Adeno-Associated Virus Activates an Innate Immune Response in Normal Human Cells but Not in Osteosarcoma Cells[▽]

Leila N. Laredj and Peter Beard*

Ecole Polytechnique Fédérale de Lausanne, Department of Life Sciences, Institute for Experimental Cancer Research, 1015 Lausanne, Switzerland

Received 14 June 2011/Accepted 22 September 2011

Adeno-associated virus (AAV) is a small, DNA-containing dependovirus with promising potential as a gene delivery vehicle. Given the variety of applications of AAV-based vectors in the treatment of genetic disorders, numerous studies have focused on the immunogenicity of recombinant AAV. In general, AAV vectors appear not to induce strong inflammatory responses. We have found that AAV2, when it infects the osteosarcoma cells U2OS, can initiate part of its replicative cycle in the absence of helper virus. This does not occur in untransformed cells. We set out to test whether the cellular innate antiviral defenses control this susceptibility and found that, in nonimmune normal human fibroblasts, AAV2 induces type I interferon production and release and the accumulation of nuclear promyelocytic leukemia bodies. AAV fails to mobilize this defense pathway in the U2OS cells. This permissiveness is in large part due to impairment of the viral sensing machinery in these cells. Our investigations point to Toll-like receptor 9 as a potential intracellular sensor that detects AAV2 and triggers the antiviral state in AAV-infected untransformed cells. Efficient sensing of the AAV genome and the ensuing activation of an innate antiviral response are thus crucial cellular events dictating the parvovirus infectivity in host cells.

The adeno-associated virus (AAV) is a small icosahedral nonenveloped, parvovirus that contains a single-stranded DNA (ssDNA) genome of ~4.7-kb (kb). It is a member of the genus *Dependovirus*, so named because it requires coinfection with a helper virus for efficient viral replication (7). The AAV2 DNA termini consist of a 145-nucleotide inverted terminal repeat (ITR), the first 125 nucleotides of which form a palindromic hairpin sequence. The ITRs flank the two viral genes *rep* (replication) and *cap* (capsid) encoding four nonstructural (Rep78, Rep68, Rep52, and Rep40) and three structural (VP1, VP2, and VP3) proteins, respectively.

Initial experiments demonstrated that AAV2 utilizes as primary attachment to the cell receptor heparin sulfate proteoglycans (40). The discovery of two further coreceptors for AAV, namely, $\alpha_v\beta_5$ integrin (39) and fibroblast growth factor receptor type 1 (34), indicated that AAV may have multiple mechanisms for cell entry. The parvovirus hence enters the cell through receptor-mediated endocytosis in clathrin-coated pits (5) and then follows a number of multistep intracellular events that include trafficking through endosomal compartments, a step that was suggested to be important for priming the parvovirus for transduction, nuclear import, virion uncoating, and viral genome replication. The question of whether intact AAV particles enter the nucleus intact or partially uncoat in the cytoplasm or in the endosome is not yet resolved. Previous reports have suggested that either AAV escapes early endosomes into the cytosol or moves to late endosomes (8) or perinuclear recycling endosomes (43). From the late endosome, AAV can be further channeled to the lysosome (8) or

the *trans*-Golgi (4). Movement to the different compartments is facilitated by a family of small GTPases, called Rab proteins (33). It was further documented that these trafficking events are cell type dependent (31), which argues that these findings are representative of the movement of the bulk of AAV virions and does not address the functional significance of every route.

One of the features of AAV biology that turned the virus into an attractive tool for gene therapy is its lack of pathogenicity and low immunogenicity (37). Indeed, AAV-mediated gene transfers proved to be successful and resulted in sustained expression of therapeutic genes in a number of murine, canine, and simian tissues (1, 10, 23, 27, 30). Initial studies on the immune responses elicited by AAV-based vectors have focused on transgene product-specific immune responses and have indicated that although AAV is considered a poor activator of both innate and adaptive immunity (49), this feature highly depends on the transgene itself, the target tissue, the choice of promoter, and most importantly the host species (29). Viral capsids were also shown to be a source of antigens that activate T cell priming (45, 47).

Innate immunity forms the first line of defense, and is orchestrated by membrane-bound and cytoplasmic pathogen-recognition receptors (PRRs) that recognize well-conserved microbial or viral structures, known as pathogen-associated molecular patterns (PAMPs). PRRs form at least four major families: the Toll-like receptor (TLR) system, the RIG-I-like receptors (RLRs), the nucleotide-binding domain leucine-rich repeats (NLRs), and the absent in melanoma 2-like receptors (ALRs).

TLRs were the first family of PRRs identified and are composed of endosomal and membrane-bound members that specifically recognize a plethora of PAMPs, ranging from bacterial agents (TLRs 1, 2, 4, 5, and 6) to nucleic acids (TLRs 3, 7, 8, and 9). TLRs are expressed not only in macrophages and

* Corresponding author. Mailing address: EPFL SV ISREC, SV 1532, Station 19, CH-1015 Lausanne, Switzerland. Phone: 41(0)21 693 0731. Fax: 41(0)21 693 0985. E-mail: peter.beard@epfl.ch.

[▽] Published ahead of print on 28 September 2011.

dendritic cells but also in nonspecialized immune cells (42). The remaining three families are all cytosolic and specialize in sensing nucleic acids (RLRs and ALRs) and glycopeptides (NLRs).

AAV2 has been reported to activate the innate immune response in murine plasmacytoid dendritic cells (50), but little is known of whether or how wild-type AAV stimulates a response in cells that are distinct from specialized immune cells. The work we present here provides evidence that AAV can activate an innate immune response in untransformed human fibroblasts, which exhibit an intact immune detection system by activating the interferon (IFN) response. Such a response is not seen in U2OS osteosarcoma cells, which are partially permissive for the early steps of AAV infection and which seem to lack an intact viral detection system. We also show that detection of AAV genome is probably achieved via endosomal TLR9 and that inhibition of this pathway significantly increases the permissiveness of these cells to the parvovirus.

