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The Nasal Epithelium as a Factory for Systemic Protein Delivery

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We have previously shown that recombinant Sendai virus (SeV) produces efficient *in vivo* airway epithelial gene transfer. The ability to produce therapeutic levels of circulating proteins following noninvasive gene transfer would have widespread clinical application. Here, we compared nose, lung, and skeletal muscle for the ability to produce circulating levels of the secreted mouse anti-inflammatory cytokine interleukin-10 (IL10) following SeV-mediated gene transfer. High levels of serum IL10 were obtained from each site with a potency order of lung > nose > muscle for a given viral titer. Serum levels from each site were within the likely required range for anti-inflammatory effects. The combination of a high-efficiency gene transfer agent (SeV) and sites that can be assessed noninvasively (nose or lung) may circumvent several current challenges to gene therapy.

Key Words: Sendai virus, gene therapy, interleukin-10

INTRODUCTION

The use of protein replacement therapy for inherited and acquired diseases is often problematic, related to the short half-life of most proteins and the high costs associated with production. Gene therapy is a potentially attractive option to overcome these difficulties. Considerable effort has therefore been committed to the local and systemic production of such secreted proteins as factors VIII and IX, erythropoietin, and apolipoprotein [1–3]. A variety of viral and nonviral vector systems have been used, as well as a number of "factory" organs, predominantly skeletal muscle and liver [4,5]. Proof of principle for this concept has been established in many studies, but generally gene transfer efficiency, and therefore protein production, has been very low. Preliminary human trials are underway, with a recent report of the use of intramuscular injection of an adeno-associated virus (AAV) expressing human factor IX [6].

Recombinant Sendai virus (SeV), a member of the paramyxoviridae [7,8], is a single-stranded RNA virus which we have previously shown produces efficient gene transfer and expression in the airway epithelium *in vivo* [9]. Given this, we hypothesized that the nose or lung may be useful as easily accessible factories for SeV-mediated production of circulating proteins and we have compared this with the current "gold standard"—skeletal muscle.

A further application for the nose as a site of gene transfer may be to produce therapeutic levels of secreted protein for the lung. It seems more logical to administer directly to the latter, but two factors may preclude this in practice. First, for some diseases such as cystic fibrosis (CF), topical application to the lungs is considerably hindered by the characteristic purulent secretions. Second, all gene transfer agents we have examined so far induce a proinflammatory reaction to some extent. In the setting of preexisting inflammatory lung disease, the addition of a further inflammatory burden would seem inadvisable. For both these reasons, the nose may also be an attractive option to target secreted proteins to the lung.

Interleukin-10 (IL10) is a potent anti-inflammatory and immunosuppressive cytokine that inhibits various T-cell and antigen-presenting cell functions [10], and has received growing attention for its therapeutic potential [11–13]. Because of our interest in the use of IL10 for respiratory and cardiovascular diseases, we have assessed a SeV carrying IL10 (SeV-IL10) as a prototype for these

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studies. The findings should be relevant to other candidate proteins such as factors VIII and IX, erythropoietin, insulin, and many others.

RESULTS AND DISCUSSION

In Vitro Gene Transfer and Determination of Biological Activity of SeV-Induced IL10

Recombinant SeV is a cytoplasmic gene transfer agent. To determine if IL10 is efficiently produced in the cytoplasm and subsequently secreted, we transfected Cos7 and HeLa cells with SeV-IL10. Twenty-four hours after transfection of Cos7 cells with SeV-IL10 or a SeV-luciferase (SeV-luc) control at a multiplicity of injection (MOI) of 0.1 to 10, a dose-related increase of IL10 was detected in the medium (Fig. 1A). At the highest MOI, SeV-IL10 produced 50 times more secreted IL10 than an optimized formulation of DNA/liposome complexes in these cells, known to be particularly susceptible to liposome-mediated transfection. To determine if the IL10 produced by SeV is functional, a biological activity assay based on IL10-dependent costimulation of interleukin-9 (IL9) secretion in murine bone-marrow-derived mast cells [14] was used. Biological activity was determined 24 hours after *in vitro* transfection of HeLa cells and IL10 levels were calculated using known amounts of recombinant IL10 protein as standards. IL10 levels obtained by the biological activity assay were twofold higher than levels obtained with a standard IL10 ELISA (Fig. 1B). Slight variations in absolute values generated by these assays are generally expected, because they rely on the affinity of different antibodies to their respective substrates. Pretreatment of samples with a neutralizing anti-mouse IL10 antibody completely blocked biological activity of the supernatant. No IL10 activity was seen in SeV-βgal transfected cells (data not shown).

