A Novel Hybrid Adenoretroviral Vector with More Extensive E3 Deletion Extends Transgene Expression in Submandibular Glands

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Abstract

Salivary glands are an attractive target for gene transfer. Salivary epithelial cells are considered to be highly differentiated and have low rates of cell division (~6 months), affording the opportunity to obtain relatively long-term transgene expression in the absence of genomic integration. Here, we report a novel modified hybrid adenoretroviral vector, which provides stable transgene expression in salivary epithelial cells *in vivo* for up to 6 months in the absence of genomic integration. This modified hybrid vector, Ad Δ E1/3LTR₂EF1 α -hEPO, encodes human erythropoietin (hEPO) and differs from a previously developed hybrid vector, AdLTR₂EF1 α -hEPO, by having more extensive E3 gene deletion. Following direct salivary gland gene transfer by retroductal cannulation, rats transduced with Ad Δ E1/3LTR₂EF1 α -hEPO had sustained, elevated serum hEPO levels and hematocrits for 6 months (length of experiment), as compared with ~2 months for animals administered the AdLTR₂EF1 α -hEPO vector. Immunohistochemistry demonstrated that this novel vector could transduce both acinar and ductal cells. Interestingly, the Ad Δ E1/3LTR₂EF1 α -hEPO vector evoked much weaker local (salivary gland) immune responses than seen after AdLTR₂EF1 α -hEPO vector delivery, which likely permits its significantly lengthened transgene expression in this tissue.

Introduction

OUR EARLIER STUDIES have shown that salivary glands are an attractive, though uncommon, target site for gene transfer (Baum *et al.*, 2004, 2009). Salivary gland epithelial cells present multiple advantages for gene transfer, including easy accessibility, a relatively stable target of well-differentiated epithelial cells for nonintegrating vectors, and the ability to produce significant amounts of protein for export in both exocrine and endocrine directions (O'Connell *et al.*, 1996; Baum *et al.*, 1999). Thus, the secretion of therapeutic transgenic proteins from salivary glands may be useful for many clinical applications, *e.g.*, locally to manage oral candidiasis (O'Connell *et al.*, 1996) and oral mucositis (Zheng *et al.*, 2009) and systemically to manage certain systemic single-protein deficiency disorders (Voutetakis *et al.*, 2005; Passineau *et al.*, 2011).

Salivary glands mainly consist of two types of epithelial cells: acinar and ductal. Both are highly differentiated and have low rates of cell division (Redman, 1995). A successful gene therapy with any tissue requires efficient, nontoxic

vectors to provide adequate transgene expression for an appropriate length of time. We previously have shown that serotype 5 adenovirus (Ad5) and serotype 2, 4, and 5 adeno-associated virus (AAV) vectors are useful for this slowly dividing epithelial tissue (Adesanya *et al.*, 1996; Delporte *et al.*, 1997; Voutetakis *et al.*, 2004, 2007; Katano *et al.*, 2006; Gao *et al.*, 2011; Zheng *et al.*, 2011a,b). However, all AAV vectors tested thus far only transduce ductal cells (*e.g.*, Katano *et al.*, 2006; Gao *et al.*, 2011). We have focused a considerable effort into improving existing gene transfer vectors for use in salivary glands with a goal of extending transgene expression in acinar cells (Zheng *et al.*, 2000, 2003a,b, 2008; Zheng and Baum, 2002). To do this, we created a hybrid vector using Ad5 and Moloney murine leukemia virus (MoMLV) elements. First-generation Ad5 vectors offer ease of production, high titers, efficient transduction of many cell types, and infrequent genomic integration (Schaack *et al.*, 1990; Greber *et al.*, 1996, 1997; Harui *et al.*, 1999), but Ad5 vectors provoke vigorous immune responses resulting in short-term transgene expression (typically ≤ 14 days) (Yang *et al.*, 1995; Kagami *et al.*, 1998; Jooss and Chirmule, 2003).

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MoMLV vectors provide long-term transgene expression, but titers are low, efficient transduction requires cell division, and at present genomic integration is uncontrolled, risking insertional mutagenesis (Harel *et al.*, 1981; Springett *et al.*, 1989; Miller *et al.*, 1990; Hacein-Bey-Abina *et al.*, 2003; Baum *et al.*, 2006).

Initially, we reported the construction of the AdLTR-luc vector, which was capable of transducing several nondividing cell types leading to longer transgene expression, yet able to mediate integration of the transgene cassette into genomic DNA with high efficiency (Zheng *et al.*, 2000, 2003a; Zheng and Baum, 2002). During our vector preparations, we recognized that the two MoMLV elements in our constructs were involved in homologous exchange, resulting in difficulty in amplifying the vectors. When shorter MoMLV elements were used in the constructs, homologous exchange was reduced. Furthermore, using the shorter MoMLV elements helped to minimize the risk of insertional mutagenesis. Thus, we further modified the AdLTR-luc vector using smaller MoMLV elements, resulting in a hybrid vector that was not efficiently integrated into the genome of targeted cells, but that still provided longer transgene expression than a conventional Ad5 vector, *i.e.*, ~2 months [AdLTR₂EF1 α -hEPO; encoding human erythropoietin (hEPO)] (Zheng *et al.*, 2008). However, this vector still elicited strong immune responses in salivary glands that likely contributed to the limitations observed in transgene expression. To address the latter problem and extend transgene expression, we hypothesized that the expression of Ad5 E3 proteins from the AdLTR₂EF1 α -hEPO vector could paradoxically affect the immune responses locally in salivary glands, because there would be minimal opportunity for vectors to transduce immunomodulatory cells in this tissue. Vectors delivered intraductally have direct access only to gland parenchymal cells lining the duct and acinar lumens (Baum *et al.*, 2002). Herein, we report the initial characterization of a new hybrid vector, Ad Δ E1/3LTR₂EF1 α -hEPO, which has extensive E3 gene deletion and mediates long-term transgene expression in salivary glands.

