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Constitutive expression of murine CTLA4Ig from a recombinant adenovirus vector results in prolonged transgene expression

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The administration of soluble muCTLA4Ig around the time of adenovirus vector mediated gene transfer into murine hepatocytes has been shown to markedly prolong transgene expression, diminish the formation of adenovirus neutralizing antibody, decrease T cell proliferative response and infiltration into the liver without causing irreversible systemic immunosuppression. In this study, an E1/E3-deleted adenovirus vector constitutively expressing murine CTLA4Ig (Ad.RSV-muCTLA4Ig) was constructed in order to determine if production of muCTLA4Ig from within transduced cells (ie hepatocytes) would provide a more specific/localized interference with the CD28/B7–1 and B7–2 signaling pathways, and thus result in prolonged transgene expression in vivo at nonimmunosuppressive serum concentrations. In contrast to C3H mice receiving a control adenovirus, transduction with 6×10^9 p.f.u. of Ad.RSV-muCTLA4Ig and a reporter adenovirus (2×10^9 p.f.u. of Ad.PGK-hAAT) resulted in prolonged reporter gene expression, reduced anti-adenovirus and anti-hAAT antibody production, and attenuated T cell proliferation and IFN-γ production in response to adenoviral vector. Mice given a constant total amount of adenovirus with diminishing amounts of Ad.RSV-muCTLA4Ig and a constant amount of reporter virus (2×10^9 p.f.u. of Ad.PGK-hAAT) demonstrated prolonged reporter gene expression and decreased anti-adenovirus and anti-hAAT antibody production only when high serum levels of muCTLA4Ig were produced. Taken together, these findings suggest that a certain threshold of muCTLA4Ig must be achieved to alter the immune responses and prolong transgene expression from adenoviral vectors.

Keywords: *recombinant adenovirus; liver; CD28/B7–1 or B7–2; immune evasion; human alpha-1-antitrypsin inhibitor; canine factor IX*

Introduction

Major obstacles in the use of adenovirus vectors for *in vivo* gene therapy are the host's immune response towards the virus, cells infected with virus, and in some cases the newly expressed transgene.[1–5] While it remains unclear whether the development of a cytotoxic T lymphocyte (CTL) response towards viral antigens or the transgene products always correlates with a loss of transgene expression, it is generally accepted that these CTLs do affect the duration of transgene expression in some systems.[4,6] The development of a soluble neutralizing anti-adenoviral antibody response following administration of adenovirus has clearly been shown to inhibit a successful secondary transduction with adenovirus.[1,2]

A significant prolongation of transgene expression as well as a decrease in the magnitude of the neutralizing antibody titer has been found following coadministration of adenovirus vector with two immune regulatory agents: muCTLA4Ig,[7] MR1 (anti-CD40 ligand antibody),[5] or a combination of the two.[8] Murine CTLA4Ig interferes with an antigen presenting cell (APC) initiated signaling pathway involving CD80 (B7–1) and CD86 (B7–2) molecules on the APC and T cell CD28 molecules.[9] The normal B7–1 or B7–2/CD28 costimulating signal is critical in the optimal response of T cells to antigens.[9–13] Once activated, T cells express CTLA4 (CD52), a molecule which binds to B7–1 and B7–2 with a >500-fold avidity than CD28 and which transmits a signal inhibiting further T cell activation.[14,15] Murine CTLA4Ig (muCTLA4Ig) is the fusion product of the extracellular domain of murine CTLA4 fused to the murine IgG2a Fc domain.[16,17] Intraperitoneal treatment with 200 μg of muCTLA4Ig in one or three doses around the time of intravenous adenoviral vector administration, prolonged transgene expression, inhibited T cell infiltrates into the liver, inhibited T cell proliferation in response to adenovirus antigens *in vitro*, and decreased the amount of anti-adenovirus neutralizing antibodies produced.[7] Secondary gene transfer with another adenovirus vector was not successful in the animals treated with soluble muCTLA4Ig after the compound was cleared even though only low levels of neutralizing antibodies were produced.[7]

While the systemic administration of muCTLA4Ig was well tolerated by the mice in these prior studies, it was of interest to determine if production of muCTLA4Ig

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from the transduced cells (ie hepatocytes) would be advantageous when compared with systemic administration. To this end, a recombinant adenovirus constitutively expressing muCTLA4Ig was prepared and administered to C3H mice along with the reporter adenovirus Ad.PGK-hAAT, which expresses human alpha-1-antitrypsin protein.

