Adenovirus serotype 3 utilizes CD80 (B7.1) and CD86 (B7.2) as cellular attachment receptors

Joshua J. Short,[a] Alexander V. Pereboev,[a] Yosuke Kawakami,[a] Chenthamarakshan Vasu,[b] Mark J. Holterman,[b] and David T. Curiel[a,*]

[a] *Division of Human Gene Therapy, Departments of Medicine, Pathology, and Surgery, and the Gene Therapy Center, University of Alabama at Birmingham, Birmingham, AL 35294-2172, USA*
[b] *Departments of Surgery, and Microbiology and Immunology, University of Illinois at Chicago, Chicago, IL 60612, USA*

Abstract

Most viruses exploit a variety of host cellular proteins as primary cellular attachment receptors in the context of successful execution of infection. Furthermore, many viral agents have evolved precise mechanisms to subvert host immune recognition to achieve persistence. Herein we present data indicating that adenovirus (Ad) serotype 3 utilizes CD80 (B7.1) and CD86 (B7.2) as cellular attachment receptors. CD80 and CD86 are co-stimulatory molecules that are present on mature dendritic cells and B lymphocytes and are involved in stimulating T-lymphocyte activation. To our knowledge, this is one of the first demonstrations of a virus utilizing immunologic accessory molecules as a primary means of cellular entry. This finding suggests a mechanism whereby viral exploitation of these proteins as receptors may achieve both goals of cellular entry and evading the immune system.
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Introduction

Adenoviruses (Ads) are nonenveloped, double-stranded DNA viruses with icosahedral symmetry. There are 51 different human Ad serotypes, classified into six different subgroups (A–F) based on their biological and genetic

Abbreviations: Ad, adenovirus; Ad2, adenovirus serotype 2; Ad3, adenovirus serotype 3; Ad5, adenovirus serotype 5; Ad7, adenovirus serotype 7; Ad11, adenovirus serotype 11; Ad37, adenovirus serotype 37; CAR, coxsackie and adenovirus receptor; Ad5Luc1, adenovirus serotype 5 vector containing an expression cassette for luciferase transgene; Ad5/3Luc1, adenovirus serotype 5 retargeted to serotype 3 receptor containing an expression cassette for luciferase transgene; vp, viral particles; 6-HIS, six consecutive histidine residues; CHO, Chinese hamster ovary cells; sCD80, extracellular domains of CD80; sCD86, extracellular domains of CD86; DC-sign, dendritic cell-specific intercellular adhesion molecule-grabbing; UAB, The University of Alabama at Birmingham.

* Corresponding author. Division of Human Gene Therapy, Departments of Medicine, Pathology, and Surgery, and the Gene Therapy Center, University of Alabama at Birmingham, 901 South 19th Street, BMR2-508, Birmingham, AL 35294-2172. Fax: +1-205-975-2961.

E-mail address: david.curiel@ccc.uab.edu (D.T. Curiel).

properties (Russell, 2000). Most Ad serotypes infect cells via a two-step process: (i) Ad binding to a primary cellular attachment receptor mediated by the distal knob domain of one of the 12 fiber proteins extending from the Ad capsid vertices (Louis et al., 1994), (ii) followed by the interaction of α_V integrins on the cell surface and the Ad penton base RGD motif (Wickham et al., 1993). The initial binding of Ad to its primary receptor is thought to be one of the key determinants of virus tropism. The vast majority of the serotypes, including Ad serotypes 2 (Ad2) and 5 (Ad5), utilize the coxsackie and adenovirus receptor (CAR) as their primary cellular attachment receptor (Bergelson et al., 1997; Roelvink et al., 1998; Tomko et al., 2000). Ad2 and Ad5 are the most thoroughly studied serotypes and the most commonly used Ad serotypes in gene therapy applications.

Of note in this regard, Ad have been exploited as a gene transfer vector. Unfortunately, gene therapy protocols employing Ad5-based vectors have resulted in limited efficacies (Douglas et al., 2001). This may be in part due to deficient/low CAR expression or inaccessibility of CAR on the cell surface. CAR has been identified as a tumor suppressor protein (Kim et al., 2003) and a member of the
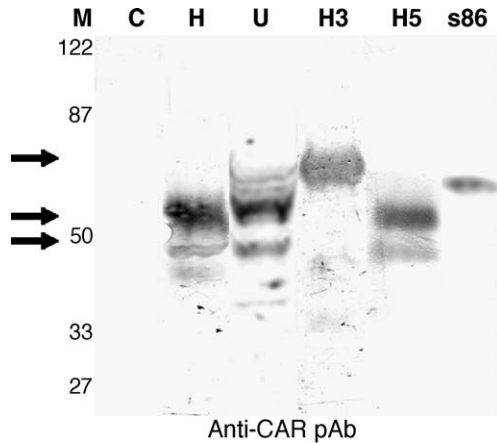
Fig. 1. Identification of an 80-kDa protein band that selectively interacts with the Ad3 fiber-knob domain. Three sample groups were generated and ran on 10% SDS-PAGE: (1) CHO (C), HeLa (H), or U118-hCAR (U) cell membranes; (2) the eluates from lysed HeLa cell membranes incubated with an Ad3 fiber-knob domain-conjugated sepharose resin (H3), or an Ad5 fiber-knob domain-conjugated sepharose resin (H5); and (3) soluble CD86 (s86). Purified proteins were checked for the presence of CAR by Western blot analysis with CAR extracellular domain specific rabbit polyclonal antibodies (anti-CAR pAb). CAR (double arrows) was present in the unpurified HeLa (H) and U118-hCAR (U) cell membranes and in the Ad5 fiber-knob domain-resin purified sample (H5). An unidentified protein (single arrow) cross-reacted with polyclonal antibodies specific to the extracellular domain of CAR. The polyclonal anti-CAR antibody also cross-reacted with 3.75 μg of the extracellular portion of CD86 (s86). Protein marker (M, kDa).

adherens junction (Cohen et al., 2001; Walters et al., 2002). Therefore, CAR is often down-regulated in the context of neoplastic conversion, and when expressed, its location basal to the tight junction may limit its accessibility. Efforts to circumvent these deficiencies have prompted the investigation of other non-CAR-binding Ad serotypes. One method of accomplishing this is by the construction of pseudotype Ad vectors (Krasnykh et al., 1996). Pseudotype Ad vectors replace the fiber-knob domain of the Ad5 vector with the fiber-knob domain from a non-CAR binding serotype, thereby achieving serotype-specific infection of cells. Vectors pseudotyped with the fiber-knob domain from Ad serotype 3 (Ad3), a member of subgroup B, efficiently transduce malignant cells in a variety of neoplastic contexts (Davidoff et al., 1999; Kanerva et al., 2002; Von Seggern et al., 2000), as well as many other target cells of interest (Stevenson et al., 1997). However, further development and characterization of Ad3-based vectors have been hindered by the failure to identify its primary cellular attachment receptor.

None of the subgroup B Ads, and several serotypes from the other subgroups, utilize CAR as their primary cellular attachment receptor (Roelvink et al., 1998). Subgroup B is subdivided into two groups: B1, consisting of serotypes 3, 7, 16, 21, and 50, and B2, consisting of serotypes 11, 14, 34, and 35. Recently, it has been shown that there are at least two subgroup B Ad receptors. Viruses from subgroup B2 can fully inhibit the binding