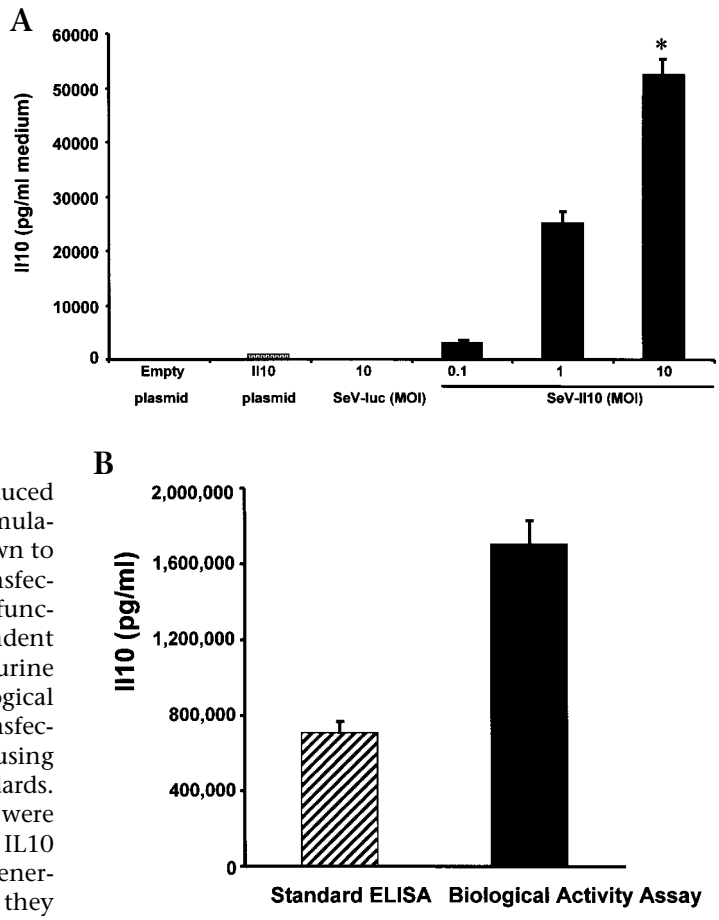

FIG. 1. *In vitro* production of IL10 and its biological activity. (A) Cos7 cells were transfected with SeV-IL10, control virus, plasmid IL10, or plasmid control (n = 6 each group). *P < 0.005 compared with plasmid IL10 production. (B) Standard IL10 ELISA (hatched bar) compared with a biological activity assay (filled bar) for IL10 using BMMC. IL10 levels were calculated by comparing the BMMC response to test samples with that to known amounts of recombinant protein standard (n = 5 all groups).

Intramuscular Gene Transfer

Transfection efficiency in skeletal muscle was initially determined using a SeV-β-galactosidase vector and was compared with optimized doses of naked plasmid DNA. SeV produced a dose-related increase in β-galactosidase expression ranging from 116 ± 49 to 61,672 ± 11,508 pg βgal/mg protein at titers of 7×10^4 and 7×10^8 plaque forming units (pfu) per mouse, respectively. At the highest titer, expression was more than 2 logs greater than plasmid-mediated expression. SeV-IL10 produced a dose-related increase in IL10 in muscle homogenate, with little or none detectable following IL10 plasmid application (Fig. 2A). These findings were mirrored by serum levels (Fig. 2B), with none detected following plasmid IL10 compared with a dose-related increase with SeV, producing maximum serum levels of approximately 800 pg/ml. Expression in the muscle was transient, with peak expression at day 2. At 9 days after injection, SeV-βgal expression was still 35-fold above background and fivefold higher than peak expression of naked plasmid DNA. At 4 weeks after injection expression had returned to baseline values.

