IFN- γ is a markedly different cytokine than the type I IFN, but it was originally classified in the IFN family because of its antiviral properties (Isaacs and Lindenmann, 1957). Recently, a new class of IFN or IFN-like molecules has emerged, the IFN- λ s: IFN- λ 1, - λ 2 and - λ 3, which are also known as interleukin-29 (IL-29), IL-28A and IL-28B, respectively (Kotenko et al., 2003). They also have antiviral properties, but they are distinct from the type I and type II IFNs and bind a different cell-surface receptor, which is composed of two chains, IFNLR1 (also known as IL-28 receptor- α , IL-28R α) and IL-10R β . IFN- λ s might ultimately be classified and accepted as type III IFN (*Table I*).

Table I

The IFN: a heterogeneous family

Type I	Species(mammals)	Function
12 IFN- α s (- α 1/13, - α 2, - α 4, - α 5, - α 6, - α 7, - α 8, - α 10, - α 14, - α 16, - α 17, - α 21)	All	Immune system, antiviral effects
IFN- β	All	Immune system, antiviral effects
IFN- δ	Pigs	reproductive system
IFN- ϵ	Eutherian mammals	reproductive system
IFN- κ	All (?)	Immune system
IFN- τ	Ungulates	reproductive system
IFN- ω	Eutherian mammals	Immune system
IFN- ζ	Mouse	Immune system
Type II	Species(mammals)	Function
IFN- γ	All	Immune system
Type III	Species(mammals)	Function
3 IFN- λ s (- λ 1, - λ 2, - λ 3)	All (?)	Immune system, antiviral effects

Apart from the antiviral effects, type I IFN may also exert antiproliferative, cytotoxic, or anti-tumoral effects depending on the cell type (Belardelli and Ferrantini, 2002; Biron et al., 1999; Stark et al., 1998), and a large number of immunoregulatory effects not only on the innate immune system but also on the adaptive immune responses (Akbar et al., 2000; Belardelli and Ferrantini, 2002; Biron, 2001; Biron et al., 1999; Bogdan, 2000). IFN- α has maturing effects on the

immature myeloid DC, enhancing their ability to present antigen and activate T cells (Gallucci et al., 1999; Le Bon and Tough, 2002; Luft et al., 2002; Santini et al., 2000). Type I IFN also enhance the activity of cytotoxic T lymphocytes (CTL) by inducing up regulation of MHC class I on virus infected cells, and increase the cytotoxic effects of Natural Killer-cells. Furthermore, type I IFN can increase the numbers of Fc-receptors on macrophages, amplifying their phagocytic activity (Bogdan et al., 2004). Moreover, it has been described that type I IFN, especially IFN- α , can have an important role in the regulation of the Th1 or Th2 types of immune responses. IFN- α may promote the development of a Th1 type of immune response by up-regulation and maintenance of a functional IL-12 receptor on Th cells in human (Rogge et al., 1998). In addition, type I IFN can promote survival and differentiation of B cells and enhance B cell receptor-dependent responses (Braun et al., 2002; Ruuth et al., 2001) (*Table II*).

Table II

Stimulatory (↑) and suppressive (↓) effects of Type I IFN on different leukocyte populations:

Population	Phenomenon	Type I IFN Effect
DC	generation	↑ or ↓
	maturation	↑
	Cross-priming of CD8+T cells	↑
	IL-12 production	↑ or ↓
	IL-15 production	↑
	B cell stimulatory capacity	↑
T cells	Neonatal IL-7 driven T lymphopoiesis	↓
	proliferation	↑ or ↓
	CTL cytotoxicity	↑
	Longevity of T _H and T _{REG}	↑
	Motility (chemokinesis)	↑
B cells	Neonatal IL-7 driven B lymphopoiesis	↓
	Differentiation into plasma cells	↑
	Isotype Switching (IgG)	↑
NK cells	Cytotoxicity	↑
	IFN-γ production	↑
Macrophages	Expression of iNOS and anti-microbial activity	↑ or ↓
	IL-12 production	↑ or ↓
	MHC class II expression	↓
	Apoptosis	↓

Type I IFN gene induction

As partly already described, the transcription of type I IFN genes is regulated by several stimuli, including viral and bacterial infections.

The regulation of the IFN- β gene it has been very well characterized. A 200 bp virus-inducible enhancer sequence (VRE) upstream of its Transcription Starting Site (TSS) is important in the regulation of the induction of the gene (*Figure D*).

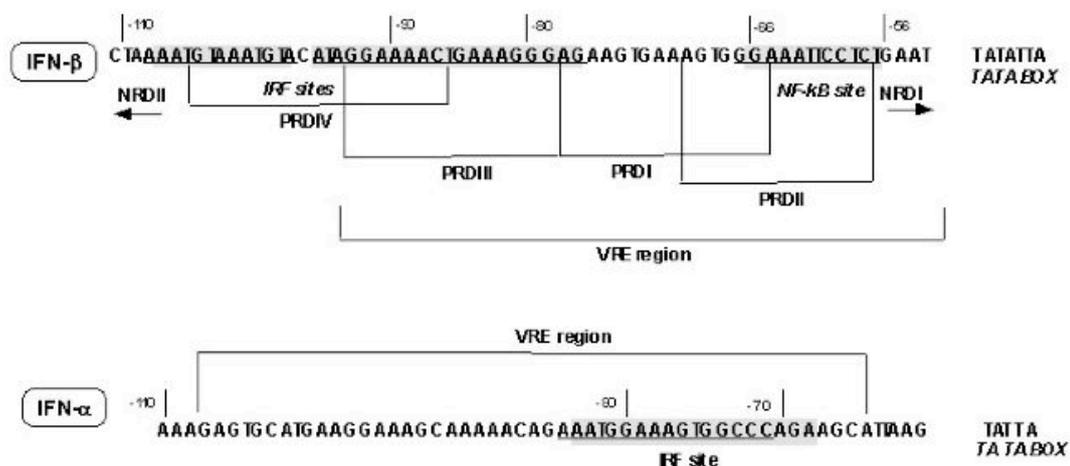


Figure D. Regulatory sequences and transcription factor binding sites within the human IFN- β and IFN- α promoters. TATA box and transcription factor binding sites are indicated. Numbers refer to distance in nucleotides from the transcription starting site.

This sequence contains two negative regulatory domains (NRD-I and -II), which are responsible for the constitutive repression of this gene prior to the addition of inducers (Goodbourn and Maniatis, 1988; Tanaka and Taniguchi, 1992). Located between the NRD there are four positive regulatory domains (PRD-I, -II, -III, -IV). Transcriptional regulation of the IFN- β gene requires the activation of IRF3/7, NF- κ B and ATF-2/c-Jun, which bind to PRDI-III, PRD-II and PRD-IV respectively. These transcription factors form a multiprotein complex, the enhanceosome, on the IFN- β enhancer (Maniatis, 1986). In the resting state, IRF3 is localized to the cytoplasm. In response to a viral challenge, IRF3 is phosphorylated on multiple serine/threonine residues, which control its dimerization. In this active form IRF3 then translocates to the nucleus and associates with the coactivators CREB-binding protein (CBP)/p300 on the IFN- β enhancer. The I κ B-related kinases, inhibitory IKK ϵ (also called IKK i (Shimada et al., 1999) and TANK binding kinase (TBK1) (also called NAK (Tojima et al., 2000) or T2K (Bonnard et al., 2000),

phosphorylate IRF3 (Fitzgerald et al., 2003a; Sharma et al., 2003) (*see Figure C*). IKK ϵ and TBK1 are structurally related to IKK α and IKK β but, unlike IKK α or IKK β , do not appear to be involved in NF- κ B activation (McWhirter et al., 2004; Sharma et al., 2003). Sharma *et al.* and Fitzgerald *et al.* showed that blocking IKK ϵ and TBK1 activity using RNA interference prevented SV-induced IRF3 phosphorylation and subsequent activation of the IFN promoter (Fitzgerald et al., 2003a; Sharma et al., 2003). Fitzgerald *et al.* also described a requirement for IKK ϵ and TBK1 in poly (I:C)-induced IRF3 activation *via* TLR3 and TLR4 (Fitzgerald et al., 2003a) (McWhirter et al., 2004). TBK1^{-/-} embryonic fibroblasts fail to activate IRF3 and induce IFN- β , IFN- α or ISGs in response to virus, LPS or poly (I:C) (McWhirter et al., 2004). TBK1 is ubiquitously expressed, while IKK ϵ expression is restricted to lymphoid cells, even if it can be inducible in several other cell types. Moreover, IKK ϵ may be functionally redundant with TBK1 in cells where both are expressed (Hemmi et al., 2004; Perry et al., 2004). Perry *et al.* showed that the SV-induced IFN response in TBK1^{-/-} embryonic fibroblasts could be partially restored by reconstitution with wild-type IKK ϵ but not with a mutant lacking the kinase activity (Perry et al., 2004).

Regulation of IFN- α genes is less well characterized though, like IFN- β , IFN- α expression is regulated at the transcriptional level. The VRE in the IFN- α promoter contains PRD-I-like sequences suggesting inducibility by various agents such as infection or IRF proteins (Au et al., 1993) (*Figure D*). The VRE contains no NF- κ B binding sites. Studies have suggested that IRF7 may have an important role in regulation of IFN- α (Au et al., 1998). Interestingly, the expression of IRF7, similar to the expression of IFN- α genes is limited to cells of lymphoid origin and is also activated by viral infection, LPS and IFN- α and IFN- β treatments. In addition, overexpression of IRF7 exhibits the same specificity in the transcriptional induction of IFN- α 4 and IFN- α 6 as viral infection. Also, IRF7 specifically binds the IFN-regulatory and stimulatory element (ISRE) of the IFN- α 4 promoter *in vitro* (Au et al., 1998).

Type I IFN signaling and ISGs

Once secreted from infected cells, type I IFN bind to cognate receptors. There is one common receptor for the type I IFN that is termed IFNAR and consists of two subunits, IFNAR-1 and IFNAR-2, members of the class II cytokine receptor (CRF2) family (Langer et al., 2004; Uze et al., 1995). A relation between the different mode of receptor engagement and IFN subtype differential signaling has not been clearly established. It is well recognized that IFN- α 2 and IFN- β engage the two receptor chains in different ways (Lewerenz et al., 1998; Piehler and Schreiber, 1999; Plataniias et al., 1996). Biophysical studies have shown that IFN- β possesses a 50 fold higher affinity than IFN- α 2 towards IFNAR1 and thus generates a more stable ternary complex at low IFNAR1 concentrations (Lamken et al., 2004). Gene expression profiling of human cells stimulated with different IFN subtypes suggested quantitative rather than qualitative differences (da Silva et al., 2002; Hilkens et al., 2003; Rani et al., 1996).

The binding of type I IFN to IFNAR1/2 induces homo- or heterodimerization of the receptor, resulting in the autophosphorylation and activation of receptor associated Janus kinase (JAK) 1 and TYK2 (Shuai and Liu, 2003; Taniguchi et al., 2001; Taniguchi and Takaoka, 2002). Upon activation, the JAK kinases phosphorylate conserved tyrosine residues in the cytoplasmic tail of the receptors, thereby creating docking sites for the SH2-domain of the signal transducer and activator of transcription (STAT) -1 and STAT-2. The activated STAT-1 and STAT-2 together with IRF-9, form a transcriptional activation complex, the IFN stimulated gene factor 3 (ISGF3) (*Figure E*). Furthermore STAT1 homodimers can also bind IFN- γ activated sequence elements (Levy and Darnell, 2002).

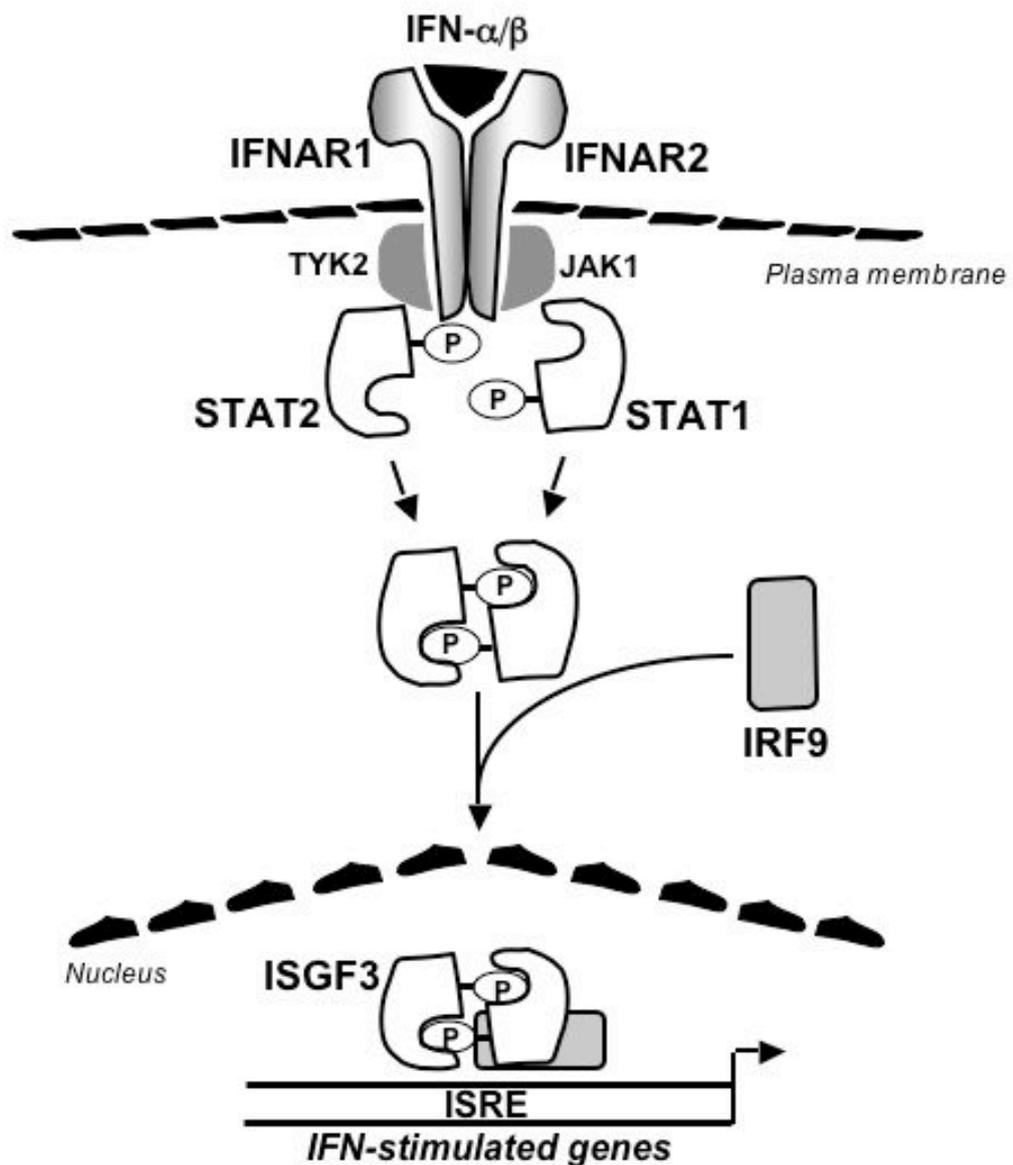


Figure E. Signal transduction by the IFN receptors.

Binding of IFN- α/β to their receptor complex formed by two different subunits, IFNAR1 and 2, activates the Janus kinases (JAK1 and TYK2) permanently bound to the cytoplasmic domains of the receptor subunits. Activation of the JAKs results in tyrosine phosphorylation of STAT1 and 2 that in their active form associate with IRF9 leading to the formation of the ISGF3 complex. This complex translocates to the nucleus where it binds ISRE elements on the promoter of the ISGs

Both ISGF3 and STAT1 homodimer translocate to the nucleus where bind promoters of a large number of ISGs, the products of which mediate the many effects of type I IFNs previously described (Der et al., 1998; Schlaak et al., 2002).

