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Maturation of Dendritic Cells Accompanies High-Efficiency Gene Transfer by a CD40-Targeted Adenoviral Vector[1]

Bryan W. Tillman,* Tanja D. de Gruijl,† Sylvia A. Luykx-de Bakker,† Rik J. Scheper,‡ Herbert M. Pinedo,†§ Tyler J. Curiel,¶ Winald R. Gerritsen,†§ and David T. Curiel[2]*

Important therapeutic applications of genetically modified dendritic cells (DC) have been proposed; however, current vector systems have demonstrated only limited gene delivery efficacy to this cell type. By means of bispecific Abs, we have dramatically enhanced gene transfer to monocyte derived DC (MDDC) by retargeting adenoviral (Ad) vectors to a marker expressed on DC, CD40. Adenovirus targeted to CD40 demonstrated dramatic improvements in gene transfer relative to untargeted Ad vectors. Fundamental to the novelty of this system is the capacity of the vector itself to modulate the immunological status of the MDDC. This vector induces DC maturation as demonstrated phenotypically by increased expression of CD83, MHC, and costimulatory molecules, as well as functionally by production of IL-12 and an enhanced allostimulatory capacity in a MLR. In comparing this vector to other Ad-based gene transfer systems, we have illustrated that the features of DC maturation are not a function of the Ad particle, but rather a consequence of targeting to the CD40 marker. This vector approach may thus mediate not only high-efficiency gene delivery but also serve a proactive role in DC activation that could ultimately strengthen the utility of this vector for immunotherapy strategies. ***The Journal of Immunology*****, 1999, 162: 6378–6383.**

A growing body of evidence suggests that dendritic cells (DC)[3] play a pivotal role in the immune system (1–4). Foremost, DC are recognized to serve as central mediators of T cell-based immunity. Stemming from this key function, DC have been proposed for use in a number of clinical immunotherapy strategies. In this context, it has become clear that genetic modification of these cells can promote immunity against pathogenic entities, both infectious and tumorigenic (5–8). Importantly, all of these strategies are predicated upon efficient vectors for gene delivery to DC. A number of approaches have been investigated for this purpose, albeit generally with poor efficiency of gene delivery (9, 10). One candidate explored for this application has been the replication-defective adenoviral (Ad) vector (9, 11). This vector has been suggested to be well suited for DC-based clinical applications by virtue of its high titer vector production and exuberant gene expression.

In spite of these theoretical advantages, the relative resistance of DC to Ad vectors has confounded the realization of gene-based immunotherapy strategies (9, 11, 12). We hypothesized that DC resistance to Ad-mediated gene transfer may stem from a paucity of the cellular receptors that mediate Ad entry. In permissive cells, the projecting Ad fiber-knob protein mediates binding to the Coxsackie-adenovirus receptor (CAR) on the cell surface, followed by internalization of the virion facilitated by interaction of Ad penton base with either of the α_v integrins, $\alpha_v\beta_3$ or $\alpha_v\beta_5$ (13–15).

We have determined that while monocyte-derived DC (MDDC) exhibit adequate levels of the α_v integrins, they do not express CAR. We and others have previously shown the utility of redirecting Ad binding to alternate cellular receptors so as to mediate enhanced gene transfer (16–18). The CD40 receptor has been reported to play an essential role in promoting both DC activation as well as Ag-presenting function. We reasoned that by targeting Ad to CD40, this vector approach might simultaneously mediate changes that would augment the immunostimulatory function of genetically modified DC. Here, we report that an Ad targeted to CD40 mediates both high-efficiency gene transfer as well as phenotypic and functional maturation of MDDC. We propose that such a vector may have utility in DC-based vaccination strategies.