Materials and Methods

Construction of recombinant vectors

Plasmid pBHG10 was obtained from Microbix Biosystems Inc. (Toronto, ON, Canada). Plasmid pACLTR₂EF1 α -hEPO was constructed as previously described (Zheng *et al.*, 2008) and uses the human elongation factor 1 α (EF1 α) promoter to drive hEPO cDNA expression. The recombinant adenoviral vector, Ad Δ E1/3LTR₂EF1 α -hEPO (Fig. 1a), was generated by homologous recombination of pACLTR₂EF1 α -hEPO with pBHG10 in C7 cells. Long terminal repeats (LTRs) from MoMLV were located upstream and downstream of this expression cassette (Zheng *et al.*, 2008). The upstream LTR also acted as an enhancer, increasing transgene expression approximately sevenfold (Zheng *et al.*, 2008). The recombinant adenoviral vector, AdLTR₂EF1 α -hEPO, was generated by homologous recombination of pACLTR₂EF1 α -hEPO with pJM17 in C7 cells, as previously described (Zheng *et al.*, 2008). A 2,685-base pair (bp) sequence was deleted in pBHG10 from nucleotide (nt) 28,133 to nt 30,818 in the E3 region of Ad5 genome (Bett *et al.*, 1994). This includes the 42-bp coding sequence of E3 12.5k and all other coding sequences

of the other six E3 proteins (E3 7.1k, E3 gp19k, E3 10.5k, truncated E3 10.4k, E3 14.5, and E3 14.7k) (Fig. 1b) (Human Adenovirus, 2007). A 747-bp sequence, from nt 30,000 to nt 30,745 in the E3 region of the Ad5 genome, was deleted in pJM17. This includes the E3 14.5k and E3 14.7k coding sequences only (Fig. 1b) (Bett *et al.*, 1995; Human Adenovirus, 2007). A 643-bp sequence from the E3 region of the Ad5 mutant dl309 (GeneBank accession no. U22898) was inserted in this E3 region (Bett *et al.*, 1995). Thus, the genomic differences between Ad Δ E1/3LTR₂EF1 α -hEPO and AdLTR₂EF1 α -hEPO exist only in the Ad5 E3 region. Both vectors have the same expression cassette and E1 deletion. A single plaque from the Ad Δ E1/3LTR₂EF1 α -hEPO or AdLTR₂EF1 α -hEPO plaque assays (see below; limiting dilution) was picked and amplified in C7 cells (a gift from Dr. J. Chamberlain), and then purified by cesium chloride density-gradient ultracentrifugation (Baum *et al.*, 2010). These purified vectors were titered at the same time by quantitative PCR (qPCR) with primers from the EF1 α promoter and hEPO cDNA in the expression cassette: EF1 α F4 (5'-GTTGCGTGACGCGAAAGATG-3') and hEPOB6 (5'-CGGAAGAGTTTTCGGAAGTG-3'). Titers were expressed as vector genomes (vg) per milliliter. To compare directly the physical titers and infectious titers of the purified vectors, we performed standard plaque assays to obtain multiplicities of infection (MOIs). In brief, 293 cells were plated in six-well plates (1.7×10^6 293 cells/well) 1 day before infection. The next day, we carefully removed media from each well, and added 1 mL of IMEM (Invitrogen, Carlsbad, CA) with 2% fetal bovine serum and 0.1 mL of vector serial dilutions (10^6 – 10^{11}) made with IMEM. We incubated the plates at 37°C for 1 hr with gentle rocking every 15 min and then removed all media from each well. Thereafter, we carefully added 2 mL of 1% agarose with MEM (Invitrogen) plus 7.5% fetal bovine serum and overlaid this with 2 mL of 1% agarose with MEM plus 7.5% fetal bovine serum every 5 days. Plaques were counted at day 14.

To determine the ratio of hEPO transgene and MoMLV element sequences present in the expression cassette and the vector backbone, we performed three qPCR assays using the following primers: hEPOtaq1 (5'-GCAGCTGCATGTGGATAAAGC-3') and hEPOtaq2 (5'-GCAGCTGCATGTGGATAAAGC-3') for the hEPO cDNA; LTRtaq1 (5'-GTCCAGCCCTCAGCAGTTTCTA-3') and LTRtaq2 (5'-GCAGCTGCATGTGGATAAAGC-3') for the MoMLV LTR₂ element; and E2q1 (5'-GCAGAACCAACCAGCACAGTGT-3') and E2q2 (5'-TCCACGATTTCCCTCTAAGCTA-3') for the Ad5 E2 region. These qPCR assays used SYBR Green PCR Master Mix and an ABI Prism 7700 sequence detector (Applied Biosystems, Foster City, CA) as follows: stage 1, 95°C for 2 min; stage 2, 95°C for 10 min; stage 3, 95°C for 15 sec, 60°C for 1 min, repeated 40 times.

To further characterize these two vector preparations, we tested for the presence of replication-competent Ad5 (RCA) by two methods. First, we used the primers E1q1 (5'-TGTTGCCCATTAACACAGTTG-3') and E1q2 (5'-TCCTCGATACATTCCACAGCCT-3') and E1 probe 1 (5'-/56-FAM/CGTGAGAGTTGGTGGGCGTCGC/36-TAMTSp/-3') to amplify part of the Ad5 E1 region from these two vector preparations. For this assay, 10^{10} vg/vector was used. The qPCR assay used TaqMan Universal PCR Master Mix and an ABI Prism 7700 sequence detector (Applied Biosystems) as follows: stage 1,

Finally, we examined both Ad_{ΔE1/3}LTR₂EF1 α -hEPO and AdLTR₂EF1 α -hEPO by restriction analyses using *NotI* restriction endonuclease. For these assays, viral DNA was obtained from purified vectors by extraction with the QIAmp DNA Micro Kit (QIAGEN, Germantown, MD).

C7 cells are derived from 293 cells, stably express the Ad5 preterminal protein and DNA polymerase (Amalfitano and Chamberlain, 1997), and were grown in high-glucose Dulbecco's modified Eagle's medium (Invitrogen). A549 cells are