Results

Preparation and characterization of the adenovirus Ad.RSV-muCTLA4Ig

The recombinant adenovirus, Ad.RSV-muCTLA4Ig, was prepared using standard cloning techniques as outlined in Materials and methods. The *Hin*dIII restriction enzyme digestion pattern of Ad.RSV-muCTLA4Ig DNA was as expected, and is summarized in the schematic diagram shown in Figure 1. The production of an immunoreactive protein following *in vitro* and *in vivo* transduction of Ad.RSV-muCTLA4Ig in ELISA assays also supports the appropriate construction of Ad.RSV-muCTLA4Ig (Figures 2 and 3).

Effects of Ad.RSV-muCTLA4Ig transduction with or without coadministration of soluble muCTLA4Ig on transgene expression and humoral immunity

Since it typically takes several days to obtain robust transgene expression following intravenous infusions of recombinant adenovirus vectors using the Rous sarcoma virus (RSV) LTR as a promoter,[1] and we have considerable knowledge about transgene expression following administration of soluble muCTLA4Ig,[7] a first experiment was designed to compare reporter gene expression in C3H mice following administration of Ad.RSV-muCTLA4Ig with and without coadministration of soluble muCTLA4Ig. Four different test groups of C3H mice were transduced with a mixture of 8×10^9 total p.f.u. of recombinant adenovirus vectors. The first two groups of mice were both infused with 6×10^9 p.f.u. of Ad.RSV-muCTLA4Ig and 2×10^9 p.f.u. of Ad.PGK-hAAT, but only the second group were given a single intraperitoneal (i.p.) injection of 200 μg of muCTLA4Ig at the time of virus administration. The last two groups were similar to the first two except the control virus Ad.RSV-cFIX was utilized in place of the 6×10^9 p.f.u. of Ad.RSV-muCTLA4Ig. Shown in Figure 2 are the serum levels of muCTLA4Ig for each mouse (a) and the average group human alpha-1 antitrypsin (hAAT) serum protein levels (b). Consistent with our other studies,[7] the serum level of muCTLA4Ig in all of the three mice receiving IP muCTLA4Ig and control adenovirus (Ad.RSV-cFIX) declined at least two orders of magnitude by day 56. Of those mice also receiving Ad.RSV-muCTLA4Ig, seven of eight had serum muCTLA4Ig levels >1 μg/ml at 112 days, with six of these having detectable muCTLA4Ig levels (range 131–469 ng/ml) for the duration of the experiment (210 days). Comparison of the serum hAAT levels (Figure 2b) demonstrates that while the four mice receiving only Ad.PGK-hAAT had a two order of magnitude loss of serum hAAT levels within 60 days, there was a similar prolongation of serum hAAT expression in those animals receiving Ad.RSV-muCTLA4Ig and/or soluble muCTLA4Ig (groups 1, 2 and 4). No clear benefit on the duration of adenovirus-mediated transgene expression was evident by administering both soluble muCTLA4Ig and Ad.RSV-muCTLA4Ig over the administration of soluble muCTLA4Ig or Ad.RSV-muCTLA4Ig independently. Preliminary results from a repeat experiment show a similar duration of serum hAAT levels for each group (data not shown).

Serum neutralizing anti-adenovirus antibodies and anti-hAAT antibodies were determined for each of the animals 4 and 13 weeks after virus administration (Table 1). Compared with the control animals (group 3), those receiving soluble muCTLA4Ig, Ad.RSV-muCTLA4Ig, or both (groups 1, 2 and 4) had low or undetectable levels of adenovirus neutralizing antibodies 4 weeks after infection. Seven of eight animals receiving Ad.RSV-muCTLA4Ig alone or with soluble muCTLA4Ig (groups 1 and 2) had similarly low or undetectable adenovirus neutralizing antibodies at week 13. In contrast, by week 13 all of the animals receiving soluble muCTLA4Ig alone developed high titers of neutralizing anti-adenovirus antibodies similar to the controls (groups 3 and 4). Since by 13 weeks most animals had developed detectable neutralizing antibody titers, secondary adenovirus administration was not attempted. In addition, all animals receiving soluble muCTLA4Ig, Ad.RSV-muCTLA4Ig, or both (groups 1, 2 and 4) had no detectable anti-hAAT antibodies compared with those animals receiving control adenovirus (group 3). Taken together, these antibody data suggest one difference following Ad.RSV-muCTLA4Ig administration compared with administration of only soluble muCTLA4Ig is a decrease in the formation of late (week 13) anti-adenovirus neutralizing antibodies.

