Review

Innate Immune Recognition of Viruses and Viral Vectors

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Abstract

Recombinant viral vectors such as adenovirus and adenovirus-associated virus have been used widely as vehicles for gene therapy applications because of the high efficiency with which they transfer genes into a wide spectrum of cells *in vivo*. However, enthusiasm for the use of viral vectors in gene therapy has been tempered by significant problems of attendant host cellular and humoral immune responses that limit their safety and efficacy *in vivo*. Advances in immunology have suggested a crucial role for the innate immune system in the induction of immune responses to viruses. Thus, a better understanding of the mechanisms by which the host's innate immune system recognizes viruses and viral vectors will help in the design of effective strategies to improve the outcome of viral vector-mediated gene therapy. In this review we first discuss our current understanding of innate immune recognition of viruses in general, and then focus on the innate immune responses to viral vectors for gene therapy.

Innate Immune Response to Viruses

THE INNATE IMMUNE SYSTEM is phylogenetically conserved and is present in almost all multicellular organisms (Hoffmann *et al.*, 1999). It is the first line of defense against invading pathogens through recognition of conserved microbial structures or products known as pathogen-associated molecular patterns (PAMPs) by a set of receptors called pattern-recognition receptors (PRRs) (Akira *et al.*, 2006). The best-studied family of PRRs consists of the Toll-like receptors (TLRs), which are expressed on various immune cells including macrophages and dendritic cells (DCs). TLRs are transmembrane proteins characterized by the presence of extracellular leucine-rich repeats and a cytoplasmic signaling domain homologous to that of the *Drosophila* Toll protein and the interleukin-1 receptor (IL-1R), termed the Toll/IL-1 receptor (TIR) domain (Bowie and O'Neill, 2000). So far, 13 TLRs have been identified in mammals, and each TLR appears to recognize a unique set of PAMPs that is distinct in chemical structure (Akira *et al.*, 2006). In addition, studies have revealed the existence of TLR-independent pathways mediated by cytosolic PRRs such as retinoic acid-inducible gene I (RIG-I) and melanoma differentiation-associated gene 5 (Mda5) (Akira *et al.*, 2006).

On recognition of viral PAMPs, PRRs trigger a series of signaling cascades leading to induction of antiviral genes and inflammatory cytokines, which results in direct killing of the

invading virus as well as promotion of the initiation of adaptive immune responses (Kawai and Akira, 2006). In particular, type I interferons (IFNs), including IFN- α and IFN- β , the key cytokines produced in response to viral infection, play an essential role in both innate and adaptive immune responses to viruses (Iwasaki and Medzhitov, 2004; Theofilopoulos *et al.*, 2005). Type I IFNs induce DC maturation by upregulating expression of costimulatory molecules such as CD80, CD86, and CD40, which in turn leads to efficient homing to secondary lymphoid organs and priming of CD8⁺ and CD4⁺ T cell responses (Hoebe *et al.*, 2003; Honda *et al.*, 2003). Type I IFNs also promote cross-priming of virus-specific CD8⁺ T cells by DCs (Le Bon *et al.*, 2003). In addition, type I IFNs can promote the effector function of virus-specific CD8⁺ T cells (Cousens *et al.*, 1999; Nguyen *et al.*, 2002). Furthermore, we have demonstrated that type I IFN signaling directly on T cells can promote the survival of activated CD8⁺ T cells, leading to the formation of long-lived memory cells in response to vaccinia viral infection *in vivo* (Quigley *et al.*, 2008).

TLR-Dependent Detection of Viral Nucleic Acids in Endosomes

As most viruses encounter the endocytic pathway on entering and leaving cells (Brandenburg and Zhuang, 2007), the endosomal compartment has evolved as a key intracellular

FIG. 1. Innate immune pathways for virus sensing. ?, unknown cytosolic sensor for viral DNA.