Materials and Methods

Culture of MDDC

PBMC were isolated from heparinized peripheral blood of normal human volunteer donors by density centrifugation over Lymphoprep (Nycomed AS, Oslo, Norway). These cells were cryopreserved until use in RPMI 1640 medium supplemented with 12.5% dimethyl sulfoxide and 25% FCS (19). PBMC were suspended at a concentration of 3–5 million cells per ml in IMDM containing 50 U/ml penicillin-streptomycin, 1.6 mM L-glutamine, 0.01 mM 2-ME (complete medium), and 10% FCS and were allowed to adhere to the bottom of plastic culture flasks (NUNC, Intermed, Denmark) at 37°C. The adherent cells were cultured for an additional 6 days in medium supplemented with 1000 U/ml rIL-4 (Centraal Laboratorium van de Bloedtransfusiedienst, Amsterdam, The Netherlands) and 100 ng/ml GM-CSF (Schering-Plough, Madison, N.J.). Adherent MDDC released by 0.5 mM EDTA were pooled with nonadherent MDDC. These cells were characterized by presence of CD1a and CD11c expression as well as the absence of lineage markers, such as CD3, CD14, CD19, and CD56, by FACS. These cells also demonstrated typical DC cell morphology, such as dendritic processes and clustered cells.

Viruses and cell lines

AdCMV luciferase (AdCMVLuc), a first generation E1-, E3-deleted vector expressing firefly luciferase from the CMV immediate early promoter, was obtained from Robert Gerard (University of Leuven, Leuven, Belgium). Ad

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[3] Abbreviations used in this paper: DC, dendritic cell; Ad, adenovirus; CAR, Coxsackie-adenovirus receptor, MDDC, monocyte-derived DC; RLU, relative light units; MOI, multiplicity of infection; EGFR, epidermal growth factor receptor; Luc, luciferase; GFP, green fluorescent protein.

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green fluorescent protein (AdGFP) was obtained from Corey Goldman (University of Alabama, Birmingham, AL).

Viruses were propagated and plaque-titered on the 293 Ad propagation line and purified by double centrifugation on CsCl gradients. All virus aliquots were stored at −80°C until use. The neutralizing murine mAb 1D6.14 specific for the carboxy-terminal, receptor-binding knob domain of Ad serotype 5 fiber has been previously described (17). The pharyngeal epithelial line KB was purchased from American Type Culture Collection (ATCC; Manassas, VA) and was maintained in DMEM with 4.5 g/L glucose.

Abs and conjugates

The anti-CAR mAb RmcB (a generous gift from Robert W. Finberg, Harvard Medical School, Boston, MA) has been previously described (13). Murine mAb LM609 to $\alpha_v\beta_3$ integrin and P1F6 to $\alpha_v\beta_5$ integrin were purchased from Chemicon (Temecula, CA) and Life Technologies (Gaithersburg, MD), respectively. For maturational and lineage marker analysis, Abs used were directly conjugated to FITC or PE. These included: HB15a (anti-CD83), MAB89 (anti-CD40) (Immunotech, Marseille, France), L243 (anti-HLA-DR) (Becton Dickinson, San Jose, CA), 2331 (anti-CD86), HA58 (anti-CD54), and TU169 (anti-HLA-DQ) (PharMingen, San Diego, CA). The hybridomas G28.5, TS2/16.2.1, and 528, producing mAbs against CD40, the β_1 integrins, and epidermal growth factor receptor (EGFR), respectively, were purchased from ATCC. These hybridomas were used to generate ascites in SCID mice. Abs were purified on a fast protein liquid chromatography system using HiTrap protein A columns (Pharmacia, Piscataway, NJ) and the mAb purification system binding buffer system (Bio-Rad, Hercules, CA). The 1D6.14 mAb was digested with immobilized papain (Pierce, Rockford, IL), and Fab fragments were purified by negative selection of Fc fragments using the HiTrap protein A columns. Bispecific Abs consisting of the 1D6.14-neutralizing anti-Ad knob Fab fragment and one of several anti-receptor Abs (G28.5, TS2/16.2.1, or 528) were prepared by chemical cross-linking with *N*-succinimidyl 3-(2-pyridldithio) propionate (SPDP), as previously described (20). Conjugates using the G28.5 mAb, TS2/16.2.1 mAb, and 528 mAb are henceforth designated as Fab-anti-CD40, Fab-anti-β_1-integrins, and Fab-anti-EGFR, respectively. Lipofectamine (Life Technologies) was used for liposome-complexed Ad, as previously described (11).

