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## The contribution of ABC transporters to dendritic cell development and function

van de Ven, R.

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**Chapter 8**

**Selective transduction of mature DC in human skin and lymph nodes by  
CD80/CD86-targeted fiber-modified Adenovirus-5/3**

*Submitted*

Rieneke van de Ven

Jelle J. Lindenberg

Dinja Oosterhoff

M.Petrousjka van den Tol

Rodney A. Rosalia

Miho Murakami

Maike Everts

George L. Scheffer

Rik J. Scheper

Tanja D. de Gruijl

David T. Curiel

## Chapter 8

### Abstract

*In vivo* targeting of dendritic cells (DC) represents an attractive alternative to currently applied *ex vivo* DC-based genetic tumor vaccination protocols. Finding the optimal vector for *in vivo* targeting of DC is important for such strategies. We therefore tested a panel of subgroup C/B chimeric and fiber-modified adenoviruses (Ad) for their relative capacity to transduce human DC. We made use of *in vitro* generated Langerhans Cells (LC) as well as of *ex vivo* human skin and melanoma-draining lymph node (LN) derived DC. Of the tested viruses the C/B-chimeric Ad5/3 virus most efficiently transduced *in vitro* generated LC. In addition, Ad5/3 preferentially targeted mature myeloid DC from human skin and draining LN and transduced them at significantly higher frequencies than Ad5. In addition, Ad5/3 was more specific for mature human skin-derived CD1a<sup>+</sup> CD83<sup>+</sup> DC than the previously reported DC-transducing C/B-chimeric vector Ad5/35, infecting less bystander cells. It was previously reported that Ad5/3 transduced human monocyte-derived DC by binding to the B7 molecules CD80 and CD86. High-efficiency transduction of mature skin-derived DC was similarly shown to be mediated through binding to CD80/CD86 and not to interfere with subsequent T cell priming. We conclude that Ad5/3, in combination with DC-activating adjuvants, represents a promising therapeutic tool for the *in vivo* transduction of mature DC, and may be less likely to induce unwanted side effects such as immune tolerance through the infection of non-professional antigen-presenting cells.

### Introduction

Dendritic cells (DC) are a promising target for gene therapeutic strategies to induce anti-tumor responses, as DC initiate immune responses by presenting tumor associated antigens (TAA) to naïve T cells. Therapeutic strategies that target DC should aim for high transduction efficiency and specificity, as optimal TAA delivery to DC is critical for the therapeutic efficacy of DC-based vaccination strategies. Adenovirus (Ad)-based therapies are ideal for *in vivo* targeting and infection of DC, as adenoviruses can infect non-replicating cells and can be genetically modified to express a TAA of choice. It is well known that the commonly used subgroup C adenovirus serotype 5 (Ad5) is of limited utility for transduction of human DC, both *in vitro* and *in vivo*, as DC lack expression of the adenovirus binding receptor CAR (Coxsackie-and Adenovirus Receptor). Other Ad types without pre-existent immunity and with a natural tropism for DC-associated molecules may be more readily translated into the clinic as vaccination vehicles, without a further need for genetic modification of their fiber knob to enable DC targeting.<sup>1-3</sup> We previously showed more effective skin DC targeting using the subgroup B2 virus Ad35 or a chimeric Ad5/35 virus containing the Ad5 fiber and Ad35 knob.<sup>4</sup> This virus enters cells via CD46<sup>5,6</sup>, which is a complement regulatory molecule, present on many cells including DC.<sup>7</sup> Although Ad5/35 was shown to be superior over Ad5 in terms of DC transduction, a more specific and truly DC-targeted virus might be preferable, as CD46 is expressed ubiquitously in the skin. Infection, and subsequent antigen presentation on MHC class I molecules by other cells than professional antigen-presenting cells (APC), might result in sub-efficient T cell activation, or even T cell anergy or tolerance induction, as a strong co-stimulatory signal via CD80 and CD86 is required to fully activate T cells. We therefore aimed to find a more DC-specific adenovirus with high-efficiency infection characteristics for future *in vivo* DC-targeting strategies. Short et al. previously reported on the binding of the subgroup B1 adenovirus Ad3 to the co-stimulatory molecules CD80 (B7.1) and CD86 (B7.2) on human monocyte-derived DC.<sup>8</sup> Since expression of these markers is abundant on activated DC, we set out to investigate whether a virus containing the Ad3 knob domain would effectively transduce human skin DC *in situ*.

## Selective transduction of mature DC in human skin and LN by CD80/CD86-targeted fiber-modified Ad5/3

The skin presents a readily accessible and highly efficient conduit for the delivery of Ad-based vaccines and subsequent activation of specific T cells.<sup>4,9</sup> Specialized DC subsets line both the epidermis (i.e. Langerhans Cells [LC]) and the dermis, which, upon transduction and appropriate activation, can induce TAA-specific T cell responses in the draining lymph nodes (LN).<sup>10</sup> A panel of genetically modified adenoviruses was therefore analyzed for their relative capacity to infect *in vitro* cultured LC and interstitial (dermal) DC. Amongst these were three different subgroup C/B chimeras, i.e. Ad5/3, Ad5/11 and Ad5/35 as well as three fiber modified viruses i.e. Ad5.RGD<sup>11</sup>, Ad5.pK7 and Ad5.RGD.pK7.<sup>12</sup> The RGD motif binds integrins and the pK7 polylysine motif can bind heparin sulfate containing receptors.<sup>12</sup> Replication deficient Ad5 virus was employed for comparison. The most efficient *in vitro* transduction was observed with Ad5/3 and Ad5.RGD, in addition to the previously reported Ad5/35. Ad5/3, and not Ad5.RGD, was found to enhance transduction of DC in the context of intact skin in human explant cultures. Conflicting data were previously reported on the tropism of Ad3 (a B1-type virus): whether it binds cells primarily via the costimulatory molecules CD80 and CD86<sup>8</sup> or, like Ad35 (a B2-type virus) and other type B viruses, via CD46.<sup>5,13-16</sup> Here we show that Ad5/3 is a high-efficiency DC-transducing adenovirus that binds DC through the B7-family members both in MoDC and human skin-migrated DC without interfering with their function. Moreover, the ability of Ad5/3 to selectively target mature human myeloid DC in the context of skin and in melanoma skin-draining LN, confirms its possible utility as an *in vivo* DC-targeted tumor vaccine vector.

### Materials and Methods

#### DC, cell line and tissue cultures

PBMC from healthy donors were isolated from buffycoats obtained from Sanquin blood supply foundation (Amsterdam, The Netherlands) by density gradient centrifugation over Hpaque lymphoprep (Nycomed AS, Oslo, Norway). Monocytes were obtained by plastic adherence and were cultured into MoDC in Iscove's modified Dulbecco's medium (IMDM) (Lonza, Verviers, Belgium), supplemented with 10% fetal calf serum (FCS) (HyClone), 100 IU/ml sodium-penicillin, 100µg/ml streptomycin, 2 mM L-glutamine, 50 µM β-mercaptoethanol (2ME) (complete IMDM) and 100ng/ml recombinant human granulocyte colony stimulating factor (rhGM-CSF) (Sagramostim, Berlex) and 10ng/ml interleukin-4 (IL-4) (R&D systems) for 5-6 days.

