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Long-term hepatic adenovirus-mediated gene expression in mice following *CTLA*4Ig administration

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Recombinant adenovirus vectors are efficient at transferring genes into somatic tissues but are limited for use in clinical gene therapy by immunologic factors that result in the rapid loss of gene expression and inhibit secondary gene transfer. This study demonstrates that systemic coadministration of recombinant adenovirus with soluble CTLA4Ig, which is known to block co-stimulatory signals between T cells and antigen presenting cells, leads to persistent adenoviral gene expression in mice without long-term immunosuppression. This form of immunotherapy greatly enhances the likelihood that recombinant adenovirus vectors will be useful for human gene therapy.

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Recombinant E1 deficient adenoviral vectors have been used in animals to transduce a number of different tissues *in vivo* with relatively high efficiencies[1]. The liver is an excellent target organ for studying adenovirus mediated gene transfer because of the large number of medical disorders that are potentially treatable and because 100% of hepatocytes can be transduced *in vivo*[2]. Adenovirus-mediated hepatic gene transfer has resulted in complete amelioration of the clinical phenotype in a number of disease models such as haemophilia B in dogs[3] and familial hypercholersterolaemia in mice[4] and rabbits[5]. Gene expression is transient, generally lasting for no more than several weeks because of a cellular immune response directed against transduced cells[6,7]. Moreover, systemic secondary gene transfer to the liver is not possible, probably due to the production of neutralizing antibodies[8,9]. Studies in immunodeficient mice[6,7,9] have demonstrated that in the absence of antigen-specific immunity, gene expression is indefinite and secondary gene transfer is possible. The immune response leading to rapid loss of gene expression most likely results from the low level production of viral antigens from the vector[6,10].

Initiation of a T cell-mediated immune response requires antigen presenting cells (APCs) which present short peptides in association with major histocompatibility (MHC) molecules to the T-cell receptor to elicit the appropriate response. Recently, it has been found that additional signals are needed for proper stimulation of the T-cell response. One such signal is provided by the B7-1 (CD80) and B7-2 (CD86) ligands present on APCs, which bind to the CD28 and CTLA4 receptors on T cells and elicit a co-stimulatory response needed for this activation[11-14]. Antigen-MHC engagement in the absence of a co-stimulatory signal produces T-cell anergy or prolonged unresponsiveness *in vitro*[13] and prolonged, perhaps indefinite survival of allografted organs[15,16]. We set out to determine whether temporary inhibition of the CD28-B7 co-stimulatory response using a soluble form of murine CTLA4Ig (muCTLA4Ig, a chimaeric protein of murine immunoglobulin Cγ2a fused to murine CTLA4)[17] would lead to persistent gene expression following adenovirus-mediated gene transfer and allow secondary gene transfer.

CTLA4Ig prolongs gene expression

The first experiment was designed to determine whether soluble muCTLA4Ig would result in prolonged gene expression after adenovirus mediated gene transfer. C3H/HeJ mice[9] were infused with Ad/RSVhAAT, an adenovirus that directs expression of human α1-antitrypsin[18] in transduced hepatocytes, and treated with either muCTLA4Ig or L6 (an isotype matched control monoclonal antibody that recognizes a human tumour antigen). Persistence of gene expression in individual animals was determined by periodic serum quantitation of human α1-antitrypsin (hAAT). Human α1-antitrypsin was used as a marker protein because of its general lack of immunogenicity in mice[19,20]. After Ad/RSV-hAAT administration in C3H mice about 60% of the animals develop detectable antibodies to hAAT. The presence or absence of antibodies to hAAT did not correlate with the persistence of hAAT in mice (not shown). Gene expression was greatly extended in animals that received muCTLA4Ig compared to the controls (Fig. 1). Each of the L6 control animals (n=8) had a greater than 100-fold decline in serum hAAT concentration between 2 and 7 weeks (Fig. 1*a*) after adenovirus administration to values that were

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not above background; these data are similar to those seen in a previous study[9]. All the muCTLA4Ig treated animals maintained high levels of hAAT expression for at least 14 weeks (n= 8), and 6 out of 8 mice expressed high levels of hAAT for >5 months, the length of the experiment (Fig. 1*a*). There was no difference in hAAT persistence in mice given 1 or 3 doses each of 200 μg of muCTLA4Ig. A second set of experiments (Fig. 1*b*) gave similar results with persistent expression for the 5-month duration of the experiment so far. Strikingly, the prolonged and persistent adenovirus-mediated gene expression was similar to that seen in our previous studies in congenic C3H *scid* mice that lack antigen specific immunity[9], and to adenovirus transduced β-galactosidase or factor IX expression observed in T-cell deficient nude mice[6,10,21].