FIG. 1. Characterization of the Ad $_{\Delta E1/3}$ LTR $_2$ EF1 α -hEPO genome. **(a)** Schematic diagram of Ad $_{\Delta E1/3}$ LTR $_2$ EF1 α -hEPO. See text for additional details. Ad5, serotype 5 adenovirus; env, Moloney murine leukemia virus (MoMLV) envelope; LTR, MoMLV long terminal repeat; EF1 α , elongation factor 1 α promoter; polyA, simian virus 40 polyadenylation signal; ITR, Ad5 inverted terminal repeat. **(b)** Examination of the AdLTR $_2$ EF1 α -hEPO and Ad $_{\Delta E1/3}$ LTR $_2$ EF1 α -hEPO genomes by *NotI* restriction endonuclease digestion using DNA samples from both purified vectors. **(c)** Assessment of expression cassette by PCR assays with DNA samples from rat submandibular glands 6 months after transduction using Ad $_{\Delta E1/3}$ LTR $_2$ EF1 α -hEPO. **(d)** Diagram of differences in Ad5 E3 gene region between AdLTR $_2$ EF1 α -hEPO ($\Delta E1$) and Ad $_{\Delta E1/3}$ LTR $_2$ EF1 α -hEPO ($\Delta E1/E3$). The Ad5 E1 and part of E3 were deleted in the AdLTR $_2$ EF1 α -hEPO, which still contained the Ad5 E3 12.5k, E3 7.1k, E3 gp19k, and E3 10.5k coding sequences and a truncated E3 10.4k protein coding sequence, *i.e.*, all except E3 14.5k and E3 14.7k. **(e)** Determination of differences in the Ad5 E3 region for pure AdLTR $_2$ EF1 α -hEPO and Ad $_{\Delta E1/3}$ LTR $_2$ EF1 α -hEPO vector genomes by PCR assays using pJM17 (17) and pBHG10 (10) as controls. **(f)** Assessment of E3 genome by PCR assays with DNA samples from rat submandibular glands 6 months after transduction using Ad $_{\Delta E1/3}$ LTR $_2$ EF1 α -hEPO at 10^{10} vg/gland. **(g)** Plasmid-Safe DNase assays to determine the general vector DNA structure (linear double-stranded or circular) in rats whose submandibular glands were transduced with Ad $_{\Delta E1/3}$ LTR $_2$ EF1 α -hEPO at 2.4×10^8 pfu/gland (equal to 10^{10} vg/gland) 6 months previously. The "+" indicates the presence of Plasmid-Safe DNase in the incubation mixture, whereas the "-" indicates the absence of Plasmid-Safe DNase. *Upper*: Treated samples were directly electrophoresed. *Lower*: The same samples were used in a PCR assay to amplify the PCR2 amplicon (see panels a and b). Please see the text for additional details regarding PCR amplicons 1-5.

derived from human lung carcinoma and were grown in RPMI 1640 medium. The following supplements were included: 10% fetal bovine serum, 100 U/mL penicillin G, and 100 μ g/mL streptomycin (all from Invitrogen). Cells were incubated at 37°C in a humidified 5% CO₂ atmosphere.

In vivo animal experiments

Animal experiments were approved by the NIDCR Animal Care and Use Committee and the National Institutes of Health Biosafety Committee. Male Wistar rats (250–350 g, ~3 months old) were anesthetized with ketamine (60 mg/kg) and xylazine (8 mg/kg) intramuscularly. Vectors, at either 2.1×10^7 or 2.1×10^8 pfu/gland (equal to 10^9 or 10^{10} vg/gland) for AdLTR₂EF1 α -hEPO and at either 2.4×10^7 or 2.4×10^8 pfu/gland (equal to 10^9 or 10^{10} vg/gland) for Ad Δ E1/3LTR₂EF1 α -hEPO, typically were administered to the right submandibular gland by retrograde ductal instillation (Baum *et al.*, 2002). In some experiments, vectors were delivered by a single intramuscular injection into the quadriceps muscle. Blood samples were collected from the tail vein or the retro-orbital sinus at different time points. Hematocrits were measured using microhematocrit capillary tubes (Fisher Scientific, Pittsburgh, PA).

Measurement of serum hEPO levels

hEPO levels in rat serum were determined by ELISAs using hEPO ELISA kits from either StemCell Technologies (Vancouver, BC, Canada) or R & D Systems (Minneapolis, MN).

Measurement of serum neutralizing antibodies

Anti-Ad5 neutralizing antibodies were assayed in serum as previously described (Sprangers *et al.*, 2003).

PCR and qPCR assays

Genomic DNA for most PCR reactions was extracted with the Wizard Genomic DNA Purification Kit (Promega, Madison, WI). High-molecular-weight (HMW) genomic DNA was extracted with a Non-Organic DNA Extraction Kit (Chemicon, Temecula, CA). Low-molecular-weight (LMW) genomic DNA was extracted by the Hirt method (Hirt, 1967). Four hundred nanograms of DNA was used for PCR reactions, and 100 ng of DNA was used for qPCR reactions. The primers LTRF109 (5'-AAAGACCCACCTGTAGTTTGGC-3') and EF1 α B1 (5'-AACTCTCCACCCACTTCCAAC-3') were used to amplify PCR1 (see Fig. 1a). PCR2 (Fig. 1a) was amplified using EF1 α F4 (5'-GTTGCGTGAGCGGAAAGATG-3') and hEPOB6 (5'-CGGAAGAGTTTGCAGGAAAGTG-3'). PCR3 (Fig. 1a) was amplified using hEPOF2 (5'-AATCACTGCTGACACTTTCCGC-3') and E2B22 (5'-TCCATCAAACGAGTTGGTGCTC-3'). PCR4 (Fig. 1c) was amplified with AdE3F323 (5'-AAAACACCACCCTCCTTACCTGCC-3') and AdE3B325 (5'-ACAGAGAATAACCCCAACCCC-3'). PCR5 (Fig. 1c) was amplified with AdE3F340 (5'-AGAGATGACCAACACAACCAACG-3') and AdE3B348 (5'-ATGAGCGTGAGGGTGACAAATCCG-3'). Primers and probes used for qPCR were hEPOtaq1 (5'-GCAGCTGCATGTGGATAAAGC-3'), hEPOtaq2 (5'-GCAGCTGCATGTGGATAAAGC-3'), and hEPOtaqprobe (5'-/56-FAM/CAGTGCCCTTCGCAGCCTCACC/36-TSMTSp/-3') for the hEPO cDNA.

Plasmid-Safe DNase assays

Genomic DNA (800 ng) extracted with the Wizard Genomic DNA Purification Kit (Promega) was digested with 10 μ L of Plasmid-Safe DNase (Epicentre, Madison, WI) plus 20 μ L of 25 mM ATP and 10 μ L of buffer for 16 hr. Thereafter, 200 ng of the DNA sample was subjected to electrophoresis in 1% agarose. PCR reactions for DNase assays used 200 ng of genomic DNA.

Cytokine and chemokine assays

Either saline, AdLTR₂EF1 α -hEPO, or Ad Δ E1/3LTR₂EF1 α -hEPO (2.1×10^8 pfu or 2.4×10^8 pfu, equal to $\sim 10^{10}$ vg of each) was administered to the right submandibular glands of male Wistar rats by retrograde ductal instillation, and the submandibular glands were removed at either day 2, 16, or 30. Glands were then homogenized in phosphate-buffered saline (PBS) at pH 7.4, containing 13 μ L/mL protease inhibitor cocktail (Thermo Scientific, Rockford, IL), and centrifuged for 15 min at 1,500 *g*; the resulting supernatants were transferred to a new tube. At the same time points, blood was also collected and separated to obtain serum. The tissue supernatants and serum samples were assayed for the cytokines and chemokines indicated in the text at PIERCE Biotechnology (Woburn, MA) using the Searchlight Protein Array analysis.

