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ADENOVIRUS-TRIGGERED INNATE SIGNALLING PATHWAYS

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Adenoviruses are important infectious agents and also emerging vectors in different biomedical applications. These viruses elicit a strong innate and adaptive immune response, which influences both the course of disease and the success of the applied vectors. Several Toll-like Receptor (TLR)-dependent and -independent mechanisms contribute to these responses. Understanding of the involved viral and cellular factors is crucial for the treatment of various adenovirus diseases and the optimal design of adenovirus vector applications. Here we summarize our current understanding of the complex nature of adenovirus-induced innate immune mechanisms.

Keywords: adenovirus, viral entry, endosomal escape, innate response, Toll-like receptors, type-I interferons, IL-1, IRF7

Introduction

Adenoviruses are important human and animal pathogens. They are also promising vectors for human or veterinary clinical science applications and biotechnology. They can be grown to high titers, can be genetically manipulated to accept large pieces of foreign DNA, and infect many different cell types and tissues with high efficiency [1]. However, adenovirus infection and particularly the entry stages elicit a strong innate immune response, which affects the course of adenoviral disease in patients, and also the therapeutic efficiency of recombinant vectors [2, 3]. Understanding how these innate responses are generated is of key importance for the treatment of adenovirus disease and vector applications. Here we discuss the mechanisms that elicit innate responses during adenovirus entry into immune and non-immune cells.

Taxonomy and structure of human adenoviruses

Adenoviruses are non-enveloped, middle-sized viruses with an icosahedral symmetry containing a linear, double-stranded DNA genome [1, 4–6]. They belong to the genus of Mastadenovirus and are subdivided to seven Ad species (A–G). Currently, there are 68 sequenced HAdVs (see http://www.vmri.hu/~harrach/ADENOSEQ.HTM). All of these viruses are closely related, but may differ in their tropism, as some lead to infections of the respiratory tract while others infect eye, kidney, liver, or the gastrointestinal tract [7]. How the different tropisms precisely relate to the viral genetics is incompletely known [8].

Human illnesses associated with adenoviral infections

In individuals with functional immune system adenovirus infections can be asymptomatic, but often cause organ-restricted illnesses such as upper and lower respiratory tract infections (pharyngitis, bronchitis or pneumonia, species C, B and E), epidemic and follicular conjunctivitis (species D and B), and gastroenteritis (species A, E and F) [9]. The caused disease can be severe and even fatal (especially in infants) but most often self limiting with viral persistence and shedding [9].

In immuno-compromised patients such as bone marrow or solid organ transplant recipients or people suffering from AIDS, adenoviruses often cause systemic diseases with high mortality affecting various organs such as liver, heart, or brain [10].

Adenoviral vectors for the treatment and prevention of diseases

Adenoviruses are promising agents for viral gene therapy vectors because they can grow to high titres, infect various cell types, and can be easily manipulated to express relatively large genes [1, 9]. Potential applications include the delivery of curative genes (cystic fibrosis, cardiovascular and hepatic diseases), oncolytic viruses, and vaccine applications (recombinant subunit vaccines or virus-like particles) [11–13].

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Importance of Ad-induced adaptive and innate responses in viral disease and vector applications

Adenoviruses, similar to other intracellular pathogens elicit an effective humoral and cellular immune response (neutralizing anti-viral capsid antibodies and the induction of $CD8^+$ T cells) that prevents generalized disease and re-infection with the same Ad serotype [9]. Notably however, due to common exposure, most adult individuals already exhibit specific antibodies and cytotoxic T cells to prevalently used vectors of Ad species C. This prevents efficient organ targeting by such vectors and comprises major obstacles in systemic Ad gene therapy [14, 15].

In natural adenovirus infections, the elicited innate responses such as complement activation, phagocytosis and the induction of proinflammatory cytokines and interferons (IFNs) are beneficial in most cases as they result in the rapid inactivation of adenoviruses and adenovirus-producing cells and also help to build a proper antiviral-acquired immune response [9]. However, overproduction of certain proinflammatory mediators such as TNF, IL-6 and IL-8 can be harmful. In infections of lower respiratory tract such a mediator "storm" elicits symptoms analogous to septic shock caused by Gram-negative bacteria [2].

Ad vector-induced innate reactions can also be beneficial or harmful depending on the special type of therapeutic application. Using oncolytic vectors and recombinant vaccines innate responses elicited by the vector itself may be helpful to eliminate targeted tumour cells or drive an accelerated immune response to antigens of interest [16]. Nevertheless, overwhelming innate responses to the Ad vectors are harmful and constitute a major limitation in systemic gene therapy applications aiming to achieve prolonged gene expression as they lead to the destruction of the vector-transduced cells [17]. Moreover, depending on the amounts of applied vector, the induced innate reactions may even lead to life-threatening side effects [18]. Therefore, understanding and proper modification of Ad-induced innate responses is of primary interest for both adenoviral disease treatment and recombinant vector applications.

