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CHAPTER 2

Adenovirus type 35, but not type 5, stimulates NK cell activation via plasmacytoid dendritic cells and TLR-9 signaling

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ABSTRACT

In hematopoietic stem cell transplant (HSCT) recipients, disseminated adenoviral infections during the first two months after HSCT can lead to severe complications and fatal outcome. Since NK cells are usually the first lymphocytes to reconstitute after HSCT and have been implicated in the clearance of adenovirus-infected cells, it was investigated whether NK cells are activated by adenovirus *in vitro*.

Exposure of PBMC to human adenovirus type 5 (HAdV5) or HAdV35 resulted in up-regulation of the activation marker CD69 on NK cells and enhanced cytolytic activity of NK cells. HAdV5-induced NK cell activation relied on the contribution of T cells as depletion of T cells from PBMC abolished NK cell activation. In contrast, NK cell activation in response to HAdV35 occurred in the absence of T cells. Plasmacytoid dendritic cells (pDC) were necessary and sufficient to mediate NK cell activation. HAdV35 induced significantly more interferon-α (IFN-α) production by pDC than HAdV5. The increased IFN-α production and NK cell activation correlated with a higher infection efficiency of viruses with the type 35 fiber. The IFN-α response of pDC was enhanced by the presence of NK cells, suggesting a reciprocal interaction between pDC and NK cells. Incubation with a toll-like receptor 9 (TLR-9) antagonist impaired the IFN-α production by pDC as well as NK cell activation, implying that TLR-9 signaling is critically involved in the IFN-α response of pDC and NK cell activation after HAdV35 exposure.

In conclusion, two human adenovirus serotypes from two different species differ considerably in their capacity to stimulate pDC and NK cells.

INTRODUCTION

Human adenoviruses (HAdV) are non-enveloped double-stranded DNA viruses that are not considered a major risk for immunocompetent hosts. Adenoviral infections commonly occur in early childhood, are self-limiting and typically associated with asymptomatic or mild disease [1]. Among the human adenovirus family, 52 serotypes have been identified on the basis of neutralizing antibodies and are categorized in six HAdV species (A-F) according to their capacity to agglutinate erythrocytes [2].

In the first line of immune defense, dendritic cells are considered the major sentinels for viral pathogens [3;4]. Plasmacytoid dendritic cells (pDC) selectively express the intracellular Toll-like receptor 9 (TLR-9), which detects unmethylated DNA with CpG-motifs frequently found in bacteria and in double-stranded DNA viruses [5-11]. Stimulation of TLR-9 initiates a signaling cascade in association with MyD88, resulting in type I interferon (IFN-I) production as well as enhanced expression of co-stimulatory molecules such as CD80, CD86 and CD40 [10;12]. IFN-α exhibits a direct antiviral effect and has a broad impact on cellular functions, bridging innate and adaptive immunity [12-16].

In immunocompromised hosts, such as hematopoietic stem cell transplant (HSCT) recipients, adenoviral infections can disseminate and lead to severe complications and fatal outcome [17-19]. Previously, it has been reported that in pediatric HSCT patients with disseminated HAdV infection the elimination of adenovirus relied on the recovery of immunity, i.e., lymphocytes [20;21]. HAdV-specific IFN-γ–producing $CD4^+$ or $CD8^+$ T cells were detected after virus clearance from blood [20;22]. However, evidence has been provided for the clearance of HAdV in the presence of NK cells only, and since NK cells constitute the majority of lymphocytes early after HSCT [23;24], we set out to study the requirements to activate NK cells in response to adenoviruses *in vitro*. We focused on the human wild type, replication-competent adenovirus serotypes HAdV5 (species C) and HAdV35 (species B) which utilize two distinct pathways of cell entry and considerably differ in their capacity to infect cells, such as DC [2;25-29]. We hypothesized that this difference has a major influence on the IFN-α production by pDC and, therefore, determined the differential cellular requirements for these two viruses to activate NK cells.

MATERIALS AND METHODS

Cell isolations and depletions

Peripheral blood mononuclear cells (PBMC) were separated from buffy coats of healthy adult donors (Sanquin Blood bank, Region Southwest, Rotterdam, the Netherlands) by Ficoll-Hypaque density gradient centrifugation. NK cells were purified from PBMC by negative selection, depleting non-NK cells through a combination of biotin-conjugated monoclonal anti-human antibodies and MicroBeads using the "Human NK cell Isolation Kit" (Miltenyi Biotech, Bergisch Gladbach, Germany); NK cell purity was >95 % as determined by flow cytometry. NK cells were defined as NK cells as $CD56^+$, $CD3^-$, $CD14^-$ and $CD19^-$ cells. pDC were isolated from PBMC by negative selection using the "human Plasmacytoid Dendritic Cell Isolation Kit" (Miltenyi Biotech); no other cell types than pDC specifically identified by the extracellular

expression of BDCA-2 (CD303) (BDCA-2$^+$ pDC) were detectable by flow cytometry in the cell isolate; pDC purity was >99 %. pDC were depleted from PBMC by positive selection using APC-conjugated anti-BDCA-2 antibodies and anti-APC MicroBeads (Miltenyi Biotech), resulting in the depletion of >99 % of pDC. T cells were depleted from PBMC (TCD PBMC) by positive selection using anti-CD3 MicroBeads (Miltenyi Biotech); TCD PBMC contained less than 0.5 % of contaminating CD3$^+$ T cells as analyzed by flow cytometry.