of subgroup B1 viruses; however, viruses from subgroup B1 can only partially inhibit the binding of subgroup B2 viruses (Segerman et al., 2003a). CD46 was identified as a cellular attachment receptor for Ad serotypes 11 (Ad11) and 35 (Ad35), both are members of the subgroup B2 (Gaggar et al., 2003; Segerman et al., 2003b). Additionally, all of the subgroup B2 Ad as well as most of the subgroup B1 Ad, with the exception of Ad3, are capable of binding to CD46 expressed on the cell surface. However, only binding of the virions to CD46, not transduc-
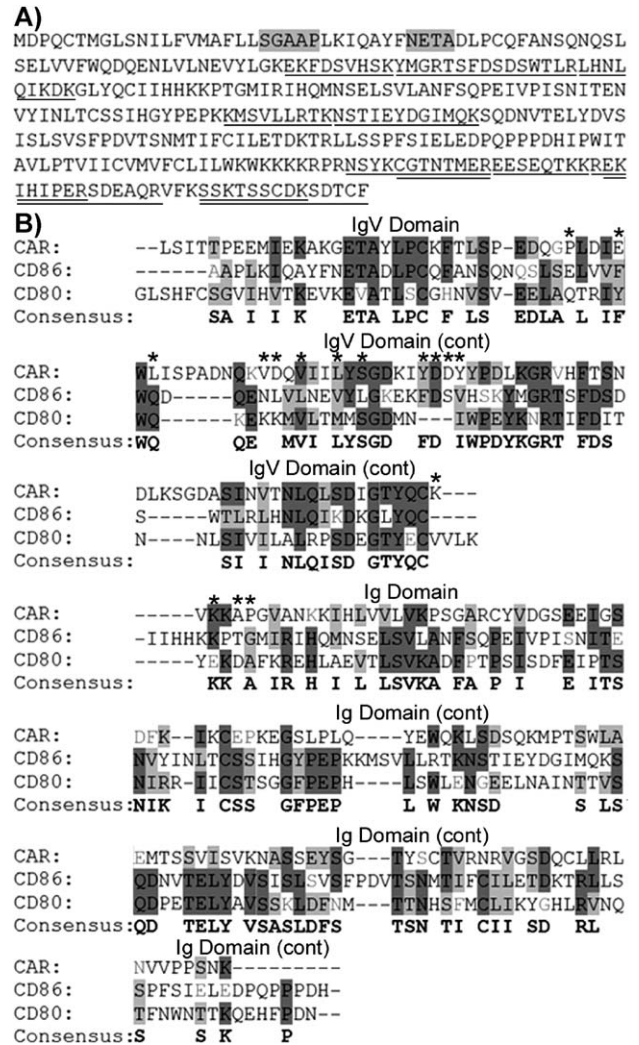

Fig. 2. Identification of CD86 and comparison with CAR and CD80. (A) Amino acid sequence of CD86. Proteins selected by affinity resins were analyzed by mass spectrometry and N-terminal peptide sequencing. CD86 was identified by the peptide sequences obtained by N-terminal sequencing (highlighted region) and by peptide fragments from the mass spectrometry (underlined regions). (B) CAR, CD86, and CD80 homology. CAR and CD86 exhibit homology (25.4% similarity, 16.4% identity) with the highest region of homology occurring in the IgV-like domains (35.3% similarity, 25.5% identity). CAR and CD80 share less homology (22.0% similarity, 12.2% identity), but again have the highest degree of homology between their IgV-like domains (34.6% similarity, 24.3% identity). * = CAR residues involved in Ad5 fiber-knob domain binding.

tion, was demonstrated (Gaggar et al., 2003). Ad serotype 7 (Ad7), a member of subgroup B1, is capable of binding to CD46; however, it cannot infect CD46-expressing Chinese hamster ovary (CHO) cells, leading to the postulation that CD46 serves as the subgroup B2-specific receptor (Segerman et al., 2003b).

Although it has been observed that Ad3 pseudotype vectors efficiently infect many cells of interest through an alternate receptor than CAR (Stevenson et al., 1995), the receptor responsible had yet to be identified (Di Guilmi et al., 1995). We hypothesized that the Ad3 receptor could be purified from susceptible cells by using recombinant produced knob domain of the Ad3 fiber (Albiges-Rizo et al., 1991) as an affinity agent. Collected proteins were analyzed by cross-reactivity with anti-CAR antibodies, mass spectrometry, and N-terminal peptide sequencing. Furthermore, identified genes were introduced into cells resistant to Ad3 infection to identify their role in conferring Ad3 infection. We identified two proteins, CD80 (B7.1) and CD86 (B7.2), which demonstrated an interaction with the knob domain of the Ad3 fiber and were capable of mediating Ad3 infection of otherwise nonpermissive cells.
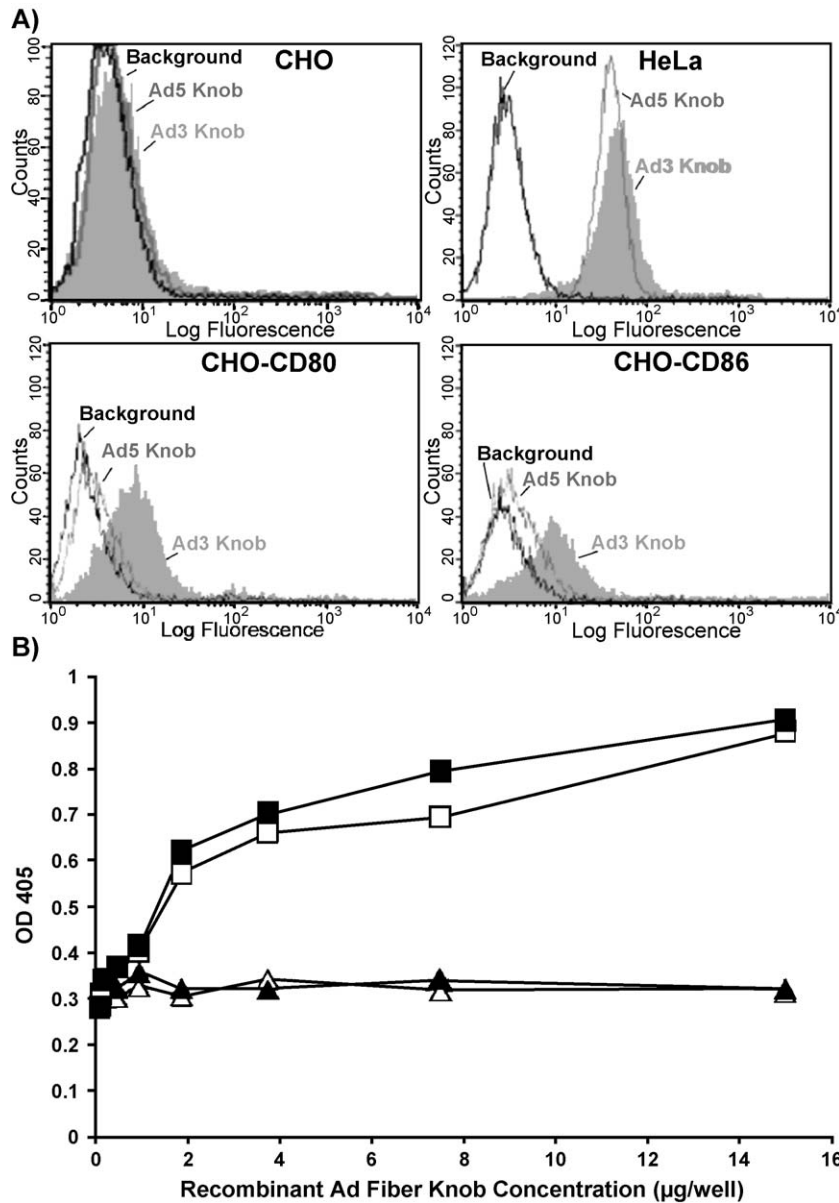

Fig. 3. The Ad3 fiber-knob domain specifically interacts with CD86 and CD80. (A) Binding of recombinant Ad3 (shaded profile) or Ad5 fiber-knob domain (gray profile) to HeLa, CHO, CHO-CD86, and CHO-CD80 cell lines detected via flow cytometry. Both Ad3 and Ad5 fiber-knob domain bound to HeLa cells, but only the Ad3 fiber-knob domain bound specifically to CHO-CD86 and CHO-CD80 cell lines. Neither Ad3 nor Ad5 fiber-knob domain bound to CHO cells. (B) ELISA demonstrating the specific interaction between the recombinant Ad3 fiber-knob domain and the soluble recombinant extracellular region of CD80 (sCD80) and CD86 (sCD86). Ad3 fiber-knob domain (■), Ad5 fiber-knob domain (▲), open symbols = sCD80, closed symbols = sCD86.