structure in detecting viruses. Several TLRs participate in the recognition of viral components, such as genomic DNA and RNA in endosomes (Fig. 1). These include TLR3, TLR7, TLR8, and TLR9 (Lund *et al.*, 2003; Diebold *et al.*, 2004; Heil *et al.*, 2004; Krug *et al.*, 2004). These endosomal TLRs have a more restricted cellular distribution. In humans, TLR7 and TLR9 are specifically expressed by plasmacytoid DCs (pDCs), also known as professional type I IFN-producing DCs. However, in mice, TLR7 and TLR9 are expressed more widely, including in nonplasmacytoid (conventional) DCs (cDCs) (Iwasaki and Medzhitov, 2004). TLR3 is preferentially expressed in cDCs although nonhematopoietic cells such as epithelial cells have been shown to express TLR3 (Akira *et al.*, 2006). Less is known about TLR8, which is thought to be nonfunctional in mice (Heil *et al.*, 2004). Although TLR3, TLR7, and TLR9 recognize viral genomes in endosomes, in the steady state, most of the receptors are localized in the endoplasmic reticulum (ER) in association with Unc93b, an ER protein that plays an essential role in regulating the recruitment of TLR3, TLR7, and TLR9 to endosomes (Tabeta *et al.*, 2006; Brinkmann *et al.*, 2007).

Recognition of viral genomes by TLR7 and TLR9 in pDCs

TLR7 senses viral genomic single-stranded RNA (ssRNA) or synthetic ssRNA oligonucleotides (ODNs) containing U or

GU repeats (Diebold *et al.*, 2004; Heil *et al.*, 2004) and plays a critical role in innate immune responses against ssRNA viruses such as influenza virus, vesicular stomatitis virus (VSV), and Sendai virus (Diebold *et al.*, 2004; Lund *et al.*, 2004). TLR9 recognizes unmethylated CpG motif-containing ssDNA ODNs (Krieg, 2002; Latz *et al.*, 2007) and detects DNA viruses such as herpes simplex virus (HSV) and murine cytomegalovirus (MCMV) (Lund *et al.*, 2003; Hochrein *et al.*, 2004; Krug *et al.*, 2004). Activation of TLR7 and TLR9 in pDCs induces type I IFN production mediated by the common TLR adaptor, MyD88 (Akira and Takeda, 2004) (Fig. 1). MyD88 then interacts with IL-1 receptor-associated receptor kinase (IRAK1) and TNF receptor-associated factor-6 (TRAF6), leading to the activation of I κ B kinase α (IKK α) and interferon regulatory factor-7 (IRF7) and the production of type I IFNs (Hoshino *et al.*, 2006; Uematsu and Akira, 2007). However, this pathway is not operative in non-pDCs such as cDCs, and TLR9 stimulation in cDCs only leads to the activation of NF- κ B and the production of proinflammatory cytokines such as IL-6 and IL-12, but not type I IFNs. Thus, pDCs have the unique ability to couple TLR9/TLR7 signaling to producing high amounts of type I IFNs. Why pDCs couple TLR9/TLR7 signaling to IRF7 remains poorly understood. It has been speculated that this might be related to a higher level of IRF7 expression in pDCs than other cell types (Izaguirre *et al.*, 2003). However, one report has suggested a role for the differential use of cofactors

such as osteopontin in pDCs (Shinohara *et al.*, 2006). Alternatively, the ability of pDCs to retain CpG DNA in the endosomal compartment may also play a role (Honda *et al.*, 2005).

How are coated viral genomes exposed in endosomes for their recognition by TLR7 or TLR9? It has been suggested that the highly acidified endosomal compartment, which contains abundant proteolytic degradation enzymes, may damage viral particles, leading to release of viral nucleic acids and recognition by TLR7 or TLR9 (Croizat and Beutler, 2004; Kawai and Akira, 2006). This process is independent of viral infection, and viruses that do not normally replicate in pDCs, as well as defective viral particles or inactivated viruses, can also be detected. Even viruses neutralized by antibody or complement can be taken up via Fc or complement receptors and become subject to recognition by TLR7 within endosomes (Wang *et al.*, 2007). Furthermore, one report has shown that autophagy in pDCs captures VSV RNA replication intermediates from the cytosol and that the resulting autophagosomes fuse with endosomes to allow for the recognition of viral RNA by TLR7 (Lee *et al.*, 2007).

