

Recombinant Adenoviral Vectors Turn on the Type I Interferon System without Inhibition of Transgene Expression and Viral Replication

Eduardo Huarte,* Esther Larrea,* Rubén Hernández-Alcoceba, Carlos Alfaro, Oihana Murillo, Ainhoa Arina, Iñigo Tirapu, Arantza Azpilicueta, Sandra Hervás-Stubbs, Sergia Bortolanza, José L. Pérez-Gracia, María P. Civeira, Jesús Prieto, José I. Riezu-Boj,[†] and Ignacio Melero^{†,‡}

Center for Applied Medical Research, School of Medicine, and University Clinic, University of Navarra, Avenida Pio XII, 55, 31008 Pamplona, Spain

*These authors contributed equally to this work.

[†]These authors share senior authorship.

[‡]To whom correspondence and reprint request should be addressed. E-mail: imelero@unav.es.

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Recombinant adenovirus administration gives rise to transgene-independent effects caused by the ability of the vector to activate innate immunity mechanisms. We show that recombinant adenoviruses encoding reporter genes trigger IFN- α and IFN- β transcription from both plasmacytoid and myeloid mouse dendritic cells. Interestingly, IFN- β and IFN- α 5 are the predominant transcribed type I IFN genes both *in vitro* and *in vivo*. In human peripheral blood leukocytes type I IFNs are induced by adenoviral vectors, with a preponderance of IFN- β together with IFN- α 1 and IFN- α 5 subtypes. Accordingly, functional type I IFN is readily detected in serum samples from human cancer patients who have been treated intratumorally with a recombinant adenovirus encoding thymidine kinase. Despite inducing functional IFN- α release in both mice and humans, gene transfer by recombinant adenoviruses is not interfered with by type I IFNs either *in vitro* or *in vivo*. Moreover, IFN- α does not impair replication of wild-type adenovirus. As a consequence, cancer gene therapy strategies with defective or replicative-competent adenoviruses are not expected to be hampered by the effect of the type I IFNs induced by the vector itself. However, type I IFN might modulate antitumor and antiadenoviral immune responses and thus influence the outcome of gene immunotherapy.

Key Words: interferon- α , interferon- β , dendritic cell, plasmacytoid dendritic cell, adenovirus, gene therapy, immunotherapy

INTRODUCTION

Gene therapy strategies have long overlooked the fact that viral vectors, either defective or replicative, might be able to induce type I interferons (IFNs). Type I IFNs are polypeptides discovered for their ability to interfere with viral infection of cell cultures [1]. In mice and humans there are 13 and 17 genes, respectively, encoding IFN- α subtypes, while there is a single gene encoding IFN- β in both species [2]. Despite this remarkable number of intronless genomic sequences within a single individual, there is evidence for only one heterodimeric receptor [3]. Slight functional differences among the IFN- α subtypes have been reported [4], but this issue remains an active and controversial area of research. The type I IFN receptor

induces biochemical pathways that include the activation of STAT-1, STAT-2, STAT-3, MAP kinases, PI₃ kinase, and NF- κ B [3,5]. Cells exposed to IFN- α / β are set in a state of viral resistance that depends on various effector molecules that interfere with viral replication and protein synthesis [1] and on increased susceptibility to the attack of cytolytic T lymphocytes (CTLs) [6,7]. It is not surprising that viral evolution has selected many mechanisms to escape type I interferon effects [8]. In addition to local antiviral effects, IFN- α manages to regulate the antibody immune response [9] and increase the activity of natural killer cells [10]. Recently IFN- α has been found to be involved in the presentation of antigens from peripheral cells by dendritic cells (DC) [11]. This cross-priming

function is believed to be crucial for the CTL immune response against viruses and possibly against tumors. It has been recently demonstrated that the expression of type I IFN receptor on CTL precursors is necessary for effector differentiation [12].

When a virus causes viremia, a specialized DC subpopulation named plasmacytoid DC (pDC) is induced to produce a high output of IFN- α and IFN- β [13,14]. In mice these cells are CD11c⁺ B220⁺ Ly6G/C⁺ and mPDCA-1⁺[15,16]. Other DC subpopulations are also known to produce IFN- α in response to proper viral molecules [17]. These cells sense the presence of virus by a number of known receptors and possibly others that remain undiscovered. Among these, TLR-9, TLR-7, and TLR-3 detect viral nucleic acids inside phagosomes [14], while cytoplasmic double-stranded RNA is detected by PKR [17] and possibly other RNA binding proteins [18]. Systemic treatment with type I IFNs is used clinically mainly in the treatment of chronic viral hepatitis [19]. Other empirical indications for this drug exist, including malignancies and multiple sclerosis, although the mechanisms of action are still poorly understood.

Type I IFN induction by viral vectors could be highly relevant for gene therapy. On the one hand, there is potential interference with viral replication or gene transfer, and on the other there are biological effects from type I IFNs to be added to those set in motion by the transgene(s) [20]. In this sense, if viral vectors were capable of triggering IFN- α/β , their effects, both as vaccines and as shuttles of immune system genes, would be greatly modified. In the case of cytokine gene therapy [20] the combined effects of the transgene expression and the innately induced type I IFN would be of obvious therapeutic importance [21].

In this study, we show that first-generation adenoviral vectors induce type I IFNs both *in vivo* and *in vitro* from myeloid DC (mDC) and pDC, even though IFN- α/β do not modify adenoviral replication or gene transfer either *in vivo* or *in vitro*.

