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*J Immunol* 2012; 188:6109-6118; Prepublished online 14 May 2012;

doi: 10.4049/jimmunol.1103717

<http://www.jimmunol.org/content/188/12/6109>

**Supplementary Material** <http://www.jimmunol.org/content/suppl/2012/05/14/jimmunol.1103717.DC1>

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*The Journal of Immunology* is published twice each month by  
The American Association of Immunologists, Inc.,  
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Print ISSN: 0022-1767 Online ISSN: 1550-6606.



# Type I IFN Induced by Adenovirus Serotypes 28 and 35 Has Multiple Effects on T Cell Immunogenicity

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Recombinant adenovirus (rAd) vectors are being investigated as vaccine delivery vehicles in preclinical and clinical studies. rAds constructed from different serotypes differ in receptor usage, tropism, and ability to activate cells, aspects of which likely contribute to their different immunogenicity profiles. In this study, we compared the infectivity and cell stimulatory capacity of recombinant adenovirus serotype 5 (rAd5), recombinant adenovirus serotype 28 (rAd28), and recombinant adenovirus serotype 35 (rAd35) in association with their respective immunogenicity profiles. We found that rAd28 and rAd35 infected and led to the *in vitro* maturation and activation of both human and mouse dendritic cells more efficiently compared with rAd5. In stark contrast to rAd5, rAd28 and rAd35 induced production of IFN- $\alpha$  and stimulated IFN-related intracellular pathways. However, the *in vivo* immunogenicity of rAd28 and rAd35 was significantly lower than that of rAd5. Deletion of IFN- $\alpha$  signaling during vaccination with rAd28 and rAd35 vectors increased the magnitude of the insert-specific T cell response to levels induced by vaccination with rAd5 vector. The negative impact of IFN- $\alpha$  signaling on the magnitude of the T cell response could be overcome by increasing the vaccine dose, which was also associated with greater polyfunctionality and a more favorable long-term memory phenotype of the CD8 T cell response in the presence of IFN- $\alpha$  signaling. Taken together, our results demonstrate that rAd-induced IFN- $\alpha$  production has multiple effects on T cell immunogenicity, the understanding of which should be considered in the design of rAd vaccine vectors. *The Journal of Immunology*, 2012, 188: 6109–6118.

Recombinant adenovirus (rAd) vectors have proved to be very effective at inducing Ag-specific, polyfunctional T cell responses (1, 2). Recombinant adenovirus serotype 5 (rAd5)-based vectors have been extensively studied as potential HIV/AIDS vaccines and tested in phase I and phase II clinical trials (3). The results of these trials, in conjunction with studies in rhesus macaques, have revealed that preexisting immunity against the rAd5 vector can reduce the immunogenicity of the vaccine and limit the memory response to the HIV-antigen insert (4). Because 40–80% of the world's population is seropositive for Ad5, the

usefulness of an rAd5-based vaccine may be compromised (5–13). To circumvent preexisting immunity, alternative adenovirus vectors from serotypes with much lower seroprevalence, such as Ad28 and Ad35, are under development (11–14). However, some vectors constructed from low-seroprevalence adenoviruses have shown poor immunogenicity *in vivo* (13). This presents a paradox whereby the usefulness of rAd5, which induces a good immune response, is limited due to widespread preexisting immunity, whereas recombinant adenovirus serotype 28 (rAd28) and recombinant adenovirus serotype 35 (rAd35) vectors, to which there is low preexisting immunity, are inherently less immunogenic. The reasons for these differences in immunogenicity are poorly understood yet critical for the future development of vaccines based on these adenoviral serotypes.

The different serotypes of rAds differ in receptor usage, cell tropism, and ability to induce cell activation (1, 15, 16). Specifically, rAd35 but not rAd5 induces maturation of dendritic cells (DCs) and high IFN- $\alpha$  production, both of which are important components of innate immunity (1). Other models have shown that differences in innate immunity can have important effects on the magnitude (17–19), Th1/Th2 distribution (20–22), and central/effector memory distribution (23–25) of the subsequent adaptive immune response. Specifically, IFN- $\alpha$ , a key cytokine involved in the innate immune response and the establishment of the antiviral state (26–29), has been shown to promote the maturation (30), proliferation (18, 31), survival (32), differentiation (18, 33), and effector function (34) of CD8 T cells. Paradoxically, IFN- $\alpha$  has also been shown to suppress the proliferation (35, 36) and limit the survival (37) of Ag-specific CD8 T cells depending on the timing, level, and duration of its production. There is little information on how rAd-induced IFN- $\alpha$  influences the development of the insert-specific adaptive immune response.

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Received for publication December 23, 2011. Accepted for publication April 16, 2012.

The microarray data presented in this article have been submitted to the Gene Expression Omnibus (<http://www.ncbi.nlm.nih.gov/geo/>) under accession number GSE37128.

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The online version of this article contains supplemental material.

Abbreviations used in this article: DC, dendritic cell; IRG, IFN-regulated gene; mDC, myeloid dendritic cell; MOI, multiplicity of infection; pDC, plasmacytoid dendritic cell; rAd, recombinant adenovirus; rAd5, recombinant adenovirus serotype 5; rAd28, recombinant adenovirus serotype 28; rAd35, recombinant adenovirus serotype 35; VA-RNA I, viral-associated RNA I; VA-RNA II, viral-associated RNA II.

In this study, we show that rAd28 and rAd35, but not rAd5, induce the production of IFN- $\alpha$  *in vitro* in cells of both human and murine origin as well as *in vivo* in mice. The induction of IFN- $\alpha$  by rAd28 and rAd35 was associated with efficient infection and phenotypic maturation of both human and mouse DCs. We further demonstrate that IFN- $\alpha/\beta$  receptor knockout (IFN $\alpha\beta$ <sup>-/-</sup>) mice vaccinated with rAd28 and rAd35 generated more Ag-specific T cells than did similarly vaccinated wild-type mice. This difference was not observed in mice immunized with rAd5. IFN- $\alpha$  signaling during immunization with rAd28 and rAd35 was also found to skew the central/effector memory distribution and functional profile of the CD8 T cell response. Finally, we show that the induction of IFN- $\alpha$  limits insert expression by rAd28 and rAd35, providing a possible mechanism for the observed effects on immunogenicity.

## Materials and Methods

### PBMC isolation

Human PBMCs from normal, healthy donors were obtained by automated leukapheresis and isolated by density gradient centrifugation using Ficoll-Plaque PLUS (GE Healthcare). PBMCs were cultured in RPMI 1640 supplemented with 2 mM L-glutamine (Invitrogen), 1% streptomycin and penicillin (Invitrogen), and 10% FCS (Life Technologies) at a density of  $1 \times 10^6$  cells/ml. Signed informed consent was obtained from all donors in accordance with the Declaration of Helsinki, and the study was approved by the relevant institutional review board.