MATERIALS AND METHODS

Cell lines and chemicals. The osteosarcoma cell line U2OS and the human lung fibroblast cell line HLF were maintained in Dulbecco modified Eagle medium (DMEM), 10% fetal calf serum (FCS), and 1% penicillin-streptomycin. z-FA-fmk (BD Pharmingen), a cathepsin inhibitor, was diluted in dimethyl sulfoxide (DMSO) and added to cells at a concentration of 10 μ M for 1 h. Recombinant human beta interferon (IFN- β ; PeproTech) was used at a concentration of 100 U/ml.

Virus production and infections. DNA-containing AAV2 particles were produced in our laboratory according to standard procedures (48). Multiplicities of infection (MOIs) were based on physical particles (22); virus was used at an MOI of 500, 1,000, 2,500, 5,000, or 10,000 as specified. After infection, DMEM supplemented with 10% FCS and antibiotics was added to the cells.

Cell transfection with synthetic dsRNA poly(I:C) and unmethylated CpG. Transfections of U2OS and HLF cells were carried out using TransLT-IT1 transfection reagents (Mirus) according to the manufacturer's instructions. HLFs and U2OS cells were transiently transfected with synthetic double-stranded RNA (dsRNA) poly(I:C) (InvivoGen) at a final concentration of 50 μ g/ml. In parallel experiments, the same concentration of poly(I:C) was added to the medium. Similarly, U2OS were either treated or transfected with the TLR9-ligand, oligodeoxynucleotide 2006 (ODN 2006) (InvivoGen), at a concentration of 2 μ M.

Western blotting. Whole-cell extracts were prepared by resuspending cell pellets in reporter lysis buffer (Promega) with proteinase inhibitors and incubation for 30 min on ice, followed by centrifugation. Protein samples were resolved on sodium dodecyl sulfate-polyacrylamide gels, transferred to nitrocellulose membranes using an iBlot dry blotting system (Invitrogen). Subsequently, the membranes were blocked and incubated with primary antibodies and horseradish peroxidase-conjugated secondary antibodies using the SNAP i.d. protein detection system (Millipore). Membranes were then processed for detection by ChemiGlow (Alpha Innotech) and exposed to a Fluor Chem 8900 camera.

The primary antibodies used were mouse anti-PML (clone PML97; Sigma), rabbit anti-Rep (P. Saudan), mouse anti- β actin (Abcam), and anti-hTLR9 (eBioscience).

Immunofluorescence staining and microscopy. For immunofluorescence experiments, cells were plated onto glass coverslips 1 day before AAV2 infection. Cells were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) for 10 min, followed by permeabilization with 0.25% Triton X-100 in PBS for 5 min. Samples were subsequently blocked in 5% milk in PBS for 1 h and then incubated with the primary antibody for 1 h in 1% milk in PBS. After three rounds of washes with PBS, samples were incubated with the secondary antibody for 30 min in 1% milk in PBS and then washed again. Finally, coverslips were incubated with DAPI (4',6'-diamidino-2-phenylindole) for 1 min, washed twice with distilled H₂O, and mounted in DABCO-glycerol. The antibodies and concentrations used were as follows: anti-assembled AAV particles (Progen, 1:50), anti-PML (Santa Cruz Biotechnology, 1:250), and anti-Rep (generated against GST-Rep fusion protein according to a standard protocol by Eurogentec, by P. Saudan, 1:1,000). The secondary antibodies used were Alexa Fluor 488-chicken

anti-rabbit IgG, Alexa Fluor 568-donkey anti-goat IgG, Alexa Fluor-goat anti-rat IgG, and Alexa Fluor-goat anti-mouse IgG (Molecular Probes).

Images were collected with an Axioplan 2 microscope (Zeiss) equipped with an Axiocam MRm camera (Zeiss) using plan Apochromat 1.4 oil objective lenses ($\times 63$) and an air 0.75 objective lens ($\times 20$). Confocal images were collected by using a Zeiss LSM 710.

Detection of IFN production. Secretion of type I IFNs by AAV-infected and/or poly(I:C)-transfected cells was determined by bioassay. Briefly, culture supernatants of stimulated U2OS or HLF cells were collected at 24 h postinfection and cleared of cell debris by brief centrifugation. IFN- β levels were measured by using a human IFN- β ELISA kit (PBL Biomedical Laboratories) according to the manufacturer's instructions.

RT-PCR. Total RNAs of mock-treated, adenovirus- and/or AAV-infected, and poly(I:C)-transfected HLF cells were isolated by using TRIzol and purified using an RNeasy minikit (Qiagen) according to the manufacturer's instructions. Isolated RNAs (2 μ g of total RNA) were used for reverse transcription (RT) using Ready-to-Go You-Prime first-strand beads (GE Healthcare). cDNA samples were then used as a template for PCR using Taq DNA polymerase (Invitrogen) and the following specific sets of primers: hTLR9 (5'-GTGCCCCACTTCTCC ATG-3' and 5'-GGCACAGTCATGATGTTGTTG-3') and glyceraldehyde-3-phosphate dehydrogenase (GAPDH; 5'-ATCTTCCAGGAGCGAGATCCC-3' and 5'-CGTTCGGCTCAGGGATGACCT-3'). PCR products were then analyzed by electrophoresis through 2% agarose gels.