Results

Purification and identification of proteins that interact with the knob domain of the Ad3 fiber

Membrane fractions from two cell lines that are efficiently infected by Ad3 and Ad5, a human cervical carcinoma cell line (HeLa) and a human malignant glioma cell line that heterologously expresses CAR (U118-hCAR) (Kim et al., 2003), were used as positive sources of Ad receptors. The CHO cell line, which is poorly susceptible to Ad3 and Ad5 infection, was used as a negative control. Affinity resins were generated by conjugating the recombinant knob domain of Ad3 fiber or the Ad5 fiber to a sepharose resin, and proteins were purified by incubating the resins with the cell membrane fractions. To verify the technique, the resin conjugated to the recombinant knob domain of the Ad5 fiber was used to purify CAR. Western blot analysis of the affinity-purified proteins demonstrated the isolation of CAR by the Ad5 fiber affinity

resin (Fig. 1, double arrows). However, unexpectedly, an approximately 80-kDa protein purified by the Ad3 fiber affinity resin cross-reacted with the anti-CAR antibodies (Fig. 1). The protein band specifically reacted with polyclonal antibodies directed against the extracellular portion of CAR and with a monoclonal anti-CAR antibody specifically directed toward the CAR IgV-like domain (data not shown). This protein was isolated and analyzed by mass spectrometry and N-terminal peptide sequencing and identified as CD86 (Fig. 2A). CD86, also known as B7.2 (Caux et al., 1994; Engel et al., 1994), is a member of the Ig superfamily and is a co-stimulatory molecule that interacts with CD28 and CTLA-4 (Azuma et al., 1993). It has homology to CAR (16.4% identity, 25.4% similarity) with the highest homology (25.5% identity, 35.3% similarity) occurring between the IgV-like domains (Fig. 2B).

CD80, also known as B7.1, is a member of the Ig superfamily (Freeman et al., 1989) and interacts with the same ligands as CD86 (Lanier et al., 1995). In this regard
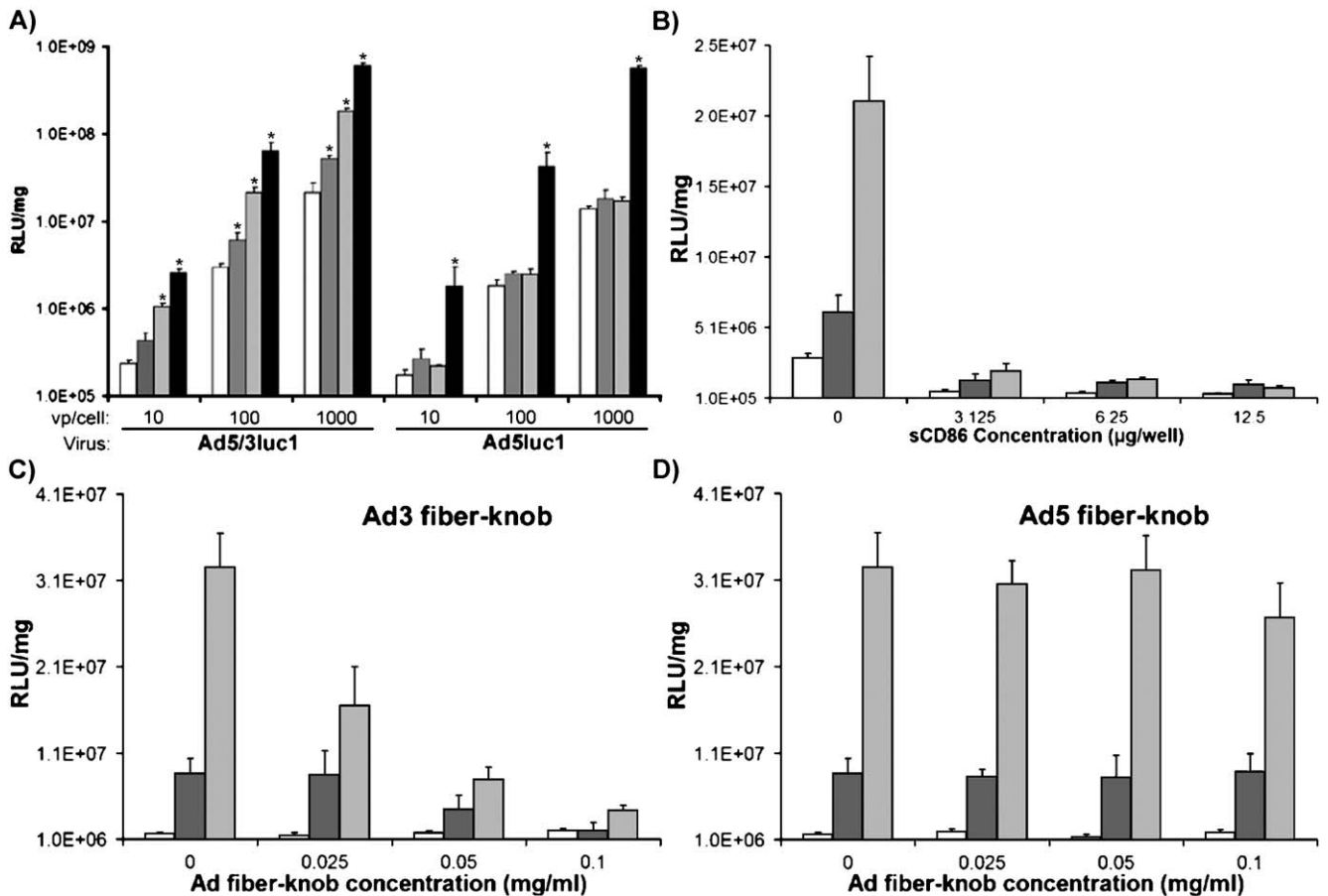

Fig. 4. CD80 and CD86 expression results in specific and enhanced gene transfer from an Ad3 pseudotype vector. (A) Luciferase gene expression from vectors containing the Ad3 fiber-knob domain (Ad5/3luc1) or the Ad5 fiber-knob domain (Ad5luc1). CHO (white bars), CHO-CD80 (dark gray bars), CHO-CD86 (light gray bars), HeLa (black bars). * = significant difference vs. CHO cells ($P < 0.05$). (B) The extracellular domain of CD86 (sCD86) specifically inhibits luciferase gene expression of vectors containing the Ad3 fiber-knob domain on CHO cells (white), CHO-CD80 cells (dark gray), and CHO-CD86 cells (light gray). (C) Recombinant Ad3 fiber-knob domain specifically blocks Ad5/3luc1 gene transfer. CHO cells (white), CHO-CD80 cells (dark gray), CHO-CD86 cells (light gray). (D) Recombinant Ad5 fiber-knob domain has no effect on Ad5/3luc1 gene transfer. CHO cells (white), CHO-CD80 cells (dark gray), CHO-CD86 cells (light gray). Relative light units (RLU), Error bars = ±SD.

and on the basis of the homology between CD80, CD86, and CAR (Fig. 2B), we analyzed the role of CD80 in Ad3 infection. CD80 also exhibits homology to CAR (12.2% identity, 22.2% similarity), with the highest degree of homology seen between the IgV-like domains (Fig. 2B). However, only 1 of the 16 amino acid residues critical for the Ad5 fiber-knob binding to CAR (Bewley et al., 1999) is conserved in each of CD80 and CD86, and only 3 residues show similarity (Fig. 2B).

The knob domain of the Ad3 fiber specifically interacts with CD80 and CD86

Using flow cytometry, the interaction of the knob domain of the Ad3 fiber with CD80 and CD86 was demonstrated by the ability of the recombinant knob domain of the Ad3 fiber to specifically bind to CHO cells which heterologously

express CD80 (CHO-CD80) or CD86 (CHO-CD86) (Vasu et al., 2003) but not to the parent CHO cells (Fig. 3A). The recombinant Ad5 fiber-knob domain showed no binding to CHO, CHO-CD80, or CHO-CD86 cells (Fig. 3A). The specificity of the Ad3 fiber-knob domain interaction with CD80 and CD86 was additionally demonstrated by ELISA (Fig. 3B), wherein a concentration-dependent binding to the extracellular portion of CD80 (sCD80) and to the extracellular portion of CD86 (sCD86) was seen for the Ad3 fiber-knob domain and no binding was detected between sCD80 or sCD86 and the knob domain of the Ad5 fiber.

