A Recombinant Adenovirus Expressing Ovine Interferon Tau Prevents Influenza Virus-Induced Lethality in Mice

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Ovine interferon tau (IFN- τ) is a unique type I interferon with low toxicity and a broad host range *in vivo*. We report the generation of a nonreplicative recombinant adenovirus expressing biologically active IFN- τ . Using the B6.A2G-Mx1 mouse model, we showed that single-dose intranasal administration of recombinant Ad5-IFN- τ can effectively prevent lethality and disease induced by highly virulent hv-PR8 influenza virus by activating the interferon response and preventing viral replication.

Type I interferons (IFN) are crucial inducers of the antiviral response that act by binding to their universally expressed receptor (IFNAR) and regulating the expression of a large set of genes known as interferon-stimulated genes (ISGs) (1). Collectively, these ISGs mediate the establishment of an antiviral state directly precluding or diminishing viral replication within the cell (1, 2). Additionally, IFN modulates the deployment of a correct adaptive antiviral immune response by acting on cells of the immune system. For these reasons, IFN has long been thought of as an important antiviral treatment option (3, 4). However, its use in clinical settings as a broad-spectrum antiviral agent has been delayed due to its short half-life and the toxicity associated with its systemic or sustained delivery (5–8).

Therefore, vectors for a localized delivery and tailoring of the signaling properties of IFN are important goals. Recently, adenoviral vectors expressing type I IFN have been generated and shown to be efficient in the prevention of a number of viral diseases in vertebrate hosts, including SARS coronavirus infections in ferrets and Ebola virus infection in macaques (9–12). IFN- τ is a type I interferon expressed in ruminants only which is known to induce low toxicity while maintaining its antiviral capacities (6, 13–18). In contrast to other type I IFNs, IFN- τ can act on receptors from a broad range of species (17, 19), and it has shown efficacy in reducing replication of different viruses, including human and feline immunodeficiency viruses (HIV and FIV), human papillomavirus (HPV) (7, 20, 21), and bovine leukemia virus (BKV) (8).

Thus, we generated a recombinant nonreplicative second-generation human adenovirus (method previously described [22]), termed Ad5-IFN- τ , as a vector for the local delivery of IFN- τ *in vivo* (23). We first tested the ability of Ad5-IFN- τ to drive the expression and secretion of active IFN- τ . To do so, we assayed increasing amounts of medium from Vero cells, which lack endogenous type I IFN genes, infected with Ad5-IFN- τ or the parental control Ad5-DsRed virus and harvested at 48 h postinfection (hpi) in an antiviral activity assay. Vero cells were protected in a dose-dependent manner from vesicular stomatitis virus (VSV) infection when incubated with different amounts of the conditioned medium from Ad5-IFN- τ -infected cells (Fig. 1A), indicating the presence of a secreted antiviral activity most likely corresponding to IFN- τ . As expected, control medium did not prevent VSV infection and showed no toxicity to the cell monolayer. As a further control, the conditioned media were preincubated with the broad-spectrum type I IFN-inhibitory protein B18 from vac-

cinia virus (24), which we had previously demonstrated to inhibit ovine IFN- τ (data not shown). This resulted in a dose-dependent abrogation of the capacity of the IFN- τ -containing media to induce an antiviral state (Fig. 1A), indicating the specificity of the IFN- τ action. To confirm the induction of a type I IFN response by the secreted IFN- τ , we transfected HEK293T cells with luciferase reporters under the control of interferon-stimulated response elements (ISRE) or an NF- κ B promoter as a control. Treatment with either purified recombinant IFN- τ or conditioned media from Ad5-IFN- τ infected cells, as described before, induced a specific response (Fig. 1B, top) that was not obtained with either the control medium or the NF- κ B reporter. Furthermore, preincubation of the supernatants obtained from Ad5-IFN- τ -infected Vero cells (s-IFN- τ) with B18 protein inhibited ISRE-dependent luciferase expression 10-fold (Fig. 1B, bottom). One of the first events in the type I IFN-induced signaling is the phosphorylation of signal transducer and activator of transcription 1 (STAT1). Western blot analyses were used to show that treatment of thymus- and spleen-derived ovine cell lines obtained in our laboratory with supernatant from Ad5-IFN- τ -infected Vero cells induced a specific and time-dependent phosphorylation of STAT1 (Fig. 1C). This was also observed when the cells were treated with recombinant purified IFN- τ . As expected, no phospho-STAT1 was detected at any time in control cells incubated with medium or with supernatant from Ad5-DsRed-infected Vero cells. Finally, using the VSV antiviral assay and purified IFN- τ as a standard, we calculated that Ad5-IFN- τ is able to express and secrete 3×10^5 U/ml of conditioned medium under the conditions mentioned before. Altogether, these results show that the newly generated Ad5-IFN- τ expresses and secretes active IFN- τ that can activate the IFN-I response in cells of human, monkey, and ovine origin.