The acute myeloid leukaemia cell line MUTZ3 was cultured in Minimum essential medium-α (MEM-α) (Lonza, Verviers, Belgium) containing 20% FCS, 100 IU/ml sodium-penicillin, 100µg/ml streptomycin, 2 mM L-glutamine and 50 µM 2ME and 10% conditioned medium of the 5637 renal cell carcinoma cell line (MUTZ3 routine medium) in 12-well plates (Co-star) at a concentration of 0.2 million cells/ml and were passaged twice weekly as described.<sup>17</sup> MUTZ3-LC were cultured by seeding MUTZ3 precursor cells in 12-well plates at 0.1 million cells/ml in MEM-α supplemented with 20% FCS, 100 IU/ml sodium-penicillin, 100µg/ml streptomycin, 2 mM L-glutamine and 50 µM 2ME (complete MEM-α). Differentiation medium contained 100ng/ml rhGM-CSF, 10ng/ml TGFβ1 (Biovision, Mountain View, CA) and 120 IU/ml TNFα (Miltenyi Biotec) and cells were differentiated into immature LC for 9-10 days.<sup>18</sup> Immature MoDC and MUTZ3-LC were matured by adding a maturation cocktail containing 2400 IU/ml TNFα, 100ng/ml IL-6 (R&D systems), 25ng/ml IL-1β (Miltenyi Biotec) and 1µg/ml prostaglandin E2 (PGE2) (Sigma Aldrich) for 2 days.

HEK-293 cells (Microbix, Toronto, Canada) and QBI-293A cells (Qbiogene, Heidelberg, Germany) were maintained in Dulbecco's modified Eagle's medium (DMEM) (Lonza, Verviers, Belgium), supplemented with 10% FCS and 100 IU/ml sodium-penicillin, 100µg/ml streptomycin, 2 mM L-glutamine.

Human skin tissue was obtained after informed consent from patients undergoing corrective breast or abdominal plastic surgery at the VU University medical center (Amsterdam, The Netherlands), the Tergooi hospital (Hilversum, The Netherlands) or the Slotervaart hospital (Amsterdam, The Netherlands), following hospital guidelines. Human melanoma lymph node material was obtained with informed consent from clinically Stage I/II patients undergoing the sentinel lymph node procedure at the VU university medical center, without interference with pathological diagnostics and following hospital guidelines.

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### Viruses

All luciferase encoding Ad5.RGD, Ad5.pK7, Ad5.RGD.pK7, Ad5, Ad5/3, Ad5/11 and Ad5/35 vectors were E1- and E3-deleted<sup>12</sup> [Murakami *et al.*, manuscript submitted for publication]. Ad5/35.eGFP was a kind gift from Prof. A. Lieber (University of Washington, Seattle, USA) and was described previously.<sup>19</sup> Ad5.eGFP was cloned by homologous recombination of the linearized pAdtrack.CMV shuttle vector with pAdEasy transfected BJ5183 cells. Correct homologous recombination was analyzed by PacI enzyme digestion. Virus was propagated in either HEK-293 or QBI-293A cells. For Ad5/3.eGFP production, homologous recombination of linearized pAdTrack.CMV was performed in BJ5183 cells co-transfected with pAdEasy 5/3 (a kind gift of Prof. M Yamamoto, DHGT, Birmingham, AL, USA). This homologous recombination gave rise to an eGFP-expressing, Ad5-based virus with the knob-domain of Ad3. Generated vectors were analyzed for transgene expression, amplified in T175-cm2 flasks, purified by double CsCl gradient ultracentrifugation, and dialyzed against PBS containing 5% sucrose. Viral titers were determined by spectrophotometry (Viral particles (Vp)/ml)<sup>20</sup> and functional titers in plaque forming units (pfu) were determined by limiting dilution titration following standard techniques on HEK-293 cells.

### Preparation of human skin and lymph node single cell suspensions

Full thickness skin layers (2-3mm), consisting of dermis and epidermis, were collected from healthy donor skin with a dermatome (Aesculap, Tuttlingen, Germany). Skin was incubated in 50ng/ml dispase II (Roche, Penzberg, Germany) for 20 minutes at 37°C, 5% CO<sub>2</sub> after which the epidermal and dermal layers were separated with tweezers. Epidermal sheets were incubated in 0.05% trypsin at 37°C for 15 min and resuspended in IMDM + 10% FCS. Dermal skin sheets were incubated in collagenase (200mg collagenase diluted in 26.6ml PBS and 6.6ml dispase II) for 90 minutes at 37°C. Single cell suspensions were made by filtering twice through 100µm sterile filters. Viable cells were counted by trypan blue exclusion. Skin single cell suspensions, taken up in IMDM with 5% human pooled serum (HPS), 100 IU/ml sodium-penicillin, 100µg/ml streptomycin and 2 mM L-glutamine, were activated with 100ng/ml GM-CSF and 10ng/ml IL-4 in T75 culture flasks for 24 hours prior to Ad infection.

Human sentinel LN single cell suspensions were prepared as described previously.<sup>21</sup> In short, LN were cut in half. From one half, 10 scrapes were made with a scalpel, rinsing the blade after each scrape in a tube filled with 15ml of 0.1% DNase and 0.14% collagenase A (Roche Nederland B.V., Woerden, The Netherlands). The cell suspension was then transferred to a sterile flask in a total volume of 30ml DNase/collagenase, after which the suspension was incubated in a 37°C waterbath on a magnetic stirrer for 45 minutes. The cell suspension was filtered through a 100µm cell sterile cell strainer and the flask and strainer were rinsed with 20ml IMDM with 10% FCS. Cells were centrifuged at 1500rpm, 4°C for 5 minutes, washed with 10ml IMDM with 10% FCS and taken up in 3ml IMDM with 10% FCS for viable cell count by trypan blue exclusion.

### Flow cytometric phenotypic analyses

DC were phenotyped using FITC- / PE- or APC-conjugated Mabs directed against: CD1a (1:25), DC-SIGN (1:10), CD86 (1:25), CD80 (1:25), CD40 (1:10) (PharMingen, San Diego, CA), CD14 (1:25), HLA-DR (1:25) (BD Biosciences, San Jose, CA), Langerin (CD207), CD83 (1:10) (Immunotech, Marseille, France) and CD34 (1:10) (Sanquin, Amsterdam, The Netherlands); 2.5 to 5·10<sup>4</sup> cells were washed in FACS buffer (PBS supplemented with 0.1% BSA and 0.02% Na<sub>2</sub>S<sub>2</sub>O<sub>3</sub>) and incubated with specific or corresponding control Mabs for 30 minutes at 4°C. The LN single cell suspension were stained with CD123-PerCP\_Cy5 (1:10), CD11c-APC (1:10), CD14-PerCP\_Cy5 (1:10), CD1a-PE (1:25), CD19-PE (1:25), CD3-APC (1:10) (BD Biosciences, San Jose, CA) and BDCA2-PE (1:25) (Miltenyi, Bergisch Gladbach, Germany) antibodies. Cells (1-2·10<sup>5</sup>) were analyzed on a FACS-Calibur flow cytometer (Becton and Dickinson, San Jose, CA) equipped with CellQuest analysis software.

