# Activity of different vaccine-associated promoter elements in human dendritic cells

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**Abstract**

Vaccine design approaches that target dendritic cells (DC) aim at achieving high levels of transgene expression. Careful selection of the promoter element driving the foreign gene is therefore important. We have constructed adenovirus vectors carrying the gene for enhanced green fluorescent protein (eGFP) driven by three different promoters, CMV, CMV5 and Ubiquitin C (UbC) promoter, and analysed their activity in different populations of human DC, namely blood plasmacytoid (pDC) and myeloid DC (mDC), monocyte-derived DC (moDC), Langerhans (LC) and dermal type DC (dDC). Although the CMV5 promoter was more active than the other two promoters in the HeLa and 911HER cell lines, in human DC the highest level of transgene expression was seen with the CMV promoter. There was very low level eGFP expression in all cell types transduced with the UbC promoter. Highest eGFP expression levels were observed in moDC, cultured mDC and LC and the lowest levels in pDC. Expression of eGFP was augmented in all DC populations upon stimulation with CD40 ligand (CD40L). These findings demonstrate that the CMV promoter is the most effective of the three promoters tested in a range of different human DC populations.

Running title: Promoter activity in human dendritic cells

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## 1. Introduction

Vaccination is the most important public health measure for maintaining protection against infectious diseases. Immune responses, whether stimulated by vaccination or by infection, are initiated by dendritic cells that are found in large numbers at sites where pathogens enter the body. In the peripheral tissues DC are present as immature cells that are highly efficient in the capture of foreign antigens which they process into short peptides for presentation on MHC class I and II molecules to $CD8^+$ and $CD4^+$ T cells respectively [1]. The release of inflammatory molecules and/or the recognition of microbial conserved molecular motifs through pattern recognition receptors (PRR), particularly toll-like receptors (TLR), induce DC maturation and migration to the draining lymph node where they cluster and stimulate T cells [2].

Blood DC are divided into two major subpopulations, myeloid (mDC) and plasmacytoid DC (pDC), exhibiting different phenotypic and functional properties [3]. The precursors of both types are found in the blood constituting approximately 1% of blood mononuclear cells in humans. Myeloid DC can be identified by the expression of MHC class II DR and CD11c molecules and the absence of lineage markers associated with T, B, natural killer (NK) and monocytic cells. Precursors of pDC are identified by the absence of CD11c and lineage markers and by expression of HLA-DR and CD123, the interleukin 3 (IL-3) receptor. Plasmacytoid DC migrate directly from blood to secondary lymphoid tissue where they secrete large amounts of interferon $\alpha$ in response to virus infection or CpG DNA sequences that are recognised through TLR9 [4]. Blood precursors of mDC, on the other hand, migrate to peripheral tissues where their function is to intercept and process antigen from invading pathogens. Tissue mDC are further divided into Langerhans cells, dermal

and interstitial DC [5,6]. Langerhans cells are characterised by the expression of Langerin and Birbeck granules and located in the epidermal layer of the skin and in the superficial mucosal epithelial layer of the genital tract. Dermal DC and the related interstitial DC are found in the deeper dermal tissue of the skin and throughout other body tissues respectively, and typically express DC-SIGN, CD1a and CD11b. Langerhans and dermal/interstitial DC are reported to differ in their biological properties with only dermal/interstitial DC being able to secrete IL-10, cluster B cells and mediate immunoglobulin class switching whereas Langerhans cells were found to more potently stimulate CTL responses [7,8]. For experimental and therapeutic studies DC have been isolated directly from blood, generated from $CD34^+$ stem cells by culture in appropriate growth factors and cytokines or derived by culturing monocytes for 5-7 days in the presence of GM-CSF and IL-4 [7,9,10].

There is currently significant interest in developing vaccines that stimulate $CD8^+$ cytotoxic T cells (CTL) to provide protection from persistent viral infections such as HIV and hepatitis C. Viral vectors are now widely used as vaccine agents to deliver and express pathogen genes in DC. It is thus important to select promoter elements that result in a high level of transgene expression in DC. Since different vaccination strategies may target different DC populations, efficient transgene transcription should be confirmed on the DC population that is targeted. A number of different promoters are being considered to drive expression of vaccine genes. The CMV promoter has been shown to lead to high levels of protein production in a large number of studies. The UbC promoter, whose native role is to drive the expression of the polyubiquitin C gene [11], has been shown to express transgenes in a wide range of tissues, with levels of expression comparable to those of CMV [12]. The CMV5 promoter, constructed by insertion of the adenovirus 5 major late promoter tripartite

leader sequence and splicing sites downstream of the transcription site of the CMV promoter, has been shown to express proteins at higher levels, a feature attributed to the dual nature of this promoter element [13-15]. In this report we have used adenovirus vectors carrying a reporter gene (e*gfp*) to measure the activity of these three commonly used promoters. For all DC populations tested, we have found that, although the CMV5 promoter stimulated higher levels of eGFP expression in cell lines, the CMV promoter performed the best whereas only low levels of transgene expression were seen with the UbC promoter.

## 2. Materials and methods

### 2.1. Bioinformatics analysis of the three promoter elements.

The nucleotide sequences of CMV, CMV5 and UbC promoters were obtained from sequences supplied for pAdTrack (Clontech), pAdenovator-CMV5-IRES-GFP (QBiogene) and pUb6V5/His(A) (Invitrogen) respectively and manipulated using Vector NTI viewer 4.0.1 (InforMax, Inc.) and BioEdit [16] software. Predicted transcription factor binding motifs were located on each respective sequence for both positive (+) and negative (-) DNA strands using the web-based matInspector program [17].

### 2.2. Plasmids

*2.2.1. pAdTrack*

pAdTrack plasmid vector (Clontech) carries the expression cassette (CMV-eGFP) from pEGFP-C1 (Clontech). This vector is described elsewhere [18].

*2.2.2. pShuttle-CMV5-GFP*

We constructed a new AdEasy "shuttle" vector, pShuttle-CMV5, and used it for generation of pShuttle-CMV5-GFP. CMV5 is a modified version of the human CMV immediate/early enhancer promoter, described elsewhere [14]. pShuttle-CMV5 was constructed through cohesive ligation of two fragments, derived from pAdenoVator-CMV5-IRES-GFP and pShuttle-CMV (Qbiogene). Appropriate amounts of both plasmids were digested with *Bsp*HI/*Bgl*II restriction endonucleases, run on a 0.8% TBE 1x agarose gel, gel-purified (BIO101 GeneClean kit), dissolved in de-ionised filter sterilised $H_2O$ at a concentration of 50 ng/μl and finally stored at -80$^{o}$C. The two fragments were ligated using the Rapid DNA Ligation Kit (Roche Diagnostics). The ligation products were used to transform DH5α *E Coli* and the bacteria were streaked on Kanamycin (50μg/ml) Luria-Bertani (LB) agar plates.