While the function of many of these genes has not been elucidated yet, some of them are known to play a role in innate antiviral responses, apoptosis and cell cycle inhibition. The largest groups of genes stimulated by IFN are genes encoding components of cellular signaling pathways (de Veer et al., 2001; Frucht et al., 2001). Many of these genes are involved in pro- or anti-inflammatory responses. Several pro-inflammatory ISG, such as MyD88 and TNF-receptor stimulating factor 1-associated via death domain are part of the TLR- and IL-1-induced signaling pathways respectively (Baker and Reddy, 1998; Kawai et al., 1999). Moreover, a large group of immune modulating genes are induced by type I IFN: for instance, the chemokines CX chemokine ligand (CXCL) -9, CXCL-11 and CXCL-10, which are involved in the recruitment of CXCR-3+ activated T cells into inflamed tissues (Lande et al., 2003a; Matikainen et al., 2000; Moser and Loetscher, 2001). CXCL-10 was originally identified in human U937 monocytic leukaemia cells as an IFN- γ inducible product (Luster, A.D., 1985) and subsequently in murine peritoneal macrophages on the basis of LPS sensitivity (Ohmori and Hamilton, 1990; Tannenbaum et al., 1988). Analysis of the promoter region of CXCL-10 revealed three conserved enhancer elements: an ISRE site and two NF- κ B sites positioned within 250 nt upstream of the TSS (Luster et al., 1985). LPS induction required that two of the three elements remained intact, either two NF- κ B sites or one NF- κ B and the ISRE sites. The ISRE-dependent effects of LPS are probably due to induction by LPS of type I IFN (Ohmori and Hamilton, 1993).

IFN stimulate also the expression of a number of transcription factors, the majority of which are transcription activators and only few are repressors. Among the transcription factors are the genes of IRF family (*Table III*). Nine IRF family members, including IRF-1, -3, -4, -5, -6, -7, IFN consensus sequence binding protein (ICSBP/IRF-8), IRF-9 and four viral homologs from the Kaposi's sarcoma herpes virus (KSHV) have been described. Of the 9 members the IRF-1, -3, -5 and -7 have been identified as positive regulators of IFN- α/β genes (Barnes et al., 2002a; Sato et al., 2001; Taniguchi and Takaoka, 2002). While IRF-3 is

constitutively expressed in most cell types, IRF-7 expression is induced by IFN-

Table III

Interferon Regulatory Factor family members and their role In immune responses:

IRF	Target gene	Main immune function of target gene	Phenotype of gene knock-out mice
IRF1 [Constitutive and Inducible by type I IFNs]	<i>NOS2, GBP1 and gp91^{PHOX}</i>	Promote anti-bacterial and Antiviral innate immunity	Susceptibility to infection with the intracellular bacteria <i>M.bovis</i> and <i>B.abortus</i> ; reduced resistance to infection with the virus EMCV
	<i>IL-12</i>	Promote Th1-cell responses	Defective Th1-cell responses; susceptibility to infection with the parasite <i>L.major</i> and the intracellular bacterium <i>L.monocytogenes</i>
	<i>IL-15</i>	Controls haematopoietic-cells development	Defective NK-cell, NKT-cell and IEL development
	<i>TAP1 and LMP2</i>	Provide MHC class I molecules with short peptides	Reduced CD8 ⁺ T-cell numbers
	<i>CDKN1A</i>	Controls the cell cycle and apoptosis	Reduced DNA-damage-induced Cell cycle arrest
IRF2 [Constitutive]	<i>Caspase-1</i>	Controls apoptosis and Processes IL-1 β	Reduced DNA-damage-induced apoptosis of mitogen-activated mature T cells
	ISGs (such as <i>OAS, PKR and IRF7</i>)	Attenuate type I IFN responses	Severe skin inflammation mediated By CD8 ⁺ T cells; defective CD4 ⁺ DC development
IRF3 [Constitutive]	<i>IL-12</i>	Promote Th1-cell responses	Susceptibility to infection with the parasite <i>L.major</i>
	<i>IFNB, IFNA4, IFIT1, CXCL9, CXCL10 and CCL5</i>	Promote antibacterial and Antiviral innate immunity	Reduced resistance to infection with the virus EMCV; increased ability to clear infection with the intracellular bacterium <i>L.monocytogenes</i> ; resistance to LPS-induced endotoxic shock
IRF4 [Constitutive and Inducible by TLR Ligation]	<i>IL4, and GATA3</i>	Controls T cell function	Defective Th2-cell differentiation
	<i>IGK and CD23</i>	Control B cell development and function	Defective mature B-cell function; defective immunoglobulin production
	<i>ND</i>	Controls DC development	Defective CD4 ⁺ DC development
IRF5 [Constitutive and Inducible by type I IFNs and TLR ligation]	<i>IL-6, IL-12, TNF, NFKBIZ and CXCL12</i>	Promote inflammation	Resistance to LPS- or CpG-containing DNA-induced shock
IRF6 [ND]	ND	ND	ND
IRF7 [Constitutive and Inducible by type IFNs]	<i>IFNB and IFNA</i>	Promote antiviral innate immunity	Susceptibility to infection with the viruses EMCV, VSV and HSV; reduced efficiency of CD8 ⁺ T-cell cross priming
IRF8 [Constitutive]	<i>IL-12 and IL-18</i>	Promote Th1-cell responses	Defective Th1-cell differentiation; susceptibility to infection with the intracellular bacterium <i>L.monocytogenes</i> and the parasites <i>L.major</i> and <i>T.gondii</i>
	ND	Controls haematopoietic-cell development	Development of chronic-myeloid-Leukaemia-like disease; defective pDC and CD8 ⁺ DC development
IRF9 [Constitutive]	ISGs (such as <i>OAS, PKR and IRF7</i>)	Mediate type I IFN responses	Susceptibility to infection with the viruses EMCV, VSV and HSV

α/β . Type I IFN can also upregulate expression of IRF-1 (Taniguchi et al., 2001; Taniguchi and Takaoka, 2002). Indeed, the produced IFN- β activates the transcription of IRF-1 and -7, and newly synthesized IRF-1 and -7 proteins are positively involved in the second wave of transcription leading to IFN- α expression (Kadowaki et al., 2001b; Sato et al., 2000). In this way, type I IFN induces and amplifies its own synthesis. The different IFN- α genes are expressed at different levels in different cell types and this may be due to differences in expression of the relevant IRF (Barnes et al., 2002a).

MATERIAL AND METHODS

Generation of human monocyte derived DC and murine bone marrow marrow derived macrophages

DC were prepared as previously described (Giacomini et al., 2001). Briefly, peripheral blood mononuclear cells were isolated from freshly collected buffy coats obtained from healthy voluntary blood donors (Blood Bank of University "La Sapienza", Rome, Italy) by density gradient centrifugation using Lympholyte-H (Cedarlane, Hornby, Ontario, Canada). Monocytes were purified by positive sorting using anti-CD14 conjugated magnetic microbeads (Miltenyi, Bergisch Gladbach, Germany). The recovered cells were >99% CD14+ as determined by flow cytometry with anti-CD14 antibody. DC were generated by culturing monocytes in 6-well tissue culture plates (Costar Corporation, Cambridge, MA) with 25 ng/ml GM-CSF and 1000 U/ml of IL-4 (R&D Systems, Abingdom, U.K) for 5 days at 5×10^5 cells/ml in RPMI 1640 (Biowhittaker Europe, Verviers, Belgium) supplemented with 2 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin and 15% fetal calf serum. At day 5 the cells were tested for their differentiation through the expression of CD1a (70-90% CD1a+) and the lack of CD14 (5% CD14+) and then DC were starved from IL-4 and GM-CSF for 20 h before their stimulation.

Mouse bone marrow (BM)-derived macrophages were generated as described (Severa et al., 2006a). Briefly, bone marrow was isolated and cultured for 8 days using L929 conditioned medium spontaneously producing M-CSF. The maturation state of the macrophages was monitored by FACS using Abs against F4/80 (Caltag, Burlingame CA) and CD11b (Pharmingen, San Diego, CA) as pure Abs or as direct conjugates to FITC or PE.

Mice, cell lines, viruses, and reagents

MyD88^{-/-}, TRIF^{-/-}, TRAM^{-/-}, and Mal^{-/-} mice were from S. Akira (Osaka University, Osaka, Japan). IRF3^{-/-} mice were from T. Taniguchi, (University of Tokyo, Tokyo, Japan). IFN α / β R^{-/-} mice were from J. Sprent (Scripps Research Institute, San Diego, CA). TBK1^{-/-}-TNFRI^{-/-} mice were from T. Mak and W-C. Yeh, (University of Toronto, Canada) and IKK ϵ ^{-/-} mice were from Millennium Pharmaceuticals (Cambridge, MA). Mouse embryonic fibroblasts (Mefs) from BLIMP1^{flox/flox} mice and Mefs targeted by Cre-recombinase (BLIMP1^{-/-}) were prepared as followed by J. Ye and T. Maniatis (Harvard, MA) from embryos provided by K. Calame (Columbia University, NY). Internal organs were completely removed from murine embryos and embryos were incubated at 4°C overnight with 0.25% Trypsin/EDTA (GIBCO, Invitrogen Life Technologies). The day after the cells were resuspended in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% Cosmic Calf Serum and, after vigorously pipetting up and down, cells were seeded overnight at a density of 10⁶ cells/cm². The day after, the dead cells were removed by washing twice the adherent cells with DMEM/CCS and the live cells were expanded to start the process of immortalization established after about 20 passages.

HEK293, used in Luciferase Assays, HEK293T, used in overexpression experiments, the Huh7.5 hepatoma cell line and the Huh7 parental cell line were maintained in DMEM supplemented with 5% FCS and 10 μ g/ml of Cyprofloxacin. TNF- α and IL1- β , used at 100 ng/ml, were from R&D Systems. LPS from *Escherichia coli* 0111:B4 (Sigma Chemical Company, St. Louis, MO) was used at a concentration of 1 μ g/ml. Poly (I:C) was from Amersham (Piscataway, NJ) and used at 100 μ g/ml and Pam2cysk4, used at 10 nM, was from EMC Microcollections (Tuebingen, Germany). TLR7 (3M-001) and TLR8 (3M-002) ligands (3M Pharmaceutical, St.Paul, MN) were used at 25 μ M and 5 μ M, respectively (Gorden et al., 2005). These optimal doses of TLR7 and TLR8 ligands

to induce DC maturation were evaluated by titrating different concentrations. IFN- β (Avonex, Biogen Inc., Cambridge, MA) was used at a final concentration of 200 pM. Sheep antiserum raised against human leukocyte IFN (Mogensen et al., 1975) was used at a 1:100 dilution (a kind gift of Dr. Gilles Uzé, CNRS UMR, Montpellier, France). Sendai virus (Cantrell strain) was from Charles River Laboratories (Boston, MA) and it was used at 300 hemagglutination units (HAU)/ml. H3N2 influenza A (Flu) strain A/Beijing/353/89 virus was grown in 11 day old embryonated chicken eggs and used at a concentration of 12.8 HAU/ml (a kind gift of Dr. I Julkunen, National Public Health Institute, Helsinki, Finland).

Flow cytometry analysis

Approximately $1-2 \times 10^5$ cells were aliquoted into tubes and washed once in PBS containing 2% FCS. The cells were incubated with mAbs at 4°C for 30 min. The DC were then washed and fixed with 2% paraformaldehyde before analysis on a FACSCAN using CellQuest software (Becton Dickinson, Mountain View, CA). The change in expression of the cell surface molecules was determined by subtracting the median fluorescent intensity (MFI) of the isotype control Ab from the MFI from the specific Ab.

The maturation and activation state of DC were monitored using Abs against CD80, CD86, CD83, and HLA-DR antigen as previously described (Giacomini et al., 2001). mAbs specific for CD1a, CD14, CD86, CD83, CD38, IgG1, (BD, Pharmingen) were used as direct conjugates to FITC or PE.

Quantitative real-time PCR

DNase I-treated total RNAs from human monocyte-derived DC and murine BM-macrophages were prepared using the RNeasy kit (Qiagen Inc., Valencia, CA) according to the manufacturer's instructions. Reverse transcriptions were primed

with oligo (dT) and performed using the murine leukemia virus reverse transcriptase. Quantitative PCR assays were done at least in triplicates using the Platinum Taq DNA Polymerase (Invitrogen Life Technologies, Carlsbad, CA) and the SYBR Green I (BioWittaker Molecular Applications, Rockland, ME) on a LightCycler (Roche Diagnostics, Mannheim, D). The standard errors (95% confidence limits) were calculated using the Student's t-test. Quantification standard curves were obtained using dilutions (4 log range) of the PCR products in 10 µg/ml sonicated salmon sperm DNA. For type I IFN that are devoid of introns in their genes, PCR products used for the standard curves have been quantified against human genomic DNA, thus allowing to compare the level of expression of type I IFN mRNAs between each other. The specificities of the 20 primer pairs used were validated by DNA sequencing. The specificity of amplification was assessed for each sample by melting curve analysis. With the exception of PCR reaction that amplifies both IFN-λ2 and -λ3 cDNAs, all primer pairs generated a single product, as checked in all experiments by the analysis of the melting curves. The human quantification data are presented as a ratio to the GAPDH level and the murine ones were normalized with β-actin. All gene expression data are presented as a ratio of gene copy number per 100 copies of GAPDH or β-actin (as indicated) ± SD.

The sequences of the primer pairs used for the quantification of the type I IFN have been described (Coccia, E.M., 2004).

A list of all the other primer pairs used follows.

For human cells:

GAPDH_for 5-ACAGTCCATGCCATCACTGCC-3,

GAPDH_rev 5-GCCTGCTTCACCACCTTCTTG-3;

Viperin/cig5_for 5-CTTTGTGCTGCCCTTGAGGAA-3,

Viperin/cig5_rev 5-CTCTCCCGGATCAGGCTTCCA-3;

OAS2_for: 5-AACTGCTTCCGACAATCAAC-3;

OAS2_rev: 5-CCTCCTTCTCCCTCCAAAA-3;

SOCS1_for: 5-AACTGCTTTTTCGCCCTTA-3;

SOCS1_rev: 5-GCCACGTAGTGCTCCA-3;
TLR7_for: 5-TTACCTGGAT GGAAACCAGCTACT-3,
TLR7_rev: 5-TCAAGGCTGAGAAGCTGTAAGCTA-3;
CD38_for: 5-GGCCTGGGTGATACATGGTGGA-3,
CD38_rev: 5-ACAGCGACTG GCTCAGATCTCA-3.
For murine cells:
 β -actin_for 5-TTGAACATGGCATTGTTACCAA-3,
 β -actin_rev 5-TGGCATAGAGGTCTTTACGGA-3;
Viperin/vig1_for 5-AACCCCCGTGAGTGT CAACTA-3,
Viperin/vig1_rev 5-AACCAGCCTGTTTGAGCAGAA-3.

cDNA and aRNA synthesis

Five μ g of total RNA were used for each analyzed condition: not stimulated (ns), 4 h IFN- β , 24 h LPS, 24 h LPS+Antibodies neutralizing type I IFNs (α -IFNs). As internal reference for Microarray analysis, a pool of RNA from immature DC collected from 5 healthy donors was used. First and second strand cDNA synthesis and the synthesis of antisense-RNA (aRNA) were performed using the Amino-Allyl MessageAmp™ aRNA Amplification Kit (Agilent, Palo Alto, CA). The quality of aRNA was tested by using Agilent Bioanalyzer 2100 (Agilent). The reaction allows the incorporation of Amino-Allyl modified NTPs that are subsequently labeled with the NHS ester labeled Cy3/5 dye (Amersham). The reference aRNA and the aRNAs from the different samples were labeled with Cy5 and Cy3 respectively. The labeled aRNAs (750 ng) were fragmented and prepared for the hybridization using the Agilent in situ Hybridization Kit Plus (Agilent).

