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Adenoviruses Activate Human Dendritic Cells without Polarization toward a T-Helper Type 1-Inducing Subset

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Human monocyte-derived dendritic cells (DC) infected with recombinant adenoviruses (rAd) are promising candidate vaccines for inducing protective immunity against pathogens and tumors. However, since some viruses are known to negatively affect DC function, it is important to investigate the interactions between rAd and DC. We now show that infection by rAd enhances the immunostimulatory capacity of immature human monocyte-derived DC through the upregulation of the costimulatory molecules CD80, CD86, and CD40 and the major histocompatibility complex class I and II molecules. Although rAd infection fails to induce the secretion of interleukin-12 (IL-12) and only marginally induces the expression of the DC maturation marker CD83, it acts in synergy with CD40 triggering in rendering DC fully mature. rAd-infected DC triggered through CD40 produce more IL-12 and are more efficient in eliciting T-helper type 1 responses than DC activated by CD40 triggering only. rAd lacking one or more of the early regions, E1, E2A, E3, and E4, which play an important role in virus-host cell interactions are equally capable of DC activation. Efficient DC infection requires a high multiplicity of infection (>1,000), a fact which can be attributed to the absence of the coxsackievirus and adenovirus receptor on this cell type. Despite the poor ability of DC to be infected by rAd, which may be improved by targeting rAd to alternative DC surface molecules, DC infected with all currently tested rAd constitute potent immunostimulators. Our study provides new insights into the interactions between two highly promising vaccine components, rAd and DC, and indicates that their combination into one vaccine may be very advantageous for the stimulation of T-cell immunity.

Dendritic cells (DC), the most potent antigen-presenting cells (APC) of the immune system, are crucial initiators of T-lymphocyte responses against pathogens and tumors. However, immature DC, which are specialized in antigen capture in peripheral tissues, are poor stimulators of T cells. The development of DC into immunostimulatory APC depends on their activation into mature cells, characterized by high costimulatory and antigen-presenting functions, loss of endocytic activity, secretion of interleukin-12 (IL-12), and ability to migrate to T-cell areas in the lymph nodes (2). Stimuli capable of triggering DC maturation include inflammatory cytokines, such as tumor necrosis factor alpha (TNF-α) and IL-1; bacterial products, such as lipopolysaccharide (LPS) (40, 41); and ligation of CD40 at the DC surface with soluble CD40 ligand (CD40L) or upon interaction with CD40L-expressing $CD4^+$ T-helper (Th) type 1 (Th1) cells (8, 9, 29).

Given their remarkable immunostimulatory properties, DC are highly promising vaccines against infectious diseases and cancer. Strategies aiming at modifying DC to express foreign antigens utilize the uptake of RNA, proteins, or peptide epitopes or the introduction of specific genes. A major advantage of gene transfer over loading of DC with proteins or peptides resides in the sustained production of the antigen of choice over time, allowing DC to acquire the capacity to trigger T cells while continuously presenting specific peptide epitopes. Several groups have used recombinant adenoviruses (rAd) as vehicles for foreign gene transfer into murine DC and have shown the value of these infected DC as a preventive and therapeutic vaccine against cancer (6, 35, 46, 50). Furthermore, human DC engineered with rAd producing melanoma antigens were found to elicit melanoma-specific cytotoxic T lymphocytes (CTLs) in vitro (7).

Viruses capable of infecting DC exert contrasting effects on APC function, leading either to immunity or to immunosuppression. DC infected with influenza virus are able to bypass the requirement for CD40 signals provided by $CD4^+$ Th cells for the generation of CTL responses (36). Influenza virus can indeed mimic CD40 signals and promote DC maturation in a fashion comparable to that of CD40 ligation (10). In contrast, measles virus and human immunodeficiency virus negatively interfere with DC function (5, 17, 20, 43), and measles virus-infected DC undergo apoptosis upon CD40 ligation (44).

Because rAd-modified DC have been proposed as candidate vaccines against pathogens and cancer, it is of crucial importance to explore the DC-modulating effects of rAd. Human monocyte-derived DC obtained by culturing of peripheral blood monocytes with granulocyte-macrophage colony-stimulating factor (GM-CSF) and IL-4 can be easily generated in sufficient numbers for clinical use (3, 38, 39) and, upon CD40 ligation, become one of the most immunostimulatory human DC types (2, 9, 37). We explored the impact of rAd infection on human monocyte-derived DC biology and examined whether rAd could interfere with CD40-mediated DC maturation. Our results show that rAd enhance the immunostimulatory functions of DC by increasing their costimulatory and antigen-presenting functions but do not trigger the secretion of IL-12. Moreover, we show that rAd act in synergy with CD40 signals to promote the full maturation of rAd-infected DC.

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MATERIALS AND METHODS

Generation of DC from peripheral blood monocytes. Human peripheral blood mononuclear cells (PBMC) obtained from healthy donors and isolated through Ficoll-Hypaque density centrifugation were plated at 1.5×10^7 per well in six-well plates (Costar Corp., Cambridge, Mass.) containing RPMI 1640 medium (Life Technologies, Paisley, Scotland) supplemented with 2 mM glutamine and 10% fetal calf serum. After 2 h of incubation at 37°C, the nonadherent cells were removed. The adherent fraction was cultured in the presence of 800 U of GM-CSF (kindly provided by S. Osanto, LUMC, Leiden, The Netherlands) per ml and 500 U of IL-4 (Pepro Tech Inc., Rocky Hill, N.J.) per ml for a total of 7 days. After 7 days, the DC obtained were 80 to 95% pure and expressed typical markers of immature cells, being $CD14^-$, $CD1a^+$, $CD80^+$, $CD86^+$, $HLA\text{-}DR^+$, HLA class I^+, and $CD83^-$. Maturation of DC was carried out by activation through CD40 with a CD8-CD40L fusion protein made of the extracellular domain of human CD40L and of the murine CD8α chain (kindly provided by Pierre Garrone, Schering Plough, Dardilly, France) (18). Immature DC were collected and incubated at 5×10^5 cells/ml in 24-well plates in the presence of CD8-CD40L for 48 h. In some experiments, lipopolysaccharide (LPS) (100 ng/ml) or poly(I · C) (50 μg/ml) (both from Sigma), monocyte-conditioned medium (MCM; 30% final volume), or TNF-α (100 ng/ml) (Pepro Tech Inc.) was used as a DC maturation agent.