Selected colonies were grown in LB growth medium supplemented with Kanamycin as before and the DNA was extracted and purified using the Qiagen Spin Miniprep Kit. Construct integrity was verified through restriction digests. Subsequently, pShuttle-CMV5-GFP was constructed through cohesive ligation of the e*gfp* gene from pEGFP-N3 (Clontech) and pShuttle-CMV5 using *Kpn*I/*Not*I restriction enzymes. Colony isolation and DNA preparation and characterisation were carried out as previously described.

*2.2.3. pAdTrack-UbC-GFP construction*

pAdTrack-UbC-GFP was constructed by substitution of the CMV promoter in pAdTrack, with the UbC promoter from pUb6/V5-His(A) vector (Invitrogen). Five μg of each vector were digested with *Bgl*II/*Age*I and run on a 0.8% TBE 1x agarose gel. The fragments representing the pAdTrack plasmid backbone and the UbC promoter were isolated, purified and subsequently ligated as previously described. Analysis of the derived constructs was carried out using appropriate restriction enzymes.

**2.3. Viruses**

*2.3.1. Virus construction, purification and storage (Ad1-CMV-GFP, Ad1-CMV5-GFP, Ad1-UbC-GFP)*

In our studies we have used a 1st generation rAd plasmid, termed pAdEasy-1, with deletions in the E1 and E3 regions. A complementary cell line, 911HER (Human Embryonic Retinoblastoma), stably transformed with the adenovirus deleted genes was used for virus propagation. We modified the traditional AdEasy system [18] in order to achieve higher numbers of "positive" clones during the homologous recombination step as described elsewhere [19]. The generated bacteria carrying the adenoviral genome plasmid pAdEasy-1 were named BJ5183-pAdEasy-1.

Three hundred ng of *Pme*I-digested pAdTrack, pShuttle-CMV5-GFP and pAdTrack-UbC-GFP vectors were used to transform BJ5183-pAdEasy-1 bacteria using electroporation. TOP10F' *E Coli* bacteria were transformed with plasmid extracted from positive clones of transformed BJ5183-pAdEasy-1 cells, grown in the presence of Kanamycin and DNA was extracted and analysed through restriction digests (*Pac*I and *Bst*XI).

911HER cells were used for the propagation of the viruses as described in the AdEasy protocol. Cells were grown in Dulbecco's Modified Eagles Medium (DMEM) supplemented with 10% Foetal Calf Serum (FCS) (Invitrogen, UK), 100 IU/mL Penicillin, 0.1 mg/mL Streptomycin and 2 mM L-Glutamine, all from Sigma Aldrich, Poole UK. The Adenopure adenovirus purification kit (Puresyn Inc) was used for purification of the three viruses, according to the manufacturer's instructions, from five 175 $cm^2$ infected flasks, exhibiting total cytopathic effect (CPE). Viruses were aliquoted in 50 μl aliquots and stored at -20$^oC$.

*2.3.2. Virus titration*

HeLa cells were seeded in 24-well plates at equal numbers and cultured overnight in DMEM supplemented with 10% FCS, Penicillin/Streptomycin and L-Glutamine, at 37$^oC$ and 5% $CO_2$. Different dilutions of each viral preparation were prepared in DMEM 10% FCS. Cells were infected at a minimum volume of 100 μl of medium for one hour and then 900 μl of medium were added to each well. The following day the cells were harvested and analysed by FACS. The viral titre estimation was based on the number of $eGFP^+$ cells for each viral dilution and was expressed as transducing units per ml.

## 2.4. Transfection experiments using the eGFP-expressing "shuttle" vectors in cell lines

*2.4.1. Transfection with Lipofectamine 2000*

911HER and HeLa cells were seeded at equal numbers in 24-well plates and cultured overnight. The following day, 1.5 μg of pAdTrack, pShuttle-CMV5-GFP and pAdTrack-UbC-GFP were complexed with Lipofectamine 2000 (LF2000) according to supplier's instructions. The DNA/Lipofectamine mixture was then applied to the cells (>90% confluency) with subsequent 48 hour culture at 5% $CO_2$ / 37$^oC$ in DMEM supplemented as previously stated. LF2000 alone was used as negative control. Following the incubation period, the cells were harvested, fixed using 4% paraformaldehyde (PFA) and read by FACS.

## 2.5. Infection of HeLa cells with rAds carrying different promoters

HeLa cells were seeded at equal numbers in a 24-well plate. The following day appropriate volumes of each adenovirus preparation containing an equal number of infectious virions ($10^5$ and 2 x $10^5$) were resuspended in 100 μl of DMEM supplemented as before, and added to the HeLa cell monolayer for one hour. Subsequently, 900 μl of medium were added to each well and the cells incubated at 37$^oC$/5% $CO_2$ for 48 hours. Monitoring through fluorescence microscopy was carried out at 24 and 48 hours. At that point, cells were harvested, fixed with 4% PFA and analysed by FACS.

## 2.6. Dendritic cell preparation

*2.6.1. Plasmacytoid DC*

Buffy coats were obtained from the UK National Blood Transfusion Unit, Colindale, London, UK. PBMC were isolated by centrifugation over Histopaque®-

1077 (Ficoll) (Sigma Aldrich), at 2000 rpm for 20 minutes at room temperature, and then separated into a low density fraction enriched in DC and monocytes by centrifuging over a 50% percoll gradient for 30 min at 300g. Plasmacytoid dendritic cells were isolated from the low density fraction using BDCA-4 immunomagnetic beads (Miltenyi) and two positive selection columns (LS followed by MS) according to manufacturer's instructions. DC were cultured in round-bottomed 96-well plates in $NaHCO_3$ buffered RPMI-1640 containing 10% heat inactivated FCS, 100 IU/ml penicillin, 0.1mg/ml streptomycin and 2mM L-glutamine (all from Sigma-Aldrich, Poole, UK). Purified pDC culture medium was supplemented with 10ng/ml IL-3.

*2.6.2. Blood Myeloid DC*

After depletion of pDC, immunomagnetic beads (Miltenyi Biotec) were used to remove CD19-expressing B cells from the percoll interface fraction. Myeloid dendritic cells were then isolated using CD1c immunomagnetic beads (BDCA-1) and two MS selection columns. Purified mDC were cultured with 100ng/ml GM-CSF (Immunex, USA) and 1,000 IU/ml IL-4 (R&D).

