Cell Defence against Viral/Bacterial Infections: Closer Mechanism than Anticipated?

(innate immunity / IRF / NFκB / Toll receptors / signalling pathways)

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Recognition of an infectious agent

The ability of the host to detect invasion by a pathogenic intruder and to activate the defence mechanism to eliminate the infection is essential for survival. The host defence system against the invading pathogen is composed from the innate and adaptive immunity. While the adaptive response is based on the recognition of antigen-specific lymphocytes and their clonal selection, the innate immune response is not pathogen-specific and provides rapid response that is not long-lasting; however, this response is essential for the generation of the adaptive immunity. An innate immune response has developed as a rapid and regulated defence mechanism in which the recognition of an invading pathogenic organism can occur upon binding to specific viral receptors, where many of these are signalling molecules or Toll-like receptors (TLRs) that can recognize the conserved patterns of proteins, lipoproteins, dsRNA, or unmethylated CpG DNA (Kopp and Medzhitov, 1999; Akira et al., 2001; Underhill and Ozinsky, 2002). While originally discovered by their ability to recognize bacterial and parasite invasion, it has been shown recently that mammalian TLRs can also recognize the patterns on the surface of virions such as the respiratory syncial virus-encoded F fusion protein (Kurt-Jones et al., 2000) and viral RNA or DNA. Altogether, the broad array of cellular responses to invading pathogens can include phagocytosis, induction of inflammatory cytokines, chemokines and co-stimulatory molecules. Among the inflammatory cytokines, type I interferons (IFNs) play a unique role in the antiviral innate immune response, since they have not only the ability to directly inhibit viral replication, but also induce differentiation of dendritic cells, activation of natural killer (NK) cells and macrophages. However, aberrant expression of IFNα genes has been shown to be associated with several chronic viral infections and autoimmune diseases (Neighbor et al., 1981; Preble et al., 1982; Rhodes-Feuillette et al., 1983; Arvin and Miller, 1984; Fitzgerald-Bocarsly et al., 1991; Kunzi et al., 1995). There is also accumulating evidence that inappropriate or deregulated expression of chemokines or their receptors can play a role in the autoimmune response (Karpus et al., 1995) and rheumatoid arthritis (Nanki et al., 2000). Thus, the identification of factors that regulate the inducible and timely expression of these inflammatory proteins is of primary importance.

The interactions of pathogens with cellular receptors induce multiple signalling pathways leading to the activation of transcription factors that control the expression of a diverse set of genes coordinating the immune responses. Although cascades of multiple kinases mediate activation of these transcription factors, their functional diversity is modulated by interaction with other transcription factors and co-factors. Indeed, these regulatory networks are critical components of the host defence against invading pathogens, including viruses.

Two families of cellular factors play a critical role in the transcriptional activation of the early inflammatory genes. One is the well-characterized family of NFκB factors (Lenardo and Baltimore, 1989); the other is a newly emerging family of interferon response factors (IRFs) (Nguyen et al., 1977). The IRFs have been first identified by their role in the virus-mediated induction of type I IFN genes, but later were shown to also participate in the induction of chemokines (e.g. Rantes and MIP-1), cytokines (e.g. IL-12 and IL-15), genes involved directly in the antiviral response (PKR, 2,'5'OAS), antibacterial response (iNOS and $gp91^{phox}$), inflammation (cox2) and MHC class II gene expression.

IRF family

The identification of IRFs as direct transducers of virus-mediated signalling truly revolutionarized our understanding of the molecular mechanisms involved in viral pathogenicity and inflammatory responses. To date, nine human IRFs were identified (IRF-1, IRF-2, IRF-3, IRF-4/Pip/ICSAT, IRF-5, IRF-6, IRF-7, ICSBP/IRF-8 and ISGF3γ/p48/IRF-9), and they all share significant homology in the N-terminal 115 amino acids, which comprise the DNA-binding domain

Received May 3, 2004.

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Abbreviations: IFN – interferon, IRF – interferon response factor, KSHV – Kaposi sarcoma herpes virus, NK – natural killer, ORF – open reading frame, TLR – Toll-like receptor.

(Nguyen et al., 1995), characterized by five tryptophane repeats. Three of these repeats contact the DNA-binding domain with a specific recognition of the GAAA and AANNNGAA sequence (Escalante et al., 1998). These factors can function as transcriptional activators (e.g. IRF-1, 3, 7 and 9), repressors (IRF-8) or both (IRF-2, 4, and 5). The unique function of a particular IRF can often be accounted for by cell type-specific expression, its intrinsic trans-activation potential, and ability to interact with other members of the IRF family or other transcription factors and co-factors (Nguyen et al., 1997; Taniguchi et al., 2001). Three IRFs (IRF-3, IRF-5 and IRF-7) play a critical role in the antiviral innate immune response and in the expression of type I IFN genes and chemokines (Au et al., 1995, 1998; Marie et al., 1998; Lin et al., 1999; Barnes et al., 2001).