### In vitro Ad transduction

*In vitro* generated MoDC, MUTZ3-LC, or GM-CSF + IL-4 activated skin single cell suspensions were *in vitro* transduced with the indicated adenoviruses at a MOI of 1000 viral particles (Vp). 0.05-0.1 million cells were generally used per transduction and transductions were performed in duplicate or triplicate. 250µl of cell suspension in serum free DMEM/ F12 (Cambrex, Verviers, Belgium), supplemented with 100 IU/ml sodium-penicillin, 100µg/ml streptomycin and 2 mM L-glutamine was seeded in either 24-well plates (Co-star) or 4PP-tubes (Greiner). 250µl of Ad-suspension in serum free DMEM/F12 was added to the cells and plates or tubes were incubated at 37°C and 5% CO<sub>2</sub> for 3 hours. 1ml of complete culture medium, depending on the cell type, was added after these 3 hours. For luciferase transgene read-outs, cells were harvested 24 hours after transduction and pellets were lysed in 100µl 1x reporter lysis buffer (Promega, Madison, WI) and frozen at -80°C. Lysates were thawed and 10µl lysate

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was added to 50 $\mu$ l luciferase substrate (Promega, Madison, WI) and luciferase activity was measured on a luminometer. LN single cell suspensions were transduced with a MOI of 1000 (based on approx. 5% DC (including myeloid and plasmacytoid subsets) within the total cell population). 1-2 million cells were transduced, as described above. For eGFP read-out, cells were harvested 48 hours after transduction and were analyzed for eGFP expression in conjunction with PE-, PerCP\_Cy5 or APC-labeled markers on a FACS-Calibur flowcytometer as described above.

For the CD80 and CD86 blocking experiments, mature MoDC or migrated skin DC were incubated with 10  $\mu$ g/ml control-Ig or CTLA4-Ig (kind gifts from Dr. L. Chen, Ann Arbor, MI) for 1 hour on ice. Cells were washed to remove excess fusion-proteins and were transduced with Ad5/35.eGFP or Ad5/3.eGFP (MOI 1000) as described above.

### In situ Ad transduction of human skin DC

6mm human skin biopsies, pre-activated with GM-CSF and IL-4, were injected with  $1 \times 10^9$  Vp of Ad5.eGFP, Ad5/35.eGFP or Ad5/3.eGFP in a total volume of 10 $\mu$ l as described previously.<sup>22</sup> Cells were allowed to migrate from the biopsies for 48 hours, after which they were harvested, quantified by trypan blue exclusion and flowcount fluorospheres, and analyzed for eGFP expression and DC marker expression by flow cytometry.

### Mixed leukocyte reaction (MLR)

$1 \cdot 10^2$  –  $3 \cdot 10^4$  migrated skin DC, *in situ* or *in vitro* transduced with Ad5.eGFP, Ad5/35.eGFP or Ad5/3.eGFP, were co-cultured with  $1 \cdot 10^5$  peripheral blood lymphocytes for 4 days in 96-wells plates in IMDM containing 10% HPS, 100 IU/ml sodium-penicillin, 100 $\mu$ g/ml streptomycin and 2 mM L-glutamine and 50  $\mu$ M 2ME. At day 4, 2.5 $\mu$ Ci/ml [<sup>3</sup>H]-thymidine (6.7Ci/mmol, MP Biomedicals, Irvine, CA) was added per well for 16 hours. Plates were harvested onto glass fiber filtermats (Packard Instruments, Groningen, The Netherlands) using a Skatron cell harvester (Skatron Instruments, Norway), and [<sup>3</sup>H]-thymidine incorporation was quantified using a Topcount NXT Microbetacounter (Packard, Meriden, CT).

### Statistical analysis

The paired two-tailed student's T-test was used for statistical analysis, except for analysis of DC-targeting indices, where a non-parametric analysis was used instead (two-tailed Wilcoxin) due to a lack of a normal distribution. Differences were considered statistically significant when  $p < 0.05$ .

## Results

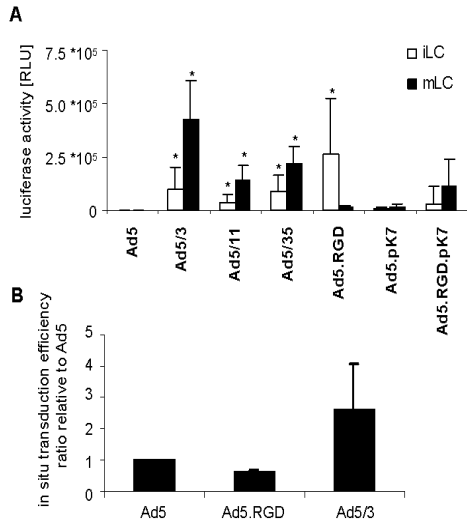
### *In vitro* cultured DC are efficiently transduced by subgroup C/B chimeric- and fiber-modified viruses

Seven replication-deficient adenoviruses (Ad) with embedded distinct tropism profiles were analyzed for their capacity to infect LC, as the major skin-resident DC subset. To this end we employed immature and mature *in vitro* cultured LC derived from the CD34<sup>+</sup> acute myeloid leukemia-derived cell line MUTZ3.<sup>17,23</sup> The viruses tested in comparison to the subgroup C virus Ad5 (Ad5) included three subgroup C/B chimeras, i.e. Ad5/3, Ad5/11, Ad5/35 and three fiber-modified viruses, i.e. Ad5.RGD, Ad5.pK7 and Ad5.RGD.pK7.<sup>12</sup> All viruses contained a luciferase reporter gene under a cytomegalovirus (CMV) promoter. The Ad5.RGD, Ad5.pK7 and Ad5.RGD.pK7 viruses in addition contained a GFP reporter gene driven by a CMV promoter. To compare the data with the C/B chimeras, luciferase expression was used as reporter assay. An isogenic Ad5 with double-reporter genes was included as a control for the RGD and pK7 viruses (data not shown).

The type C/B chimeras gave higher transgene expression levels than Ad5 in LC, with the highest levels in mature LC (mLC) [Figure 1A]. Ad5/3 infection was specifically more effective in mLC than in iLC ( $p < 0.002$  for iLC vs mLC and  $p < 0.003$  compared to mLC transduction levels by Ad5/11 or Ad5/35). Viruses containing the RGD motif only showed enhanced transduction efficiency compared to Ad5 in immature LC (iLC) ( $p < 0.004$ ). No advantage was observed with the pK7 insert alone or in combination with RGD, compared to Ad5 infectivity of iLC or mLC. Similar results were obtained with *in vitro* cultured interstitial DC (IDC) generated from human monocytes

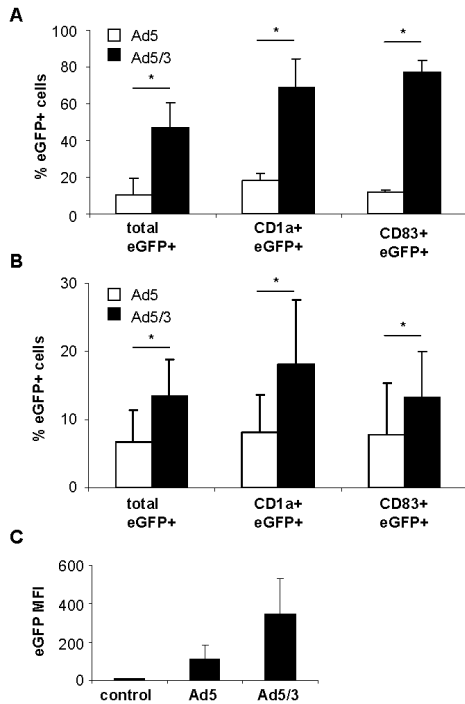
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(data not shown). Based on the fact that they induced significantly higher transgene levels compared to Ad5, Ad5.RGD and Ad5/3 were selected for further studies in human skin.