RESULTS

Recombinant Adenovirus Induces IFN- α and IFN- β Release From Murine Plasmacytoid and Myeloid DC Cultures

In vitro culture of mononucleated mouse bone marrow cells for 10–12 days in the presence of 100 ng/ml sFLT-3L leads to the enrichment of DC of both plasmacytoid (mPDCA-1⁺) and myeloid (mPDCA-1⁻ CD11c⁺) lineages [22]. In our cultures, CD11c⁺ cells routinely ranged from 40 to 70% and plasmacytoid mPDCA-1⁺ cells from 20 to 30% (data not shown). As shown in Fig. 1A, exposure to recombinant adenovirus encoding enhanced green fluorescent protein (EGFP) under the control of the CMV promoter (AdCMVGFP) gave rise to induction of IFN- α and - β mRNA in bulk cultures. When we sorted each cell

subtype sequentially to >95% purity using immunomagnetic beads, we found that both subtypes of DC were transcribing type I IFNs. Moreover, we found that this phenomenon occurred in a wide range of adenoviral m.o.i., without need for productive gene transfer of the pDC, as documented by lack of EGFP expression in FACS studies (Fig. 1B). By contrast, a fraction of mPDCA-1⁻ CD11c⁺ mDC was transfected by AdCMVGFP (Fig. 1B). These IFN- α and IFN- β inducing effects were independent of the transgene encoded in the adenovirus, since they were also observed with an adenovirus encoding β -galactosidase (AdCMVLacZ) (data not shown).

Although we had expected a broader spectrum of IFN- α genes to be transcribed, IFN- α 5 was the one that was earlier and more intensely expressed along with IFN- β (Fig. 1C). The polymerase chain reaction (PCR) cloning technique used to study the IFN- α genes has been validated, and consequently all 16 IFN- α sequences had an equal chance to be detected. It is of note that the majority of IFN- α mRNA produced under baseline conditions in DC is IFN- α 2, which has been described as a pseudogene [23].

Type I IFN was secreted into the supernatant of these bulk cultures and we assessed its biological activity in a standard encephalomyocarditis virus (EMCV) protection bioassay quantified with titrated standards of commercial mouse IFN- α (Fig. 1D).

AdCMVGFP was virtually lipopolysaccharide (LPS) free (<0.15 EU/ml). However, to exclude the effects of other possible contaminants in the adenovirus preparation, we used an adenovirus encoding the HSV-TK (AdTK), which was manufactured and quality controlled under GMP conditions [24]. As shown in Fig. 1E, this clinical-grade recombinant adenovirus induced the transcription of IFN- α and - β as efficiently as AdCMVGFP tested in parallel at the same m.o.i.

These results suggested that recombinant adenoviruses could act as inducers of IFN- α and - β from DC and that this mechanism, if operational *in vivo*, could alter the results of gene therapy strategies involving adenoviral vectors.

IFN- α and IFN- β Transcription is Induced by Intravenous Injection of Recombinant Adenovirus in Splenic Myeloid and Plasmacytoid DC

To investigate if recombinant adenovirus would induce IFN- α and IFN- β production from DC *in vivo*, we used a procedure to enrich the spleen populations of DC *in vivo*. As described [25], we performed a hydrodynamic injection of a plasmid encoding the DC differentiation factor sFLT-3L. As a result, 1 week after hydrodynamic injection the spleen of mice contained up to 10% total DC. This represented a homogeneous 10-fold enrichment of the various DC populations.

We injected doses of 10⁸ pfu AdCMVGFP or clinical grade AdTK into these mice via the tail vein. Twenty-