Intranasal Gene Transfer

To assess whether the nose could also serve as a factory for serum proteins, a technique was first devised to selectively transfect the nasal epithelium, without significant expression being induced in the lung. Using a fine-catheter perfusion system, the local production of luciferase was > 400-fold higher in the turbinate than in the lung (Fig. 3A). Further, transfection of the nasal epithelium lining the turbinate was confirmed with X-gal staining in SeV-βgal infected mice (Fig. 3B). There was an absence of staining in the nasal epithelium of mice transfected with the SeV-luc control virus, confirming the specificity of the reaction. Following administration of intranasal SeV-IL10 (7×10^7

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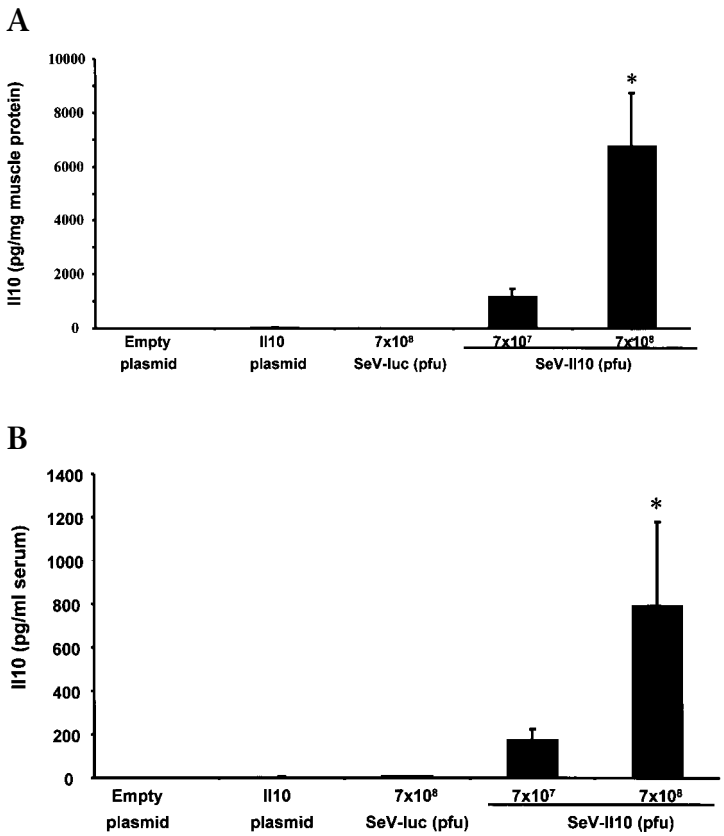

FIG. 2. Intramuscular injection of SeV-IL10. Both tibialis anterior muscles were injected with SeV-IL10 or a SeV-luc control virus (7×10^7 and 7×10^8 pfu/mouse) or "naked" plasmid DNA expressing IL10 (100 μg/mouse). IL10 was measured in muscle homogenate (A) and serum (B) 2 days after gene transfer ($n = 6$ each group). *$P < 0.05$ compared with plasmid IL10.

and 7×10^8 pfu/mouse), we obtained a dose-related increase in both local tissue (Fig. 4A) and serum IL10 levels (Fig. 4B), with maximal levels in serum reaching approximately 1700 pg/ml.

Intrapulmonary Gene Transfer

Finally, we determined the capacity of SeV-IL10 to produce local (lung homogenate and BALF) and serum IL10 when administered topically to the lung. At 2 days after gene transfer a dose-related increase in IL10 was detected in lung homogenates (Fig. 5A), bronchoalveolar lavage fluid (Fig. 5B), and serum (Fig. 5C). Maximum serum levels were approximately 60,000 pg/ml. IL10 could not be detected above background levels in any of the above tissues using optimized lipid/DNA complexes (data not shown). Administration of SeV led to inflammation in the airways (interstitial inflammation, mainly lymphocytes, monocytes, and few neutrophils and plasma cells) and muscle (dose-dependent inflammation and epimyceal thickening).

