The Maturation of Murine Dendritic Cells Induced by Human Adenovirus Is Mediated by the Fiber Knob Domain*

Valérie Molinier-Frenkel‡‡, Armelle Prévost-Blondel§§, Saw-See Hong**, Renée Lengagne‡, Sarah Boudaly††, Maria K. Magnusson§§, Pierre Boulanger***, and Jean-Gérard Guillet‡‡

From the ‡Département d’Immunologie, Institut Cochin, INSERM U567, CNRS UMR 8104, Laboratoire membre de l’Institut Fédératif de Recherche 116, Université R. Descartes, 27 rue du Faubourg Saint-Jacques, 75014 Paris, France, the §§Laboratoire de Virologie et Pathogénèse Virale, CNRS UMR 5537, Faculté de Médecine R.T.H. Laennec, 7, Rue Guillaume Paradin, 69372 Lyon Cedex 08, France, ‡‡Laboratoire de Pathologie Métabolique et Hormonale du Développement, INSERM U342, Hôpital Saint Vincent de Paul, 75014 Paris, France, and the §§Got-A-Gene AB, Stena Center 1B, SE 41292 Göteborg, Sweden

We investigated the mechanism of adenovirus serotype 5 (Ad5)-mediated maturation of bone marrow-derived murine dendritic cells (DC) using (i) Ad5 vectors with wild-type capsid (AdE1+, AdGFP); (ii) Ad5 vector mutant deleted of the fiber C-terminal knob domain (AdGFPΔknob); and (iii) capsid components isolated from Ad5-infected cells or expressed as recombinant proteins, hexon, penton, penton base, full-length fiber, fiber knob, and fiber mutants. We found that penton capsomer (penton base linked to its fiber projection), full-length fiber protein, and its isolated knob domain were all capable of inducing DC maturation, whereas no significant DC maturation was observed for hexon or penton base alone. This capacity was severely reduced for AdGFPΔknob and for fiber protein deletion mutants lacking the β-stranded region F of the knob (residues Leu-485–Thr-486). The DC maturation effect was fully retained in a recombinant fiber protein deleted of the HI loop (FiΔHI), a fiber (Fi) deletion mutant that failed to trimerize, suggesting that the fiber knob-mediated DC activation did not depend on the integrity of the HI loop and on the trimeric status of the fiber. Interestingly, peptide-pulsed DC that had been stimulated with Ad5 knob protein induced a potent CD8+ T cell response in vivo.

Adenovirus (Ad)1 vectors induce a vigorous stimulation of the innate immune system very rapidly after their administration followed by the induction of the cognate immune response mediated by T and B lymphocytes. The early inflammatory response is independent of the transgene expression (1), but the antigen-specific response can be directed to both viral and transgenic proteins (2, 3). As a consequence, transgene expression is only transient, and the occurrence of anti-Ad neutralizing antibodies is in part responsible for the low efficiency of gene transfer using Ad vectors in preimmunized subjects (4, 5).

Several studies have attempted to elucidate the mechanism of Ad-triggered activation and the antigenic targets of the immune response against Ad vectors. Increasing evidence has emerged which shows that capsid components of the vector play a major role in this process. The Ad virion is composed of capsomers of two different types. Hexon (Hx) capsomers form the facets and edges of the icosahedral capsid, whereas penton (Pn) capsomers, located on the 5-fold axis of the virus, comprise a protruding fiber (Fi) protein (a homotrimer) linked via non-covalent bonds to the capsid-anchored penton base (Pb, a homopentamer). The fiber protein consists of three structural domains, the N-terminal tail, which binds to the penton base, the central shaft, and the C-terminal spherical domain, called the knob. The capsid proteins of the input virus are not only targets for the humoral and T helper responses (5–7), but they are also recognized by cytotoxic CD8+ T lymphocytes (8, 9). The capsid components may also directly interact with cells of the immune system, and our previous data indicated that hexon stimulates helper T cell responses (10).