Cell cultures and stimulations

All cell cultures were performed in RPMI 1640 medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10 % FCS and penicillin/streptomycin at 37 ºC under humidified and 5 % CO_2-containing conditions. PBMC or TCD PBMC were cultured in round-bottomed 96-well plates (2x10^5 per 200 µl) to study NK cell activation, or in 24-well plates (2x10^6 per ml) to study pDC intracellular IFN-α production and maturation. Isolated NK cells and purified pDC were co-cultured in round-bottomed 96-well plates in RPMI medium at indicated cell ratios and cell concentrations. PBMC, isolated PBMC subsets and the co-cultures were stimulated with human wildtype adenovirus serotype 5 (HAdV5) and HAdV35 or with recombinant rAd5f35 at multiplicity of infection (MOI) 1 (at least one infectious virus particle per cell), 10 and 100. In rAd5f35 the type 5 shaft and head sequences are replaced by the type 35 shaft and head sequences, which are fused to the type 5 fiber tail [27;30]. In addition, PBMC were stimulated with the recombinant replication-deficient, E1-deleted, GFP-encoding adenovirus variants rAd5-GFP, rAd35-GFP and rAd5f35-GFP at MOI 10 and 100 [27;31]. All adenovirus preparations were kindly provided by Drs. Jan de Jong (Erasmus Medical Centre, Rotterdam, Netherlands), Menzo Havenga (TNO Biosciences, Leiden, The Netherlands), and purified on a CsCl-gradient. Additional exogenous stimuli were as follows: IL-15 (10 ng/ml, Bender Med. Sys, Vienna, Austria); IFN-α (ranging from 125 to 25,000 IU/ml (Roche, Basel, Switzerland); inhibitory CpG TTAGGG (iCpG) '5 -ttt agg gtt agg gtt agg gtt agg g -3' (12.6 µM, Invivogen, San Diego, CA, USA). Blocking experiments were performed with the following antibodies: rabbit polyclonal anti-IFN-α, mouse monoclonal anti-interferon-Alpha/Beta Receptor 1 (both PBL Interferon Source, Piscataway, NJ, USA; cat.no. 31101-1 and 21385-1, respectively) and mouse monoclonal anti-IL-2 (R&D Systems, Minneapolis, MN, USA; clone 5334). As the kinetics of activation between pDC (i.e., intracellular IFN-α production and maturation) and NK cells (i.e., considerably induced levels of CD69 expression) in response to HAdV were different, pDC and NK cells were analyzed 18 h and 40 h following virus exposure, respectively.

Flow cytometry

pDC and NK cells were analyzed phenotypically by staining with fluorescently labeled antibodies and flow cytometry. The following antibodies were applied according to the manufacturer's instructions: BDCA-2/CD303^{APC}, BDCA-1/CD1c^{APC} (Miltenyi Biotech); CD3$^{PerCP\text{-}Cy5.5}$, CD11c^{PE}, CD14$^{PerCP\text{-}Cy5.5}$, CD19$^{PerCP\text{-}Cy5.5}$, CD20$^{PerCP\text{-}Cy7}$, CD40^{FITC}, CD69^{PE}, HLA-DR^{FITC} (Becton Dickinson, Franklin Lakes, NJ, USA); CD19$^{\text{Pacific Blue (PB)}}$ (Dako, Glostrup, Denmark); CD3^{PB} (BD Biosciences, San Jose, CA, USA); CD56^{APC},CD56$^{PE\text{-}Cy7}$ (IOTEST Immunotech, Marseille, France). After extracellular staining, intracellular staining of IFN-α with IFN-α^{FITC} (PBL Interferon Source; cat.no. 21112-3) was performed in PBMC using the "FoxP3 Staining Buffer Set" (eBiosciences, San Diego, CA, USA). FACS measurements were performed

with the FACSCalibur or the LSR II (BD Biosciences) and analyzed with the "BD Cell Quest Pro^{TM}" software (version 5.2.1) or "BD FACSDiva^{TM}" software (version 6.1.2.).

Cytolytic activity of NK cells – ^{51}chromium release assay

Cytolytic activity of NK cells against the cell lines K562 (sensitive to unstimulated and activated NK cells) and Daudi (sensitive to activated NK cells only) was measured in 4-hour ^{51}chromium (^{51}Cr) release assays. Effector cells were cultured for 72 h under conditions as indicated. K562 and Daudi target cells (ATCC, Manassas, VA, USA) were labeled with 100 µl Na-chromate (^{51}Cr, 3.7 MBq) for 1 hr. After washing, 2.5x10^{3} target cells were added to effector cells in triplicate at the indicated effector-target (E:T) ratios and incubated for 4 h at 37°C. Supernatants were collected, and the release of ^{51}Cr was measured with a gamma-counter (Wallac/PerkinElmer, Waltham, MA, USA). Spontaneous and maximum release were obtained by incubation with medium and 2N HCl, respectively. The specific lysis was calculated by the following formula: percentage of specific lysis = 100 x (experimental release–spontaneous release / maximum release–spontaneous release).

Enzyme-linked immunosorbent assay (ELISA)

Concentrations of IFN-α were determined in supernatants from cultures of PBMC, TCD PBMC or purified PBMC subpopulations using the "ELISA assay for human interferon-α pan" (Mabtech, Nacka Strand, Sweden) according to the manufacturer's instructions.

RESULTS

Adenovirus serotype 5 and type 35 stimulate activation and cytolytic activity of NK cells in unseparated PBMC

To investigate the capacity of wildtype adenovirus serotypes 5 (HAdV5) and 35 (HAdV35) to induce activation of NK cells in PBMC *in vitro*, CD69 expression and the cytolytic activity against the HLA class I negative cell lines Daudi and K562 were assessed. Analogous to the effect of interleukin-15 (IL-15), 40 h stimulation of PBMC with escalating viral loads of HAdV5 and HAdV35 increased the percentage of NK cells expressing the activation marker CD69 as compared to the unstimulated medium control (Figure 1, panel A, i). HAdV5 induced CD69 expression only at MOI 100, whereas HAdV35 produced appreciable activation of NK cells at a 10-fold lower MOI. Similarly, HAdV35 has previously been shown to be more effective than HAdV5 in eliciting T cell responses [25]. Notably, stimulation with HAdV35 at MOI 100 appeared to exhibit cytopathic effects because the percentage of CD14^{+} monocytes in PBMC was strongly reduced, whereas lower viral loads of HAdV35 or HAdV5 did not alter the percentage of monocytes (Figure 1, panel A, ii). For that reason, virus stimulations were performed with HAdV5 at MOI 100 and HAdV35 at MOI 10 in subsequent experiments.