Microarray analysis

Microarrays were purchased from Agilent. Agilent's Human 1A Oligo Microarray (V2) contains 20,173 oligonucleotide probes (60-mers) spanning conserved exons across the transcripts of the targeted full-length genes. Microarrays were hybridized at 60°C in the Agilent Oligo Microarray Hybridization chamber (22K format) for 17 h under constant rotation. Following hybridization, slides were washed once for 1 min in 6x SSPE-0.005% N-Lauroylsarcosine at room temperature, then washed for 1 min with 0.06x SSPE-0.005% N-Lauroylsarcosine. Microarrays were stabilized and dried with the Stabilization and Drying Solution (Agilent). Using a ScanArray lite scanner and ScanArray Express software (Packard Biochip technologies, Perkin-Elmer Wellesley, MA) microarrays were scanned at 532 and 633 nm. The results and images were quantified using Quantarray software 2.1 (Packard Biochip technologies, Perkin-Elmer). Raw data and sample information were then entered into Genespring (Silicon Genetics, Agilent Technologies). The LOWESS normalization was performed because in experiments where two fluorescent dyes (red and green) are used, intensity-dependent variation in dye bias may introduce spurious variations in the collected data. LOWESS normalization merges two-color data, applying a smoothing adjustment that removes such variation. Moreover, the Student's *t*-test was applied to compare the different conditions.

Cell lysates

Whole cell extracts were prepared as previously described (Remoli, M.E., 2002). Briefly, cells (1×10^7) were lysed in 30-50 μ l of ice cold whole cell extraction buffer (20 mM Hepes pH 7.9, 50 mM NaCl, 0.5% NP-40, 1 mM DTT, 10 mM EDTA and 2 mM EGTA, 10 μ g/ml leupeptin, 100 mM NaF, 0.5 mM PMSF, 10 mM sodium orthovanadate and sodium molybdate). The lysate was incubated 30 min on a

shaker at 4°C, insoluble debris was removed by centrifugation (13 000 rpm, 4°C, 10 min) and the lysate was stored at -80°C.

Nuclear cell extracts were prepared as previously described (Severa et al., 2006a). Briefly, cell pellets (5×10^6) were resuspended in 1 ml of buffer A (0.5 % Nonidet P-40, 10 mM EDTA, 10 mM EGTA, 10 mM KCl, 10 mM Hepes pH 7.9) to which 1 mM DTT, 0.5 mM PMSF, 10 µg/ml leupeptin, 1 µg/ml pepstatin, 1 µg/ml trypsin inhibitor, and 1 µg/ml antipain were freshly added and incubated on ice for 10 min. Nuclei were sedimented by centrifuging the lysates at 1200 x g for 10 min. The nuclear pellets were resuspended in 30-40 µl with buffer C (1 mM EDTA, 1 mM EGTA, 0.4 M NaCl, 20 mM HEPES (pH 7.9), 5 mM MgCl₂, 25% glycerol, with fresh addition as above) and incubated for 10 min on ice with occasional mixing. The suspensions were clarified by centrifugation at 15 000xg for 10 min. The supernatants were recovered as nuclear extracts and were rapidly frozen on crushed dry ice and stored at -80°C.

DNA affinity purification assay

Nuclear lysates were prepared from 293T transiently transfected with 4 µg (or the indicated amounts) of the HA-tagged IRF9, flag-tagged STAT1 or flag-tagged PRDI-binding factor I (PRDI-BF1) expression vectors using Genejuice (Novagen) in 10-cm plates. Where indicated, the cells were treated for 16 h with SV.

Biotinylated oligonucleotide of the multiple ISREs (5-GTTTCACATGTGGA AAAATCGAAACTCTAAC-3) designed from the human promoter of Viperin gene was annealed with the corresponding antisense oligonucleotide in 1X STE buffer, containing 10 mM Tris-HCl (pH 8), 50 mM NaCl, and 2 mM EDTA. Biotinylated DNA oligonucleotides were mixed with 500 µg of nuclear extracts in 500 µl of binding buffer containing 4 % glycerol, 1 mM MgCl₂, 0.5 mM EDTA, 0.5 mM DTT, 50 mM NaCl, 10 mM Tris-HCl (pH 7.5), 20 µg poly(dI)-poly(dC) (Amersham Pharmacia Biotech) and incubated for 25 min at room temperature.

Then streptavidin magnetic beads (Promega, Madison, WI), washed three times with 400 μ l of 1X binding buffer, were added to the reaction mixture and incubated for 30 min at 4°C and for 10 min at room temperature with mixing by rotation. The beads were collected with a magnet and washed three times with 500 μ l of binding buffer. The bound proteins were eluted from the beads by boiling in sample buffer and were resolved on 7.5% SDS-PAGE followed by immunoblotting with the indicated specific Abs: anti-HA-HRP (Roche Applied Science), anti-Flag-HRP (Sigma Chemical), anti-STAT1 (Santa Cruz Biotechnology, Santa Cruz, CA) anti-PRDI-BF1 (Novus Biologicals Inc., Littleton, CO). The level of the nuclear lysates used in the assay was verified by immunoblotting with the anti-USF2 Ab (Santa Cruz Biotechnology).

Western blot analysis

For TLR7 detection, whole cell lysates were separated by 10% SDS-PAGE gel and blotted onto nitrocellulose membranes. Blots were incubated with a polyclonal Ab against TLR7 (Novus Biologicals Inc.) and reacted with an anti-rabbit HRP-coupled secondary Ab (Amersham Pharmacia Biotech). Bands were revealed with an ECL detection system (Amersham Pharmacia Biotech) and quantified with the Kodak Image Station 440CF.

EMSA

EMSA was conducted as previously described (Severa et al., 2005). Briefly, to measure the association of DNA-binding proteins with different DNA sequences, synthetic double-stranded oligonucleotides were end-labeled with [γ -³²P] ATP by T4 polynucleotide kinase. For the analysis of IRF-1 complexes, nuclear cell lysates (15 μ g) were used in EMSA experiments.

Binding reaction mixture (20 µl final volume) contained labeled oligonucleotide probes (50 000 cpm) in binding buffer [4 % glycerol, 1 mM MgCl₂, 0.5 mM EDTA, 0.5 mM DTT, 50 mM NaCl, 10 mM Tris-HCl (pH 7.5), 1 µg poly(dI)-poly(dC)]. Nuclear lysates were added and the reaction mixture was incubated for 30 min at room temperature. The samples were analyzed on 5% PAGE gels with 0.25 x TBE (1 x TBE is 50 mM Tris-borate (pH 8.2) and 1 mM EDTA) for 3 h at 150 V at 18°C. For supershift analysis, 1 µg of anti-IRF-1 Ab (Santa Cruz Biotechnology) was added to the reaction for 30 min at room temperature. For competition analysis, a 100-fold molar excess of cold oligonucleotides was added to the binding reaction. The oligonucleotide used was as follows: IRF-1 site-TLR7 5'-CTATAAAAACGAAAGAAA TTTGGT-3'.

***In silico* analysis of human TLR7 and Viperin promoters**

The analysis of the human promoters for TLR7 (accession NM_016562.3) and Viperin (accession NM_080657.3) genes was done by using the Transcription Factor Binding Site (TFBS) prediction program MatInspector (online at <http://www.genomatix.de/matinspector.html>). MatInspector uses informations of MatInd (that generates Position Weight Matrices, PMW) to scan nucleotide sequences for matches to this pattern by calculating a matrix similarity score that reaches 1 only if the test sequence corresponds to the most conserved nucleotide at each position of the matrix. The analyzed sequences were regions spanning 1,000 bp upstream the TSS of the two genes (Cartharius et al., 2005). Moreover, no enhancer was found to regulate the transcription of the genes of interest.

Plasmid constructs

The human and mouse Viperin promoters were cloned from human THP-1 and mouse ES cell (129SV) genomic DNA, respectively, and inserted into pGL3-basic (Severa et al., 2006b). pEF-BOS-RIG-I, Mda-5 and the IFN- β luciferase reporter (p125Luc) construct were from T. Fujita (Tokyo Metropolitan Institute of Medical Science, Tokyo, Japan) (Yoneyama et al., 2004). pEF-Bos MAVS was cloned from human 293T total cDNA. pEF-BOS-Lgp2 and the NF- κ B luciferase reporter were as previously described (Rothenfusser et al., 2005). The ISG54-ISRE was purchased from Stratagene (La Jolla, CA). pCDNA3-Flag-PRDI-BF1, pCDNA3-Flag-PRD1-BF1 Δ 398-789 and pCMV-Flag-p65 were from T. Maniatis (Harvard University, Cambridge, MA). pRc-CMV-flag-STAT1 was from G. Stark (Cleveland Clinic, Cleveland, OH). pCDNA3-HA-IRF9 and pCMV-Flag-IRF3 were from Paula M. Pitha (John Hopkins University, Baltimore, MD); and pRK-HA-IRF1 from H-B Shu (National Jewish Medical and Research Center, Denver, CO).

Reporter assays

For reporter assays, HEK293 (seeded 10^5 cells/ml in 96-well plates) were transfected with 40 ng of the indicated luciferase reporter gene together with 40 ng of thymidine kinase *Renilla reniformis* (*R.reniformis*)-luciferase reporter gene and the indicated amount of expression plasmids using Genejuice (Novagen, San Diego, CA). Where indicated SV or IFN- β were added for 16 h, and luciferase activity was measured as previously described (Fitzgerald et al., 2003a).

DC were transfected with Rc-CMV empty vector and Rc-CMV IRF-1 (Sgarbanti et al., 2002) using Nucleofector technology according to the manufacturer's instruction (Amaya Nucleofector, Koeln, D) as previously described (Remoli et al.,

2006). Twenty h after transfection, total RNA was extracted and analyzed for TLR7 expression by Real-Time RT-PCR.

In all experiments, data were normalized for transfection efficiency with *R. reniformis* luciferase.

Cytokine and chemokine determinations

Supernatants from DC cultures were harvested 24 h after treatments, and stored at -80°C. CXCL-10 specific ELISA kit was obtained from R&D Systems. IL-6, IL-10, IL-12p70 and TNF- α were measured with the human inflammation cytometric bead assay (CBA) (Becton Dickinson). All assays were conducted in triplicates according to manufacturers' instructions.

Statistical analysis

Statistical analysis was calculated using a two-tailed for paired data Student's *t*-test. A *p* value < 0.05 was considered statistically significant.

RESULTS

Induction of type I IFN subtypes and IFN- λ s upon viral infections and TLR triggering.

The profile of pro- and anti-inflammatory cytokines released from maturing DC as well as their impact on the immune response have been extensively described (Lande et al., 2003a; Moser and Loetscher, 2001; Proietto et al., 2004). Conversely, very little is known about the specific expression profile of the different type I IFN subtypes and IFN- λ genes following virus infections or TLR triggering in monocyte-derived DC.

In our study, the expression of several IFN- α subtypes, IFN- β and IFN- λ genes was analyzed by real-time RT-PCR using primers specific for sequences within the coding region of each isotype (Coccia et al., 2004). Since all standards used for the quantitation of type I IFN subtypes have been standardized against human genomic DNA (see Material and Methods), the IFN/GAPDH ratios are directly comparable between the different IFN subtypes.

Total RNA was isolated from DC infected with Flu or SV at different time points (0, 1, 3, 6, 8 and 16 h) (*Figure 1A*). IFN- α 1, - α 2, - α 8 and - β together with IFN- λ 1 subtype (also called IL-29) and IFN- λ 2 and IFN- λ 3 (IL-28A and B respectively), which are highly homologous, were chosen as the representative subtypes to be studied.

Both Flu and SV infection induced IFN- β gene expression within 1 h, whereas the expression of IFN- α 1, - α 2, - α 8 mRNA was slightly delayed, increasing 3 h after virus infection (*Figure 1A*). In contrast to Flu-infected DC, where the IFN induction reached the highest level of induction at 8 h and remained at moderately high levels also at 16 h, in SV-infected cells the expression of IFN- β , IFN- α 1, - α 2, - α 8 mRNA peaked at 8 h after infection, decreasing thereafter. Interestingly, IFN-

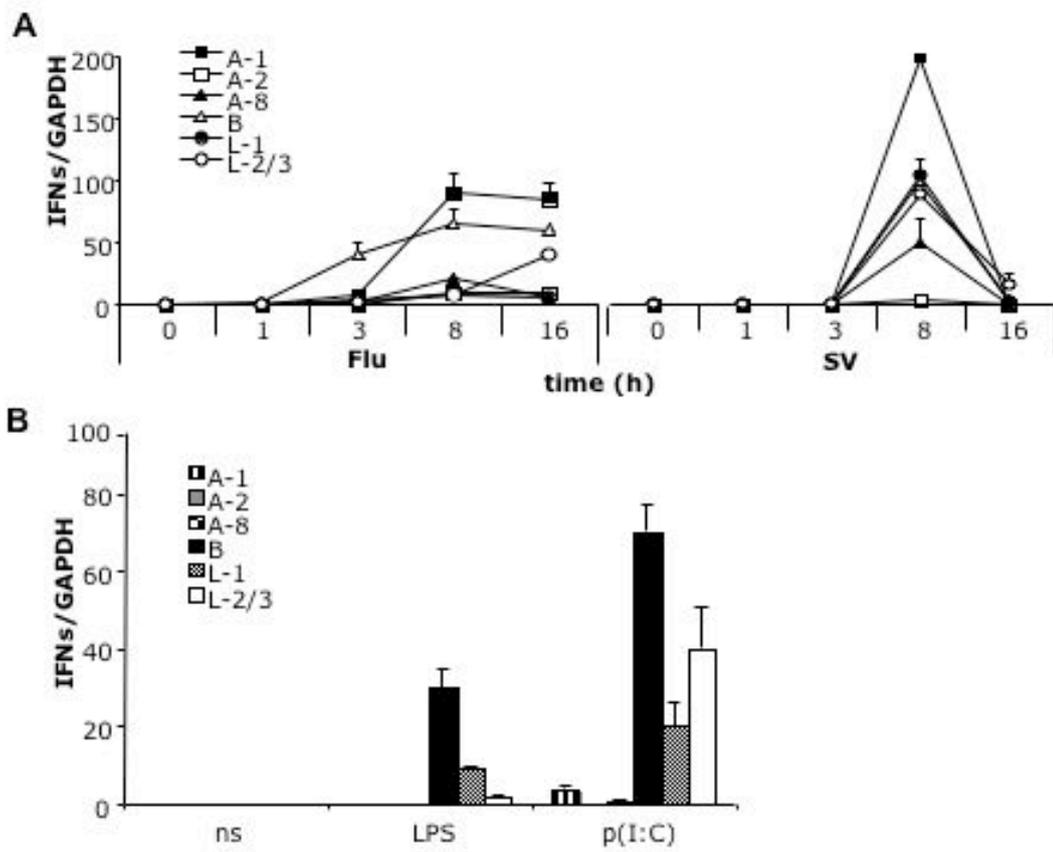


Figure 1. Expression of type I IFN and IFN- λ s in monocyte-derived DC. (A) DC were infected with Influenza A (Flu) or Sendai virus (SV) for the indicated time points and total cellular RNA was extracted. The expression of IFN- α 1, - α 2, - α 8, IFN- β , IFN- λ 1 and IFN- λ 2/3 was analyzed by Real-time RT-PCR. (B) Cells were treated 4 h with LPS (100 ng/ml) or poly(I:C) (100 μ g/ml) and the expression of the indicated IFN genes tested by Quantitative PCR.

λ 1 and IFN- λ 2/3 mRNAs were induced by Flu or SV infection. Maximal levels of IFN- λ 1 and IFN- λ 2/3 expression were reached within 8 h or 16 h after Flu infection, respectively. SV-induced IFN- λ mRNA expression peaked at 8 h post-infection and then sharply decreased. The kinetics of IFN- λ expression are thus similar to those of IFN- β , IFN- α 1, - α 2, - α 8.

Human monocyte-derived DC express TLR-3 and TLR-4 and it is known that the stimulation of these receptors with their ligands, poly (I:C) and LPS respectively, triggers type I IFN induction (Barnes et al., 2002b; Coccia et al., 2004; Kadowaki

et al., 2001a; Toshchakov et al., 2002). In order to investigate the specific expression pattern of type I IFN subtypes and IFN- λ s induced following TLR triggering, DC were stimulated 4 h with LPS or poly (I:C) (*Figure 1B*). TLR-4 or TLR-3 stimulation drove a rapid and robust expression of IFN- β and IFN- λ genes. Among the IFN- α subtypes, only a slight IFN- α 1 expression in poly (I:C)-treated DC was seen.