**Figure 1: In vitro transduction of immature and mature LC with different fiber-modified Ad5 viruses**

**A)** Luciferase expression levels in immature and mature MUTZ-3-derived LC (iLC (white bars) and mLC (black bars)) 24h after infection with type C/B chimeric adenoviruses and fiber-modified viruses at an MOI of 1000 Vp per cell. \*  $p < 0.05$  for modified/chimeric vs. Ad5 (n=3). **B)** *in situ* human skin DC transduction efficiency ratio relative to Ad5-mediated human skin DC transduction for Ad5.RGD and Ad5/3 (n=3).



**Figure 2: Ad5/3 mediates enhanced transduction of cutaneous DC in vitro and in situ.**

Transduction efficiencies upon **A)** *in vitro* infection of migrated skin DC or **B)** *in situ* infection of skin DC with Ad5 and Ad5/3, represented by the percentage of eGFP+ cells. The total percentage of eGFP+ cells, as well as the percentage of CD1a+eGFP+ and CD83+eGFP+ cells are shown. **C)** eGFP transgene expression levels in the total population of migrated human cutaneous DC after Ad5 or Ad5/3 transduction (n=3). P values obtained by two-tailed, paired Student's T test. MFI; mean fluorescence intensity.

## Selective transduction of mature DC in human skin and LN by CD80/CD86-targeted fiber-modified Ad5/3

To study the percentage of infected DC in conjunction with DC surface marker expression, enhanced GFP (eGFP)-encoding Ad5 and Ad5/3 viruses were constructed for further studies. Ad5.RGD and Ad5/3 were analyzed for their capacity to target and transduce human skin DC *in situ* compared to Ad5 control viruses. Intra-dermal (i.d.) injection of Ad5.RGD did not enhance the percentage of GFP-positive migrated human skin DC compared to injection of Ad5. In contrast, i.d. injection of Ad5/3 resulted in a marked enhancement of the transduction efficiency of migrated human skin DC compared to Ad5. Figure 1B shows the transduction efficiency ratios for *in situ* skin DC transduction relative to Ad5 for both Ad5.RGD and Ad5/3 (n=3). In light of its superior performance Ad5/3 was further investigated.

### ***In vitro* and *in situ* skin DC infection: Ad5/3 superior to Ad5**

Pre-conditioning of human skin with GM-CSF and IL-4 leads to strong activation of skin resident DC.<sup>24</sup> Since the *in vitro* infection data showed enhanced luciferase expression levels in mature LC compared to immature LC after transduction with Ad5/3 [Figure 1A], human abdominal skin was pre-conditioned with the cytokines GM-CSF and IL-4 prior to Ad injection. For the *in vitro* Ad transduction of migrated human skin DC, *in situ* GM-CSF and IL-4 activated and subsequently migrated cells over a period of two days, were harvested and transduced *in vitro* with Ad5 or Ad5/3. The transduction efficiencies, represented by the percentage of eGFP-positive cells are shown in figure 2A. Ad5/3 transduced significantly more of the total ( $p < 0.04$ ), CD1a<sup>+</sup> ( $p < 0.03$ ) and CD83<sup>+</sup>-i.e. mature- ( $p < 0.04$ ) skin-emigrated DC as compared to Ad5. No further activation of the DC was observed upon transduction with either of the adenoviruses (data not shown). Enhanced *in situ* transduction capacity by Ad5/3 over Ad5 was observed, as shown in figure 1B. To more closely look at the subsets of DC transduced by Ad5/3 during *in situ* targeting of human skin explants, we gated either on the total DC population based on scatter profiles, the cells expressing the DC marker CD1a, or the maturation marker CD83 [Figure 2B]. Additional gating was performed on CD80 and CD86 expressing cells (data not shown). Significantly more eGFP<sup>+</sup> cells were present after Ad5/3 injection as compared to Ad5 injection within all groups ( $p < 0.05$ ). Figure 2C shows the transgene expression levels in the *in situ*-transduced migrated skin DC. Although not significantly so (most likely due to inter-experimental variation), transduction of human skin DC with Ad5/3 led to considerably increased transgene expression levels as compared to Ad5 (n=3).

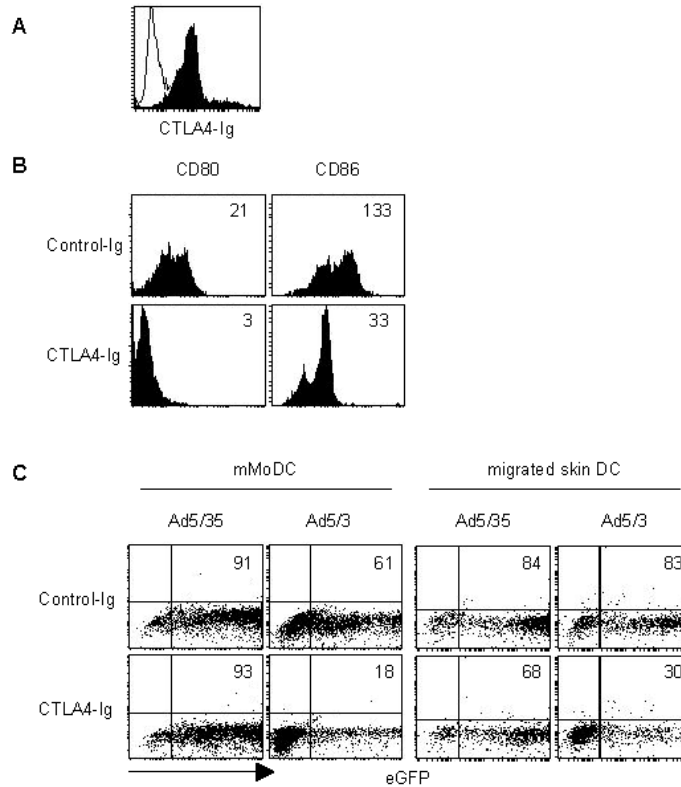
### **Ad5/3 binds human skin DC primarily via CD80 and CD86**