Heterologous expression of CD80 and CD86 permits infection by Ad3-psuedotype vectors

The ability of CD80 and CD86 to confer susceptibility to Ad3 infection was evaluated in CHO cells or CHO cells
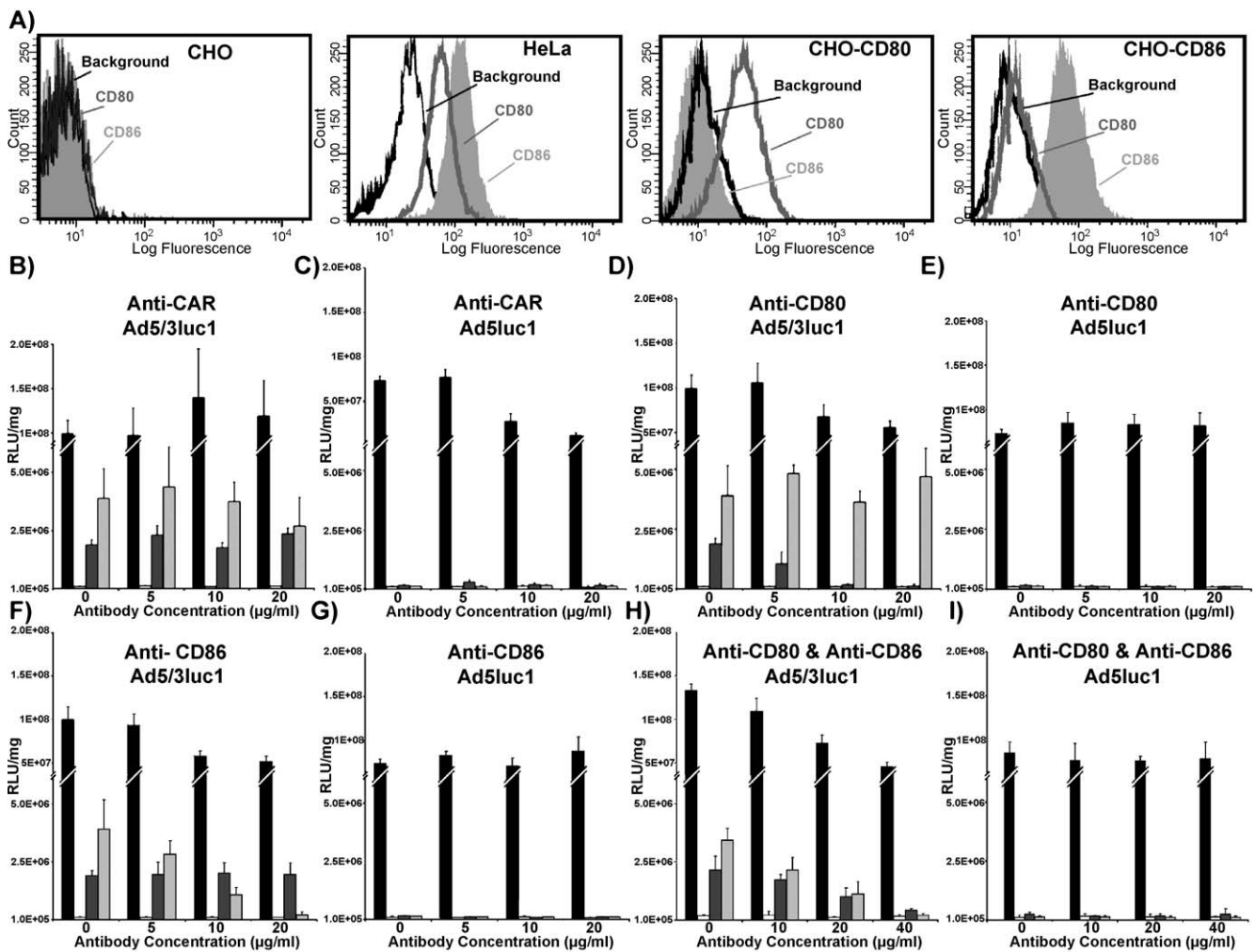

Fig. 5. CD80 and CD86-dependent infection by Ad3 pseudotype vectors. (A) CD80 (dark gray, empty pattern) and CD86 (gray, filled pattern) expression on CHO, HeLa, CHO-CD80, and CHO-CD86 cells as assayed by flow cytometry. (B) Anti-CAR antibodies do not block Ad5/3luc1 luciferase gene transfer, but anti-CAR antibodies block Ad5luc1 (C) gene transfer on HeLa (black), CHO (white), CHO-CD80 (dark gray), and CHO-CD86 cells (light gray). (D) Anti-CD80 or (F) anti-CD86 antibodies block CD80 or CD86-mediated gene transfer by Ad3 pseudotype vectors, but have no effect on Ad5luc1 gene transfer (E, G). (H) Concurrent incubation with anti-CD80 and anti-CD86 antibodies results in an increased inhibition of infection by Ad5/3luc1, but has no effect on Ad5luc1 (I). (B–I) HeLa (black), CHO (white), CHO-CD80 (dark gray), and CHO-CD86 cells (light gray). Relative light units (RLU), Error bars = ±SD.

expressing either CD80 or CD86. An Ad5-based vector pseudotyped with the serotype 3 fiber-knob domain, thereby specifically infecting via the Ad3 receptor and not via CAR, and encoding the firefly luciferase transgene (Ad5/3luc1) was used for the gene transfer analysis (Kanerva et al., 2002). Infection of CHO-CD86 cells with the Ad5/3luc1 vector resulted in 10-fold greater reporter gene expression compared to the parental CHO cells at all three MOIs: 10, 100, and 1000 virus particles per cell (vp/cell) (Fig. 4A). The enhanced gene transfer was highly significant for all three MOIs ($P = 0.005, 0.011, 0.003$, respectively). CHO-CD80 cells showed an enhanced reporter gene expression of 4- to 5-fold, though never as high as the CHO-CD86 cells. At an MOI of 10 vp/cell, the CHO-CD80 cells showed a trend toward significance ($P = 0.095$), and a significant

difference at 100 and 1000 vp/cell for the Ad5/3luc1 vector ($P = 0.031$ and 0.005, respectively). No significant differences in reporter gene expression were seen between the CHO, CHO-CD80 ($P = 0.115, 0.12, 0.151$), or CHO-CD86 ($P = 0.103, 0.161, 0.169$) cells infected with an Ad5 vector with an Ad5 fiber-knob domain (Ad5luc1) (Fig. 4A). The low level of infection seen with the CHO cells, which do not express the CD80, CD86, or CAR, is presumably due to the secondary entry mechanism mediated by the RGD motif in the Ad capsid and α_V integrins on the cell surface, because there is no difference in infectivity of the CHO cells by the Ad5luc1 or Ad5/3luc1 vectors (Fig. 4A).