Ad infectious entry pathways in different cell types

Ad entry, the earliest step in the viral replication cycle seems to be predominantly responsible for the virus-induced innate responses [19]. As innate virus sensing and signalling can be initiated at various steps of this process, it is of crucial importance to understand the mechanisms of the initial events of virus–cell interaction during Ad infection.

Entry in non-immune cells

Adenovirus entry is relatively well characterized in non-immune cells *(Fig. 1)*. HAdVs attach to high affinity receptors, such as Coxsackie Adenovirus Receptor (CAR, species A, C–F) [20], CD46 (species B2), or to low affinity high avidity receptors, such as desmoglein 2 and CD46 (species

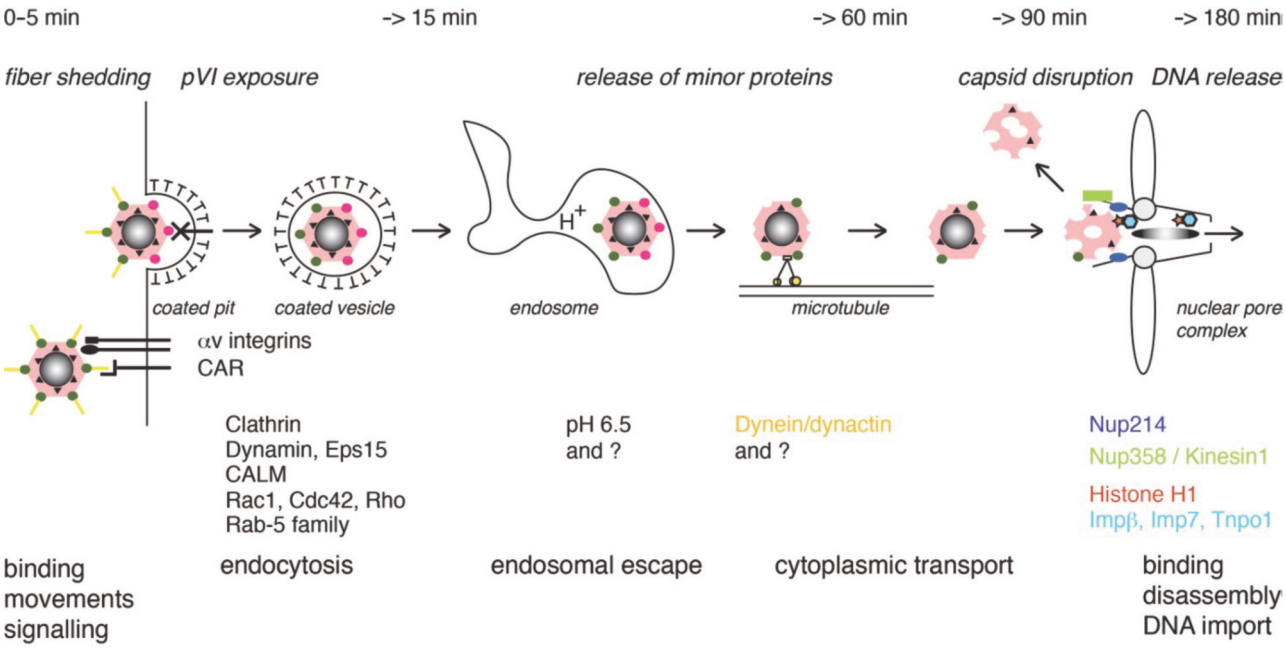

Fig. 1. Ad entry into non-immune cells, HAdVs attached to CAR and to integrins promotes endocytosis. CAR receptors and alpha v integrin coreceptors trigger the initial uncoating, leading to exposure of protein VI that facilitates endosomal penetration. Ad receptor-mediated endocytosis also involves clathrin, dynamin, Eps15, Rab5 GTPase subfamily proteins, and actin dynamics by Rho GTPases. Ad particles that escaped are then transported by the dynein motor on microtubules to the nuclear pore complex, where viral DNA is injected in and transcription begins

B1) [21–25], or very low affinity sialic acid residues [26, 27]. Additionally, association of the blood coagulation factor X with blood-delivered HAdVs mediates effective virus uptake into hepatocytes of the liver in mice [28]. The binding of species C and species B HAdV to integrins promotes endocytosis and signalling, which can result in macropinocytic stimulation, and enhances infection [29–33].