Overall, these data indicate that, among all the type I IFN subtypes, IFN- β , together with IFN- λ , is highly induced along DC maturation irrespectively of the stimuli. Since DC, besides representing one of the major sources of type I IFN, are also key responders, IFN- β production would be of crucial importance in addressing the DC differentiation status.

Analysis of IFN- β -induced gene expression in LPS-matured DC.

Every TLR triggers a specific cellular activation program through the induction of different profiles of gene expression. We focused our attention on determining the contribution of IFN- β along the LPS-induced DC maturation using α -IFNs Antibodies.

Before analyzing the IFN- β -related functional and phenotypical modifications occurring in DC following LPS treatment, initial studies were designed to test the efficacy of type I IFN neutralization in our experimental model. To this aim, we monitored IRF-7 expression by Western Blotting since it is known that IRF-7 expression occurs following type I IFN release during most viral and bacterial infections. Whole lysates were prepared from untreated DC, or DC treated for 24 h with IFN- β or LPS in presence or absence of α -IFNs Abs. As expected, IRF-7 protein expression was strongly upregulated after IFN- β and LPS treatments (*Figure 2A*). Moreover, the presence of α -IFNs Abs completely inhibited the LPS-

induced IRF-7 expression, proving the requirement of IFN- β production for its induction and the ability of these neutralizing Abs to dramatically inhibit the

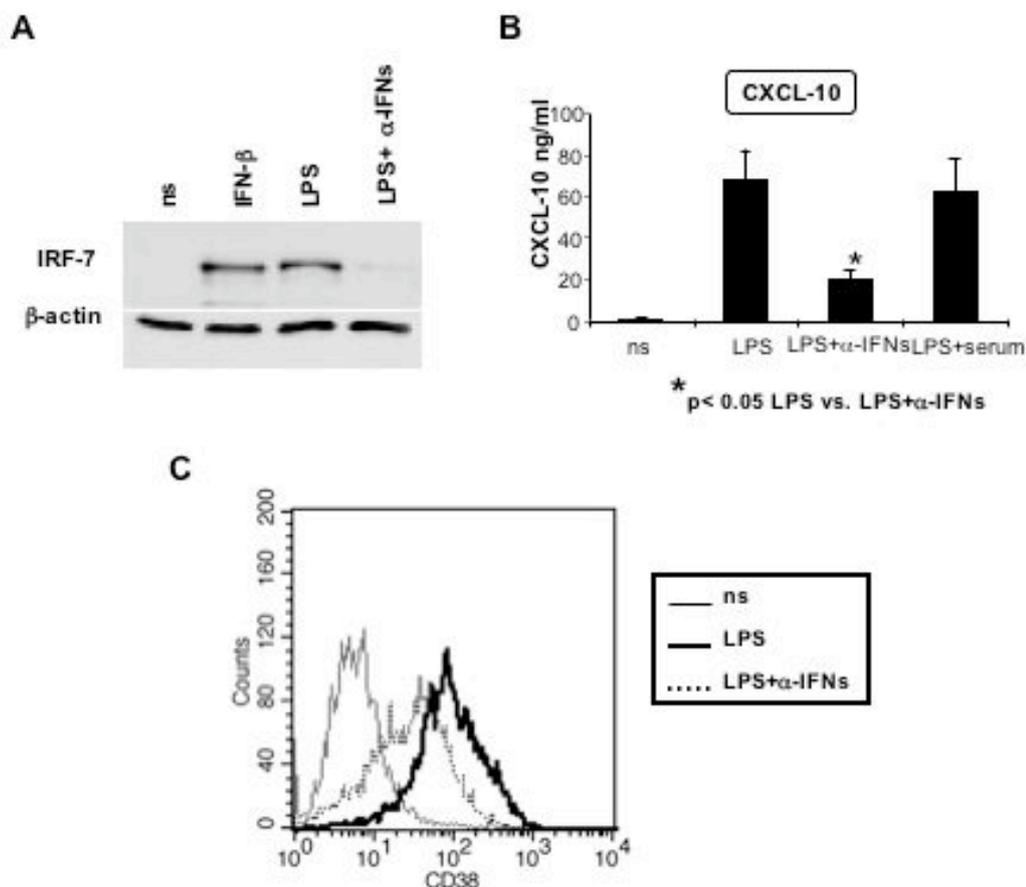


Figure 2. Type I IFN-dependent functional modifications in DC during LPS-induced maturation. (A) DC were left untreated, stimulated for 24 h with IFN- β (200 pM) with LPS (100 ng/ml) in presence or absence of α -IFNs Abs. Whole cell extracts were prepared and 30 μ g of lysates subjected to immunoblot analysis using anti-IRF-7 or anti- β -actin specific Abs. This is a representative experiment performed with DC obtained from other 2 donors. (B) Supernatants were harvested from non stimulated or LPS treated-DC with or without α -IFNs Abs or sheep anti-serum (serum) as control for 24 h. The level of CXCL-10 was measured by ELISA. The results represent the means \pm SE of 3 different experiments. $P < 0.05$ LPS vs LPS + α -IFNs Abs. (C) Surface expression of CD38 was evaluated by FACS in non stimulated or LPS-matured DC in presence or absence of α -IFNs Abs. A total of 5000 cells were tested per sample. A representative FACS profile is shown. The analysis was repeated 3 additional times, using DC from a total of 4 different blood donors.

autocrine/paracrine type I IFN-mediated intracellular signaling. No reduction of IRF-7 expression was observed by the addition of sheep control serum to LPS-

treated cultures (data not shown). Equal loading of all samples was verified by immunoblotting with an anti- β -actin Ab (*Figure 2A, lower panel*).

We next monitored the production of the IFN-inducible CXCL-10 by ELISA. Matured DC produce CXCL-10 and this has been shown to be IFN-regulated (Hilkens et al., 2003; Lande et al., 2003a; Walker and Tough, 2006). DC were matured with LPS, with or without α -IFNs Abs, for 24 h and supernatants were then harvested. CXCL-10 release, observed following LPS treatment, was significantly decreased in DC matured with LPS in presence of α -IFNs Abs (*Figure 2B*). To further confirm that the activity of these neutralizing Abs was specific, DC were treated with LPS in presence of sheep control serum; in this condition no decrease of CXCL-10 production was observed (*Figure 2B*).

Furthermore, we investigated whether the profile of CD38, a surface molecule known to be induced also by type I IFN (Pogue et al., 2004; Tliba et al., 2004) was affected by α -IFNs Abs. Surface expression of CD38 was examined using flow cytometry analysis and as expected a marked reduction in the LPS-induced increase in CD38 expression was observed (*Figure 2C*).

Once we had been proved the efficacy of type I IFN neutralization, we performed microarray analysis in DC following 4 h of treatment with IFN- β or 24 h of treatment with LPS in the presence or absence of the α -IFNs Abs. These time points were judged to provide the best snapshot of IFN-mediated changes in LPS-matured DC and were used for all subsequent experiments. The RNA was collected from 4 different healthy donors and similar gene expression profiles were found among donors. aRNAs (synthesized as described in Material and Methods) were hybridized to Agilent's Human 1A Oligo Microarray (V2) that contains 22 K oligonucleotide probes and the data were analyzed with Genespring Software.

Since one of the principal goals of our study was to find a specific list of genes regulated by type I IFN production during LPS-induced DC maturation, we began our analysis by profiling the changes in gene expression that occur in DC in response to 4 h IFN- β treatment compared to that observed after 24 h stimulation with LPS. Two hundred eighteen genes were found to be modulated by IFN- β and

315 genes by LPS. We focused our attention on the genes that were up-regulated by the two different treatments and which were down-modulated in the presence of α -IFNs Abs. The expression profile of 23 genes followed this criteria. A hierarchical clustering analysis of this set of genes is shown in *Figure 3A*.

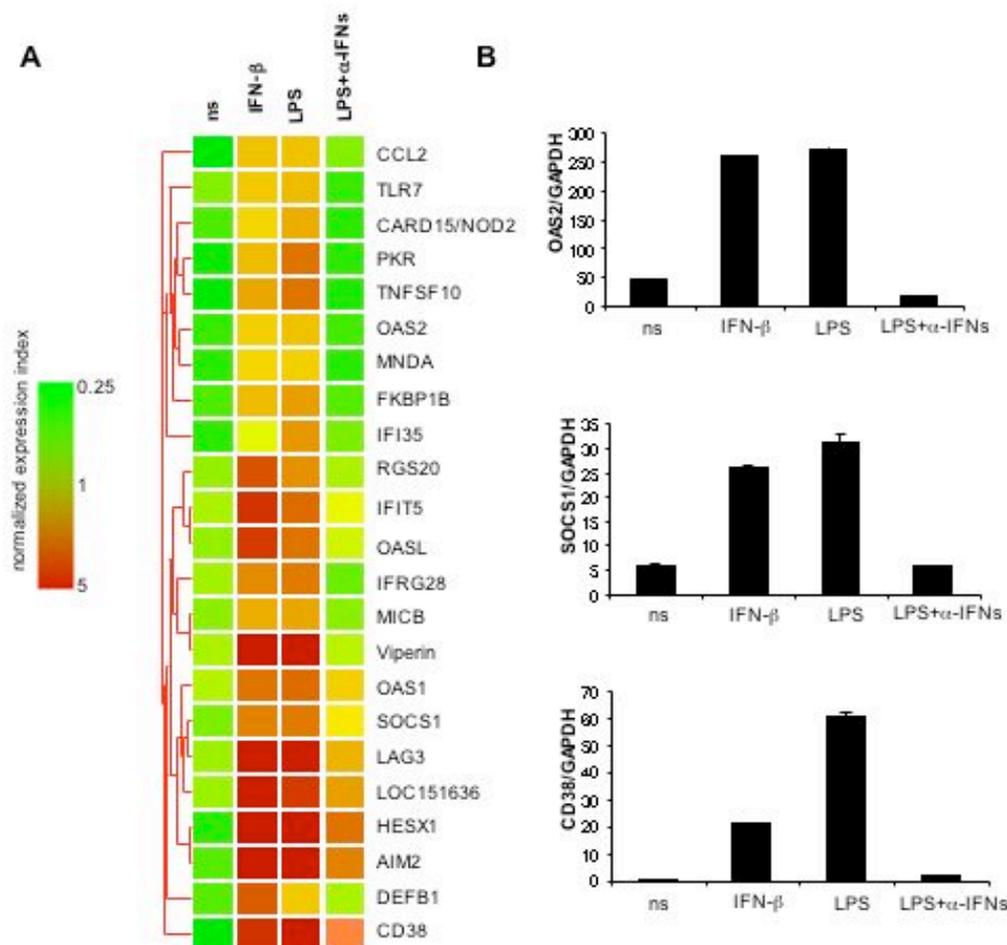


Figure 3. Specific transcriptional profile induced by type I IFN release along LPS-induced DC maturation. Total RNA was isolated from DC either following 4 h IFN- β or 24 h LPS treatment in presence or absence of α -IFNs Abs. (A) Hierarchical clustering of gene expression data by 4 different healthy donors is shown. Each row represents a separate gene and each column a different condition, as indicated. The normalized expression index for every transcript sequence (rows) in each sample (columns) is indicated by a color code. Red, yellow and green squares indicate that expression of the gene is greater than, equal to or less than the mean level of expression across the different donors. The scale extends from fluorescent ratios of 0.25 to 5.0. (B) mRNA expression of OAS2, SOCS1, CD38 and GAPDH was analyzed by Real-time RT-PCR. Data were then normalized on GAPDH level of expression. Results are means \pm SE of triplicate values. This is a representative experiment of 4 different analyzed donors.

Some of these genes are part of the so called “IFN signature”, a set of genes known to be regulated by type I IFN signaling (de Veer et al., 2001), such as the 2'-5'-oligoadenylate synthetase (OAS) 1 and 2 (Floyd-Smith et al., 1981), suppressor of cytokine signaling (SOCS) 1 (Crespo et al., 2000; Saito et al., 2000), PKR (Meurs et al., 1990) and the surface molecule CD38 (Pogue et al., 2004; Tliba et al., 2004). Others were ISGs with unknown function but which have been shown to be induced via IFN production including IFN-induced protein 35 and IFN-induced protein with tetratricopeptide repeats 5 (de Veer et al., 2001). We validated the results obtained by microarray analysis by quantifying the mRNA level of OAS2, SOCS1 and CD38 by Real-time RT-PCR (*Figure 3B*).

Interestingly, IFN- β and LPS treatments also induced the expression of a subset of genes which have not previously been reported to be IFN-inducible in DC. Importantly, two of these were PRR including NOD2 and TLR7 (*Figure 3A*). Whereas NOD2 (also called CARD15) plays a central role in the immune response to intracellular bacteria by recognizing muramyl dipeptide (MPD), a derivative of peptidoglycan (Strober et al., 2006), TLR7 recognizes ssRNA and is activated by infections with ssRNA viruses (Diebold et al., 2004a; Heil et al., 2004). The TLR7 gene has been reported to be induced by type I IFN in monocytes/macrophages or in B cells (Bekeredjian-Ding et al., 2005; Siren et al., 2005), but its inducibility by type I IFN has never been characterized in the context of DC maturation.

The expression of Defensin- β 1 (DEFB1) was also found to be IFN-regulated. DEFB1 is an antimicrobial peptide implicated in the resistance of epithelial surfaces to microbial colonization and it has already been described that IFN- γ treatment or TLR3 and 4 triggering can drive its expression (Duits et al., 2002; Schaefer et al., 2005).

Another gene that was found to be IFN-regulated was Viperin (virus inhibitory protein, endoplasmic reticulum-associated, interferon- γ inducible), an anti-viral gene whose function is still unclear (Chin and Cresswell, 2001).

The picture that the microarray analysis depicted highlighted a particular capacity of the released type I IFN along DC maturation to activate a specific pattern of

genes that “alerts” the cell to future infections. Among the IFN-induced genes, we characterized the IFN-mediated activation of TLR7 and Viperin genes since their regulation has never been studied in DC.

Transcriptional regulation of Viperin gene expression in human DC.

Viperin was initially identified as a human CMV- and IFN γ -inducible protein in fibroblasts (Chin and Cresswell, 2001) and was shown to inhibit productive HCMV infection by down-regulating several HCMV structural proteins critical for viral assembly and maturation. Viperin (known as cig5 in humans, vig1 in mouse and also RSAD2 in both human and mouse) has also been shown to be induced after infection with VSV, Yellow Fever virus, human polyomavirus JC, Hepatitis C virus (HCV) (Boudinot et al., 2000; Helbig et al., 2005; Khaiboullina et al., 2005; Verma et al., 2006) or after the lipofection-mediated delivery of bacterial or viral DNA into the cytoplasm (Ishii et al., 2006). The rapid and robust induction of the Viperin gene by a range of different viruses and microbial products such as LPS and DNA suggests that it is an important component of innate immunity to diverse pathogens. For this reason, in this study, we have examined the molecular mechanisms regulating Viperin gene expression (Severa et al., 2006a).