RESULTS

Early steps of AAV infection are restricted in HLFs. AAV appears to have a broad host range, and different AAV serotypes are able to replicate *in vitro* in many human, simian, and rodent cell lines provided a helper virus is present. Interestingly, initial experiments in our laboratory indicated that AAV2 was able to efficiently express Rep proteins and in a few cells produce capsid protein following infection of the osteosarcoma cell line, U2OS, without the need of a helper virus (Fig. 1A, row c). Hence, in order to understand what makes a cell line permissive or not to AAV, we performed double immunofluorescence staining for Rep and capsid proteins in human primary fibroblasts and U2OS cells. As illustrated in Fig. 1, Rep expression was clearly present in U2OS following infection with AAV2 and with Ad5 as a helper and reached an expression level of over 80% 24 h postinfection (p.i.) (Fig. 1B, left panel). In contrast, HLF cultures sustained only a low level of Rep expression (7%) (Fig. 1B, right panel). AAV2 uptake clearly occurred in these cells, as the nucleoli of the cells showed aggregated particles that are believed to be incoming virus in the absence of Rep expression (Fig. 1A, row f). Newly formed capsid proteins, characterized by a pan-nuclear staining using the A20 antibody, were detected solely in U2OS cells (Fig. 1A, rows b and c), in an MOI-dependent manner.

Altogether, these data confirm the oncotropic nature of AAV2, which seemingly depends more on the ability of the virus to express replication proteins than on uptake. These observations also bear out earlier reports that nuclear transport is not rate-limiting for the transduction of AAV (17) and suggest the presence of a defense mechanism in normal but not transformed cells.

AAV infection of HLFs leads to both IFN production and activation of IFN-inducible genes. Having found that HLF cells are refractory to AAV2 infection, we postulated that these primary cells mount an antiviral response against AAV2, a response that is most likely weak or inhibited in the tumor cells. In order to test this hypothesis, we measured, using enzyme-linked immunosorbent assay (ELISA), the levels of IFN- β at 24 h p.i. in AAV-infected HLFs and U2OS superna-

FIG. 1. U2OS cells, unlike HLFs, are partially permissive to AAV2. (A) Immunofluorescence analysis of the kinetics of Rep and CAP expression in AAV2-infected and mock-treated U2OS (a to c) and HLF cells (d to f) 24 h p.i. Cells were grown on 19-mm coverslips and then infected or not with AAV2 at an MOI of 2,500 and incubated for 24 h p.i. A positive control included coinfection with Ad5 at an MOI of 5. U2OS and HLF cells were double stained for Rep and CAP as described in Materials and Methods. Nuclei were visualized using DAPI. The data show representative images for each condition. Bar, 10 μ m. (B) Quantitation of the percentage of Rep and capsid expression in U2OS and HLF cells. For each MOI, 10 randomly chosen fields of infected or mock-treated cells stained for Rep, CAP, and DAPI were counted. The relatively muted action of Ad5 in this experiment was due to the Ad5 stock used; we found that quantitatively the response to Ad5 can be quite variable. The values shown are means with standard deviation bars from three independent experiments analyzed.

tants (Fig. 2A). Indeed, IFN- β was previously shown to be the primary cytokine secreted by fibroblasts to mediate the immediate cellular antiviral response in infected cells (35). AAV infection was found to induce HLFs to release about 60 pg of

IFN- β molecules/ml into the culture medium and 180 pg/ml when coinfecting with Ad5. In contrast, U2OS cultures failed to produce this cytokine upon AAV2 infection, raising the possibility that this cell line may have accumulated in the course of

FIG. 2. AAV infection of leads to both IFN production and activation of IFN-inducible genes in HLFs but not U2OS cells. (A) IFN- β production and release upon AAV2 infection was assessed using ELISA. Cells grown in a 12-well plate were infected at an MOI of 2,500. Medium was collected at 24 h p.i. and centrifuged to discard cellular debris. IFN- β concentration was estimated based on the standard curve generated with recombinant IFN β . The data reflect the means with standard deviation bars of one experiment performed in quadruplicate. (B) Confocal microscopy analysis (using z-stacks) of AAV2-infected U2OS (MOI of 2,500, 48 h p.i.) double-stained for Rep and PML. Bar, 1 μ m. (C) Immunofluorescence analysis of mock-treated and AAV2-infected U2OS cells at 24 h p.i. Cells were grown in a 12-well plate on 19-mm coverslips and infected with AAV2 at an MOI of 5,000 before being double stained for PML and Rep 24 h p.i. Bar, 5 μ m. (D) Similarly, HLFs were infected with AAV2 at an MOI of 2,500 and stained for PML at 24 h p.i. Nuclei were visualized by using DAPI. Bar, 5 μ m. (E) Quantitative analysis of PML-NB number following infection of HLFs with AAV (MOI of 2,500). The cumulative frequency curve is representative of three experiments. The median PML-NB number is the intercept of the curve with the 50% level on the vertical axis. Mann-Whitney statistical analysis shows that the difference between the PML-NB populations before and after infection is highly significant ($P < 0.001$).

its transformation mutations that compromised its ability to detect and respond to viral invasion. It remained to be determined, however, whether the osteosarcoma cell line was deficient in virus detection or the ensuing activation of the adaptive immune response. It is also firmly established that treatment of cells with type I IFNs upregulates the expression of several hundred genes, one of which is the promyelocytic leukemia (PML) protein, the principal component of sub-nuclear structures termed PML nuclear bodies (NBs), that act as a protein depot for a plethora of proteins, including DNA repair proteins, transcription factors etc. Associations between the PML-NB and the parental genomes of DNA viruses and replication centers have been reported (14). Figure 2B shows a

clear juxtaposition between the PML body and Rep foci in AAV-infected U2OS cells. Quantification of this apparent proximity between the NBs and Rep foci revealed that ca. 10% of Rep-expressing cells show juxtaposition between the two nuclear entities. An increase in size and number of the sub-nuclear NB structures is indicative of an antiviral response. Surprisingly, however, immunofluorescence analyses of PML protein expression revealed that infection with AAV dramatically increased the number and average size of the PML-NBs in HLFs but not U2OS cells (Fig. 2D and C, respectively), suggesting that the NBs might exert different actions in the two cellular contexts, with a protective, antiviral role in the first instance and a more cooperative function in the second, pro-

FIG. 3. HLFs, but not U2OS cells, activate the production of type I IFN and IFN-inducible genes. (A and B) Western blot analyses of PML and/or Rep expression in U2OS cells and HLFs, respectively. Cells were grown in 10-cm dishes, mock treated or infected with AAV2 at an MOI of 2,500 or 5,000, and incubated for the indicated times. Ad5 was used as a helper virus at an MOI of 5. The cells were subsequently processed for Western blotting as indicated in Materials and Methods. β -Actin was used as a loading control. Each blot is representative of three independent experiments that all showed similar results.

viding the virus with factors necessary to accomplish replication. Interestingly, the median PML-NB number in HLFs also increased >2-fold (from 11 to 25, $P < 0.001$) after infection with AAV2 (Fig. 2E) and remained unchanged in U2OS cells (data not shown).