Ad5/3luc1 infection could be inhibited by 90% by preincubation of the virus with the soluble CD86 (sCD86) (Fig. 4B). This inhibition occurred by sCD86 binding to the
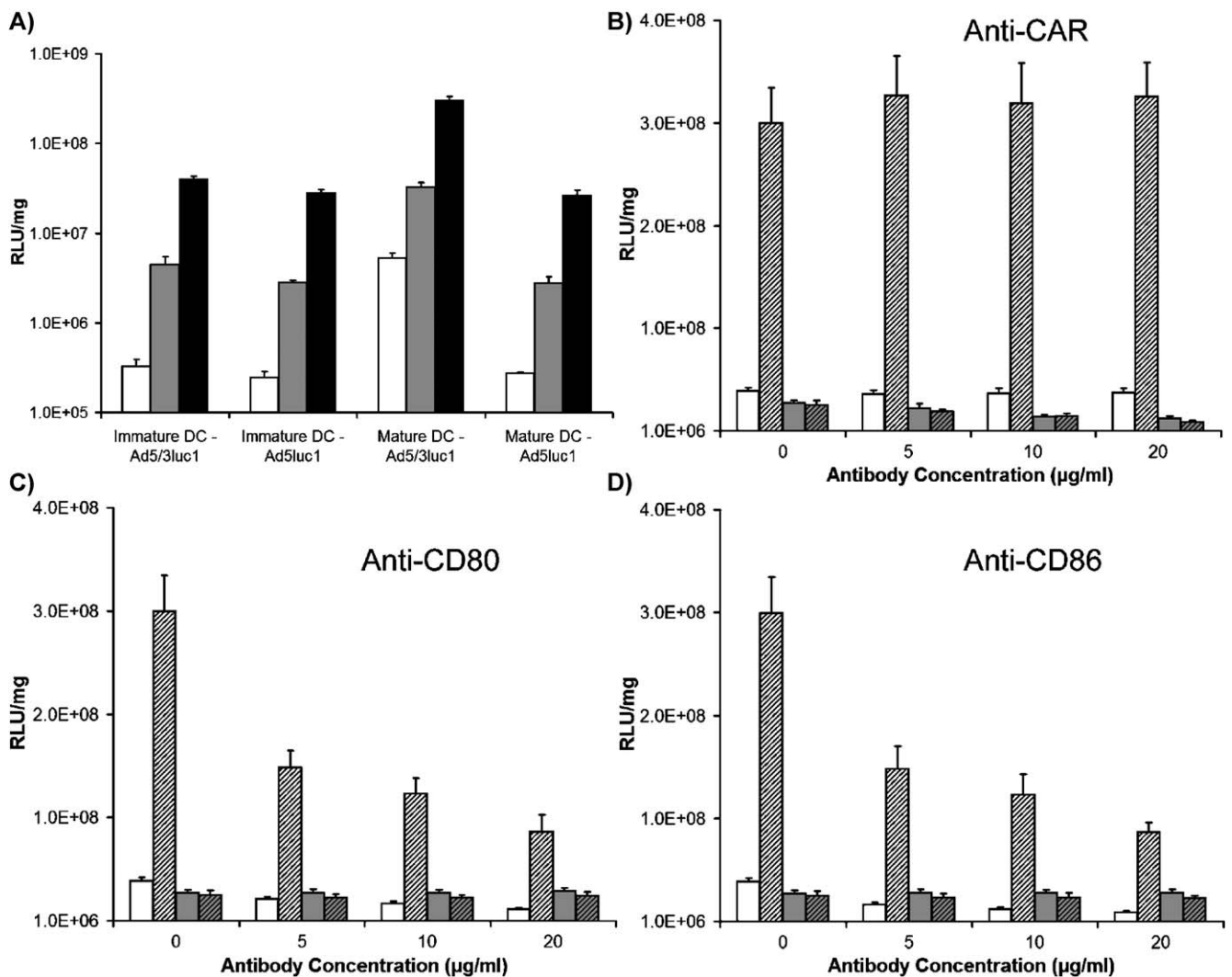

Fig. 6. CD80 and CD86-dependent infection of human dendritic cells. (A) Luciferase gene expression from vectors containing the Ad3 fiber-knob domain (Ad5/3luc1) or the Ad5 fiber-knob domain (Ad5luc1) on immature and mature human dendritic cells. 1 vp/cell (white bars), 10 vp/cell (gray bars), 100 vp/cell (black bars). (B) Anti-CAR antibodies specifically inhibit luciferase gene expression of Ad5 vectors. (C) Anti-CD80 and (D) anti-CD86 antibodies specifically inhibit luciferase gene expression of vectors containing the Ad3 fiber-knob domain on immature and mature dendritic cells. (white background) Ad5₁/luc1, (gray background) immature dendritic cells (open symbols) dendritic cells (diagonal-lined symbols). Relative light units (RLU), Error bars = ±SD.

virus, thereby blocking the interaction of the knob domain of the fiber with any cellular receptors. The inhibition seen on the CHO cells is presumably due to the added steric hindrance of the sCD86 attached to the virion, interfering with penton base and integrin interactions. Finally, Ad5/3luc1 gene transfer was specifically inhibited by preincubation of the cells with recombinant Ad3 fiber-knob domain (Fig. 4C), but not by recombinant Ad5 fiber-knob domain (Fig. 4D). Therefore, Ad vectors targeted with the knob domain of the Ad3 fiber specifically utilize CD80 and CD86 as primary cellular attachment receptors.

To further characterize the utilization of CD80 and CD86 by Ad3 pseudotype vectors (Ad5/3luc1), the ability to specifically block infection by antibodies was investigated. On the CHO cell derivatives, the anti-CAR antibodies had no effect on either virus or any of the cell lines because CAR is not expressed on any of the cells. Of note in this regard, the monoclonal antibody used in these experiments, RmcB, did not cross-react with CD80 or CD86 by Western blot analysis (data not shown). Preincubation of HeLa cells with anti-CAR antibodies effectively blocked Ad5 gene transfer (Fig. 5C), but not Ad5/3luc1 gene transfer (Fig. 5B). Anti-CD80 and anti-CD86 antibodies were able to specifically block the gene transfer of Ad3 pseudotype vectors (Figs. 5D, 5F, and 5H) on the respective CHO derivative and showed no effect on Ad5 vectors or the CHO parental cells (Figs. 5E, 5G, and 5I). The Ad5/3luc1 gene transfer to the CHO cells correlated with the expression of CD80 and CD86 on the cell surface (Fig. 5A). Preincubation of HeLa cells with anti-CD80 antibodies blocked Ad5/3luc1 gene transfer by about 10% (Fig. 5D) and preincubation with anti-CD86 antibodies blocked Ad5/3luc1 gene transfer by about 25% (Fig. 5F). These inhibitions corresponded with the levels of receptor expression on HeLa cells gathered by flow cytometry (Fig. 5A) and RT-PCR (data not shown). Concurrent incubation of the HeLa cells with anti-CD80 and anti-CD86 antibodies more effectively blocked Ad5/3luc1 gene transfer but only by about 55% at the highest antibody concentration (Fig. 5H). It appears that Ad3 pseudotype vectors may utilize CD80 and CD86 on HeLa cells, but there remains a high probability of a second primary receptor on these cells.

Adenoviral vectors containing the fiber-knob domain of serotype 3 infect human dendritic cells through CD80 and CD86

Adenoviral vectors containing the fiber-knob domain of serotype 3 exhibited an enhanced infectivity compared to Ad5 vectors on immature dendritic cells as well as an 8-fold enhancement of Ad5/3luc1 infection of mature dendritic cells vs. immature dendritic cells (Fig. 6A). Gene transfer could also be partially inhibited by anti-CD80 and anti-CD86 antibodies, about 40% reduction by preincubation with anti-CD80 antibodies and about 60% reduction by preincubation with anti-CD86 antibodies. However, gene transfer mediated by the Ad5 vectors was unaffected by preincubation of the cells with anti-CD80 or anti-CD86 antibodies (Figs. 6C and 6D). However, the infection of Ad5 vectors could be inhibited by preincubation of the cells with anti-CAR antibodies, which had no effect on the Ad3 pseudotype vectors (Fig. 6B). Here it appears that CD80 and CD86 serve as cellular receptors utilized by Ad3 pseudotype vectors for infection of human dendritic cells.

Discussion

Our major finding is that Ad3 can utilize both CD80 and CD86 as cellular attachment receptors. It appears that CD80 and CD86 may not be the only receptor utilized by Ad3 in the infection of HeLa cells, whereas with human dendritic cells, CD80 and CD86 provide a primary means of cellular attachment. CD80 and CD86 are co-stimulatory molecules that are primarily expressed on mature dendritic cells and mature B lymphocytes. Additionally, they have been shown to possess dysregulated expression on a variety of neoplasms (Brouwer et al., 2000; Koyama et al., 1998; Maeda et al., 2000; Mutti et al., 1998; Tatsumi et al., 1997). Our finding reconciles several recent reports in the literature. First, it is consistent with the observation that the Ad fiber-knob domain can elicit the maturation process of immature dendritic cells (Rea et al., 1999) and that subgroup B vectors are significantly better able to infect mature dendritic cells compared to immature dendritic cells (Rea et al., 2001), because both CD86 and CD80 are up-regulated during dendritic cell maturation (Lapteva et al., 2001). Second, Ad3 efficiently infects EBV-transformed B-cell lymphomas (Von Seggern et al., 2000), which often overexpress CD80 and CD86 (Suvas et al., 2002; Widney et al., 2003). Third, the subgroup B viruses efficiently infect cells of hematopoietic origin (Marini et al., 2002; Sakurai et al., 2003; Segerman et al., 2000).