As Ad5/3 was reported to bind the B7 family members CD80 and CD86 on *in vitro* generated human monocyte-derived DC (MoDC),<sup>8,25</sup> but has also been reported to bind CD46<sup>14,16</sup>, we decided to examine the targeted surface molecules by Ad5/3 on human skin-DC. Monocyte-derived DC (MoDC) and skin-emigrated DC were incubated with a control-Ig or CTLA4-Ig fusion protein, the latter to prevent CD80 and CD86 binding by the virus. As a control, Ad5/35 was taken along, as this subgroup B virus is believed to transduce MoDC through binding of CD46<sup>26</sup> -although binding to CD80 and CD86 has also been reported.<sup>25</sup> Figure 3A shows control-Ig and CTLA4-Ig binding to mature MoDC. All CD80 molecules were blocked after CTLA4-Ig incubation of MoDC, as a PE-labeled anti-CD80 antibody was unable to bind the cells [Figure 3B]. In contrast, binding of a PE-labeled anti-CD86 antibody was reduced, but not completely blocked after CTLA4-Ig treatment [Figure 3B]. In the MoDC cultures, blocking of CD80 and CD86 binding sites did not affect Ad5/35 transduction of the cells (91% vs. 93% of the cells were transduced after control-Ig or CTLA4-Ig pre-incubation, respectively) [Figure 3C]. Among the skin-emigrated DC, some Ad5/35 transduction was blocked (20% reduction) by pre-incubating the cells with CTLA4-Ig (transduction efficiency was decreased from 84% to 68% after control-Ig or CTLA4-Ig incubation, respectively). Ad5/3 infection was reduced from 61% to 18% in control-Ig vs. CTLA4-Ig treated MoDC (70% reduction) [Figure



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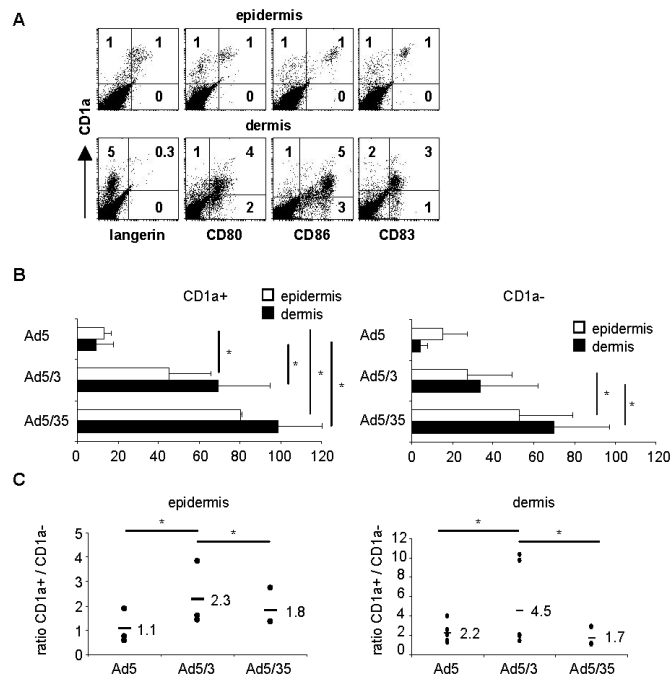
3C] and from 83% to 30% in control-Ig vs. CTLA4-Ig treated skin DC (64% reduction) [Figure 3C]. These data clearly show that Ad5/3 primarily uses CD80 and CD86 to bind human DC, whereas Ad5/35 does not.



**Figure 3: Ad5/3, but not Ad5/35, transduces MoDC and human skin DC through binding of CD80 and CD86**

**A)** Binding of control-Ig (open histogram) and CTLA4-Ig (closed histogram) fusion protein to mature MoDC, visualized with a FITC-labeled anti-IgG. **B)** *In vitro* cultured MoDC and migrated skin DC were incubated with either the control-Ig (top row) or the CTLA4-Ig fusion protein (bottom row) to block the CD80/CD86 binding-site. The ability of PE-labeled CD80 and CD86 Mabs to bind to the CD80 and CD86 surface molecules after control-Ig or CTLA4-Ig incubation are shown. **C)** Percentages of Ad5/3- or Ad5/35-transduced DC pre-incubated with control-Ig (top row) or CTLA4-Ig (bottom row) (data shown are representative of 3 experiments). Pre-incubation of the DC with CTLA4-Ig prevented transduction by Ad5/3, but not Ad5/35, in both MoDC and human skin DC.

Selective transduction of mature DC in human skin and LN by CD80/CD86-targeted fiber-modified Ad5/3



**Figure 4: Transduction of dermal and epidermal single-cell suspensions**

**A)** DC marker expression within CD1a<sup>+</sup> single-cell suspensions of human epidermis (top) and dermis (bottom) twenty-four hours after GM-CSF+IL-4 treatment. **B)** Transduction efficiencies for Ad5, Ad5/3 and Ad5/35, represented by the percentage of eGFP<sup>+</sup> cells within the CD1a<sup>+</sup> and CD1a<sup>-</sup> populations in both the epidermis and the dermis. White bars represent data obtained for epidermal single-cell suspension and black bars represent data obtained for dermal single-cell suspension and. In the epidermal samples Ad5/3 performed significantly better than Ad5 within the CD1a<sup>+</sup> cell subset ( $p < 0.03$ ), while Ad5/35 performed significantly better than Ad5 and Ad5/3 in all populations ( $p < 0.02$ ). In the dermal samples, Ad5/3 performed significantly better than Ad5 and Ad5/35 performed significantly better than Ad5 and Ad5/3 in all defined subpopulations ( $p < 0.05$  for eGFP<sup>+</sup> cells within the CD1a<sup>+</sup> and CD1a<sup>-</sup> populations). P values obtained by two-tailed, paired Student's T test. P-values for transduction efficiencies within CD1a<sup>+</sup> versus CD1a<sup>-</sup> subsets within the epidermis  $p=0.59$  for Ad5,  $p<0.0005$  for Ad5/3 and  $p<0.00004$  for Ad5/35 and within the dermis were  $p<0.003$  for Ad5,  $p<0.0006$  for Ad5/3 and  $p<0.05$  for Ad5/35. **C)** CD1a<sup>+</sup> DC-targeting indices (calculated by dividing the percentage of transduced cells within the CD1a<sup>+</sup> population by the percentage of transduced cells within the CD1a<sup>-</sup> population) are presented. Mean values are indicated. Indices for Ad5/3 were significantly higher than the corresponding indices for Ad5 and Ad5/35 in the epidermis and the dermis (both  $p<0.04$ ).

**Ad5/3 less efficient but more selective for mature skin DC than Ad5/35**

Since Ad5/3, unlike Ad5/35, was shown to bind to the DC-specific markers CD80 and CD86 in order to transduce human skin DC [Figure 3C], we analyzed whether this influenced the selectivity of these viruses for mature DC within human skin single cell suspension. Single cell suspensions were made from dermal or epidermal sheets to investigate the transduction specificity of Ad5, Ad5/3 and Ad5/35 for the DC populations within human skin. Figure 4A displays the percentages and expression levels of Langerin, CD80, CD86 and CD83 on CD1a<sup>+</sup> DC within epidermal- and dermal single cell suspensions, twenty-four hours after GM-CSF and IL-4 treatment. These GM-CSF + IL-4 activated, total cell suspensions were *in vitro* transduced with the different Ads.