### Isolation of primary DC subsets from blood

The sorting processes for direct isolation of CD11c<sup>+</sup> myeloid dendritic cells (mDCs) and CD123<sup>+</sup> plasmacytoid dendritic cells (pDCs) from blood have been described previously (1, 38, 39). Briefly, PBMCs collected from healthy blood donors were further enriched for monocytes/DCs by semi-automatic counterflow centrifugation (Elutra Cell Separation System). CD1c and BDCA4 isolation kits (Miltenyi Biotec) were used in conjunction with an AutoMACS magnetic cell sorter (Miltenyi Biotec) to isolate mDCs and pDCs, yielding highly enriched populations for both (>85%) (1). Flow cytometry revealed the contaminating cells to be CD14<sup>+</sup>. For microarray experiments, the respective DC populations were further purified into CD11c<sup>+</sup>CD14<sup>-</sup> populations (for mDCs) and CD123<sup>+</sup>CD14<sup>-</sup> populations (for pDCs) using a FACSARIA cell sorter (BD Biosciences). This process yielded highly pure cell populations (>99.9%). Sorted cell populations were cultured in RPMI 1640 supplemented with 2 mM L-glutamine, 1% streptomycin and penicillin, and 10% FCS at a density of  $1 \times 10^6$  cells/ml.

### Construction of rAds

Replication-deficient rAd5, rAd28, and rAd35 vectors expressing SIV Gag or eGFP were provided by GenVec. The construction of the E1/E3/E4-deleted rAd5 and rAd35 (40, 41) and the E1-deleted rAd28 (14) has been described previously.

### rAd infection and stimulation

Cells were exposed to multiple multiplicities of infection (MOIs) of rAd5, rAd28, or rAd35 encoding eGFP for 24 h. TLR7/8 ligand (1  $\mu$ g/ml) (3M Company) was used to induce DC maturation.

### CD46 blocking

Isolated mDCs were treated with anti-CD46 neutralizing Ab (clone M177; Hycult Biotechnology) or an isotype-matched control (clone P3; eBioscience) for 15 min at room temperature prior to rAd exposure.

### DC infection and phenotype assessment by polychromatic flow cytometry

Isolated and rAd-infected DCs were washed with PBS and incubated with a viability marker (ViViD; Molecular Probes) and FcR blocking reagent (Miltenyi Biotec) for 10 min at room temperature. Cells were then washed with PBS containing 0.5% BSA and stained with fluorescently labeled Abs to CD11c, CD123, CD14, and CD80 for 15 min at room temperature. Similarly, PBMCs were washed and incubated with ViViD and FcR blocking reagent before the cells were stained with fluorescently labeled Abs to CD3, CD4, CD8, CD14, CD19, CD56, and HLA-DR for 15 min at room temperature. All samples were analyzed using an LSR II flow

cytometer (BD Biosciences). Data analysis was performed using FlowJo version 9.3.2 (Tree Star).

### Measurements of human and mouse IFN- $\alpha$ release

The supernatants from human and mouse DC cultures as well as serum collected from mice 6 h postimmunization were analyzed using VeriKine Human or Mouse IFN- $\alpha$  ELISA Kits (PBL Laboratories), respectively, in accordance with the manufacturer's instructions.

### Microarrays

Purified DC populations were either left unexposed or incubated with rAd5, rAd28, rAd35, or TLR7/8 ligand for 24 h. The cells were then harvested and placed at  $-80^\circ\text{C}$  in RLT buffer. Total RNA was isolated using RNeasy Micro Kits (Qiagen) according to the manufacturer's protocol and checked for quantity and quality using a NanoDrop 2000c spectrophotometer (Thermo Fisher Scientific) and an Experion Automated Electrophoresis System. Samples with an RQI classification  $\geq 7.0$  were normalized at 150 ng for tRNA input and amplified using Illumina TotalPrep RNA amplification kits (Ambion) according to the manufacturer's protocol. Microarray analysis was conducted using 750 ng of biotinylated cRNA hybridized to Human RefSeq-8 V3 BeadChips (Illumina) at  $58^\circ\text{C}$  for 20 h. The hybridized BeadChips were washed, blocked, stained, and scanned according to the manufacturer's protocol. The arrays were scanned using an Illumina BeadStation 500GX scanner and quantified using GenomeStudio software (Illumina). Analysis of the GenomeStudio output data was conducted using the R statistical language (R Development Core Team; www.R-project.org) (42) and various software packages from Bioconductor (43). Quantile normalization was applied, followed by a  $\log_2$  transformation. The LIMMA package from Bioconductor (44) was used to fit a single linear model to each probe and to perform (moderated) *t* tests on the incubation effects for a given DC population. Specifically, the fitted linear model assigned mock samples as DC population-specific and individual-specific intercepts (multivariate analog of paired samples). The list of IFN-regulated genes (IRGs) employed to filter genes was defined in previous work (45, 46). Ingenuity Pathway Analysis software (Ingenuity Systems, www.ingenuity.com) was used to test for overrepresentation of canonical pathways in the lists of genes differentially expressed upon incubation. For this purpose, statistical significance for gene differential expression was defined as  $|FC| > 1.5$ ,  $p < 0.01$  at FDR < 30%, whereas statistical significance for overrepresentation (Fisher's exact test) was defined at FDR < 5%. The expected proportions of false positives (FDR) were estimated from the unadjusted *p* values using the Benjamini and Hochberg method. The microarray data are available through the National Center for Biotechnology Information Gene Expression Omnibus database (accession number GSE37128).

### Mice

C57BL/6 mice purchased from The Jackson Laboratory were maintained in the Vaccine Research Center Animal Care Unit. IFN $\alpha\beta$ <sup>-/-</sup> mice were provided by Brian Kelsall (National Institutes of Health) and bred and maintained in the animal facility at the National Institutes of Health. All experiments were conducted following the guidelines of, and with approval from, the Vaccine Research Center Animal Care and Use Committee.