*2.6.3. Monocyte-derived DC (moDC)*

Monocytes were isolated from percoll interface cells depleted of pDC and mDC by culturing in gelatin-coated flasks [20] for 2h at 37°C. After washing to remove non-adherent cells, adherent monocytes were cultured for seven days in medium supplemented with IL-4 and GM-CSF as described elsewhere [21].

*2.6.4. Langerhans and Dermal DC from $CD34^+$ cells*

Permission for the use of umbilical cord blood was given by the local ethics committee. Umbilical cord blood samples were obtained from consented mothers in full-term labour. $CD34^+$ cells were isolated using magnetic beads (Miltenyi Biotech) and cultured in complete RPMI-1640 with 10% FCS supplemented with 100ng/ml of

GM-CSF, 100ng/ml tumour necrosis factor alpha (TNF-α), 25ng/ml Stem Cell Factor (SCF), 25ng/ml Flt-3L all from R&D (R&D Systems Abingdon, UK). After 5 days two distinct populations were present, $CD14^+$ $CD1a^-$ and $CD14^-$ $CD1a^+$. $CD14^+$ cells were removed using magnetic beads (Miltenyi Biotech) and cultured independently for a further five to seven days in GM-CSF (100ng/ml) and IL-4 (1,000 units/ml) differentiating into dDC. The remaining cells were cultured for the same period in complete media supplemented with 100ng/ml GM-CSF, TNFα (100ng/ml) and 1ng/ml of transforming growth factor beta (TGF-β) (R&D) before Langerhans cells were finally isolated based on their expression of CD1a using magnetic bead selection (Miltenyi Biotech). The purity of all cells isolated by positive selection was increased to over 95% by passing over a second positive selection MS column.

*2.6.5. Infection procedure*

DC were seeded in 100 μl aliquots in round bottomed 96-well plates using the appropriate growth factors for each cell type. Viruses were added at multiplicities of infection (MOI) of 3 and 30 for 1.5h. Subsequently medium was added to a final volume of 200μl and the cells were incubated at 37$^o$C overnight. Equal numbers of an irradiated murine myeloma cell expressing human CD40 ligand [22] were then added to half of the wells. Cells were harvested after 48 hours in culture, washed, labelled with CD83-PE, CD86-APC and HLA-DR-PerCP (Becton Dickinson), fixed with 4% PFA and analysed by FACS. Analysis was performed using WinMDI software.

## 3. Results

### *3.1. Promoter sequence comparison*

Transcription factor (TF) binding site prediction showed the presence of 4 NFκB and 2 Ap1 binding sites on the CMV promoter whereas 5 NFκB and 2 Ap1 predicted binding sites were found on the CMV5 promoter, on the positive strand of both sequences. On the other hand, the UbC promoter contains only one predicted NFκB binding site and one binding site for each of Ap1 and Sp1 factors on its positive strand. Identification of these TF binding sites on the UbC promoter is also reported elsewhere [23]. The position of these predicted TF binding sites is shown in Table 1.

### *3.2. Transfection of cell lines*

Total eGFP content was calculated for both cell types by multiplying the values for the percentage of eGFP-expressing cells and MFI. For both HeLa and 911HER cells transfection with pShuttle-CMV5-GFP resulted in greater production of eGFP as shown in Figure 1. However, the relative difference between eGFP expression by pAdTrack (CMV-eGFP) and pShuttle-CMV5-GFP in HeLa and in 911HER cells indicates that CMV5 and CMV promoters behave differently in those two cell lines. The UbC promoter drives the expression of eGFP at low levels in both cell lines tested.

### *3.3. HeLa cells transduction*

Transduced with Ad1-CMV5-GFP HeLa cells, were found to exhibit higher levels of eGFP fluorescence than cells transduced with the other two viruses. This finding agrees with our plasmid transfection studies and indicates that the CMV5 promoter is stronger than the CMV promoter in the cell lines studied. Total eGFP

content was also higher for cells transduced with the Ad1-CMV5-GFP vector (Figure 2).

*3.4. DC transduction studies*

Expression of eGFP driven by the three different promoters was measured in five different populations of DC 48h after transduction either in the absence of an exogenous maturation stimulus or with CD40L added 24h after transduction. For all DC types maturation caused a marked up-regulation of eGFP expression.

*3.5. Plasmacytoid DC*

Plasmacytoid DC were transduced poorly by all three adenoviruses used. Low levels of eGFP expression were observed, accompanied by low MFI values, resulting in low levels of total eGFP content (Figure 3A). CD40L stimulation caused both the percentage of $eGFP^+$ cells and the relative MFI values to increase. Ad1-CMV-GFP gave the best results with the highest average total eGFP content value when it was used at an MOI of 30 and in the presence of CD40L.

*3.6. Myeloid DC*

Freshly isolated blood mDC were more easily transduced by the adenoviruses and showed a higher survival rate accompanied by higher levels of eGFP expression than pDC. CD40L stimulation resulted in enhanced activity by the CMV and CMV5 promoters, as indicated by increased eGFP expression, and a small increase in activity of the UbC promoter. Ad1-CMV-GFP once again gave the best results with regards to total eGFP content at an MOI of 30 in the presence of CD40L (Figure 3B).

Blood $CD11c^+$ DC are the precursors of immature tissue myeloid DC. Our previous studies have shown that after culture for two days in the presence of GM-CSF and IL-4, blood mDC differentiate and acquire markers such as CD1a and DC-SIGN, characteristic of tissue DC [24]. Transduction of 48h cultured mDC led to

higher levels of eGFP expression, with and without CD40L stimulation, than freshly isolated cells for all three vectors. Furthermore, these cells responded better upon CD40L stimulation as can be seen in Figure 3C. Once again, total eGFP content was highest in cells transduced with Ad1-CMV-GFP and then stimulated with CD40L.

*3.7. Monocyte derived DC (moDC)*

Compared to blood DC, moDC were more easily transduced with a greater percentage of eGFP positive cells with higher MFI and total eGFP values (Figure 4A). In addition, relative MFI and total eGFP content increase upon CD40L stimulation was much higher than the corresponding values obtained for freshly isolated pDC and mDC. The MFI and percentage of eGFP expression for moDC was only slightly higher than the range of the values obtained with the cultured mDC, indicating similarities in the protein production potential and CD40L activation of these cells.