Stimulation of PBMC with either HAdV5 (MOI 100) or HAdV35 (MOI 10) induced NK cell cytolysis of Daudi cells, a target cell line which is naturally not lysed by resting but only by activated NK cells [32-34] (Figure 1, panel B). Additionally, NK cell-mediated lysis of K562 cells was slightly enhanced in HAdV5-stimulated PBMC (Figure 1, panel B).

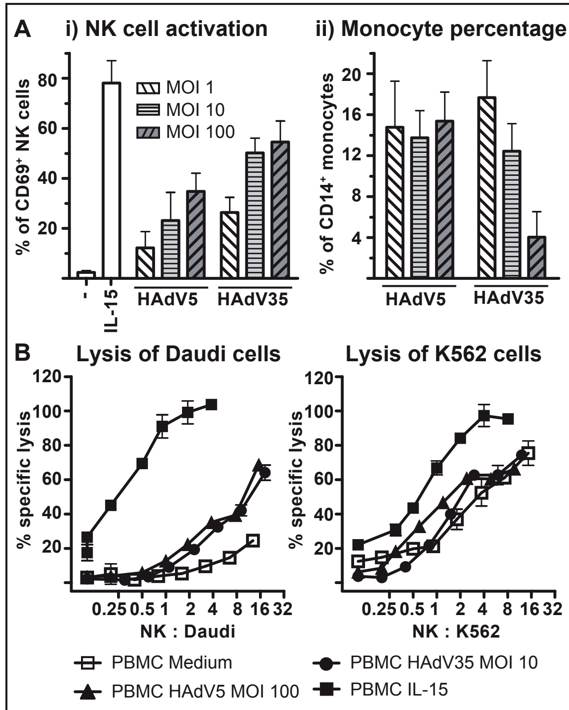

Figure 1: HAdV5 and HAdV35 induce NK cell activation in PBMC

(A) PBMC were stimulated with HAdV5 and HAdV35 at MOI 1, 10 and 100 and controls (unstimulated medium as negative control or IL-15 as positive control) for 40 h. (i) NK cell activation was measured by the increased expression of the activation marker CD69 on NK cells. NK cells were gated as lymphocytes based on FSC/SSC plots and defined as the cell population positive for CD56 expression but devoid of CD3, CD14 and CD19 expression. Data depicted are combined from 3 independent experiments (error bar represents SEM). (ii) The percentage of viable $CD14^+$ monocytes was compared among virus stimulations. Data depicted are combined from 3 independent experiments (error bar represents SEM). **(B)** After stimulation of PBMC with HAdV5 (MOI 100), HAdV35 (MOI 10) and controls for 72 h, the cytolytic potential of NK cells against Daudi and K562 cells was tested in triplicate in a 4 h ^{51}chromium release assay. Effector:target (E:T) ratios were two-fold dilutions of effectors, starting with $5x10^5$ PBMC and $2.5x10^4$ target cells. PBMC E:T ratios were adjusted to the frequency of NK cells in PBMC measured by FACS to obtain actual NK:T ratios. Data are representative for 2 independent experiments.

Hence, stimulation of PBMC with HAdV5 and HAdV35 resulted in enhanced expression of the NK cell activation marker CD69 and concomitantly increased the cytolytic activity of NK cells.

HAdV5-induced NK cell activation requires the presence of T cells

To study whether the activation of NK cells by HAdV was due to a direct effect or required other cell populations of blood mononuclear cells, purified NK cells were stimulated with HAdV5 and HAdV35 for 40 h. On purified NK cells alone CD69 expression was not increased after HAdV5 or HAdV35 exposure as compared to NK cells in HAdV-stimulated PBMC (Figure 2, panel A, i and ii). In contrast, IL-15 stimulation induced CD69 up-regulation, demonstrating that the purified NK cells were functional and responsive. Therefore, it can be concluded that at least one additional cell type of blood mononuclear cells was essential to mediate HAdV-induced NK cell activation.

Since most healthy individuals have HAdV-specific T cells recognizing multiple serotypes, we reasoned that the activation of NK cells in HAdV-stimulated PBMC might be induced via the production of IL-2 by HAdV-specific T cells [29;35-38]. In order to investigate whether NK cell activation requires T cells, PBMC were depleted of $CD3^+$ T cells (TCD PBMC). CD69 up-regulation on NK cells in response to HAdV5 was abolished by T cell depletion. In contrast, HAdV35 still induced CD69 up-regulation in the absence of T cells (Figure 2, panel A, iii). Notably, HAdV5 and HAdV35 exposure of PBMC induced CD69 expression on both the $CD56^{dim}$ and the $CD56^{bright}$ NK cell population, whereas in the absence of T cells CD69 up-regulation was mainly observed in the $CD56^{dim}$ NK cell population after HAdV35 exposure.