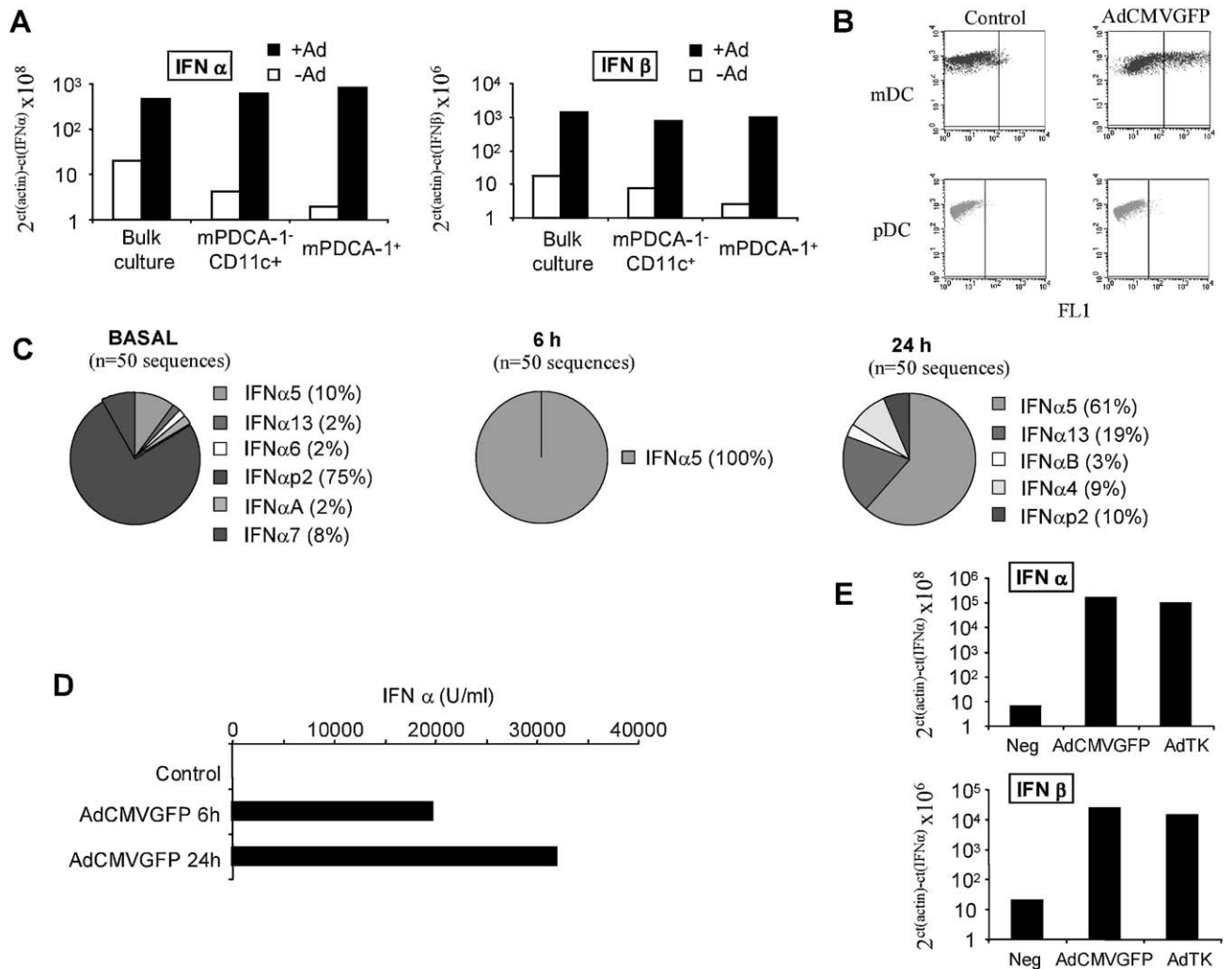


FIG. 1. IFN- α and IFN- β induction by adenovirus in murine DC. (A) Bone marrow mononuclear cells were cultured for 12 days in the presence of 100 μ g/ml sFLT-3L giving rise to cultures containing \approx 60% different subpopulations of DC. Bulk cultures and mPDCA-1⁻ CD11c⁺ or mPDCA-1⁺ positively selected cells by immunomagnetic methods were exposed to AdCMVGFP at m.o.i. 500. mRNA encoding IFN- α and IFN- β was quantified in comparison to β -actin mRNA by quantitative RT-PCR before (white bars) and 24 h after (black bars) exposure to recombinant adenovirus. (B) EGFP expression measured by FACS analysis of purified mPDCA-1⁺ DC and mDC from sFLT-3L bone marrow cultures transfected with AdCMVGFP at m.o.i. 1000. (C) IFN- α subtype mRNA amplified by PCR from bone marrow cell culture was cloned in the TOPO TA cloning kit. DNAs from 50 single clones at 0, 6, and 24 h after exposure to AdCMVGFP were completely sequenced and compared to reported GenBank IFN- α sequences to identify the different IFN- α subtypes. Results are expressed as the relative frequency of clones from each IFN- α gene at each given time point. (D) Quantitative bioassay of type I IFN activity present in supernatants of sFLT-3L bone marrow cultures at 0, 6, and 24 h after exposure to AdCMVGFP at m.o.i. 1000. (E) Experiment similar to that in A with dendritic cells in culture stimulated for 6 h as indicated by AdCMVGFP or AdTK at m.o.i. 500. Data in A, B, and E are representative of five experiments performed independently.

four hours later we removed the spleens surgically and extracted RNA from immunomagnetically purified mPDCA-1⁺ plasmacytoid and mPDCA-1⁻ CD11c⁺ myeloid DC. Purity was as high as 90–100% in each separation procedure.

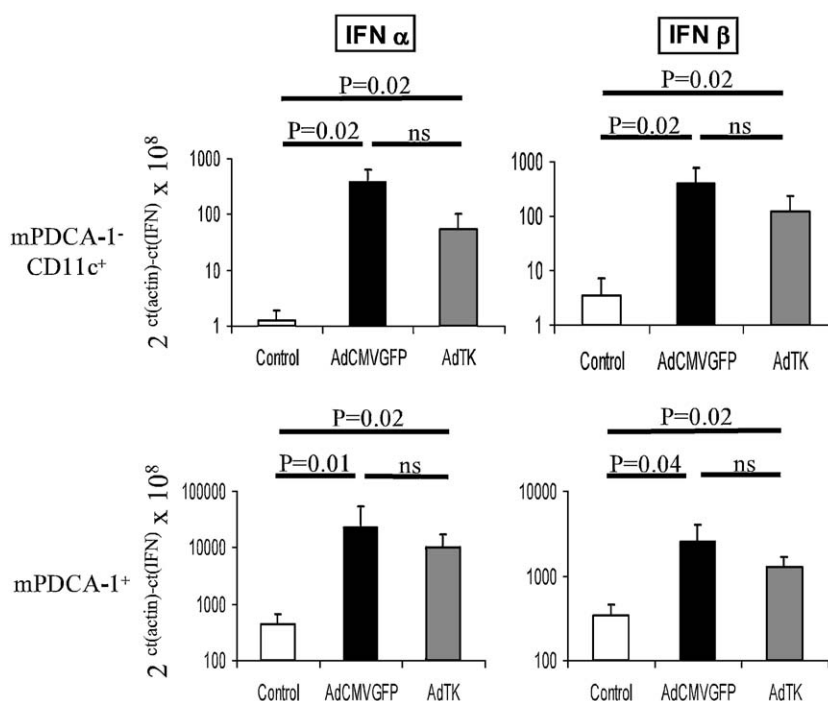
As can be observed in Fig. 2, both mPDCA-1⁻ CD11c⁺ and mPDCA-1⁺ splenocytes readily transcribed IFN- α and IFN- β mRNAs with high intensity as assessed by quantitative RT-PCR. It is of note that levels of mRNA expression were apparently much higher in the

mPDCA-1⁺ cells, indicating their prominent role in IFN- α production.

Once again, in the AdCMVGFP or AdTK-treated mice the majority of the detected IFN- α corresponded to the IFN- α 5 gene (8 of 15 cloned sequences), indicating a selective induction of this particular IFN- α gene (data not shown).

These *in vivo* results imply that systemic adenovirus administration in mice causes the activation of the type I IFN system in DC.