We first confirmed the microarray results measuring Viperin mRNA level by Real-time RT-PCR either in immature DC or IFN- β and LPS-treated DC in presence or absence of α -IFN Abs (*Figure 4A*). Then, we examined Viperin gene expression in DC in response to various stimuli. DC were treated for 3 h with the synthetic mycobacterial lipoprotein, Pam2Cysk4, poly I:C and LPS, ligands for TLR2, TLR3 and TLR4, respectively. As seen in *Figure 4B*, quantitative real-time RT-PCR measurements demonstrate that both poly (I:C) and LPS induced a robust induction of Viperin. In contrast Pam2Csk4 or the cocktail of pro-inflammatory cytokines

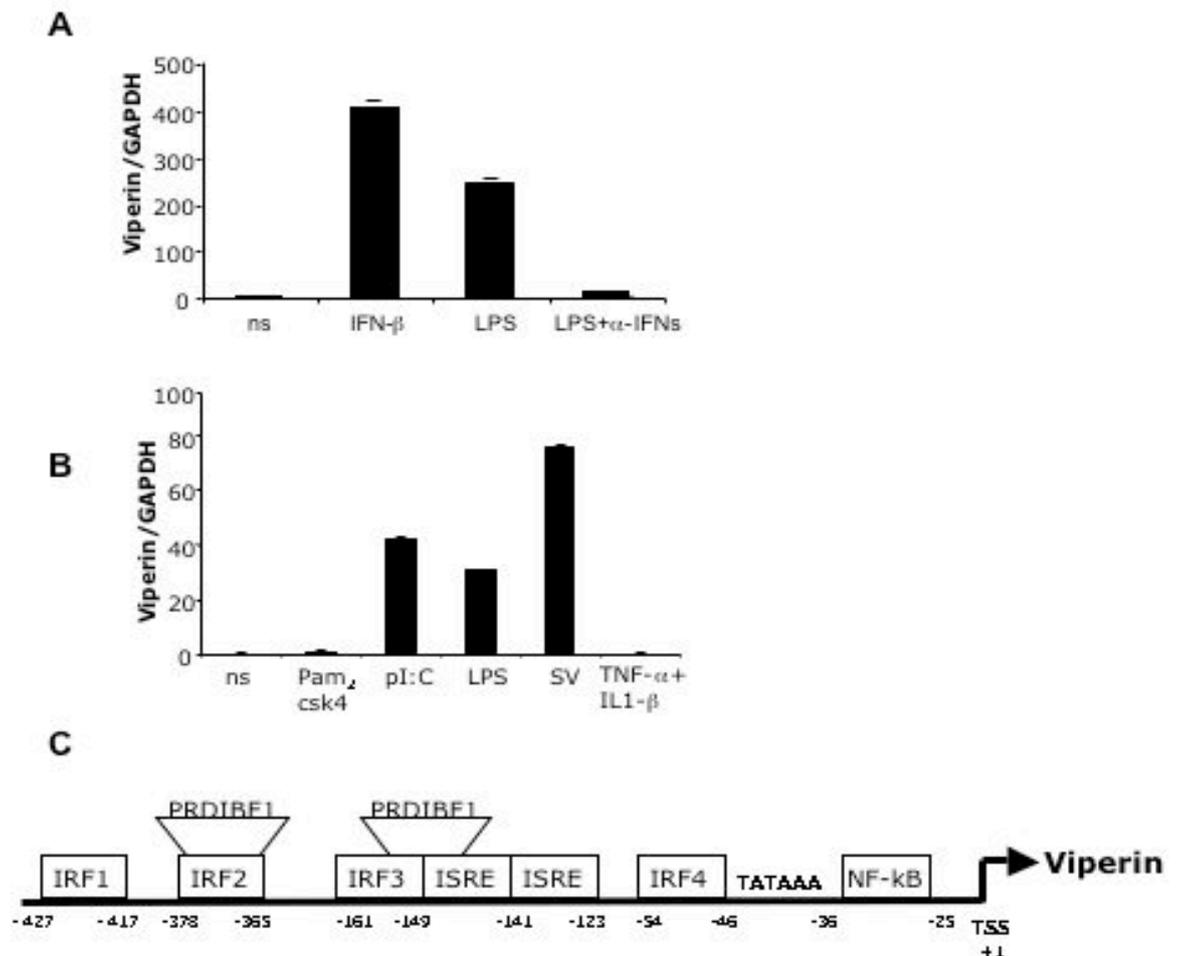


Figure 4. Regulation of Viperin gene expression in DC. (A) Total RNA was isolated from human DC following LPS treatment in presence or absence of α -IFNs Abs. mRNA expression of Viperin gene was analyzed by real-time RT-PCR. Data were then normalized with GAPDH level of expression. (B) Total RNA was extracted from non stimulated, Pam2cysk4- (10 nM), poly(I:C)- (100 μ g/ml), LPS- (100 ng/ml) and TNF- α (50 ng/ml) +IL1- β (50 ng/ml)- treated DC upon 3 h treatment. Viperin and GAPDH mRNA expressions were analyzed by Real-Time RT-PCR. (C) Schematic map of ~500 bp of the human Viperin promoter. TSS, transcription starting site. TATAAA is the consensus sequence for the TATA box.

TNF- α and IL1- β did not induce this gene although these stimuli induced efficiently DC maturation. SV also induced a strong expression of Viperin in DC. This differential inducibility of Viperin prompted us to examine its promoter region for transcription factor binding sites. Using the TFBS prediction program MatInspector (online at <http://www.genomatix.de/matinspector.html>) (Cartharius et

al., 2005), a region spanning 1,000 bp upstream of the TSS and 500 bp downstream was analyzed. Several transcription factor binding sites for IRFs (IRF1, IRF2, IRF3 and IRF4), as well as two ISRE sites, which can be bound by the IRFs and the ISGF3 complex, were identified within 500 bp of the TSS. An NF- κ B binding site in close proximity to the TSS was also detected (*Figure 4C*). Interestingly, we also found binding sites for PRDI-BF1 (see below). A very similar promoter region was also found when the murine Viperin gene (*vig1*) was analyzed (data not shown).

Differential involvement of TIR and CARD-domain containing adapters in Viperin gene induction.

In 2004 Bjorkbacka H. and Fitzgerald K. examined the expression profiles induced by LPS in murine BM-derived macrophages and found that *vig1*, the murine version of the Viperin gene, was induced by LPS treatment (Bjorkbacka et al., 2004). This results prompted us to analyze in collaboration with Kate Fitzgerald (University of Massachusetts Medical school, Worcester, MA) the regulation of Viperin gene expression using knock out (ko) mice for the different components of the TLR-dependent and independent pathways.

We examined Viperin induction in murine BM-derived macrophages from C57BL/6 wild-type, MyD88-, Mal-, TRIF- and TRAM-deficient mice following LPS treatment in order to probe the adapter molecules required for this response. Macrophages from these mice were treated for 3 h with LPS and the level of Viperin mRNA was examined by quantitative RT-PCR (*Figure 5A*). Viperin gene expression was completely abrogated in LPS-treated macrophages from TRIF- and TRAM-deficient mice. In contrast, the response in both Mal- and MyD88-deficient mice was not impaired, in fact these two strains showed an even stronger induction compared to C57BL/6 wild-type mice highlighting the possible involvement of a MyD88/Mal-dependent repressor of Viperin gene expression (see below).

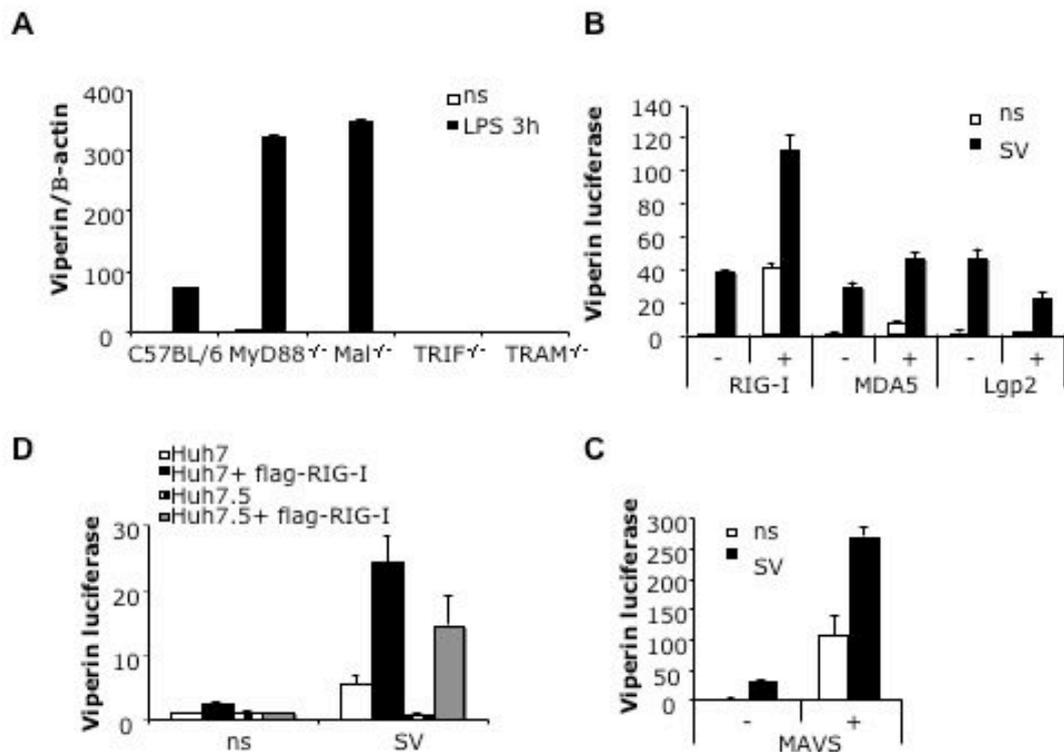


Figure 5. TIR- and CARD-domain containing adapters are differentially required for Viperin gene transcription. (A) BM- macrophages from C57BL/6, MyD88^{-/-}, Mal^{-/-}, TRIF^{-/-} and TRAM^{-/-} mice were stimulated with LPS (3 h). mRNA expression of Viperin gene was measured by Real-Time PCR. (B) HEK293 cells were transfected with the Viperin reporter gene and then cotransfected with full-length RIG-I, MDA-5 and Lgp2. Cells were infected with SV for 16 hours, and luciferase reporter activity was measured. In all cases, data are expressed as fold induction relative to the reporter only control and are the mean \pm SD. (C) Viperin reporter was transfected in Huh7 and Huh7.5 cell lines with or without 40 ng of full-length RIG-I. Luciferase reporter activity was measured in non stimulated or SV-infected cells. (D) Forty ng of an expression vector for MAVS was transfected in HEK293 together with the Viperin reporter and luciferase reporter activity was measured in SV-infected or untreated cells.

It has been previously shown that the RNA helicase RIG-I is important in sensing of SV (Rothenfusser et al., 2005). To determine whether RIG-I contributes to SV-induced Viperin gene expression, we generated both human and mouse Viperin promoters using luciferase-based reporter constructs. HEK293 cells were transfected with the reporter gene encoding the human Viperin promoter and cotransfected with an expression vector for RIG-I or Mda-5 (Figure 5B). Transfection of the full-length RIG-I alone activated the Viperin reporter and this effect was dramatically enhanced upon SV infection. Mda-5 overexpression slightly induced

Viperin promoter, however no enhancement was observed with SV. Similar results were obtained with the murine version of the Viperin reporter (data not shown). Moreover, Lgp2, a protein structurally related to RIG-I (Cui et al., 2001), that seems to be a post-induction feedback inhibitor of RIG-I signaling (Rothenfusser et al., 2005; Yoneyama et al., 2005) decreased SV-induced Viperin reporter activity (*Figure 5B*). To clearly demonstrate a role for RIG-I we used the human Huh7.5 hepatoma cell line that has a single point mutation within the CARD domain of RIG-I, which impairs its signaling (Sumpter et al., 2005). Consistent with the previous data, the Viperin promoter was induced in the parental Huh7 cell line upon SV stimulation. However, we observed an induction in Huh7.5 upon SV infection only after their complementation with wild type RIG-I (*Figure 5C*). Moreover, overexpression of MAVS in HEK293 cells potently activated the Viperin promoter, and this response was enhanced by SV infection (*Figure 5D*). SV-induced Viperin reporter activity was blocked by the HCV protease NS3/4A (data not shown), in fact it has been recently shown that NS3/4A abrogates signaling through RIG-I by cleaving MAVS from the mitochondrial membrane (Li et al., 2005).

Collectively these data support a key role for the TRIF/TRAM pathway in the induction of Viperin following LPS treatment and demonstrate that RIG-I and its downstream CARD-containing adapter MAVS regulate Viperin gene expression upon SV infection.

Regulation of Viperin Gene Expression by the TBK1/IRF3/type I IFN axis.

We next compared the induction of Viperin in macrophages from C57BL/6 wild-type, IKK ϵ -deficient and TBK1/TNFR1 double ko mice. TBK1-deficient mice are embryonic lethal, however, this lethality can be rescued by crossing these mice

onto the TNFR1^{-/-} background. C57BL/6 and TNFR1^{-/-} mice induced equivalent levels of Viperin in response to all stimuli tested (not shown). The induction of Viperin in response to both LPS and poly(I:C) was completely abrogated in TBK1/TNFR1 ko mice (*Figure 6A*). A minor defect was also seen in IKKε-deficient macrophages. In contrast, when SV was examined, the induction of Viperin was intact in both strains. This may reflect a functional redundancy between these two kinases in the SV response, since macrophages express both kinases (unpublished data). Support for this idea comes from studies in TBK1-deficient embryonic fibroblasts (MEF cells have little or no IKKε expression) that fail to induce Viperin upon SV infection (data not shown).

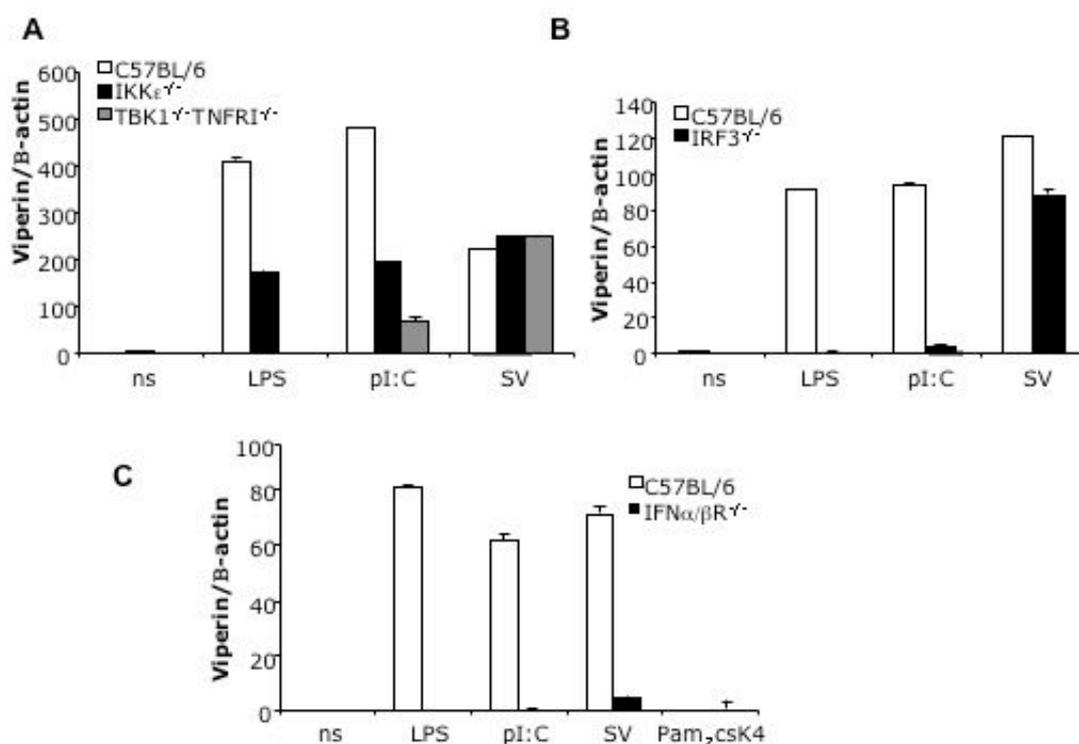


Figure 6. Viperin gene expression is regulated via the TBK1/IRF3 axis. (A-C) BM-macrophages from C57BL/6, IKKε^{-/-} or TBK1^{-/-}TNFR1^{-/-} (A) IRF3^{-/-} (B) or IFNα/βR^{-/-} (C) -mice were differentiated and treated with LPS, poly(I:C) or SV for 6 h. Total RNA was extracted and the Viperin mRNA quantified by real-time RT-PCR.

The induction of Viperin in response to LPS and poly (I:C) was also completely impaired in macrophages from IRF3-deficient mice (*Figure 6B*). Conversely, the virus-induced response in IRF3-deficient macrophages was completely normal. Compensation by other IRF may account for the normal induction of Viperin upon SV infection in IRF3-deficient cells.