Prospects for SeV-Mediated Production of Secreted Proteins

We have previously shown that SeV is the most effective gene transfer agent currently available for gene therapy to the airway epithelium [9]. This is likely due to several factors. Its receptors, sialic acid and cholesterol, are present on the surface of the conducting airway epithelium. In addition, SeV entry into the cell is not significantly affected by surface mucus, and occurs within a few minutes. Finally, SeV is a cytoplasmic expression system [15], thus avoiding the requirement for nuclear entry through the nuclear pore complex, known to be an important barrier—particularly for nonviral gene transfer agents. It is, therefore, perhaps not surprising that SeV applied to the lung mediates the highly efficient production of a secreted protein, both locally and into serum. The levels that can be generated at this site (60,000 pg/ml) were high compared with existing gene transfer strategies; reference levels of 200 pg/ml are considered sufficient for therapeutic benefit in a range of animal models. Given the well-established, simple lung-delivery devices that are widely available, this route of administration may be readily applicable for a number of extrapulmonary diseases, such as the hemophilias and diabetes. However, administration directly into the lung may not be desirable and/or feasible for the treatment of lung disease. First, it is well documented that viral and nonviral gene transfer agents trigger transient inflammation in the lung and many of the potential targets for pulmonary gene therapy already include an inflammatory component. Second, airway diseases such as cystic fibrosis, chronic obstructive pulmonary disease (COPD), and bronchiectasis are characterized by excess production of purulent airway secretions, which may cover large proportions of the airway surface and prevent gene transfer agents from reaching the epithelial cells. These factors have prompted a search for other potential "factory" sites to produce secreted proteins, which can reach the lung by means of the circulation. Because of relatively noninvasive access, skeletal muscle has been most commonly studied. SeV-mediated local IL10 production at this site was equivalent [16], but generally two- to sixfold higher than that produced by a variety of viral and nonviral gene transfer agents [17–19]. In keeping with this, respectable serum levels could be achieved, but were the lowest of the sites studied here.

The nose is a novel site for the production of serum proteins. It is a particularly attractive site because of the easy access and, as for the lung, the well-developed delivery devices. As expected, from the lung studies, SeV transfects nasal epithelium very efficiently. High serum IL10 levels were achieved following nasal SeV-mediated transfection. Values were approximately twofold greater than for muscle,