Immunofluorescence and immunohistochemistry staining

For immunofluorescence staining, submandibular glands were removed 2 days post transduction, fixed in 10% formalin, and embedded in paraffin. Sections of 5 μ m were deparaffinized and rehydrated in a gradient series of ethanol and then washed in PBS. Antigen retrieval was performed with 1 mM EDTA (pH 8), 0.05% Tween 20, in a microwave oven for 10 min. Sections were then blocked with 20% goat serum in 5% bovine serum albumin (BSA) for 1 hr, incubated with primary antibodies (either mouse monoclonal anti-rat CD4, mouse monoclonal anti-rat CD8- α , rabbit polyclonal anti-mouse CD19, or mouse monoclonal anti-rat macrophage; all from Santa Cruz Biotechnology, Santa Cruz, CA) in 5% BSA in PBS for 1 hr at room temperature, and then washed with PBS. Next, the slides were incubated with secondary antibodies, either Alexa Fluor488 donkey anti-mouse IgG (H+L) or Alexa Fluor488 donkey anti-rabbit IgG (H+L) (Invitrogen) for 1 hr, washed with PBS, and mounted with ProLong Gold Antifade Reagent with DAPI (Invitrogen). For immunohistochemistry, submandibular glands were removed 6 months post transduction, fixed in 10% formalin, embedded in paraffin, sectioned (HistoServ, Germantown, MD), and stained with anti-human EPO antibody and the rabbit ImmunoCruz Staining System, sc-2051 (Santa Cruz Biotechnology), plus streptavidin and biotin block. Sections were also stained conventionally with hematoxylin and eosin (H&E).

Cellular immune response assays

AdLTR₂EF1 α -hEPO and Ad Δ E1/3LTR₂EF1 α -hEPO were administered 2.1×10^8 pfu/gland or 2.4×10^8 pfu/gland (equal to $\sim 10^{10}$ vg/gland) to the right submandibular glands of male Wistar rats by retrograde ductal instillation, and their spleens were removed on either day 2, 16, or 30.

TABLE 1. CHARACTERISTICS OF AdLTR₂EF1 α -hEPO AND Ad Δ E1/3LTR₂EF1 α -hEPO VIRAL VECTORS

Vectors	qPCR titer ($\times 10^{12}$)	pfu titer ($\times 10^{12}$)	Ratio (qPCR/MOI)	RCA in 10^{10} vp	Endotoxin in 10^{10} vp	Ratio (hEPO/AdE2)	Ratio (LTRs/AdE2)
Δ E1	6.7	0.14	47.9	ND	ND	1.1	2.1
Δ E1/E3	2.5	0.06	41.7	ND	ND	1.1	2.2

ND, none detected; pfu, plaque-forming units; RCA, replication-competent Ad5; vp, viral particles; Δ E1, AdLTR₂EF1 α -hEPO; Δ E1/E3, Ad Δ E1/3LTR₂EF1 α -hEPO.

Splenocytes were freshly isolated as described (Hori *et al.*, 2002) and plated in a 96-well plate (10^5 cells/well) using RPMI 1640 medium (Invitrogen). The cells were incubated at 37°C in a humidified 5% CO₂ atmosphere. After 1 hr, AdLTR₂EF1 α -hEPO or Ad Δ E1/3LTR₂EF1 α -hEPO (MOI=10 particles/cell) or concanavalin (Con) A (10 μ g/mL; Sigma, St. Louis, MO) was added to the appropriate wells. After 48 hr, MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assays were performed in the 96-well plates to assess proliferative responses (MTT colorimetric kit; Chemicon) (Kruisbeek, 1997; Basagoudanavar *et al.*, 2006).

Statistical analysis

Data analyses were performed using SigmaStat version 2.0 (SPSS, Inc., Chicago, IL). Results are presented as means $\pm$ SEM. One-way ANOVAs followed by a Tukey test or Dunn's method were used as appropriate (see figure legends).

Results

Characteristics of purified vectors

Both purified vectors, AdLTR₂EF1 α -hEPO and Ad Δ E1/3LTR₂EF1 α -hEPO, were negative for RCA by qPCR assay, as well as negative for the presence of endotoxin (Table 1). To further examine if RCA was present in either the AdLTR₂EF1 α -hEPO or Ad Δ E1/3LTR₂EF1 α -hEPO preparation, we tested both vectors in A549 cells. When WT Ad5 was used to infect A549 cells as the positive control, on day 7 the cells began to show CPE at either 1,000 or 10,000 vg/well. However, no CPE were observed in cells infected with WT Ad5 (100 vg/well) or in cells transduced with AdLTR₂EF1 α -hEPO or Ad Δ E1/3LTR₂EF1 α -hEPO and observed until day 18 (assay terminated; this assay was performed twice).

The ratios of the hEPO transgene and Ad5 E2 sequences, as well as the ratios of LTR sequences and Ad5 E2 sequences (note there are two LTR elements in each construct), were ~ 1 and ~ 2 , respectively (Table 1). This indicates that there were no other contaminating vectors in either preparation.

The ratio of the qPCR titer and MOI were quite similar: 47.9 for AdLTR₂EF1 α -hEPO and 41.7 for Ad Δ E1/3LTR₂EF1 α -hEPO (Table 1). Furthermore, *NotI* restriction endonuclease digestion showed only the presence of the predicted bands for both AdLTR₂EF1 α -hEPO and Ad Δ E1/3LTR₂EF1 α -hEPO (Fig. 1b).