While IRF-3 is constitutively expressed in all cell types (Au et al., 1995), expression of IRF-5 and IRF-7 has been primarily detected in lymphoid cells and can be further stimulated by type I IFNs (Au et al., 1998; Marie et al., 1998; Lu et al., 2000, 2002). In monocytes, and particularly in precursors of dendritic cells (pDC2), that are high producers of type I IFNs, both IRF-5 and IRF-7 are expressed constitutively (Izaguirre et al., 2003). In infected cells, expression of IRF-3 is sufficient to support induction of IFNβ, while IRF-5 or IRF-7 are needed for stimulation of IFNα genes. Reconstitution of IRF-5 or IRF-7 expression in human cells that upon viral infection expressed only IFNβ resulted in virus-mediated induction of IFNα genes (Yeow et al., 2000; Barnes et al., 2001).

All these three IRFs serve as direct transducers of virus-mediated signalling. In uninfected cells these factors reside predominantly in the cytoplasm, but upon virus-induced phosphorylation of serine residues in the carboxyl-terminal regions, they translocate to the nucleus (Au et al., 1998; Lin et al., 1998; Weaver et al., 1998; Yoneyama et al., 1998; Barnes et al., 2001), where they were identified as components of the transcriptional complex-enhanceosome, assembled on the promoters of IFNα1 and IFNβ genes in infected cells (Wathelet et al., 1998; Sato et al., 2000; Au and Pitha, 2001; Barnes et al., 2002). However, functions of IRF-3, IRF-5 and IRF-7 are not redundant, since these factors are expressed in different cell types and stimulate expression of distinct profiles of IFNα, chemokine and IFN-stimulated genes (Barnes et al., 2001; Peters et al., 2002). The microarray analysis also revealed induction of common as well as distinct sets of genes in IRF-5 or IRF-7 overexpressing infected cells (unpublished results).

Role of IRFs in cancer

The functions of IRFs are not limited to innate response to viral infection, but these factors also play an important role in differentiation of lymphoid cells and apoptosis. Perturbation of these functions leads to tumorigenicity. IRF-1, identified as a pro-apoptotic gene, is localized on human chromosome 5q31.3. This region is frequently deleted in acute myelogenous leukaemia or myelodysplasia (Boultwood et al., 1993). IRF-3 is localized on human chromosome 19q13.3 (Lowther et al., 1999), a region frequently deleted in gliomas (Yong et al., 1995). IRF-4 expression in B cells is associated with the differentiation of germinal B cells into plasma cells (Falini et al., 2000). In multiple myeloma, IRF-4 is juxtaposed next to the immunoglobulin heavy chain and acts as a new oncogene (MUM1). Chromosomal translocation t(6;14) x (p25;q32) involves the MUM1/IRF-4 (Iida et al., 1997). IRF-5 functions as a regulator of cell growth and differentiation. It is localized in the imprinted region on human chromosome 7p32 (Barnes et al., 2001) and IRF-5 is not expressed in many primary leukaemias and lymphomas (Barnes et al., 2003). Furthermore, IRF-5 is a component of the p53 signalling pathway and induces G_2/M cell cycle arrest and apoptosis in a p53-independent manner (Barnes et al., 2003). Overexpression of IRF-5 in a B-cell tumour line inhibits its growth in nude mice. IRF-7 induces macrophage differentiation (Lu and Pitha, 2001) and its expression is silenced by CpG methylation in many tumour cell lines (Lu et al., 2000). IRF-7 is localized on human chromosome 11p15.5 (Lu and Pitha, 2001) and this region contains several imprinted genes. IRF-8 plays an essential role in differentiation of macrophages and dendritic cells. Mice that are lacking IRF-8 genes develop myeloid-like proliferation disease and have a defect in a subset of high interferon producing dendritic cells (Tsujimura et al., 2003). These findings indicate that, apart from their role in antiviral immune responses, IRF proteins regulate cell growth and differentiation, and that deregulation of these functions leads to tumorigenesis.

Toll receptors

The TLRs are an evolutionarily conserved family of cell surface proteins. In eukaryotic cells, at least 11 TLRs have been identified (Akira and Hemmi, 2003) and ligands that are recognized by these receptors include components of bacteria, viruses, yeast and fungi. Thus TLR2 recognizes peptidoglycan (Schwandner et al., 1999), TLR3 ssRNA (Alexopoulou et al., 2001), TLR4 LPS (Poltorak et al., 1998), TLR5 reacts with flagellin (Hayashi et al., 2001), TLR7/8 are activated by guanosine derivatives, imiquimod and ssRNA and TLR9 interacts with unmethylated CpG DNA (Hemmi et al., 2000). The recognition of these structures by TLRs triggers signalling pathways and consequently results in induction of inflammatory cytokines that bridge innate and acquired immune responses.