Sensing of viral double-stranded RNA by TLR3 in cDCs

Double-stranded RNA (dsRNA), a common viral PAMP produced by many viruses during their replication (Jacobs and Langland, 1996), is recognized by TLR3 (Alexopoulou *et al.*, 2001). Stimulation of TLR3 with poly(I:C), a synthetic analog of dsRNA, elicited similar results (Alexopoulou *et al.*, 2001). TLR3 signals via the adaptor TRIF (Toll/IL-1R domain-containing adaptor inducing IFN- β), which activates TBK-1 (TANK [Traf family member-associated NF- κ B activator]-binding kinase-1) and I κ B kinase ϵ (IKK ϵ) (Fig. 1). This allows TLR3 to couple to the IRF3 pathway, leading to production of type I IFNs (Akira *et al.*, 2006). Like other TLRs, activation of TLR3 also activates nuclear factor (NF)- κ B and production of proinflammatory cytokines such as IL-6 and IL-12 (Alexopoulou *et al.*, 2001). CD14 has been shown to enhance poly(I:C) uptake and TLR3 signaling (Lee *et al.*, 2006). TLR3 recognizes viruses with a dsRNA genome, such as reovirus, or dsRNA produced during viral replication in the cytosol (Pichlmair and Reis e Sousa, 2007). In addition, TLR3 is highly expressed in cDCs, which avidly phagocytose dying cells (Muzio *et al.*, 2000), and has been implicated in cDC recognition of phagocytosed virus-infected cells containing dsRNA (Schulz *et al.*, 2005).

TLR-Independent Detection of Viral Genomes in the Cytosol

In addition to TLR-dependent recognition of viral genomes in endosomes, accumulating evidence supports the existence of TLR-independent mechanisms of virus sensing by cytosolic PRRs such as RIG-I and Mda5 (Yoneyama *et al.*, 2004; Kato *et al.*, 2005) (Fig. 1). These PRRs also play an important role in detecting viruses and establishing potent antiviral immunity as many viruses, such as vaccinia virus, carry out their entire infectious cycle in the cytosol, and others, such as influenza virus, replicate in the nucleus but traffic through the cytosol on their way in and out of the nucleus. In fact, non-pDCs including nonhematopoietic cells such as fibroblasts rely on

the cytosolic virus-sensing pathway to produce large amounts of IFN- α and IFN- β (Akira *et al.*, 2006).

The cytoplasmic RIG-I was identified as a key molecule in dsRNA-induced production of type I IFNs (Yoneyama *et al.*, 2004) (Fig. 1). RIG-I is a member of the DExD/H box-containing RNA helicases, defined by their ability to unwind dsRNA molecules in an ATPase-dependent fashion (Tanner and Linder, 2001). It contains two copies of the caspase recruitment domain (CARD) at its N terminus and a helicase domain at its C terminus (Zhang *et al.*, 2000). The CARD domains are essential in activating both IRF3 and NF- κ B (Sato *et al.*, 2000; Yoneyama *et al.*, 2004). In one report, ubiquitination of the CARDs of RIG-I, induced by tripartite motif protein-25 (TRIM25) E3 ubiquitin ligase, resulted in a marked increase in RIG-I downstream signaling activity and type I IFN production (Gack *et al.*, 2007). The helicase domain harbors the ATP-binding site, mutation of which caused the inactivation of ATPase activity and conferred dominant negative activity to RIG-I (Yoneyama *et al.*, 2004). One report suggested that RIG-I signals as a multimeric complex regulated by an internal repressor domain at the C terminus (Saito *et al.*, 2007). The ligand for RIG-I has been identified as 5'-triphosphate RNA generated by viral polymerases, regardless of whether it is single or double stranded (Hornung *et al.*, 2006). Capping of the 5'-triphosphate end or by nucleoside modification of RNA, events occurring during posttranscriptional RNA processing in eukaryotes, abolished RNA detection by RIG-I (Hornung *et al.*, 2006).

Mda5 and laboratory of genetics and physiology-2 (Lgp2) are two other members in the RIG-I-like receptor (RLR) family. Mda5 contains two CARD-like domains and a single helicase domain, but lacks the C-terminal repression domain (Yoneyama *et al.*, 2005). Thus, overexpression of Mda5 led to enhanced induction of type I IFN production on Newcastle disease virus (NDV) infection as well as antiviral responses to infections with VSV or encephalomyocarditis virus (EMCV), whereas small interfering RNA (siRNA) targeting endogenous Mda5 blocked NDV-induced expression of type I IFNs (Yoneyama *et al.*, 2005). Lgp2 contains the helicase domain but lacks CARD domains and may act as a negative regulator of RIG-I and Mda5 (Rothenfusser *et al.*, 2005; Yoneyama *et al.*, 2005).