Consistent with the immunofluorescence data, Western blot analysis revealed that Rep proteins could readily be detected in infected U2OS cells (Fig. 3A). Bands corresponding to Rep78 and Rep52 were obtained when cells were infected with AAV2 (Fig. 3A, lane 2), and the levels of PML protein remained unchanged in the osteosarcoma cells. No Rep expression was detected in HLFs (data not shown), but the activation of an interferon response was apparent from the time-dependent upregulation of the PML protein, which peaked at 24 h p.i. and declined thereafter (Fig. 3B).

Altogether, we have established that there is type I IFN secretion upon AAV2 infection in normal HLFs, a finding that suggests that these cytokines are involved in the antiviral response to the parvovirus. In addition, these results show that AAV2 is both a trigger and a target of the type I interferon response in human primary fibroblasts.

AAV2 is sensitive to the antiviral action of type I IFNs in U2OS cells. To examine where the failure of U2OS cells to respond to AAV2 resides, we added 100 U of IFN- β to the culture medium of these cells to test whether they were capable of responding to the cytokine and noted a sharp induction of PML-NBs that persisted up to 48 h (Fig. 4A), an observation indicative of the presence of an IFN response. Furthermore, quantification of PML-NB numbers after IFN treatment revealed that the median increased from 15 in controls to 27 and 29 at 24 and 48 h after treatment, respectively ($P < 0.001$) (Fig. 4B). Based on this finding and on the inability of U2OS cells to produce IFN- β upon infection with AAV2, we concluded that the first phase of the IFN response is impaired in this cell line. One can further postulate that the virus evades innate immunity probably because an important player in the IFN pathway is inhibited or mutated in U2OS cells; hence, the production of

IFN- β is abrogated in this cell line, thus increasing permissiveness to the parvovirus.

The question remained as to whether lack of an antiviral response could account for the permissiveness of U2OS to AAV2. In order to address this point, immunofluorescence microscopy was used to quantify the number of Rep-expressing cells in the presence or absence of IFN- β treatment. As illustrated in Fig. 4C, exposure of U2OS to IFN- β significantly abrogated their permissiveness to AAV2, reducing the number of Rep-expressing cells from 63 to 32% at 24 h p.i. and from 70 to 40% at 48 h. p.i. Similar results were obtained when an MOI of 5,000 was used (data not shown). Taken together, these results reveal that U2OS cells lack the appropriate AAV-sensing machinery, which we postulate accounts to a great extent for their permissiveness to AAV2.

U2OS cells develop an antiviral response upon poly(I:C) transfection. To assess if failure to produce IFN- β is a general feature of U2OS, we challenged HLFs and U2OS cells with 50 mM poly(I:C), using either transfection or direct addition to the medium. Poly(I:C) is a synthetic analog of dsRNA, a known immunostimulant, and a ligand of TLR3. The rationale behind using both methods was to distinguish between a cytoplasmic as against endosomal recognition of poly(I:C) in the two cell lines. HLFs responded strongly to both treatments, as evidenced by a significant increase in PML-NB size and number, although transfection exerted a bigger effect than direct addition (Fig. 5A, right panel). This was corroborated with a sharp rise in IFN- β secretion by these cells (Fig. 5B). Similarly, transfection of poly(I:C) into U2OS cells elicited an increased expression of PML, demonstrating that these cells are able to mount an IFN response and secrete cytokines, and are extremely sensitive to the action of IFNs (Fig. 5A, left panel). Indeed, a significant increase in IFN- β production upon transfection was recorded in these cells (Fig. 5B). Addition of the synthetic dsRNA to the medium did not engender an antiviral response. Collectively, these data provide evidence that U2OS cells can activate both IFN production and IFN-inducible

FIG. 4. Treatment of U2OS cells with IFN- β significantly diminishes their permissiveness to AAV2. (A) Immunofluorescence images of IFN- β -treated U2OS cells at 24 and 48 h posttreatment. The cells were grown in a 12-well plate on glass coverslips and later treated with 100 U of IFN- β . The cells were then stained for PML at the indicated time points. Bar, 10 μ m. (B) Quantitative analysis of PML-NB numbers after treatment with 100 U of IFN- β at 24 and 48 h posttreatment. Mann-Whitney statistical analysis revealed that the difference between the PML-NB populations before and after treatment is significant ($P < 0.001$). (C) Quantitation of the percentage of Rep-expressing U2OS cells after treatment with IFN- β . For each condition, 10 randomly chosen fields of treated or mock-treated cells stained for Rep and DAPI were counted. The values shown are averages with standard deviation bars from three independent experiments analyzed.

genes and that the impairment in this response upon AAV infection is probably specific to the parvovirus or one of its constituents.