Conjugate titration to determine optimal conjugate to virus ratio

To determine the amount of retargeting conjugate necessary to optimally enhance gene transfer, the conjugate was titrated with AdCMVLuc at a multiplicity of infection (MOI) of 100 and used to infect MDDC by previously described methods (17). The mass of conjugate corresponding to the highest levels of luciferase gene expression was termed an "optimal ratio of conjugate to virus" and was used in all subsequent experiments. This ratio was determined to be 30.3 ng:2.4 $\times$ 10^6 PFU for the Fab-anti-CD40 and Fab-anti-β_1 integrins conjugates and 60 ng:2.4 $\times$ 10^6 PFU for the Fab-anti-EGFR conjugate. Adenovirus conjugated with the optimal ratio of Fab-anti-CD40, Fab-anti-β_1 integrins, and Fab-anti-EGFR will be referred to henceforth as CD40-targeted Ad, β_1-integrin-targeted Ad, and EGFR-targeted Ad, respectively.

AdCMVLuc infection and luciferase analysis

Twenty-four thousand MDDC in a volume of 50 μl of complete RPMI with 2.5% FCS were distributed to individual microcentrifuge tubes in triplicate for each test condition. The use of microcentrifuge tubes enabled simplified infection and washing of the pooled adherent and nonadherent MDDC. Cells were preblocked for 30 min as indicated with either media or blocking agent consisting of the unconjugated anti-CD40 Ab at 100 μg/ml. All blocking was performed at 4°C to minimize premature receptor modulation from the cell surface. Conjugate and virus were incubated for 30 min at room temperature in a volume of 20 μl per each test condition. Following incubation, the mixture was diluted such that 100 μl was used to infect each microcentrifuge tube or well of cells. The amount of virus in this volume corresponded to an MOI of 100. Following further incubation for a period of 30 min at 4°C, cells were washed, resuspended in complete RPMI with 10% FCS, and plated on polylysine-coated 24-well plates (Becton Dickinson) before transition to 37°C. Following 24 h of incubation postinfection, wells were processed using the Promega (Madison, WI) luciferase assay kit. The lysates were evaluated on a Lumat luminometer (Wallac, Gaithersburg, MD). The results were normalized for the number of cells present during infection.

Analysis of differential MOI between CD40-targeted and untargeted Ad

Fab-anti-CD40 was complexed with AdCMVLuc at a concentration corresponding to 1000 MOI. Subsequently, this mixture was serially diluted to MOIs of 100, 10, and 1. Simultaneously, samples of the same MOIs of Ad but without retargeting conjugate were prepared. MDDC were then infected and processed as described under "AdCMVLuc Infection and Luciferase Analysis" methods.

Flow cytometry analyses

For analyses of Ad entry receptors, MDDC or KB were stained using mAb with affinity for CAR, $\alpha_v\beta_3$, or $\alpha_v\beta_5$, followed by an FITC-labeled rabbit anti-mouse Fc-specific secondary Ab (Jackson Immunoresearch Laboratories, West Grove, PA). These samples were analyzed on a FACStar using Cellquest FACS analysis software (Becton Dickinson). To assess changes in expression of maturation and lineage markers, MDDC were batch-infected using AdCMVLuc complexed to media (untargeted Ad), lipofectamine (liposome-complexed Ad), or the optimal ratio of the indicated Fab-anti-receptor conjugates. Twenty-four hours postinfection, cell staining and FACS analysis were performed using mAbs with affinity to CD83, CD40, HLA-DR, CD86, CD54, or HLA-DQ directly conjugated to FITC or PE. Samples were assessed by FACS analysis. To determine the percentage of MDDC transduced, 24 h before analysis, cells were infected with AdGFP that had been complexed for 30 min with Fab-anti-CD40, Fab-anti-β_1 integrins, or liposomes. After incubation for 1 h at 37°C, cells were washed with PBS and left to incubate in microcentrifuge tubes in complete RPMI with 10% FCS for the duration of the 24-h incubation.

DC functional assays

For allogeneic MLR, MDDC were infected as described in *Materials and Methods*. Seventy-two hours after infection, these DC were added as stimulator cells to round-bottom 96-well culture plates (Nunclon Delta, Intermed, Denmark) at graded doses reflecting the indicated responder-stimulator ratios (R:S). Nonadherent lymphocyte fractions were used as a source for responder cells, and 100,000 lymphocytes were added per well to the allogeneic MDDC. The cells were cultured for 3 days in complete medium with 10% human pooled serum (CLB, Amsterdam, The Netherlands). During the last 18 h, [^{3}H]thymidine was added (0.4 μCi per well) (Amersham, Aylesbury, U.K.), after which the cells were harvested onto fiberglass filters and [^{3}H]thymidine incorporation was determined using a flatbed liquid scintillation counter (Wallac). IL-12 production was assessed using an ELISA, as described (21). Forty-eight hours postinfection, assessment was made of supernatants in which a million cells had been incubated in 1 ml of media.