Ad also activates several kinase pathways (including p38/MAPK, NF-κB, and Raf1) during the minutes following vector administration, leading to the synthesis of chemokines and inflammatory cytokines (11–14). The stimulation of intracellular signaling pathways seems to be required for the early phases of the viral cycle since activated cAMP-dependent protein kinase and p38/MAPK boost microtubule-mediated nuclear translocation of the virus (13). Ad has been found to modulate dendritic cell (DC) maturation by increasing the expression of major histocompatibility complex and costimulatory molecules and, at least for murine DC, by inducing the secretion of IL12 (15–18). We and others have shown that such activated DC are fully competent for initiating protective immune responses (19–21). Interestingly, the Ad-mediated activating effect on DC is apparently independent of viral gene expression (15–17) but correlates with NF-κB activation (16).

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† Both authors contributed equally to this work.

‡ Supported by a fellowship from VLM. To whom correspondence may be addressed: Laboratoire d’Immunologie, Hôpital Henri Mondor, 51, av du Maréchal de Lattre de Tassigny, 94010 Créteil Cedex, France. Tel.: 33-1-4981-2298; Fax: 33-1-4981-2897; E-mail: valerie.frenkel@hmn.ap-hop-paris.fr.

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¶¶¶ To whom correspondence may be addressed: Tel.: 33-4-7877-8621; Fax: 33-4-7877-8751; E-mail: Pierre.Boulanger@laennec.univ-lyon1.fr.

†† The abbreviations used are: Ad, adenovirus; Hx, hexon; Pn, penton; Fi, fiber; Pb, penton base; DC, dendritic cell(s); LPS, lipopolysaccharide; MLR, mixed lymphocyte reaction; TLR, toll-like receptor(s); CAR, Coxsackievirus adenovirus receptor; WT, wild-type; GFP, green fluorescent protein; TNF, tumor necrosis factor; IFN, interferon; CFSE, 5-carboxyfluorescein diacetate succinimidyl ester; MAPK, mitogen-activated protein kinase; NS, non-stimulated; MS, mock-stimulated; Ab, antobody; mAb, monoclonal antibody; m.o.i., multiplicity of infection(s); ELISPOT, enzyme-linked immunoSPOT; FACS, fluorescence-activated cell sorter; MFI, mean fluorescence intensity; Δ, deletion.
Flow Cytometry—DC were washed in phosphate-buffered saline with 1% fetal calf serum. After incubation with anti-FcRII/III Ab (2.4G2, Pharmingen), cells were incubated with various combinations of the following mAbs (all from Pharmingen): phosphorylcholine-conjugated anti-I-Aq (AF6-120.1) and anti-CD40 (3/23), fluorescein isothio- cyanate-conjugated anti-CD11c (HL3) and anti-CD80 (16-10A1), biotin-ylated anti-H2-D^d (28-8-6) and anti-CD86 (GL1) + streptavidin-PerCP (BD Biosciences). Anti-fiber-tail mAb mAb (4D2.5 (28)) was obtained from Jeff Engler (University of Alabama at Birmingham, Birmingham, AL). For Coxsackievirus-adenovirus receptor (CAR) staining, day 6 DC, Chinese hamster ovary and HeLa cells (obtained from ATCC) were labeled with anti-CAR mAb E1.1 (ascites fluid, kindly provided by Dr. Silvio Hemmi, University of Zurich) or a control irrelevant ascites fluid.

Counterstaining was performed using biotinylated rat anti-mouse IgG and streptavidin-phosphatidylethanolamine. Flow cytometric analyses were performed on a FACSCalibur (BD Biosciences).

In Vitro Functional Assays (MLR, IL12, and TNFa)—For mixed lymphocyte reaction (MLR), day 8 DC were distributed at increasing doses and co-cultured for 4 days with aliquots of allogeneic (BALB/c, H2-D^d, CD4^+ purified splenocytes (2 x 10^6 cells/well)); CD4^+ T cell proliferation was measured by [3H]thymidine incorporation (1 μCi/well) during the final 18 h of coculture. For IL12 and TNFa assays, day 8 DC supernatants were tested using mouse IL12p70 and TNFa enzyme-linked immunosorbent assay kits (Pharmingen).