The use of subgroup B Ad vectors offers many advantages in gene therapy and vaccine applications. The discovery of the utilization of CD80 and CD86 by Ad3 is very useful for the further development of these applications and offers a more fundamental understanding of virus–cell interactions that are critical for advancing these studies. A further implication of our finding is that Ad3 pseudotype vectors may offer an advantage in cancer gene therapy protocols based on CD86 and CD80 dysregulation in the context of neoplastic conversion. CD86 is generally up-regulated in several neoplastic contexts, including gastric carcinoma (Koyama et al., 1998), various lymphomas (Brouwer et al., 2000; Freeman et al., 1989; Suvas et al., 2002), and mesothelioma (Mutti et al., 1998), whereas CD80 expression is either unaffected or down-regulated (Koyama et al., 1998; Suvas et al., 2002).

The finding that Ad3 utilizes CD80 and CD86 as cellular attachment receptors highlights the broad variation in the functions of receptors utilized by Ad. CAR, which is utilized

by the majority of Ad serotypes, is a tumor suppressor protein (Kim et al., 2003; Okegawa et al., 2000) and a component of the adherens junction (Cohen et al., 2001; Walters et al., 2002). It is a homophilic cell adhesion molecule that is generally down-regulated in neoplastic conversion and plays a role in brain development (Honda et al., 2000). CD46 (Membrane Cofactor Protein) which is used by Ad11 and Ad35, is ubiquitously expressed and involved in the complement pathway (Gaggar et al., 2003; Segerman et al., 2003b). Ad37, which is able to bind CAR but not use it as a receptor, utilizes sialic acid residues as a receptor (Arnberg et al., 2002), as well as an additional unidentified protein (Wu et al., 2001). Ad5 can also infect cells by binding to the MHC class I $\alpha 2$ domain (Hong et al., 1997) and $\alpha 3 \beta 1$ integrins (Salone et al., 2003). There are at least two groups of subgroup B Ad receptors (Segerman et al., 2003a), therefore, there may still exist other receptors that may be utilized by subgroup B Ad. This broad variation in receptor classes may further help to explain the variation in viral tropism and pathogenesis between the different serotypes.

Finally, the finding that Ad3 uses CD80 and CD86 as cellular attachment receptors is to our knowledge one of the first examples of a virus utilizing a co-stimulatory molecule as a primary means of cellular entry. However, precedent for this scenario is set by several viruses, such as HIV-1 and hepatitis C, using the C-type lectin DC-sign (dendritic cell-specific intercellular adhesion molecule-grabbing) for entry into dendritic cells (van Kooyk and Geijtenbeek, 2003). From a biological perspective, this may allow the virus to escape the surveillance of the immune system and further provide an advantage in affecting immune response modulation. Normal virus replication biology includes the down-regulation of cell protein synthesis and disrupted cellular functions. Therefore, due to the prevalence of CD80 and CD86 on antigen presenting cells (APC), Ad3 may have evolved the utilization of these molecules to infect APC as a means of modulating an immune response to the viral infection. It is unknown at this time what effect Ad3 infection has on the native functions of CD80 and CD86. However, both molecules are critical in stimulating T-cell responses and any perturbations in their normal functions may disrupt T-cell activation.

Methods

Cell culture

Chinese hamster ovary cell line (CHO) and HeLa cell line (both from ATCC, Manassas, VA) were grown in DMEM/F12 50:50 with 10% FBS and antibiotics as recommended by the provider. Generation of CHO-CD80, CHO-CD86, and U118-hCAR cell lines was previously described (Kim et al., 2003; Vasu et al., 2003); the cells were grown in the same media in the presence of geneticin

at 400 $\mu g/ml$. All cells were cultured in a humidified incubator at 37 °C with 5% CO_2.

Recombinant Ad

Two replication-incompetent Ad5-based vectors containing a firefly luciferase transgene cassette in place of the deleted E1 region were used. Ad5luc1 contains the full Ad5 capsid and fiber proteins, whereas Ad5/3luc1 contains the Ad5 capsid with chimeric fiber proteins in which the tail and shaft domains are from Ad5 and the knob domain is from Ad3. Both vectors were generated in our laboratory and described previously (Kanerva et al., 2002).

Recombinant Ad3 and Ad5 fiber-knob domain protein production

The fiber-knob domains of Ad5 and Ad3 fibers were produced in *Escherichia coli* with N-terminal tags of six consecutive histidine residues (6-HIS), using the pQE30 expression vector (Qiagen, Valencia, CA) (Krasnykh et al., 1996). The soluble forms of the Ad3 and Ad5 fiber-knob domain proteins were isolated from the induced *E. coli* cultures using the BugBuster Protein Extraction Reagent (Novagen, Madison, WI). The proteins were further purified using the Talon metal affinity resin (Clontech, Palo Alto, CA) as recommended by the manufacturer. To further increase the protein yield, the expressed Ad3 fiber-knob domain protein was purified from inclusion bodies using the BugBuster Protein Extraction Reagent inclusion body protocol as recommended by the manufacturer. The concentration of the purified proteins was determined by the Bio-Rad protein assay (Bio-Rad, Hercules, CA). The ability of each Ad fiber-knob domain to form a homotrimer was verified by Western blot of unboiled vs. boiled samples. Furthermore, Ad fiber-knob domain function was confirmed by its ability to inhibit Ad infection of the same serotype but not affect infection of the other serotype.

Receptor purification and analysis

Affinity resins were generated by conjugating recombinantly produced Ad3 fiber-knob or Ad5 fiber-knob to a cyanogens-bromide activated sepharose resin (Sigma) as directed by the manufacturer. Cell membrane fractions were collected as previously described (Wu et al., 2001), and incubated with the resins overnight at 4 °C in PBS with Ca^{2+} and Mg^{2+} (PBSCM) plus 1% BSA plus protease inhibitor cocktail (Sigma). Resins were then washed 3 times with PBSCM and bound proteins were eluted with glycine:HCl (pH 2) for 10 min at 37 °C and then neutralized to a neutral pH (pH 7). Samples were analyzed by SDS-PAGE and Western blot with anti-CAR polyclonal antibodies [The University of Alabama at Birmingham (UAB)] and anti-CAR IgV-like domain-specific monoclonal antibody 9b3 (UAB Hybridoma Shared Facility, Birmingham, AL). Se-

lected protein bands were further analyzed by the UAB Mass Spectrometry Shared Facility and the UAB Peptide Sequencing Shared Facility.

Flow cytometry

Cells were grown to approximately 85% confluency, harvested with Versene, and incubated with Ad3 fiber-knob, Ad5 fiber-knob, or without Ad fiber-knob in growth media for 1 h at 4 °C. Cells were then washed 3 times in cold (4 °C) PBSCM. Cells were next incubated with mouse monoclonal anti-polyhistidine clone HIS-1 antibody (Sigma) in PBSCM plus 1% BSA for 1 h at 4 °C. Cells were washed 3 times in cold PBSCM and incubated with anti-mouse IgG antibodies conjugated to a fluorophore, Alexa 488 (Molecular Probes, Eugene, OR), in PBSCM plus 1% BSA for 1 h at 4 °C. Alternatively, for the expression of CD80 and CD86, specific polyclonal antibodies were used for each marker (Santa Cruz Biotechnology, Santa Cruz, CA) with incubation for 1 h at 4 °C in PBS plus 1% BSA. Cells were washed 3 times in cold PBS and incubated with anti-rabbit IgG antibodies conjugated to a fluorophore, Alexa 488 (Molecular Probes). All of the cells were washed 3 final times in cold PBSCM and analyzed by flow cytometry by the Center for Aids Research FACS Core Facility at UAB.

Human dendritic cell purification

Human dendritic cells were derived from the peripheral blood of normal donors, using a protocol approved by the UAB Institutional Review Board. Peripheral blood mononuclear cells were purified with gradient centrifugation by using Histopaque (Sigma Diagnostics, St. Louis, MO). CD14$^+$ monocytes were then isolated by using CD14 Microbeads and magnetic cell sorting (Miltenyi Biotec, Auburn, CA). They were cultured for 6 days in RPMI 1640 medium with 10% FBS, 2 mM glutamine, 100 units of penicillin/ml, 100 μg of streptomycin/ml, and 50 μM 2-mercaptoethanol containing 100 ng of recombinant human interleukin-4 (R&D Systems, Minneapolis, MN) and 100 ng of recombinant human granulocyte-macrophage colony-stimulating factor (Immunex, Seattle, WA)/ml. Maturation of a portion of the dendritic cells was induced by adding LPS at a concentration of 100 ng/ml to the media on the 5th day. Expression of molecular markers typical of immature dendritic cells (CD14$^-$ CD11c$^+$ CD40$^+$ CD86$^+$ HLADR$^+$) was confirmed by staining with relevant monoclonal antibodies (MAbs).