Analysis of effects of muCTLA4Ig

To determine whether the effects of muCTLA4Ig were due to a general blockade in antiviral defenses, we assessed the effects of muCTLA4Ig on the course of primary infection with herpes simplex virus (HSV). In mice, host defense to HSV is dependent on T-cell (particularly CD8$^+$ T cell) mediated immunity[22,23]. Mice were infected with HSV either concomitantly or 3 to 5 weeks after administration of 200 μg of CTLA4Ig. These experiments were performed both with C3H/HeJ mice and with BALB/c mice; the effects of muCTLA4Ig treatment on Ad/RSVhAAT expression were similar in these two strains (data not shown). The results of the HSV experiments in the BALB/c and C3H/HeJ mice were similar so they were combined. The survival curve of the mice is shown in Fig. 2. Coadministration of muCTLA4Ig did not affect the course of the infection whereas preadministration resulted in a slight acceleration that was not statistically significant. These results are consistent with those showing development of effective CD8 T-cell responses following infection with lymphocytic choriomeningitis virus and vesicular stomatitis virus in CD28-deficient knockout mice[24] and contrast with the importance of CD28-B7 interactions in host response to allografts, tumour cells[25] and the non-replicating adenoviral vectors used here.

In addition, the long-term adenovirus mediated gene expression in the muCTLA4Ig treated mice did not appear to be the result of persistent immunosuppression. Expression continued long after the serum concentration of muCTLA4Ig had fallen from peak concentrations of ~100 μg ml^{-1} to values less than 10 ng ml^{-1} over a period of 6 to 10 weeks (Fig. 1*c* and *d*). Immunosuppressive effects of muCTLA4Ig have in our experience required concentrations greater than 1 μg ml^{-1} (Linsley, P.S. unpublished data). In addition the muCTLA4Ig treated animals were able to mount an immune response to a different antigen. To determine this, the muCTLA4Ig and L6 treated animals were challenged with a neoantigen, bacteriophage ϕX174 (ref. 26), 10 weeks after the initial adenovirus infusion. T-cell help is required for efficient antibody production including amplification and IgM to IgG isotype switch, in response to bacteriophage ϕX174 (ref. 27). Since T cell-dependent B-cell responses are