Results

MDDC are deficient in CAR

Using Abs with affinity for CAR or specific for each of the integrin heterodimers $\alpha_v\beta_3$ and $\alpha_v\beta_5$, we assessed MDDC as compared with an Ad-permissive epithelial line, KB. This analysis revealed an absence of CAR on DC but confirmed the expression of $\alpha_v\beta_5$ (Fig. 1*A*) and $\alpha_v\beta_3$ (data not shown). This contrasts to KB cells, which express both CAR and $\alpha_v\beta_5$ (Fig. 1*B*). Based on understanding of the Ad entry pathway (13–15), these findings suggest that the absence of CAR expression is the likely cause for the resistance of DC to adenovirus.

Enhanced, CD40-specific gene transfer by CD40-targeted Ad

Based on our previous success at mediating CAR-independent gene transfer using retargeted Ad, we postulated that a similar strategy targeting CD40, a marker expressed on DC, might enable enhanced gene transfer in spite of the absence of CAR expression. For this purpose, a bispecific Ab was generated through chemical conjugation of a Fab fragment of a neutralizing anti-fiber-knob mAb to a mAb with affinity for CD40, a receptor expressed on DC. MDDC were prepared by culture of adherent monocytes in the presence of IL-4 and GM-CSF, and the DC phenotype was confirmed by characteristic receptor expression and morphology. The Fab-anti-CD40 conjugate was titered against Ad to ascertain the optimal ratio of conjugate to virus as measured by improvements

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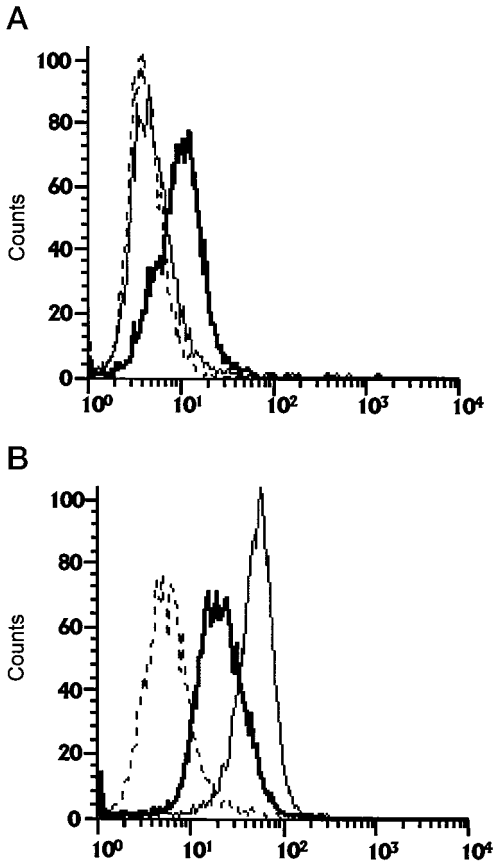

FIGURE 1. MDDC are deficient in the primary Ad-entry receptor. Relative to an isotype control (dashed line), MDDC (*A*) are negative for expression of CAR (thin line) but do express sufficient $\alpha_v\beta_5$ (thick line), as determined by flow cytometry, while the Ad-permissive epithelial line KB (*B*) is positive for both of these receptors.

in gene transfer to MDDC (data not shown). The magnitude of gene expression mediated by Ad carrying the gene for luciferase, as well as specificity for CD40, was illustrated on MDDC and also on a CD40-negative glioma line, D65, which, like MDCC, is both negative for expression of CAR and has been reported to be resistant to gene transfer by Ad (22). As illustrated in Fig. 2, compared with untargeted Ad, Ad complexed with Fab-anti-CD40 conjugate (CD40-targeted Ad) mediated several orders of magnitude enhancement in gene expression in MDDC. Furthermore, this enhancement was blocked by ~90% when cells were pretreated with an excess of the unconjugated anti-CD40 mAb. As evidence that the anti-CD40 mAb did not promote gene transfer by a mechanism independent of its association with the virion, no enhancement was observed in cells pretreated with unconjugated anti-CD40 mAb before infection with untargeted Ad. Further specificity was illustrated by failure of the Fab-anti-CD40 conjugate to enhance Ad-based gene transfer to the CD40-negative glioma, D65 (data not shown). The successful anti-CD40 blockade of enhanced gene transfer to MDDC, as well as the restriction of enhancements in gene transfer to cells expressing CD40, indicate that this vector is specifically targeted to cells by means of its affinity for CD40.