The entry mechanisms of the species C HAdV (HAdV-C) is in part known at the mechanistic level. For example, the movement of CAR receptors by actomyosin-mediated drifting motions and the stationary confinement of the alpha v integrin coreceptors trigger the initial uncoating steps for the incoming HAdV-C2 or C5 at the cell surface, the loss of the fibre proteins from the capsid [34–36]. This then leads to the exposure of the membrane lytic protein VI from the capsid interior, and thereby prepares the partly uncoated virus for membrane penetration [36–39]. HAdV-C2/5 are taken up by receptor-mediated endocytosis, and this involves clathrin, dynamin and the EGF receptor substrate Eps15, proteins from the Rab5 GTPase subfamily, and also actin dynamics indicated by the involvement of various Rho GTPases [34, 40–46].

Soon after endocytosis, the virus penetrates from an endosome to the cytosol [45, 47, 48]. Although the penetration mechanism is sensitive to lysosomotropic agents, such as ammonium chloride [34], it is independent of the protein ATPase inhibitor bafilomycin A1 [43, 49], and independent of microtubules [50] and the 19 °C block of early endosomal trafficking [43, 51]. This implies that virus penetration does not require late endosomes or lysosomes, in agreement with the observation that the membrane lytic activity of the viral protein VI is pH-independent [37]. Virus particles, which escaped from the endosome, are then transported by the dynein motor on microtubules to the nucleus, and they lead to infection [50, 52–55]. This is enhanced by the dynactin complex [50, 53, 56].

How the virus gets from the microtubules to the nuclear pore complex is still not completely understood, although this step is inhibited when nuclear export is blocked [57–59]. When the virus arrives near the nuclear envelope it docks the nuclear pore complex protein Nup214 [60]. The capsid is disassembled by the action of a kinesin motor protein, conventional kinesin-1, which binds in its inactive form to the virus particle [61]. The kinesin motor gets activated by a dual cue, the binding to the nuclear pore complex protein Nup358 and microtubules [62]. Microtubules also bind to the distal part of Nup358 [63], and hence provide a track for the displacement of the viral capsid fragments from the nuclear envelope to the periphery [61]. Surprisingly, this also leads to the disruption of the nuclear pore complex and thereby enhances the permeability of the nuclear pore. The enhanced permeability of the nuclear pore together with nuclear import factors importin beta, importin 7 and transportin, which bind to the viral DNA and associated proteins, then enhance import of the viral DNA genome into the nucleus [60, 64–66].

Ad entry in immune cells

Studies with various Ad vectors have shown that these viruses infect and activate mononuclear phagocytes very efficiently *in vivo* in a way that does not require the known high affinity virus-binding receptors (CAR, CD46 and HSPG) [19]. As these cells possess strong phagocytic activity, Ad entry into them is generally believed to be mediated by phagocytosis. Indeed, macrophage receptors, such as Fc receptor and DC-Sign have been shown to facilitate cellular binding requiring anti Ad antibodies and lactoferrin, respectively [67–69]. If modified by an Fc fusion protein, they can directly attach to the high affinity FcR CD64 [70]. In immature myeloid dendritic cells, adenoviruses escape from the phagolysosome and this requires the virion protease similarly to what was found in non-immune cells [71]; however, much less is known about additional steps of Ad entry into mononuclear phagocytes compared to other non-immune cells. Ads can also infect plasmacytoid dendritic cells (pDCs) that are non-phagocytic using CD46 as a receptor [72], but again, further details of Ad entry into these cells are not characterized.

Ad entry-induced cellular signalling and innate responses in non-immune cells

In non-immune cells signalling events activated by Ad entry are relatively well studied. High affinity receptors (e.g. CAR), necessary for cell surface binding of Ads, may initiate cellular signalling events, even in the absence of virus [73, 74]. Binding of RGD motifs of the Ad virion component penton base to cellular integrins triggers the activation of several important signalling molecules such as phosphatidyl-inositol 3-OH kinase (PI3K) and the Rho family of small GTPases and these events enhance virus internalization [8, 75]. Viruse uptake or endosomal escape of species C Ads has been shown to be associated with protein kinase C activity [35], and the p38 and ERK mitogen-activated protein kinases (MAPK) facilitate Ad movement along the microtubule network [76, 77]. The activation by the ERK and p38 MAPKs and nuclear factor kappa B (NF-κB) has also been shown upon species C Ad infection in renal epithelial cells [19]. Activation of these MAPKs and NFκB was responsible for the induction of chemokines such as IP-10 and IL-8 [78], which has also been observed in respiratory epithelial cells infected with species B Ads [79].