FIG. 2. *In vivo* induction of type I IFN in mouse DC by recombinant adenovirus. Mice enriched in splenic DC by pretreatment with sFLT-3L given by hydrodynamic delivery 7 days prior to the experiment were iv injected with 10^8 pfu AdCMVGFP or saline as control. Spleens were harvested 24 h later and mRNA was obtained from mPDCA-1⁻ CD11c⁺ or mPDCA-1⁺ immunomagnetically sorted cells. Results represent quantitative RT-PCRs measuring IFN- α and IFN- β mRNA in comparison to β -actin from three experiments independently performed.



IFN- α Does Not Interfere with Gene Transfection of Adenoviral Vectors

If exposure to adenoviral vectors induces IFN- α and IFN- β , this event could potentially interfere with their ability to infect and deliver genes to target cells. However, as shown in Fig. 3A, exposure to serial concentrations of human IFN- α 2 from 100 to 5000 IU/ml for 24 h was unable to inhibit gene transfer to HeLa cells by AdCMVGFP at various m.o.i. However, using a recombinant defective Semliki Forest virus encoding β -Gal (SFVLacZ) as a control, we observed a very strong (nearly 100%) inhibition of transgene expression in those HeLa cells infected with SFVLacZ (Fig. 3B).

The *in vitro* effects shown in Fig. 3A suggest that infection with adenovirus *in vivo*, although inducing IFN- α and - β , would not interfere with adenoviral infection or gene transfection. This is relevant either for sequential repetition of adenovirus doses or for conditional replicative adenovirus used in cancer treatment.

To explore this issue, we pretreated C57BL/6 mice intravenously with 10^8 pfu of AdCMVGFP or AdCMV-LacZ 24 h before iv administration of a replication-competent adenovirus encoding luciferase (AdWTLuc). Using this route of administration, most adenoviral-mediated gene expression is detected in the liver. At different time points, we measured the expression of luciferase by *in vivo* detection of light emission after the intraperitoneal injection of the substrate D-luciferin. Consistent with the *in vitro* results, the intra-

venous injection of two different adenoviral vectors did not reduce the infectivity and gene transfer of another adenoviral vector administered 24 h later (Fig. 3C). Our results are interpreted in the sense that the type I IFN induced by the adenovirus vectors does not interfere with subsequent adenoviral gene transfer in the liver *in vivo*. This does not preclude that such IFN- α and - β could be relevant for other *in vivo* effects of gene therapy with recombinant adenoviruses.

IFN- α Does Not Interfere with Adenovirus Replication *in Vitro*

To address whether viral replication was affected by type I IFN, we studied the influence of this molecule on the ability of the wild-type adenovirus (AdWT) to propagate its cytopathic effect on monolayers of permissive cells (human cervical cancer HeLa cells). We designed the experimental conditions (low viral load and late time points of evaluation) to ensure that the reduction in the cell number is dependent on viral replication and on cell-to-cell spread in the monolayers. Cytopathic effects of adenovirus were not altered by IFN- α (Fig. 4A), while this cytokine totally decreased the cytopathic effect of EMCV over a wide range of concentrations (Fig. 4B).

Therefore if adenovirus induced the release of IFN- α and - β when given to human subjects, it would be not expected to interfere acutely with adenoviral replication or gene transfer.