rAd vectors. rAd with deletions of E1 and E3 (AdTG6401), E1, E3, and E2A (AdTG9542), and E1, E3, and E4 (AdTG9546) have been described previously (30). rAd5CMVβ-gal (referred to here as AdCMVβ-gal), which carries the *Escherichia coli*-derived *lacZ* gene as a reporter, was obtained from Joachim Herz (21). Adenovirus vector stocks were generated and purified by double CsCl density centrifugation essentially as described previously (16). To remove the CsCl, the virus bands were mixed with 1 ml of dialysis buffer (TD buffer [25 mM Tris-Cl, 137 mM NaCl, 5 mM KCl, 0.73 mM Na_2HPO_4, 0.9 mM CaCl, 0.5 mM $MgCl_2$] [pH 7.45]) and dialyzed against 2 liters of TD buffer, which was refreshed four times, at 4°C. The final dialysis was performed with TD buffer containing 10% sucrose. Virus stocks were stored at −80°C until further use. Virus stocks were routinely tested for the presence of replication-competent adenoviruses (RCA) by PCR. In the stocks used, no RCA could be detected. The sensitivity of the assay is approximately 1 RCA per 5×10^7 PFU. Wild-type adenovirus type 5 was propagated on HEpG2 cells and purified as described above.

Infection of DC with rAd. Immature DC or mature DC (5×10^5) were resuspended in 500 μl of medium and incubated with AdCMVβ-gal at various multiplicities of infection (MOI). After 2 h at 37°C, 500 μl of medium containing GM-CSF was added. Transduction efficiency was assessed 24 h later by measuring the expression of intracellular β-galactosidase by use of a Fluoreporter *lacZ* flow cytometry kit (Molecular Probes Inc., Eugene, Oreg.) according to the manufacturer's instructions and a FACScan (Becton Dickinson, San Jose, Calif.).

Analysis of the DC surface phenotype by flow cytometry. DC were stained on ice with mouse monoclonal antibodies for 30 min in phosphate-buffered saline–1% fetal calf serum, followed by 30 min of staining with fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse Fab fragments (Immunotech, Marseille, France) when necessary, and were analyzed by flow cytometry. The following antibodies were used: phycoerythrin (PE)–anti-CD1a (Caltag Laboratories, Burlingame, Calif.); PE–anti-CD80 (BB1), PE-anti-CD86 (FUN-1), FITC–anti-CD40 (5C3), PE–anti-CD54 (HA58), and PE–anti-CD58 (1C3) (all from Pharmingen, San Diego, Calif.); PE–anti-CD14 (L243), PE–anti-HLA-DR (mφP9), and PE- and FITC-conjugated isotype controls (all from Becton Dickinson); PE–anti-CD83 (Immunotech); and FITC–anti-HLA class I (Serotec Inc., Raleigh, N.C.). Anti-αVβ3 and -αVβ5 integrin antibodies were obtained from Chemicon International, Inc. (Temecula, Calif.). The hybridoma cell line producing the OKM1 antibody, directed against αMβ2 integrins, was obtained from the American Type Culture Collection. The anti-coxsackievirus and adenovirus receptor (CAR) antibody was a kind gift from J. M. Bergelson (Dana-Farber Cancer Institute, Boston, Mass.) and has been previously described (24).

Antigen uptake experiments. DC were resuspended in medium buffered with 25 mM HEPES; FITC-bovine serum albumin (BSA) and FITC-mannosylated BSA (Sigma) were each added at 1 mg/ml (final concentration). The cells were incubated at 37 or 0°C to determine background uptake. After 1 h, the cells were washed extensively with ice-cold phosphate-buffered saline and analyzed by flow cytometry with propidium iodide to eliminate dead cells.

Cytokine detection by ELISA. Culture supernatants were analyzed in serial twofold dilutions in duplicate. For IL-12 p40 and gamma interferon (IFN-γ) detection (sensitivity, 10 pg/ml), capture monoclonal antibodies and biotinylated polyclonal antibodies were obtained from Peter van de Meijde (BPRC, Rijswijk, The Netherlands). IL-12 p70 was detected with a solid-phase sandwich enzyme-linked immunosorbent assay (ELISA) kit (Diaclone Research, Besancon, France) (sensitivity, 3 pg/ml). IL-10 was detected with a Pelikine compact ELISA kit (CLB, Amsterdam, The Netherlands) (sensitivity, 3 pg/ml).