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Transduction efficiencies were low for Ad5, whereas both Ad5/3 and Ad5/35 transduced CD1a<sup>+</sup> DC within the epidermal or dermal cell populations at significantly higher efficiencies than Ad5 [Figure 4B,  $p < 0.05$  for both Ad5/3 and Ad5/35 vs. Ad5], in keeping with our previous observations for Ad35.<sup>4</sup> While Ad5/35 in turn was more efficient in transducing LC and dermal DC than Ad5/3, it was also less specific for mature skin DC than Ad5/3, as significantly more CD1a<sup>-</sup> bystander cells were infected by Ad5/35 ( $p < 0.001$  or  $p < 0.01$  for CD1a<sup>+</sup> and CD1a<sup>-</sup> dermal and epidermal cells, respectively) [Figure 4B]. As there are far more CD1a<sup>-</sup> cells within human skin compared to CD1a<sup>+</sup> DC, the high transduction efficiency of Ad5/35 for these bystander cells makes this virus much less selective for DC. In figure 4C the DC-targeting index (% transduced cells of the CD1a<sup>+</sup> population divided by % transduced cells of the CD1a<sup>-</sup> population) is shown for the three viruses within the epidermal and dermal single-cell suspensions. While CD1a<sup>+</sup> DC-targeting with Ad5/3 was more efficient in the dermal than in the epidermal cell suspensions, the DC-targeting index for Ad5/3 was significantly higher than the index for Ad5 or Ad5/35 in both the epidermal and dermal cell suspensions ( $p < 0.04$ ).

### Specifically enhanced transduction efficiency by Ad5/3 of mature myeloid DC in melanoma skin-draining LN

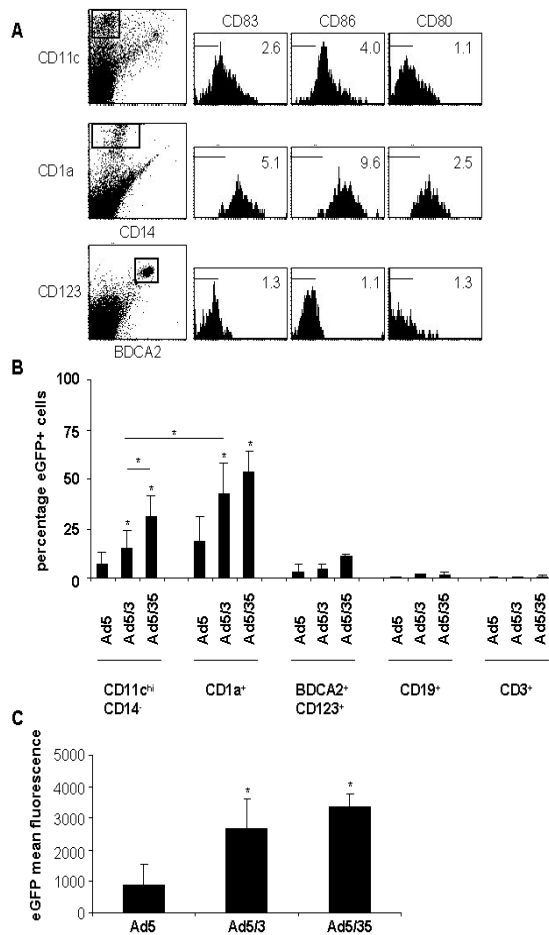
As the size of adenoviruses (approx. 80 nm) may allow for direct diffusion to draining LN upon i.d. injection<sup>27</sup>, DC selectivity and transduction efficiency were also determined for Ad5, Ad5/3 and Ad5/35 in *ex vivo* LN suspensions. We made use of first-line skin tumor-draining LN (so-called Sentinel Lymph Nodes [SLN]), from clinically Stage I/II melanoma patients. This is a particularly relevant model in view of our intent to use these Ad vectors as tumor vaccine vehicles. We previously showed CD11c<sup>+</sup> resident and CD1a<sup>+</sup> skin-derived myeloid DC subsets within these melanoma SLN to have a mature CD83<sup>+</sup> phenotype<sup>28,29</sup> and we therefore hypothesized them to be susceptible for Ad5/3 and Ad5/35 infection. Five different leukocyte subsets were analyzed, i.e. CD11c<sup>hi</sup>CD14<sup>-</sup> LN-resident myeloid DC<sup>29</sup>, skin-derived CD1a<sup>+</sup> myeloid DC, CD123<sup>+</sup>BDCA2<sup>+</sup> plasmacytoid DC (pDC), CD19<sup>+</sup> B cells, and CD3<sup>+</sup> T cells. Figure 5A shows typical dot plots (left) of the three different DC subsets within the LN and the gates used for flow cytometric marker analysis. The histogram plots show CD83, CD86 and CD80 expression levels within the CD11c<sup>hi</sup>CD14<sup>-</sup> and CD1a<sup>+</sup> myeloid DC populations and the CD123<sup>+</sup>BDCA2<sup>+</sup> pDC population. The median fluorescence indices are listed in the upper right corners. The percentage of eGFP<sup>+</sup> cells within the CD11c<sup>hi</sup>CD14<sup>-</sup>, CD1a<sup>+</sup>, CD123<sup>+</sup>BDCA2<sup>+</sup>, CD19<sup>+</sup> and CD3<sup>+</sup> subsets are shown in figure 5B ( $n=3$  for the myeloid DC subsets and  $n=2$  for pDC, T- and B-cells). Ad5/3 was significantly better than Ad5 in transducing the myeloid DC subsets ( $p < 0.03$  and  $p < 0.01$  for CD11c<sup>hi</sup>CD14<sup>-</sup> and CD1a<sup>+</sup> DC, respectively). Ad5/35 transduced significantly more CD11c<sup>hi</sup>CD14<sup>-</sup> cells as compared to Ad5 ( $p < 0.01$ ) or Ad5/3 ( $p < 0.02$ ) and more CD1a<sup>+</sup> DC than Ad5 ( $p < 0.002$ ). Ad5/35 transduced slightly more pDC compared to Ad5 or Ad5/3. In the two donors tested, hardly any B- or T cells were transduced [Figure 4B]. We showed that the Ad5/3 required binding to the co-stimulatory molecules CD80 and CD86 on human skin DC in order to transduce the cells [Figure 3C].<sup>8</sup> In line with this and in conjunction with higher expression levels of CD86 and CD80 on CD1a<sup>+</sup> DC as compared to CD11c<sup>hi</sup>CD14<sup>-</sup> DC present within the LN [Figure 5A], there was a significantly better transduction of CD1a<sup>+</sup> DC by Ad5/3 ( $p < 0.02$ ) and virtually no transduction of the CD80<sup>-</sup>CD86<sup>-</sup> immature pDC [Figure 5A and 5B]. Of note, both Ad5/3 and Ad5/35 transduction of CD1a<sup>+</sup> DC within the SLN resulted in significantly enhanced transgene expression levels as compared to Ad5-mediated transduction [Figure 5C].