### Murine DC isolation

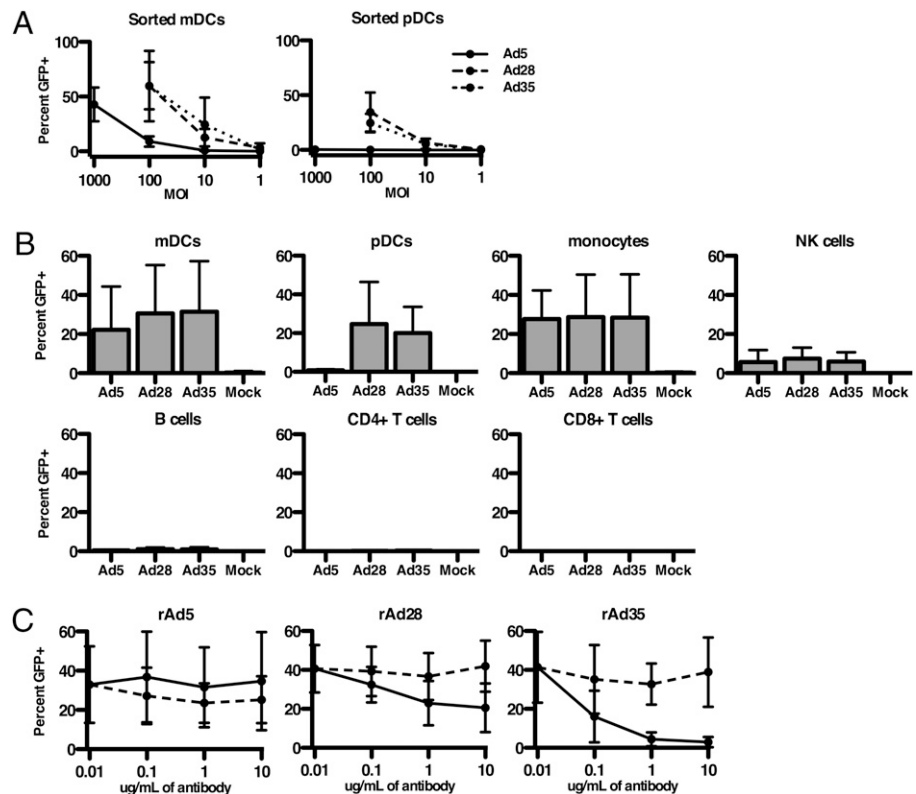
Splenocytes were harvested from wild-type mice. Total DCs were isolated to >50% purity using a pan-DC microbead kit (Miltenyi Biotec) and an AutoMACS cell sorter according to the manufacturer's protocols.

### Immunizations

Mice were immunized with rAd5, rAd28, or rAd35 in a total volume of 50  $\mu$ l by s.c. injection in each of the hind footpads.

### Polychromatic flow cytometric analysis of SIV Gag-specific responses

Blood was collected from mice into heparin-containing tubes and treated for 3 min at room temperature with ACK buffer (Lonza) to lyse the RBCs. The cells were then pelleted and washed twice with PBS. Cells were transferred to 96-well plates and incubated with a viability marker (OrViD; Molecular Probes) and FcR blocking reagent for 10 min at room temperature. After a further wash with PBS containing 0.5% BSA, cells were stained for 15 min at room temperature with fluorescently labeled AL11 (AAVKNWMTQTL) SIV Gag tetramer, constructed around H2-D<sup>b</sup> using protocols described previously (47), and Abs to CD8, CD27, CD44, CD62L, and CD127. After fixation, cells were analyzed using an LSR II flow cytometer (BD Biosciences). Viable CD8 T cells were selected within a small lymphocyte



**FIGURE 1.** rAd28 and rAd35 infect human immune cells more efficiently than rAd5. **(A)** Infection frequencies of mDCs and pDCs treated with rAd5 vector, rAd28 vector, or rAd35 vector ( $n = 5$ ). **(B)** Infection frequencies of mDCs, pDCs, monocytes, NK cells, B cells, CD4 T cells, and CD8 T cells from PBMCs treated with rAd5 vector (MOI 1000), rAd28 vector (MOI 100), or rAd35 vector (MOI 100), each with an uninfected control ( $n = 8$ ). **(C)** Infection frequencies of mDCs treated with anti-CD46 Ab (solid line) or a matched isotype control (dashed line) ( $n = 4$ ).

gate based on CD8 expression and lack of staining with OrViD. The fraction of viable CD8 T cells binding the AL11 tetramer was used to determine the percentage of SIV Gag-specific CD8 T cells. Data analysis was performed using FlowJo version 9.3.2.

#### Intracellular cytokine staining

Splenocytes isolated from mice 82 d after immunization were transferred to a 96-well plate. Cells were exposed to AL11 peptide for 30 min and treated with brefeldin A (10  $\mu$ g/ml; Sigma Aldrich) and monensin (0.7  $\mu$ g/ml; eBioscience) for 6 h. Cells were then washed twice with PBS and incubated with a viability marker (ViViD) for 10 min at room temperature. After a further wash in PBS containing 0.5% BSA, cells were stained with fluorescently labeled Abs to CD4 and CD8 for 15 min at room temperature. Cells were then permeabilized with Fix/Perm (BD Biosciences) for 20 min at room temperature, washed with Perm/Wash (BD Biosciences), and stained with fluorescently labeled Abs to CD3, IFN- $\gamma$ , IL-2, and TNF for a further 20 min at room temperature. Cells were analyzed using an LSR II flow cytometer (BD Biosciences). Viable CD8 T cells were selected using the viability marker, a small lymphocyte gate, and expression of CD3 and CD8. Frequencies of cytokine-producing cells were calculated within the viable CD3<sup>+</sup> CD8<sup>+</sup> T cell populations. Data analysis was performed using FlowJo version 9.3.2.

#### Exogenous rIFN- $\alpha$ treatment

Exogenous mouse rIFN- $\alpha$  (5 ng/ml) (PBL IFN Source) was added to cultures immediately before infection with the rAd vectors.

#### Statistical analyses

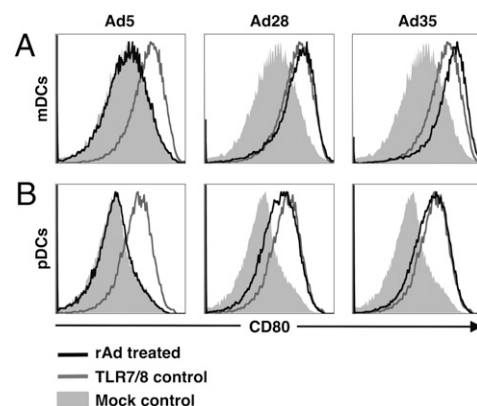
Statistical analyses were performed using the Wilcoxon signed-rank test or the Mann-Whitney  $U$  test with GraphPad Prism version 5.0c software (GraphPad Software). Analysis and presentation of distributions was performed using SPICE version 5.1, downloaded from <http://exon.niaid.nih.gov> (48). Comparison of distributions was performed using a Student  $t$  test and a partial permutation test as described previously (48). Error bars shown represent SD in all cases.