Ad entry-induced innate responses in immune cells

Although non-immune cells may contribute to certain Ad innate reactions, the majority of these responses stem from cells of the innate immune system such as various macrophage and dendritic cell populations. In the following sections, we discuss the major determinants of these responses.

Ad entry-elicited soluble mediators

In vivo studies with rodent and primate animal systems and in human clinical trials revealed the induction of a plethora of mostly pro-inflammatory mediators. These include pro-inflammatory cytokines (IL-6, TNF, IL-12, IL-1α and β), chemokines (IL-8, MIP-2, IP-10, RANTES, MIP-1α, MIP-1β and MCP-1), platelet-activating factor (PAF), and type-I interferons (IFN-αβ) [19, 71, 80–83]. Interestingly, certain Ad-induced mediators may in turn lead to the production of others, as it was shown recently that the production of Ad-induced chemokines depended on virus-induced IL-1 [84], and also that IFN-αβ signalling positively regulates the production of type I IFNs, IL-6 and IL-12 [71, 80].

Cellular sources of Ad-induced mediators

Ad entry-induced inflammatory mediators are predominantly produced by different kinds of mononuclear phagocytes [80]; however, epithelial cells and organ parenchymal cells also contribute to the production of chemokines [19]. Various Ad species have different organ tropism [85], and data available in this respect refer mostly to species C Ads. With these viruses, liver and spleen are the main targeted organs [85]. TNF has been shown to be produced by the liver resident macrophages, Kupffer cells [81], while the induction of chemokines, IL-1 and IL-6, has been demonstrated both in liver and spleen [71, 84, 86]. In the later organ, Ads induce the production of IL-1α and β in marginal zone and metallophilic macrophages [84]. Ads induce the production of IFN-αβ in various mononuclear phagocytes such as bone marrow-derived macrophages and dendritic cells or primary Kupffer cells [71, 80, 83] and in pDCs [71, 72, 80] *in vitro*. However, during systemic Ad infection, the vast majority of type I IFNs is made in spleen, predominantly in myeloid dendritic cells [71]. Interestingly, only a small part of IFN-αβ is made by pDCs [71] which are the main type I IFN producer cells in the course of infection with most other viruses [87].

Ad-induced LPS hypersensitivity

Importantly, experiments in mice revealed that infection with Ads modulates innate immune responses to other microbial agents. The best studied example is the induction of hypersensitivity to bacterial endotoxin (LPS) [88]. Other viruses such as Lymphocytic Choriomeningitis Virus (LCMV) have also been shown to induce LPS hypersensitivity. In these cases, early production of IFN-αβ 2–3 days after infection mediates a relatively weak (2–4-fold) hypersensitivity (or even downregulation of TNF production) [92], while later (after 7 days) IFN- production mediates a strong hypersensitivity to LPS, characterized by the overproduction of TNF [89–93]. In contrast, Ad-induced early-type I IFN production mediates a very strong LPS hypersensitivity characterized by the dramatic overproduction of TNF (50–100-fold) and IL-6 (5–10-fold) [88]. Furthermore, there is a strong overproduction of nitric oxide (NO), mostly in spleen [88]. Interestingly, LPS injection of uninfected control animals elicits NO production mainly in liver. Ad-induced LPS hypersensitivity also results in enhanced, TNF-mediated lethality and thus represents an important mechanism of pathologies in mixed infections caused by both viruses and bacteria [71, 88] *(Fig. 2)*.