CD40-targeted Ad reduces the viral dose for a given magnitude of gene expression

Dose-related cytotoxic effects of adenoviral vectors can compromise potential applications (23). Thus, the capacity to reduce viral dose would increase the utility of adenoviral vectors immensely.

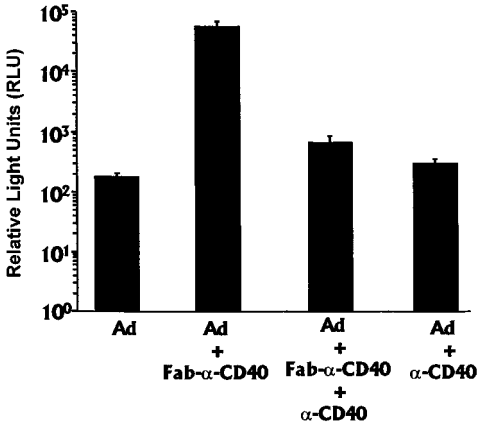

FIGURE 2. Ad targeted by Fab-anti-CD40 mediates enhanced magnitude of gene transfer that is specific for CD40. MDDC preincubated in either the presence or absence of unconjugated anti-CD40 mAb were infected with AdCMVLuc (MOI 100) either alone or complexed with Fab-anti-CD40. After 24 h of incubation, cells were assessed for expression of luciferase, measured as relative light units (RLU). The background of the luminometer is ~100 RLU in the absence of sample.

To compare the efficacy of this retargeting strategy in MDDCs, CD40-targeted Ad was compared with untargeted Ad at several MOI (Fig. 3). The results illustrated that, with increased numbers of infectious particles per cell, gene transfer was progressively increased under both conditions. Nevertheless, it is apparent that CD40-targeted Ad mediates a level of expression greater than untargeted Ad, even when 100-fold more virus is used. For example, CD40-targeted Ad attained a magnitude of gene expression at an MOI of 10 equivalent to that observed with untargeted Ad at an MOI of 1000. These findings indicate that by adenoviral targeting to CD40, the number of infectious particles required to attain a given level of gene expression is reduced appreciably.

CD40-targeted Ad enables gene transfer to increased numbers of cells

While luciferase gene transfer illustrated an overall increase in gene expression by CD40-targeted Ad, this assay can indicate only the magnitude of gene expression. To exclude the possibility that the enhancements merely reflect a small subset of DC that was transduced multiple times, we assayed the actual percent of cells transduced by Ad-mediated delivery of the gene for GFP. At this stage, we also employed two other high-efficiency Ad-based vector

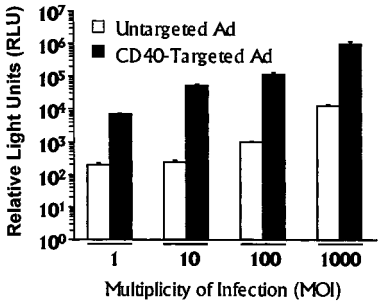

FIGURE 3. Targeting of Ad to CD40 reduces the viral MOI necessary to attain a given level of gene expression. Virus, either in the presence or absence of Fab-anti-CD40 conjugate, was incubated for 30 min and subsequently serially diluted to correspond to MOI of 1000, 100, 10, and 1. MDDC were infected for 1 h, and cells were assayed at 24 h for luciferase expression. The background of the luminometer is ~100 RLU in the absence of sample.