*3.8. Langerhans and Dermal dendritic cells (LC and dDC)*

High transduction efficiencies were observed for both cell types with LC exhibiting higher levels of eGFP expression (Figure 4C). dDC expressed eGFP at relatively high levels and responded to CD40L stimulation as expected (Figure 4B). In addition to FACS analysis, fluorescence microscopy monitoring 24hrs post-infection revealed that the levels of eGFP expression by LC were much higher than those of dDC, when the CMV and CMV5 promoters were used, with Ad1-UbC-GFP transduced cells exhibiting almost zero fluorescence (results not shown).

*3.9. Transduction by adenovirus vectors does not inhibit up-regulation of co-stimulatory molecules*

Co-stimulatory molecule (HLA-DR, CD83 and CD86) up-regulation was monitored during CD40L stimulation for all DC types tested. Minor up-regulation of

the activation markers in some samples was observed upon infection with the adenoviruses in the absence of CD40L indicating that infection alone is not sufficient to induce full maturation (Figure 5-CD86 expression levels shown, HLA-DR and CD83 data not shown). Both $eGFP^+$ and $eGFP^-$ cells showed similar up-regulation of HLA-DR suggesting that transduction does not impair the ability of DC to mature (Figure 5G). Co-stimulatory molecule expression was high on LC after isolation with CD1a beads suggesting that this procedure caused a degree of LC maturation (Figure 5F-CD86 expression shown). Non-isolated LC do not present such a maturation phenotype as has been observed in other studies conducted in our lab (data not shown).

## 4. Discussion

The aim of this report is to describe the activity of three promoters in different human DC populations in order to determine which is the most suitable for use in vaccine development. We have used first generation serotype 5 adenoviral vectors to transduce DC with an *egfp* transgene driven by three different promoters (CMV, CMV5 and UbC). Dendritic cell populations, particularly moDC and LC, are of high importance in vaccine protocols. Monocyte-derived DC have been widely used in *ex vivo* vaccinations, especially in the area of cancer research [21] and LC represent an excellent target for conventional vaccination as they are present in large numbers in the skin. A recent report suggests that these cells are more potent stimulators of cytotoxic T cell responses than either dermal or moDC [8]. Using our eGFP constructs, we have identified substantial differences in the transduction efficiencies and protein production by the DC populations we tested. Bioinformatics analyses of CMV, CMV5 and UbC promoters showed differences in the predicted pattern of transcription factor binding sites which help to explain differences in eGFP expression. The predicted presence of an additional NFκB binding site on the positive strand of the CMV5 promoter in conjunction with the lower levels of eGFP production achieved upon CD40L stimulation when compared to the CMV promoter, indicates that relative positioning of this TF binding site plays an important role in NFκB-induced activation of CMV5.

Cell lines are frequently used to test the efficiency of vector constructs, however, our studies show that they do not necessarily reflect the activity of the intended *in vivo* target. In contrast to DC, the CMV5 promoter was better than CMV at driving eGFP expression in cell lines. Other reports have also shown that the CMV5 promoter leads to much higher expression in certain cell lines than the CMV

promoter [14]. Enhanced GFP expression by all promoters was found to be higher in 911HER than HeLa cells indicating that promoter activity differs between these two cell lines. The levels of eGFP expression driven by the UbC promoter are relatively low when compared with the other two promoters in all cells tested. The activity of the CMV5 promoter varies depending on cell type and may be dependent on the replication state of the cell. This can perhaps be attributed to enhanced activation of the adenoviral sequences, particularly major late promoter sequences, contained in this promoter within the context of cell and cellular DNA replication [25,26]. Differences in eGFP expression due to nature of the vector, including size, structural elements etc., are not considered significant since the construction of each individual plasmid was based on the same backbone sequence (pShuttle) with a few modifications so that the resulting plasmids were only different in the promoter sequences they were carrying.

We have found that freshly isolated pDC were less easily transduced by the adenoviral vectors used in this study. Others have also reported that human pDC are not readily transduced by serotype 5 rAds [27-29]. Also, type I interferons (IFN) produced by pDC upon rAd infection were not found to suppress the level of transgene expression according to Huarte *et al* [30] in both the mouse and human systems [31]. Low endocytic activity or unavailability or low levels of the appropriate transcription factors for activation of the three promoters might explain the low levels of eGFP observed. The CMV promoter still behaves better than the CMV5 promoter and the UbC promoter shows the least activity. Stimulation through CD40 ligation led to increase in the levels of eGFP expression clearly supporting previous findings relating activation of the CMV promoter through NFκB action [32]. Interestingly, eGFP expression from Ad1-UbC-GFP was up-regulated upon CD40 ligation but this

difference was not found to be statistically significant. Similar results were observed in other DC types tested and agrees with the prediction of an NFκB binding site on the UbC promoter sequence. A recent publication also shows activation of UbC through glucocorticoid action on Sp1/MEK1 [33]. Sp1 has been shown to interact with NFkB in activation of the HIV-1 LTR [34] and perhaps, elevated levels of NFkB due to CD40L treatment, in association with the relevant positioning of the TF binding sites for these factors, can explain the observed increase in activity by the UbC promoter.

Myeloid DC were found to be much more easily transduced than their homologous pDC and express eGFP at higher levels. In a recently published paper, Lore *et al* [28] also report higher levels of transduction of mDC with rAds of serotypes 5 and 35 expressing GFP, when compared to pDC, although both cell types were shown to have similar levels of CAR and CD46. When mDC were cultured for two days prior to infection, we observed a remarkable difference in the percentage of eGFP-expressing cells and eGFP fluorescence intensity, compared to the freshly isolated mDC and pDC. Fortunately, with respect to vaccination, the cultured mDC would more closely resemble immature tissue DC that would be the target of a potential vaccine. Culture conditions might lead to upregulation of transcription factors or alteration of the cellular environment of the mDC, making them more susceptible to adenovirus infection and subsequent protein expression. mDC survived better in culture than pDC but even with these cells we observed increased death in some samples when MOI of 30 was used accounting for the low eGFP content value seen in Figure 3B.

Monocyte-derived DC (moDC) were easily transduced and expressed high levels of eGFP in the presence or absence of CD40 ligand. Immature and CD40L-

matured moDC have previously been shown to lack expression of CAR and $\alpha_V\beta3$ integrins [35], however, increased MOI seems to circumvent the resistance to infection due to absence of these adenovirus serotype 5 receptors. Furthermore, in our experiments, transduction efficiencies were relatively high even at the low MOI of 3. The CMV promoter was again the strongest promoter in these cells and responded better to CD40L stimulation (Figure 4A). Levels of eGFP driven by the UbC promoter were also higher in the presence of CD40L for some samples.