We next tested the ability of Ad5-IFN- τ to provide antiviral

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FIG 1 Recombinant Ad5-IFN- τ expresses and secretes active IFN- τ . (A) IFN- τ antiviral effect. Vero cells were preincubated with increasing amounts of supernatants obtained from Ad5-IFN- τ -infected Vero cells (s-IFN- τ) (10, 25, 50, and 100 μ l, as indicated) or with 100 μ l of supernatant obtained from Ad5-DsRed-infected Vero cells. The supernatants had been previously preincubated (+B18) or not (-B18) for 45 min with 20 ng of purified B18 protein at 37°C. Twenty-four hours later, the cells were washed, and 10-fold serial dilutions of VSV (starting with 10^3 PFU/cell) were added, allowing the infection to continue for an additional 48 h. The monolayers were fixed and stained with crystal violet to assess the extent of cytopathic effect. Uninfected cells (NI) were stained as controls (bottom). The experiments were performed in triplicate. (B) (Top) Capacity of the IFN- τ secreted into the medium by Ad5-IFN- τ -infected Vero cells to activate luciferase expression from an ISRE promoter. HEK293T cells were transfected with luciferase reporters under the control of interferon-stimulated response elements (ISRE) (pISRE-luc; Clontech) or an NF- κ B promoter as a control (pNF κ B-luc; Stratagene). Twenty-four hours later, cells were incubated with either purified recombinant IFN- τ (p-IFN- τ) (10,000 U) or 500 μ l of conditioned medium from Ad5-IFN- τ -infected cells (s-IFN- τ) or Ad5-DsRed-infected cells (s-DsRed). After 24 h, luminescence in each triplicate well was measured using the Dual-Glo luciferase assay system (Promega). Normalized mean luciferase stimulation values (n -fold) are shown for each condition. The assays were done in triplicate. *, $P < 0.05$ (paired t test). (Bottom) Blockade of IFN- τ induced luciferase activity by the type I IFN-inhibitory protein B18 from vaccinia virus. The experiment was performed as described above, with supernatants preincubated with 40 ng of purified recombinant B18 or not preincubated. Values are means of normalized luciferase expression induction levels (n -fold) from triplicate samples for each condition. ♦, $P < 0.0001$ (paired t test). (C) Spleen-derived (top) and thymus-derived (bottom) ovine cell lines were preincubated for 0, 15, and 30 min at 37°C with medium (M), supernatant obtained from Ad5-DsRed-infected Vero cells (C), supernatant obtained from Ad5-IFN- τ infected Vero cells (s-IFN- τ), or 10^4 U of purified recombinant IFN- τ (p-IFN- τ). Samples were analyzed by Western blotting using anti-phospho-STAT-1 (Tyr701), anti-STAT-1 and anti-actin antibodies (Sigma), as indicated. The blots are representative of three independent experiments. The position of molecular mass markers (kDa) is indicated on the left.