ELISA

Indicated amounts of recombinant Ad3 fiber-knob or Ad5 fiber-knob were adsorbed in a 96-well plate for 30 min at 37 °C. Wells were then rinsed with Tris-buffered saline with Tween 20 (TBST) and blocked with 5% casein in TBST for 30 min at 37 °C. After rinsing with TBST, 0.2 μg/well of recombinant extracellular domain of CD80 or CD86 fused

with mouse Fc region (sCD80, sCD86) (Alexis Biochemicals, Lausanne, Switzerland) in PBSCM plus 1% BSA was added to each well for 30 min at 37 °C. Wells were then washed 3 times with TBST and incubated with anti-mouse IgG antibodies conjugated to alkaline phosphatase (DAKO Corp.) in PBSCM plus 1% BSA for 30 min at 37 °C. Wells were washed 3 times with TBST and developed with *p*-nitrophenyl phosphate (Sigma). Alkaline phosphatase activity was assayed by measuring the optical density at 405 nm.

Gene transfer analysis

Cells were seeded in 12-, 24-, or 48-well plates and allowed to grow until 85% confluence. The cells were then incubated with either Ad5luc1 (1, 10, 100, or 1000 vp/cell), Ad5/3luc1 (1, 10, 100, or 1000 vp/cell), or no virus in DMEM:F12 50:50 with 2% FBS for 1.5 h at 37 °C. For blocking experiments, (1) Ad5/3luc1 virus (100 vp/cell) was preincubated in the presence of three different concentrations of sCD86 (3.125, 6.25, 12.5 μg/well) or (2) the cells were preincubated with various antibodies: anti-CAR (RmcB, ATCC), anti-CD80 (Santa Cruz Biotechnology), or anti-CD86 (Santa Cruz Biotechnology), at three different concentrations (5, 10, 20 μg/ml) or anti-CD80 and anti-CD86 (at 10, 20, 40 μg/ml), or (3) the cells were preincubated with recombinant Ad3 or Ad5 fiber-knob (0.1, 0.05, 0.025 mg/ml). For all blocking trials, incubation occurred for 10 min at 37 °C before virus was incubated with the cells for 1.5 h at 37 °C. Virus was then removed and the cells were rinsed with PBS and grown in growth media for 36 h. After 36 h, the cells were lysed and the luciferase assay was performed with the Luciferase Assay System (Promega, Madison, WI). The protein concentration of the cell lysates was determined with DC-Bio-Rad protein assay to allow normalization of the gene expression data to the number of cells. Background luciferase activities were subtracted from the readings. All of the samples were done in triplicate and the mean average, standard deviation, and *P* values were calculated using Student's *t* tests.

References

Albiges-Rizo, C., Barge, A., Ruigrok, R.W., Timmins, P.A., Chroboczek, J., 1991. Human adenovirus serotype 3 fiber protein. Comparison of native and recombinant proteins. J. Biol. Chem. 266 (6), 3961–3967.
Arnberg, N., Pring-Akerblom, P., Wadell, G., 2002. Adenovirus type 37

uses sialic acid as a cellular receptor on Chang C cells. J. Virol. 76 (17), 8834–8841.

Azuma, M., Ito, D., Yagita, H., Okumura, K., Phillips, J.H., Lanier, L.L., Somoza, C., 1993. B70 antigen is a second ligand for CTLA-4 and CD28. Nature 366 (6450), 76–79.

Bergelson, J.M., Cunningham, J.A., Droguett, G., Kurt-Jones, E.A., Krithivas, A., Hong, J.S., Horwitz, M.S., Crowell, R.L., Finberg, R.W., 1997. Isolation of a common receptor for Coxsackie B viruses and adenoviruses 2 and 5. Science 275 (5304), 1320–1323.

Bewley, M.C., Springer, K., Zhang, Y.B., Freimuth, P., Flanagan, J.M., 1999. Structural analysis of the mechanism of adenovirus binding to its human cellular receptor, CAR. Science 286 (5444), 1579–1583.

Brouwer, R.E., Zwinderman, K.H., Kluin-Nelemans, H.C., van Luxemburg-Heijs, S.A., Willemze, R., Falkenburg, J.H., 2000. Expression and induction of costimulatory and adhesion molecules on acute myeloid leukemic cells: implications for adoptive immunotherapy. Exp. Hematol. 28 (2), 161–168.

Caux, C., Vanbervliet, B., Massacrier, C., Azuma, M., Okumura, K., Lanier, L.L., Banchereau, J., 1994. B70/B7-2 is identical to CD86 and is the major functional ligand for CD28 expressed on human dendritic cells. J. Exp. Med. 180 (5), 1841–1847.

Cohen, C.J., Shieh, J.T., Pickles, R.J., Okegawa, T., Hsieh, J.T., Bergelson, J.M., 2001. The coxsackievirus and adenovirus receptor is a transmembrane component of the tight junction. Proc. Natl. Acad. Sci. U.S.A. 98 (26), 15191–15196.

Davidoff, A.M., Stevenson, S.C., McClelland, A., Shochat, S.J., Vanin, E.F., 1999. Enhanced neuroblastoma transduction for an improved antitumor vaccine. J. Surg. Res. 83 (2), 95–99.

Di Guilmi, A.M., Barge, A., Kitts, P., Gout, E., Chroboczek, J., 1995. Human adenovirus serotype 3 (Ad3) and the Ad3 fiber protein bind to a 130-kDa membrane protein on HeLa cells. Virus Res. 38 (1), 71–81.

Douglas, J.T., Kim, M., Sumerel, L.A., Carey, D.E., Curiel, D.T., 2001. Efficient oncolysis by a replicating adenovirus (ad) in vivo is critically dependent on tumor expression of primary ad receptors. Cancer Res. 61 (3), 813–817.

Engel, P., Gribben, J.G., Freeman, G.J., Zhou, L.J., Nozawa, Y., Abe, M., Nadler, L.M., Wakasa, H., Tedder, T.F., 1994. The B7-2 (B70) costimulatory molecule expressed by monocytes and activated B lymphocytes is the CD86 differentiation antigen. Blood 84 (5), 1402–1407.

Freeman, G.J., Freedman, A.S., Segil, J.M., Lee, G., Whitman, J.F., Nadler, L.M., 1989. B7, a new member of the Ig superfamily with unique expression on activated and neoplastic B cells. J. Immunol. 143 (8), 2714–2722.

Gaggar, A., Shayakhmetov, D.M., Lieber, A., 2003. CD46 is a cellular receptor for group B adenoviruses. Nat. Med. 9 (11), 1408–1412.

Honda, T., Saitoh, H., Masuko, M., Katagiri-Abe, T., Tominaga, K., Kozakai, I., Kobayashi, K., Kumanishi, T., Watanabe, Y.G., Odani, S., Kuwano, R., 2000. The coxsackievirus–adenovirus receptor protein as a cell adhesion molecule in the developing mouse brain. Brain Res. Mol. Brain Res. 77 (1), 19–28.

Hong, S.S., Karayan, L., Tournier, J., Curiel, D.T., Boulanger, P.A., 1997. Adenovirus type 5 fiber knob binds to MHC class I alpha2 domain at the surface of human epithelial and B lymphoblastoid cells. EMBO J. 16 (9), 2294–2306.

Kanerva, A., Mikheeva, G.V., Krasnykh, V., Coolidge, C.J., Lam, J.T., Mahasreshti, P.J., Barker, S.D., Straughn, M., Barnes, M.N., Alvarez, R.D., Hemminki, A., Curiel, D.T., 2002. Targeting adenovirus to the serotype 3 receptor increases gene transfer efficiency to ovarian cancer cells. Clin. Cancer Res. 8 (1), 275–280.

Kim, M., Sumerel, L.A., Belousova, N., Lyons, G.R., Carey, D.E., Krasnykh, V., Douglas, J.T., 2003. The coxsackievirus and adenovirus receptor acts as a tumour suppressor in malignant glioma cells. Br. J. Cancer 88 (9), 1411–1416.