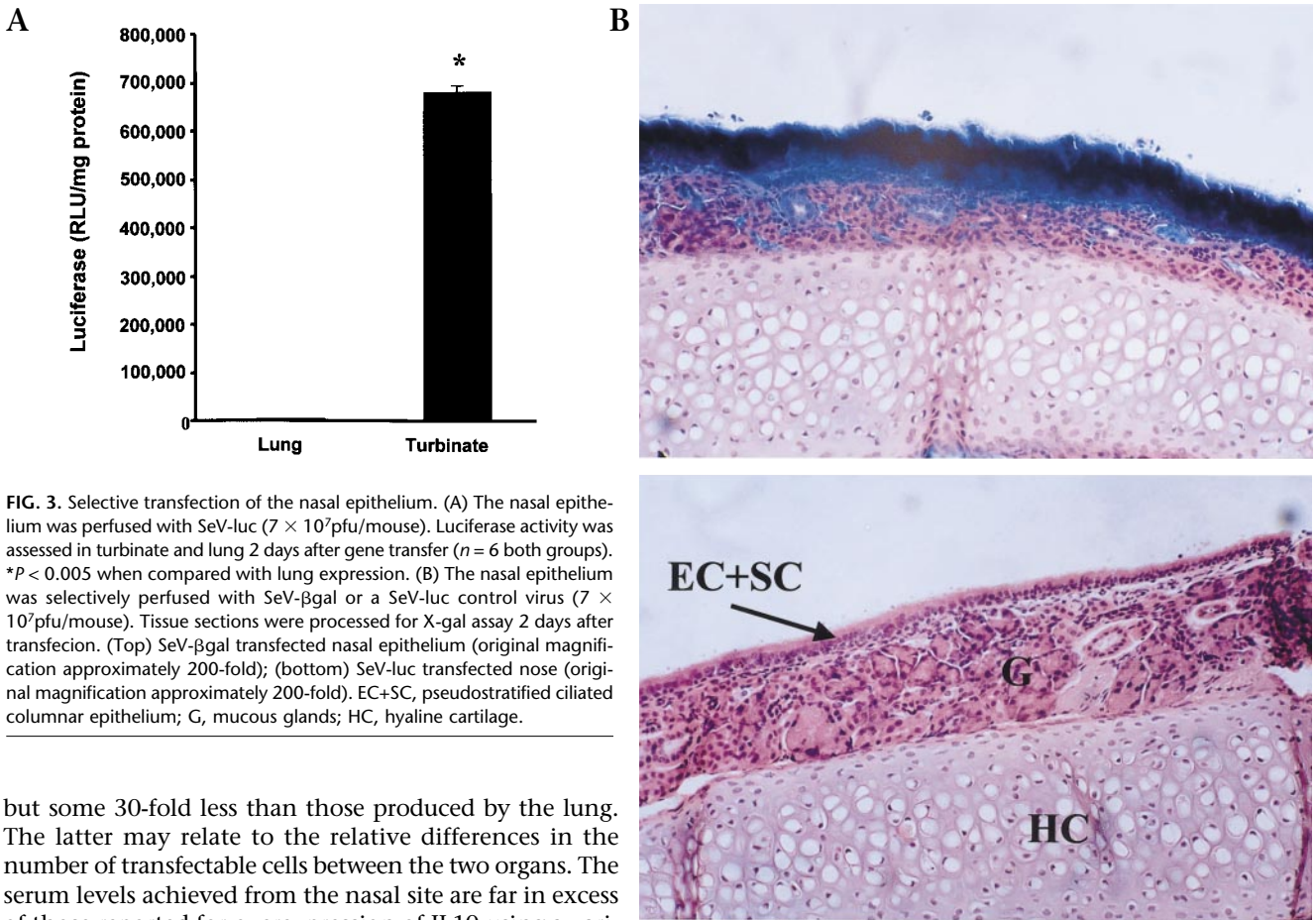

FIG. 3. Selective transfection of the nasal epithelium. (A) The nasal epithelium was perfused with SeV-luc (7×10^7pfu/mouse). Luciferase activity was assessed in turbinate and lung 2 days after gene transfer ($n = 6$ both groups). *$P < 0.005$ when compared with lung expression. (B) The nasal epithelium was selectively perfused with SeV-βgal or a SeV-luc control virus (7×10^7pfu/mouse). Tissue sections were processed for X-gal assay 2 days after transfecion. (Top) SeV-βgal transfected nasal epithelium (original magnification approximately 200-fold); (bottom) SeV-luc transfected nose (original magnification approximately 200-fold). EC+SC, pseudostratified ciliated columnar epithelium; G, mucous glands; HC, hyaline cartilage.

but some 30-fold less than those produced by the lung. The latter may relate to the relative differences in the number of transfectable cells between the two organs. The serum levels achieved from the nasal site are far in excess of those reported for overexpression of IL10 using a variety of gene transfer agents and target organs [20–24].

Here, we have demonstrated that recombinant SeV produced biologically active IL10 *in vitro*. We have also recently tested the biological efficacy of SeV-IL10 in a murine bleomycin model of lung inflammation. We have shown that administration of a SeV-control virus increased lung disease in bleomycin-treated mice, but SeV-IL10 reduced lung disease back to bleomycin-only levels (data not shown).