Peptides— Immunodominant viral peptides, peptide 33–41 from lymphocytotropic polyomavirus (KAYNNFATM) and peptide 366–374 from influenza virus nucleoprotein (ASNNEMTM), abbreviated GP33 and NP366, respectively, were purchased from NeoSystem (Strasbourg, France). Both are H2-D^d-restricted.

Immunizations and CFSE-labeled Splenocyte Rejection Assays—C57BL/6 mice were immunized subcutaneously into the flank with 3 x 10^4, 8 x 10^4, or 2.5 x 10^5 DC loaded with GP33 or NP366 (10^6 cells/mouse). Ten days later, mice received an intravenous injection of 3 x 10^7 syngeneic splenocytes labeled with the fluorescent dye 5-6-carboxyfluorescein di-acetate succinimidyl ester (CFSE, Molecular Probes) as described in Ref. 29. The percentage of CFSE^- donor cells among recipient peripheral blood lymphocytes or splenocytes was determined using FACS analysis. Donor cell rejection was calculated using the formula: [% of CFSE^- cells in immunized mice] / [% of CFSE^- cells in naive mice] x 100.

ELISPOT-IFNγ—Nitrocellulose microplates (Millipore) were coated with rat anti-mouse IFNγ antibody (R4-824, Pharmingen) and then washed and saturated with complete medium. Aliquots (in triplicate) of freshly isolated spleen cells (10^6 cells/well) or peripheral blood lymphocytes (2 x 10^6 cells/well) were added to the wells in complete medium containing 30 units/ml human rIL2 (Roche Applied Science) and 10^-7 M GP33 or NP366. After 20 h at 37 °C, the spot-forming cells were revealed and counted (10). The results obtained with NP366 were subtracted from the mean values of triplicate tests obtained with GP33.

Cytotoxicity Assay— Splenocytes from DC-immunized mice (effector cells) were added to 4 x 10^4 (H-2K^d) or 4 x 10^4 (H-2D^d) donor cells. Effector:target ratios were performed on a FACSCalibur (BD Biosciences). Then transfected with 51Cr-labeled EL4 target cells (10^4 μCi/cell) pulsed with GP33 or NP366 peptide, in duplicate and at different effector:target ratios. After 5 h at 37 °C, supernatants were collected, and radioactivity was measured (Top Count, Packard Instruments). Target cells were incubated with medium alone to determine the level of spontaneous release of 51Cr and with 2% alkyl-trimethyl-ammonium bromide (Sigma) to determine the total 51Cr release. Specific 51Cr release was calculated using the formula: (experimental - spontaneous release/total release - spontaneous release) x 100.

RESULTS

Effect of Isolated Ad5 Capsid Components on DC Phenotype—Immature DC were incubated with isolated Pn or Hx capsomers, purified from Ad5-infected HeLa cells. The major subset of Hx-treated DC showed a poorly mature phenotype, not significantly different from that shown by NS-DC, with a low expression of major histocompatibility complex class II, CD40, CD80 (B7.1), and CD86 (B7.2) molecules (Fig. 2a).

In contrast, Pn-treated DC massively expressed a mature phenotype, similar to the phenotype of LPS-stimulated DC, with a high level of expression of the different markers (Fig. 2a). Since the Ad Pn capsomer is composed of two structural entities, Pn and Fi, the following experiments were designed to determine which of these two constitutive proteins was responsible for the DC maturation. DC were then treated with recombinant Pn or...
Fi protein isolated from insect cell extracts. The results indicated that isolated Fi protein reproduced the entire stimulatory effect of Pn, whereas Pb protein alone showed no detectable effect on DC maturation (Fig. 2).

**Role of the Fiber Knob Domain in Fiber-mediated DC Maturation**—The C-terminal domain of Ad fiber has been expressed as recombinant knob protein (26, 28). DC incubated with the fiber knob protein expressed a mature phenotype similar to that observed in DC stimulated by the full-length Fi (Fig. 3).