Are U2OS cells missing an AAV sensor? How does AAV2 activate the innate immune response? To date, knowledge of intracellular DNA sensors that mediate the induction of type I IFNs and cytokines in response to DNA viruses is limited compared to the evidence compiled for RNA viruses. Notably, ALR family members AIM2 and gamma-inducible protein 16 (IFI16) were shown to be potent DNA sensors that induce the inflammasome (6) and type I IFNs (44), respectively. It seemed, however, unlikely that these two cytoplasmic DNA-binding proteins would recognize AAV2 because this virus enters the cell by endocytosis and is transported to the nucleus through endoplasmic vacuoles. This led us to explore the possibility that whatever is recognizing AAV in the cell would have to be endosomal. The only such PRRs reported to date are

TLRs 3, 7, 8, and 9, which reside within the endoplasmic reticulum and traffic to endosomal compartments in response to the presence of the corresponding ligands enclosed in such structures. TLRs 3 and 7/8 were readily discarded as potential AAV sensors since they specifically recognize dsRNA and ssRNA, respectively (3). TLR9, on the other hand, responds to unmethylated CpG residues in DNA and was shown to play an essential role in inducing type I IFNs and the ensuing adaptive response to AAV (50). Indeed, following uptake of DNA ligands, endosomes form tubular structures and translocate from the cell periphery to the juxtanuclear area, where they intersect with TLR9-containing vesicles (24). TLR9 is subsequently delivered to the endolysosomes, where it becomes active. Recent work by Park et al. demonstrated that activation of TLR9 in the endosome was subject to proteolytic cleavage using a cathepsin inhibitor (z-FA-fmk) that was later shown to be specific to TLR9 (2, 32). In order to confirm whether TLR9

FIG. 5. Activation of an antiviral response in U2OS and HLF cells by poly(I:C). (A) Immunofluorescence analysis of mock-treated and poly(I:C)-stimulated U2OS and HLF cells. Cells were grown in 12-well plates on 19-mm glass coverslips and then challenged or not with poly(I:C) (50 μ g/ml) either through the addition of the dsRNA analog directly the culture medium or by transfection with *TransLT-IT*. Cells were stained for PML at 24 h posttreatment. The presented images are representative of two independent experiments that produced similar results. Bar, 10 μ m. (B) IFN- β production and release upon poly(I:C) treatment was assessed by using ELISA. Medium was collected from cells shown in panel A at 24 h posttreatment and centrifuged to discard the cellular debris. IFN- β concentration was estimated based on the standard curve generated with recombinant IFN β . The data reflect means with standard deviation bars of two experiments performed in triplicates.

is recognizing AAV2 DNA, we inactivated this receptor by blocking its cleavage. Hence, HLF and U2OS cells were pre-incubated with the inhibitor z-FA-fmk (10 μ M) for a period of 1 h prior to infection. Cells were subsequently stained for Rep 24 h p.i., and the number of Rep-expressing cells was quantified in both cell types. Figure 6A and B reveals that abrogating TLR9 function in HLFs results in a significant increase in Rep expression from 9 to 36%. Unlike HLFs, inhibition of TLR9 in U2OS cells had no impact on Rep expression, further indicating that these cells lack this receptor (Fig. 6C). In all cases, additional controls included DMSO- and z-FA-fmk-treated

cells to rule out any possible effect of the DMSO contained in z-FA-fmk preparations and z-FA-fmk itself (data not shown). **U2OS cells lack TLR9 protein but not the corresponding mRNA.** RT-PCR analysis of TLR9 expression in HLFs and U2OS revealed that TLR9 mRNA is present in both cell lines (Fig. 6D). On the other hand, protein analysis of the two cell lines following treatment or transfection with ODN 2006, a major TLR9 agonist, failed to reveal TLR9 polypeptide in U2OS cells, while this protein was clearly detected in HLFs 24 h posttreatment (Fig. 6E), hence corroborating the hypothesis that U2OS cells lack this sensor. It seems therefore that

FIG. 6. TLR9 inhibition raises the permissiveness of HLFs for AAV2. (A) Immunofluorescence images of mock-treated and AAV2-infected HLFs that were pretreated with z-FA-fmk. Cells were grown in a 12-well plate prior to treatment with z-FA-fmk (10 μ M) for 1 h. HLFs were subsequently infected with AAV2 at an MOI of 2,500. Ad5 was used as a helper virus at an MOI of 5. The cells were then stained for Rep 24 h p.i. Nuclei were visualized with DAPI. Bar, 10 μ m. (B) Quantitation of the percentage of Rep-expressing AAV2-infected or mock-treated HLFs, in the presence or absence of pretreatment with 10 μ M z-FA-fmk at 24 h p.i. (C) Quantitation of the percentage of Rep-expressing AAV2-infected or mock-treated U2OS cells, in the presence or absence of pretreatment with 10 μ M z-FA-fmk at 24 h p.i. Low MOIs (500 and 1,000) were used to examine the effect of TLR9 inhibitor on U2OS cells, since the generally used MOI of 2,500 seemed to engender maximum Rep expression in these cells. Ad5 was used as a helper virus at an MOI of 5 and, for all of the above conditions, 10 randomly chosen fields of treated or mock-treated cells stained for Rep and DAPI were counted. The values shown are averages with standard deviation bars from three independent experiments analyzed. (D) Semiquantitative RT-PCR analysis of TLR9 transcripts in HLFs and U2OS. GAPDH was used as an internal control. (E) Western blot analysis of TLR9 expression in mock-treated or ODN 2006-stimulated HLFs and U2OS cells. Cells were grown in 10-cm dishes prior to the addition of and/or transfection with ODN 2006 for 12 h. β -Actin was used as a loading control. The data shown are representative of two independent experiments.

the osteosarcoma cells may have developed, during the course of their immortalization, mutations that led to TLR9 posttranscriptional deregulation, which resulted ultimately in making them a susceptible target for AAV.