Although we used IL10 as a prototypical secreted protein, this cytokine may have an important therapeutic role at several sites. Thus, chronic lung inflammation leads to severe tissue damage in a number of diseases including cystic fibrosis and acquired respiratory distress syndrome (ARDS). The rapid production of high levels of circulating IL10 may be particularly relevant in ARDS with its characteristic acute multisystem inflammatory component. IL10 has also been suggested to be a potential new treatment in several non-pulmonary problems including rheumatoid arthritis and inflammatory bowel disease. Application of a nasal/pulmonary IL10 inhaler may, therefore, be of widespread relevance.

There are several obvious caveats with the present technology. First, the recombinant SeV used in this study is transmission competent, potentially an obstacle to human trials. To overcome this, a nontransmissible SeV has recently been generated through deletion of the F protein, which is essential for virus uptake into cells. During virus preparation the F protein is supplied in *trans* and thus allows a one-time only entry into cells [25]. Second, the natural hosts for SeV infections are rodents and the data presented here may not be representative of other species. Against this we have previously shown that both human and ovine airway epithelium was efficiently transfected *in vitro* or *ex vivo*, respectively [9]. Third, repeated administration of all viral vectors including SeV has proven difficult, because of the immune responses to viral proteins. Several strategies based on either altering the virus (gene deletion, antigene masking) or the host response (immune suppression) are being investigated. The therapeutic relevance of SeV-mediated therapy in the settings in which repeated applications are required will likely be determined by the success or failure of these studies.

In summary, recombinant SeV mediates efficient production of a secreted protein using the airways as a "factory" for systemic distribution. Delivery devices are in clinical use to access the nose or lung and this technology may therefore be applicable to several diseases, both intrapulmonary and distant to the site of administration.

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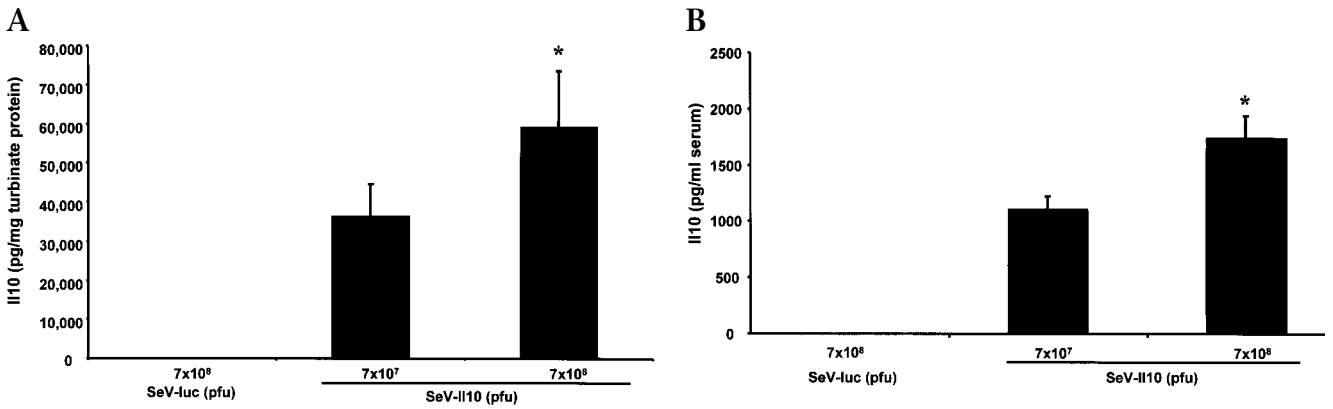

FIG. 4. Intranasal administration of SeV-IL10. The nasal epithelium was selectively perfused with SeV-IL10 or a SeV-luc control virus (7×10^7 and 7×10^8 pfu/mouse) and IL10 was measured in turbinate (A) and in serum (B) ($n = 6$ both groups). *$P < 0.005$ when compared with any other group.