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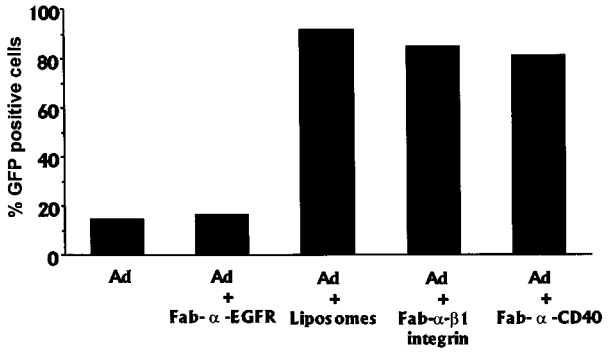

FIGURE 4. CD40-targeted, β_1 integrin-targeted and liposome-complexed Ad mediate comparable gene transfer to MDDC. MDDC were infected with Ad (MOI 100)-encoding GFP preincubated with one of the following: PBS, Fab-anti-CD40, Fab-anti-β_1 integrin conjugate, Fab-anti-EGFR conjugate or liposomes. After 24 h of incubation, the conditions were assessed using flow cytometry for expression of GFP and are displayed as percent GFP-positive cells based on analysis of 10,000 cells. This depicts a representative of two experiments.

systems for comparison with CD40-targeted Ad. The Fab-anti-β_1 integrin conjugate has a similar construction to the CD40-targeting conjugate but with affinity for the widely expressed β_1 integrin subunit. Meanwhile, Ad complexed with liposomes has been described previously for gene transfer to MDDC (11). As an additional control, a conjugate (Fab-anti-EGFR) targeted to a receptor absent from MDDC, EGFR, was included to ascertain any nonspecific binding by conjugate-based strategies. GFP expression was quantitated through use of flow cytometry and indicated that Ad vectors complexed with Fab-anti-CD40, Fab-anti-β_1 integrin, and liposomes all transduced similarly enhanced percentages of cells relative to untargeted Ad (Fig. 4). The failure of Ad targeting to EGFR to enhance gene transfer illustrates the specificity of conjugate-based targeting. In aggregate, it is apparent that the CD40-targeted, β_1-integrin-targeted, and liposome complexed Ad-based vector systems mediate comparable levels of enhancement in gene transfer efficacy.

CD40-targeted Ad induces phenotypic and functional characteristics of mature DC

The anti-CD40 mAb employed in the targeting conjugate, G28.5, has been previously described for its cross-linking-dependent partial agonist activity on CD40 in B cells (24). As activation of CD40 has been established to have profound effects on DC (3, 25, 26), we sought to evaluate the effect of CD40-targeted Ad on DC phenotype and function. To determine the effects on DC maturation by the retargeted Ad vectors or the CD40-retargeting conjugate alone, several markers were analyzed using flow cytometry. Cells infected 24 h previously were analyzed for expression of CD83, ICAM-1, CD86, HLA-DR (Fig. 5), as well as HLA-DQ (data not shown). While no alterations in DC phenotype were observed when Ad was used alone or when targeted by an irrelevant conjugate, Fab-anti-EGFR (data not shown), clear changes including augmented expression of CD86, HLA-DR, and HLA-DQ were observed with all three high-efficiency Ad gene delivery systems, as well as by the Fab-anti-β_1 integrin-targeting conjugate alone (data not shown). Unique characteristics imparted by treatment with either Fab-anti-CD40 conjugate or CD40-targeted Ad included increased expression of CD83 and ICAM-1, features seen only negligibly, if at all, in cells infected with β_1 integrin-targeted or liposome-complexed Ad. Of note, CD83 enhancement appeared more remarkable with CD40-targeted Ad than with CD40-targeting conjugate alone. As an indication of functional maturation, MDDC-treated, using the targeted Ad vectors or Fab-anti-CD40 conjugate alone, were combined with responder cells from an allogeneic donor and tested for their capacity to elicit an MLR (Fig. 6). While Ad alone did not mediate any enhancement in MLR, use of Fab-anti-CD40 conjugate either in the presence or absence of Ad was able to promote enhanced MDDC reactivity in the allo-MLR relative to uninfected cells by an average of 2-fold $\pm$ 0.6 ($n = 4$). Moreover, the effect of conjugate alone was comparable to that seen with the conjugate plus virus. One possible explanation of the maturational effects observed with CD40-targeting could have been a viral-mediated effect from high-efficiency entry of Ad particles into DC. It was for this reason that DC were infected with