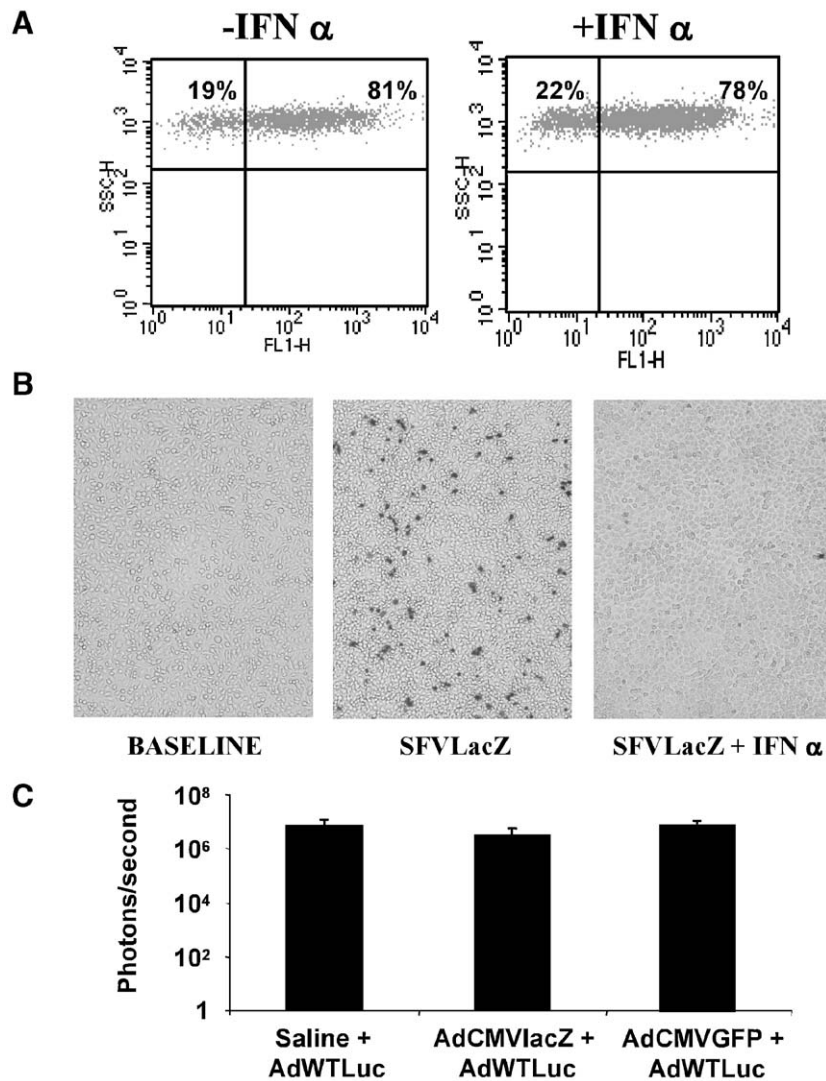


FIG. 3. IFN- α does not interfere with *in vitro* and *in vivo* gene transduction with adenoviral vectors. (A) HeLa cells were infected with AdCMVGFP (m.o.i. 500) with or without human IFN- α 2 at 5000 IU/ml. EGFP expression was quantified by FACS in gated viable cells. (B) As a positive control for the inhibitory effects, a SFV vector carrying the LacZ gene (SFVLacZ) was used to infect HeLa cells in the presence or absence of 500 IU/ml IFN- α , as indicated. The expression of the LacZ gene was visualized by X-Gal staining and microscopy. Baseline: uninfected cells. (C) C57BL/6 mice were intravenously injected with 10^8 pfu of AdCMVlacZ, AdCMVGFP, or saline as control ($n = 10$ per group). Twenty-four hours later, mice were injected intravenously with 10^8 pfu of a replication-competent adenovirus expressing luciferase (AdWT-Luc). The luciferase activity was measured *in vivo* 48 h later using an *in vivo* imaging system. The bars represent the light emission (photons/s \pm SD) in the liver of mice that were previously ip injected with the luciferase substrate D-luciferin. Results are representative of two independent experiments.

Recombinant Adenovirus Induces IFN- α and IFN- β from Human Peripheral Blood Leukocytes

Defective adenoviruses encoding different transgenes are being used for cancer immunotherapy both in animal models and in the clinic. To address if exposure of human beings to first-generation adenoviral vectors would activate the type I IFN system, we purified mononuclear cells from the peripheral blood of six healthy donors. RNA was extracted from such cells before as well as 6 and 24 h after exposure to AdCMV-LacZ at m.o.i. 500. We quantified IFN- α and IFN- β by quantitative RT-PCR.

Indeed, both IFN- α and - β mRNAs were induced in all six cases (Fig. 5). We confirmed the presence of type I IFN activity in the 24-h culture supernatant by functional bioassays (data not shown).

Strikingly, there were again two preferentially transcribed IFN- α genes in response to adenovirus in the human system: IFN- α 1/13 in four cases and IFN- α 5 in the other two cases (Fig. 5).

Type I IFN is Detected in the Sera from Human Patients after Intratumoral Injection of Recombinant Adenovirus

The *in vitro* observations in human leukocytes indicated that the type I IFN system is induced by recombinant adenoviral vectors in human beings. It could be inferred that adenovirus administration to human subjects would lead to IFN- α and IFN- β production. To explore this issue, we measured the presence of type I IFN with a functional bioassay in the sera of two human patients from a gene therapy clinical trial. These two patients

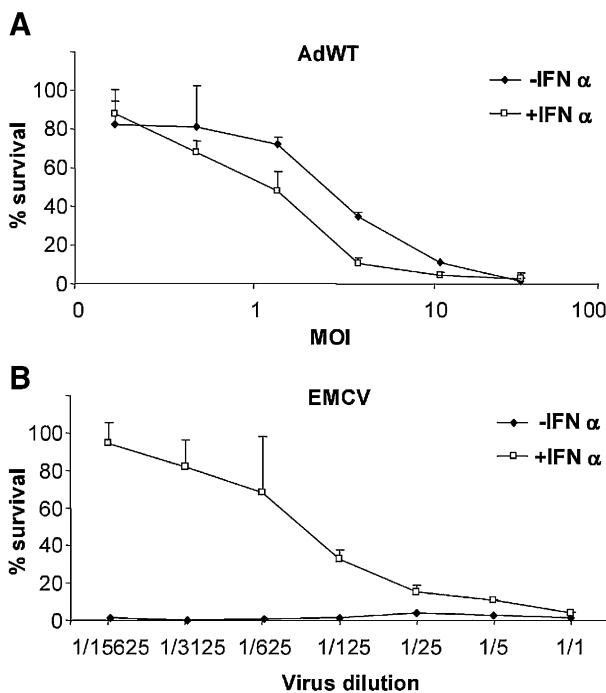


FIG. 4. *In vitro* replication of adenovirus is not interfered with by IFN- α . The viability of Huh-7 cells incubated with or without IFN- α (500 IU/ml) after infection with increasing doses of (A) wild-type serotype 5 adenovirus or (B) EMCV is presented. Four days after infection, cells were quantified and compared with uninfected cultures using the crystal violet method. The percentage of surviving cells is displayed as the mean \pm SD in quadruplicate wells. Similar results were obtained at later time points.

had received 3×10^{12} viral particles of a recombinant adenovirus encoding thymidine kinase (AdTK) administered as a single ultrasound-guided injection inside hepatocellular carcinoma lesions [24]. As can be seen in Fig. 6, type I IFN was undetectable in the baseline serum samples of these two patients, but readily detected 24 h after intratumoral adenoviral treatment, although levels fell in samples drawn 1 day later. Interestingly, transgene expression in these patients has been recently documented by positron emission tomography (PET) in the malignant lesions [24]. Some spill of viral particles had taken place from the injected tumors into the bloodstream, since 5 min after adenoviral injection quantitative PCR detected 1.2×10^5 and 5.7×10^4 adenoviral DNA copies/ml in serum samples from each patient (not shown).

As a whole, the ability of adenoviral vectors to induce type I IFNs should be taken into account as this cytokine could substantially modify both the therapeutic and the side effects of any given gene therapy regime.

DISCUSSION

The main conclusion of this study is that adenoviral vectors activate IFN- α and IFN- β synthesis from DC,

whereas paradoxically adenoviruses are not affected by the activity of this cytokine with regard to gene transfer or replication.