DISCUSSION

In the present study, we investigated the interaction of AAV2 with the innate immune machinery. We have shown by using Western blotting and immunofluorescence that infection with AAV2 induces an antiviral response in human fibroblasts, characterized by secretion of IFN- β and upregulation of the IFN-inducible gene, *PML*. This IFN response was not detected in U2OS osteosarcoma cells. These cells were shown to support the early steps the parvovirus life cycle in the absence of a helper virus, hence making them a useful cellular system to study the first stages of AAV infection. We also observed that U2OS cells were transduced particularly efficiently by AAV vectors, both single and double stranded, encoding green fluorescent protein (GFP) (data not shown). Therefore, our conclusion of the high susceptibility of U2OS cells for AAV is likely valid for AAV-based vectors too.

Our work points to TLR9 as a possible AAV2 sensor. Of the known TLRs, TLR9 is the principal candidate since it specifically recognizes CpG in DNA. TLRs 1, 2, 4, 5, and 6 recognize bacterial patterning, and TLRs 3, 7, and 8 specifically recognize RNA. Confirming this, during the reviewing of our manuscript, it was reported that AAV vectors activate TLR9-dependent innate responses (26). The presence or absence of TLR9 protein, but not TLR3 (46), is linked to the resistance or susceptibility, respectively, of cells to AAV2 infection. Inactivation of TLR9 by blocking its cleavage during 1 h renders resistant primary fibroblasts sensitive to AAV2 infection. TLR9 was initially reported to be unique among endosomal TLRs in requiring this type of proteolytic activation (32), and we based our reasoning on this. A recent report by Avelos et al. (2) confirmed the inhibitory action of α -FA-fmk on TLR9 cleavage and activation. Ewald et al. (13), however, have suggested that receptor proteolysis may be a general regulatory strategy for TLRs involved in nucleic acid recognition. It remains a possibility that the proteolysis inhibition may affect additional proteins. However, as mentioned above, TLRs 3, 7, and 8 are excluded on the basis of their ligand specificity.

Comparing the antiviral response of different cell types to a virus is often revealing about virus-host interactions and can shed light on the various pathways activated by the virus, but it can also indicate how the latter evades the immune response. AAV is a defective parvovirus that normally replicates only when coinfects with a helper virus, which is generally an adenovirus or a herpesvirus. The observation that the initial phase of the parvovirus life cycle is supported in the osteosarcoma cell line, U2OS, in the absence of a helper raised a number of questions. First, what makes these cells partially permissive to the virus, and can we uncover the mechanism underlying this?

Initial experiments demonstrated that U2OS, unlike many human tumor cells (11), are sensitive to the action of type I IFNs. Indeed, the results showed that addition of exogenous recombinant IFN- β to the medium of U2OS cells triggered an efficient antiviral response against AAV2, as evidenced by a

reduced count of Rep-expressing cells compared to unchallenged cells. This also demonstrates that the incapacity of these cells to establish an anti-AAV response is due to a defective first phase of the interferon response making their permissiveness explicable, at least in part, by their inability to produce IFNs upon infection with AAV.

The second phase of the interferon response seems to be functional in U2OS cells, as evidenced by the upregulation of the IFN-inducible gene, *PML*, and the establishment of an antiviral state when challenged with IFN β . Interestingly, however, stimulating these cells with poly(I:C) resulted in the activation of an antiviral response and production of IFN- β upon transfection but not simple addition of this stimulant, suggesting that these cells have an intact cytosolic sensing machinery for dsRNA, namely, RIG1 and MDA5, but less active membrane-bound TLR3. Similarly, HLFs released significant amounts of IFN- β upon addition and transfection of poly(I:C), although transfection had a more dramatic effect. This finding is in line with previous reports showing that TLR3 is expressed both on the cell membrane and endosomes in fibroblasts (28), and that IFN- β is a major antiviral cytokine in this cell type (16), but it also hints that normal cells sense RNA in the cytoplasm, probably through the RLR pathway.

TLR9 is the membrane-bound receptor for DNA in the cell, and responds to unmethylated CpG-containing DNA. TLR9 stimulation activates a signaling cascade that results in the transcription and regulation of proinflammatory cytokines and type I IFNs. In addition, and although our understanding of the receptors that sense DNA in the cytoplasm remains imperfect, four such intracellular TLR-independent sensors of viral DNA were identified. First, DAI (for DNA-dependent activator of IRFs, also called ZBP1) (41) and RNA polymerase III (9) were shown to be potent detectors of DNA leading to IFN- β induction. Then, AIM-2 and IFI16, both members of the PYHIN protein family, were also proposed as a new family of innate DNA sensors, engaging the inflammasome (19) and the STING-TBK1-IRF3 pathway (44), respectively. The cellular distribution of the above sensors argues that their involvement in the detection of the AAV genome is unlikely, but further studies are required to rule out completely any role for them.

Further analysis of the osteosarcoma cell line revealed that although these cells express TLR9 mRNA, they lack the corresponding protein, a conclusion corroborated by a recent preliminary report (44). This could be due to accumulation of mutations during transformation that ultimately led to gene-specific posttranscriptional deregulation. Such a mutation is most likely to be spontaneous rather than virus-driven because U2OS are reported to have no viral etiology. In fact, it is becoming increasingly clear that TLR expression is not limited to immune cells. A screening study on five human tumors revealed that TLR9 is the most strongly expressed in these cells (20). This is in line with previous reports that detected functional TLR9 in human lung cancer cells (12), gastric carcinoma cells (38), cervical tumors (25), and prostate cancer cells (21).

Interestingly, a link between TLR9 and parvoviruses was suggested in previous reports. Zipris et al. proposed that the autonomous rat parvovirus, Kilham rat virus, activates the innate immune response in splenic B lymphocytes and bone marrow-derived dendritic cells in a TLR9-dependent manner

leading to the production of the proinflammatory cytokines, interleukin 6 (IL-6) and IL-12p40 (51). Similarly, a study by Zhu et al. showed that AAV1, -2, and -9 stimulate TLR9 through their ssDNA genomes in murine immune cells (50). It is also suggested that TLR9 could act as a sensor for the autonomous parvovirus, minute virus of mice (15), although the closely related rat parvovirus H-1 showed little TLR9 activation upon infection (36).