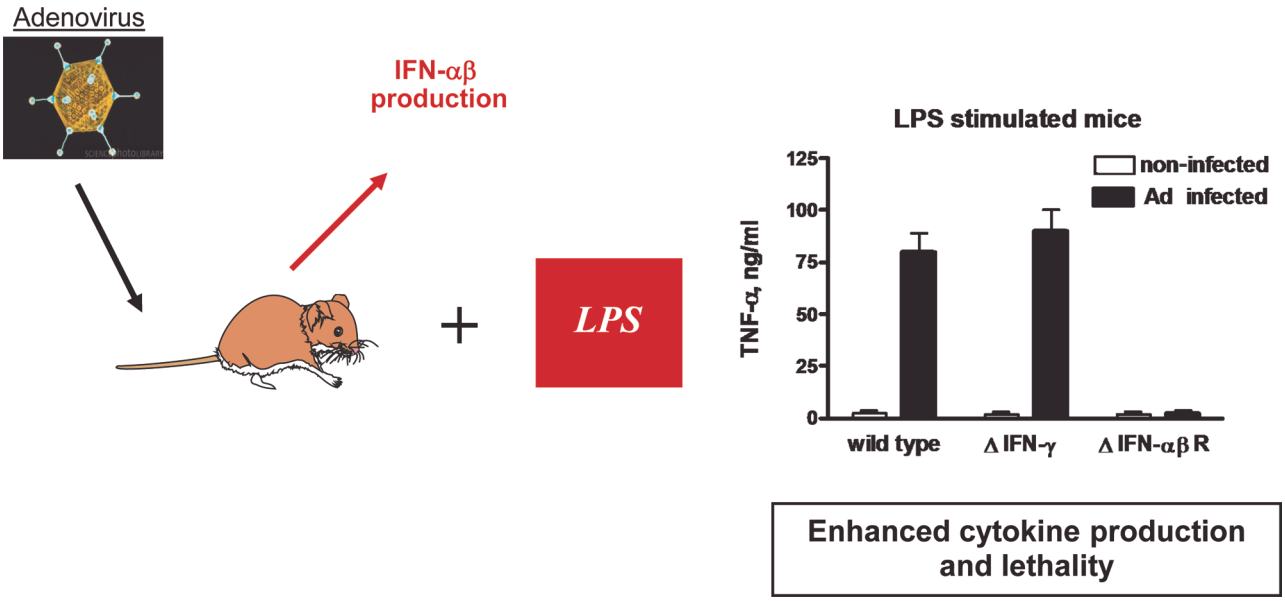

Fig. 2. Adenovirus-induced LPS hypersensitivity is mediated by type I interferons. Human species B adenovirus injected into B6 mice intraperitoneally induces the production of IFN-αβ. Sixteen hours after virus infection, LPS challenge elicits strong overproduction in wild-type and IFN-γ-deficient mice but not in IRF7-deficient mice incapable of IFN-αβ production or in animals deficient in IFN-αβ receptor

Enhancement of Ad-induced monocyte-derived innate mediators on the infectious entry into non-immune cells

In confluent polarized epithelial cells, the high affinity species C receptor CAR and the integrin coreceptors are localized to the basolateral surface and tight intercellular junctions. They are inaccessible to incoming virus particles from the airway lumen on the apical side of the epithelium [94, 95]. This arrangement constitutes a "natural resistance" to viruses using these receptors, for example, coxsackie virus B3 [96]. A recent study showed that if monocytes are also present in the epithelium, they can take up virus particles without getting infected themselves, but they respond to the viruses by releasing cytokines, such as CXCL8 [97]. CXCL8 release then leads to the redistribution of CAR and alpha v beta 3 (but not beta 5) integrin to the apical surface, and allows the viruses in the apical airway lumen to directly infect the epithelial cells from the apical side *(Fig. 3)*. Since macrophages can be pre-activated by bacterial infection, a bacterial and viral coinfection can potentially contribute to worsening the outcome of disease in mixed infections.

Molecular mechanisms mediating the Ad triggered innate responses

Several receptors and signalling pathways have been shown to play important roles in the Ad-induced innate responses. However, not much attention was paid to possible differences in causative viral ligands, receptors involved, signalling and responses in different Ad-sensitive cell types. Actually, the inducing adenoviral ligand(s) and cellular receptors are largely unknown and multiple mechanisms seem to be involved. In the following, we discuss our current knowledge of the innate molecular sensing of Ads.

Binding to Ad surface receptors

Since cellular integrins elicit well-characterized downstream signalling events (e.g. PI3K activation) which can lead to NF-κB and MAPK activation, these receptors were thought to be involved in the induction of pro-inflammatory mediators for a long time. Indeed, RGD deleting Ads not capable of binding to cellular integrins elicit weaker responses [98]. However, since integrin mediated signalling events are also