Antigen presentation assays. The *Mycobacterium tuberculosis* and *M. leprae* hsp65-specific HLA-DR3-restricted $CD4^+$ Th1 clone Rp15 1-1 used in this study has been previously described (19) and recognizes an hsp65 epitope corresponding to peptide residues 3 to 13 (referred to here as p3-13) in the context of HLA-DR3 molecules. HLA-DR-matched immature DC were pulsed with 10 μg of p3-13 per ml for 2 h at 37°C, washed extensively, and then infected with AdCMVβ-gal at 1,000 PFU/cell, incubated with CD8-CD40L, or left untreated as described above. After 48 h, Rp15 1-1 T cells (10^4) were cultured with different numbers of gamma-irradiated (3,000 rads) DC in triplicate in 96-well flat-bottom plates (Costar) for 3 days. In some experiments, IL-12 (Sigma) was added at 250 pg/ml (final concentration). [^{3}H]thymidine incorporation was measured on day 3 after a 16-h pulse. Before the addition of [^{3}H]thymidine, 50 μl of supernatant was collected from each well, and supernatants from triplicate wells were pooled to measure IFN-γ production. For the generation of p3-13-specific T cells from $HLA\text{-}DR3^+$ individuals, immature DC, rAd-infected DC, CD40-triggered DC, and rAd-infected CD40-stimulated DC pulsed with 10 μg of hsp65 per ml were cocultured with autologous nonadherent responders in 24-well plates (Costar) at a ratio of 1 to 10. Five days later, cultures were supplemented with 10% TCGF (Biotest, Dreieich, Germany), fed every other day, and tested for the presence of p3-13-specific and adenovirus-specific T cells 8 days later. In brief, 10^4 responders were cocultured with 5×10^4 gamma-irradiated autologous PBMC in triplicate, either in the absence of antigen or in the presence of 1 μg of p3-13 per ml, 1 μg of 70K peptide (a DR3-binding negative control peptide) per ml, or AdCMVβ-gal. [^{3}H]thymidine incorporation and IFN-γ production were measured as described above.

Statistical analysis. Covariance analysis was used to compare T-cell proliferation and IFN-γ production as a function of DC numbers in immature uninfected DC, rAd-infected DC, and CD40-triggered DC in the presence or absence of exogenous IL-12 (see Fig. 5).

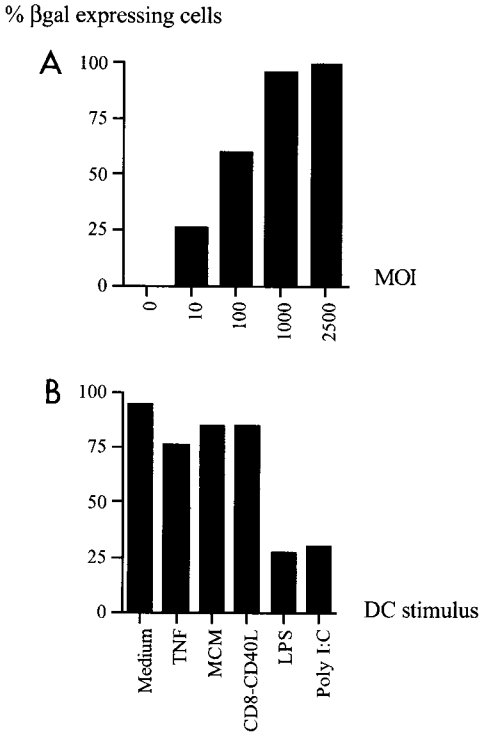

FIG. 1. Expression of β-galactosidase (βgal) by immature and mature DC after infection with AdCMVβ-gal. (A) Immature DC were incubated with AdCMVβ-gal at MOI ranging from 10 to 2,500 and tested 24 h later with uninfected controls for βgal expression. Intracellular βgal was detected with a Fluoreporter *lacZ* flow cytometry kit. Results are expressed as the percentage of βgal-expressing cells and are representative of three independent experiments. (B) DC either were allowed to mature in the presence of TNF-α, MCM, CD8-CD40L, LPS, or poly(I · C) or were cultured in control medium and then were further incubated with AdCMVβ-gal at 1,000 PFU/cell. After 24 h, DC were analyzed for βgal expression as described above. Uninfected mature DC failed to express βgal (data not shown). The data are representative of two independent experiments.

RESULTS

Poor permissiveness of immature and mature human monocyte-derived DC for rAd. The susceptibility of human monocyte-derived DC to rAd was evaluated by incubating immature DC with AdCMVβ-gal at MOI ranging from 10 to 2,500 PFU/cell. As shown in Fig. 1A, the number of infected DC increased with the amount of virus used for infection, with less than 60%