Inactivation of the innate immune response against AAV in U2OS cells is clearly accomplished through inhibition of type-I interferon release. This work suggests that a compromised posttranscriptional regulation of TLR9 is a possible explanation. However, other mechanisms of evasion can be envisaged. Indeed, other essential players in the innate response and downstream effectors of TLRs, such as the adaptor protein myeloid differentiation primary-response 88 (MyD88) predominantly used by TLRs 7, 8, and 9 (18) and the IFN regulatory factor family of transcription factors, may also be compromised in osteosarcoma cells. Further work will be required to test the overall integrity of the pathway.

The ability of AAV2 to elicit an IFN response via TLR9 would imply that either the parvovirus undergoes at least partial uncoating while in the endosome or that a few defective viral particles have their genetic material exposed in the endosome and activate the TLR9 pathway. The evidence as to whether AAV uncoating occurs before or after nuclear import is conflicting, and there exist more than one route of virus trafficking; thus, data gathered on AAV trafficking and nuclear import may reflect bulk virus and not necessarily the infectious fraction.

Several intracellular factors are needed for efficient replication of AAV. Adenoviruses and herpesviruses, each in their own way, contribute to the intracellular milieu that allows AAV growth. The work we report here shows that innate immunity of the host cell is an additional determinant that governs the ability of AAV to infect or not a given cell type. Whether helper viruses might be able to modulate this determinant of AAV infection remains an open question.

ACKNOWLEDGMENTS

This project was supported by a grant from the Swiss National Science Foundation and by the Ecole Polytechnique Fédérale de Lausanne.

We thank Nicole Paduwat for skillful technical help.

REFERENCES

- Acland, G. M., et al. 2005. Long-term restoration of rod and cone vision by single dose rAAV-mediated gene transfer to the retina in a canine model of childhood blindness. *Mol. Ther.* **12**:1072–1082.
- Avalos, A. M., and H. L. Ploegh. 2011. Competition by inhibitory oligonucleotides prevents binding of CpG to C-terminal TLR9. *Eur. J. Immunol.* **41**:2820–2827.
- Barber, G. N. Innate immune DNA sensing pathways: STING, AIM2 and the regulation of interferon production and inflammatory responses. *Curr. Opin. Immunol.* **23**:10–20.
- Barbero, P., L. Bittova, and S. R. Pfeffer. 2002. Visualization of Rab9-mediated vesicle transport from endosomes to the *trans*-Golgi in living cells. *J. Cell Biol.* **156**:511–518.
- Bartlett, J. S., R. Wilcher, and R. J. Samulski. 2000. Infectious entry pathway of adeno-associated virus and adeno-associated virus vectors. *J. Virol.* **74**:2777–2785.
- Bauernfeind, F., et al. Inflammasomes: current understanding and open questions. *Cell. Mol. Life Sci.* **68**:765–783.
- Berns, K. I., and C. Giraud. 1996. Biology of adeno-associated virus. *Curr. Top. Microbiol. Immunol.* **218**:1–23.
- Bucci, C., P. Thomsen, P. Nicoziani, J. McCarthy, and B. van Deurs. 2000. Rab7: a key to lysosome biogenesis. *Mol. Biol. Cell* **11**:467–480.
- Chiu, Y. H., J. B. Macmillan, and Z. J. Chen. 2009. RNA polymerase III detects cytosolic DNA and induces type I interferons through the RIG-I pathway. *Cell* **138**:576–591.
- Conrad, C. K., et al. 1996. Safety of single-dose administration of an adeno-associated virus (AAV)-CFTR vector in the primate lung. *Gene Ther.* **3**:658–668.
- Critchley-Thorne, R. J., et al. 2009. Impaired interferon signaling is a common immune defect in human cancer. *Proc. Natl. Acad. Sci. U. S. A.* **106**:9010–9015.
- Droemann, D., et al. 2005. Human lung cancer cells express functionally active Toll-like receptor 9. *Respir. Res.* **6**:1.
- Ewald, S. E., et al. Nucleic acid recognition by Toll-like receptors is coupled to stepwise processing by cathepsins and asparagine endopeptidase. *J. Exp. Med.* **208**:643–651.
- Geoffroy, M. C., and M. K. Chelbi-Alix. Role of promyelocytic leukemia protein in host antiviral defense. *J. Interferon Cytokine Res.* **31**:145–158.
- Grekova, S., et al. Activation of an antiviral response in normal but not transformed mouse cells: a new determinant of minute virus of mice oncoprotein. *J. Virol.* **84**:516–531.
- Haller, O., G. Kochs, and F. Weber. 2006. The interferon response circuit: induction and suppression by pathogenic viruses. *Virology* **344**:119–130.
- Hansen, J., K. Qing, and A. Srivastava. 2001. Infection of purified nuclei by adeno-associated virus 2. *Mol. Ther.* **4**:289–296.
- Honda, K., H. Yanai, A. Takaoka, and T. Taniguchi. 2005. Regulation of the type I IFN induction: a current view. *Int. Immunol.* **17**:1367–1378.
- Hornung, V., et al. 2009. AIM2 recognizes cytosolic dsDNA and forms a caspase-1-activating inflammasome with ASC. *Nature* **458**:514–518.
- Huang, B., J. Zhao, J. C. Unkeless, Z. H. Feng, and H. Xiong. 2008. TLR signaling by tumor and immune cells: a double-edged sword. *Oncogene* **27**:218–224.
- Ilvesaro, J. M., et al. 2007. Toll-like receptor-9 agonists stimulate prostate cancer invasion in vitro. *Prostate* **67**:774–781.
- Ingemarsdotter, C., D. Keller, and P. Beard. The DNA damage response to non-replicating adeno-associated virus: centriole overduplication and mitotic catastrophe independent of the spindle checkpoint. *Virology* **400**:271–286.
- Kay, M. A., et al. 2000. Evidence for gene transfer and expression of factor IX in haemophilia B patients treated with an AAV vector. *Nat. Genet.* **24**:257–261.
- Latz, E., et al. 2004. TLR9 signals after translocating from the ER to CpG DNA in the lysosome. *Nat. Immunol.* **5**:190–198.
- Lee, J. W., et al. 2007. Increased Toll-like receptor 9 expression in cervical neoplasia. *Mol. Carcinog.* **46**:941–947.
- Martino, A. T., et al. The genome of self-complementary adeno-associated viral vectors increases Toll-like receptor 9-dependent innate immune responses in the liver. *Blood* **117**:6459–6468.
- Mas, A., et al. 2006. Reversal of type 1 diabetes by engineering a glucose sensor in skeletal muscle. *Diabetes* **55**:1546–1553.
- Matsumoto, M., and T. Seya. 2008. TLR3: interferon induction by double-stranded RNA including poly(I:C). *Adv. Drug Deliv. Rev.* **60**:805–812.
- Mays, L. E., and J. M. Wilson. The complex and evolving story of T cell activation to AAV vector-encoded transgene products. *Mol. Ther.* **19**:16–27.
- Mount, J. D., et al. 2002. Sustained phenotypic correction of hemophilia B dogs with a factor IX null mutation by liver-directed gene therapy. *Blood* **99**:2670–2676.
- Pajusola, K., et al. 2002. Cell-type-specific characteristics modulate the transduction efficiency of adeno-associated virus type 2 and restrain infection of endothelial cells. *J. Virol.* **76**:11530–11540.
- Park, B., et al. 2008. Proteolytic cleavage in an endolysosomal compartment is required for activation of Toll-like receptor 9. *Nat. Immunol.* **9**:1407–1414.
- Pfeffer, S. R. 2001. Rab GTPases: specifying and deciphering organelle identity and function. *Trends Cell Biol.* **11**:487–491.
- Qing, K., et al. 1999. Human fibroblast growth factor receptor 1 is a coreceptor for infection by adeno-associated virus 2. *Nat. Med.* **5**:71–77.
- Randall, R. E., and S. Goodbourn. 2008. Interferons and viruses: an interplay between induction, signaling, antiviral responses and virus countermeasures. *J. Gen. Virol.* **89**:1–47.
- Raykov, Z., S. Grekova, B. Leuchs, M. Aprahamian, and J. Rommelaere. 2008. Arming parvoviruses with CpG motifs to improve their oncosuppressive capacity. *Int. J. Cancer* **122**:2880–2884.
- Schlehofer, J. R., M. Rentrop, and D. N. Mannel. 1992. Parvoviruses are inefficient in inducing interferon-beta, tumor necrosis factor-alpha, or interleukin-6 in mammalian cells. *Med. Microbiol. Immunol.* **181**:153–164.
- Schmausser, B., M. Andrusis, S. Endrich, H. K. Muller-Hermelink, and M. Eck. 2005. Toll-like receptors TLR4, TLR5, and TLR9 on gastric carcinoma cells: an implication for interaction with *Helicobacter pylori*. *Int. J. Med. Microbiol.* **295**:179–185.
- Summerford, C., J. S. Bartlett, and R. J. Samulski. 1999. $\alpha_v\beta_5$ integrin: a coreceptor for adeno-associated virus type 2 infection. *Nat. Med.* **5**:78–82.
- Summerford, C., and R. J. Samulski. 1998. Membrane-associated heparan