MATERIALS AND METHODS

Viral and nonviral vectors. Recombinant SeVs were constructed and propagated as described [7,8]. Viral titers were determined by a standard chicken red blood cell hemagglutination and plaque assay on CV1 cells and viruses were stored at –80°C. GL-67/DOPE/DMPE-PEG_{5000} (GL-67, Genzyme Corporation, Framingham, MA) or DC-Cholesterol/DOPE were complexed with plasmid DNA as described [26–28]. The eukaryotic expression plasmid pCI (Promega, Southampton, UK) carrying a nuclear-localized β-galactosidase reporter gene or the murine IL10 cDNA under the control of the CMV immediate-early promoter enhancer was used in all studies. The murine IL10 cDNA was purchased from the American Tissue Type Collection (ATCC, Maryland).

In vitro gene transfer and IL10 activity assay. Semiconfluent Cos7 or HeLa cells were transfected with SeV-IL10 or a control virus carrying a luciferase reporter gene (SeV-luc) at MOIs of 0.1 to 10 for 1 hour in Opti-MEM (Sigma-Aldrich, Poole, UK), after which complete medium was added (MEM + 10% FCS + 1% streptomycin/penicillin; Sigma). In other studies cells were transfected with plasmid/DC-Cholesterol:DOPE complexes under previously optimized conditions [28]. Medium was collected for IL10 ELISA and assessment of bioactivity 24 hours later.

Il-10 bioactivity was assessed using its costimulatory properties for the production of IL-9 by murine mast cells as described [14]. Briefly, murine bone marrow derived mast cells (BMMCs) were generated from bone marrow cells and stimulated with 0.75 mM ionomycin and 10 U/ml IL-1 alone, or in combination with either known amounts of recombinant murine Il-