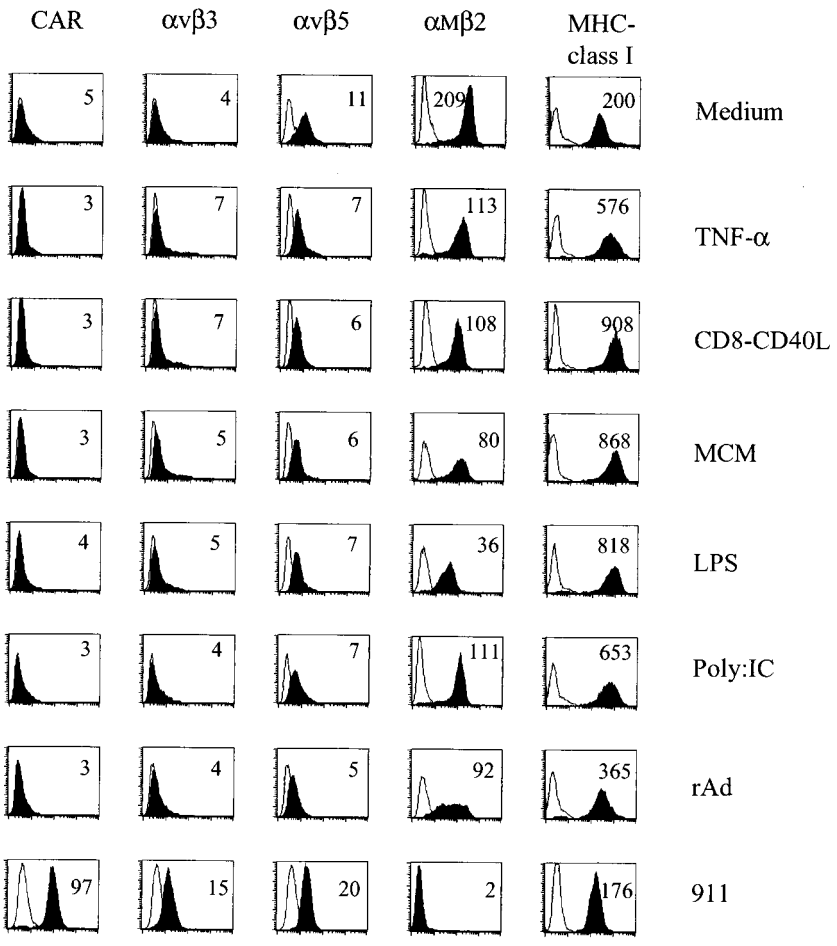

FIG. 2. Surface expression of CAR, of the αVβ3, αVβ5, and αMβ2 integrins, and of MHC class I molecules on immature DC, rAd-infected DC, and different types of mature DC. DC were cultured in control medium, infected with AdCMVβ-gal at 1,000 PFU/cell, or allowed to mature with different stimuli and were analyzed by flow cytometry 48 h later. 911 cells are shown as a positive control. White histograms show background staining with isotype control antibodies, and black histograms show specific staining. Mean fluorescence intensities are indicated. The mean fluorescence intensities of the background controls were less than 5. Comparable results were obtained in two independent experiments.

of cells infected at an MOI of <100 and more than 96% of cells infected at an MOI of >1,000. Importantly, no cytopathic effect was observed. Cell viability, as determined by trypan blue exclusion, and viable cell recovery were similar in infected and noninfected cultures for up to 4 days after infection. To assess whether the activation of immature DC into mature immunostimulatory cells could modify their sensitivity to rAd infection, DC stimulated with TNF-α, MCM, CD8-CD40L, LPS, or poly(I · C) were incubated with AdCMVβ-gal at 1,000 PFU/cell. About 80% of TNF-α-, MCM-, or CD8-CD40L-stimulated DC were infected, whereas LPS- and poly(I · C)-stimulated DC were considerably less susceptible to rAd infection, with only about 30% of cells infected (Fig. 1B).

In order to understand the mechanisms underlying the poor permissiveness of DC for rAd, we analyzed the expression of molecules involved in the binding and entry of the virus into host cells. The initial attachment of rAd to the majority of human cell types is mediated by its fiber capsid protein, which binds to the high-affinity CAR (4, 22). Major histocompatibility complex (MHC) class I molecules may also serve as receptors for the fiber protein (23). Alternatively, rAd attachment to cells of hematopoietic origin may occur upon binding of the penton base capsid protein to αMβ2 integrins (25). Subsequent virus entry occurs via internalization into clathrin-coated vesicles after interaction of the penton base protein with cellular αVβ3 or αVβ5 integrins (51). Immature DC, rAd-infected DC, and DC stimulated with TNF-α, MCM, CD40L, LPS, and poly(I · C) all lacked CAR and αVβ3 integrins (Fig. 2). The αVβ5 and αMβ2 integrins were expressed at higher levels on the surface of immature DC than in rAd-infected DC or mature DC (Fig. 2). MHC class I molecules were present in large amounts on immature DC, and their levels were further increased after rAd infection and DC maturation (Fig. 2). As a control, 911 cells (16), which are highly permissive for rAd, exhibited high levels of CAR, αVβ3 and αVβ5 integrins, and MHC class I molecules and lacked αMβ2 integrins (Fig. 2).

In conclusion, optimal infection of human monocyte-derived DC by rAd relies on the use of immature cells and requires high MOI. Mature DC are more resistant than immature DC to rAd infection, but the degree of this resistance strongly depends on the stimulus used to induce maturation. The absence of CAR and the maturation-induced downregulation of the αVβ5 and αMβ2 integrins are in accordance with the low susceptibility of DC to rAd infection but cannot account for the disparity in the degree of infection among the different types of mature DC.

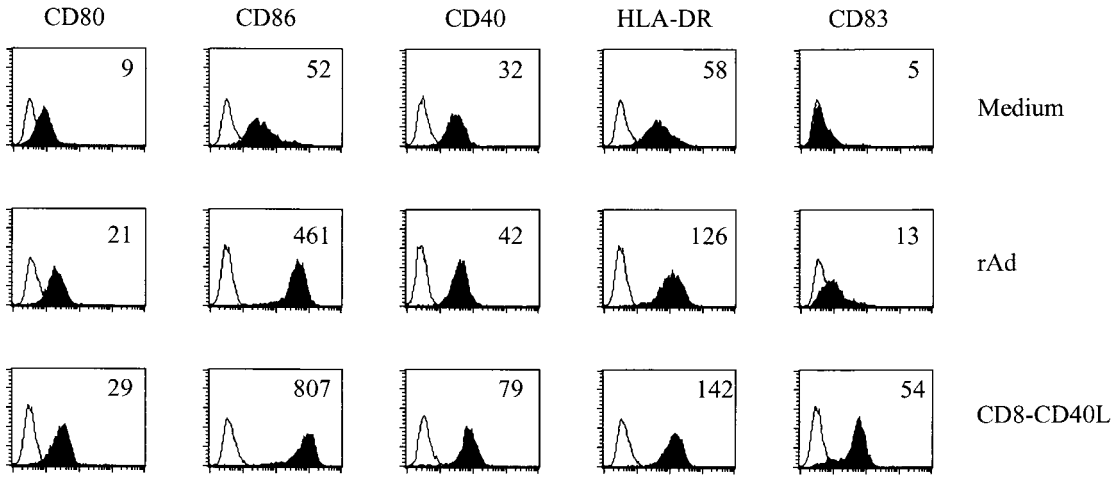

FIG. 3. rAd-mediated phenotypic changes after infection of immature DC. Immature DC were infected with AdCMVβ-gal at 1,000 PFU/cell and analyzed 48 h later by flow cytometry for surface expression of costimulatory molecules, MHC class II molecules, and DC maturation marker CD83. Uninfected immature DC (medium control) and DC allowed to mature by incubation with CD8-CD40L were tested in parallel. White histograms show background staining with isotype control antibodies, and black histograms show specific staining. Mean fluorescence intensities are indicated. The mean fluorescence intensities of background controls were less than 5. Comparable results were obtained with DC generated from five different individuals.