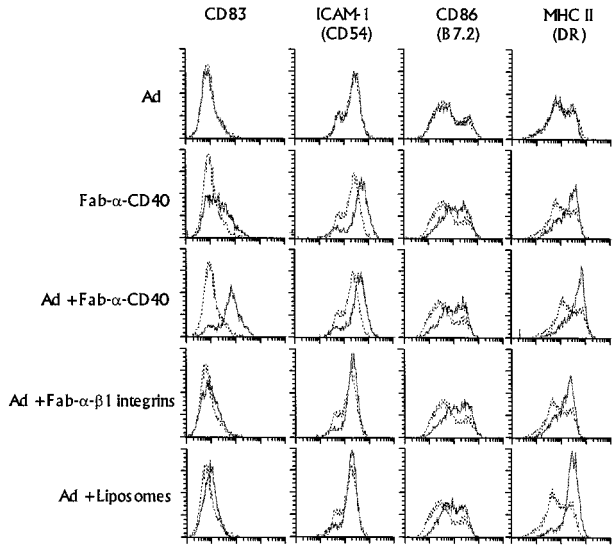

FIGURE 5. CD40 targeting induces expression of DC maturational markers. MDDC were treated as indicated and incubated for 24 h before analysis. Samples shown indicate expression of CD83, CD54, CD86, and HLA-DR (solid line) by flow cytometry and are depicted as compared with mock-transduced MDDC (dotted line). Abs directly conjugated to fluorophores were used for these analyses. An MOI of 100 was used in instances where Ad was employed. A total of 5000 per condition were counted.

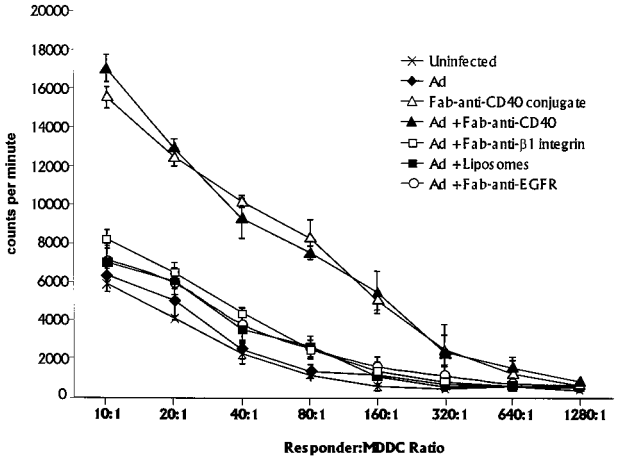

FIGURE 6. Ad targeting to CD40 mediates enhancement in the capacity to generate an allo-MLR. MDDC were infected (MOI 100) with the indicated conditions and, after 72 h of incubation, were mixed with nonadherent lymphocyte responder cells at the indicated responder-MDDC ratios. Cells were ^{3}H-labeled and assessed for cell-associated cpm after 3 days. This is a representative result from four experiments.

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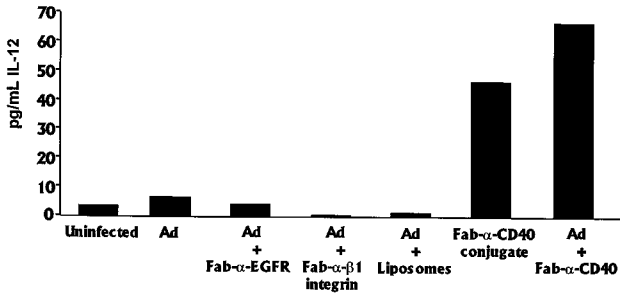

FIGURE 7. IL-12 production is enhanced after treatment with Fab-anti-CD40 conjugate or CD40-targeted Ad. MDDC were treated with the indicated retargeted Ad vectors (MOI 100) or the Fab-anti-CD40 conjugate in the absence of Ad. At 48 h, the supernatants were assessed by ELISA for production of IL-12. Values <8 pg/ml are beyond the linear range of detection by this assay. A representative experiment of two is shown.

the alternate high-efficiency Ad vectors β_1-integrin-targeted Ad or liposome-complexed Ad and tested in an MLR. The failure of these latter two vector systems to mediate notable enhancements suggests that the maturation phenomenon is CD40-associated. As further evidence of functional maturation, MDDC supernatants were tested at 48 h postinfection for production of IL-12 (Fig. 7), a cytokine for which expression is characteristic of DC maturation and which plays a critical role in the induction of cellular immunity (12, 27). The results indicated that IL-12 levels were elevated in supernatants of cells treated either with Fab-anti-CD40-retargeting conjugate alone or with CD40-targeted Ad. In contrast, IL-12 augmentation was not observed for untargeted, EGFR-targeted, β_1-integrin-targeted, or liposome-complexed Ad.