RIG-I and Mda5 share a common signaling pathway involving an adaptor molecule, IFN- β promoter stimulator-1 (IPS-1) (Kawai *et al.*, 2005), also called mitochondrial antiviral signaling protein (MAVS) (Seth *et al.*, 2005), CARD adaptor inducing IFN- β (CARDIF) (Meylan *et al.*, 2005), or virus-induced signaling adaptor (VISA) (Xu *et al.*, 2005). IPS-1 contains an N-terminal CARD-like domain that mediates interaction with the CARDs of RIG-I and Mda5, which results in the initiation of a downstream signaling cascade that culminates in the activation of IRF3, IRF7, and NF- κ B transcription factors, leading to production of type I IFNs and inflammatory cytokines.

It has been demonstrated that cytosolic sensing of double-stranded B-form DNA (B-DNA) derived from microbes can also activate IRF3 and induce type I IFNs and other cytokines independently of TLRs or helicase RIG-I (Ishii *et al.*, 2006; Stetson and Medzhitov, 2006). Indeed, many DNA viruses can trigger TLR-independent production of type I IFNs (Hochrein *et al.*, 2004; Zhu *et al.*, 2007a,c). One candidate for sensing B-DNA receptor is the DNA-dependent activator of

IFN-regulatory factors (DAI). Overexpression of DAI in mouse fibroblasts enhances the DNA-mediated induction of type I IFNs, whereas knockdown of endogenous DAI by siRNA inhibits it (Takaoka *et al.*, 2007). However, the generation of DAI-deficient mice showed that DAI was not essential for either innate or adaptive responses to B-DNA or DNA vaccination *in vitro* or *in vivo* (Ishii *et al.*, 2008). Therefore, the sensor for cytosolic dsDNA still remains elusive; further studies are needed to identify such DNA sensors that mediate DNA-activated innate immunity.

In summary, there is a striking similarity between the endosomal and cytosolic pathways of virus sensing. They both recognize viral nucleic acids and activate parallel downstream pathways for induction of type I IFNs. Thus, the innate immune system senses viral nucleic acids as an invariant determinant of viral presence.

TLR-Dependent Recognition of Viral Envelope Proteins

In addition to TLR-dependent and -independent recognition of viral nucleic acids, TLR2 and TLR4 have been shown to play a role in the sensing of viral envelope proteins. TLR2 has been shown to recognize CMV (Compton *et al.*, 2003), HSV (Kurt-Jones *et al.*, 2004), and vaccinia virus (Zhu *et al.*, 2007c), whereas TLR4 can sense respiratory syncytial virus (RSV) (Kurt-Jones *et al.*, 2000) and mouse mammary tumor virus (MMTV) (Rassa *et al.*, 2002). Interestingly, most of these viruses also induce type I IFNs via TLR-dependent and -independent sensing of viral genomes. Because both TLR2 and TLR4 signaling triggers production of proinflammatory cytokines via the MyD88 pathway (although TLR4 signaling can also induce type I IFNs via the TRIF pathway), it is thought that sensing of viral envelope proteins represents an additional mechanism to enhance innate immune responses to viruses.

Innate Recognition of Adenoviral Vectors

Adenovirus is a nonenveloped, dsDNA virus with a genome of 35 to 40 kb. The experience with adenoviral vectors in various animal models and in human clinical trials has consistently demonstrated that transgene expression from adenoviral vectors *in vivo* usually extinguishes within 2 to 3 weeks, concurrent with the development of inflammation (Yang *et al.*, 1994b; Dai *et al.*, 1995; Kay *et al.*, 1995). This is caused by the rapid activation of potent CD8⁺ and CD4⁺ T cell responses against both the viral antigens and the transgene (Yang *et al.*, 1994a, 1996b; Dai *et al.*, 1995). In addition, activation of B cells by viral capsid proteins, leading to the production of neutralizing antibodies, limits effective readministration of the vector (Dai *et al.*, 1995; Yang *et al.*, 1996a).