- sulfate proteoglycan is a receptor for adeno-associated virus type 2 virions. *J. Virol.* **72**:1438–1445.
41. **Takaoka, A., et al.** 2007. DAI (DLM-1/ZBP1) is a cytosolic DNA sensor and an activator of innate immune response. *Nature* **448**:501–505.
42. **Takeda, K., T. Kaisho, and S. Akira.** 2003. Toll-like receptors. *Annu. Rev. Immunol.* **21**:335–376.
43. **Trischler, M., W. Stoorvogel, and O. Ullrich.** 1999. Biochemical analysis of distinct Rab5- and Rab11-positive endosomes along the transferrin pathway. *J. Cell Sci.* **112**(Pt. 24):4773–4783.
44. **Unterholzner, L., et al.** IFI16 is an innate immune sensor for intracellular DNA. *Nat. Immunol.* **11**:997–1004.
45. **Vandenberghe, L. H., and J. M. Wilson.** 2007. AAV as an immunogen. *Curr. Gene Ther.* **7**:325–333.
46. **Wang, Z. J., K. Zhang, F. Yu.** 2010. Toll-like receptors in neuroblastoma and osteosarcoma. *J. Clin. Oncol.* **28**:e20006.
47. **Xin, K. Q., et al.** 2006. Induction of robust immune responses against human immunodeficiency virus is supported by the inherent tropism of adeno-associated virus type 5 for dendritic cells. *J. Virol.* **80**:11899–11910.
48. **Yakobson, B., T. Koch, and E. Winocour.** 1987. Replication of adeno-associated virus in synchronized cells without the addition of a helper virus. *J. Virol.* **61**:972–981.
49. **Zaiss, A. K., et al.** 2002. Differential activation of innate immune responses by adenovirus and adeno-associated virus vectors. *J. Virol.* **76**:4580–4590.
50. **Zhu, J., X. Huang, and Y. Yang.** 2009. The TLR9-MyD88 pathway is critical for adaptive immune responses to adeno-associated virus gene therapy vectors in mice. *J. Clin. Invest.* **119**:2388–2398.
51. **Zipris, D., et al.** 2007. TLR9-signaling pathways are involved in Kilham rat virus-induced autoimmune diabetes in the biobreeding diabetes-resistant rat. *J. Immunol.* **178**:693–701.