Characteristics of vector genomes in transduced submandibular glands

The plasmid pBHG10, which contains the Ad5 genome except for the E1 gene and most of the E3 gene (Fig. 1), was used to construct Ad Δ E1/3LTR₂EF1 α -hEPO. The comparative vector used for these studies, AdLTR₂EF1 α -hEPO, was constructed with a similar Ad5 plasmid, pJM17, which lacks the E1 gene, but contains most of the E3 gene (see Materials and Methods). PCR assays, with genomic DNA samples extracted from rat submandibular glands 6 months post transduction, confirmed that the novel Ad Δ E1/3LTR₂EF1 α -hEPO vector retained its intact expression cassette (Fig. 1c). Additionally, PCR assays confirmed that most E3 sequences were absent in Ad Δ E1/3LTR₂EF1 α -hEPO DNA, as well as in DNA samples extracted from glands 6 months post transduction (Fig. 1e and f). At this time, qPCR assays showed that genomic DNA samples extracted from glands transduced with either vector contained $\sim 5\text{--}7 \times 10^3$ vector particles/100 ng of DNA (Table 2), corresponding to $\sim 10^5$ pfu/gland (equal to $\sim 10^7$ particles/gland), *i.e.*, $\sim 0.1\%$ of the delivered vector dose. Furthermore, the ratio of vector genome found in HMW and LMW DNA fractions from these extracted DNA samples after administration of either vector was similar: 1.3 for AdLTR₂EF1 α -hEPO and 1.2 for Ad Δ E1/3LTR₂EF1 α -hEPO (Table 2). We also examined whether the Ad Δ E1/3LTR₂EF1 α -hEPO genome was present in glands as circular or linear double-stranded DNA using Plasmid-Safe ATP-Dependent DNase, which can efficiently digest linear double-stranded DNA to mononucleotides. Figure 1g shows that vector DNA from rat submandibular glands 6 months after administration was completely digested by the

TABLE 2. VECTOR COPIES AND hEPO EXPRESSION IN TRANSFUSED RAT SUBMANDIBULAR GLANDS

Vectors	hEPO in serum (mU/mL)	hEPO in saliva (mU/mL)	hEPO in SG (mU/mg protein)	Median NAb titer	HMW ($\times 10^3/100$ ng DNA)	LMW ($\times 10^3/100$ ng DNA)	Ratio (HMW/ LMW)
AdLTR ₂ EF1 α -hEPO	0	1.6 $\pm$ 0.8	104.8 $\pm$ 55.3	1:512	5.6 $\pm$ 1.1	4.7 $\pm$ 1.2	1.3 $\pm$ 0.2
Ad Δ E1/3LTR ₂ EF1 α -hEPO	3.8 $\pm$ 1.2	132.8 $\pm$ 50.3	608.1 $\pm$ 62.7	1:4	7.6 $\pm$ 1.0	6.5 $\pm$ 0.8	1.2 $\pm$ 0.2

HMW, high-molecular-weight; LMW, low-molecular-weight; NAb, neutralizing antibody; SG, submandibular gland.

The numbers of vector copies were measured as hEPO sequences present in HMW and LMW genomic DNA obtained from rat submandibular glands 6 months post transduction with the indicated hybrid vectors. hEPO was measured by an ELISA in serum, saliva, and gland extracts. Data shown are mean $\pm$ SEM ($n=5$). See Materials and Methods for additional details.

Plasmid-Safe DNase. As previously reported (Zheng *et al.*, 2008), similar results were found in the AdLTR₂EF1 α -hEPO-transduced glands (data not shown). These findings suggest that both vectors exist as nonintegrated, linear double-stranded DNA in an extrachromosomal site after administration to submandibular glands.

hEPO expression from transduced submandibular glands

Initial *in vitro* experiments demonstrated that C7 cells, transduced by either Ad Δ E1/3LTR₂EF1 α -hEPO or AdLTR₂EF1 α -hEPO, expressed and secreted hEPO into culture media (data not shown). Next, we extensively tested both vectors *in vivo* following retrograde delivery to rat submandibular glands in two separate experiments. The first experiment was a limited dose-response study, in which 2.4×10^7 or 2.4×10^8 pfu/gland (equal to 10^9 or 10^{10} vg/gland) of Ad Δ E1/3LTR₂EF1 α -hEPO or 2.1×10^7 or 2.1×10^8 pfu/gland (equal to 10^9 or 10^{10} vg/gland) of AdLTR₂EF1 α -hEPO was delivered to right submandibular glands ($n=5$). Serum hEPO levels from the two AdLTR₂EF1 α -hEPO-treated dose groups were not different on day 7 or 180. Similarly, the hematocrit levels obtained after both vector doses were comparable (Fig. 2a and c). In animals that were Ad Δ E1/3LTR₂EF1 α -hEPO-treated, the higher vector dose resulted in significantly greater hEPO levels on days 7 and 180 ($p<0.05$), with corresponding significant hematocrit increases ($p<0.05$; Fig. 2b and d). Note that at day 7 AdLTR₂EF1 α -hEPO resulted in high serum hEPO levels at both doses, whereas by day 180 Ad Δ E1/3LTR₂EF1 α -hEPO treatment yielded the higher serum hEPO level.

Thereafter, for all experiments, we used the higher vector dose [2.4×10^8 pfu/gland (equal to 10^{10} vg/gland) of Ad Δ E1/3LTR₂EF1 α -hEPO or 2.1×10^8 pfu/gland (equal to 10^{10} vg/gland) of AdLTR₂EF1 α -hEPO].

Figure 3 shows combined data from the high-dose group for the experiment shown in Fig. 2 ($n=5$), with that from a second separate experiment, with $n=10$ rats/group. On day 7

after AdLTR₂EF1 α -hEPO delivery, hEPO found in saliva and serum averaged 30,802 mU/mL and 305 mU/mL, respectively. With Ad Δ E1/3LTR₂EF1 α -hEPO-treated rats, these values were significantly less: 2,117 mU/mL saliva and 20 mU/mL serum (Fig. 3a and b). Thus, as noted above, initially hEPO expression from AdLTR₂EF1 α -hEPO was much (~ 15 -fold) higher in both saliva and serum than that from Ad Δ E1/3LTR₂EF1 α -hEPO (Fig. 3a and b). However, hEPO expression from AdLTR₂EF1 α -hEPO decreased quickly and dramatically thereafter compared with expression seen after Ad Δ E1/3LTR₂EF1 α -hEPO administration (Fig. 3a and b). For example, at 2–3 weeks post transduction, there was little difference in hEPO expression from the two vectors, whereas by 8–10 weeks post administration, expression from glands transduced with Ad Δ E1/3LTR₂EF1 α -hEPO was much higher. Indeed, from 30 days post transduction, hEPO expression from the Ad Δ E1/3LTR₂EF1 α -hEPO vector was relatively stable until day 180, when the experiment was terminated (Fig. 3a and b). Furthermore, hematocrit levels in the Ad Δ E1/3LTR₂EF1 α -hEPO-treated rats were increased from normal levels (47–50%) to $\sim 65\%$ by day 30, and stayed between 60 and 70% until day 180. Conversely, hematocrit levels in the AdLTR₂EF1 α -hEPO-treated rats also increased by day 30, but thereafter decreased to normal levels by day 90 and remained stable until the conclusion of the experiment (Fig. 3c). Finally, as shown in Fig. 3d, hEPO expression was detectable in both ductal and acinar cells of the Ad Δ E1/3LTR₂EF1 α -hEPO-transduced submandibular glands at day 180. These aggregate results demonstrate that Ad Δ E1/3LTR₂EF1 α -hEPO-mediated transgene expression in rat submandibular glands is markedly more stable than that derived from the AdLTR₂EF1 α -hEPO vector in this tissue.