### Ad5/3-mediated transduction does not alter the T-cell stimulatory capacity of skin DC

It was previously shown that CD46 surface expression was down-regulated upon Ad35 binding and did not recover for up to 96 hours after removal of the virus.<sup>30</sup> If a similar down-regulation of CD80/CD86 occurs or if CD80/CD86 are effectively blocked upon Ad5/3 binding, this may confound T cell activation by Ad5/3-targeted DC

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and disqualify this virus for vaccination purposes. Therefore, an allogeneic mixed leukocyte reaction (MLR) was performed with migrated, *in situ* transduced skin DC to show that binding of Ad5/3 did not interfere with the capacity of the DC to induce T cell proliferation. Figure 6A shows that i.d. transduction with Ad5 or Ad5/3 did not reduce the expression levels of either CD80 or CD86 on subsequently human skin-emigrated DC (48h after Ad injection). In addition, there was no difference in DC-induced T cell proliferation using these DC in an allogeneic MLR (Fig. 6B) or indeed, using skin-emigrated DC which were transduced *in vitro* with Ad and added to MLR cultures 4h later (data not shown). Finally, we also investigated the effect of the different Ad types on cytokine release by DC. Supernatants were harvested from skin-migrated DC two days after i.d. Ad transduction or from MoDC, 24 hours after transduction. No significant alterations in IL12p70, TNF $\alpha$ , IL-1 $\beta$ , IL-6, IL-8 or IL-10 levels were observed in a flow cytometric cytokine bead array, although there was a reduction in IL-10 levels after Ad5/3 transduction in both skin DC and MoDC samples (data not shown), similar to our previous findings for Ad5/35.<sup>4</sup> Thus, transduction of DC by Ad5/3 through binding of CD80 or CD86 does not hamper DC-induced T cell activation or DC cytokine release.



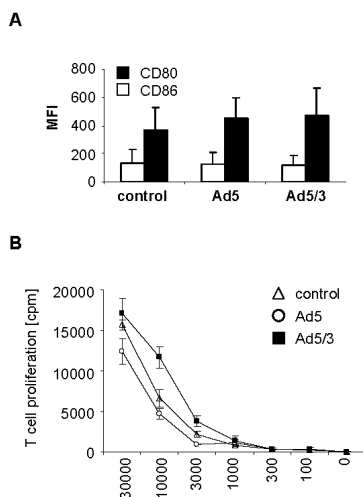
**Figure 5: Transduction of single-cell suspensions derived from human melanoma-draining Sentinel Lymph Nodes (SLN) by Ad5, Ad5/3 and Ad5/35**

(A) Dot plots (left) showing gating of the different DC subsets within the SLN, i.e. CD11c<sup>hi</sup>CD14<sup>-</sup> and CD1a<sup>+</sup> myeloid DC subsets and the CD123<sup>+</sup>BDCA2<sup>+</sup> pDC subset. The histogram plots (right) show the CD83, CD86 and CD80 levels expressed on the respective DC subsets indicated by the gates in the dot plots. Median fluorescence indices (median of the specific marker divided by the median value of the isotype control) are indicated in the upper right corners. The position of the isotype controls are indicated in the plots by markers. (B) The percentages of transduced myeloid CD11c<sup>hi</sup>CD14<sup>-</sup> DC and CD1a<sup>+</sup> DC, pDC (CD123<sup>+</sup>BDCA2<sup>+</sup>), B cells (CD19<sup>+</sup>) and T cells (CD3<sup>+</sup>) are shown (n=3 for CD11c<sup>hi</sup>CD14<sup>-</sup> and CD1a<sup>+</sup> DC and n=2 for pDC, B cells, T cells). Ad5/3 and Ad5/35 transduced significantly more CD11c<sup>hi</sup>CD14<sup>-</sup> myeloid DC than Ad5 (\* p < 0.03 and p < 0.01, respectively). Ad5/3 and Ad5/35 transduced significantly more CD1a<sup>+</sup> DC compared to Ad5 (\* p < 0.01). Ad5/3 transduced significantly more CD1a<sup>+</sup> DC than CD11c<sup>hi</sup>CD14<sup>-</sup> DC (p < 0.02). (C) Transgene expression levels after transduction in the CD1a<sup>+</sup> myeloid DC population are shown. Transgene levels were significantly higher in Ad5/3 (p < 0.05) and Ad5/35 (p < 0.02) transduced cells as compared to Ad5-transduced cells.

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### Discussion

The aim of this study was to examine in detail whether Ad5/3, which was previously shown to be more efficient than Ad5 in transducing human MoDC due to binding to the co-stimulatory molecules CD80 and CD86, was also efficient in transducing human cutaneous DC *in situ*. This would make Ad5/3 particularly suitable for immunotherapeutic vaccination purposes. We showed that from a panel of seven modified adenoviruses, Ad5/3 most efficiently transduced *in vitro* generated mature MUTZ3-LC. Similar as in human MoDC, Ad5/3 specifically bound human emigrated skin DC through interaction with the B7-molecules CD80 and CD86 and was capable of efficient transduction of mature human skin IDC and LC both *in vitro* and *in situ* and of human LN DC *ex vivo*. Next to Ad5/3, the fiber-modified Ad5.RGD displayed significantly enhanced transduction over Ad5 of, predominantly immature, IDC (data not shown) and LC *in vitro*. However, unlike with *in vitro* generated MoDC and LC, Ad5.RGD performed far less efficiently in the *ex vivo* skin explant system, showing no benefit over the use of unmodified Ad5. Ad5.RGD was previously shown to efficiently transduce primary human keratinocytes and fibroblasts.<sup>31</sup> Possibly, the majority of i.d. injected Ad.RGD viral particles transduced keratinocytes and fibroblasts within the explant, reducing the chances of effective DC transduction. We conclude therefore that viruses with sole RGD-modifications are not suitable for human DC-targeting strategies based on intradermal delivery.



**Figure 6: Ad5/3 transduction does not hamper DC-induced allogeneic T cell proliferation**

*In situ* Ad5 and Ad5/3 transduction of skin DC had no effect on expression levels of CD80 or CD86 and did not affect the T cell stimulatory capacity of subsequently skin-emigrated DC. **A)** CD80 and CD86 mean fluorescence intensities on skin-emigrated DC, 48h after i.d. injection and *in situ* transduction with Ad5 or Ad5/3 (n=3). **B)** DC were *in situ* transduced by Ad5 or Ad5/3 and were used upon skin emigration in an MLR two days after Ad injection (data from one representative experiment out of two are shown).

Since Ad5/3, and not Ad5/35, used the B7 molecules CD80 and CD86 to bind to and transduce human skin DC, we examined whether this would result in enhanced DC-targeting specificity of Ad5/3 over Ad5/35. We had previously identified Ad5/35 as an efficient DC-targeting adenovirus.<sup>4</sup> Comparing Ad5/3 and Ad5/35 with each other and with Ad5 in the selective targeting of DC within human epidermal and dermal single cell suspensions, Ad5/3 displayed enhanced specificity for DC, as less CD1a<sup>-</sup> non-DC bystander cells within human skin cell suspensions were transduced by Ad5/3 compared to Ad5/35. Ad5/3 was on average 2.5-fold more targeted than Ad5/35 in dermal cell suspensions, as determined through comparing the index of CD1a<sup>+</sup> transduction efficiency vs CD1a<sup>-</sup> transduction efficiency, making Ad5/3 more selective for mature human skin DC than Ad5/35. This might reduce the chances of any untoward side effects associated with bystander infection,

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such as cytopathic effects on keratinocytes or stromal cells, or suboptimal T cell activation or tolerance induction through preferential transduction of non-professional over professional antigen-presenting cells.