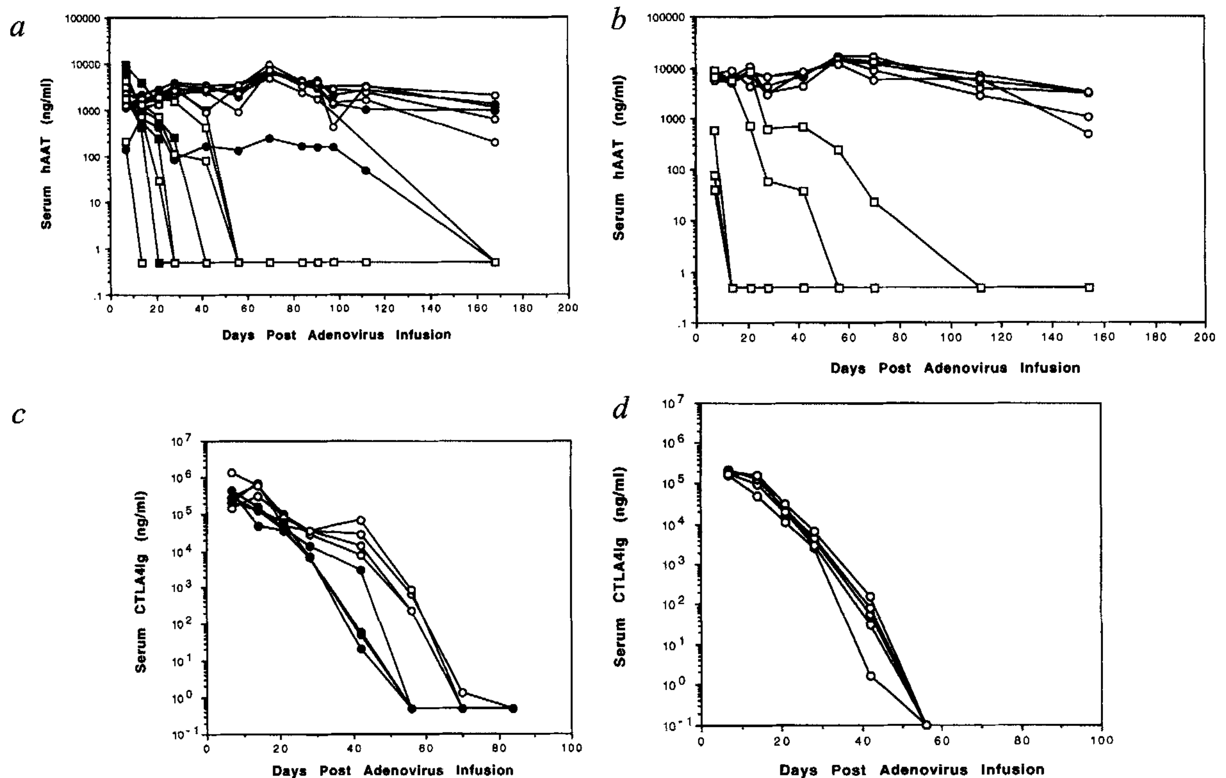

Fig. 1 Adenovirus mediated gene expression with coadministration of soluble CTLA4Ig. Sixteen female C3H/HeJ mice 6 to 8 weeks of age were infused with 5 × 10^9 pfu of Ad/RSVhAAT adenovirus by tail vein on day 0. *a*, *c*, The animals received 200 μg of murine CTLA4Ig (IP) on day 2 (n = 4, solid circles) or days 0,2,10 (n = 4, open circles). Control animals received equivalent amounts of a control antibody (L6) on day 2 (n = 4) (solid squares) or days 0,2,10 (open squares) (n = 4). *b*, *d* Repeat experiment. Ten mice were infused with Ad/RSVhAAT adenovirus on day 0. Animals received either soluble CTLA4Ig (n = 5, open circles) or L6 (n = 5, open squares) on days 0, 2 and 10. Periodic serum samples were analyzed for *a*, *b*, hAAT or (*c*,*d*) CTLA4Ig by ELISA. Each line represents an individual animal. ELISA assays were performed in at least duplicate for each measurement.

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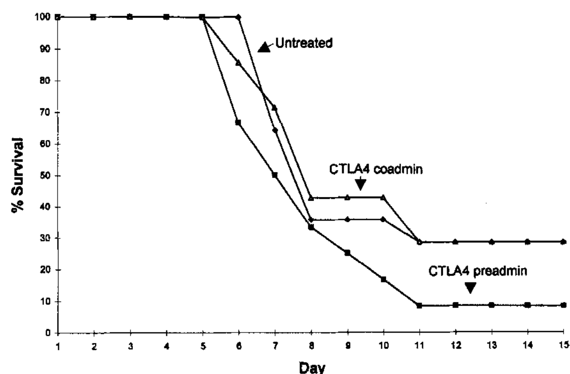

Fig. 2 Effects of muCTLA4Ig on survival of mice infected with HSV. Mice were infected in the hind footpads with 2×10^5 pfu/footpad of virulent HSV type I (KOS) either concomitantly or 3 to 5 weeks after administration of 200 μg of muCTLA4Ig. In the latter group the concentrations of muCTLA4Ig had diminished to values ranging from < 20 ng ml^{-1} to ~2 μg ml^{-1} (median 292 ng ml^{-1}) of serum. Presented are the combined results in BALB/c and C3H/HeJ mice. Untreated (*n* = 14), muCTLA4Ig coadministration (*n* = 14), muCTLA4Ig preadministration (*n* = 12).

blocked by muCTLA4Ig[13,17], this provided a sensitive measure of immune competence. 2.5×10^8 pfu of bacteriophage ϕX174 was infused I.V., 10 and 14 weeks after adenovirus transduction. Phage neutralizing antibody titers were determined weekly for a total of eight weeks after initial phage infusion. The titers were expressed as Kv or rate of phage inactivation over time. In muCTLA4Ig and L6 treated mice, the geometric mean peak Kv reached two weeks post primary infusion was 2.1 (range 0.9 to 14.3) and 3.9 (1.5 to 33.8), respectively. Two weeks post secondary phage infusion the geometric mean Kv for muCTLA4Ig treated animals was 49 (range 3.6 to 1043) and for L6 treated animals was 100.5 (range 15 - 1469), while the percentage of phage specific IgG antibody was 87% +/- 13% and 75% +/- 18%, respectively. Thus, each of the muCTLA4Ig and L6 treated animals produced normal amounts of neutralizing antibodies in response to primary and secondary bacteriophage ϕX174 immunization, including a switch from IgM to IgG during the secondary response[26]. The efficient development of an antibody response to ϕX174 is associated with clearance of the bacteriophage. These results suggest that immunological competence sufficient for the muCTLA4Ig treated mice to mount an effective *de novo* T-cell dependent antibody response was intact within 10 weeks of the initial adenovirus administration. Together with the studies of HSV, our results suggest that this form of treatment prolonged adenoviral transduced gene expression without inducing a general impairment in antiviral defenses or persistent impairment in the immune response.