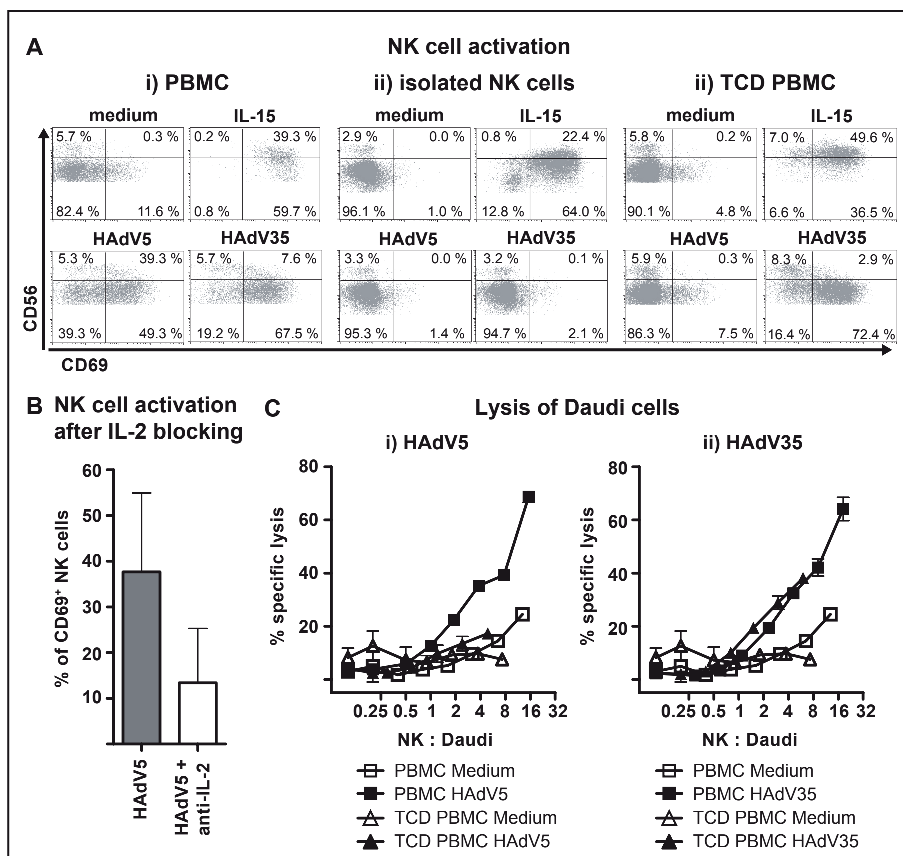

Figure 2: HAdV5, but not HAdV35, requires T cells to activate NK cells

(A) NK cells were isolated from PBMC by negative selection, i.e., depleting non-NK cells, with a purity of >95%. T cells were depleted from PBMC by positive selection (TCD PBMC); TCD PBMC contained less than 0.5% of contaminating $CD3^+$ T cells. PBMC ($2x10^5$) (panel i), isolated NK cells ($1x10^5$) (panel ii) and TCD PBMC ($2x10^5$) (panel iii) were stimulated with HAdV5 (MOI 100), HAdV35 (MOI 10) and controls for 40 h. NK cell activation was measured by up-regulation of CD69 expression on $CD56^{dim}$ and $CD56^{bright}$ NK cell subsets. NK cells were gated as lymphocytes based on FSC/SSC plots and defined as the cell population positive for CD56 expression but devoid of CD3, CD14 and CD19 expression. Data are representative for 3 independent experiments. **(B)** CD69 expression on NK cells was assessed in PBMC exposed to HAdV5 with or without the addition of neutralizing antibodies against IL-2. Data depicted are combined from 3 independent experiments (error bar represents SEM). **(C)** Cytolytic potential of NK cells against Daudi cells was tested after stimulation of PBMC and TCD PBMC with HAdV5 (panel i) and HAdV35 (panel ii) and compared to unstimulated medium controls. The ^{51}Chromium release assay was performed in triplicate as described in Figure 1.

Neutralization of IL-2 reduced CD69 expression on NK cells of HAdV5-exposed PBMC, suggesting that NK cell activation in response to HAdV5 is, at least partially, induced by IL-2 production (Figure 2, panel B).

Cytolysis of Daudi cells by NK cells after exposure to HAdV5 was strongly reduced in TCD PBMC as compared to non-depleted PBMC (Figure 2, panel C, i), whereas HAdV35-stimulated lysis of Daudi cells was unaffected after T cell depletion (Figure 2, panel C, ii). These results suggest that HAdV5 and HAdV35 can induce NK cell activation in PBMC by different mechanisms. HAdV5 activates NK cells via a T cell-dependent mechanism, whereas the activation of NK cells in response to HAdV35 can occur independently of T cells.

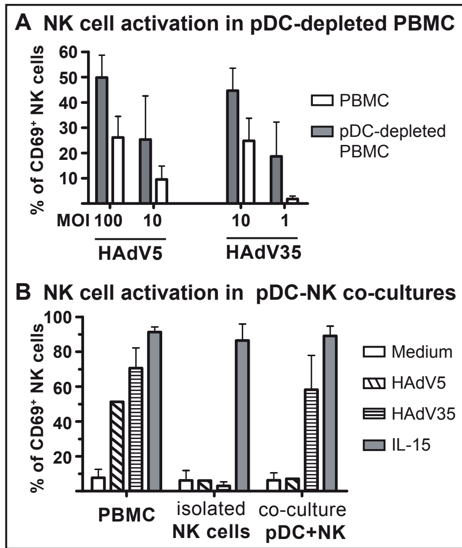

Figure 3: Plasmacytoid dendritic cells directly mediate NK cell activation after HAdV35 exposure

(A) pDC were depleted from PBMC by positive selection, resulting in the depletion of >99% of pDC. PBMC and pDC-depleted PBMC (each $2x10^5$) were stimulated with HAdV5 (MOI 10 and 100) and HAdV35 (MOI 1 and 10) for 40 h. NK cell activation was measured by up-regulation of CD69 expression. Data depicted are combined from 4 independent experiments (error bar represents SEM). **(B)** pDC were purified from PBMC by negative selection with a purity of >99 % and no $CD3^+$, $CD14^+$, $CD19^+$ cells detectable. PBMC, isolated NK cells ($1x10^5$) and co-cultures of NK cells ($1x10^5$) and pDC ($1x10^4$) (10:1 ratio) were stimulated with HAdV5 (MOI 100), HAdV35 (MOI 10) and controls for 40 h. NK cell activation was measured by up-regulation of CD69 expression. Data depicted are combined from 4 independent experiments (error bar represents SEM).

Plasmacytoid dendritic cells mediate HAdV35-induced NK cell activation

NK cell activation in response to HAdV35 occurred in the absence of T cells but still required another cell population of PBMC as purified NK cells were not activated. Since pDC are known to sense viruses, it was investigated whether pDC were required for NK cell activation following HAdV35 exposure. Depletion of pDC from PBMC led to reduced CD69 expression on NK cells after both HAdV5 and HAdV35 stimulation, in particular at lower viral loads, indicating that pDC were involved (Figure 3, panel A). To explore whether pDC were sufficient to elicit NK cell activation following HAdV35 exposure, pDC were isolated by negative selection, added to purified NK cells and stimulated with both HAdV serotypes. HAdV35, but not HAdV5, was capable of inducing NK cell activation in the presence of pDC and, apparently, did not require the contribution of other cell types (Figure 3, panel B). Consequently, pDC were both necessary and sufficient for the activation of NK cells following the exposure to HAdV35.