rAd infection increases the expression of costimulatory and MHC molecules but does not polarize DC toward a Th1-inducing phenotype. The use of rAd-infected DC as potential vaccines in human cancers requires that the virus does not subvert DC immunostimulatory properties. We thus compared immature DC infected with AdCMVβ-gal with their uninfected counterparts and with CD8-CD40L-stimulated DC for their cell surface phenotype. rAd infection induced a marked upregulation of the costimulatory molecules CD80, CD86, and CD40 and a significant increase in the expression of surface MHC class II molecules, but the levels of expression of these markers were lower than those obtained by stimulation with CD8-CD40L (Fig. 3). The expression of the adhesion markers CD54 and CD58 was also increased by rAd infection (data not shown). rAd infection induced only low levels of the DC maturation marker CD83 (Fig. 3). These rAd-mediated phenotypic changes were already detectable 24 h postinfection, peaked after 48 h, and remained stable for at least 4 days (data not shown).

Immature DC efficiently capture antigens mainly through macropinocytosis and mannose receptor-mediated endocytosis and lose this property upon maturation (15, 40, 41, 48). When rAd-infected DC were analyzed with respect to antigen uptake capacity, the internalization of FITC-BSA and FITC-mannosylated BSA was found to be significantly decreased in comparison with that in immature noninfected cells, but this downregulation was less pronounced than that observed in CD8-CD40L-stimulated DC (Fig. 4A).

A key feature of mature DC resides in their ability to produce IL-12, a crucial cytokine for the priming of $CD4^+$ Th1 responses and CD4-dependent CTL responses (33). However, in contrast to CD40 stimulation, rAd infection did not trigger IL-12 production by DC (Fig. 4B). The possibility that rAd infection could promote an alternative cytokine profile was also explored. Like immature noninfected DC, rAd-infected DC did not produce IL-10, whereas low levels of IL-10 could be detected after CD40 triggering (Fig. 4B), as reported earlier (13).

The early regions of the adenovirus genome play an important role in host immunosurveillance and virus-cell interactions (11, 26, 45, 52). Since in many of the recently developed rAd vectors, one or more of these early regions have been deleted (30), we analyzed the contributions of the different early genes to adenovirus-mediated phenotypic changes in DC. The triple-deletion mutants (deletions of E1, E2A, and E3 and of E1, E3, and E4) and the wild-type virus induced phenotypic changes similar to those observed with the double-deletion mutant (deletion of E1 and E3) (AdCMVβ-gal virus) (Table 1). Of note, phenotypic changes were not observed when DC were infected with rAd inactivated by visible light in the presence of methylene blue. Such virus is still capable of infecting DC but is incapable of expressing genes due to multiple double-stranded DNA breaks (42) (data not shown). This latter observation suggests that the expression of one or more viral gene products is required for DC activation.

In conclusion, all currently tested rAd activate DC by upregulating their surface expression of costimulatory and MHC molecules and by downregulating their antigen uptake machinery. However, the lack of a high level of CD83 expression and of IL-12 production indicates that the activation of DC by rAd does not lead to their full maturation and to their polarization toward a Th1-inducing phenotype.

rAd infection increases T-cell stimulatory functions of DC. We next studied the consequences of rAd-induced phenotypic changes for DC immunostimulatory properties. Uninfected immature DC, rAd-infected DC, and DC allowed to mature in the presence of CD8-CD40L were pulsed with the p3-13-specific peptide epitope and tested for their ability to stimulate the hsp65-specific $CD4^+$ Th1 clone RP15 1-1. Consistent with the phenotypic analysis, rAd infection of DC significantly (P, <0.001) enhanced the levels of T-cell proliferation in an antigen-specific manner. However, CD40 ligation on DC was a more potent stimulus than rAd infection (P, <0.001) (Fig. 5A). In contrast to CD40 ligation, rAd infection only weakly increased the T-cell-dependent production of IFN-γ (Fig. 5B). We analyzed whether the addition of exogenous IL-12 would further increase rAd-infected DC stimulatory potential. Figure 5A shows that exogenous IL-12 did not have any impact on DC-induced T-cell proliferation. However, in combination with rAd-infected DC, IL-12 significantly enhanced the secre-