Discussion

Despite enormous clinical potential, widespread application of genetically modified DC has been hindered by several obstacles. Among these are the extensive handling required for ex vivo transduction, the poor gene transfer efficacy by existing vectors, and the necessity to mature DCs to an optimal T cell activation status subsequent to gene transfer (2, 28). With regard to the latter, peripheral DC active in the process of Ag capture are referred to as "immature DC." In spite of active Ag retrieval, these cells do not express the adequate panel of costimulatory molecules and cytokines necessary to activate immune effector cells. Therefore, immature DC must be differentiated to an immunologically potent "mature" state that can optimally stimulate immune cells (3, 25, 26, 29). For this reason, we sought to understand what effects this CD40-targeted Ad vector would have on the maturation status of DC.

The ability of the anti-CD40 conjugate to mediate DC maturation in the absence of virus clearly indicates that the maturation phenomenon is Ad-independent. Further, expression of CD83 and ICAM-1, production of IL-12, and improved MLR were observed almost exclusively with treatment of MDDC by Fab-anti-CD40 conjugate or CD40-targeted Ad but not with other Ad vectors tested. Based on this finding, it seems fairly certain that the observed DC maturation is a direct and specific result of CD40 engagement.

In summary, it appears from our results that Ad, as reported for several other viruses (3), mediates minor effects on DC phenotype, but that these effects are seen only when a sufficient number of particles enter each cell, such as by the high-efficiency Ab-targeted or liposome-complexed Ad-based vectors. It is interesting to speculate as to whether the enhanced expression of some costimulatory molecules seen with β_1 integrin-targeted or liposome-complexed Ad is a consequence of the capsid itself entering the cell, of reporter gene expression, or of background Ad gene expression. Since the conjugate, or even the unconjugated anti-CD40 mAb, can mediate agonistic DC maturation in an Ad-independent fashion, it would at first seem needless to introduce an adenovirus into the formula. This would indeed be the case if maturation were the only desired outcome. In instances where delivery of an Ag-encoding gene is desired, however, the only way to attain the requisite level of gene transfer is by appending a targeting conjugate to overcome the deficiency of native Ad-binding receptors on DC. Thus, it seems clear that the conjugate forms a fundamental link between the maturational features of an agonistic anti-CD40-Ab and the gene transfer capacity of an adenoviral vector in a manner that exceeds the value of either component individually.

A CD40-targeted Ad vector exhibits several features that may prove attractive for Ad-based vaccination. By virtue of its highly efficient gene delivery, it should be possible to deliver cryptic Ags that might otherwise not be accessible to the immune system. Also, the restricted gene delivery to cells expressing CD40 may prove advantageous for in vivo approaches when compared with less specific vectors like Ad alone or Ad complexed with liposomes. With regard to the maturational capacity of this vector, it has been reported that activation of DC to maturity renders them resistant to both the effects of DC inhibitory cytokines like IL-10 (2, 30, 31) as well as to direct tumor-induced apoptosis (32). Further, the capacity with which murine DC can generate an immune response in vivo has been shown to correlate with the degree of their maturation (33). Moreover, based on proposals that CD40 activation may bypass $CD4^+$ T cell help (3, 25, 26), a CD40-targeted Ad might also have applications in cases of $CD4^+$ dysfunction. The dual role of CD40 in this schema as both a surrogate Ad receptor and a powerful trigger of DC maturation may prove useful as a retargeting strategy to this central cell type of the immune system. Regardless, we have recognized the limitations of this Ab-based targeting strategy for intensive clinical applications. For this reason, we are currently pursuing a genetic fusion strategy between the trimeric Ad fiber and the natural ligand of CD40, CD40L, which is also trimeric (34).

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