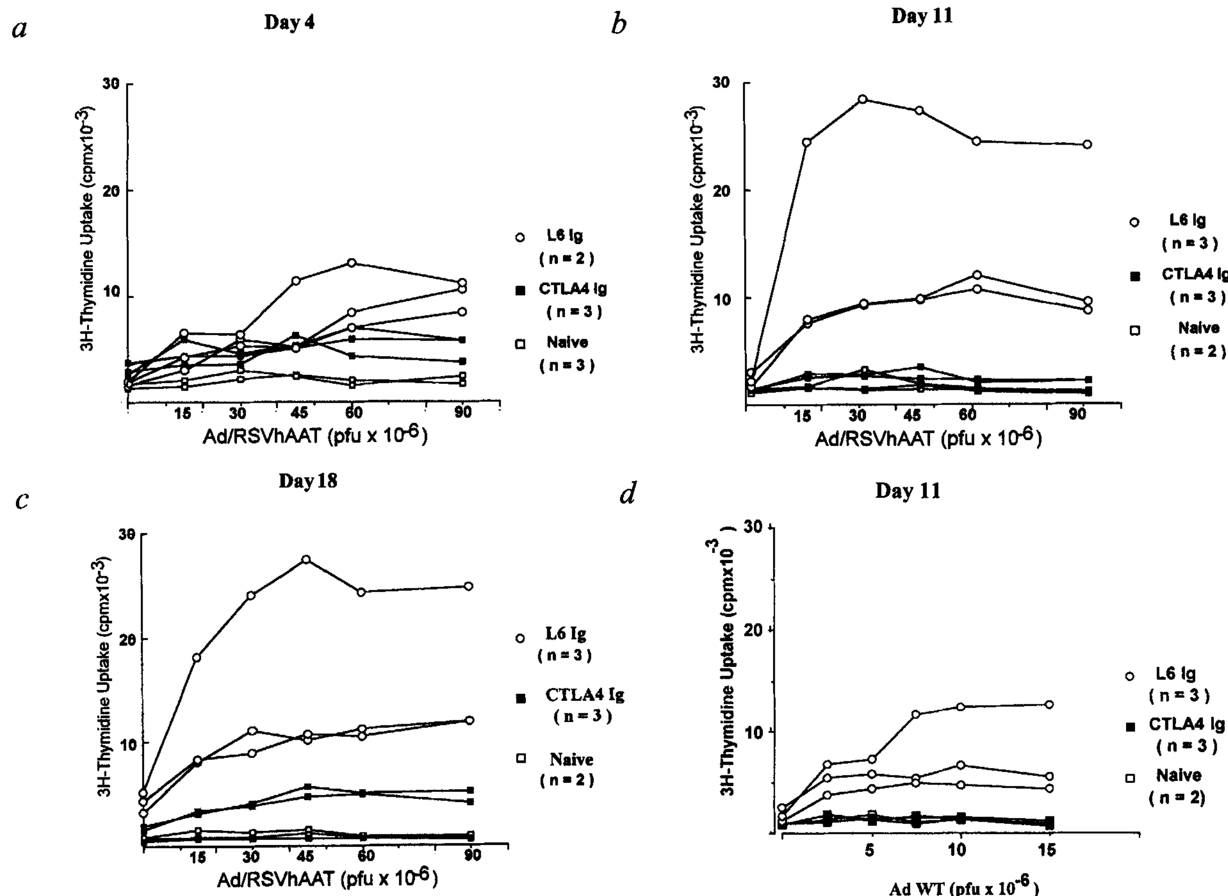

Fig. 3 Spleen proliferation assays. On days 4 (*a*), 11 (*b*), (*d*) or 18 (*c*) after the administration of Ad/RSVhAAT (*a-c*) or wild-type Ad 5 (WT) (*d*). 6×10^5 splenocytes were cultured and incubated with varying concentrations of UV inactivated Ad/RSVhAAT or wild-type Ad 5 as indicated. Supernatants were harvested at 72 h for lymphokine assays or cells were pulsed with ^3H-thymidine and harvested 24 h later for determination of proliferation.

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Table 1 Liver cell infiltrate identification

	Day 4							Day 11							Day 18						
	#	CD3	CD4	CD8	I	II	NK	#	CD3	CD4	CD8	I	II	NK	#	CD3	CD4	CD8	I	II	NK
L6	1	0	0	0	3	0	0	11	2	2.5	3	3	3	1.5	21	4	2.5	3	3	2	1
L6	2	0	0	0	3	0	0	12	4	1.5	4	2.5	3	2.5	22	4	1	3	2.5	2.5	0
L6	3	0	0	0	1.5	0	0	13	3	0	2	2.5	2	2	23	0	0	0	0	0	0
L6	4	1.5	0	0	3	0	0	14	0	0	0	1.5	1.5	0	24	2	1.5	2	1.5	2	0
L6	5	0	0	2	1.5	0	0	15	2.5	1.5	0	2.5	1.5	0	25	3	2	2.5	3	2	0
	mean	0.3	0	0.4	2.4	0	0	mean	2.3 (2.9)	1.1 (1.4)	1.8 (2.3)	2.4 (2.6)	2.2 (2.4)	1.2 (1.5)	mean	2.6 (3.3)	1.4 (1.8)	2.1 (2.6)	2.0 (2.5)	1.7 (2.1)	0.2 (0.3)
CTLA4	6	0	0	0	1	1	0	16	0	0	0	1	1	0	26	0	0	0	2.5	0	1
CTLA4	7	2	0	0	2.5	0	1	17	0	0	0	2	0	1	27	0	0	0	2.5	0	1
CTLA4	8	0	0	1	2.5	0	0	28	1	0	0	2.5	2	1	28	0	0	0	1.5	2	0
CTLA4	9	0	0	0	2	0	0	19	0	0	0	2	2	0	29	0	0	0	1.0	2.5	0
CTLA4	10	0	0	0	1.5	0	0	20	0	0	0	2	1	1	NA						
	mean	0.4	0	0.2	1.9	0	0.2	mean	0.2	0	0	1.9	1.2	0.6	mean	0	0	0	1.9	1.1	0.5