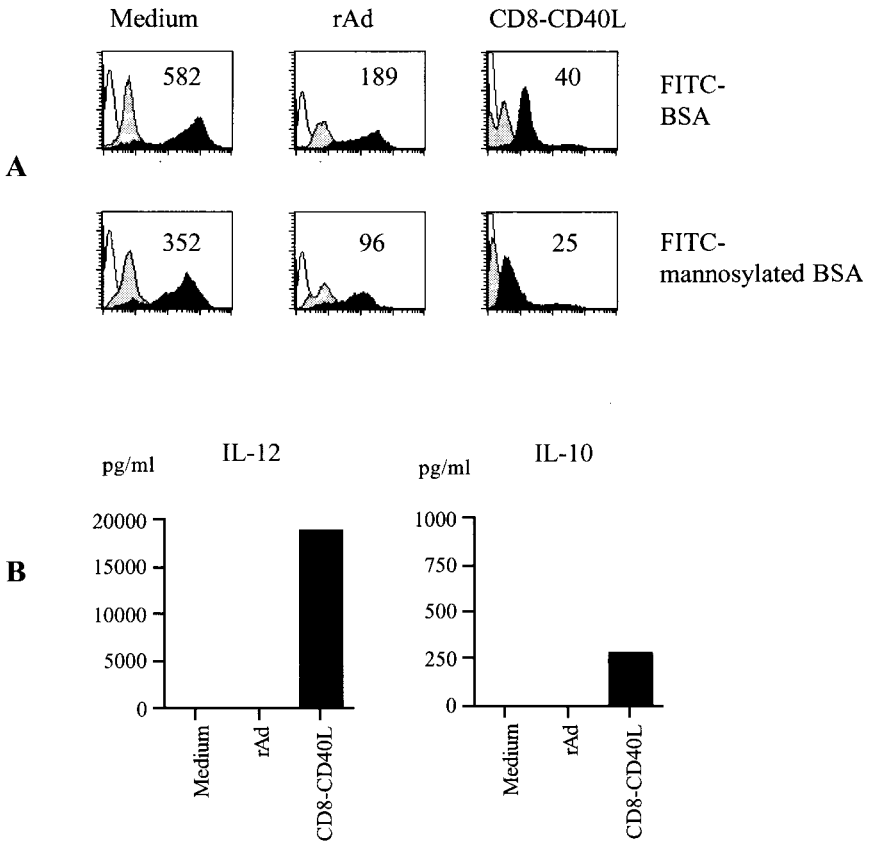

FIG. 4. rAd decrease the capacity of DC to internalize antigens but do not trigger the secretion of IL-12 and IL-10. (A) Immature DC were infected with AdCMVβ-gal at 1,000 PFU/cell, incubated with CD8-CD40L, or cultured in medium alone. DC were tested 48 h later for their ability to internalize FITC-BSA and FITC-mannosylated BSA and were analyzed by flow cytometry. White histograms show background autofluorescence, grey histograms show background uptake at 0°C, and black histograms show specific uptake at 37°C, for which mean fluorescence intensities are indicated. The results are representative of two independent experiments. (B) DC were prepared as described above, and culture supernatants collected 48 h later were tested for the presence of IL-12 p40 and IL-10 by an ELISA. The results are representative of five independent experiments.

tion of T-cell-derived IFN-γ (P, <0.001) (Fig. 5B). Exogenous IL-12 did not have any beneficial effect on IFN-γ production by T cells in the presence of CD40-triggered DC, which already secrete IL-12, and only slightly increased IFN-γ production induced by uninfected immature DC at high T-cell/DC ratios. In conclusion, the upregulation of costimulatory and MHC molecules on rAd-infected DC correlates with increased T-cell stimulatory functions. The inability of rAd to trigger IL-12 secretion by DC is responsible for the poor induction of IFN-γ secretion by activated T cells.

TABLE 1. Effect of different adenoviruses on DC surface phenotypic changes[a]

Virus	Mean fluorescence intensity of:				
	Isotype control	CD80	CD86	HLA-DR	HLA class I
None (uninfected)	2.2	12	39.2	48	226
AdCMVβ-gal (E1 and E3)	2.5	34.8	521.7	63.2	539.8
AdTG9542 (E1, E2A, and E3)	2.6	22.7	320.9	59.9	371.1
AdTG9546 (E1, E3, and E4)	2.5	25.7	363.3	53.7	434.4
Wild type	2.5	19.2	281.8	65.4	331.8

[a] Immature DC were infected with different viruses at 1,000 PFU/cell and analyzed by use of a FACScan for cell surface phenotype 48 h later. Data from one of two independent experiments are shown.

rAd infection and CD40 ligation act in synergy to trigger DC maturation. The intermediate activation state of rAd-infected DC suggested either that the activation signals provided by rAd were insufficient to trigger full DC maturation or that full DC maturation was prevented by a second, counteracting rAd effect. To discriminate between these possibilities, DC infected with AdCMVβ-gal were stimulated through CD40 with the CD8-CD40L fusion protein. DC viability and recovery were comparable in infected and noninfected cultures (data not shown). CD40 ligation on rAd-infected DC resulted in the strong upregulation of the DC maturation marker CD83 (Fig. 6A) and in the secretion of IL-12 (Fig. 6B, left panel). The level of production of IL-12 by rAd-infected CD40-triggered DC was even higher than that observed with noninfected CD40-triggered DC (up to twofold). In contrast, the secretion of IL-10 by CD40-triggered DC was not enhanced by the presence of rAd (Fig. 6B, right panel). Similar results were obtained when rAd-infected DC pulsed with the p3-13-specific peptide epitope were activated by Th1 clone RP15 1-1, a process that involved CD40-CD40L interactions (8, 32). The synergistic effect of rAd infection and CD40 ligation on DC maturation was also reflected by the capacity of DC pulsed with hsp65 to stimulate both secondary p3-13-specific and rAd-specific $CD4^+$ Th1-type responses in cultures from HLA-