**Fig. 1. Schematic structure of Ad5 fibers, WT, and mutants, as virion components (a and b) or recombinant proteins (c).** a, WT Ad5 fiber with its three structural domains, the tail, which is non-covalently linked to the penton base, the shaft and the knob, which is responsible for the attachment of Ad5 to the cell receptor CAR. WT Ad5, AdE1°, and AdGFP carried long shafted fibers with 22 repeats of a 15-residue β-sheet motif. b, the knobless fiber vector AdGFPknob carried a shorter fiber shaft (7 β-repeats), which terminates by the extrinsic trimerization motif neck region peptide from the human lung surfactant protein D, and an opale-stop codon. c, diagrammatic representation of the conformational structure of Ad5 fiber knob (35), showing the different regions in β-sheet structure (A to J) and their connecting loops, aligned with a linear representation of the fiber knob deletion mutants FIΔ402-481, FIΔHI, FIΔEF, and FIΔ485-486. The deleted region of the knob sequence is represented by a thin line, and the intact region is represented by a solid box. The numbers indicate the amino acid numbers, the numbering starting from the initiator methionine of full-length Ad5 fiber. The bold letters m and t indicate the monomeric or trimeric status of the recombinant fibers, respectively, and the symbols +, −, and ∼ refer to their degree of affinity for CAR (24, 34). Knob regions interacting with CAR (30, 31) are represented by stippled boxes.
ated DC activation since a detectable effect was only observed at the dose of 0.2 μg of knob protein, i.e. 5 × 10⁶ molecules of fiber knob per DC. Interestingly, the MLR values for 0.2 μg of knob protein were similar to that of 1 μg of Fi, a result consistent with the fact that the quantity of knob protein present in the 0.2-μg knob sample roughly corresponded to the knob protein content of the 1-μg Fi sample (180 amino acid residues for the knob domain versus 581 amino acid residues for the full-length fiber). The knob protein also induced IL12 and TNFα secretion in a dose-dependent manner (Fig. 3c).

We then investigated whether the knob was capable of directly targeting DC and induce their maturation, without the participation of any other intermediate cells. To this aim, CD11c+ cells were purified using anti-CD11c magnetic beads and tested in maturation assays. Although NS-DC showed some up-regulation of expression of co-stimulatory molecules, knob-stimulated DC expressed a significantly more mature phenotype than control cells (Fig. 4a). Moreover, recombinant Fi protein was able to bind to immature DC, with 63% fiber-positive cells at day 6 of culture (Fig. 4b). In this assay, cell-bound Fi was detected using anti-fiber tail mAb, implying that the epitope in the tail was accessible and that the fiber attachment to the cell surface had occurred via the knob domain. These data strongly suggested that DC were direct targets for the fiber knob domain.

To assess whether the effect of Ad5 virion on DC activation was also directly mediated by the fiber knob domain, comparative experiments were carried out using virions of AdE1a, carrying WT Fi projections, and of AdGFPΔknob, a knobless Ad5 vector (Fig. 1, a and b). Upon incubation with AdE1a, there was an up-regulation of the expression of maturation markers on DC (Fig. 4c), as expected from previous observations (15, 16). With the same doses of AdGFPΔknob virions, the DC showed a significantly less mature phenotype. These results confirmed that the fiber knob was the common stimulating factor in all the active viral components assessed, Ad5 virion, Pn capsomer, Fi, and knob proteins, and that the knob domain carried most of the determinants responsible for the stimulation of murine DC.

Absence of CAR Expression at the Surface of Murine DC and Low Degree of Permissiveness to Ad5 Infection—The cell entry of Ad5 results from the attachment of the fiber knob to a high affinity primary receptor, identified as the CAR in most Ad-permissive cells (24, 30, 31), followed by the binding of the RGD motifs in Pb to αvβ3/5 integrins, which promotes virus endocytosis (32). It has already been shown that human and murine DC do not express CAR (17, 33) but do express αvβ3/5 integrins at their surface, and this probably explains their low permissiveness to Ad. We verified by FACS analysis the level of CAR expression at the surface of DC differentiated in our culture conditions. HeLa cells, which are known to be high CAR expressors, were used as positive control, whereas Chinese hamster ovary cells, which do not express CAR, were used as negative control. As expected, no CAR signal above the background level could be detected on immature DC collected before stimulation (Fig. 5a), confirming previous observations.