## Results

#### Infectivity of rAd5, rAd28, and rAd35 vectors

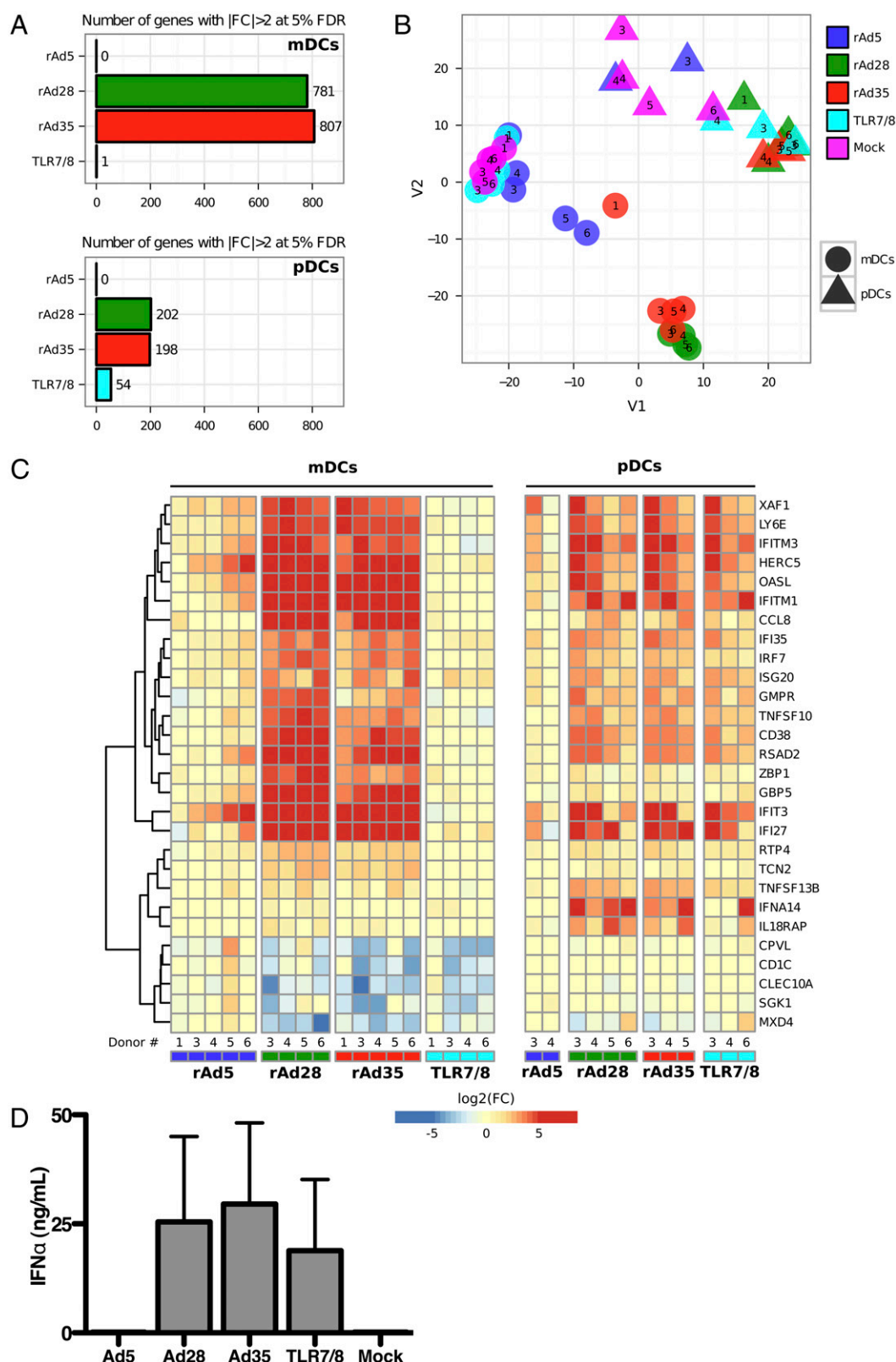
We compared the infectivity of rAd5, rAd28, and rAd35 on isolated human DCs. rAd28 and rAd35 efficiently infected mDCs ( $59 \pm$

21% and  $59 \pm 32\%$  eGFP<sup>+</sup> cells at MOI 100, respectively). In contrast, rAd5 required a 10-fold higher virus inoculum to show similar infectivity ( $43 \pm 15\%$  eGFP<sup>+</sup> cells at MOI 1000) (Fig. 1A). Moreover, rAd28 and rAd35 infected  $>25\%$  of pDCs at MOI 100, whereas rAd5 exposure did not show detectable infection at any MOI tested. This disparity between rAd5 and rAd35 infection of DCs is in line with prior observations (1, 49, 50). To test the ability of rAd5, rAd28, and rAd35 to infect other human immune cells, we performed similar infections on unfractionated PBMCs. At an MOI of 100, rAd28 and rAd35 infected mDCs ( $31 \pm 24\%$ ,  $31 \pm 26\%$ ), pDCs ( $24 \pm 21\%$ ,  $20 \pm 13\%$ ), monocytes ( $28 \pm 21\%$ ,  $28 \pm 22\%$ ), and NK cells ( $7 \pm 5\%$ ,  $6 \pm 5\%$ ) (Fig. 1B). A 10-fold greater dose (MOI 1000) of rAd5 was needed to infect mDCs ( $22 \pm 20\%$ ), monocytes ( $27 \pm 14\%$ ), and NK cells ( $6 \pm 5\%$ ) at a similar level. As expected, rAd5 did not infect pDCs. The rate of



**FIGURE 2.** rAd28 and rAd35 induce DC maturation. CD80 expression in mDCs **(A)** and pDCs **(B)** treated with rAd5 vector (MOI 1000), rAd28 vector (MOI 100), or rAd35 vector (MOI 100) (black line) compared with a TLR7/8 ligand-treated (gray line) control and an uninfected control (gray background).





**FIGURE 3.** Transcriptional profiling by gene array of DC populations under different incubation conditions. **(A)** Number of genes differentially expressed relative to the uninfected control ( $|FC| > 2$  at 5% FDR). **(B)** Two-dimensional multidimensional scaling. Genes were filtered for IRGs prior to applying the algorithm. **(C)** Heat map representation of  $(\log_2)$  fold-changes top most significant IRGs relative to the uninfected control from the same individual. Specifically, genes were selected as the top 10 upregulated and top 10 downregulated IRGs for each incubation separately, subsequently applying a cutoff for statistical significance at  $|FC| > 5$  at 5% FDR to limit the number of genes shown. **(D)** The level of IFN- $\alpha$ , measured by ELISA, in the supernatant of pDCs treated with rAd5 vector (MOI 1000), rAd28 vector (MOI 100), rAd35 vector (MOI 100), or TLR7/8 ligand, together with uninfected controls ( $n = 5$ ).

infection of B cells, CD4 T cells, and CD8 T cells was negligible with all rAd vectors.