Immune reactivity in animals following salivary gland vector delivery

We hypothesized that the differences observed in hEPO expression described above were a consequence of lessened

FIG. 2. Effect of two vector doses on serum hEPO levels and hematocrits in rats. (a) The hEPO expression in serum after AdLTR₂EF1 α -hEPO (2.1×10^7 or 2.1×10^8 pfu, equal to 10^9 or 10^{10} vg) delivery to one submandibular gland. On day 7, there is no significant difference between the two doses. (b) The hEPO expression in serum after Ad Δ E1/3LTR₂EF1 α -hEPO (2.4×10^7 or 2.4×10^8 pfu, equal to 10^9 or 10^{10} vg) delivery to one submandibular gland. Differences between the two dose groups are significant ($p<0.05$) on days 7 and 180 using a Tukey test. (c) Hematocrit levels measured for the same rats shown in panel a. Differences between the two dose groups are not significant on days 7 and 180. (d) Hematocrit levels measured for the same rats shown in panel b. Differences between the two dose groups are significant ($p<0.05$) on days 7 and 180 using a Tukey test. Data shown are the means $\pm$ SEM for one experiment ($n=5$).

local host immune reactivity following gland transduction with the Ad Δ E1/3LTR Δ EF1 α -hEPO vector. To test this hypothesis, we first examined cytokine and chemokine profiles in submandibular gland extracts and serum from days 2, 16, and 30 post transduction with either AdLTR Δ EF1 α -hEPO or Ad Δ E1/3LTR Δ EF1 α -hEPO ($n=4$ rats/group, 2.1×10^8 pfu or 2.4×10^8 pfu, equal to 10^{10} vg/gland), or following administration of saline to the glands of control animals. As an example of data obtained from individual rats, Fig. 4a shows the interleukin (IL)-1 α profile in gland extracts for every rat (4/group/time point) in this experiment. IL-1 α was dramatically increased in the submandibular glands of three of the four treated rats at 2 days post transduction with

AdLTR Δ EF1 α -hEPO. One animal showed no change, possibly due to individual animal differences in immune reactivity or to an inadequate cannulation of the targeted gland. Conversely, the IL-1 α levels in glands treated with either saline or Ad Δ E1/3LTR Δ EF1 α -hEPO were markedly lower than those of the AdLTR Δ EF1 α -hEPO-treated group. Similar results were observed for most other cytokines and chemokines measured, *i.e.*, IL-1 β , interferon (IFN)- γ , IL-10, monocyte chemotactic protein 1 (MCP1), macrophage inflammatory protein 1 α (MIP1 α), IL-6, but not regulated upon activation normal T-cell expressed and secreted (RANTES) protein (Fig. 4b-i). Although most immune mediators were dramatically increased in the submandibular glands at 2 days post transduction with the AdLTR Δ EF1 α -hEPO vector, the comparable values for the saline- and Ad Δ E1/3LTR Δ EF1 α -hEPO-treated glands consistently were much lower. Interestingly, there were no changes in serum IL-1 α , IL-1 β , IFN γ , IL-10, MCP1, and MIP1 α levels (data not shown) 2 days post transduction, *i.e.*, the only changes in the measured immune mediators following vector treatment were local, within the targeted submandibular glands.

Next, we directly examined gland tissue, at 2 days post saline or vector administration, for general pathology, and to determine if specific inflammatory cell infiltrates were present (CD4-, CD8-, and CD19-positive lymphocytes; macrophages; by immunofluorescence). With H&E staining, the submandibular glands of saline-treated rats showed normal gland structure (Fig. 5). Conversely, in submandibular glands of AdLTR Δ EF1 α -hEPO-treated rats, there was a strong acute inflammatory infiltrate present (Fig. 5), whereas in the submandibular glands of Ad Δ E1/3LTR Δ EF1 α -hEPO-treated rats, only a mild acute inflammation was observed (Fig. 5). Following immunofluorescence staining, we saw no consistent difference between the three animal study groups in the amount of CD4-positive T lymphocytes infiltrating gland tissue (Fig. 5). However, CD8-positive T-lymphocyte staining, CD19-positive B-lymphocyte staining, and macrophage staining were consistently higher in the submandibular glands of AdLTR Δ EF1 α -hEPO-treated rats when

FIG. 3. Effects of vector administration on serum hEPO levels and hematocrits in rats. **(a)** Time course of hEPO expression in saliva after vector [AdLTR Δ EF1 α -hEPO (Δ E1) or Ad Δ E1/3LTR Δ EF1 α -hEPO (Δ E1/3); 2.4×10^8 pfu, equal to 10^{10} vg] delivery in rats to one submandibular gland. Data shown are the means $\pm$ SEM for two experiments ($n=15$). Differences between two vectors at each time point are significant ($p<0.05$) using a Tukey test or Dunn's method. **(b)** Time course of hEPO expression in serum from the same experiments as shown in panel a. Differences between two vectors at each time point are significant ($p<0.05$) using a Tukey test or Dunn's method, except for days 14 and 60. **(c)** Time course of hematocrit levels (means $\pm$ SEM) measured for the same rats shown in panels a and b. The control rats exhibited hematocrits of 47–50% over this time (data not shown). Differences between two vectors after day 90 are significant ($p<0.05$) using a Tukey test or Dunn's method. **(d)** Immunohistochemistry staining to detect hEPO in submandibular glands 6 months post transduction with the Ad Δ E1/3LTR Δ EF1 α -hEPO. *Left:* Stained with a rabbit IgG control. *Right:* Stained with anti-hEPO antibody, as described in **Materials and Methods**. a, acinar cells; d, ductal cells.