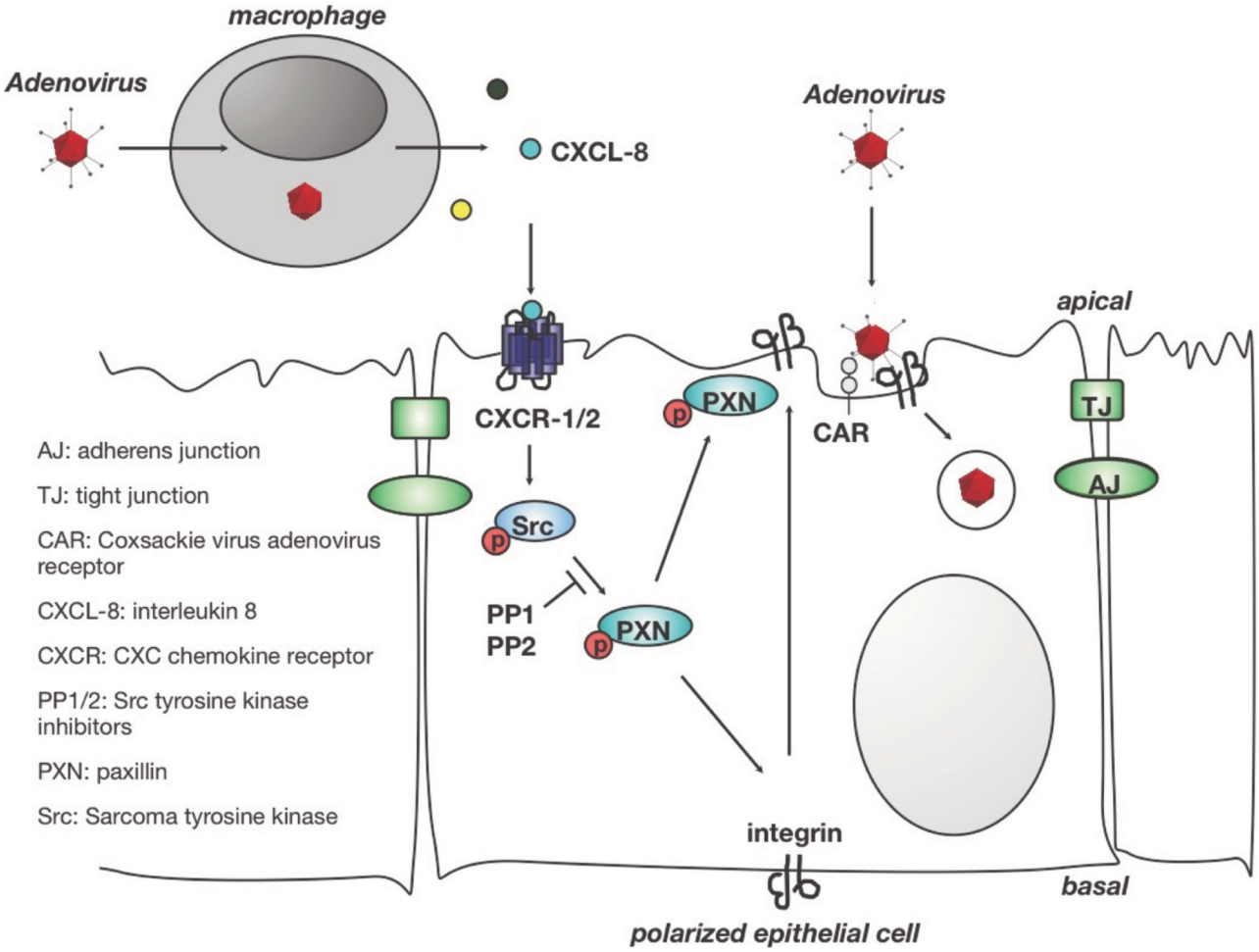

Fig. 3. Ad-induced enhancement of monocyte-derived innate mediators on the infectious entry into epithelial cells. CAR and the integrin coreceptors are localized to the basolateral surface and tight intercellular junctions in confluent polarized epithelial cells, preventing species C Ad entry. Ad-infected monocytes produce IL-8 that induces redistribution of CAR and alpha v beta 3 integrin to the apical surface and allows virus infection from the apical airway lumen

required for virus internalization many of these effects can reflect delayed activation of other sensors at later stages of Ad entry [8]. Nevertheless, at least in Ad infected spleen marginal zone macrophages integrin mediated signalling by itself is sufficient to elicit the production of IL-1α and β because Ad2 ts1, a mutant endocytosed normally but incapable of escaping from the endosome [43, 46, 99] induces IL-1 production requiring Ad RGD motifs and cellular integrins but not Toll-like receptors (TLRs) [84].

Ad-induced TLR activation

Cell surface TLRs 2 and 4 and endosomal TLR7, 8 and 9) are implicated in the innate sensing of different types of viruses [87, 100]. Also, in the case of Ads, TLRs have been shown to be involved in innate sensing. In pDCs, TLR9 plays a crucial role in Ad-induced type I IFN production [71, 72, 80]. Furthermore, TLR2, 4 and 9 were reported to play a role in the elicitation of IL-12, MCP-1 and RANTES responses in mononuclear phagocytes in Ad-infected mice or *in vitro* [86, 101]. However, the induction of IL-1, type I IFNs was predominantly independent of TLRs [71, 84].