#### Receptor usage

The entry receptors used by Ad5 and Ad35 are CAR and CD46, respectively (1). The entry receptor for Ad28 is less clear. An anti-CD46 Ab was unable to block infection of mDCs by rAd5 but efficiently blocked infection by rAd35 (Fig. 1C). In contrast, the anti-CD46 Ab only modestly reduced infection of mDCs by rAd28, and only at high concentrations. The isotype-matched control Ab showed no reduction in infectivity at any dose for any vector. Although we cannot rule out that Ad28 may be using CD46 as one of several cellular receptors, we can conclude that Ad28 is not as dependent as Ad35 on CD46 for infection of human mDCs.

#### rAd vector-induced maturation

To determine the degree of maturation and activation induced by rAd vectors, we analyzed costimulatory receptor CD80 expression on mDCs (Fig. 2A) and pDCs (Fig. 2B) 24 h postinfection. In both mDCs and pDCs exposed to rAd28 and rAd35, CD80 expression was upregulated similar to that induced by TLR stimulation. In contrast, expression of CD80 in either of the DC subsets exposed to rAd5 was unchanged compared with the uninfected control.

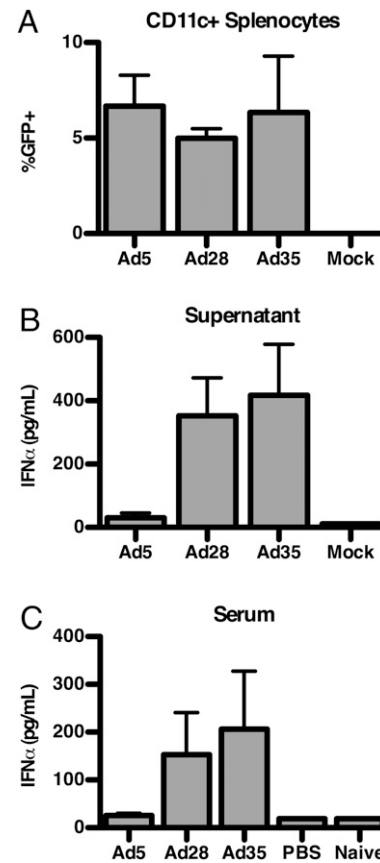
#### mRNA expression profile

To characterize further the effects of rAd exposure on human DCs, we performed gene array analysis on sorted populations of rAd-infected mDCs and pDCs. Microarrays of mDCs infected with rAd28 and rAd35 showed that the expression levels of 781 and 807 genes were either upregulated or downregulated compared with those of donor-matched mock-infected controls, respectively ( $|FC| > 2$  at 5% FDR) (Fig. 3A). Conversely, no genes were differentially expressed upon infection with rAd5. This difference in effect was also observed with pDCs, where expression levels of 202 and 198 genes were significantly altered upon infection with rAd28 and rAd35, respectively, compared with no changes postinfection with rAd5. Many of the genes altered upon infection with rAd28 and rAd35 related directly to type I IFN and type I IFN signaling (Supplemental Fig. 1). Pathway analysis with Ingenuity Pathway Analysis software confirmed that activation of type I IFN was a major effect of infection with rAd28 and rAd35 in both mDCs and pDCs (Supplemental Fig. 2). Additionally, two-dimensional multidimensional scaling focusing on IRGs revealed that samples infected with rAd28 and rAd35 generally clustered together in mDC and pDC samples, away from the clusters formed by samples infected with rAd5 and mock-infected controls (Fig. 3B).

Many of the genes upregulated by infection with rAd28 and rAd35, but not by rAd5, are directly involved in the type I IFN response in both mDCs and pDCs (Fig. 3C). We therefore compared IFN- $\alpha$  production induced by these vectors. pDCs infected with rAd28 and rAd35 produced large amounts of IFN- $\alpha$  ( $25 \pm 19$  ng/ml and  $29 \pm 19$  ng/ml, respectively), whereas pDCs infected with rAd5 and uninfected controls did not produce IFN- $\alpha$  (Fig. 3D). Notably, rAd28 and rAd35 infection led to levels of IFN- $\alpha$  production that were similar or higher than those induced by TLR7/8 ligation ( $19 \pm 16$  ng/ml).

#### Effects of rAd vectors on IFN- $\alpha$ induction in mice

The expression of the receptors for Ad5 (CAR) and Ad35 (CD46) differs between humans and mice (1, 49, 51, 52). We therefore evaluated the ability of rAd5, rAd28, and rAd35 to infect and induce IFN- $\alpha$  by isolated mouse DCs. Although higher inocula of rAd28 and rAd35 were required to achieve infection, all three vectors were able to infect murine DCs (Fig. 4A). Importantly, the



**FIGURE 4.** rAd28 and rAd35, but not rAd5, upregulate IFN- $\alpha$  production in mice in vivo and in vitro. **(A)** Infection frequencies of sorted murine CD11c $^{+}$  splenocytes treated with rAd5 vector (MOI 1000), rAd28 vector (MOI 10,000), or rAd35 vector (MOI 10,000), together with uninfected controls ( $n = 5$ ). **(B)** Levels of IFN- $\alpha$ , measured by ELISA, in the supernatant of sorted murine CD11c $^{+}$  splenocytes treated with rAd5 vector (MOI 10,000), rAd28 vector (MOI 10,000), or rAd35 vector (MOI 10,000), together with uninfected controls ( $n = 5$ ). **(C)** Serum levels of IFN- $\alpha$ , measured by ELISA, in mice immunized with rAd5 vector ( $1 \times 10^9$  VP/mouse), rAd28 vector ( $1 \times 10^9$  VP/mouse), or rAd35 vector ( $1 \times 10^9$  VP/mouse), together with PBS-injected and naive controls, at 6 h postvaccination ( $n = 5$ ).

pattern of IFN- $\alpha$  induction by these vectors held in mouse DCs. As observed in human DCs, only rAd28 ( $352 \pm 121$  pg/ml) and rAd35 ( $417 \pm 161$  pg/ml) were able to induce IFN- $\alpha$  production in mouse DCs, and neither rAd5 nor the uninfected control produced detectable IFN- $\alpha$  (Fig. 4B). Even when the same inocula of all three vectors were used, rAd28 and rAd35, but not rAd5, induced IFN- $\alpha$  production (data not shown). To confirm the differential induction of IFN- $\alpha$  production in vivo, we injected mice s.c. with the rAd vectors ( $1 \times 10^9$  VP/mouse). Mice injected with rAd28 and rAd35 vectors had serum IFN- $\alpha$  levels of  $153 \pm 88$  pg/ml and  $206 \pm 121$  pg/ml, respectively, 6 h postinjection (Fig. 4C). Serum IFN- $\alpha$  levels in the rAd5-treated, PBS-treated, and naive mice were all below the detection limit of the kit.