As intradermally delivered Ad viruses may also drain directly to LN and infect DC populations there, we decided to study transduction of single-cell suspensions derived from skin-draining LN. In single-cell suspensions of melanoma-draining SLN, Ad5/35 and Ad5/3 showed significantly enhanced transduction of both CD11c<sup>hi</sup>CD14<sup>-</sup> and CD1a<sup>+</sup> myeloid DC subsets as compared to Ad5. A low percentage of pDC (CD123<sup>+</sup>BDCA2<sup>+</sup>) were transduced by Ad5/35 but none by Ad5/3. This is in keeping with a previous report showing Ad35 to infect both human myeloid and plasmacytoid DC, but myeloid DC with a higher efficiency.<sup>32</sup> Neither of the viruses transduced B- or T cells in the LN samples, confirming their relative specificity for DC and precluding the possibility of B cell-mediated antigen presentation which was previously implicated in CD8<sup>+</sup> T cell tolerance induction.<sup>33</sup> The observed DC transduction efficiencies for Ad5/3 correlated with the detected CD80/86 expression levels on the respective LN-DC subsets: high on CD1a<sup>+</sup> DC (which were transduced at a three-fold higher efficiency than the other CD11c<sup>hi</sup> DC), intermediate on the CD11c<sup>hi</sup>CD14<sup>-</sup> DC, and absent from pDC. As the levels of these important co-stimulatory molecules likely correspond to T cell stimulatory capacity, this may point to an attractive potential of Ad5/3 viruses to target the most potent T cell stimulatory DC subsets. Unlike the CD11c<sup>hi</sup>CD14<sup>-</sup> DC, which may mostly consist of LN-resident DC with an unknown capacity for T cell activation, we previously showed that CD1a<sup>+</sup> DC (with intermediate CD11c expression levels -data not shown) in these melanoma-draining LN most likely correspond to LC and dermal DC migrating from the skin.<sup>21</sup> Upon i.d. GM-CSF administration, frequencies of these CD1a<sup>+</sup>CD83<sup>+</sup> DC in the melanoma SLN correlated to melanoma-specific CD8<sup>+</sup> T cell rates<sup>34</sup> and we recently confirmed the capacity of both mature CD1a<sup>+</sup> epidermis- and dermis-emigrated DC to prime CD8<sup>+</sup> T cells specifically recognizing the melanoma-associated antigen MART-1 *in vitro*.<sup>35</sup> Thus, preferential transduction of this skin-derived DC subset by Ad5/3 may be conducive to the subsequent priming of TAA-specific T cells. The fact that we observed this preferential and enhanced CD1a<sup>+</sup> DC transduction by Ad5/3 in melanoma-conditioned SLN bodes well for its possible use as vaccine vehicle in cancer patients.

As many contradictory reports can be found in literature with respect to the attachment receptors of subgroup B viruses<sup>14-16,25</sup>, we analyzed whether Ad5/3 and Ad5/35 both used the B7-molecules CD80 and CD86 to bind target cells. Ad5/3, and not Ad5/35, was shown to primarily use these co-stimulatory molecules to bind human DC, as blockade of the binding sites of these molecules by means of pre-incubation with a CTLA4-Ig fusion protein, drastically reduced MoDC and skin DC transduction by Ad5/3 by 60-70%. This preference of Ad5/3 for CD80 and CD86 can explain why it is more specific for mature DC and transduces less bystander cells than Ad5/35, which was previously shown to transduce DC through CD46.<sup>6,26</sup> Data by DiPaolo *et al.* showed that a soluble CD46 (sCD46) could completely inhibit human DC transduction by Ad5/35, suggesting that CD80 and CD86 do not play a role during the transduction of human *in vitro* cultured DC by Ad5/35. Previously, Short *et al.* reported that all type B adenoviruses can bind CD80 and CD86.<sup>25</sup> Although their data suggest that Ad5/35 might be able to bind these molecules, our data indicate that Ad5/35 does not primarily use CD80 or CD86 to transduce human DC. From our data we cannot conclude that interaction of Ad5/3 with CD46 (or another surface molecule) is not involved in Ad5/3-based DC transduction, as the blockade of CD86 expression by the CTLA4-Ig was not complete. The remaining transduced DC after CTLA4-Ig treatment (18% in MoDC and 30% in skin DC) could thus occur through CD86 binding or binding of Ad5/3 to another surface receptor, for instance CD46. As different CD46 isoforms have been characterized,<sup>36</sup> it could be that Ad3, which is a subgroup B1 virus, and Ad35, which belongs to subgroup B2, bind different CD46 isoforms and that the isoform present on human DC is preferentially bound by Ad35.

High transduction efficiency and Ad-binding after retargeting should not interfere with DC functionality. CD46 surface expression was reported to be down-regulated upon binding of Ad35.<sup>30</sup> As CD80 and CD86

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expression are crucial for the activation of T cells by DC, and hence for the initiation of a proper (anti-tumor) immune response, the T cell stimulatory capacity of the DC after transduction by Ad5/3 needed to be addressed. Transduction with Ad5 or Ad5/3 did not reduce expression levels of CD80 or CD86 and had no effect on the capacity of the DC to induce T cell proliferation in an allogeneic MLR or on the secreted cytokine profile of the infected cells. These data thus indicate that Ad5/3 can be used to target mature human DC *in situ*, while preserving the capacity of the DC to migrate and subsequently stimulate T cells.

As Ad5/3 transduced less bystander cells in the skin compared to Ad5/35, there may be a reduced likelihood of causing unwanted side effects, but whether in a therapeutic setting one would benefit more from a virus with somewhat higher efficiency but lower specificity (Ad5/35) or vice versa (Ad5/3) remains to be further examined in relevant *in vivo* models. Based on data by Ophorst *et al.*, these comparative *in vivo* studies, or to that respect clinical trials, could best be carried out with Ad35- and Ad3-based vaccines rather than the respective Ad5 Fib35 or Fib3 chimeras, as they found that an Ad5/35 chimera could not circumvent Ad5-directed pre-existing immunity *in vivo* in BALB/c mice or non-human primates.<sup>37</sup> The same would likely hold true for an Ad5/3 chimera. Later studies by the same group showed that within a population of healthy blood donors, there was a far lower prevalence of anti-group B immunity than anti-Ad5 immunity.<sup>38,39</sup> In a murine vaccination setting, Ad35-based therapeutic vaccines were not affected by pre-existing anti-Ad5 immunity. Wu *et al.* showed that replacing the Ad5 hexon with an Ad3 hexon already prevented neutralization in mice with pre-existing anti-Ad5 immunity.<sup>40</sup> In addition, it would be interesting to analyze whether Ad3-based vaccines are hampered by anti-Ad35 immunity and vice versa. If there is no efficient cross-neutralization, Ad35 and Ad3 could be used in a prime-boost therapeutic strategy in hosts with pre-existing anti-Ad5 immunity. Ophorst *et al.* also showed that a prime-boost strategy with Ad5 and Ad35 alone already dramatically enhanced the generation of antigen-specific CD8<sup>+</sup> T cells in mice.<sup>38</sup> Similar to CD80/86-targeted Ad5/3, we previously showed the ability of CD40-targeted Ad5 (CD40-Ad) to target mature DC in the context of human skin.<sup>22</sup> With a panel of different, efficiently DC-targeting adenoviruses, e.g. Ad35, Ad3 and CD40-Ad, it may become feasible to design sequentially varying prime-boost regimens that facilitate the generation of an even more robust antigen-specific T cell response while circumventing anti-Ad immunity. This would certainly benefit future immunotherapeutic strategies.

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The authors have no financial conflict of interest to disclose.

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