Early studies have shown that adenoviral vectors can activate innate immune responses immediately after infection, leading to the secretion of proinflammatory cytokines and chemokines in mice, humans, and nonhuman primates (Schnell *et al.*, 2001; Zhang *et al.*, 2001; Raper *et al.*, 2003). Studies have indicated that in addition to proinflammatory cytokines, high levels of type I IFNs are induced on infection with adenoviral vectors both *in vitro* and *in vivo* (Basner-Tschakarjan *et al.*, 2006; Zhu *et al.*, 2007a). Activation of innate immunity is associated with a reduction in efficacy of gene transfer (Worgall *et al.*, 1997; Zhang *et al.*, 2001), as well as profound damage to healthy tissue and significant morbidity

in transduced hosts (Schnell *et al.*, 2001; Raper *et al.*, 2003). Although newer generations of helper-dependent, gutted adenoviral vectors, which are deleted of almost all viral coding sequences (Parks *et al.*, 1996), have diminished adaptive immune responses to these vectors and improved the duration of gene transfer (Muruve *et al.*, 2004), the acute toxicity provoked by innate immunity remains the most significant barrier associated with clinical application of this otherwise promising technology (Brunetti-Pierri *et al.*, 2004; Muruve *et al.*, 2004).

How do adenoviral vectors activate the innate immune system? Several groups have demonstrated that the innate immune recognition of adenovirus is mediated by both TLR-dependent and -independent pathways (Hensley *et al.*, 2005; Basner-Tschakarjan *et al.*, 2006; Cerullo *et al.*, 2007; Hartman *et al.*, 2007; Iacobelli-Martinez and Nemerow, 2007; Nociari *et al.*, 2007; Zhu *et al.*, 2007a). Sensing of adenovirus or adenoviral DNA by pDCs is mediated by TLR9 and MyD88, leading to production of type I IFNs (Basner-Tschakarjan *et al.*, 2006; Zhu *et al.*, 2007a). The mechanism(s) underlying this cell type-specific involvement of the TLR9–MyD88 pathway in innate sensing of adenovirus remains unclear. pDCs have the unique ability to couple TLR9 signaling to the production of high amounts of type I IFNs, possibly due to high levels of IRF7 expression and/or the use of cofactors such as osteopontin (Izaguirre *et al.*, 2003; Shinohara *et al.*, 2006). In addition, studies have suggested that CpG DNAs are retained longer in pDC endosomes but are rapidly transferred to the lysosome for degradation in non-pDCs (Honda *et al.*, 2005; Guiducci *et al.*, 2006). How is the encapsidated adenoviral DNA exposed in endosomes for its recognition by TLR9? As discussed previously, the highly acidified endosomal compartment that contains abundant proteolytic degradation enzymes may damage viral particles and release some viral DNA for recognition by TLR9 (Croizat and Beutler, 2004; Kawai and Akira, 2006). This process is independent of viral infection. Indeed, we have found that pDCs are poorly transduced by adenoviral vectors (Zhu *et al.*, 2007a), which may be related to the preferential retention of CpG DNA in the endosome by pDCs (Honda *et al.*, 2005; Guiducci *et al.*, 2006). Alternatively, viral DNA could be detected by capturing fragments of virus-infected apoptotic cells by pDCs. Thus, further investigation is needed to define TLR9 recognition of adenoviral DNA in the endosome.

In addition to TLR9-dependent, endosomal sensing of adenovirus by pDCs, recognition of adenovirus by non-pDCs such as cDCs, macrophages, and hepatic Kupffer cells is independent of TLR, leading to secretion of both type I IFNs and proinflammatory cytokines such as IL6, IL-12, and TNF- α (Nociari *et al.*, 2007; Zhu *et al.*, 2007a). This is mediated by cytosolic sensing of adenoviral DNA and dependent on IRF3 (Nociari *et al.*, 2007; Zhu *et al.*, 2007a), although the cytosolic sensor for adenoviral DNA is yet to be identified. Why does adenovirus adopt both TLR-dependent and -independent pathways to induce type I IFNs? It is well established that type I IFNs play an essential role in mediating potent antiviral responses (Garcia-Sastre and Biron, 2006). Although pDCs are the most potent producer of type I IFNs compared with other cell types, studies have shown that other cell types such as cDCs and macrophages can produce type I IFNs on viral infection (Diebold *et al.*, 2003; Jiang *et al.*, 2005). This would suggest that, although on a per-cell basis pDCs secrete much