We thus checked whether knob stimulation of DC was associated with effective cell entry of Ad and Ad-mediated...
gene expression. AdGFP was used at two different m.o.i., 1 × 10^4 virions/cell, which was sufficient to induce DC maturation (Fig. 4c), and 3 × 10^4 virions/cell. At 48 h after infection at an m.o.i. of 10^5, only 12% of DC expressed GFP, and GFP expression slightly increased (19% of DC) at an m.o.i. of 3 × 10^4 (Fig. 5b), confirming that murine DC were poorly permissive to Ad5.

Mapping of the Fiber Knob Region Involved in DC Maturation—Four Ad5 Fi protein mutants carrying deletions in the knob domain (Fig. 1, c and d) were assayed for DC maturation. The large deletion in recombinant FiΔ402–481 overlapped the AB loop, the β-strands B and C, the CD loop, and the N-terminal moiety of the DG loop, whereas FiΔHI mutant lacked only the HI loop. Two other recombinant Fi proteins, FiΔEF,
and FiΔ485–486, carried deletions involving the short double β-stranded region EF, and the two residues Leu-485 and Thr-486, which form the β-sheet F (34, 35), respectively. Both FiΔEF and FiΔ485–486 occur as fiber trimers, whereas Δ402–481 and ΔHI are defective in fiber trimerization (24). We compared the DC maturation activity of WT knob and fiber mutant proteins by evaluating their capacity to induce allogeneic CD4+ splenocyte proliferation using MLR assays, as above. Since the knob domain represents approximately one-third of the whole fiber protein bulk, we used three to four times less WT knob protein than fiber mutant proteins to stimulate DC, to normalize the samples to similar knob contents. As shown in Fig. 6, mutant FiΔHI retained its full DC maturation activity, implying that the distal HI loop and the trimeric structure of the knob were not required for induction of DC maturation. The three other mutants, FiΔ402–481, FiΔEF and FiΔ485–486, did not change significantly the DC phenotype, suggesting that the double-stranded region EF, and more specifically the short β-strand F, was responsible for the maturation effect.

**Knob-stimulated DC Induced a Specific CD8+ T Cell Response in Vivo**—To compare the in vivo functioning of knob-stimulated DC with that of DC stimulated with other Ad capsid components, we tested their ability to generate a cellular immune response. We immunized mice with DC pulsed with a model antigen and tested their ability to generate a cellular immune response.

DC were incubated with AdGFP at m.o.i. of 10,000 or 30,000 virions/cell (dotted and solid lines, respectively) or with empty vector AdE1Δ at 10,000 virions/cell (control, gray area), and then analyzed by FACS at 48 h after infection. Numbers in parenthesis (12 and 19%) represent the level of the GFP signal over the background fluorescence of empty vector-infected cells.

was observed in mice treated with knob-stimulated DC, at a rate similar to that observed in mice immunized with GP33 emulsified in incomplete Freund’s adjuvant. The curve of rejection in mice immunized with knob-stimulated DC loaded with the irrelevant NP366 peptide was similar to the curve observed in mice immunized with GP33-loaded DC stimulated by Pb or Hx or non-stimulated (Fig. 7a). In the latter mice, the CFSE+ cell diminution in blood was likely due to a non-specific redistribution of splenocytes in the secondary lymphoid organs. Consistent with this hypothesis, analysis of CFSE-labeled cells in spleens of recipient mice 10 days after adoptive transfer showed that only 5% of CFSE+ cells persisted in mice immunized with 3 × 10⁴ knob-stimulated DC, whereas they still represented 72 and 60% in mice immunized with 2.5 × 10⁵ Pb- or Hx-treated DC, respectively (Fig. 7a). The effect obtained using 3 × 10⁴ knob-stimulated DC, as compared with that obtained using 2.5 × 10⁵ Pb- or Hx-treated DC, implied that the knob-treated DC were at least 10 times more efficient in inducing splenocyte rejection than DC treated by other Ad capsid proteins.