The cytoplasmic region of all TLRs contains a common peptide domain called TIR that mediates interaction of TLRs with the adaptors such as MyD88, recruited to TLRs upon ligand binding. Binding of a lig-

and to TLRs also leads to activation of the serine/threonine protein kinases of the IRAK family and consequently activation of TRAF6 factor and NFκB. In addition, another adaptor, Mal/Tollip (Burns et al., 2000; Fitzgerald et al., 2001; Kaisho et al., 2001) that associates with TLR2, is also required for activation of the NFκB.

Binding of ligands to two TLRs, TLR3 and TLR4, activates signalling not only by MyD88-dependent, but also by the MyD88-independent pathway, which leads to activation of IRF-3 and IRF-7 factors. The activation of IRF-3 and IRF-7 mediated by binding of LPS to TLR4 requires two adaptors: TRIF/TICAM (Yamamoto et al., 2002; Oshiumi et al., 2003) and TRAM (Fitzgerald et al., 2003b). In contrast, binding of dsRNA to TLR3, which is believed to mimic viral infection, activates IRF-3 and IRF-7 only through the TRIF adaptor (Fitzgerald et al., 2003b; Yamamoto et al., 2003). It is noteworthy that activation of the antiviral response and type I IFNs upon binding of ssRNA, imiquimod or CpG DNA to TLR7 or TLR9, respectively, is MyD88-dependent and that these two TLRs do not associate with TRIF. Altogether, these observations show that the initial recognition of the pathogen by the TLRs is quite diverse, but that all signals merge into the activation of IRFs and NFκB factors.

NFκB and IRFs are activated by distinct kinases. While IKKγ and IKKα play a major role in the activation of NFκB by the MyD88-dependent pathway, two non-canonic IκB kinases, IKKε and TBK-1, were implicated in the phosphorylation and activation of IRF-3 in cultured human cell lines *in vitro* (Fitzgerald et al., 2003a; Sharma et al., 2003). There is high homology between these two kinases (Pomerantz and Baltimore, 1999; Peters et al., 2000). Both kinases are synergistic with TANK (TRAF family-associated NFκB activator), which links them to the IKK complex by TANK association with IKKγ/nemo (Pomerantz and Baltimore, 1999). Both IKKε or TABK-1 kinase also phosphorylate IkBα, but only on serine 36, and since the degradation of IkBα by the ubiquitination pathway requires phosphorylation of serines 32 and 36, neither IKKε nor TABK-1 is able to target IκBα for degradation and activate NFκB.

The essential role of TBK-1 in the activation of IRF-3 in response to viral infection and TLR3 and TLR4 ligands was demonstrated by using TBK-1 null mice (McWhirter et al., 2004). IRF-3 was not activated and translocated to the nucleus in TBK-1-deficient cells, and expression of IRF-3-targeted genes such as type I IFNα and Rantes was profoundly inhibited in virus-infected, polyIC-treated, or LPS-stimulated TBK-1 null cells. However, TNFα-mediated induction of the Rantes gene was the same in wt and TBK-1 null cells, indicating that TBK-1 is not required for activation of the IKKα, β, γ complex and IkBα degradation.

Altogether these results indicate that multiple factors play a critical role in the antiviral response to infectious agents. Mutations or defect in any of these factors leads to defects in the immune response to infection as shown in mice that are TRIF-deficient (Yamamoto et al., 2003) or have a mutation in the *TRIF* gene (Hoebe et al., 2003a). These mice have not only a profound defect in IFNβ and chemokine production, but also in upregulation of co-stimulating proteins on antigen-presenting cells (Hoebe et al., 2003b).

Finally, it is noteworthy that several virus-specific autosomal loci, named If loci, were previously identified by a genetic analysis (DeMaeyer and DeMaeyer-Guignard, 1988). These If loci, which have high and low alleles expressed on macrophages and haematopoetic cells, determine the levels of IFN production in infected and polyIC-treated inbred strains of mice. Although about 10 different If loci were found, only two of them were mapped. Thus, the If-1 locus influencing IFN induction by Newcastle disease virus (NDV) was assigned to chromosome 3, and HSV-induced IFN production was shown to be X-linked and assigned to the If-x locus. Clearly, it will be of great interest to determine whether the virus-specific If loci represent distinct TLRs or a critical component of the TLR signalling pathway.