In order to determine if Viperin was a direct IRF3 target gene or if this gene was induced as a result of IFN- β signaling we examined C57BL/6 and IFN α/β R ko macrophages. Viperin was not induced in response to LPS, poly (I:C) or SV in macrophages from IFN α/β R ko (*Figure 6C*), suggesting that Viperin induction mainly relies on IFN- β signaling regardless of the stimuli. Pam2CSK4 fails to induce Viperin mRNA expression consistent with a failure of TLR2 ligands to induce IFN- β (Toshchakov et al., 2002).

ISGF3 is the direct transducer of Viperin transcription.

Since our *in silico* analysis identified two putative ISREs in the Viperin promoter and type I IFN target genes are known to be regulated via ISGF3-dependent ISRE binding, we next examined the effect of overexpressing IRF9 and STAT1, which are the two ISGF3 factors responsible for DNA binding within the ISRE sites (Bluyssen and Levy, 1997; Paulson et al., 1999; Qureshi et al., 1996). We also analyzed the involvement of IRF1, IRF3 and the NF- κ B subunit p65 (*Figure 7A*). While p65 failed to induce the Viperin reporter, an NF- κ B reporter was strongly induced in response to p65 overexpression (data not shown). IRF1 induced the Viperin reporter gene, however there was no enhancement by SV under these conditions. The ability of IRF1 to induce the reporter is consistent with a role for IRF1 in mediating the IFN- γ induced Viperin induction (Chin and Cresswell, 2001). IRF3 also induced a very weak Viperin promoter activity and this response

was not dramatically enhanced upon SV infection. Most significantly, when either

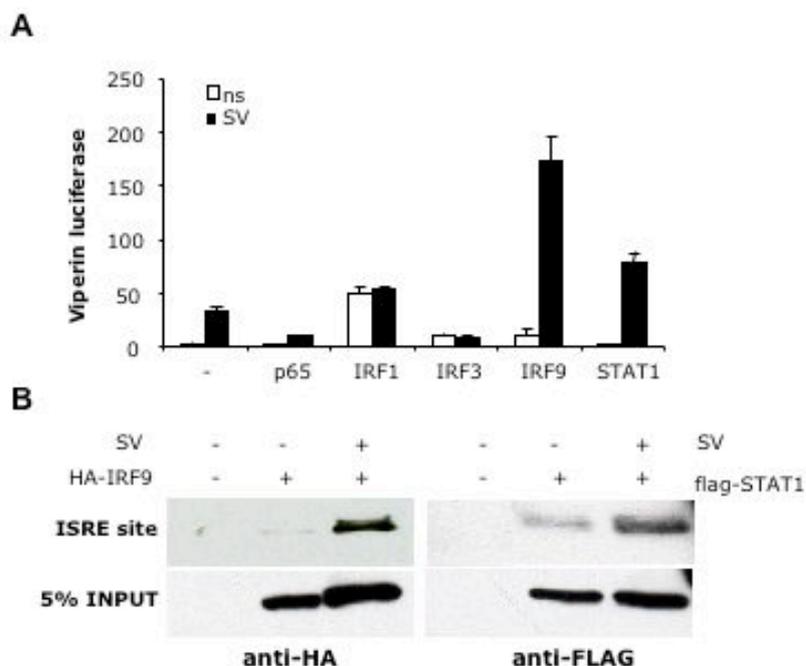


Figure 7. The ISGF3 complex is the key transducer of Viperin transcription. (A) Forty ng of expression vectors for p65, IRF1, IRF3, IRF9 or STAT1 were transfected in HEK293 with vector encoding a reporter for Viperin promoter. The cells were then infected by SV or left untreated. Luciferase activity was measured following 24 h. (B) HEK293T were transiently transfected with expression vectors encoding HA-tagged IRF9 and flag-tagged STAT1 and then infected with SV. Nuclear cell extracts were prepared after 16 hours of infection. Nuclear lysates (500 µg) were incubated with streptavidin magnetic beads to which the biotinylated oligonucleotide corresponding the two ISREs of Viperin promoter was bound. After extensive washing, the pulled-down ISGF3 complex was identified by Western blot with anti-HA or anti-flag Abs. Input indicates the levels of the transfected IRF9 and STAT1 tagged proteins detected in the nuclear extracts.

IRF9 or STAT1 were expressed a clear enhancement was detected upon SV infection (Figure 7A).

We next performed DNA affinity purification assays to examine ISGF3 complex binding to the ISRE sites. For this purpose, biotinylated oligonucleotides corresponding to the two tandem ISRE sites within the Viperin promoter were incubated with nuclear extracts from HEK293 cells transiently transfected with IRF9 or STAT1, and infected or not with SV. Both IRF9 and STAT1 binding to the ISRE sites could be detected post-virus infection (Figure 7B). We could also detect

endogenous STAT2 binding to this site (data not shown). Equivalent levels of transfected proteins were observed (*Figure 7B, lower panels*).

PRDI-BF1/BLIMP1 competes with the ISGF3 complex for the ISRE sites and acts as a negative regulator of Viperin gene transcription.

The *in silico* analysis of the Viperin gene promoter also led to the identification of two binding sites for the transcriptional repressor PRDI-BF1 (also called B lymphocyte-induced maturation protein 1, BLIMP1), in the same region as the binding sites for IRF2 and overlapping IRF3/ISRE sites. PRDI-BF1 is a member of the PRDM gene family of transcriptional repressors which recruits corepressor proteins of the Groucho family, that function at least in part through association with histone deacetylases (Chen et al., 1999; Ren et al., 1999). PRDI-BF1 can also assemble a silent chromatin architecture over its target genes recruiting the histone H3 lysine methyltransferase G9a (Gyory et al., 2004). We therefore assessed the effect of PRDI-BF1 on the regulation of Viperin gene transcription. Exogenous expression of PRDI-BF1 completely abolished SV-induced Viperin reporter gene activation (*Figure 8A*). Consistent with published data, PRDI-BF1 also blocked SV-induced IFN- β reporter gene expression. Notably, NF- κ B activation was unaffected. In addition the induction of Viperin by IFN- β was also dose-dependently inhibited by PRDI-BF1 (*Figure 8B*), suggesting that Viperin repression is not indirect via inhibition of IFN- β production. Consistent with its ability to block IFN- β -induced Viperin activation, PRDI-BF1 binding to the ISRE sites was detected in a virus inducible manner by DNA affinity purification Assay (*Figure 8C*). Furthermore, the *in silico* analysis suggested that the PRDI-BF1 sites overlapped the consensus sites for IRF3, which in turn overlaps in part with one of

the two ISRE sites. PRDI-BF1 could also be detected on the IRF3 site (Figure 8C, upper panel).

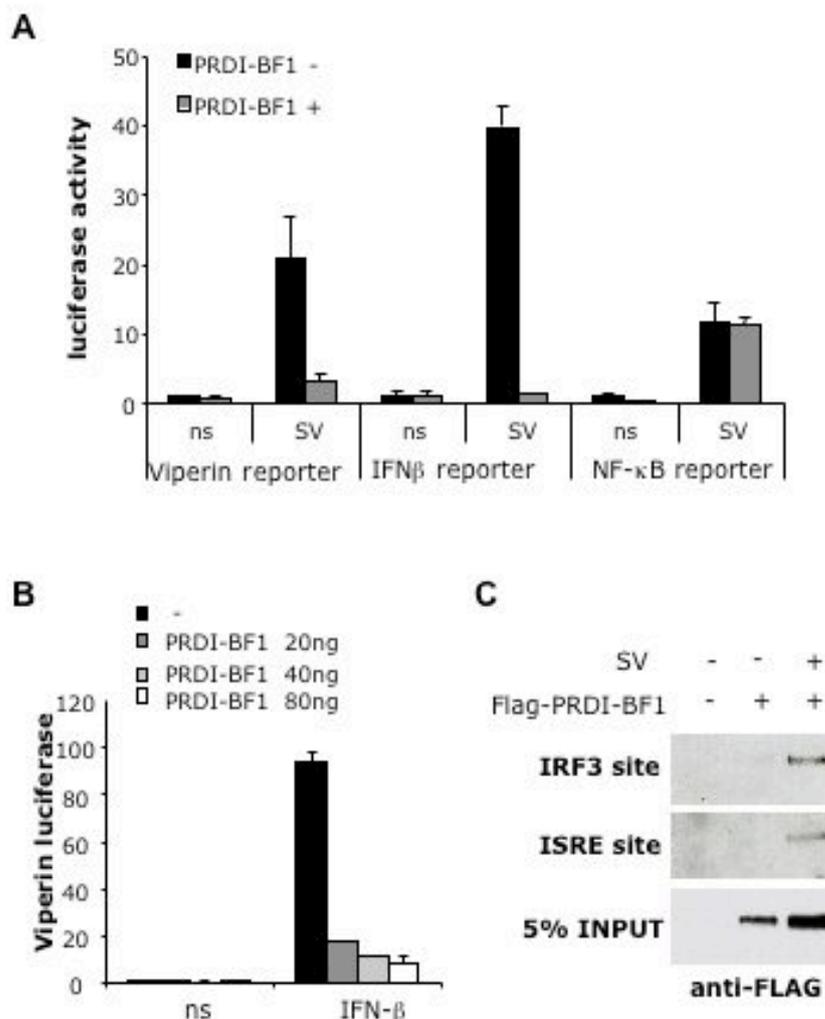


Figure 8. PRDI-BF1/BLIMP1 negatively regulates Viperin gene expression. (A) HEK293 cells were transfected with a vector encoding the human PRDI-BF1/BLIMP1 and cotransfected with Viperin, IFN- β or NF- κ B reporter genes, as indicated. The cells were then infected with SV or left untreated and lysates were assayed for luciferase activity. (B) Viperin reporter activity in IFN- β -treated HEK-293 cells cotransfected with serial doses of PRDI-BF1 expression vector. (C) Nuclear cell extracts from HEK293T cells transfected with flag-tagged PRDI-BF1 expression vector, with or without SV infection, were prepared. Binding of 500 μ g of nuclear extracts to biotinylated oligonucleotide corresponding to the ISREs or IRF3-binding site present on Viperin promoter were tested by DNA-binding Assay, as described in the legend of Figure 7. Specifically bound PRDI-BF1 (upper panels) and the relative level of transfected protein (bottom panel) were detected by Western blotting with anti-flag Ab.

Interestingly, the virus induced binding of STAT1 to these sites was dose-dependently inhibited in the presence of increasing amounts of PRDI-BF1 (*Figure 9A*). The relative amounts of the proteins present in each cell lysate before pull down was examined by Western Blotting (lower panels) and the quantity of the nuclear lysates used in the assay was verified by immunoblotting with the ubiquitously expressed nuclear protein USF-2 (*Figure 9A, lowest panel*). Previous

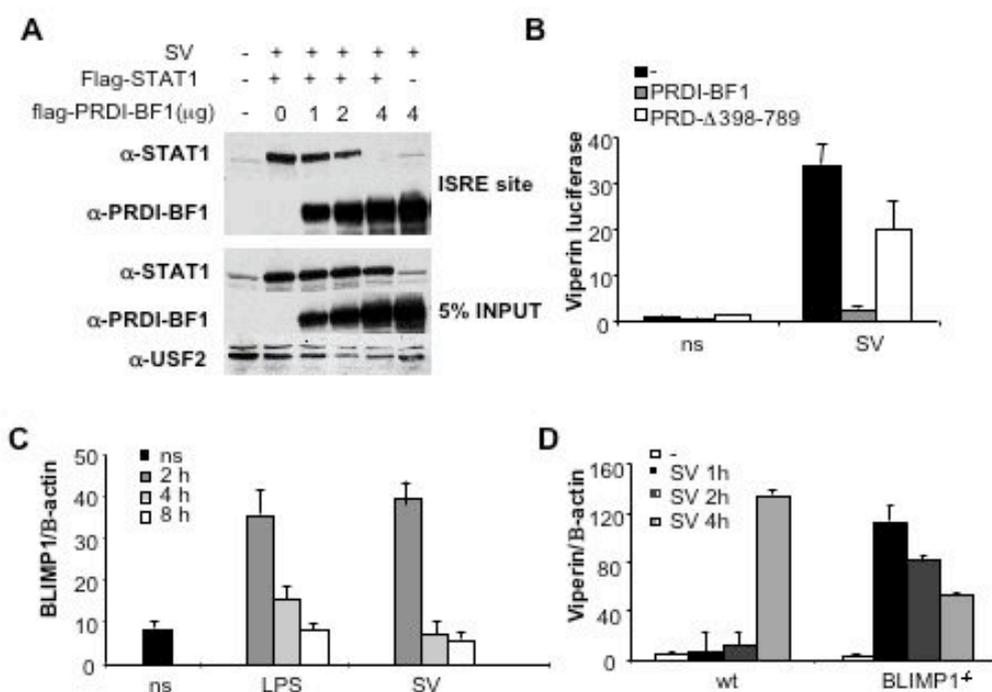


Figure 9. PRDI-BF1/BLIMP1 negatively regulates Viperin gene expression competing with the ISGF3 complex for the ISRE sites. (A) Nuclear extracts were obtained from HEK-293T cells transfected with flag-tagged STAT1 (4 μg) and then cotransfected with increasing amounts of vector encoding flag-tagged PRDI-BF1 (1, 2 and 4 μg) and infected with SV. The extracts were used for the oligonucleotide pull-down assay where the relative levels of STAT1 and PRDI-BF1 bound to the ISREs were detected by immunoblotting with anti-STAT1 and anti-PRDI-BF1 Abs, respectively. The relative level of the overexpressed proteins and of the lysates used for the Assay were tested by Western Blotting with α-STAT1 and α-PRDI-BF1 Abs and with an Ab against the ubiquitously expressed nuclear protein USF-2. (B) Viperin reporter activity in SV-stimulated or unstimulated HEK293 over-expressing either PRDI-BF1 full-length or deleted in the amino-acids 1-398 (PRD-Δ398-789). (C) BLIMP1 gene expression in BM-macrophages derived from C57BL/6 mice and treated with either LPS or SV at the indicated time points. BLIMP1 mRNA was quantified by real-time RT-PCR. (D) Quantification of Viperin gene expression in wt or BLIMP1^{-/-} MEFs.

published studies have defined a region within PRDI-BF1 between amino acids 331 and 429, which are important for the inhibition of IFN- β gene transcription (Keller and Maniatis, 1992). To determine if this same region was critical for suppression of Viperin induction, we examined virus induced reporter gene induction in cells co-expressing either the full length PRDI-BF1 or a deletion mutant lacking the repression domain. Importantly, while the full-length PRDI-BF1 inhibited Viperin induction, the deletion mutant (PRD- Δ 398-789) failed to repress Viperin transcription (*Figure 9B*).

To define the role of BLIMP1 in the transcriptional regulation of Viperin gene expression *in vivo*, we measured BLIMP1 mRNA levels in BM-derived macrophages from C57BL/6 wild-type mice upon LPS and SV treatments at different time points (*Figure 9C*). We observed a strong induction of the BLIMP1 gene very early within 2 hrs post-infection/treatment. We therefore next employed mouse embryonic fibroblasts (Mefs) lacking BLIMP1 to further understand the relevance of this gene on Viperin gene expression following SV infection. Maximal levels of Viperin mRNA were reached at 4 h post-infection in wild type Mefs (*Figure 9D*). In contrast in BLIMP1^{-/-} Mefs, the Viperin expression profile was dramatically altered and the maximal induction was observed much earlier, at 1 hr post-infection, proving that the presence of BLIMP1 negatively affects Viperin transcription, blocking it in the early phases of virus infection. Collectively, these data are consistent with a model whereby PRDI-BF1/BLIMP1 competes with the ISGF3 complex and binds to the Viperin promoter in a virus inducible manner and mediates its transcriptional repression *in vivo*.