At day 20 after DC immunization, which corresponded to 10 days after the adoptive transfer of CFSE-labeled splenocytes, the number of IFN-γ-secreting CD8+ T cells was determined by ex vivo ELISPOT assays. GP33-specific T cells were not significantly induced in mice immunized with Hx- or Pb-stimulated and GP33-loaded DC or in mice immunized with knob-stimulated DC loaded with control peptide NP366 (Fig. 7b). In contrast, significant numbers of IFN-γ-secreting T cells were detected in both spleen and blood of mice immunized with knob-stimulated and GP33-loaded DC, and the induction of the GP33-specific T cell response seemed to correlate with the number of knob-stimulated DC injected (Fig. 7b). However, no T cell response was detectable after immunization with 3 × 10⁴ DC despite the rejection of CFSE+ cells shown in Fig. 7a. The cytolytic activity of in vitro restimulated spleen cells was then tested. No specific lytic activity was observed in effector cells from mice immunized with 3 × 10⁴ NS-DC, but effector cells from mice immunized with knob-stimulated DC were found to lyse GP33-loaded EL4 cells with a high efficiency (Fig. 7c).

**DISCUSSION**

DC are at the interface between innate and adaptive immunity and thus play a crucial role in interpreting the stimuli delivered by their environment. Their resulting maturation status is critical for the development of the immune response.
Fig. 7. Knob-stimulated DC primed GP33-specific CD8^+ T cells in vivo. a, rejection of donor cells by recipient mice vaccinated with DC. CFSE-labeled splenocytes from C57BL/6 mice were loaded with GP33 and transfused into C57BL/6 recipient mice that had been immunized with (i) GP33-loaded DC (2.5 x 10^9, 8 x 10^8 and 3 x 10^7 cells, respectively) stimulated with knob; (ii) GP33-loaded DC (2.5 x 10^9) stimulated with Pb or Hx, or NS; (iii) NP366-loaded DC (2.5 x 10^9) stimulated with knob; or (iv) 50 μg of GP33 emulsified in incomplete Freund’s adjuvant (IFA). The rejection level (expressed as the percentage of CFSE-positive cell population in naïve mice) was determined by FACs analysis in blood (left part of panel a) and spleen (right part of panel a) at different time points after adoptive transfer. b, ex vivo ELISpot-IFNγ response of the same mice. Freshly isolated spleen cells or peripheral blood lymphocytes were incubated with GP33, and results were expressed as spot-forming cells/10^4 CD8^+ T cells (mean from triplicate tests ± S.D.). c, cytolytic activity of effector cells derived from mice vaccinated with GP33-loaded and NS or knob-stimulated DC (3 x 10^6 cells). After in vitro restimulation with GP33, spleen cells were incubated with 51Cr-labeled EL4 target cells loaded with GP33 or NP366 at different effector-to-target (E:T) ratios and assayed for 51Cr release. Data shown are mean of duplicate experiments ± S.D.

In the present study, we provide evidence showing that fiber knob protein, the 180-residue C-terminal domain of Ad5 fiber capsomer, interacts with DC and activates signals that render DC phenotypically and functionally mature. Using deletion mutants in the knob domain of recombinant Fi protein, we found that short deletions that altered the double β-strand EF, as in mutants FiΔEF and FiΔ485–486, resulted in the loss of their capacity to induce DC maturation. It is noteworthy that FiΔHI, a Fi protein deletion mutant that occurs in a monomeric form (24), retained its full DC-maturation activity. This suggested that the effect of the knob on DC maturation depended upon the integrity of the β-strand F (residues 485–486), but not upon the HI loop, and did not require the trimeric structure of the knob.