Langerhans and dermal DC were generated from $CD34^+$ cord blood cells, transduced with the adenoviruses at an MOI of 30 and subsequently stimulated with CD40L. Levels of eGFP expression were much higher in LC than in dDC, irrespective of CD40L stimulation, although levels of integrins and CAR, required for virus binding and internalisation, were shown to be similar in both these cell types by a recent study from our group [36]. We hypothesise that the differences are due to transcription potential differences between these two cell types. Transduced cells responded as expected to CD40L treatment, and in accordance with our findings with the other DC types, i.e. up-regulation of eGFP MFI. Interestingly, the number of eGFP-expressing DC increased when they were treated with CD40L (data not shown) indicating that a number of "dormant" adenoviral transcripts probably exist inside $eGFP^-$ cells, which are activated due to the increase in levels of NFκB and perhaps other transcription factors.

Levels of HLA-DR, CD83 and CD86 were increased upon stimulation with CD40 ligand, coinciding with the increase in eGFP expression by these cells. This finding indicates that transduced cells respond normally to a maturation stimulus. Myeloid DC cultured for 48h before transduction had elevated expression of CD86, CD83 and HLA-DR (data not shown) when compared with their freshly isolated

counterparts. A small increase in the levels of co-stimulatory molecules was observed in transduced cells (particularly at the MOI of 30) but the levels were well below those observed after CD40L stimulation. Transduction of dDC and LC with higher MOIs (up to 100 transducing units) was not found to induce their maturation whereas when an MOI of 500 transducing units was used, CD83 upregulation was observed [36].

Although we have examined promoter activity in DC as vaccine target cells, cross-priming should not be overlooked as means of generation of good memory responses. This process is dependent on exogenous antigen entering the Class I pathway in DC and thus non-DC cell types can also act as antigen producers. Cross-priming depends on the nature of antigen (i.e. secreted or not) and on the whether there is apoptosis of the transfected cell. At present there is no evidence for or against induction of apoptosis in cells transduced with replication defective adenovirus vectors. Additionally, there could be differences in expression driven by these promoters in a non-DC population.

To our knowledge, this study is the first that has investigated a wide range of different human DC populations with respect to promoter activity. It has demonstrated the importance of testing promoter function in the proposed target DC populations rather than relying on data obtained only from cell lines. We hope that our findings will help facilitate the design of more effective DC-targeted vaccines.

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| **CMV promoter (575 nt)** | **Position** | **Sequence [(+) strand]** |
|---|---|---|
| NF-κB | 159 - 173 | ta**GGGActttcc**att |
| | 310 - 324 | at**GGGActttcc**tac |
| | 415 - 429 | acg**ggg**at**TTCC**aag |
| | 478 - 492 | ac**GGGActttcc**aaa |
| Activator protein 1 | 335 - 359 | atctacgta**TTAGtca**tcgctatta |
| Activator protein 1 | 407 - 417 | tt**tga**C**TCA**cg |
| | | |
| **CMV5 promoter (1048 nt)** | | |
| NF-κB | 101 - 115 | ta**GGGActttcc**att |
| | 252 - 266 | ac**GGGActttcc**tac |
| | 357 - 371 | acg**ggg**at**TTCC**aag |
| | 420 - 434 | ac**GGGActttcc**aaa |
| | 599 - 613 | cgc**gg**tct**TTCC**agt |
| Activator protein 1 | 277 - 301 | atctacgta**TTAGtca**tcgctatta |
| Activator protein 1 | 349 - 359 | tt**tga**C**TCA**cg |
| | | |
| **UbC promoter (1209 nt)** | | |
| c-Rel (NF-κB) | 549 - 563 | ggc**gg**ctg**TTCC**cga |
| Activator protein 1 | 191 - 201 | gg**TGAC**tctag |
| Sp1 | 868 - 882 | tgca**gGG**T**Gg**ggcca |

**Table 1**

Predicted TF binding sites on the positive strand of the three promoters tested.

Bold characters represent nucleotides at positions where the matrix exhibits a high conservation profile. Capital characters denote the core sequence (ie the 4 more highly conserved consecutive bases) used by the MatInspector software to identify the site.

## Titles and Legends to Figures

**Figure 1**

Plasmid transfection of 911HER and HeLa cell lines at 48h.

The pattern of eGFP expression was similar for both cell lines transfected with the gene for eGFP controlled by different promoters. Total eGFP content levels were highest with the CMV5 promoter (pShuttle-CMV5-GFP) followed by CMV (pAdTrack) with the UbC promoter (pAdTrack-UbC-GFP) giving the lowest levels of expression. Bars represent standard mean errors. Differences in the levels of eGFP among all three promoters were statistically significant with $p<0.05$ (Mann-Whitney test).

**Figure 2**

Adenoviral transduction of 911HER and HeLa cell lines.

HeLa cells transduced with the 1$^{st}$ generation eGFP rAds exhibited high levels of fluorescence. Differences in eGFP production among the promoters were statistically significant with $p<0.05$ (Mann-Whitney test) for both doses of virus used. Bars represent standard mean errors.

**Figure 3**

Enhanced GFP levels produced by human DC upon transduction with the three adenoviral vectors

**(A)** pDC exhibited low levels of eGFP production with Ad1-CMV-GFP giving the best results either with or without CD40L stimulation. CD40L addition led to a significant increase ($p<0.05$, Mann-Whitney test) in eGFP levels only in the case of

the CMV promoter. **(B)** mDC exhibited relatively high eGFP levels. Addition of CD40L led to significant increase in eGFP by CMV and CMV5 at the low MOI of 3 used ($p<0.001$) and by CMV5 at the MOI of 30 ($p<0.05$). No statistically significant difference was observed with MOI: 30 using Ad1-CMV-GFP although there was an evident increase in fluorescence. **(C)** Cultured for 48 hours prior adenoviral transduction mDC produced more eGFP than their counterparts transduced shortly after isolation. Again, addition of CD40L increased the levels of eGFP produced, with the CMV promoter responding more efficiently to the stimulus at both MOI used than the other two promoters.

**Figure 4**

**(A)** moDC produced high levels of eGFP with the CMV and CMV5 promoters responding significantly ($p<0.05$) to CD40L treatment at both MOI used. No statistical difference was observed between CMV and CMV5 irrespective of MOI used or of presence of CD40L. However, CMV-driven eGFP expression levels were higher than CMV5-driven eGFP levels. Both these promoters were significantly ($p<0.05$) stronger than the UbC promoter at the MOI of 30. **(B)** dDC transduced with Ad1-CMV-GFP produced higher eGFP levels than dDC transduced with Ad1-CMV5-GFP or Ad1-UbC-GFP. **(C)** Enhanced GFP expression in Langerhans cells was higher than in dDC. There were no statistically significant differences observed between CMV and CMV5 promoter strength irrespective of the presence of CD40L, although eGFP levels were higher with CMV. Both these promoters, drove eGFP production significantly ($p<0.05$) more than the UbC promoter when CD40L was added. Enhanced GFP levels were significantly ($p<0.05$) increased by all three promoters

upon addition of CD40L. Mann-Whitney t-test was used for statistical analysis-bars represent standard mean errors.