#### Role of IFN- $\alpha$ in the immunogenicity of rAd vectors in mice

Because the data generated both with human and murine cells showed that high levels of IFN- $\alpha$  were induced by rAd28 and rAd35, but not by rAd5, we investigated the impact of this differential IFN- $\alpha$  induction on vector immunogenicity. Previous studies have shown that rAd5 is significantly more immunogenic at lower doses than either rAd28 or rAd35 (13). We therefore immunized both wild-type and IFN- $\alpha$  receptor-deficient ( $IFN\alpha R^{-/-}$ ) mice with a low dose

( $5 \times 10^7$  VP/mouse) of each rAd vector encoding an SIV Gag insert and compared the magnitude of the induced T cell response by quantifying the number of tetramer<sup>+</sup> CD8 T cells specific for a dominant epitope derived from SIV Gag (AL11) (53). Only rAd5 immunization was able to induce a large AL11-specific T cell response in both wild-type (peak  $21.0 \pm 7.4\%$ ) mice and IFN $\alpha$  receptor-deficient (peak  $25.3 \pm 4.5\%$ ) mice (Fig. 5A, *top panel*). Both rAd28 (peak  $22.5 \pm 7.4\%$ ) and rAd35 (peak  $18.0 \pm 5.8\%$ ) immunization induced a frequency of AL11-specific T cells similar to that induced by rAd5 in the IFN $\alpha$  receptor-deficient mice, but not in the wild-type mice (Fig. 5A, *middle and bottom panels*). Wild-type mice developed a small AL11-specific CD8 T cell response after immunization with rAd28 (peak  $6.4 \pm 2.2\%$ ), and no measurable response was generated by rAd35 immunization. This difference in immunogenicity between wild-type and IFN $\alpha$  receptor-deficient mice immunized with rAd28 and rAd35 could be overcome by increasing the immunization dose. At a 20-fold higher inoculation ( $1 \times 10^9$  VP/mouse), all three vectors showed similar immunogenicity in wild-type and IFN $\alpha$  receptor-deficient mice (Fig. 5B).

#### rAd vector-induced IFN- $\alpha$ signaling and CD8 T cell memory phenotype and function

Next, we determined the impact of excess IFN- $\alpha$  production on long-term memory differentiation as defined by CD127 (IL-7 receptor) expression (54) in the high-dose immunized mice. Groups with no induction of IFN- $\alpha$  (rAd5-wild type, rAd5-IFN $\alpha$  receptor-deficient) and groups with interrupted IFN- $\alpha$  signaling (rAd28-IFN $\alpha$  receptor-deficient, rAd35-IFN $\alpha$  receptor-deficient) failed to establish high percentages of AL11<sup>+</sup> CD8 T cells that expressed CD127 ( $32.4 \pm 15.4\%$ ). In contrast, groups with intact IFN- $\alpha$  induction and signaling (rAd28-wild type, rAd35-wild type) maintained a much higher percentage of AL11<sup>+</sup> CD8 T cells that expressed CD127 ( $59.1 \pm 8.8\%$ ) (Fig. 6A).

To determine cytokine production profiles in SIV Gag-specific CD8 T cells from high-dose immunized mice, we measured the frequency of IFN- $\gamma$ , IL-2, and TNF producing CD8 T cells by flow cytometry after stimulation with the AL11 peptide. Overall, mice

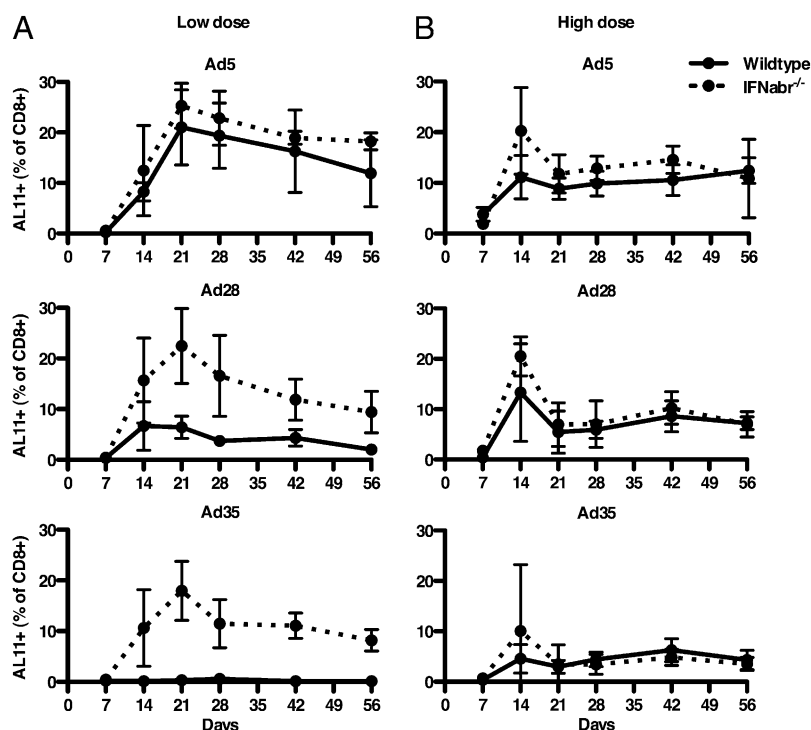
vaccinated with the rAd5 vector had slightly greater numbers of cytokine-producing cells than those of mice vaccinated with the rAd28 or rAd35 vectors (Fig. 6B, *top left panel*). This observation, although not statistically significant, is consistent with the larger AL11-specific CD8 T cell populations detected after high-dose vaccination. No significant differences in the frequency of cytokine-producing CD8 T cells between wild-type and IFN $\alpha$  receptor-deficient mice were observed in any of the groups (Fig. 6B).

When measured as a fraction of the total AL11-specific CD8 T cell response, wild-type mice vaccinated with rAd28 or rAd35 exhibited higher proportions of IL-2-producing cells ( $57.2 \pm 6.3\%$  and  $60.6 \pm 10.0\%$ , respectively) compared with their IFN $\alpha$  receptor-deficient counterparts ( $29.22 \pm 2.9\%$  and  $32.7 \pm 13.2\%$ , respectively) (Fig. 6B, *bottom panels*). Both wild-type and IFN $\alpha$  receptor-deficient mice vaccinated with rAd5 had low percentages of IL-2-producing cells ( $21.7 \pm 15.2\%$  and  $30.3 \pm 10.7\%$ , respectively) (Fig. 6B, *upper right panel*). Wild-type mice vaccinated with rAd28 and rAd35 had a higher proportion of their response dedicated to the coexpression of three cytokines (IFN- $\gamma$ <sup>+</sup> TNF<sup>+</sup> IL-2<sup>+</sup>) than that of IFN $\alpha$  receptor-deficient mice (Fig. 6C). This difference was mostly due to the higher proportion of IL-2-producing cells in the wild-type mice vaccinated with rAd28 and rAd35. Both wild-type and IFN $\alpha$  receptor-deficient mice vaccinated with rAd5 displayed a relatively lower proportion of triple-cytokine-positive AL11-specific CD8 T cells.