HAdV35 induces IFN-α production by plasmacytoid dendritic cells

As pDC were able to mediate NK cell activation by HAdV35, activation of pDC was assessed by analyzing intracellular IFN-α production following virus exposure. In contrast to HAdV5, HAdV35 exposure of PBMC resulted in a higher percentage of IFN-α–producing cells which expressed the pDC marker BDCA-2 (Figure 4, panel A). There were practically no IFN-α^+ cells detectable in the non-BDCA-2^+ cell fraction. Detailed analysis of the phenotype of all IFN-α^+ cells (Q2 + Q4; Figure 4, panel A) revealed a specific increase of pDC after HAdV35 exposure (Table 1). The percentage of other PBMC subsets, such as myeloid dendritic cells (mDC), T cells, B cells, NK cells and monocytes, did not alter or decreased after HAdV35 exposure. In addition, HAdV35, but not HAdV5, induced up-regulation of HLA-DR and CD40 as well as down-regulation of BDCA-2 on pDC, illustrating activation and maturation of pDC by HAdV35 (Figure 4, panel B). HAdV35 exposure resulted in considerable IFN-α levels in culture supernatants of unseparated PBMC (Figure 4, panel C). The depletion of pDC from

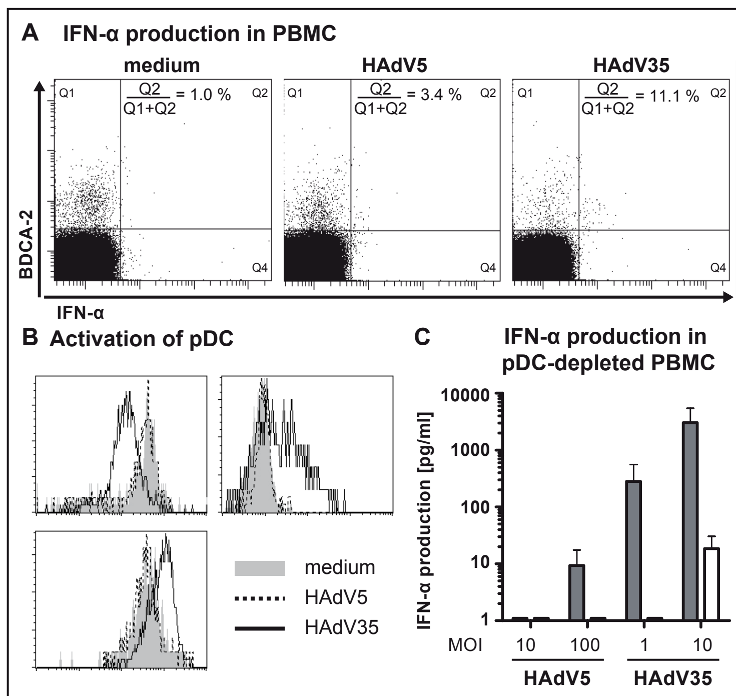

Figure 4: HAdV35 induces IFN-α production and maturation of plasmacytoid dendritic cells

(A) PBMC (2x10^{6}) were cultured with HAdV5 (MOI 100), HAdV35 (MOI 10) and the medium control for 18 h. pDC were identified in PBMC as BDCA-2^{+} cells by extracellular staining followed by intracellular staining for IFN-α. The percentage of IFN-α^{+} cells was determined within the BDCA-2^{+} pDC population. Data are representative for 3 independent experiments. **(B)** Activation and maturation of pDC was assessed by the expression of CD40 and HLA-DR as well as down-regulation of BDCA 2 on BDCA 2^{+} purified pDC after the exposure to HAdV5 (MOI 100) and HAdV35 (MOI 10) for 18 h. Data are presented as histograms, depicting the mean fluorescent intensity, MFI, of the four markers and the three conditions: medium, grey filled histogram, HAdV5, dotted line; HAdV35, black solid line. Data are representative for 3 independent experiments. **(C)** IFN-α levels were measured in culture supernatants of HAdV5 (MOI 10 and 100) and HAdV35-exposed (MOI 1 and 10) PBMC and pDC-depleted PBMC by ELISA. Data depicted are combined from 4 independent experiments (error bar represents SEM).

Table 1: The percentage of pDC within the IFN-α^{+} cells increases following HAdV35 exposure

	Percentage of PBMC subsets within IFN-α^{+} cells		
	Medium	HAdV5	HAdV35
pDC	7.7	11.1	73.0
mDC	5.1	0.0	0.2
T cells	43.6	13.9	13.5
B cells	10.3	30.6	0.0
NK cells	18.0	11.1	3.8
monocytes	2.6	13.8	0.0

PBMC were cultured with HAdV5 (MOI 100), HAdV35 (MOI 10) and medium for 18 h. Extracellular stainings of PBMC subsets were followed by intracellular staining for IFN-α. Within all IFN-α^{+} cells, pDC were identified as BDCA-2^{+} cells which were negative for CD11c^{-} and devoid of CD3^{+}/14^{+}/19^{+}/20^{+}/56^{+} cells; mDC as cells double positive for BDCA-1 and CD11c and devoid of CD3^{+}/14^{+}/19^{+}/20^{+}/56^{+} cells; T cells as CD3^{+} cells devoid of CD20^{+}/56^{+} cells; NK cells as CD56^{+} cells devoid of CD3^{+}/19^{+} cells; B cells as CD19^{+} and CD20^{+} cells devoid of CD3^{+}/56^{+} cells; monocytes as cells double positive for CD14 and CD11c. The percentage of the various PBMC subpopulations within IFN-α^{+} PBMC (equals 100%) was determined.