**Figure 5**

Effect of adenovirus and CD40L on blood DC maturation

**(A)** & **(B)** Adenoviral transduction did not affect maturation of blood DC (A= pDC and B= mDC) as indicated by minor up-regulation of CD86 by these DC populations and comparison with the uninfected control-left column. On the other hand, addition of CD40L caused up-regulation of co-stimulatory molecules (data for CD86 shown). Also, unstimulated pDC exhibited relatively high levels of HLA-DR after 48 hours in culture, which is expected as these cells can undergo a certain degree of maturation differentiating from the freshly isolated phenotype (data not shown). **(C)** 48h cultured mDC exhibited higher levels of expression of HLA-DR, CD83 and CD86, compared to their "immature" mDC counterparts (data for CD86 shown). Again, these cells can mature in culture, acquiring a phenotype that resembles that of moDC. Addition of CD40L further increased expression of the maturation markers (data for CD86 shown).

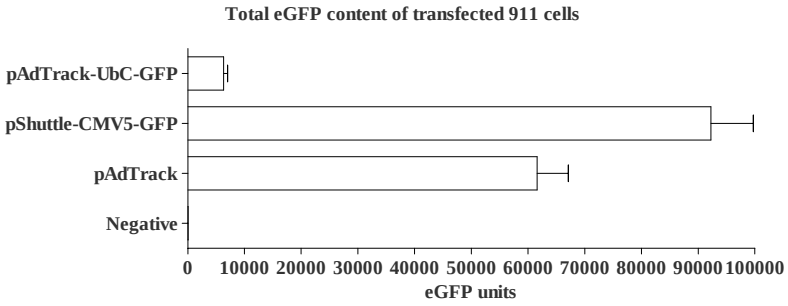
Effect of adenovirus and CD40L on moDC maturation

**(D)** *In vitro* generated moDC did not mature upon rAd transduction, exhibiting only minor upregulation of CD86 compared to the uninfected control sample. CD40L addition upregulated expression of the co-stimulatory molecules (data for CD86 shown). Furthermore, $eGFP^-$ cell populations of transduced samples exhibited minor up-regulation of HLA-DR in the absence of CD40L with its expression increasing upon addition of the CD40L stimulus **(G)** (representative sample).

Effect of adenovirus and CD40L on CD34-derived DC maturation

**(E)** CD34-derived dDC responded as expected to CD40L treatment by up-regulating expression of co-stimulatory molecules. Adenoviral transduction had minimal effect on marker expression (CD86 expression shown). **(F)** Isolated LC exhibited a degree of maturation, compared with the uninfected control, most presumably caused by the use of CD1a beads for their isolation. This maturation effect was further enhanced by CD40L (CD86 expression shown).

Grey histograms represent isotype controls, black line histograms show molecule expression in the absence of CD40L and bold black line histograms show expression in DC stimulated with CD40L after 24h. Samples were transduced at an MOI of 3.


Total eGFP content of transfected 911 cells
pAdTrack-UbC-GFP
pShuttle-CMV5-GFP
pAdTrack
Negative
0
10000
20000
30000
40000
50000
60000
70000
80000
90000
100000
eGFP units

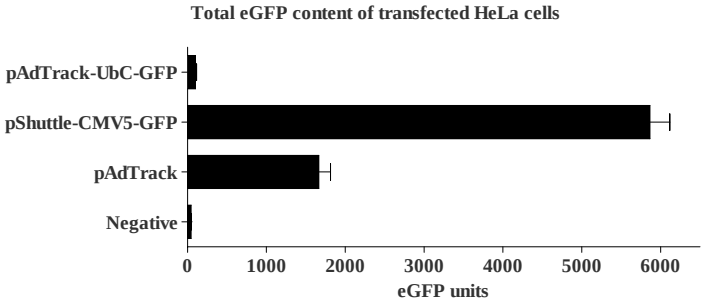

Total eGFP content of transfected HeLa cells
pAdTrack-UbC-GFP
pShuttle-CMV5-GFP
pAdTrack
Negative
0
1000
2000
3000
4000
5000
6000
eGFP units

Figure 1

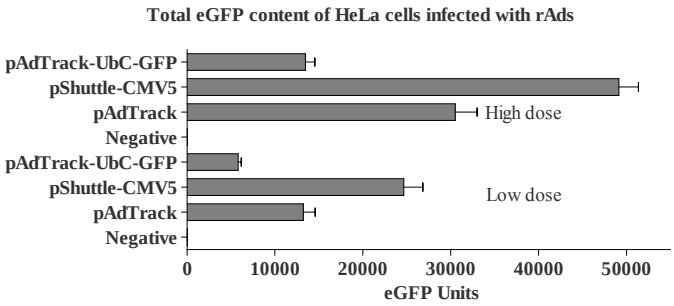

Total eGFP content of HeLa cells infected with rAds
pAdTrack-UbC-GFP
pShuttle-CMV5
pAdTrack
Negative
pAdTrack-UbC-GFP
pShuttle-CMV5
pAdTrack
Negative
High dose
Low dose
0
10000
20000
30000
40000
50000
eGFP Units