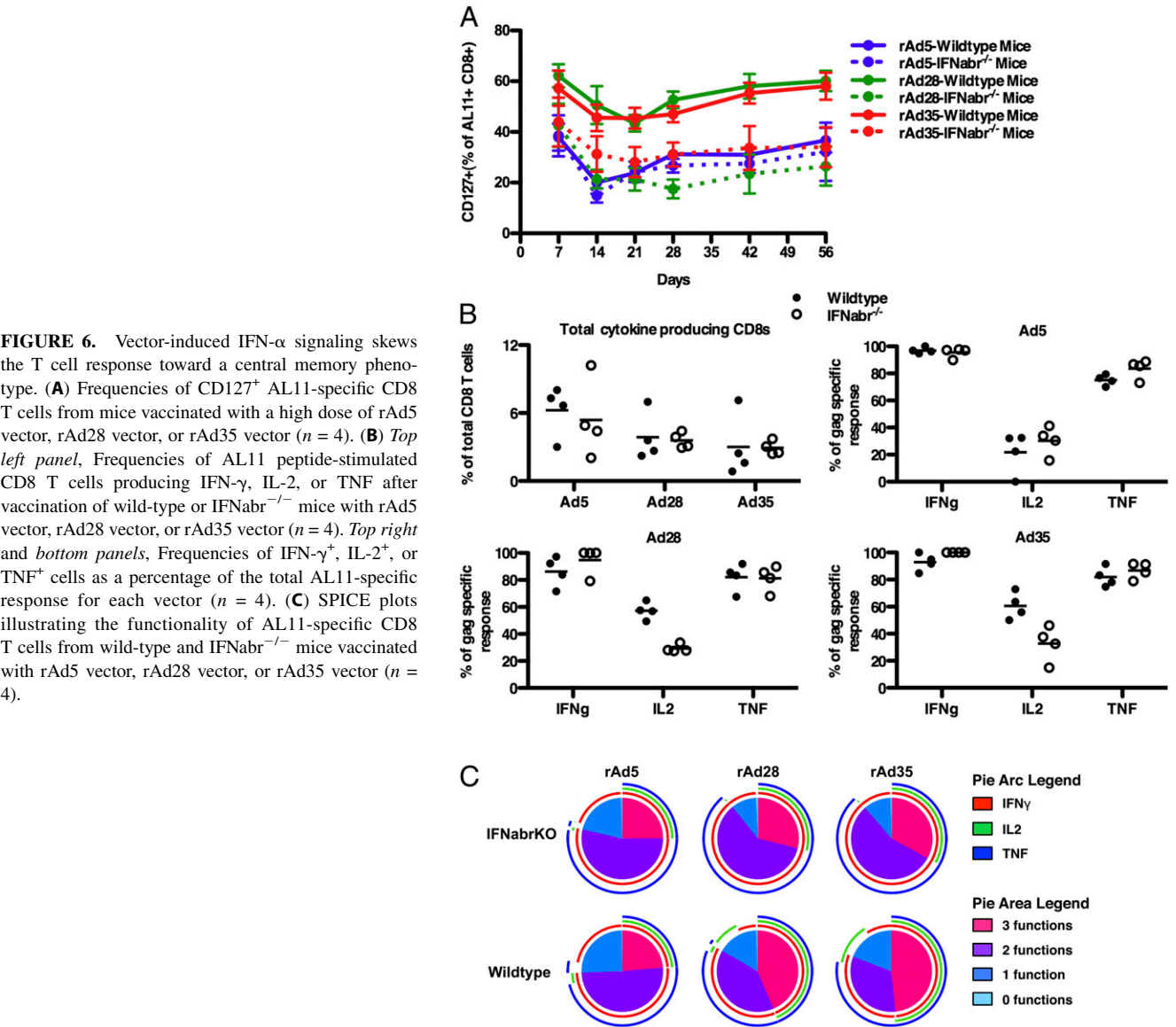
#### Impact of IFN- $\alpha$ on insert expression

We next examined the role of IFN- $\alpha$  production on insert expression by the rAd vectors to determine a possible mechanism for the differing immunogenicity. To determine this, we first compared the infectivity of each vector in wild-type mouse DCs exposed or not exposed to exogenous IFN- $\alpha$ . rAd5-infected DCs treated with IFN- $\alpha$  were found to have a 1.8-fold decrease in GFP expression compared with untreated rAd5-infected DCs (Fig. 7A). No difference in GFP expression was detected between IFN- $\alpha$ -treated or untreated DCs infected with rAd28 or rAd35.

We also compared the infectivity of the rAd vectors in DCs isolated from IFN $\alpha$  receptor-deficient mice with that in DCs from wild-type

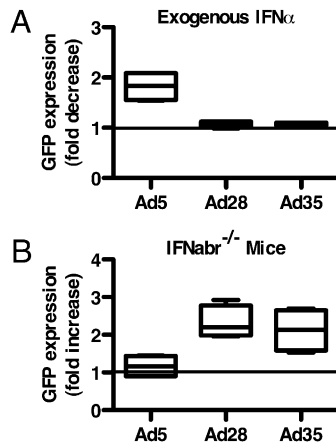


**FIGURE 5.** IFN $\alpha$  receptor-deficient mice develop greater insert-specific CD8<sup>+</sup> T cell responses to rAd28 and rAd35 than wild-type mice. Frequencies of AL11-specific CD8 T cells, quantified by tetramer staining, after low-dose ( $5 \times 10^7$  VP/mouse) (A) ( $n = 5$ ) and high-dose ( $1 \times 10^9$  VP/mouse) (B) ( $n = 4$ ) vaccination of wild-type and IFN $\alpha$  receptor-deficient mice with rAd5 vector, rAd28 vector, or rAd35 vector, all encoding SIV Gag.



mice. In rAd5-infected samples, we found no difference in GFP expression between wild-type and IFN $\alpha$ R<sup>-/-</sup> DCs (Fig. 7B). In

rAd28- and rAd35-infected samples, DCs from IFN $\alpha$ R<sup>-/-</sup> mice expressed GFP at more than twice the rate of that of wild-type DCs.