higher levels of type I IFNs, biologically relevant levels of type I IFNs can also be achieved by much higher numbers of non-pDCs to ensure a potent antiviral response, which may explain why adenoviral vectors are so potent in activating innate immune responses. Furthermore, nonhematopoietic cells such as fibroblasts can also produce type I IFNs via the cytosolic virus-sensing pathway (Akira *et al.*, 2006). Thus, this TLR-independent, cytosolic sensing pathway may function to ensure effective viral control at the site of infection as many viruses carry out their entire infectious cycle in the cytosol, and even others that replicate in the nucleus need to traffic through the cytosol on their way in and out of the nucleus.

The TLR-dependent and -independent induction of type I IFNs is pivotal for innate immune defense against adenovirus *in vivo* (Zhu *et al.*, 2007a). We have further demonstrated that this is mediated by the activation of natural killer (NK) cells, which are critical in the elimination of adenoviral vectors (Zhu *et al.*, 2008). Type I IFNs also play an important role in regulating the induction of proinflammatory cytokines on adenoviral infection *in vivo* (Zhu *et al.*, 2007a). Adaptive T cell responses to adenoviral vectors are also dependent on type I IFNs *in vivo*. Besides promoting DC maturation by upregulating costimulatory molecules such as CD80 and CD86 (Zhu *et al.*, 2007a), our data suggest that type I IFNs act directly on virus-specific T cells to promote the survival of activated T cells in response to adenoviral infection (J. Zhu and Y. Yang, unpublished observation). Furthermore, type I IFN signaling on both B cells and CD4⁺ T cells is required for the formation of neutralizing antibodies to adenoviral vectors *in vivo* (Zhu *et al.*, 2007b). This is achieved by promoting multiple stages of B cell responses to adenoviruses. Taken together, these observations suggest that strategies to interfere with the type I IFN pathway may improve the outcome of adenovirus-mediated gene therapy. Indeed, neutralizing antibodies to IFN- α and IFN- β were effective in blocking innate and adaptive immune responses to adenoviral vectors, leading to improved transgene expression and reduction of inflammation *in vivo* (Zhu *et al.*, 2007a).

Besides TLR-independent induction of type I IFNs and proinflammatory cytokines mediated by IRF3 and NF- κ B, respectively, cytosolic sensing of adenoviral DNA can also activate NALP3 (NACHT-, LRR-, and PYD-containing protein-3; cryopyrin) and ASC (apoptosis-associated speck-like protein containing a CARD), components of the inflammasome (Martinon *et al.*, 2002), leading to caspase-dependent activation of IL-1 β (Muruve *et al.*, 2008). However, only partial reduction of proinflammatory cytokines was observed in mice deficient in inflammasome components (Muruve *et al.*, 2008). It is not clear whether this represents a redundant mechanism to augment the proinflammatory response. Thus, the biological significance of the inflammasome pathway in regulating innate and adaptive immune responses to adenoviral vectors *in vivo* remains to be seen.

Whether capsid components of adenoviral vectors can activate innate immune responses remains unknown. Besides TLR9-dependent recognition of adenoviral DNA by pDCs, sensing of adenovirus by non-pDCs is TLR independent, suggesting that capsid proteins are not involved in TLR recognition (Nociari *et al.*, 2007; Zhu *et al.*, 2007a). It has been proposed that adenoviral penton-integrin interactions might be responsible for the activation of a TLR-independent pathway mediated by phosphatidylinositol-3-kinase (PI3K)

(Philpott *et al.*, 2004). This was based on observations that infection of DCs with adenoviral vectors deleted of the penton base RGD motif or with adenoviral vectors in the presence of PI3K inhibitor significantly reduced TLR-independent DC maturation and TNF- α production (Philpott *et al.*, 2004). However, direct evidence to support this model is lacking. Because both the penton RGD motif-integrin interaction and PI3K activation are critical for adenoviral entry, the observed inhibition of DC maturation and TNF- α production could be due to a reduction of adenoviral DNA targeted to the cytosol, leading to limited cytosolic sensing of viral DNA. Indeed, the viral DNA copy number per cell was significantly reduced with the RGD deletion mutants compared with the wild-type controls (Philpott *et al.*, 2004). Thus, further investigation is required to determine whether purified, endotoxin-free penton proteins can promote DC maturation and trigger the production of proinflammatory cytokines.