Immunohistochemical staining for CD3, CD4, CD8, MHC class I and II and NK cells was performed on liver sections obtained from the animals studied in Fig. 3. Eight normal animals were used as controls and scored as 0. Two of the authors masked as to the experimental groups scored the relative staining on an arbritary 0 to 4 scale relative to the normal animals paralleling a scoring system used by others to grade portal inflammation (7,32). Induction of MHC class I staining was observed in hepatocytes. The average of the two scores for each liver section is listed above. Two mice, #14 and #23 had less than 50 ng/ml of serum hAAT probably due to an inadequate infusion of Ad/RSVhAAT virus. The numbers in parentheses represent the mean without animals 14 and 23. NA=not available.

CTLA4Ig therapy interferes with T-cell responses

The long-term persistence of hAAT gene expression from muCTLA4Ig-treated animals suggested that inhibition of the costimulatory activation of T cells was responsible for inhibiting cellular immunity directed against viral antigens. To determine whether T-cell recognition of adenovirus antigens was inhibited with muCTLA4Ig therapy, spleen cell proliferation assays were performed in animals transduced with Ad/RSVhAAT and treated with muCTLA4Ig or L6; the results from one representative experiment from two performed are shown in Fig. 3. On day 4, 11 or 18 after adenovirus infusion, mice were sacrificed and splenocytes were isolated and stimulated with varying concentrations of Ad/RSVhAAT (Fig. 3). On day 4 there was minimal ^{3}H-thymidine incorporation in either group (Fig. 3*a*). On days 11 and 18 dose-dependent, antigen-induced splenocyte proliferation was detected in L6-treated controls, but was absent or markedly reduced in the CTLA4Ig treated animals (Fig. 3*b*,*c*). The responses in the muCTLA4Ig treated mice were similar to those observed in naive animals that did not receive adenovirus at day 11 and were markedly attenuated at day 18. A similar proliferation profile was detected when splenocytes from Ad/RSV-hAAT treated animals were exposed to varying doses of wild-type adenovirus (Fig. 3*d*); this suggests that the splenocyte response to Ad/RSV-hAAT resulted from T-cell priming after exposure to adenovirus antigens produced from the recombinant adenovirus. Furthermore, the impairment of the response in the muCTLA4Ig treated mice was specific, since proliferation in response to anti-CD3 stimulation was similar in muCTLA4Ig treated, L6 treated and naive control mice (data not shown). Yang *et al.*[7] found that the immune response to adenoviral vector transduced hepatocytes had the characteristics of a type 1 or TH1 lymphokine response, in that interferon-γ and IL-2 but not IL-4 were produced by antigen-stimulated splenocytes. Our data are consistent with this in that interferon-γ was induced in Ad/RSVhAAT stimulated cultures from 7 of 11 L6 treated mice (9.4-63 ng ml^{-1}), whereas interferon-γ was detected in cultures in only two of 12 muCTLA4Ig treated mice and in these two was present in low concentrations (7.1, 7.3 ng ml^{-1}). Interferon-γ was not detected in cultures from naive mice and IL-4 was not detected (<100 pg ml^{-1}) in cultures from any of the groups of mice.

Consistent with the results of the *in vitro* assays, the T-cell response to adenovirus transduced hepatocytes was markedly attenuated *in vivo*. In assays performed in parallel with the lymphocyte proliferation studies, liver sections were examined from normal control animals and from mice that received Ad/RSVhAAT 4, 11 and 18 days earlier were examined. To determine the nature of the cellular infiltrate, sections were reacted with a monoclonal antibody to CD3, which detects all T cells, with monoclonal