protection *in vivo*. To do so, we inoculated B6.A2G-Mx1 mice (25) intranasally with a single dose (5×10^6 PFU) of Ad5-IFN- τ or either Ad5-DsRed or phosphate-buffered saline (PBS) as a control. At 24 h postinoculation, we challenged the mice with increasing doses of the highly virulent influenza variant hv-PR8 (26–29),

closely related to the Cambridge strain of A/PR/8/34 (H1N1) virus. As shown in Fig. 2A, infection of PBS-treated or Ad5-DsRed-treated mice resulted in mortality rates of 100% at 20 times the 50% lethal dose (LD_{50}). Treatment with Ad5-IFN- τ completely abrogated infection-associated mortality, thus providing 100%

FIG 2 Intranasal Ad5-IFN- τ inoculation prevents murine influenza virus-induced disease and mortality *in vivo*. B6.A2G-Mx1 mice were treated with either PBS or Ad5-DsRed (Red) as controls or with Ad5-IFN- τ (IFN- τ) 24 h before infection with three different hv-PR8 influenza virus doses: 4 $\times$, 20 $\times$, and 100 $\times$ the LD₅₀ (LD₅₀ = 10² PFU [26]). The different mouse groups were named according to the treatment and the viral dose received. (A) Survival graph for mice inoculated with 4 $\times$ LD₅₀ or 20 $\times$ LD₅₀ of hv-PR8. Four animals per group were used, except that three were used in the PBS-20PR8 group. (B) Survival graph for mice inoculated with 100 $\times$ LD₅₀ of hv-PR8. Five animals per group were used. Data were statistically analyzed by a log-rank Mantel-Cox test. Relative significance was obtained at 20 $\times$ LD₅₀ and 100 $\times$ LD₅₀ of hv-PR8 between Ad5-IFN- τ -treated and untreated groups, with *P* values of 0.01 and 0.0062 and *P* values of 0.004 and 0.02 for the Ad5-DsRed and PBS groups, respectively. (C and D) Body weight loss in B6.A2G-Mx1 mice after inoculation with hv-PR8. Body weights were taken every day after challenge. Each value is the average percentage change in weight and the standard error of the mean for all the mice per group. Animals with a weight loss of a 25% relative to the initial weight were euthanized, following ethics protocols. Statistically significant differences, as determined by *t* test, are marked in panel C: *, *P* < 0.03 and *P* < 0.003 between the IFN- τ -20PR8 treated and untreated groups (PBS and Red, respectively); ♦, *P* < 0.05 between IFN- τ -4PR8 treated and untreated groups (Red). (D) *, *P* < 0.05 between IFN- τ -100PR8 treated and untreated groups (PBS). Arrows indicate the day of challenge, 24 h after the treatments.

protection. Consistently, at a lower infection dose (4 $\times$ LD₅₀), no animals from the Ad5-IFN- τ -treated group succumbed to infection, while control groups exhibited 50% mortality. Weight loss typically associated with murine influenza virus infections was not

detected in Ad5-IFN- τ -treated mice, in stark contrast to the control groups (Fig. 2C). When mice were infected with a much higher dose of influenza virus, corresponding to a 100-fold excess of the calculated LD₅₀ (Fig. 2B), Ad5-IFN- τ treatment was still

FIG 3 Analysis of expression of IFN-stimulated genes (ISGs) by shqRT-PCR in lungs of treated mice. Mice were divided into three groups (*n* = 3) and inoculated with PBS, Ad5-DsRed, and Ad5-IFN- τ . ISG gene expression from total RNA isolated from lung samples 24 h after intranasal inoculation was measured by quantitative real-time PCR (qRT-PCR) in an ABI 7900HT system (Applied Biosystems, Foster City, CA) using primers and probes previously described (Life Technologies). Each individual sample was analyzed in duplicate. Relative levels of expression of ISG mRNA were determined by comparative threshold cycle ($\Delta\Delta C_T$) analysis, utilizing GAPDH (glyceraldehyde-3-phosphate dehydrogenase) mRNA expression (internal control) as a reference. Statistical analysis was performed by a moderated *t* test modification of the Student *t* test adapted to a low number of replicates (StatMiner; PerkinElmer): *, *P* = 0.001; **, *P* = 0.02. Relative RNA quantity (RQ) = 2^{− $\Delta\Delta C_T$} .