**FIGURE 7.** IFN- $\alpha$  signaling reduces the frequency of insert-positive cells. **(A)** Fold decrease in GFP expression in rAd-infected, IFN- $\alpha$ -treated DCs from wild-type mice compared with rAd-infected, IFN- $\alpha$ -untreated DCs. **(B)** Fold increase in GFP expression in rAd-infected DCs from IFN $\alpha$ R<sup>-/-</sup> mice compared with rAd-infected DCs from wild-type mice.

## Discussion

rAd vectors have been studied for potential use in vaccination strategies because of their ease of construction and ability to infect a large number of cells. Vectors constructed from Ad5 are efficient at eliciting large T cell responses but are limited clinically by extensive preexisting immunity in the general population. Rare serotype adenoviral vectors, such as rAd28 and rAd35, circumvent issues related to preexisting immunity but are less immunogenic than rAd5. The goal of this study was to determine what accounts for some of the differences in the immunogenicity of rare serotype rAd vectors.

DCs play an important role in both the initiation of innate immunity and the development of adaptive immunity (55). This dual function, combined with the important role that DCs play in anti-viral immunity, led us to investigate the impact of rAd exposure on two blood DC subsets, mDCs and pDCs (56). Notably, both rAd28 and rAd35 exerted a substantial effect on both mDCs and pDCs, particularly with respect to IFN- $\alpha$  production and maturation. In striking contrast, rAd5 did not infect pDCs and had



almost no detectable impact on either DC population, including any effects on IFN-related pathways. It has been proposed that vectors that induce innate immunity might perform better as vaccines due to potential adjuvant effects (1). Alternatively, vectors that do not alter the host cell or activate innate immunity are considered ideal for gene therapy, where immunity can prove detrimental (57, 58).

In this study, we show that increased innate immune activation, notably IFN- $\alpha$  signaling, by rAd vectors can have detrimental effects on the immunogenicity of the insert, reducing the magnitude of the resulting CD8 T cell response. IFN $\alpha$ <sup>-/-</sup> mice, when vaccinated with rAd28 and rAd35, could not respond to the IFN and developed more robust CD8 T cell responses compared with those of wild-type mice and similar to those observed after rAd5 vaccination.

The mechanisms responsible for reduced immunogenicity in the presence of IFN- $\alpha$  are not well understood. IFN- $\alpha$  can act in an autocrine manner, upregulating and activating RNase L and PKR, two potent host antiviral proteins (59). RNase L and PKR shut down host transcription and translation, respectively, and could potentially reduce insert expression or increase apoptosis in infected APCs, thereby reducing vector immunogenicity. Notably, our observations that IFN- $\alpha$  reduces the number of insert-positive cells after rAd exposure strongly support this possible mechanism. Additionally, the fact that the addition of exogenous IFN- $\alpha$  to rAd28- and rAd35-exposed DCs does not further decrease the fraction of insert-positive cells suggests that the amount of vector-induced IFN- $\alpha$  produced is already a maximally suppressive dose and that this suppression is limited to a 2-fold reduction of insert expression *in vitro*. This incomplete reduction in vector expression by IFN- $\alpha$  signaling may explain why immunogenicity can be restored by increasing the vaccine dose. Despite the partial suppression of insert expression by IFN- $\alpha$ , the magnitude of insert-positive cells can still attain a necessary immunogenicity threshold.

Additionally, IFN- $\alpha$  has been shown to have multiple paracrine activities. At a high inoculum of rAd28 and rAd35, we observed that vaccine-elicited CD8 T cells from wild-type mice were more polyfunctional and had a more pronounced long-term memory phenotype than the vaccine-elicited CD8 T cells from IFN $\alpha$ <sup>-/-</sup> mice or mice vaccinated with rAd5. These findings are consistent with prior observations showing an increased long-term memory phenotype in CD8 T cells stimulated with IFN- $\alpha$  (31). It is also feasible that CD8 T cell expansion in the presence of IFN- $\alpha$  leads to a predominantly central memory-like phenotype, which has previously been associated with enhanced polyfunctionality due to the retention of IL-2 production (60). Alternatively, separate mechanisms may be operating to mediate these distinct effects. IFN- $\alpha$  has also been shown either to promote or suppress T cell proliferation and survival depending on the timing, level, and duration of exposure. The effects of IFN- $\alpha$  signaling on CD8 T cell proliferation and survival after vaccination with rAd vectors are not fully understood. However, the inherent paradox of our results is that vector-induced IFN- $\alpha$  production can lead to a reduction in the magnitude of the CD8 T cell population while simultaneously increasing the quality and longevity of the response.

The mechanism(s) underlying the differential induction of IFN- $\alpha$  by rAd5 and the lower seroprevalence vectors, rAd28 and rAd35, is currently unknown. All three vectors used in our study are E1-deleted, rendering them replication-deficient and greatly reducing the transcription of the viral genome. Importantly, expression of two non-translated viral RNA sequences, viral-associated RNA I (VA-RNA I) and viral-associated RNA II (VA-RNA II), transcribed by host RNA polymerase III, are only partially down-

regulated (61, 62). These sequences are likely transcribed in infected host cells. All Ad serotypes contain a VA-RNA I sequence, and most encode a VA-RNA II (63). rAd5 VA-RNA I has been shown to inhibit RNase L and PKR function, as well as alter gene expression by functioning as microRNAs (64, 65). The function of Ad5 VA-RNA II is not well understood, but it may play a compensatory role in VA-RNA I-deleted vectors (66). The sequences of VA-RNAs vary greatly between rAd5, rAd28, and rAd35, and it is unknown if VA-RNAs from rAd28 or rAd35 function in a similar manner (63). It is known, however, that rAd5 VA-RNAs induce moderate IFN- $\alpha$  production at high doses (MOI 10,000) by stimulating host pattern recognition receptors (62). It is possible that rAd28 and rAd35 stimulate IFN- $\alpha$  production through a similar mechanism at lower MOI due to differences in VA-RNA sequence or expression levels, rendering them more efficient vectors for inducing IFN- $\alpha$ . Alternatively, different factors, such as surface receptor engagement, route of entry, intracellular trafficking patterns, and CpG content of the genome, may also be involved in the induction of IFN- $\alpha$  (67, 68). Further research is required to determine which of these factors are responsible for the differential induction of IFN- $\alpha$  by various rAd vectors.

In conclusion, our results show that, in stark contrast to rAd28 and rAd35, rAd5 fails to induce significant changes in DC mRNA expression or maturation. IFN- $\alpha$  production and induction of IFN-related pathways is characteristic of rAd28 and rAd35 infection of DCs, and this leads to a decrease in the magnitude but an improvement in the long-term memory potential (CD127 expression) and cytokine polyfunctionality of the insert-specific CD8 T cell response. The presence and impact of IFN- $\alpha$  production and signaling should therefore be taken into account when designing future rAd vectors.

## Disclosures

The authors have no financial conflicts of interest.

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