Signalling and cytokine responses requiring Ad endosomal escape

IL-1β induction. Cell surface-initiated integrin signalling was shown to contribute to IL-1 production [84], but the same study has also shown that Ads capable of endosomal escape induce much more IL-1 than the escape-deficient ts1 mutant [84]. Two other studies also emphasized the crucial role of endosomal Ad escape in the induction of IL-1β. Muruve et al. demonstrated that cytosolic sensing of Ad DNA is capable of activating the inflammasomes in a manner requiring NALP3 and ASC and thus promoting IL-1β secretion [102]. As an alternative mechanism, the release of cathepsin B from phagolysosomes during Ad escape and the consequential activation of caspase-1 and the NLRP3 inflammasome has also been suggested for the production of this cytokine [103].

IFN-αβ induction. The Ad entry-mediated production of type I IFNs has been recognized only recently. The importance of IFN-αβ production was indicated by clinical trials with oncolytic Ads, where a better prognosis was associated with situations in which recombinant Ad vectors induced robust IFN-αβ responses [104]. While pDCs can recognize Ad DNA via TLR9 *in vitro* [71, 72, 80], *in vivo* studies in Ad-infected mice have shown that the majority of IFN-αβ is produced in myeloid DCs *(Fig. 4)* in a TLR-independent manner [71]. So far reported, cytosolic induction of IFN-αβ by bacterial and viral DNA strictly requires IRF3 but not the activation of MAP kinases [105, 106]. In contrast, Ad-induced type I IFN production strictly requires endosomal escape-mediated IRF7 (but not necessarily IRF3), JNK MAP kinase and TBK1 activation. But the cytosolic DNA sensor DAI, the RNA helicases RIG-I/MDA-5 [71, 107] are not required for IFN-αβ induction and adenoviral gene expression inhibits their production [71]. Therefore, Ad-induced type I IFN production appear to represent a novel, distinct viral induction pathway, besides the previously described ones mediated by TLRs, RIG-I/MDA5 and free cytosolic DNA recognition *(Fig. 5)*.

Concluding remarks and further questions

The adenovirus entry-elicited innate responses represent a very complex example of virus-induced immune reactions. Although many individual aspects of the mechanisms involved are known, our understanding is still rather incomplete. We need more information about the key viral and host elements involved such as the main cellular sensors of Ads and the inducing cognate viral ligands. By studying these questions, further experiments will probably increase our understanding not only on adenovirus-generated responses, but also on general aspects of host-pathogen relationship.

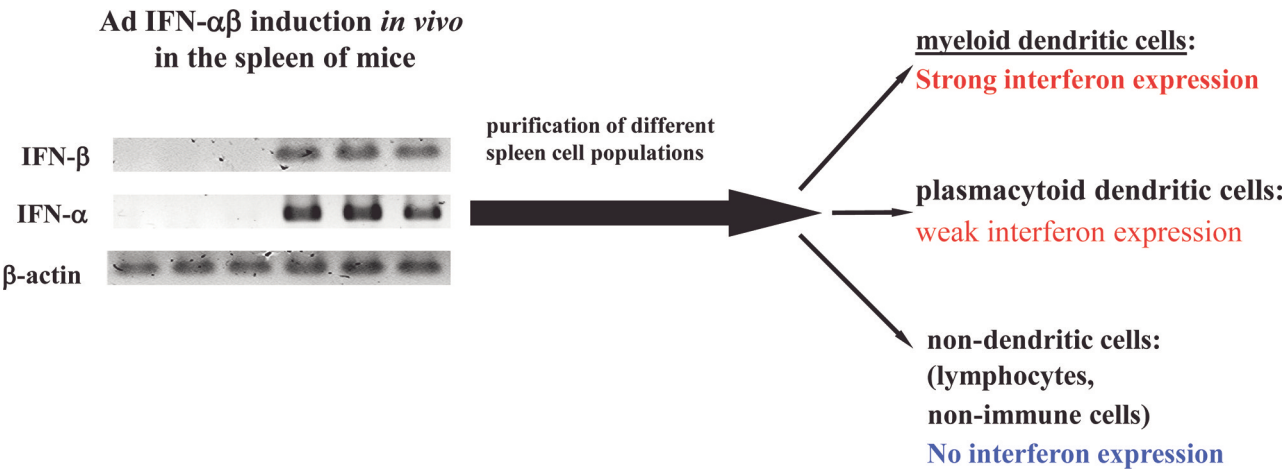

Fig. 4. Splenic myeloid dendritic cells are the major cellular sources of type-I interferons in response to Ad infection in vivo. IFN-αβ is produced in the spleen of Ad-infected mice. Detection of IFN-αβ in FACS-sorted splenocytes reveals weak induction in pDCs, strong production in myeloid DCs and the absence of IFN-αβ synthesis in all other spleen cell populations