Innate Immune Response to AAV

In contrast to adenoviral vectors, whether and how AAV activates innate immunity remains unknown. Infections of HeLa cells with AAV vectors do not lead to the production of detectable levels of inflammatory cytokines (Zaiss *et al.*, 2002). Similarly, robust transcriptome responses associated with adenoviral vectors by microarray studies were not observed with AAV vectors (Stilwell and Samulski, 2004; McCaffrey *et al.*, 2008). These observations are consistent with the notion that AAV is a weak immunogen compared with adenoviral vectors (Zaiss and Muruve, 2005). Indeed, studies in various animal models have demonstrated that AAV vectors can achieve long-term expression of the transgene product (Flotte *et al.*, 1993; Fisher *et al.*, 1997; Herzog *et al.*, 1997, 1999; Song *et al.*, 1998; Greelish *et al.*, 1999; Snyder *et al.*, 1999; Wang *et al.*, 1999; Gregorevic *et al.*, 2006).

However, AAV vectors are not intrinsically inert in activating host immune responses. AAV vectors can efficiently activate the B cell response, leading to the production of neutralizing antibodies against viral capsids, which limit effective readministration of the vector (Chirmule *et al.*, 2000; Peden *et al.*, 2004; Scallan *et al.*, 2006). AAV vectors can also elicit cellular immune responses against the vector and the transgene product depending on the vector dose and serotype, the nature of the transgene, the route of administration, and the host species, leading to the destruction of AAV-transduced target cells *in vivo* (Vandenberghe and Wilson, 2007). These concerns have been substantiated by the outcome of a clinical trial in hemophilia B patients (Manno *et al.*, 2006). In this trial, hepatic delivery of AAV2 vectors encoding factor IX led to therapeutic levels of transgene-encoded factor IX in one patient. However, the therapeutic levels of factor IX were only transient, and were accompanied by a transient transaminitis and the detection of T cell responses to the AAV2 vector. Thus, the previously made observations in mice and humans suggest overall that adaptive immune responses to AAV vector also pose a major challenge in AAV-mediated gene therapy *in vivo*.

Given that innate immunity plays a crucial role in activating adaptive T and B cell responses in other models of viral infection (Iwasaki and Medzhitov, 2004; Pulendran and Ahmed, 2006), it is conceivable that AAV also activates the innate immune system. Because DCs play a pivotal role in the

innate immune sensing of invading pathogens (Kawai and Akira, 2006), future studies should focus on the ability of DCs, particularly pDCs, to detect AAV via TLR9, as AAV has an ssDNA genome. It is also possible that AAV capsid proteins may activate other TLRs or PRRs.

Future Perspectives

In conclusion, the innate immune system detects viral presence through the TLR-dependent endosomal and TLR-independent cytosolic pathways. Both pathways sense viral nucleic acids and activate downstream pathways for induction of type I IFNs. In fact, adenoviral vectors trigger both pathways for efficient activation of the innate immune system. Thus, it imposes tremendous challenges for viral vector-mediated gene therapy. TLR9-dependent recognition of viral DNA by pDCs suggests that strategies to block the TLR9 signaling pathway may improve the outcome of gene therapy with DNA viral vectors. Similarly, novel approaches need to be developed in order to abrogate TLR-independent sensing of viral DNA in the cytosol. Future studies are required to identify the cytosolic innate sensor(s) for viral DNA, which may shed light on the mechanism of viral DNA recognition in the cytoplasm in general, but also on the design of strategies to interfere with viral DNA-triggered innate immunity. Alternatively, because type I IFNs are the critical mediators that link innate immunity to adaptive immune responses, the development of strategies to interfere with the type I IFN signaling pathway may also improve the outcome of gene therapy with viral vectors.

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