FIG. 4. Cytokine and chemokine profiles in extracts of vector- [AdLTR₂EF1 α -hEPO (Δ E1) or Ad _{Δ E1/3}LTR₂EF1 α -hEPO (Δ E1/3); 2.1×10^8 pfu or 2.4×10^8 pfu, equal to 10^{10} vg] or saline- (Control) treated rat submandibular glands 2 days post transduction. **(a)** Example showing IL-1 α levels in submandibular gland extracts from each rat studied in this experiment (*i.e.*, $n=4$ /treatment group/time point), showing individual animal variation. **(b–i)** Panels show the time course (days 2, 16, and 30) for measured cytokine and chemokine levels, as indicated in each panel, in gland extracts from all four rats in each treatment group. The following cytokines and chemokines were measured as indicated: IL-1 α , IL-1 β , IFN γ , IL-10, MCP1, MIP1 α , IL-6 and RANTES (for the latter two, gland extracts only were measured). Data shown are means $\pm$ SEM.

FIG. 5. General pathology and immunofluorescence staining of rat submandibular glands 2 days post transduction. Vectors (indicated at the top) or saline was administered to rat submandibular glands. On day 2 post administration, glands were removed and processed for general pathologic evaluation (H&E) or stained for one of four markers of inflammatory cells: CD4 and CD8 (subpopulations of T cells), macrophages and CD19 (B cells). Representative results, of those found with the four rats studied in Fig. 4, are shown. In the H&E panels, black arrows indicate areas of acute inflammatory infiltrates (shown in the AdLTR₂EF1 α -hEPO panel only): a, acinar cells; d, ductal cells (shown in the saline control panel only). See the text for additional details.

compared with the glands from Ad Δ E1/3LTR Δ EF1 α -hEPO- and saline-treated glands (Fig. 5).

We next measured the levels of Ad5 neutralizing antibodies present in rat serum samples. As shown in Fig. 6a, following vector administration (2.1×10^8 pfu or 2.4×10^8 pfu, equal to 10^{10} vg) to submandibular glands, the titers of serum Ad5 neutralizing antibodies in AdLTR Δ EF1 α -hEPO-treated rats were markedly (19-fold) higher than those found in Ad Δ E1/3LTR Δ EF1 α -hEPO-treated rats at day 30. By day 180, the median titer of Ad5 neutralizing antibodies in Ad Δ E1/3LTR Δ EF1 α -hEPO-treated rats was 1:4, whereas that from

AdLTR Δ EF1 α -hEPO-treated rats was 1:512 (Table 2). Interestingly, we observed no difference in the serum Ad5 neutralizing antibody titers between AdLTR Δ EF1 α -hEPO- and Ad Δ E1/3LTR Δ EF1 α -hEPO-treated rats following intramuscular vector delivery at day 30 (Fig. 6a). These results suggest the presence of an organ-specific difference in immune reactivity after administration of the Ad Δ E1/3LTR Δ EF1 α -hEPO vector.

Finally, we evaluated cellular immune responses using splenocytes prepared from the same rats used for the cytokine/chemokine experiments shown in Fig. 4. Con A treatment was used as a positive control for T-cell activation, and medium alone was used as a negative control (Fig. 6b). Splenocytes obtained from the AdLTR Δ EF1 α -hEPO-treated rats on day 2 post transduction showed a much stronger ($p < 0.05$) proliferative response to vector addition than splenocytes from Ad Δ E1/3LTR Δ EF1 α -hEPO-treated rats (Fig. 6b).

Discussion

As with any tissue, in order to use salivary glands efficiently as a gene transfer target site, an optimal vector is needed. Conventional Ad5 vectors have the advantage of transducing both acinar and ductal cells in salivary glands quite efficiently, but they can only mediate significant transgene expression for ~ 2 weeks (Kagami *et al.*, 1998). To overcome this disadvantage, we began to develop a hybrid adenoretroviral vector system for use in salivary glands. The initial version of this hybrid vector was useful conceptually, but posed (i) a risk for insertional mutagenesis and (ii) difficulties in constructing therapeutic vector cassettes (Zheng *et al.*, 2000, 2003a; Zheng and Baum, 2002). A second version of the hybrid adenoretroviral vector circumvented both of these problems and has been useful in gene transfer to salivary glands for up to 2 months with preclinical models (Zheng *et al.*, 2009, 2011b). Herein, we have described a fairly simple modification in hybrid-vector construction, more extensive deletion of the Ad5 E3 gene, which extends the length of useful transgene expression from the hybrid vector in salivary glands to at least 6 months, while still retaining the previously reported version's beneficial features of nonintegration and the ability to transduce both acinar and ductal cells. Indeed, hEPO transgene expression from the Ad Δ E1/3LTR Δ EF1 α -hEPO was more stable, higher (after 30 days), and functional as compared with results obtained with the previous vector, AdLTR Δ EF1 α -hEPO (Fig. 3).

The specific genomic difference between the two vectors was a 2,685-bp sequence deleted from nt 28,133 to nt 30,818 in the E3 region of Ad Δ E1/3LTR Δ EF1 α -hEPO, in addition to inclusion of a mutated 643-bp E3 sequence (see Materials and Methods). This deletion includes 42 bp of coding sequence for the E3 12.5k protein and all coding sequences for the other six Ad5 E3 proteins (Bett *et al.*, 1994; Human Adenovirus, 2007). Conversely, only the E3 14.5k and E3 14.7k coding sequences were deleted in the E3 region of AdLTR Δ EF1 α -hEPO (Bett *et al.*, 1995; Human Adenovirus, 2007). Thus, the AdLTR Δ EF1 α -hEPO vector can still lead to expression of the E3 12.5k, E3 7.1k, E3 gp19k, and E3 10.5k proteins, as well as a truncated E3 10.4k protein, in transduced cells (Bett *et al.*, 1995; Human Adenovirus, 2007). These E3 proteins are thought to protect infected or transduced cells from killing mediated by cytotoxic T cells and

FIG. 6. Evaluation of Ad5 neutralizing antibody levels and splenocyte proliferative responses following transduction. **(a)** Ad5 neutralizing antibody levels in serum samples following transduction [AdLTR Δ EF1 α -hEPO (Δ E1) or Ad Δ E1/3LTR Δ EF1 α -hEPO (Δ E1/3); 2.1×10^8 pfu or 2.4×10^8 pfu, equal to 10^{10} vg] of submandibular glands or quadriceps muscle at day 30 ($n = 5$ animals). For salivary gland (SG) administration, differences between the AdLTR Δ EF1 α -hEPO-treated rats and the Ad Δ E1/3LTR Δ EF1 α -hEPO-treated rats are significant ($p < 0.05$) using a Tukey test, whereas there are no differences after intramuscular (IM) injection. **(b)** Proliferative response *in vitro* 2 days post transduction using splenocytes. Differences between the medium and Con A groups, between the medium- and AdLTR Δ EF1 α -hEPO-treated groups, and between the two vector-treated groups are significant using a Tukey test ($p < 0.05$). See text for additional details on neutralizing antibody and cellular immune response assays. Data shown are means $\pm$ SEM.