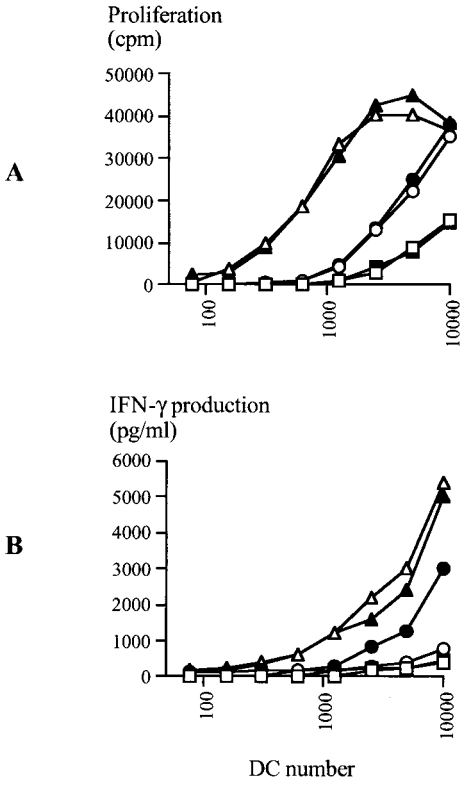

FIG. 5. rAd-infected DC are more potent immunostimulatory APC than their noninfected counterparts, and exogenous IL-12 further increases their T-cell stimulatory functions. HLA-DR3-matched immature DC were pulsed with 10 μg of p3-13 per ml for 2 h at 37°C, washed extensively, and subsequently cultured in medium control (□), infected with AdCMVβ-gal at 1,000 PFU/cell (○), or allowed to mature with CD8-CD40L (△). After 48 h, various numbers of DC were incubated with Th1 clone Rp15 1-1 in the absence (open symbols) or presence (solid symbols) of exogenous IL-12 (■ [immature DC], ● [infected DC], and ▲ [mature DC]). T-cell proliferation (A) and T-cell-derived IFN-γ production (B) were measured after 3 days. DC pulsed with a control DR3-binding peptide failed to induce T-cell proliferation and IFN-γ production (data not shown). The results are representative of three independent experiments.

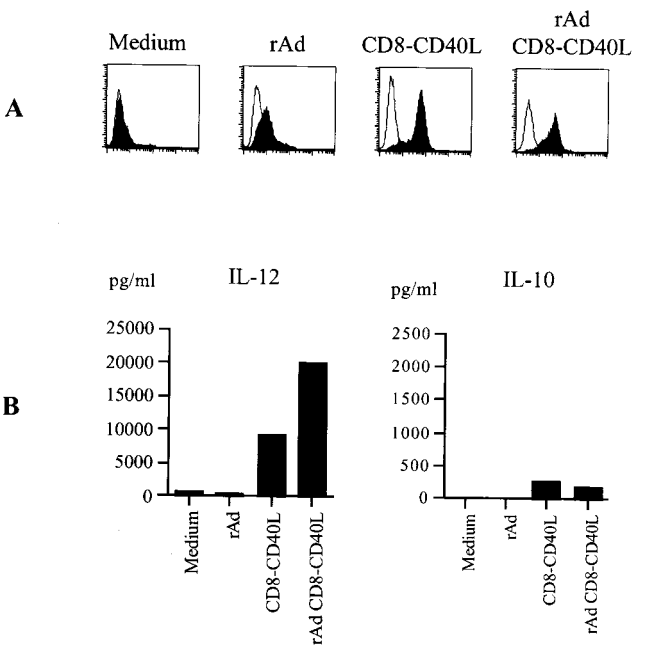

FIG. 6. CD40 triggering of rAd-infected DC induces CD83 expression and high levels of IL-12 production. Immature DC were infected with AdCMVβ-gal at 1,000 PFU/cell, further incubated with CD8-CD40L (rAd CD8-CD40L histogram) or not so incubated (rAd histogram), and compared with their uninfected counterpart (medium and CD8-CD40L histograms). (A) CD83 expression was analyzed 48 h later by flow cytometry. White histograms show background staining with isotype control antibodies, and black histograms show specific staining. (B) IL-12 and IL-10 were detected in the culture supernatants after 48 h by an ELISA. The results are representative of five independent experiments.

$DR3^+$ individuals (Fig. 7). Both proliferation and IFN-γ production were optimally induced by rAd-infected DC triggered through CD40.

In conclusion, rAd do not counteract CD40-induced DC maturation, and rAd-derived signals even act in synergy with CD40 signals to promote the full maturation of rAd-infected DC.

DISCUSSION

We demonstrate that rAd infection induces partial maturation of human monocyte-derived DC, thereby increasing the immunostimulatory functions of these APC. However, efficient infection requires high MOI, in agreement with previous reports by others (1, 14). This finding can be readily explained by our observation that DC lack surface expression of CAR, which serves as a high-affinity receptor for the adenovirus fiber protein and through which this virus can efficiently attach to cells (4). MHC class I molecules and αMβ2 integrins may serve as alternative receptors for fiber-mediated and penton-mediated rAd attachment, respectively (23, 25). However, since DC express considerable levels of these molecules, these alternative adhesion pathways do not appear to efficiently compensate for the lack of CAR, as observed for hamster cells (12). On the other hand, a major blockade for DC infection may also lie downstream of the attachment stage, at the internalization stage, for which αVβ3 and αVβ5 integrins are important (51). Immature DC express αVβ5 but lack αVβ3. Interestingly, αVβ5 expression decreases upon DC maturation, a result which correlates with our observation that mature DC are even less permissive to rAd. Taken together, our data indicate that the low permissiveness of DC results from poor adhesion and internalization of rAd.

At present, we cannot exclude the possibility that the poor permissiveness of DC to rAd infection reflects additional hurdles in the infection pathway. In fact, a comparison of different types of mature DC shows that αVβ5 integrin expression is downmodulated in all cases, whereas the permissiveness of only poly(I · C)- and LPS-activated DC is severely reduced. Poly(I · C)- and LPS-stimulated DC produce high levels of IFN-α/β prior to viral infection (10). IFN-α/β may provide an early and efficient line of defense of these DC against rAd, before virus-associated RNAs can inhibit the antiviral effects of IFN-α/β (28).

Irrespective of these considerations, our data clearly argue that immature DC rather than mature DC constitute the target cell of choice for rAd-mediated gene transfer. It will be important to search for means to increase the efficiency of the attachment and internalization steps. The development of rAd with modified fiber and penton proteins which can target surface proteins that are abundantly available at the DC surface or of dual-specificity antibodies which can link the rAd capsid proteins to such DC surface molecules (49) represents a logical step in this direction.