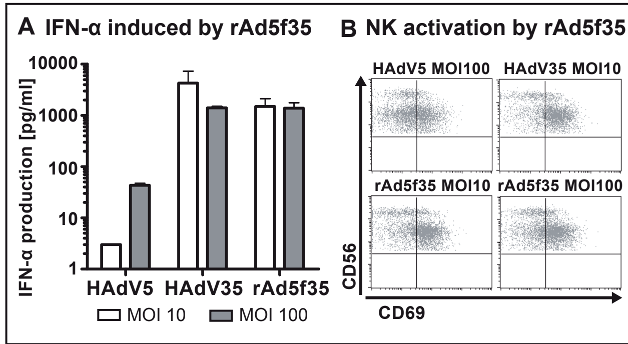

Figure 5: rAd5f35 induces high IFN-α production and activates NK cells

(A) PBMC were stimulated with HAdV5, HAdV35 and rAd5f35 (all MOI 10 and 100) for 40 h. IFN-α levels were measured in culture supernatants by ELISA. Data depicted are combined from 3 independent experiments (error bar represents SEM). **(B)** PBMC were stimulated with HAdV5 at MOI 100, HAdV35 at MOI 10 and rAd5f35 at MOI 10 and 100 for 40 h. NK cell activation was measured by up-regulated expression of CD69 on NK cells. Data are representative for 3 independent experiments.

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PBMC substantially diminished the IFN-α levels, confirming that IFN-α production was almost exclusively attributed to pDC (Figure 4, panel C).

Adenovirus strains from different species utilize distinct surface molecules to enter cells. Internalization of HAdV5 involves binding of the HAdV5 fiber to the CAR receptor on target cells, while the HAdV35 fiber engages CD46 for cell entry [2]. To investigate the role of the fiber and its potential role in the way of entry into pDC, a recombinant rAd5f35 virus, which is a HAdV5 virus equipped with a HAdV35 fiber [27;30], was tested for its ability to induce IFN-α production and NK cell activation. At identical MOI, IFN-α production following rAd5f35 stimulation of PBMC was similar to the induction by HAdV35 and much higher than IFN-α production after HAdV5 exposure (Figure 5, panel A). Correspondingly, exposure of PBMC to rAd5f35 resulted in CD69 expression on NK cells comparable to HAdV35 exposure (Figure 5, panel B). To investigate whether the higher NK cell activation of type 35 fiber-equipped viruses was dependent on a higher infection rate of PBMC and in particular pDC, PBMC were stimulated with recombinant, replication-deficient rAd5-GFP, rAd35-GFP and rAd5f35-GFP and the percentage of GFP-expressing cells was assessed per PBMC subset. Exposure of PBMC with rAd5f35-GFP and rAd35-GFP resulted in a higher percentage of GFP-expressing pDC than rAd5-GFP in particular at high viral loads (Table 2). Infection of mDC and monocytes was also higher by rAd35-GFP and rAd5f35-GFP viruses, whereas only a minor percentage of T cells, NK cells and B cells (except for rAd5f35-GFP at MOI 100) was infected by both viruses.

Table 2: rAd5f35 and rAd35 but not rAd5 are able to infect pDC

	Percentage of GFP$^+$ cells among PBMC subsets						
	Medium	rAd5-GFP		rAd35-GFP		rAd5f35-GFP	
MOI		10	100	10	100	10	100
pDC	0.0	0.0	0.2	4.5	6.1	0.5	6.1
mDC	0.0	22.3	22.5	17.8	30.4	42.5	70.9
T cells	0.0	0.1	0.4	0.5	0.8	1.0	4.5
B cells	0.0	0.2	1.8	1.4	1.7	3.5	11.6
NK cells	0.0	0.1	0.4	0.2	0.8	0.6	1.8
monocytes	0.0	2.2	16.5	8.9	26.0	31.8	70.8

PBMC were cultured with replication-deficient GFP-encoding rAd5-GFP, rAd35-GFP and rAd5f35-GFP at MOI 10 and 100 for 18 h. pDC, mDC, T cells, B cells, NK cells and monocytes were identified in PBMC as described in Table 1. To determine infection by the adenoviruses, the percentage of GFP-expressing cells per PBMC subset was assessed.

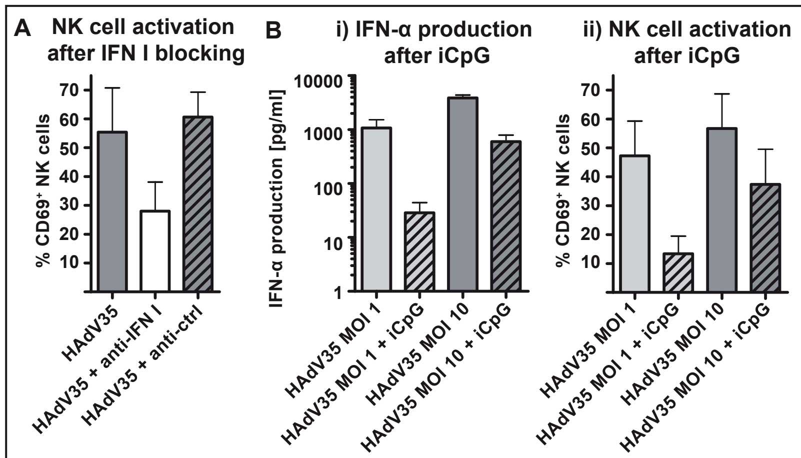

Figure 6: Both NK cell activation and IFN-α production require TLR9 signaling

(A) TCD PBMC were stimulated with HAdV35 at MOI 10 for 40 h in the presence or absence of neutralizing antibodies against IFN-α and the IFN I receptor or an irrelevant antibody. CD69 expression on NK cells was measured. Data depicted are combined from 4 independent experiments (error bar represents SEM). **(B)** TCD PBMC were stimulated with HAdV35 at MOI 1 and MOI 10 for 40 h in the presence or absence of iCpG. (i) IFN-α levels in supernatants of these cultures as well as (ii) CD69 expression on NK cells was measured. Data depicted are combined from 5 independent experiments (error bar represents SEM).