Figure 2

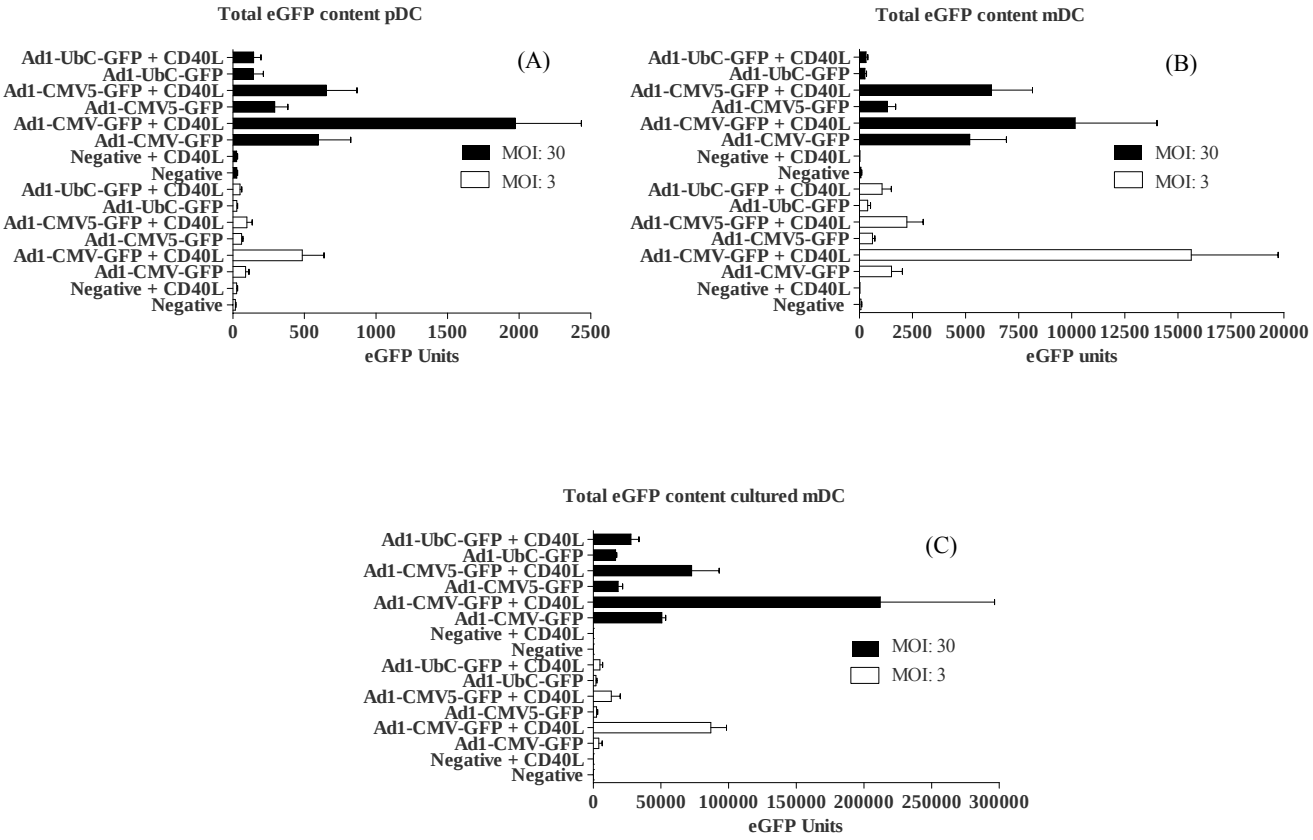

Total eGFP content pDC
(A)
Ad1-UbC-GFP + CD40L
Ad1-UbC-GFP
Ad1-CMV5-GFP + CD40L
Ad1-CMV5-GFP
Ad1-CMV-GFP + CD40L
Ad1-CMV-GFP
Negative + CD40L
Negative
Ad1-UbC-GFP + CD40L
Ad1-UbC-GFP
Ad1-CMV5-GFP + CD40L
Ad1-CMV5-GFP
Ad1-CMV-GFP + CD40L
Ad1-CMV-GFP
Negative + CD40L
Negative
MOI: 30
MOI: 3
0 500 1000 1500 2000 2500
eGFP Units
Total eGFP content mDC
(B)
Ad1-UbC-GFP + CD40L
Ad1-UbC-GFP
Ad1-CMV5-GFP + CD40L
Ad1-CMV5-GFP
Ad1-CMV-GFP + CD40L
Ad1-CMV-GFP
Negative + CD40L
Negative
Ad1-UbC-GFP + CD40L
Ad1-UbC-GFP
Ad1-CMV5-GFP + CD40L
Ad1-CMV5-GFP
Ad1-CMV-GFP + CD40L
Ad1-CMV-GFP
Negative + CD40L
Negative
MOI: 30
MOI: 3
0 2500 5000 7500 10000 12500 15000 17500 20000
eGFP units
Total eGFP content cultured mDC
(C)
Ad1-UbC-GFP + CD40L
Ad1-UbC-GFP
Ad1-CMV5-GFP + CD40L
Ad1-CMV5-GFP
Ad1-CMV-GFP + CD40L
Ad1-CMV-GFP
Negative + CD40L
Negative
Ad1-UbC-GFP + CD40L
Ad1-UbC-GFP
Ad1-CMV5-GFP + CD40L
Ad1-CMV5-GFP
Ad1-CMV-GFP + CD40L
Ad1-CMV-GFP
Negative + CD40L
Negative
MOI: 30
MOI: 3
0 50000 100000 150000 200000 250000 300000
eGFP Units