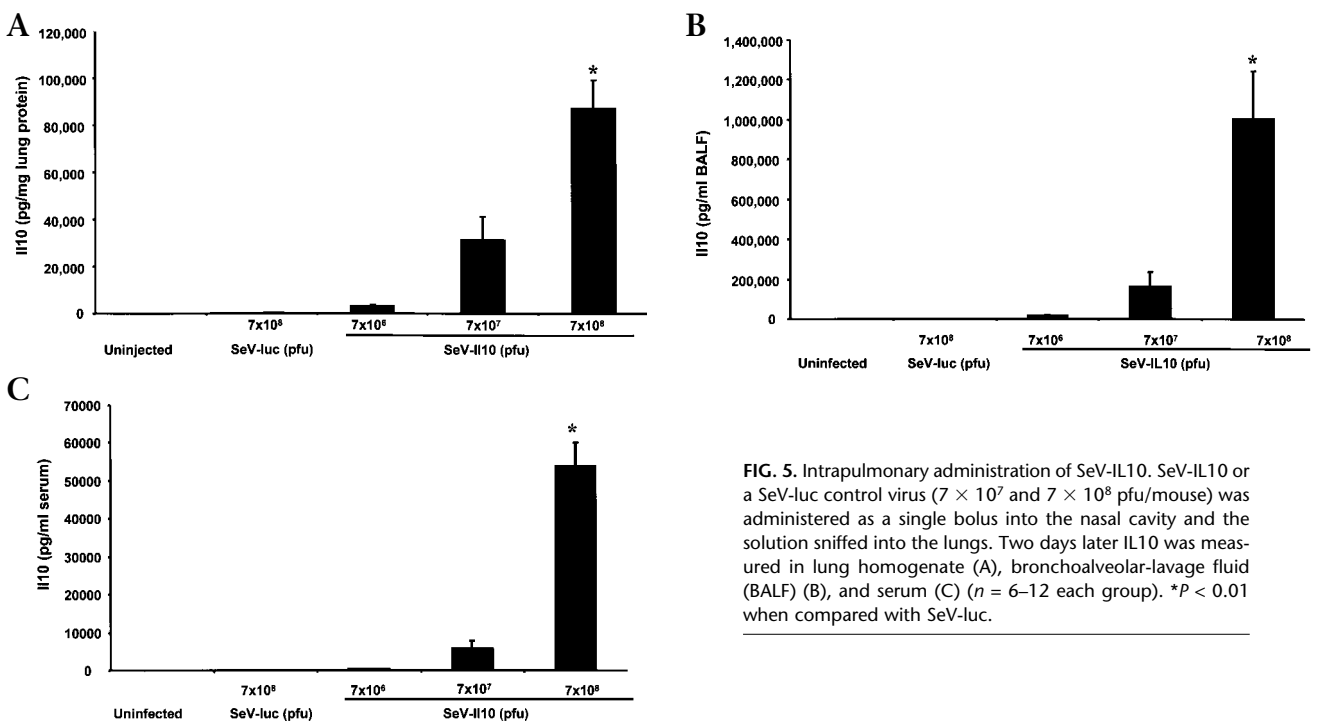

FIG. 5. Intrapulmonary administration of SeV-IL10. SeV-IL10 or a SeV-luc control virus (7×10^7 and 7×10^8 pfu/mouse) was administered as a single bolus into the nasal cavity and the solution sniffed into the lungs. Two days later IL10 was measured in lung homogenate (A), bronchoalveolar-lavage fluid (BALF) (B), and serum (C) ($n = 6$–12 each group). *$P < 0.01$ when compared with SeV-luc.

10 as a standard or supernatant from SeV-IL10 transfected HeLa cells. After 24 hours, IL-9 was measured in the medium using an ELISA. IL9 production was converted into levels of biologically active IL10 by comparing the response of the test samples to known amounts of recombinant IL10 standard. The specificity of IL10-mediated stimulation of IL9 was determined in control reactions containing anti-murine IL10 antibodies and in reactions using supernatant from SeV-βgal infected cells. All experiments were carried out in triplicate and the mean values determined for each sample. The biological activity assay measured 2.4-fold higher values than the ELISA in the same samples.

In vivo gene transfer. All *in vivo* experiments were carried out in C57Bl/6 mice (5–8 weeks old). Intramuscular injection was carried out as follows: Recombinant SeV (7×10^4 to 7×10^8 pfu/mouse) in 100 μl balanced salt solution (137 mM NaCl, 5.4 mM KCl, 10 mM Tris-HCl, pH 7.6) or naked plasmid DNA (100 μg/mouse in 100 μl saline) was injected into both tibialis anterior muscles (TA) of C57Bl/6 mice. Intranasal administration was carried out as follows: a fine-tip catheter (outer diameter: 0.5 mm) was placed 5 mm within the nasal cavity and 100 μl of the appropriate vector solution perfused at a rate of 10 μl/min using a peristaltic pump (P1 pump, Pharmacia, Herts, UK). During the procedure the mice were kept in an inverted position to avoid transfection of the lung. Intrapulmonary administration was carried out as follows: Recombinant SeV (7×10^6 to 7×10^8 pfu/mouse) or plasmid DNA complexed to the cationic liposome GL-67 (80 μg DNA/mouse) was placed as a single 100 μl bolus into the nasal cavity and the solution was sniffed into the lungs [29].

Tissue preparation. At 2 days after transfection, TA muscle, lung, and turbinate were harvested and homogenized in 0.25 M Tris-HCl, pH 8. Following three freeze/thaw cycles the tissue lysates were spun for 10 minutes at 13,000 rpm and the supernant frozen at –80°C. (b) For the bronchoalveolar lavage (BAL), a catheter (18G, Ohmeda, Sweden) was placed into the trachea and the lungs washed with 3×0.5 ml of PBS. The retrieved BAL fluid (BALF) was spun for 5 minutes at 4000 rpm to remove cellular debris and then frozen at –80°C. (c) Blood was collected by direct cardiac puncture. Whole blood was incubated at 37°C for 2 hours and spun for 10 minutes at 4000 rpm. The serum was frozen at –80°C.

Reporter gene and IL10 ELISA assays. β-Galactosidase activity was determined using a chemiluminescent assay (Clontech, Hampshire, UK). Murine IL10 was measured using a mouse IL10-specific ELISA assay (R&D, Oxon, UK).

Statistical analysis. All values are expressed as the mean ± SEM for convenience and *n* refers to the number of animals or tissue culture samples used. Data were compared using the Mann-Whitney U-test, and the null hypothesis rejected at $P < 0.05$.

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