Viral mimicry

The effective innate antiviral response is able not only to block the majority of viral infections, but also stimulate the acquired immune responses. To be able to overcome this cellular blockade, many viruses developed mechanisms to inhibit or eliminate the antiviral response. Among various mechanisms of viral mimicry (Katze et al., 2002), there is an increased evidence that many viruses encode proteins that can inhibit the induction of type I IFNs by targeting the function of cellular IRFs, namely IRF-3 and IRF-7, thus directly indicating the importance of these IRFs in the innate antiviral response and induction of IFN genes (Au et al., 1998; Burysek et al., 1999b; Juang et al., 1999; Lubyova and Pitha, 2000; Talon et al., 2000).

The herpes γ virus, Kaposi sarcoma herpes virus KSHV/HHV-8 has employed a different mechanism and inserted modified analogues of human IRFs into its genome.

KSHV-encoded vIRFs

KSHV encodes a cluster of open reading fames (ORFs) that encode at least four proteins with similarity to the cellular IRFs. From these, three vIRFs have been cloned and characterized. In contrast to cellular IRFs, vIRFs are not DNA-binding proteins, since the essential binding domains in the N-terminal part of the protein are deleted. However, like cellular IRFs, the KSHV vIRFs are able to interact with the cellular IRFs and other transcriptional factors and co-factors through their C-terminal regions.

VIRF-1 (ORF K9) expression is induced in KSHV-positive B-cell lymphoma cells (PEL cells) treated with 12-O-tetradecanoylphorbol-13-acetate (TPA). This vIRF has been studied extensively by several groups: it inhibits both the virus-mediated induction of type I IFN genes and IFN-induced genes; vIRF-1 overexpression in NIH/3T3 cells confers tumorigenicity when these cells are injected into nude mice (Gao et al., 1997; Flowers et al., 1998; Li et al., 1998; Burysek et al., 1999b). However, targeted expression of vIRF-1 to B cells did not lead to formation of B-cell tumours in the transgenic mice (unpublished results). It was also shown that vIRF-1 binds to cellular IRFs and to CBP/p300 and inhibits its acetyltransferase activity (Burysek et al., 1999b; Li et al., 2000) resulting in global inhibition of acetylation of histones H3 and H4 (Li et al., 2000).

VIRF-2 (ORF K11.1) encodes a small nuclear protein (163 aa) that is constitutively expressed in PEL cells (Burysek et al., 1999a). VIRF-2 specifically associates with several cellular IRFs and with p300 (Burysek et al., 1999a). It also binds dsRNA-activated protein kinase, PKR, inhibits its kinase activity, and blocks the phosphorylation of the PKR substrate, eukaryotic translation initiation factor 2α (Burysek and Pitha, 2001). An additional transcript, encompassing vIRF-2 spliced to the ORF K11, has been identified; it has been detected only in TPA-treated PEL cells and its function has not yet been characterized (Jenner et al., 2001; Pitha, unpublished results).

VIRF-3/LANA2 is encoded by ORFs K10.5 and K10.6 (Lubyova and Pitha, 2000; Jenner et al., 2001; Rivas et al., 2001). It is a constitutively expressed nuclear protein that shows close similarity to IRF-4. It was shown that LANA2 binds to IRF-1, IRF-3, IRF-7, and CBP/p300 and modulates IRF-7-mediated activation of IFNα1, IFNβ, and Rantes promoters in infected cells (Lubyova et al., 2004). The constitutive expression of LANA2 and its ability to bind several growth-regulatory genes (unpublished results) suggest that LANA2 may play a role in KSHV-associated neoplasia.

Thus, virus-mediated mimicry allows the virus not only to conquer the immune defence, but has also a pathogenic consequence. KSHV-encoded vIRF-1 and vIRF-2 target the function of innate antiviral defence systems. However, nuclear antigen LANA2/vIRF-3 that is constitutively expressed in B-cell lymphoma (PEL) seems to modulate function of the growth-stimulatory and tumour-suppressing genes and as such it may play a role in KSHV-associated tumorigenicity. The observation that regression of some of the KSHV-associated malignancies can be induced by treatment with IFN suggests that the function of a KSHV oncogene(s), and perhaps other viral oncogenes as well, may be suppressed by the innate immune response.

Conclusion and significance

There are two important conclusions that can be made from these studies. One is that the innate antiviral response plays a key role in the immune response to pathogens, the second is that although the recognition of the invading pathogens employs a distinct set of receptors and adaptors, the final steps by which virus, bacteria or parasite signal their presence to the nucleus is mediated by the same mechanism – activation of NFκB and IRF families of transcription factors. As the impact of genetic variability and alternative pathways in the signalling mechanisms is characterized in more details, therapeutic intervention of these processes that would either stimulate the immune response to vaccine antigens or inhibit this pathway when induction of the inflammatory response is detrimental should become apparent.

Acknowledgements

Author regrets that because of space limitation many important references could not be included.

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