Figure 3

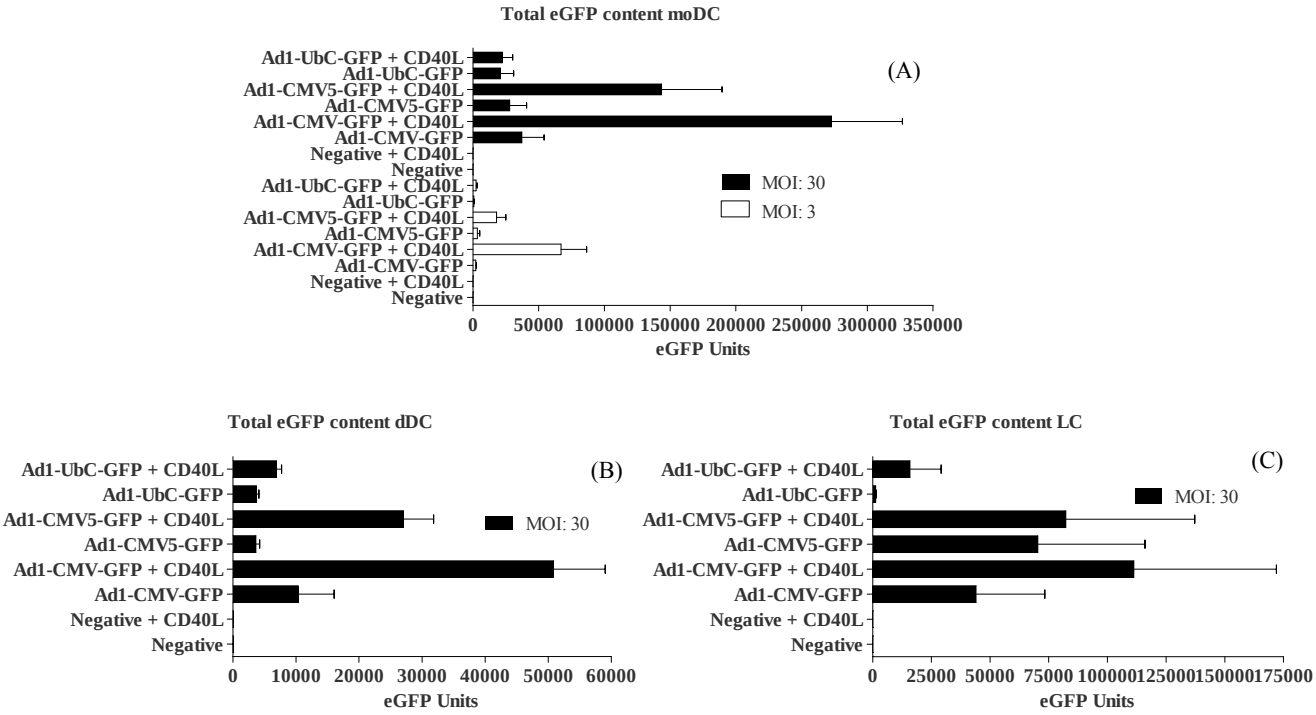

Total eGFP content moDC
(A)
Ad1-UbC-GFP + CD40L
Ad1-UbC-GFP
Ad1-CMV5-GFP + CD40L
Ad1-CMV5-GFP
Ad1-CMV-GFP + CD40L
Ad1-CMV-GFP
Negative + CD40L
Negative
Ad1-UbC-GFP + CD40L
Ad1-UbC-GFP
Ad1-CMV5-GFP + CD40L
Ad1-CMV5-GFP
Ad1-CMV-GFP + CD40L
Ad1-CMV-GFP
Negative + CD40L
Negative
MOI: 30
MOI: 3
0 50000 100000 150000 200000 250000 300000 350000
eGFP Units
Total eGFP content dDC
(B)
Ad1-UbC-GFP + CD40L
Ad1-UbC-GFP
Ad1-CMV5-GFP + CD40L
Ad1-CMV5-GFP
Ad1-CMV-GFP + CD40L
Ad1-CMV-GFP
Negative + CD40L
Negative
MOI: 30
0 10000 20000 30000 40000 50000 60000
eGFP Units
Total eGFP content LC
(C)
Ad1-UbC-GFP + CD40L
Ad1-UbC-GFP
Ad1-CMV5-GFP + CD40L
Ad1-CMV5-GFP
Ad1-CMV-GFP + CD40L
Ad1-CMV-GFP
Negative + CD40L
Negative
MOI: 30
0 25000 50000 75000 100000 125000 150000 175000
eGFP Units

Figure 4

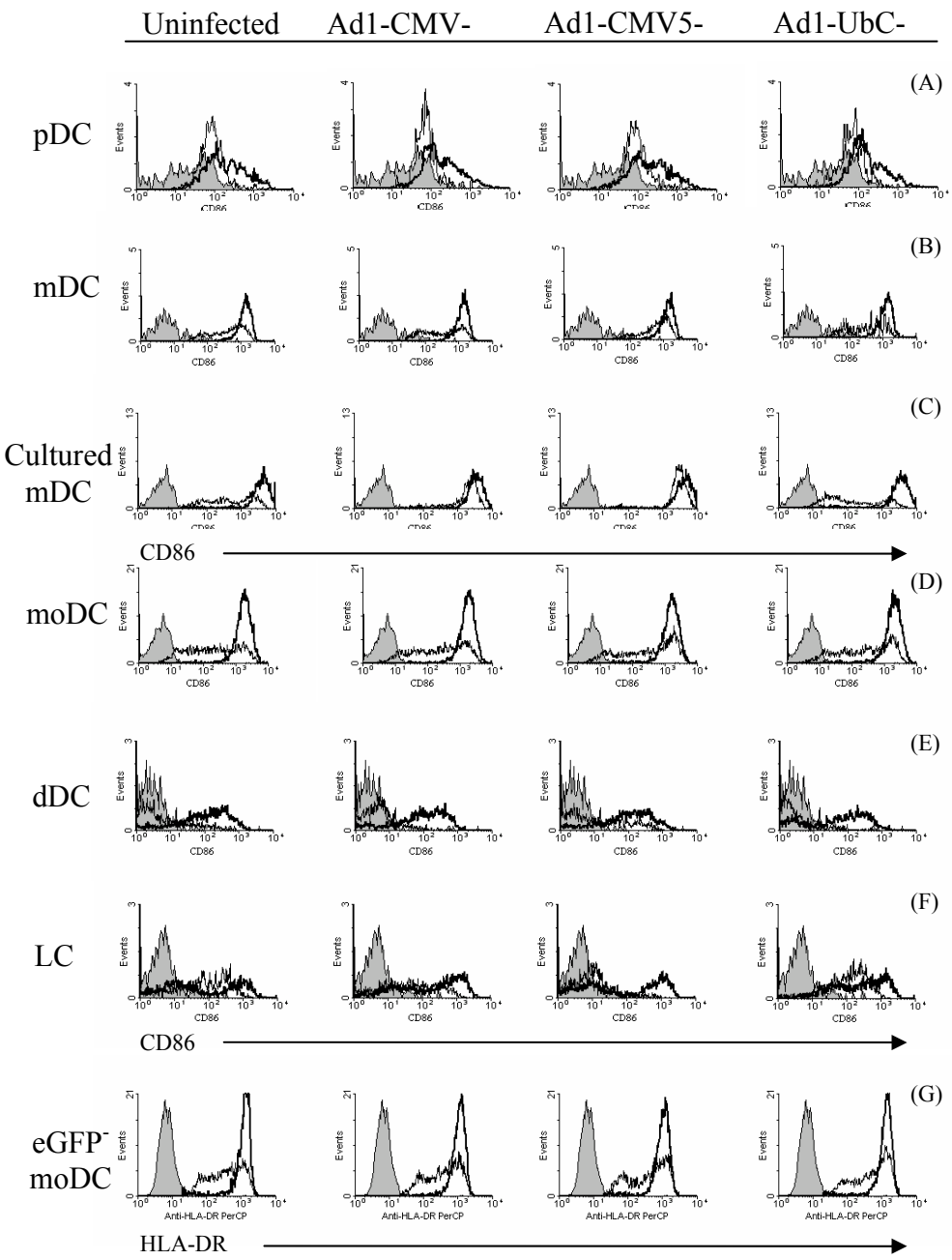

Uninfected
Ad1-CMV-
Ad1-CMV5-
Ad1-UbC-
pDC
(A)
mDC
(B)
Cultured mDC
(C)
CD86
moDC
(D)
dDC
(E)
LC
(F)
CD86
eGFP- moDC
(G)
HLA-DR
Events
CD86
Anti-HLA-DR PerCP

Figure 5