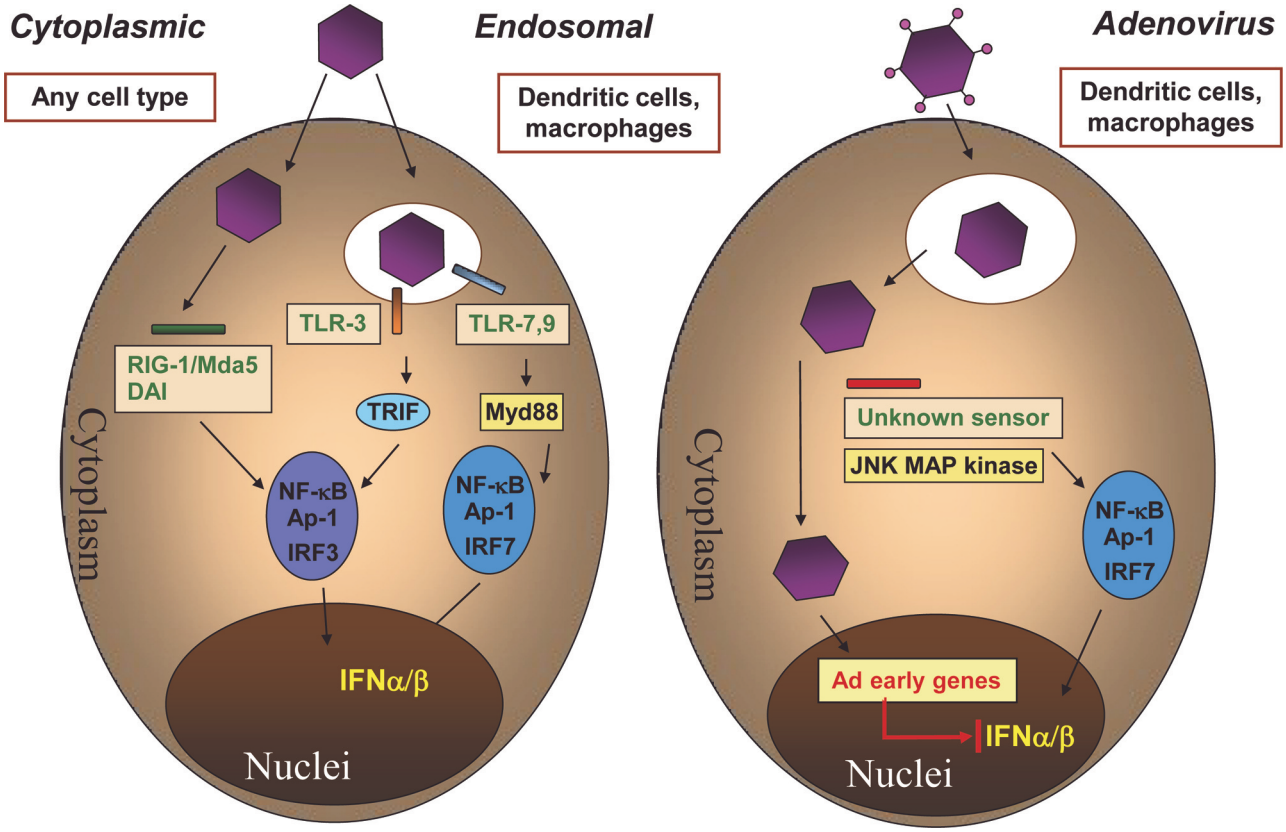

Fig. 5. Adenovirus elicits IFN-αβ production by a novel viral induction pathway. Left: Type-I IFN induction pathways by viruses other than Ads. Recognition of viral RNAs or DNA in the cytoplasm leads to IFN-αβ production via the RNA helicases RIG-I/MDA-5 and the DNA sensor DAI requiring IRF3 in any infected cell type. Alternatively, in dendritic cells and macrophages, endosomal TLR3 or TLR7-9 activation leads to the induction of IFN-αβ by activating IRF3 or IRF7, respectively. Right: Adenoviruses are recognized in mononuclear phagocytes after endosomal escape in the cytoplasm by a so far unknown receptor requiring JNK MAP kinases and IRF7. This activates type-I IFN production independent of TLRs, RIG-I/MDA-5, DAI, and IRF3. Ad-induced IFN-αβ production is inhibited by viral early gene expression

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