Type I IFN-dependent regulation of TLR7 expression in LPS-treated DC

As mentioned before, TLR7 expression was also modulated along DC maturation by the autocrine release of type I IFN, thus we focused our attention on TLR7 to

understand its regulation in monocyte-derived DC. Indeed, in humans the expression of TLR7, together with TLR9, is known to be restricted only to the endosomes of the pDC but not of the conventional DC, whereas TLR8 expression is constitutively expressed in myeloid DC, albeit at low levels (Gorden et al., 2005; Hornung et al., 2002). We confirmed the results obtained by microarray analysis measuring TLR7 mRNA level by Real-time RT-PCR either in untreated or IFN- β and LPS-treated DC in presence or absence of α -IFN Abs (*Figure 10A*). Whole cell

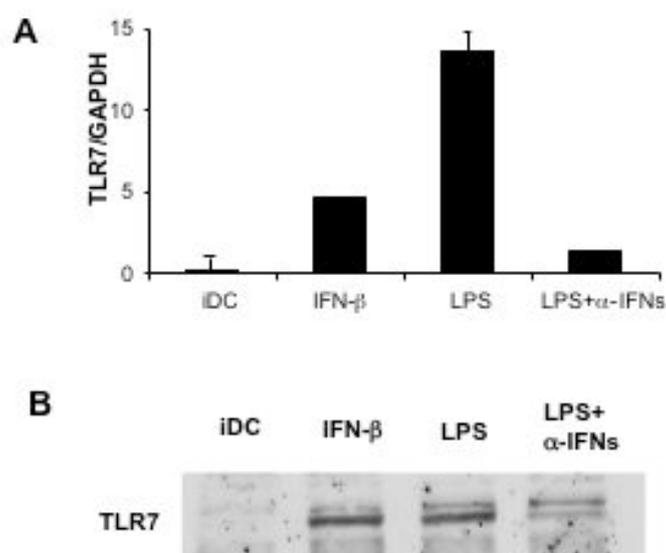


Figure 10. TLR7 expression by LPS-induced IFN- β production. (A) TLR7 gene expression was quantified by Real-time RT-PCR in total RNA extracted from non stimulated or IFN- β (200pM)-, LPS (100 ng/ml)- and LPS+ α -IFNs Abs-treated DC. (B) Whole cell lysates collected from cells treated for 24 h as described in (A) were analyzed by Western Blotting using a specific anti-TLR7 Ab. This analysis was repeated 2 additional times using DC from a total of 3 different blood donors.

lysates were prepared from DC following 24 h of treatment with IFN- β or with LPS in the presence or absence of α -IFNs Abs. TLR7 protein expression was quantified by Western Blotting (*Figure 10B*). Consistent with mRNA expression data, TLR7 protein level was also induced following stimulation with LPS and

inhibited when α -IFNs Abs were added to the cultures, further proving a clear dependence on IFN production and signaling in the regulation of TLR7 by LPS.

We next examined whether the activation of other TLR expressed in DC could modulate TLR7 gene expression as we had observed following LPS stimulation. DC were stimulated for 24 h with synthetic bacterial lipopeptide (Pam2cysk4) and with synthetic dsRNA [poly (I:C)] which engage TLR2 and 3, respectively. Total RNAs were prepared and TLR7 mRNA levels were analyzed by quantitative PCR (*Figure 11A*). Both poly(I:C) and LPS treatments strongly induced TLR expression, in contrast to Pam2Cysk4, which did not stimulate TLR7 expression. This differential activation of the TLR7 gene prompted us to examine its promoter region to identify the presence of regulatory sequences involved in this regulation. Using the TFBS prediction program MatInspector, transcription factor binding sites for IRF-1, STAT1 and NF- κ B were detected within 600 bp of the TSS (*Figure 11B*). To probe the involvement of each of these factors to the regulation of TLR7 gene induction, oligonucleotides corresponding to the binding sites for IRF-1, STAT1 and NF- κ B from the TLR7 promoter were designed. Nuclear extracts were prepared following stimulation of DC with IFN- β and LPS in the presence or absence of α -IFNs Abs and analyzed by EMSA for the specific binding of each factor to the respective site. No STAT1 binding and only a slight signal in the case of NF- κ B were detected (data not shown). Conversely, a clear induction of IRF-1 DNA-binding activity was observed in LPS- and IFN- β -treated cells and this binding activity was completely lost when α -IFNs Abs were added (*Figure 11C*). The identity of the complexes was confirmed by supershift experiments using antibodies raised against IRF-1. Moreover, complex formation was inhibited by the addition of an excess of unlabeled oligonucleotide containing the putative IRF-1 consensus sequences within TLR7 promoter, but not by an excess of an unrelated sequence (*Figure 11D*).

To confirm the role of IRF-1 on TLR7 regulation in DC, we transiently transfected a vector expressing the human IRF-1 or an empty vector by nucleofection in immature DC. The nucleofection is usually employed to efficiently transfect

primary cells, as DC. As shown in *Figure 11E*, the expression of the TLR7 mRNA measured by Real-time RT-PCR was clearly increased by the ectopic expression of IRF-1 compared to DC transfected with an empty vector or untransfected. All together, these results indicate a key role for IRF-1 in the regulation of TLR7 along LPS-induced DC maturation.

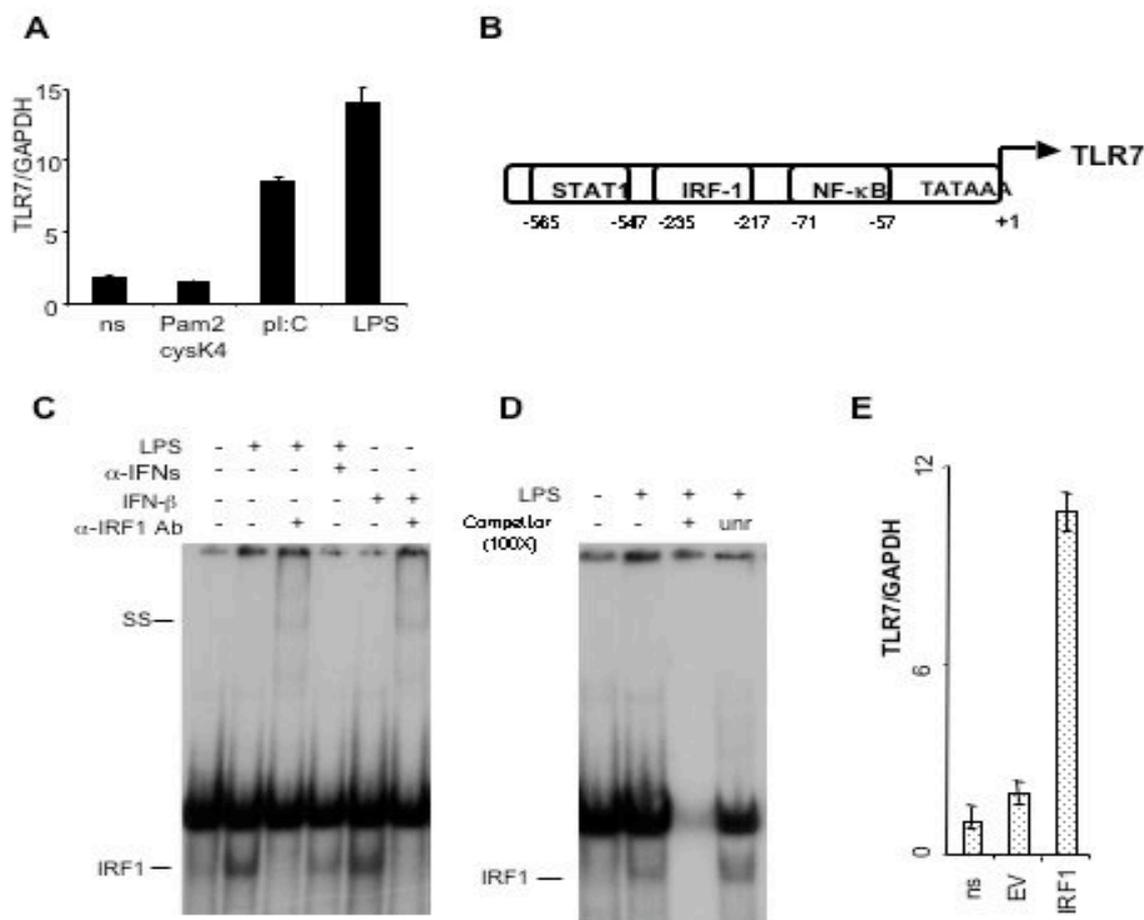


Figure 11. Transcriptional regulation of TLR7 gene. (A) Total RNA was extracted from non stimulated (ns), Pam2cysk4-, poly(I:C)- and LPS-treated DC upon 24 h treatment. Real-Time RT-PCR was performed to measure TLR7 mRNA level. One representative out of 3 different experiments is shown. (B) Schematic map of ~600 bp of the human TLR7 promoter. TATAAA is the consensus sequence for the TATA box. (C) DC were treated with IFN-β or LPS with or without α-IFNs Abs for 4 h. Nuclear extracts (15 μg) were prepared and analyzed by EMSA using a specific radiolabeled oligonucleotide corresponding to the IRF-1 binding site present within TLR7 promoter. Supershift assay (SS) was performed using an anti-IRF-1 Ab, where indicated. This experiment was repeated two additional times. (D) For competition studies 100X excess of cold oligonucleotide containing the putative IRF-1 consensus sequence within the TLR-7 promoter and a sequence unrelated (unr) to IRF-1 were added to the binding reaction. (E) Total RNA was also extracted from DC nucleo-transfected with vectors expressing IRF-1 or with an empty vector (EV). TLR7 expression was evaluated by quantitative PCR.

Maturation of IFN- β -pretreated DC by a specific TLR7 agonist

Although TLR7 and TLR8 are phylogenetically and structurally related, their selective activation leads to completely different immune responses. It has been shown that the small molecule imidazoquinoline analogues 3M-001 and 3M-002 are selective ligands for TLR7 and TLR8, respectively (Gorden et al., 2005). 3M-001 directly activates pDC and, to a lesser extent, monocytes; conversely, 3M-002 activates monocytes and monocyte-derived DC. Since our findings support a model whereby TLR7 gene and protein expression are induced following type I IFN release, we examined whether a 24 h pre-treatment of DC with IFN- β would enable these cells to respond to TLR7 ligands. To this aim, IFN- β -pretreated DC were stimulated for 24 h with 3M-001, a small molecule imidazoquinoline analogues which is the selective ligand for TLR7 (Gorden et al., 2005). As control, the stimulation of DC with TLR8 agonist, 3M-002, was also performed. The expression of CD83, CD86 and CD38 surface markers were evaluated by Flow Cytometry Analysis to assess the activation status of the cells (*Table IV*).

Table IV
Induction of surface marker expressions by TLR-7 triggering in IFN- β pre-treated immature DC:

Ligand	IFN- β	MFI +/- SE		
		CD83	CD86	CD38
none	-	7+/-0.6	12+/-6	11+/-1
none	+	5+/-1.5	29+/-12	79+/-18
3M001	-	8+/-4	12+/-3	12+/-4
3M001	+	21+/-12	80+/-20	153+/-41
3M002	-	10+/-5	21+/-9	75+/-12

Immature DC were pre-treated 4 h with IFN- β (200 pM) and then stimulated 20 h either with 3M001 (25 μ M) or 3M002 (5 μ M). Results are means \pm SE from four separate experiments.

We observed that a pre-stimulation of DC with IFN- β provoked the maturation of the 3M-001-treated DC, phenomenon not observed in the absence of IFN- β treatment. As expected, TLR8 triggering alone induced an up-regulation of the considered markers and IFN- β by itself caused an increase of CD38 and CD86. Furthermore, the secretion of the pro-inflammatory cytokines, such as TNF- α and IL-6, and of the regulatory cytokines, IL-12p70 and IL-10, was analyzed. Supernatants were harvested from DC treated for 24 h with the TLR7 agonist 3M-001 with or without 24 h IFN- β pre-treatment. 3M-002-stimulated DC were used as our internal control and a strong production of all the analyzed cytokines was observed upon TLR8 triggering (*Figure 12*). Conversely, in the IFN- β -pre-treated DC a clear induction of IL-12, IL-6, TNF- α and IL-10 production was detected following TLR7 activation compared to DC treated with 3M-001 alone.

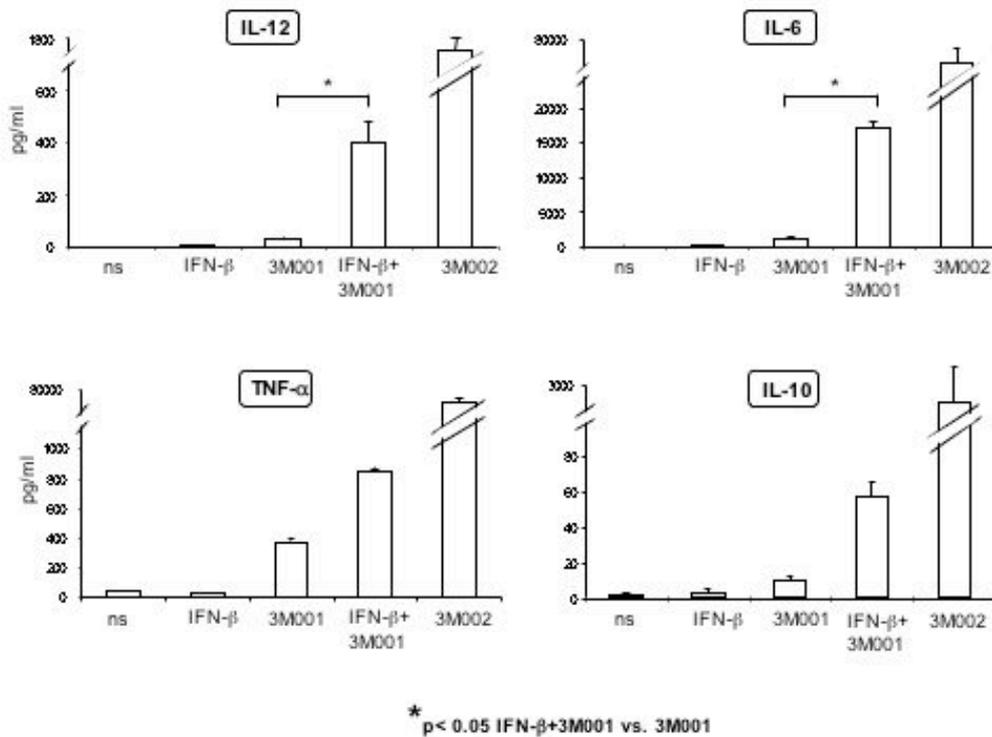


Figure 12. Analysis of cytokine production in IFN- β pre-treated DC following TLR7 triggering. Cell culture supernatants were harvested from DC treated for 24 h with the TLR7 agonist 3M-001 with or without 24 h pre-treatment with IFN- β , or stimulated either with 3M-002 or IFN- β alone. The levels of IL-12, IL-6, TNF- α or IL-10 were measured by CBA. Results are means \pm SE from 4 different experiments. $p < 0.05$ 3M-001 vs IFN- β + 3M-001.

DISCUSSION

DC play a critical role in initiating and modulating the immune responses elicited upon recognition of infectious agents. Indeed, DC respond to microbes by triggering a complex maturation program that results in their migration from tissue to the draining lymph nodes and in an enhanced T cell stimulatory capacity (Steinman, 2003). During the maturation process and in a finely regulated fashion, DC produce several cytokines and chemokines that act sequentially in different micro-environments and on different leukocyte populations (Huang et al., 2001; Langenkamp et al., 2000). The cytokines and chemokines produced by maturing DC immediately after contacting the pathogens may in turn regulate, in an autocrine and paracrine fashion, the production of other soluble mediators critical for the establishment of an inflammatory and innate immune response and for the recruitment of monocytes, macrophages, DC and neutrophils. In particular, it has been demonstrated that type I IFN regulate the production of CXCL-10 (Hilkens et al., 2003; Lande et al., 2003b) as well as members of the IL-12 family (McRae et al., 1998; Nagai et al., 2003; van Seventer et al., 2002).

Type I IFNs are cytokines that play a central role in host immunity, in fact they are expressed by many cell types in response to viral or microbial infections (Bogdan et al., 2004; Coccia et al., 2004; Remoli et al., 2002). By binding to specific transmembrane receptors, they trigger a response that culminates in the induction of a large number of genes modulating and linking the innate and the adaptive immune responses.

The main objective of this thesis has been defining the type I IFN expression profile in DC in the context of viral infection or upon stimulation of TLR and the effects that the production of these cytokines has along the process of DC maturation.