Koyama, S., Maruyama, T., Adachi, S., Nozue, M., 1998. Expression of costimulatory molecules, B7-1 and B7-2 on human gastric carcinoma. J. Cancer Res. Clin. Oncol. 124 (7), 383–388.

Krasnykh, V.N., Mikheeva, G.V., Douglas, J.T., Curiel, D.T., 1996. Gen-

eration of recombinant adenovirus vectors with modified fibers for altering viral tropism. J. Virol. 70 (10), 6839–6846.

Lanier, L.L., O'Fallon, S., Somoza, C., Phillips, J.H., Linsley, P.S., Okumura, K., Ito, D., Azuma, M., 1995. CD80 (B7) and CD86 (B70) provide similar costimulatory signals for T cell proliferation, cytokine production, and generation of CTL. J. Immunol. 154 (1), 97–105.

Lapteva, N., Nieda, M., Ando, Y., Ide, K., Hatta-Ohashi, Y., Dymshits, G., Ishikawa, Y., Juji, T., Tokunaga, K., 2001. Expression of renin–angiotensin system genes in immature and mature dendritic cells identified using human cDNA microarray. Biochem. Biophys. Res. Commun. 285 (4), 1059–1065.

Louis, N., Fender, P., Barge, A., Kitts, P., Chroboczek, J., 1994. Cell-binding domain of adenovirus serotype 2 fiber. J. Virol. 68 (6), 4104–4106.

Maeda, T., Towatari, M., Kosugi, H., Saito, H., 2000. Up-regulation of costimulatory/adhesion molecules by histone deacetylase inhibitors in acute myeloid leukemia cells. Blood 96 (12), 3847–3856.

Marini, F.C., Shayakhmetov, D., Gharwan, H., Lieber, A., Andreeff, M., 2002. Advances in gene transfer into haematopoietic stem cells by adenoviral vectors. Exp. Opin. Biol. Ther. 2 (8), 847–856.

Mutti, L., Valle, M.T., Balbi, B., Orengo, A.M., Lazzaro, A., Alciato, P., Gatti, E., Betta, P.G., Pozzi, E., 1998. Primary human mesothelioma cells express class II MHC, ICAM-1 and B7-2 and can present recall antigens to autologous blood lymphocytes. Int. J. Cancer 78 (6), 740–749.

Okegawa, T., Li, Y., Pong, R.C., Bergelson, J.M., Zhou, J., Hsieh, J.T., 2000. The dual impact of coxsackie and adenovirus receptor expression on human prostate cancer gene therapy. Cancer Res. 60 (18), 5031–5036.

Rea, D., Schagen, F.H., Hoeben, R.C., Mehtali, M., Havenga, M.J., Toes, R.E., Melief, C.J., Offringa, R., 1999. Adenoviruses activate human dendritic cells without polarization toward a T-helper type 1-inducing subset. J. Virol. 73 (12), 10245–10253.

Rea, D., Havenga, M.J., van Den Assem, M., Sutmuller, R.P., Lemckert, A., Hoeben, R.C., Bout, A., Melief, C.J., Offringa, R., 2001. Highly efficient transduction of human monocyte-derived dendritic cells with subgroup B fiber-modified adenovirus vectors enhances transgene-encoded antigen presentation to cytotoxic T cells. J. Immunol. 166 (8), 5236–5244.

Roelvink, P.W., Lizonova, A., Lee, J.G., Li, Y., Bergelson, J.M., Finberg, R.W., Brough, D.E., Kovesdi, I., Wickham, T.J., 1998. The coxsackievirus–adenovirus receptor protein can function as a cellular attachment protein for adenovirus serotypes from subgroups A, C, D, E, and F. J. Virol. 72 (10), 7909–7915.

Russell, W.C., 2000. Update on adenovirus and its vectors. J. Gen. Virol. 81 (Pt. 11), 2573–2604.

Sakurai, F., Mizuguchi, H., Hayakawa, T., 2003. Efficient gene transfer into human CD34+ cells by an adenovirus type 35 vector. Gene Ther. 10 (12), 1041–1048.

Salone, B., Martina, Y., Piersanti, S., Cundari, E., Cherubini, G., Franqueville, L., Failla, C.M., Boulanger, P., Saggio, I., 2003. Integrin alpha3-beta1 is an alternative cellular receptor for adenovirus serotype 5. J. Virol. 77 (24), 13448–13454.

Segerman, A., Mei, Y.F., Wadell, G., 2000. Adenovirus types 11p and 35p show high binding efficiencies for committed hematopoietic cell lines and are infective to these cell lines. J. Virol. 74 (3), 1457–1467.

Segerman, A., Arnberg, N., Erikson, A., Lindman, K., Wadell, G., 2003a. There are two different species B adenovirus receptors: sBAR, common to species B1 and B2 adenoviruses, and sB2AR, exclusively used by species B2 adenoviruses. J. Virol. 77 (2), 1157–1162.

Segerman, A., Atkinson, J.P., Marttila, M., Dennerquist, V., Wadell, G., Arnberg, N., 2003b. Adenovirus type 11 uses CD46 as a cellular receptor. J. Virol. 77 (17), 9183–9191.

Stevenson, S.C., Rollence, M., White, B., Weaver, L., McClelland, A., 1995. Human adenovirus serotypes 3 and 5 bind to two different cellular receptors via the fiber head domain. J. Virol. 69 (5), 2850–2857.

Stevenson, S.C., Rollence, M., Marshall-Neff, J., McClelland, A., 1997. Selective targeting of human cells by a chimeric adenovirus vector containing a modified fiber protein. J. Virol. 71 (6), 4782–4790.

Suvas, S., Singh, V., Sahdev, S., Vohra, H., Agrewala, J.N., 2002. Distinct role of CD80 and CD86 in the regulation of the activation of B cell and B cell lymphoma. J. Biol. Chem. 277 (10), 7766–7775.

Tatsumi, T., Takehara, T., Katayama, K., Mochizuki, K., Yamamoto, M., Kanto, T., Sasaki, Y., Kasahara, A., Hayashi, N., 1997. Expression of costimulatory molecules B7-1 (CD80) and B7-2 (CD86) on human hepatocellular carcinoma. Hepatology 25 (5), 1108–1114.

Tomko, R.P., Johansson, C.B., Totrov, M., Abagyan, R., Frisen, J., Philipson, L., 2000. Expression of the adenovirus receptor and its interaction with the fiber knob. Exp. Cell Res. 255 (1), 47–55.

van Kooyk, Y., Geijtenbeek, T.B., 2003. DC-SIGN: escape mechanism for pathogens. Nat. Rev. Immunol. 3 (9), 697–709.

Vasu, C., Wang, A., Gorla, S.R., Kaithamana, S., Prabhakar, B.S., Holterman, M.J., 2003. CD80 and CD86 C domains play an important role in receptor binding and co-stimulatory properties. Int. Immunol. 15 (2), 167–175.

Von Seggern, D.J., Huang, S., Fleck, S.K., Stevenson, S.C., Nemerow, G.R., 2000. Adenovirus vector pseudotyping in fiber-expressing cell lines: improved transduction of Epstein-Barr virus-transformed B cells. J. Virol. 74 (1), 354–362.

Walters, R.W., Freimuth, P., Moninger, T.O., Ganske, I., Zabner, J., Welsh, M.J., 2002. Adenovirus fiber disrupts CAR-mediated intercellular adhesion allowing virus escape. Cell 110 (6), 789–799.

Wickham, T.J., Mathias, P., Cheresh, D.A., Nemerow, G.R., 1993. Integrins alpha v beta 3 and alpha v beta 5 promote adenovirus internalization but not virus attachment. Cell 73 (2), 309–319.

Widney, D., Boscardin, W.J., Kasravi, A., Martinez-Maza, O., 2003. Expression and function of CD28 on Epstein-Barr virus-positive B cell lines and AIDS-associated non-Hodgkin's lymphoma cell lines. Tumour Biol. 24 (2), 82–93.

Wu, E., Fernandez, J., Fleck, S.K., Von Seggern, D.J., Huang, S., Nemerow, G.R., 2001. A 50-kDa membrane protein mediates sialic acid-independent binding and infection of conjunctival cells by adenovirus type 37. Virology 279 (1), 78–89.