Therefore, the type of fiber and, likely, the rate of infection appeared to be responsible for the higher IFN-α production after exposure to HAdV35 than to HAdV5.

IFN-α production in response to HAdV35 relies on TLR-9 signaling and mediates NK cell activation

To further confirm whether IFN-α did mediate NK cell activation, CD69 expression on NK cells was assessed in the presence of neutralizing antibodies against soluble IFN-α and the IFN-I receptor [14;39]. In TCD PBMC exposed to HAdV35 at MOI 10, blockage of IFN-I reduced CD69 expression on NK cells by approx. 50 %, indicating that IFN-I was at least in part responsible for this activation (Figure 6, panel A). As far as other cytokines are involved, the presence of IL-12 or IL-15 in supernatants of HAdV35-exposed pDC-NK cell cultures was excluded (data not shown).

To explore whether IFN-α production by pDC requires sensing of adenovirus via the intracellular double-stranded DNA receptor TLR-9, the effect of inhibitory CpG (iCpG) was addressed. iCpG neutralizes the stimulatory effect of the TLR-9 agonist CpG and blocks IFN-α production by pDC in response to CpG and viruses [40-43]. IFN-α levels in supernatants of HAdV35-stimulated TCD PBMC were considerably reduced by iCpG (Figure 6, panel B, i). Likewise, iCpG inhibited NK cell activation following exposure to HAdV35 (Figure 6, panel B, ii). The inhibiting effect of iCpG was most apparent when TCD PBMC were exposed to lower viral loads of HAdV35, i.e., MOI 1 compared to MOI 10.

Overall, TLR-9 signaling appeared to be involved in the IFN-α production by pDC in response to HAdV35 which could mediate the activation of NK cells in response to HAdV35.

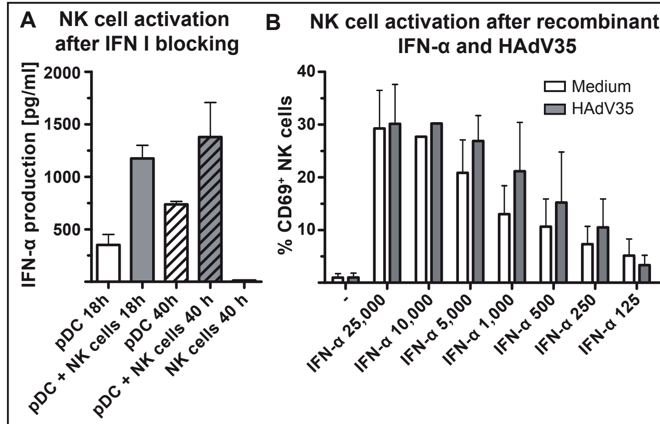

Figure 7: IFN-α production by plasmacytoid dendritic cells is enhanced in the presence of NK cells
(A) Purified pDC, purified NK cells and pDC-NK cell co-cultures (pDC: NK cells = 1:4) were stimulated with identical virus concentrations as for pDC-NK cell cultures (MOI 10) for 18 h and 40 h, respectively. IFN-α levels were measured in the supernatants of these cultures. Data depicted are combined from 2 independent experiments (error bar represents SEM). **(B)** Purified NK cells were stimulated with decreasing amounts of recombinant IFN-α with or without the addition of HAdV35 (MOI 10) for 40 h, and CD69 expression on NK cells was analyzed. Data depicted are combined from 2 independent experiments (error bar represents SEM).

The IFN-α production by plasmacytoid dendritic cells is amplified by NK cells

It was examined whether NK cells contribute to the IFN-α response of pDC [14]. Indeed, IFN-α levels were augmented in HAdV35-stimulated co-cultures of pDC and NK cells as compared to pDC alone (Figure 7, panel A). NK cells alone failed to produce IFN-α. After 18 h stimulation with HAdV35, the increase of IFN-α levels by the presence of NK cells was superior to the increase after 40 h stimulation, suggesting that the reciprocal interaction between pDC and NK cells boosted the IFN-α response of pDC in particular early after exposure to HAdV35.

To investigate whether the increased response could be due to the NK cells becoming directly responsive to virus after exposure to pDC derived IFN-α, purified NK cells were stimulated with different concentrations of recombinant IFN-α in the presence and absence of HAdV35. However, HAdV35 hardly increased the CD69 expression on IFN-α–induced NK cells, suggesting that NK cells themselves cannot sense HAdV35 (Figure 7, panel B).

It can be concluded that there was cross-talk between NK cells and pDC characterized by the activation of NK cells through pDC-derived IFN-α and the amplification of IFN-α production by pDC through NK cells.

DISCUSSION

In this study we demonstrate that HAdV35 exposure results in considerable production of IFN-α by pDC, leading to NK cell activation. Conversely, under the same conditions HAdV5 does not induce significant IFN-α production and requires the contribution of T cells to activate NK cells. The difference in IFN-α production is specifically related to the virus fiber and, therefore, most likely due to the way of cell entry and infection efficiency of the virus. The IFN-α response of pDC to HAdV35 is mediated by TLR-9 signaling and is further enhanced by the reciprocal interaction of NK cells and pDC.

In patients post HSCT, the interplay between pDC and reconstituting NK cells may play a role in the immune response to HAdV early after HSCT, especially in the period when T lymphocytes are not yet detectable [20]. The presumed NK cell response to adenovirus in humans, however, has not yet been described as detailed as the NK cell response to other viruses, such as herpes simplex virus type 1, influenza virus or reovirus [14;39;44;45]. In mice, IFN-I and dendritic cell-dependent activation of NK cells has been implicated in the clearance of cells infected with adenoviral vectors [46;47]. However, since human adenovirus is known to result in only low yields of productive virus after infection of murine cells due to limited viral replication, imperfect protein synthesis and an abortive virus life cycle, mouse models are not optimal for studying immune responses to human adenovirus [48-51]. After infection with recombinant adenoviral vectors, human fibroblasts were more susceptible to lysis by NK cells in association with the NK cell-activating receptor NKG2D [52]. Inspired by these observations, we set out to study the activation of NK cells in PBMC of healthy adult donors in response to human adenovirus.