Ad5 uptake by permissive cells is a two-step process consisting of (i) the binding of the Fi terminal knob to the attachment receptor CAR, a high affinity receptor for most Ad subgroups (e.g. subgroups C Ad2 and Ad5 and subgroup A Ad12), except for subgroup B serotypes such as Ad3 and Ad7 (36). This step is followed by (ii) the endocytosis of cell-bound virions, mediated by the Pb RGD motifs and α5β1 integrins (32). In the initial step of virus-cell binding, the β-stranded region EF represents one of the critical regions involved in the interaction of the knob with CAR (Fig. 1c), and mutant fibers FiΔEF and FiΔ485–486 have been shown to bind to CAR with a significantly lower affinity than WT fiber (24, 30, 31, 34). Since the DC do not express CAR (Fig. 5a) (17, 33), our data suggested that Ad5- and Fi-mediated DC maturation resulted from a direct interaction between knob and DC via another receptor (Fig. 4b). However, the partial overlapping of the DC-binding site(s) on the knob with those specific for CAR is not coincidental, if one considers that the regions of the knob involved in the interaction with cell components must be very exposed on the protein surface and thus accessible to many other receptors and/or ligands. For example, a CAR-blocking anti-knob monoclonal antibody has been found to inhibit the binding of Ad5 virions to alternative receptors, heparan sulfate proteoglycans and sialoglycoproteins (37).

Immature DC are instructed of danger through TLR-mediated signaling. TLR belong to a family of receptors that recognize molecular motifs conserved among large groups of microorganisms. Due to conserved domains in their cytoplasmic tail, TLR share a common signaling pathway leading to the activation of MAPK and to the translocation of NF-κB in the nucleus, where it directs the expression of a number of genes involved in inflammatory and immune responses (38). Interestingly, previous data have shown that Ad induced maturation of DC via early activation of NF-κB independently of viral gene expression (16). However, with the exception of TLR4, which mediates RSV protein F signaling and activation of B cells by murine retroviruses, the role of TLR in immune responses to viruses in mammals remains poorly understood (39). Réa et al. (40) recently reported that chimeric Ad5 virions carrying fibers from subgroup B virus induced DC maturation. We also observed DC maturation upon stimulation with an Ad vector carrying chimeric fibers composed of tail and shaft from serotype 5 and knob from serotype 3 (data not shown). These data suggest that DC surface molecules could recognize phylogenetically distant Ad knobs and that the DC-knob interaction probably involves conserved secondary structures, rather than conserved individual amino acid residues in the knob domain.

Many studies have focused on recombinant Ad as vaccine vectors for viral and tumor antigens. One of the major reasons of their vaccine efficacy has been attributed to their natural ability to infect DC in vivo (41). If the Ad Fi knob carries peptide motif(s) and/or conformational structure(s) that medi-
Dendritic Cell Maturation Effect of Ad Fiber Knob

In vivo Dendritic Cell Maturation Effect of Ad Fiber Knob

The results presented here should also have implications in gene therapy as prolonged transgene expression requires minimal vector immunogenicity. In the last generations of Ad vectors, most of the regulatory viral genes, or even the whole viral genome, have been deleted, in part to prevent expression of immunogenic viral proteins. This has led to some prolonged transgene expression in mouse models, although cellular and humoral responses to the vector and the transgenic protein have still been detected. Thus, persistent cellular and humoral responses to the vector and the transgene expression in mouse models, although of immunogenic viral proteins, have been deleted, in part to prevent expression of immunogenic viral proteins (43, 44). This has led to some prolonged transgene expression in mouse models, although cellular and humoral responses to the vector and the transgenic protein have still been detected (43, 44). Thus, persistent immunostimulatory properties have been assigned to capsid proteins of the input virions (8). Deletion of the entire knob domain, or replacement of knob regions by cellular ligands with new cell-binding specificities, might confer upon the vectors both retargeting properties and a decrease in their immunogenicity, as suggested by the low level, if any, of the DC maturation effect of our knobless virions AdGFPknob (Fig. 4c). However, it has to be kept in mind that fiber knob constructs must respect several biological functions that are carried by the knob domain and are essential to the viability and growth of Ad (24, 45, 46). In this case, the use of Ad5 vectors carrying cleavable knob domains by means of the insertion of a restriction protease site in the fiber shaft (23) might represent a possible alternative to reconcile contradictory requirements such as a low immunostimulation and a high gene transfer efficiency.

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