death-inducing cytokines such as tumor necrosis factor (TNF), Fas ligand, and TNF-related apoptosis-inducing ligand (TRAIL) (Lichtenstein *et al.*, 2004a). For example, the E3 gp19k protein blocks major histocompatibility complex class I-restricted antigen presentation, which diminishes killing by cytotoxic T cells (Tollefson *et al.*, 1996; Lichtenstein *et al.*, 2004a). The receptor internalization and degradation (RID) complex, formerly termed E3 10.4k/14.5k, stimulates the clearance from the cell surface, and subsequent degradation, of the receptors for Fas ligand and TRAIL, thereby preventing the action of these important immune mediators (Lichtenstein *et al.*, 2004a,b). Somewhat contradictory results, however, have been reported (Schowalter *et al.*, 1997; Gantzer *et al.*, 2002), which suggest that the E3 proteins cannot inhibit acute inflammatory responses.

Interestingly, as shown herein, AdLTR₂EF1 α -hEPO, which retains most E3 coding sequences, induced much stronger immune responses after administration to rat submandibular glands than seen following delivery of Ad Δ E1/3LTR₂EF1 α -hEPO, which lacked most E3 coding sequences. Multiple cytokines and chemokines were markedly increased in submandibular gland extracts 2 days post transduction with AdLTR₂EF1 α -hEPO compared with levels measured in the saline control and Ad Δ E1/3LTR₂EF1 α -hEPO-treated glands (Fig. 4), whereas such differences were not found in serum. Furthermore, H&E staining clearly demonstrated that the AdLTR₂EF1 α -hEPO-treated glands exhibited a much stronger local inflammatory response (Fig. 5) than seen in control and Ad Δ E1/3LTR₂EF1 α -hEPO-treated glands. This impression was confirmed with immunofluorescence staining, which showed that the AdLTR₂EF1 α -hEPO-treated glands exhibited more infiltrates of CD8 T lymphocytes, CD19 B lymphocytes, and macrophages than observed in the Ad Δ E1/3LTR₂EF1 α -hEPO-treated glands (Fig. 5). Additionally, Ad Δ E1/3LTR₂EF1 α -hEPO-treated rats displayed lower levels of serum Ad5 neutralizing antibodies (Fig. 6a), and splenocytes prepared from these animals gave much weaker proliferation responses *in vitro* in response to vector (Fig. 6b). These aggregate results show that the Ad Δ E1/3LTR₂EF1 α -hEPO vector elicits weaker immune responses in rat submandibular glands than the AdLTR₂EF1 α -hEPO vector, and that this difference in their elicited immune reactivity most likely led to the former vector mediating much longer transgene expression in this tissue. Interestingly, we also found that the two vectors, when delivered intramuscularly, showed no difference in Ad5 neutralizing antibody levels elicited (Fig. 6a).

As indicated above, our aggregate results were not necessarily what would be expected when comparing E1- and E3-deleted vectors based on most existing studies, and there is as yet no clear explanation for them. However, Ad5 E3 proteins are not secreted proteins (Horwitz, 2004), so understanding their mechanisms of action must in part be considered in the context of to what cells or tissues the vectors/viruses are administered. Indeed, it is well recognized that immunogenicity can be route-of-antigen-dependent (*e.g.*, Foged and Sundblad, 2008; Peng *et al.*, 2009; Murphy *et al.*, 2011). Almost all previous studies examining the immunomodulatory functions of Ad5 E3 proteins were performed either in cell lines (*e.g.*, Friedman and Horwitz, 2002; Lesokhin, *et al.*, 2002), in transgenic animals (*e.g.*, von Herrath *et al.*, 1997; Efrat *et al.*, 2001), or delivered by intra-

venous injection as vectors encoding Ad5 E3 genes (*e.g.*, Poller *et al.*, 1996; Ilan *et al.*, 1997). In the present study, however, vectors primarily were delivered locally by salivary duct cannulation and retrograde infusion, *i.e.*, the vectors thus delivered do not have a significant opportunity to transduce and modulate immune cells directly, unlike the circumstances of earlier *in vivo* studies. As we have shown, the salivary glands provide an enclosed, somewhat protected mucosal immune system target for the Ad5 vectors with little to no access to the circulation (Zheng *et al.*, 2010). Conversely, following use of intramuscular injections, no differences were found when comparing E1- and E3-deleted vectors. After intramuscular injections, vectors can easily transduce multiple cell types, including skeletal muscle cells, endothelial cells, and dendritic cells. Dendritic cells are professional antigen-presenting cells, and their transduction likely also contributes to the observed differences herein.

A second consideration for immunogenicity related to nonsecreted proteins is the extent of local tissue damage present. As we have clearly shown (Fig. 5), there is more inflammation present in glands following AdLTR₂EF1 α -hEPO versus Ad Δ E1/3LTR₂EF1 α -hEPO administration in the targeted submandibular glands. Furthermore, it is recognized that such local tissue damage (cell death, inflammation) can serve as an adjuvant driving the adaptive immune response (Chen *et al.*, 2007), something that likely also contributed to results observed following intramuscular injections.

A third consideration related to the differences in vector immunogenicity involves the secreted transgenic protein tested, hEPO. Following AdLTR₂EF1 α -hEPO administration, initial hEPO serum levels were dramatically higher (>10-fold) than seen after Ad Δ E1/3LTR₂EF1 α -hEPO delivery (Fig. 3). The increased presence of CD8-positive T lymphocytes in AdLTR₂EF1 α -hEPO-transduced glands, and consequent tissue damage and inflammation, in part may be in response to the extremely high levels of this secreted foreign protein.

In summary, we have shown that local delivery to salivary glands of the novel Ad Δ E1/3LTR₂EF1 α -hEPO hybrid vector was associated with significantly lengthened transgene expression and reduced immunogenicity, which may be particularly beneficial for clinical applications that use salivary gland targeting. Furthermore, we have observed paradoxically that deletion of most Ad5 E3 coding sequences in this hybrid vector leads to an abrogation of innate and cell-mediated immune reactivity in this same tissue. A complete understanding of the likely multiple mechanisms involved in these unexpected results requires further detailed study.

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