Dendritic cells behave as sentries of the immune system [26]. Many DC subpopulations with different phenotypic features are scattered throughout lymphoid and nonlymphoid tissues [27]. By means of sensing pathogens through innate mechanisms [14], they are key players in the initiation of the innate and adaptive immune response against viral infections.

Viral vectors such as those commonly used in gene therapy are not an exception and they have been shown to activate DC both to produce proinflammatory cytokines and to mediate productive antigen presentation to T lymphocytes [28–30]. Viruses often encode genes that restrain the activation/maturation of DC to inhibit the antiviral immune response at its very beginning [31,32].

In the past 5 years a specific DC subpopulation has been extensively studied as the main cell subtype responsible for the activity performed by the until recently elusive natural interferon-producing cells [33]. These cells are named plasmacytoid dendritic cells. Detection of viruses by means of TLRs induces a high

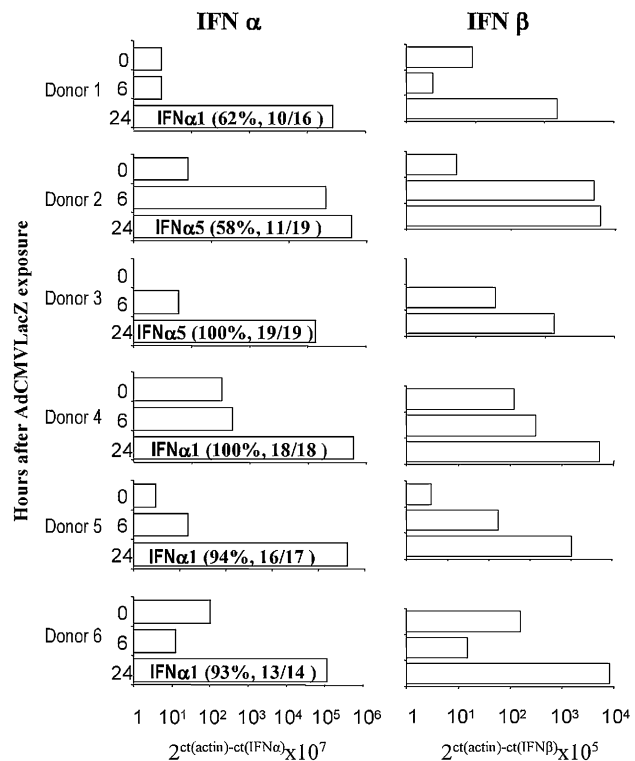


FIG. 5. Human leukocytes transcribe IFN- α and - β in response to adenovirus exposure. Real-time RT-PCR quantification of IFN- α and IFN- β resulting from *in vitro* exposure of human PBMC from six healthy donors to AdCMVlacZ at m.o.i. 500 at the given time points. The most frequently transcribed IFN- α gene is shown inside the 24-h bar as the number of sequences (% and fraction) among the sequenced colonies.

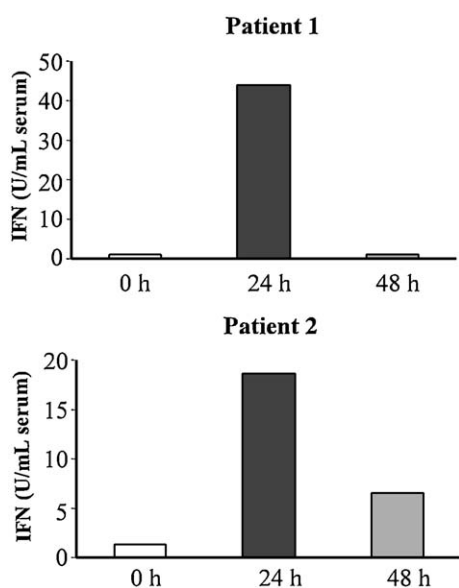


FIG. 6. Type I IFN is detected in the sera from human hepatocellular carcinoma patients after intratumoral injection of recombinant adenovirus encoding TK (AdTK). Biofunctional type I IFN concentrations assessed in serum samples from two patients who had received an ultrasound-guided injection of 3×10^{12} viral particles of AdTK inside a intrahepatic hepatocellular carcinoma lesion are presented. Peripheral blood samples were drawn either immediately before or 24 and 48 h after viral injection as indicated.

output of IFN- α and - β from these pDC both in humans and in mice [14]. Accordingly, it has been proposed that this cell population plays a crucial role in antiviral defense.

Our experiments with cultures enriched in those cells show that recombinant adenoviruses trigger IFN- α and IFN- β biosynthesis both from pDC differentiated in sFLT-3L cultures [22] and from pDC sorted out from mouse splenocytes. It was observed that such a phenomenon did not require adenoviral transfection, which was found to be extremely inefficient in this particular subset of DC, as opposed to the relatively efficient gene transfection reported for mDC [34,35]. Inefficiency of gene transfer by adenovirus to pDC has been also observed in a recent report [36] and is reminiscent of the resistance of this cell type to viral infection with only a few exceptions [14].

Adenoviruses are known to trigger mDC maturation both in mice and in human beings [28–30] through an activity mediated by the capsid knob protein and viral DNA [37]. Very recently it has been found that human DC such as those differentiated from human monocytes with GM-CSF and IL-4 produce IFN- α in response to incubation with recombinant adenovirus and other viral vectors such as retrovirus and lentivirus [35].

From the point of view of translational gene therapy with adenovirus these findings are very important, since type I IFNs are readily detected in DC obtained from mice that have been exposed to adenovirus *in vivo*. Given the

fact that adenoviral vectors induce IFN- α and - β , there was a potential for self-interference. Contrary to our expectations, we found that first-generation defective adenoviruses and replication-competent adenoviruses were not interfered with by IFN- α either *in vitro* or *in vivo*. Resistance of adenovirus to IFN- α has been reported in the literature [38] and attributed to activities encoded in the E1a and E3 regions [39]. Deletion of E3 and E1a in our defective adenoviruses seems to question this and suggests that other factors might exist to explain the observed resistance of adenovirus to the type I IFN system.