FIG 4 Photomicrographs of hematoxylin-and-eosin-stained lung sections from B6.A2G-Mx1 after intranasal application of Ad5-IFN- τ and challenge with hv-PR8 virus. (A) Representative lung section from an Ad5-DsRed inoculated mouse and euthanized at 7 days after inoculation with $20\times$ LD₅₀ hv-PR8, presenting severe consolidation with the classical pathological signs of murine influenza virus infection (bar, 1 mm), inflammatory interstitial infiltration, alveolar epithelial hyperplasia, vacuolation, disorganization, denudation, and degenerative/regenerative bronchial epithelial hyperplasia. (B) Bronchial epithelial hyperplasia (magnification, $\times 40$; bar, 50 μ m). (C) Inflammatory interstitial infiltration (predominately mononuclear cells) (magnification, $\times 40$; bar, 50 μ m). (D) Representative lung section from a surviving Ad5-IFN- τ -pretreated mouse that was inoculated with $20\times$ LD₅₀ hv-PR8, exhibiting much lighter pathological signs of murine influenza virus infection (bar, 1 mm), with much less interstitial pneumonia and minimum alveolar epithelial hyperplasia. (E) Bronchiole with close-to-normal epithelium (magnification, $\times 40$). (F) Close-to-normal lung parenchyma (magnification, $\times 40$; bar, 50 μ m). Asterisks indicate magnified regions. Histological samples were digitalized with a NanoZoomer 2.QRS scanner from Hamamatsu.

able to reduce virus-induced mortality from 100% to 40%. This was accompanied by a significant reduction of the disease-associated weight loss from days 4 to 8 postinfection (Fig. 2D). Analysis by quantitative real-time reverse transcription-PCR (RT-PCR) of lung tissue before the challenge at 24 h after adenovirus inoculation showed a statistically significant increase in RNA expression of three ISGs—ISG15, MX1, and OAS1—in Ad5-IFN- τ -treated mice compared to either control group (PBS- or Ad5-DsRed-treated mice) (Fig. 3). While the mechanisms by which ISG15 exerts its antiviral activity are unclear, ISG15 is one of the most abundantly induced transcripts upon IFN treatment (30). In the case of influenza A virus infection, the IFN antagonist NS1 is a known target of ISGylation (31, 32), which inhibits viral replication. Altogether, these results support the notion that an IFN-induced antiviral response mediates the protective effect of Ad5-IFN- τ treatment *in vivo*.

Histopathological examination of lung samples from nonpretreated and infected mice showed typical lesions associated with influenza virus infection (Fig. 4A, B, and C), and viral RNA was readily detected by RT-PCR of the influenza NP gene in lung samples from 11 out of 16 of these animals by their time of death.

Using immunofluorescence virus titration assays, infectious virus was detected in lung homogenates from 14 out of the 16 mice that had not been pretreated with Ad5-IFN- τ . However, surviving animals that had been exposed to Ad5-IFN- τ showed a marked reduction of histopathological effects in the lung (Fig. 4D, E, and F), and neither infectious virus nor viral RNA was recovered from these samples, indicating almost complete recovery from infection by day 15 after infection. To analyze if Ad5-IFN- τ treatment was able to block influenza virus replication at early time points, we measured viral replication in lung samples of three animals from groups infected with the highest PR8 dose. While all individuals from the control group had detectable virus, with a mean titer of 4×10^4 PFU/ml, in the Ad5-IFN- τ -treated group, virus was detected in only two animals, with a mean titer of 5×10^3 PFU/ml. This suggests that even at the higher influenza virus dose tested, IFN- τ exerts its activity by controlling viral replication *in vivo*. Additional effects of IFN- τ on development of lung injury cannot be excluded and deserve further analysis.

Altogether, our results show that a single intranasal dose treatment with a nonreplicative recombinant human adenovirus expressing ovine IFN- τ can prevent the development of fatal influ-

enza in a murine infection model. Given the described low toxicity and broad host range of IFN- τ compared to other type I IFN variants, this might represent a safer option for first line antiviral treatments as well as a vector with broader host applicability and easy administration, which might be especially useful for the treatment of zoonotic diseases.

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