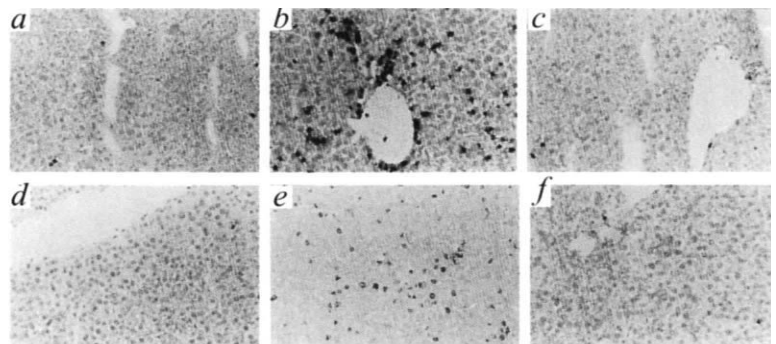

Fig. 4 Immunohistochemical identification of hepatic inflammatory cells. Animals were infused with 5×10^9 pfu of Ad/RSVhAAT on day 0. The animals were treated with either L6 (*b*, *e*) or muCTLA4Ig (*c*, *f*) on days 0, 2 and 10. Liver from normal animals were processed in parallel (*a*, *d*) Frozen liver sections from the animals sacrificied on day 18 were incubated with anti-CD3 (*a*-*c*) or anti-CD8 (*d*-*f*) antibodies. Original magnification is 50×.

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Table 2 Antibody titres

Type of antibody	Week 4		Week 6		Week 10	
	CTLA4Ig	L6	CTLA4Ig	L6	CTLA4Ig	L6
Neutralizing	<16 (<16)	32 (16–128)	<16 (<16–64)	64(16–128)	<16 (<16–64)	32 (<16–64)
IgG2a ELISA	nd	nd	<100 (<100–400)	6400 (1600–>6400)	100 (<100–6400)	400 (1600–>6400)

Neutralizing and isotype-specific antibodies directed against adenovirus were determined from mice studied in Fig. 1*a*,*c*. All the L6 controls that received adenovirus had neutralizing titres greater than or equal to 1/16, whereas only two of the muCTLA4Ig mice had neutralizing titres that were equal to or greater than 1/16. The serum from two muCTLA4Ig treated animals had no inhibition of Ad.RSVβgal transduction at a 1/16 dilution, whereas the serum from four of these animals had some inhibition of Ad.RSVβgal transduction (less than 75% inhibition of βgal staining) at a 1/16 serum dilution, indicating the presence of low titre adenovirus neutralizing antibody. The neutralizing antibody and IgG2a titres are listed as the median reciprocal (1/x) titre. The range for each group is given in parentheses. nd = not determined.

antibodies to the CD4 and CD8 T cell subsets, class I and class II MHC and with a polyclonal antiserum to NK cells. Representative sections reacted with antibodies to CD3 and CD8 are shown in Fig. 4 and all results are summarized in Table 1. There was little or no cellular infiltrate in either group after 4 days. However, after 11 and 18 days there was a robust (primarily periportal) infiltrate of T cells in the L6 animals but little or no infiltrate in mice that received muCTLA4Ig. The T cells were predominantly of the CD8 subset. Induction of class II MHC expression was also diminished in the muCTLA4Ig treated group relative to the L6 treated group, but the results were more variable than for the T-cell markers. There was little NK cell infiltration in either group. The animals that were transduced with adenovirus had increased hepatocellular staining for class I MHC regardless of whether they received muCTLA4Ig or L6.

Effects of CTLA4Ig on antibody response

While cellular immunity is believed to be the predominant mechanism limiting the duration of adenovirus-transduced gene expression, antibody-mediated humoral immunity is believed to be the predominant mechanism limiting secondary adenovirus transduction[6-9,21]. When antibody production was determined in the mice shown in Fig. 1*a*, muCTLA4Ig was found to impede markedly but not to ablate production of antibodies to the adenovirus vector. All L6 treated animals developed neutralizing antibodies to adenovirus by 4 weeks after the infusion, whereas the response was delayed and attenuated in the muCTLA4Ig treated mice (Table 2). Nonetheless, two of the CTLA4Ig treated mice developed neutralizing titres greater than or equal to 16 by 10 weeks after infusion, and only two of the mice failed to develop any detectable neutralizing antibody. Virtually all of the virus-specific antibody detected by isotype-specific ELISA at 6 and 10 weeks was of the IgG2a isotype. Each of 8 L6 treated mice developed high titres of IgG2a antibodies. The IgG2a response was markedly diminished in the muCTLA4Ig treated mice, although 6 of 8 ultimately developed detectable antibody titers of ≥100. One L6 treated mouse had measurable antibody of the IgG1 isotype (1:400) and one muCTLA4Ig treated mouse had measurable IgM antibody (1:400) detected on the 6- and 10-week samples. The IgG2a isotype predominance is consistent with a type I or TH1 lymphokine response by T cells, since such T cells produce interferon-γ, which favours the production of this isotype while inhibiting IgG1 production, whereas IL-4 produced by type II or TH2 T cells has the opposite effect[28,29]; these results are consistent with the lymphokine results presented above. The preponderance of IgG2a antibodies in both groups of mice, albeit in much reduced amounts in the muCTLA4Ig treated group, suggests that this agent markedly attenuates but does not alter the nature of the humoral immune response that develops.