Interestingly, the mechanisms of NK cell activation in response to the two tested HAdV serotypes, which belong to different species of the HAdV family, differed substantially. Most healthy individuals possess a highly cross-reactive T cell repertoire, recognizing a variety of different HAdV serotypes and species [29;35-37]. The addition of HAdV to PBMC results in T cell activation and, thereby, is likely to cause IL-2 production. As the T cell repertoire is highly cross-reactive this T cell activation occurs in response to both HAdV5 as well as HAdV35 [36;53]. It is known that T cell-derived IL-2 stimulates NK cell proliferation, activation and IFN-γ production [38;54-56]. Neutralization of IL-2 indeed inhibited at least partially the NK cell activation in response to HAdV5. A similar mechanism was shown to account for NK cell activation after exposure to influenza, a virus that also elicits a strong T cell response in most healthy donors [38].

Surprisingly, although the depletion of T cells did abrogate NK cell activation in response to HAdV5, it did not abrogate HAdV35-induced NK cell activation. Accordingly, an additional mechanism for HAdV35-induced NK cell activation is operational that is independent of T cells. Compared to HAdV5, HAdV35 evoked considerably stronger IFN-α production by pDC which may be sufficiently high to induce NK cell activation via pDC [14;25].

IFN-I production by pDC in response to viral DNA or stimulatory CpG motifs requires TLR-9 signaling [15]. IFN-α production in response to HAdV35 was also mediated by TLR-9 as most of the response was eliminated by the TLR-9 antagonist iCpG [41;57]. Since IFN-α was almost exclusively derived from pDC upon HAdV35 exposure, TLR-9-independent pathways of other cells, such as mDC or monocytes, do not seem to contribute to the IFN-α response as supported by the data on intracellular IFN-α in various subsets of mononuclear cells [14;39;58]. NK cell activation following HAdV35 exposure was at least partly mediated by IFN-α as the presence of neutralizing antibodies against IFN-α and the IFN-I receptor reduced CD69 expression on NK cells by approx. 50 % [14;39]. The lack of total inhibition suggested that either IFN-α was produced in large excess or that besides IFN-α additional (soluble) factors may be involved in mediating NK cell activation by pDC. While incubation of purified NK cells with IFN-α–containing supernatants of HAdV35-stimulated pDC-NK cell co-cultures induced NK cell activation, neither IL-12 nor IL-15, two other NK cell-activating cytokines, were detected in these supernatants (data not shown) [14].

Finally, we observed that cross-talk between NK cells and pDC boosted the IFN-α production of pDC in response to HAdV35, suggesting a reciprocally amplified antiviral innate immune response. In previous studies, cross-talk between NK cells and DC via direct cell-cell contact, such as NKG2D-NKG2D ligand interaction, has been associated with NK cell activation and can enhance IFN-α production [14;39;47]. Interestingly, the DNAM-1 ligand CD112 was expressed but not up-regulated on pDC after virus exposure whereas NKG2D ligands were not detectable on pDC (data not shown). Hence, interaction between NK cells and pDC could theoretically occur via DNAM-1 and CD112.

Our findings raised the question why HAdV35 was so much more effective than HAdV5 in inducing IFN-α production and maturation of pDC. The two virus serotypes differ in the type of cellular receptor necessary for cell entry. The HAdV5 fiber binds to the CAR (coxsackie adenovirus receptor) receptor which is absent from pDC, while the type 35 fiber binds to the cellular surface protein CD46 (complement regulatory protein [59]) which is abundantly expressed on pDC [2;25]. As reported previously, especially myeloid cells such as mDC and monocytes were infected by rAd5-GFP, rAd35-GFP and rAd5f35-GFP (rAd5 equipped with the type 35 fiber), while lymphocytes such as T cells, B cell and NK cells were weakly infected [25-28]. Moreover, we show that rAd5f35 induced IFN-α levels similar to HAdV35 and likewise HAdV35 and rAd5f35 were able to infect pDC at comparable efficiencies, whereas HAdV5 failed to infect pDC. Thus, the increased IFN-α production of rAd5f35 may be a result of higher infection rates of pDC due to the type 35 fiber. Correspondingly, the much higher infection efficiency of HAdV5f16 (HAdV5 equipped with the species B type 16 fiber) than HAdV5 has been associated with high IFN-α production by pDC [41]. The percentage of IFN-α producing pDC was higher than the percentage of GFP-expressing pDC, suggesting that also non-infected pDC might produce IFN-α (data not shown).

The clinical relevance of NK cells with respect to HAdV clearance in humans is unresolved. In pediatric HSCT patients, the kinetics of lymphocyte recovery is different, i.e., NK cells usually reconstitute earlier than T cells. During the early post-HSCT period, HAdV may (re)activate and potentially cause a disseminated infection with a fatal course [36;60;61]. HAdV5 and other species C HAdV are predominantly encountered in these patients in our center and elsewhere [62-64]. In comparison to HAdV5, antibodies specific for the species B virus HAdV35 are rarely detected in sera of healthy donors, suggesting that the virus is not ubiquitously present in the human population [65;66]. In immunocompromised patients, HAdV35 causes disease only infrequently although it has been reported in HSCT centers in the USA [67]. The low prevalence of the virus, absence of functional immune evasion mechanisms or a strong response of the innate immune system to HAdV35 may provide an explanation for the low occurrence of disseminated HAdV35 infections in HSCT patients.

The phenomenon that these two adenovirus serotypes exhibit substantial differences in their capacity to evoke an innate immune response is relevant because HAdV35-derived vectors are recently being explored for the delivery of vaccines [66;68]. Most experience with adenovirus-derived vectors has been acquired in experimental models or clinical trials using HAdV5-derived vectors. Based on the present findings, it may be anticipated that the HAdV35-derived vectors behave differently in their efficacy and/or their degree of side effects in clinical trials.

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