Another practical point to be considered is that many actions attributed to immune-potentiating transgenes (such as cytokines) could result from additive or synergistic effects of the transgene product acting in conjunction with type I IFN. Although experiments set up to define the role of each therapeutic transgene have been controlled with empty adenoviral vectors or vectors encoding reporter genes, the need of transgene-independent mechanisms for the therapeutic effects has been overlooked. Experiments in type I IFN receptor KO mice [7] and the recent availability of a monoclonal antibody blocking this receptor [40] will address this possibility in the future.

The multiplicity of type I IFN genes in the genome still defies explanation. In mice there is a single gene encoding IFN- β and multiple genes encoding 17 IFN- α subtypes including 4 pseudogenes with subtle sequence differences among them [41]. In humans there is also a single gene for IFN- β as well as 13 genes encoding different IFN- α subtypes plus 3 pseudogenes [2] along with another member of the family named IFN- ω [2]. A PCR cloning technique set up to study the IFN- α genes involved showed that IFN- β and IFN- $\alpha 5$ are the type I IFN transcripts activated by adenovirus in mouse DC. Although in our hands IFN- $\alpha 1/13$ and IFN- $\alpha 5$ are the predominant subtypes in humans, nomenclature correspondences with rodents are not possible, since gene duplication [2] has occurred independently in primates [42], and therefore subtype numbers merely tend to reflect the arbitrary order of discovery. The reason IFN- $\alpha 5$ is so selectively induced by adenovirus in the mouse system is enticing and deserves further study. According to previous reports [43], there is a likely involvement of a first wave of autocrine IFN- β in the induction of IFN- α .

Human leukocytes such as those met by adenovirus during treatment of patients in clinical trials with adenoviral vectors also produce IFN- α and IFN- β . This finding indicates that the ability of adenovirus to induce type I IFN is not species-specific and could be an important factor in the design and interpretation of clinical trials. We took advantage of the availability of serum samples from patients who had received an injection of adenovirus encoding thymidine kinase to explore this issue [24]. Indeed, type I IFN was detectable in

serum after adenovirus injection into liver tumor lesions. Recorded data showed that a part of the adenoviral dose given inside liver tumors reached the bloodstream and viral DNA was detected in the serum of these two patients 5 min after adenoviral treatment. This is consistent with reports that show that intratumoral treatment of adenovirus gives rise to a certain degree of viral spill to systemic circulation [44]. Leukocytes including dendritic cells can be easily reached at/from the bloodstream.

In conclusion, this study reveals that IFN- α and IFN- β are turned on by adenoviral vectors in DC. The fact that adenovirus itself is resistant to the immediate antiviral actions of type I IFNs does not preclude that IFN- α could shape a subsequent specific antiadenoviral immune response. Moreover, innate type I IFNs elicited by viral vectors might play a pivotal role in the therapeutic activity attributed to several immunostimulating transgenes.

MATERIAL AND METHODS

Mice and cells. Female 4- to 6-week-old C57BL/6 mice were purchased from Harlan (Barcelona, Spain), housed in appropriate animal care facilities during the experimental period, and handled following the institutional guidelines required for experimentation with animals. HeLa (human cervix carcinoma), Huh-7 (human hepatoma), and BNL (BALB/c mice hepatoma) cell lines were cultured at 37°C and 5% CO₂ in DMEM supplemented with 10% fetal calf serum (FCS) and antibiotics. All reagents were purchased from Cambrex (Verviers, Belgium).

Recombinant virus and cell transfection. Defective recombinant adenoviruses AdCMVGFP [45] and AdCMVLacZ [46] were produced using standard conditions. AdWTLuc is a replication-competent adenovirus that expresses luciferase under the control of the endogenous adenoviral late promoter. The reporter gene was inserted in the E3 region, replacing the 6.7K/gp19K genes, as described by Hawkins *et al.* [47]. SFVLacZ [48] and EMCV [4] were produced and titers assessed as described in each case. LPS concentrations in adenovirus batches, including clinical grade adenovirus, were below the detection levels of a sensitive commercial kit that detects up to 0.15 EU/ml (Cambrex *Limulus* amoebocyte lysate QCL-1000). Replication-competent adenovirus contamination in AdCMVGFP was 1.42×10^5 as assessed by the ratio of E1 versus E4 genes measured by quantitative PCR.

DC or HeLa cells were plated at 10^7 cells/ml in serum-free medium and infected for 2 h with the indicated adenovirus at m.o.i. ranging from 50 to 3000. After infection, RPMI supplemented with 10% FCS was added to the wells and cells were subsequently incubated at 37°C for 24 h. After incubation, adenovirus-infected cells were harvested, washed three times with saline buffer, resuspended to a cell density of 5×10^6 cells/ml in PBS, and analyzed by flow cytometry (FACSCalibur; Becton-Dickinson, San Jose, CA, USA).

Bone marrow cultures enriched in DC. Cultures of sFLT-3L-supplemented mouse bone marrow cells were generated as described [22]. Briefly, bone marrow cells were cultured after erythrocyte lysis at a density of 1.5×10^6 cells/ml in RPMI 1640, 10% FCS, 100 ng/ml sFLT3-L (R&D) for 10 to 12 days.

The phenotype of pDC was assessed by flow cytometry (FACSCalibur) upon staining with FITC- or PE-conjugated monoclonal antibodies against Ly6C, mPDCA-1, CD80, CD86, CD11c, CD11b, B220, CD40, CD14, H-2K^b, and I-A^b [22] (Pharmingen). A nonreactive fluorochrome-tagged rat monoclonal antibody was used as control.