In the first part of our study, we investigated the IFN gene regulation in monocyte-derived DC either upon Flu or SV infections or following TLR-3 and TLR-4 triggering with their respective ligands LPS and poly (I:C) (*Figure 1*). The

expression of several IFN- α subtypes, IFN- β and IFN- λ genes was analyzed by real-time RT-PCR (Coccia et al., 2004). IFN- α 1, - α 2, - α 8 and - β together with IFN- λ 1 subtype (also called IL-29) and IFN- λ 2 and IFN- λ 3 (IL-28A and B respectively), which are highly homologous, were chosen as the representative subtypes to be studied.

The kinetics of type I IFN subtypes measured in our experimental model upon virus infection are not reminiscent of a two-wave expression profile described in virus-infected fibroblasts, where IFN- β production is followed by a delayed induction of specific IFN- α genes (Marie et al., 1998). Indeed, the expression of all examined IFN was rapid and started as early as 1 to 3 h after infection, indicating that DC may differ from other cell types in the regulation of IFN production by viral infection (*Figure 1A*). Conversely, TLR-3 stimulation by poly(I:C) or TLR-4 stimulation by LPS induced a fast and robust expression of IFN- β and IFN- λ mRNA (*Figure 1B*). With the exception of IFN- α 1, which showed a poor induction, none of the other IFN- α subtypes was induced by poly(I:C) or LPS treatment.

These results indicated that, among all the type I IFN subtypes, IFN- β , together with IFN- λ , was highly induced along DC maturation irrespectively of the stimuli. Since DC, besides representing one of the major sources of type I IFN, are also key responders, we wanted to elucidate the role that the IFN- β plays in addressing the DC differentiation status.

Since TLR4, triggered by its ligand LPS, induces IFN- β as a 'signature' cytokine (Coccia et al., 2004; Thomas et al., 2006; Toshchakov et al., 2002), we chose LPS as stimulus to investigate the IFN- β -related transcriptional and subsequent functional consequences that can occur in human monocyte-derived DC along LPS-induced maturation. To this end, using gene expression profiling, we identified a set of 23 genes that were induced in DC following IFN- β and LPS treatments and downregulated by neutralizing the autocrine IFN- β produced following LPS stimulation by adding α -IFNs Abs (*Figure 3A*). Among the various

induced genes, several of these were well known ISGs with anti-viral activity. Additional genes were identified, which have not previously been characterized as ISGs. We found that different sensors for pathogen-derived molecules, such as TLR7 as well as NOD2 and DEFB1, were induced in a type I IFN-dependent manner. Both NOD2 and DEFB1 are involved in the resistance to bacterial pathogens. NOD2 (also called CARD15) is a member of the phylogenetically conserved NLR (NACHT-LRR) family and plays a role in the immune response to intracellular bacteria by recognizing MPD, a peptidoglycan component (Strober et al., 2006). DEFB1 is an antimicrobial peptide implicated in the resistance of epithelial surfaces to microbial colonization and belongs to the family of defensins, microbicidal and cytotoxic peptides made by neutrophils (Kluver et al., 2006). TLR7 recognizes ssRNA and is activated by infections with ssRNA viruses (Diebold et al., 2004a; Heil et al., 2004). The TLR7 gene has been reported to be induced by type I IFN in monocytes/macrophages or in B cells (Bekeredjian-Ding et al., 2005; Siren et al., 2005), but its inducibility by type I IFN has never been characterized in the context of DC maturation. Moreover, Viperin, an anti-viral gene whose function is still unclear, was found to be IFN-regulated.

Despite the great importance of the NOD pathway and the role of defensins in the innate immune response, our interest especially focused on characterizing TLR7 and Viperin gene regulation in DC, since very little is known about it.

It is known that, in the case of human CMV infection, Viperin inhibits viral assembly and maturation by downregulating the viral proteins gB, pp28 and pp65 (Chin and Cresswell, 2001). Viperin is highly conserved between species and contains an MoaA/PQQIII motif, shown to be important for iron-sulfur cluster coordination associated with protein radical formation, and the biosynthesis of cofactors, which may influence anti-microbial defenses (Menendez et al., 1996). Further studies are required to establish the importance of this region and the mechanisms by which Viperin contributes to mammalian host-defenses.

This study provides clear evidence that type I IFN signaling and STAT activation are essential for Viperin gene induction. Consistent with their role in the regulation of IFN- β , induction of Viperin upon LPS or poly(I:C) treatment was TRIF-mediated (*Figure 5*) and completely dependent on the TBK1/IRF3 axis (*Figure 6*), as experiments with macrophages deficient in TRIF, TBK1 or IRF3 showed. Furthermore, as expected, Viperin was not induced upon LPS treatment in IFN α/β R ko cells (*Figure 6C*), consistent with a model whereby TBK1 and IRF3 control type I IFN production, which then induce Viperin *via* the IFN α/β R.

Analysis of the Viperin promoter revealed the existence of putative binding sites for multiple transcription factors including IRF1, IRF2, IRF3, IRF4, ISGF3 and NF- κ B (*Figure 4C*). We provided clear evidence that the ISRE elements which bind ISGF3, and not the other sites, are important in the transcriptional regulation of the Viperin gene. In fact, DNA Affinity purification Assays demonstrated that ISGF3 binding to biotinylated oligonucleotides corresponding to the two tandem ISRE sites present within the Viperin promoter could be detected post-virus infection (*Figure 7B*).

Although advantageous to the host by inducing genes like Viperin, type I IFNs are not always beneficial. Left uncontrolled excessive IFN production can contribute to autoimmune-like symptoms resembling those of systemic lupus erythematosus, thyroiditis, rheumatoid arthritis or psoriasis (Kunzi and Pitha, 2003) (Theofilopoulos et al., 2005). Like other cytokines therefore, the actions of IFN- α/β and target gene induction must be controlled (Coccia et al., 2006). We provided evidence that PRDI-BF1/BLIMP1 acts as a negative regulator of Viperin gene expression (*Figure 8*). We showed that PRDI-BF1 inhibits not only virus-induced IFN- β gene expression, but also virus-induced and IFN β -induced Viperin expression. Moreover, PRDI-BF1 competes with the ISGF3 complex for the ISRE sites (*Figure 9A*). PRDI-BF1 binding to the IRF3 site may also enhance the inhibitory effect on the ISRE sites. PRDI-BF1 is a member of the PRDM gene family of transcriptional repressors which recruits corepressor proteins of the Groucho family, that function at least in part through association with histone

deacetylases (Chen et al., 1999; Ren et al., 1999), and the H3 histone methyltransferase G9a (Gyory et al., 2004). We have also detected PRDI-BF1 mRNA induction upon either virus infection or LPS treatment in mouse macrophages, consistent with PRDI-BF1 acting as a post-induction feedback regulator of gene expression (*Figure 9C*). Indeed, Mefs lacking BLIMP1 showed an altered profile of virus-induced Viperin gene induction (*Figure 9D*). These results suggest that BLIMP1 is an important negative regulator of Viperin gene regulation *in vivo*. How the BLIMP1 gene is induced is unclear at present. TLR may activate PRDI-BF1 gene expression via NF- κ B activation (Johnson et al., 2005). These interesting observations may implicate PRDI-BF1 as a target of the MyD88-dependent pathway for TLR4 signaling and provide a molecular basis for the elevated induction of Viperin observed in MyD88-deficient macrophages (*Figure 5A*).

Another important aspect of our study, as previously highlighted, was the IFN-dependent induction of TLR7 in monocyte-derived DC.

It has been described that, in different human DC subpopulations, only pDC express TLR7, whereas myeloid DC constitutively express TLR8 (Hornung et al., 2002; Jarrossay et al., 2001; Kadowaki et al., 2001b).

TLR7 and TLR8 belong to the ‘TLR9 subfamily’ (Wagner, 2004). The members of this subfamily of TLR (TLR3, TLR9, TLR7 and TLR8) are evolutionary related and sense viral and bacterial nucleic acids at the endosomal subcellular compartment. This is in contrast to the other TLR, such as TLR2 and TLR4, which are localized at the cell surface. Initially, TLR7 and 8 were shown to trigger IFN production in response to the imidazoquinolines, imiquimod and resiquimod (or R-848), low molecular weight immune response modifiers with potent anti-viral and anti-tumor properties (Hemmi et al., 2002). Subsequently, the immunostimulatory action of several additional guanine nucleoside analogs has been shown to be controlled exclusively *via* TLR7 (Lee et al., 2003). Only later, then, the natural ligands of TLR7 and 8 were identified in the virus-derived ssRNAs, specifically

guanosine- and uridine-rich ssRNA oligonucleotides derived from HIV-1 (Heil et al., 2004) or ssRNA derived from wild-type Influenza virus and synthetic ssRNA (polyU) (Diebold et al., 2004b). Moreover, only very recently, the possible role of TLR7 in recognizing ‘self’ RNA, acting in this way as a sensor of endogenous “danger signals”, has been described. In fact, the recognition of U1 small nuclear RNA, that is part of the small nuclear ribonucleoproteins, one of the major component of the immune complexes associated with the pathogenesis of the autoimmune disease Systemic Lupus Erythematosus, is dependent on TLR7 (Savarese et al., 2006). All these findings highlight the importance of understanding the regulation of expression and the consequent activity of TLR7.

We showed that TLR7 gene expression can be activated in myeloid DC upon TLR4 stimulation in a type I IFN-dependent manner *via* a mechanism which is dependent upon IRF-1. Consistent with these findings TLR3 triggering was also able to increase TLR7 mRNA levels, likely through the release of IFN- β , whereas TLR2 stimulation failed to do so (*Figure 11*). These findings are consistent with a failure of TLR2 to activate IFN- β production (Toshchakov et al., 2002).

A key question arises from these observations: can IFN- β priming of DC render them responsive to specific TLR7 agonists? To answer this question, we pre-treated immature DC with IFN- β and then stimulated with the small molecule imidazoquinoline analogues 3M-001 and 3M-002, selective ligands for TLR7 and TLR8, respectively (Gorden et al., 2005). We demonstrated that the TLR7 gene is not only induced in these conditions, but is also functional. In fact, 3M-001 treatment, which selectively activates TLR7, was able to induce the expression of maturation and activation markers, like CD83, CD86 and CD38, in IFN- β -treated DC but not in immature DC (*table IV*). Therefore, a robust enhancement in pro-inflammatory as well as regulatory cytokines (IL-12, IL-6, TNF- α and IL-10) was observed (*Figure 12*). Importantly, the acquisition of TLR7 expression by myeloid DC did not confer to these cells the ability to produce significant amounts of type I IFN following stimulation with specific agonists (data not shown).

The scenario detailed in our study is consistent with recent reports demonstrating that combined TLR ligation of DC triggers distinct signaling pathways resulting in an enhanced activation of these cells in a synergistic manner in terms of cytokine production and expression of costimulatory molecules (Gautier et al., 2005; Napolitani et al., 2005; Warger et al., 2006). The exogenous IFN- β can mimic, in our case, the endogenous IFN- β production stimulated by TLR3 and TLR4 triggering, which has been shown in other studies to potently act in synergy with endosomal TLR (Napolitani et al., 2005). This synergy can also be explained by our findings that highlight the ability of TLR3 and TLR4-activated pathways to induce, in a type I IFN-dependent manner, sensors normally absent in immature DC and ensuring in this way that powerful effectors will be generated to respond combinatorially to microbial products in different cellular compartments. Another explanation supporting this hypothesis is that TLR4 can participate in the immune response to certain viruses (Burzyn et al., 2004; Haynes et al., 2001; Kurt-Jones et al., 2000; Otten et al., 2002; Rassa et al., 2002; Triantafilou and Triantafilou, 2004); the virus-induced triggering of TLR4 can mediate the transcriptional activation of certain genes encoding intracellular sensors for viruses, such as TLR7, to amplify the response to specific viral infections.

All our findings indicate that the release of IFN- β during the maturation process of DC activates specific transcription programs amplifying the status of alert of these cells through the expression of pathogen sensors (such as TLR7, DEFB1 and NOD2) or factors able to fight the viral replication and spreading (as Viperin). For an overview of our study see *Figure 13*.

Overall, our results might be relevant in understanding the complex activation of the IFN-induced intracellular pathways occurring in DC during pathogen infections and might be helpful in developing new DC-based strategies against persistent viral infections.

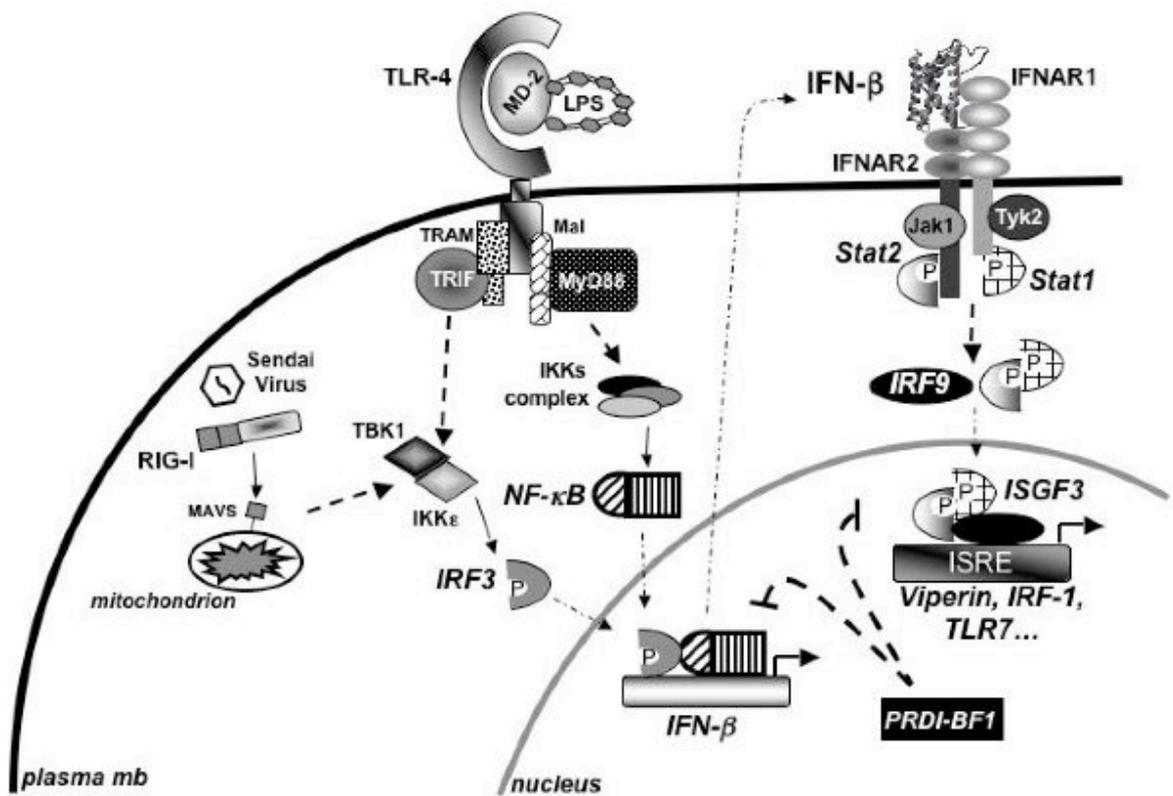


Figure 13. A summary.

TLR4 activation or virus infection induce IFN- β release along DC maturation and the IFN- β acts in an autocrine and paracrine manner to activate a specific gene expression profile.

AKNOWLEDGMENTS

First and foremost, I would like to thank Eliana M. Coccia (Istituto Superiore di Sanita`, Rome, Italy) and Kathrine A. Fitzgerald (University of Massachusetts Medical School, Worcester, MA) for allowing me to work with them and for their ability to teach and motivate. Thanks a lot for making me a curious and passionate researcher!

I would like also to thank all the adorable people that are working with me at ISS. Maria Elena Remoli *in primis* for her priceless help and for the good times we had together, Elena Giacomini for her knowledge on the cell biology and for her beautiful songs and Roberto Lande...the only -patient- man in our lab!

Thanks a lot also to the very international LRB, 3rd floor at Umass: the precious support of you all and your friendship made my life much easier in the US!

Last but not least my family and in particular my parents for all the sacrifices made to support me in my never easy choices!

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