To determine whether the low levels of antibodies in muCTLA4Ig treated mice were sufficient to impair gene expression with a secondary adenovirus vector, another experiment similar to those shown in Fig. 1 was carried out. Nine weeks after the first administration of Ad/RSVhAAT (when muCTLA4Ig was no longer detectable), the animals received an infusion of Ad/RSVcFIX that expresses canine factor IX[3], with or without a second treatment with muCTLA4Ig. Despite continued expression of the initial hAAT gene product, the factor IX gene product from the second vector was not detected (data not shown) suggesting that the low levels of neutralizing antibody present in the muCLTA4Ig treated mice was sufficient to block secondary gene transfer.

Discussion

Our results indicate that administration of muCTLA4Ig for a brief period near the time of systemic vector administration leads to persistent adenovirus-mediated gene expression. The transient immunosuppression by muCTLA4Ig markedly attentuated T-cell infiltration into the liver, the principal target organ, *in vivo* and antigen-induced T-cell responses assayed *in vitro*. It is likely that these effects on T-cell reactivity are causally related to the long-term persistence of adenovirus transduced hepatocytes, in contrast to immune-mediated clearance observed in controls.

Our results with muCTLA4Ig most closely parallel those achieved with a persistent genetic defect in T-cell or T-cell and B-cell function due to the *nude* or *scid* mutations[6,9,10,21]; mice with these mutations continue to express adenovirus-transduced genes for the duration of the periods of observation (5 to 10 months). Two published studies have attempted to modulate the immune response of mice to achieve more sustained adenovirus expression: Englehardt *et al.*[10] used continuous daily administration of the potent T-cell immunosuppressive agent, cyclosporin A, but produced minimal enhancement of gene expression from the liver. Dai *et al.*[21] also found that cyclosporin A had little effect on adenovirus mediated gene expression from muscle. However, these investigators found that a combined regimen of 8 weeks of cyclosporin A and the cytotoxic agent cyclophosphamide resulted in expression of the factor IX transgene for 5 months (the period of observation reported), although in all but one animal, expression declined by about 10-fold over this period. Our study, which used a much shorter and non-cytotoxic regimen, provides evidence that a more focused and transient period of immunomodulation can result in substantive

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therapeutic enhancement of adenovirus mediated gene expression *in vivo*. Strikingly, the transient, non-cytotoxic nature of the muCTLA4Ig treatment contrasts to the continuous immunosuppression provided by the genetic defects and with the more protracted and cytotoxic therapy used by Dai *et al.*[21] and suggests that persistent gene expression may be achieved without long term or cytotoxic immunosuppression and its resultant adverse effects.

Despite inhibition sufficient to lead to long-term expression of the transduced gene, secondary gene transduction was not possible. This likely reflects the efficient neutralization of the secondary vector by low levels of residual circulating neutralizing antibodies. Optimization of CTLA4Ig dosing and/or other immunologic based therapies, possibly in combination with less antigenic adenovirus vectors[10,30] may be necessary for the development of true antigenic tolerance that will allow for repeat systemic adenovirus delivery.

These studies must be pursued with cautious optimism as adjunct therapeutic approaches for adenovirus-mediated gene therapy are developed. The effects of such therapy during an exposure to wild-type adenovirus is not predictable. The production of humoral immunity as observed here would be sufficient to protect an individual from a wild-type infection. However, if humoral immunity is eventually blocked by such therapy, it is unclear whether production of the immunodominant E1 proteins[31] would allow an individual to eliminate selectively the wild-type virus. Overwhelming adenovirus infection or clearance of both the recombinant vector and wild-type virus are possible outcomes. These possible scenarios are not currently testable in the mouse.

The liver is a quiescent tissue under normal conditions and it is not possible to predict accurately how long the nonchromosomal, nonintegrated adenovirus genome will persist in hepatocytes or other tissues in the absence of immune destruction. Studies comparing the persistence of adenovirus mediated gene expression in immunodeficient animals with immunocompetent animals undergoing immunotherapies such as the one described here will be helpful in determining the limitations imposed by the immune system on the duration of gene expression as compared to non immune mechanisms of genome turnover. Nonetheless, our study is an important step towards using adenovirus vectors for long-term gene expression *in vivo* and may be useful for developing future clinical gene therapy trials because of the availability of human CTLA4Ig.

Methods

Animal studies. Animal studies were performed in accordance with the institutional guidelines set forth by the University of Washington. All animals were housed in SPF facilities. Mice were injected with recombinant adenovirus diluted to 100 μl in DMEM media (Hyclone) by tail vein infusion. Our previous studies have shown that 80% to 90% of hepatocytes are transduced with this dose of adenovirus[2]. Murine CTLA4Ig or L6 were diluted in pyrogen free physiologic saline and administered in 200 to 400 μl by IP injection. Blood samples were obtained by retroorbital technique. Animals were killed by cervical dislocation.