Our study reveals that rAd infection leads to the upregula-

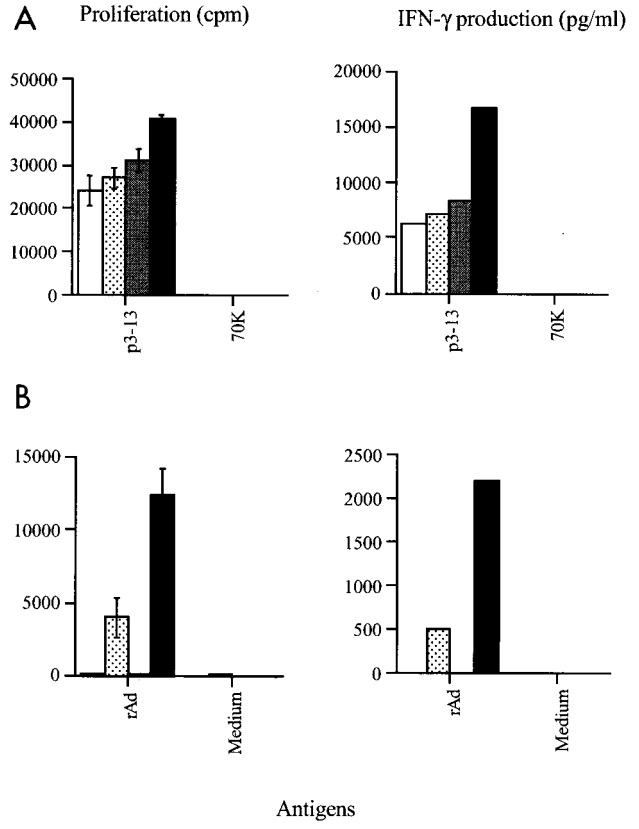

FIG. 7. rAd acts in synergy with CD40 triggering for the development of Th1 immunostimulatory DC. Immature DC from HLA-DR3$^+$ individuals were pulsed with 10 μg of the hsp65 protein per ml and further cultured in medium only (white bars), infected with AdCMVβ-gal (stippled bars), allowed to mature with CD8-CD40L (grey bars), or infected with AdCMVβ-gal and allowed to mature with CD8-CD40L (black bars). DC were cocultured with autologous responders 48 h later. After 5 days, T cells were expanded with TCGF for 8 additional days. Responders were subsequently cocultured with autologous feeders and tested for the presence of p3-13-specific (A) and rAd-specific (B) T cells by measuring proliferation and IFN-γ production. The 70K peptide is a DR3-binding negative control peptide. For proliferation, results are the mean ± standard deviation cpm in triplicate wells. Comparable results were obtained with cells from two different HLA-DR3$^+$ individuals.

tion of costimulatory and MHC molecules and increases the T-cell stimulatory functions of DC. However, rAd-infected DC are not fully mature, as shown by the very low levels of CD83 and the absence of IL-12 secretion. These results contrast with the recent report of Zhong et al. (53), who interpreted rAd infection as a neutral event for DC. A careful analysis of their data reveals that the cells used as immature DC already expressed low levels of the maturation marker CD83. The moderate effects of rAd on such DC may be difficult to discriminate from spontaneous activation due to culture conditions. It is important to note that the capacity of rAd to induce the expression of the adhesion molecule CD54 has been reported for human airway epithelial cells (32, 34, 47). Combined with our results, these studies indicate that rAd upregulate the expression of molecules involved in immune and inflammatory responses in both APC and non-APC populations.

We show that rAd-infected DC are less potent APC than CD40-triggered DC, a result which correlates with the partial upregulation of costimulatory and MHC molecules on rAd-infected DC and with the lack of IL-12 production. We found that exogenous IL-12 enhanced the secretion of IFN-γ by T cells stimulated with rAd-infected DC but did not affect T-cell proliferation. Our results differ from earlier reports showing that IL-12 upregulates both proliferation and IFN-γ secretion by Th1 clones (27, 31); this discrepancy in results may be explained by different experimental conditions. These reports indeed made use of total spleen cells as APC instead of DC. Importantly, we show that rAd-infected DC do not produce the immunoregulatory cytokine IL-10. In addition, unlike other viruses, rAd do not counteract the full maturation of DC through CD40 and even act in synergy with CD40 signals to promote the development of rAd-infected DC into typical Th1-inducing DC.

Most of the recently developed rAd vectors not only lack the E1 region but have a deletion of one or more of the other early regions (30). Since efficient infection of DC requires high MOI, whereas expression of the viral genome at high MOI is not strictly dependent on a functional E1 protein, we tested whether the presence or absence of the different early regions affected rAd-induced DC activation. Our data show that all rAd with deletions induce DC activation comparable to that of wild-type adenovirus, indicating that the E1, E2A, E3, and E4 regions have neither a negative nor a positive effect on this activation process. This finding implies that rAd-induced DC activation involves other mechanisms which may relate to late-gene expression. Testing this hypothesis will require the analysis of mutants carrying deletions in the late regions of the adenovirus genome. More importantly, our data indicate that rAd infection enhances the immunostimulatory characteristics of DC regardless of the type of rAd tested so far, a finding which implies that all of these rAd vectors can be safely used for this application.

Our findings provide new insights into the interactions between rAd and human DC and are important for the use of rAd-infected DC in immune-intervention strategies. The fact that rAd infection enhances DC function supports the suitability of rAd-infected DC as vaccines against infectious agents or tumor antigens. The failure of rAd to trigger the activation of DC into fully mature Th1-inducing APC indicates that optimization requires additional triggering of DC through their CD40 receptor. Importantly, rAd infection and CD40 triggering act in synergy, in that this combination results in fully mature DC that show a higher level of IL-12 secretion and T-cell stimulatory potential than DC triggered either by rAd or through CD40 alone. The application of rAd-infected DC as vaccines should therefore preferentially involve CD40 triggering prior to administration in vivo. On the other hand, further in vivo activation of rAd-infected DC may be driven by the interaction with Th cells against rAd-derived antigens, for which memory exists in most individuals.

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