Immunomagnetic bead separation. Mouse pDC were isolated using immunomagnetic mPDCA-1 beads (Miltenyi Biotec, Bergisch Gladbach,

Germany). mDC were isolated from the mPDCA-1 negative fractions using CD11c-conjugated beads (Miltenyi) according to manufacturer's instructions using an AutoMACS device (Miltenyi). Yield and purity of each cell separation were monitored immediately after each immunomagnetic cell sorting procedure by immunofluorescence and FACS analysis.

Analysis of mRNA expression by quantitative real time-PCR. Total RNA from the cells was extracted using Ultraspec Reagent (Biotex, Houston, TX, USA). One microgram of total RNA was treated with DNase (Gibco BRL, Paisley, UK) prior to reverse transcription with M-MLV reverse transcriptase (Gibco BRL) in the presence of RNaseOUT (Gibco BRL). Mouse and human IFN- α , IFN- β , and β -actin expression was measured by quantitative real-time PCR using an ICycler and the IQ SYBR Green Supermix (Bio-Rad Laboratories, Hercules, CA, USA). Aliquots of 2 μ l of the cDNA pool were used for each PCR, containing upstream and downstream primers specific for each gene in a 20- μ l final volume (Supplementary Table 1). Degenerate primers designed to recognize all IFN- α subtypes amplified all subtypes from both humans and mice with similar efficiency. To determine the PCR specificity, the final PCR products were analyzed by melting curves and electrophoresis. Results were normalized according to β -actin quantification in the same sample. The amount of each transcript was expressed by the formula $2^{c_t(\text{actin}) - c_t(\text{gene})}$, with c_t the point (PCR cycle) at which the fluorescence rises appreciably above the background fluorescence [49]. Statistical analyses were performed using nonparametric (Kruskal-Wallis and Mann-Whitney *U*) tests.

IFN- α subtypes analysis. For sequence analysis of IFN- α subtypes, RNA extracted from the cells was treated with DNase (Gibco BRL) prior to reverse transcription and was amplified by RT-PCR using Expand High Fidelity *Taq* polymerase (Roche Diagnostics, Mannheim, Germany). IFN- α PCR products were cloned using the TOPO TA cloning kit (Invitrogen, Paisley, UK) according to the manufacturer's protocol. At least 20 clones from each transformation were purified with the Templiphi amplification kit (Amersham Biosciences, Barcelona, Spain). Plasmids obtained were sequenced by fluorescence-based *Taq* Dye Deoxy-terminator cycle sequencing system using the BigDye Terminator Cycle Sequencing Ready Reactions Kit (ABI Prism, Applied Biosystems, Foster City, CA, USA) and M13 universal primers in an automated sequencer (ABI Prism 310 genetic analyzer; Applied Biosystems). Sequences were checked against the GenBank database by using BLAST to determine the IFN- α subtype.

IFN- α bioassay. The antiviral activity of mouse IFN was determined by measuring its ability to protect murine BNL cells against the cytopathic effect of EMCV. The assay was performed in a 96-well microtiter plate. First, 2×10^4 BNL cells per well were seeded in 150 μ l of medium containing serial dilutions of DC supernatants and incubated for 24 h. EMCV (5×10^6 pfu per well) were added, and 24 h later the cytopathic effect was measured by staining with crystal violet dye solution (0.5% in 1/4 v/v methanol/water). The optical density was read at 595 nm. At the same time, serial dilutions of mouse IFN- α (PBL Biomedical Laboratories, Piscataway, NJ, USA) were tested to obtain a standard curve. Results are expressed as units/ml and were calculated interpolating the optical density of each sample in the standard curve. IFN activity in human serum samples was determined using the above conditions with HeLa cells and serial dilutions of human IFN- α (Intron A; Schering-Plough, Madrid, Spain) as standard curve.

Hydrodynamic injection. Hydrodynamic injections of a plasmid encoding mouse sFLT-3L were performed in a volume of 100 ml/kg using a 27-gauge needle at a rate of 0.4 ml/s as previously described [25]. Increases in DC numbers were checked in the spleen of a sample of the mice.

In vivo detection of luciferase activity. C57BL/6 mice were pretreated intravenously with 10^5 pfu of AdCMVGFP or AdCMVLacZ 24 h before being intravenously injected with a replicative adenovirus encoding luciferase. Forty-eight hours later mice were anesthetized by intraperitoneal injection of ketamine (100 mg/kg) and xylazine (5 mg/kg), and their abdomen was shaved. The luciferase substrate D-luciferin (3 mg dissolved in 100 μ l PBS) was administered intraperitoneally (In Vivo Imaging

System (IVIS); Xenogen, Alameda, CA, USA). Five minutes later, light emission was quantified using an *in vivo* imaging system based on a CCD camera (IVIS; Xenogen) [50]. The activity is represented as photons/s.

Viral cytotoxicity assays. The cytotoxicity of adenovirus was analyzed by crystal violet staining of virus-infected cell monolayers. Huh-7 cells were seeded at a density of 5×10^3 cells/well in 96-well plates. The following day, cells were infected with increasing amounts of AdWT or EMCV, starting with very low m.o.i. (0.12 in the case of AdWT) to ensure that the destruction of the monolayer depended on viral replication. At least 4 days later, dead cells were removed by PBS washes. The unbound dye was removed by washing several times with water, and the plate was air-dried. Finally, the crystal violet incorporated in the cellular DNA was dissolved in 10% acetic acid and the absorbance at 595 nm was measured in a spectrophotometer.

Patients. Clinical-trial-authorized serum samples stored for 8 months at -80°C were taken from two advanced hepatocellular cancer patients before and after intratumoral treatment with 3×10^{12} viral particles of a clinical-grade adenovirus manufactured and quality controlled by Molecular Medicine LLC (Los Angeles, CA, USA) encoding the herpes virus thymidine kinase (AdTK) [24]. Transgene expression was confirmed in these patients by PET imaging as recently published [24].

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APPENDIX A. SUPPLEMENTARY DATA

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jymthe.2006.02.015.

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