Virus preparation. The construction, production and purification of the recombinant E1 deficient Ad5 vector (Ad/RSVhAAT) was as described[18]. A single recombinant virus preparation was used for L6 controls and muCTLA4Ig experimental animals. All virus preparations were found to be negative for the presence of contaminating wild-type virus as described[9]. Bacteriophage φX174 was prepared and purified as described[26].

Immunohistochemistry. Immunohistochemical staining was carried out on liver tissues that were frozen in OCT and sectioned. The following antibodies were used: CD3-biotin (1452C11, Pharmingen, San Diego, CA), CD 4 (GK1.5, ATCC), CD8 (53-6.7 ATCC), NK (rabbit anti-asialoGM1, Dako), anti-class I-biotin (anti-H2K^k, 11-4.1 Pharmigen), anti-class II biotin (anti-I-E^k 14-4-45, provided by Dr. A. Rudensky, Univ. Washington). Biotinylated antibodies were detected with avidin horseradish peroxidase, the anti-CD4, anti-CD8 and anti-asialo GM1 antibodies with horseradish conjugated goat anti-rat or anti-rabbit as appropriate. Matched irrelevant rat or rabbit antibodies were used as controls for non-specific staining. The scoring system for portal inflammation was based on that described[32].

Spleen proliferation assays. Splenocytes were isolated and cultured as described[30]. 0.2 ml of cell suspension containing 3×10^6 cells per ml was added to each microtitre well. Varying concentrations of UV inactivated Ad/RSVhAAT or wild-type type 5 adenovirus were added in replicates of 3 to 4. After 72 h incubation the cells were pulsed with ^{3}H-thymidine for 24 h and uptake determined as described[33].

Antibody determinations. Antibodies to φX174 were assayed as described and the titre is expressed as Kv[26]. The assay to detect neutralizing antibody directed against adenovirus was modified from[9]. Briefly, 5×10^4 293 cells were plated in 96 well plates. Serum samples were heat inactivated at 55 °C for 40 min and 100 μl of four fold serum dilutions (the least dilution assayed was 1/16) in DMEM/1% fetal calf serum were mixed with 10 μl of Ad.RSVβgal (3×10^5 pfu) for 1 h at 37 °C. The mixture was layered onto the 293 cells for 60 min before replacing the virus mixture with fresh medium. The cells were stained with X-gal 16 to 24 h later and the titre was defined as the dilution that inhibited staining of 293 cells by 75%. IgM, IgG1 and IgG2a antibodies directed against the adenovirus were determined by ELISA. Wells of microtiter plates (Dynatech Immunolon) were coated with the optimal amount (50 ng/well) of UV-inactivated Ad/RSVhAAT in carbonate buffer (pH 9.6). After the plates were blocked, they were incubated sequentially with serum samples (diluted from 1:100 to 1:6400 for IgG1 and IgG2a, diluted 1:100 to 1:800 for IgM), then reacted with horseradish peroxidase-conjugated goat antibodies to mouse IgG1, IgG2a (Southern Biotechnology, Birmingham, AL) or IgM (Tago) and developed with ABTS reagent (Kirkegaard and Perry). The lowest dilution yielding an optical density (405 nm) >0.100 (IgG1 and IgG2a) or >0.050 (IGM) above that obtained with serum from naive control was defined as the titer.

hAAT and CTLA4Ig assays. hAAT was assayed by ELISA as previously described[18]. Murine CTLA4Ig serum concentrations were also assayed by ELISA. Human B7-1-Ig (50 ng in 50 ml bicarbonate buffer pH 9.6) was coated onto 96-well Immulon-2 plates. Diluted serum samples or diluted standard muCTLA4Ig were added to the washed and blocked wells. After binding and washing, 50 ml (1/5000 dilution) of horseradish peroxidase labelled goat anti-mouse IgG2a (Southern Biotechnology) was incubated at r.t. for 1 h. After washing, the substrate 3,3',5,5' tetramethylbenzidine (Sigma) was added and the reaction was stopped using 50 μl of 1 N sulphuric acid. Absorbance at 450 nm (reference 630nm) was performed using a microtitre plate reader (Biorad). The concentrations were determined using a linear regression of the standard curve. The limit of detectability was 0.1 ng ml^{-1}.

Murine interferon-γ was determined by ELISA using the monoclonal antibodies R46A2 and XMG1.2 as capture and detection reagents respectively. Murine IL-4 was determined by ELISA using monoclonal antibody pairs from Pharmingen. Recombinant murine IFN-γ and IL-4 were used as standards in each assay.

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