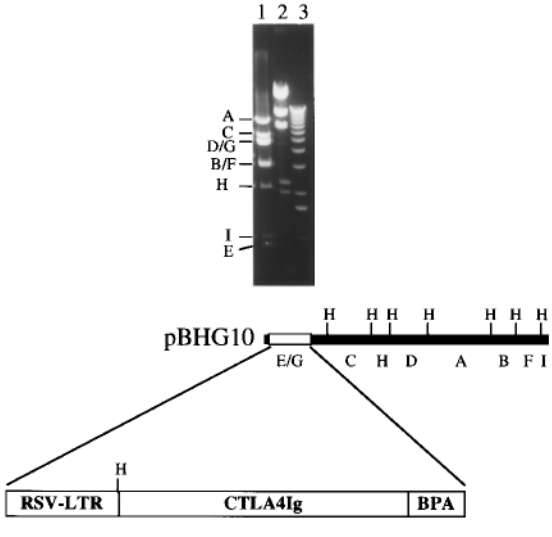


Figure 1 *Schematic diagram and restriction digestion map of Ad. RSV-muCTLA4Ig DNA. Ethidium bromide-stained agarose gel of HindIII digested DNA of Ad.RSV-muCTLA4Ig (lane 1) electrophoresed on a 0.8% agarose gel. Molecular weight markers include lambda HindIII DNA (lane 2) and the BRL 1 kb ladder (lane 3). Shown below the figure is a schematic diagram showing the expected fragment sizes of the recombinant virus labeled A–I.*

The effects of diminishing amounts of Ad.RSV-muCTLA4Ig on transgene expression and humoral immunity

A second experiment was designed to determine if a prolongation in transgene expression similar to that shown

Table 1 Anti-human alpha-1-antitrypsin (hAAT) and adenovirus neutralizing antibodies for each mouse from Figure 2

Group	*200μg muCTLA4Ig IP*	*Ad. RSV-muCTLA4Ig (p.f.u.)*[a]	*Anti-hAAT IgG (12 weeks)*	*Ad/Neutralizing (4 weeks)*	*Ad/Neutralizing (13 weeks)*
1	–	6×10^9	–, –, –	⩽16, ⩽16, 16	<16, ⩽16, 64
2	+	6×10^9	–, –, –, –, –	<16, <16, ⩽16, ⩽16, 16	<16, <16, ⩽16, 16, ⩽16
3	–	0	+, +++, n/a, ++	64, 256, 64, 64	64, 256, 64, 64
4	+	0	–, –, –	16, ⩽16, ⩽16	64, 64, 1024

Neutralizing antibody titers for each animal are expressed as the median reciprocal (1/x) dilution yielding 75% neutralization. Values listed as ⩽16 indicate that antibodies were detected but resulted in less than 75% neutralization at a 1:16 dilution, and <16 indicates no antibodies were detected. Serum anti-hAAT IgG is qualitatively reported for each animal in comparison to a standard curve of monoclonal anti-hAAT antibody as: –, not detected; +, detected similar to standard Mab diluted >1:1000; ++, detected similar to standard Mab diluted ⩾1:100; or +++, detected greater than lowest dilution of Mab standard. The order of the data are the same within each group for all three determinations. n/a indicates there was insufficient sample to perform the test.
[a]Additional viruses are as noted in the legend for Figure 2.

for high-dose Ad.RSV-muCTLA4Ig can occur with lower systemic levels of muCTLA4Ig produced by Ad. RSV-muCTLA4Ig-transduced hepatocytes. In this experiment, a mixture of recombinant adenoviruses was administered to five groups of C3H mice, each differing in the amount of Ad.RSV-muCTLA4Ig (4×10^9, 2×10^9, 4×10^8, 4×10^7 and 0 p.f.u.) administered. Along with Ad.RSV-muCTLA4Ig, a constant amount of a reporter adenovirus Ad.PGK-hAAT (2×10^9 p.f.u.) was included. A third adenovirus, Ad.RSV-cFIX (recombinant adenovirus which produces canine factor IX under the control of the RSV-LTR promoter), was included to keep the total amount of transduced adenovirus at 6×10^9 p.f.u., an amount of adenovirus previously shown to have minimal toxicity and the incorporation, on average, of 15–50 adenovirus genomes per murine hepatocyte.[7,18,19] Shown in Figure 3a are the serum levels of muCTLA4Ig. Following administration of 4×10^9 p.f.u. of Ad.RSV-muCTLA4Ig, the maximum serum levels of muCTLA4Ig ranged from 2000 to 5000 ng/ml, while following administration of 4×10^7 p.f.u. the maximum levels were approximately 10 ng/ml (Figure 3a).

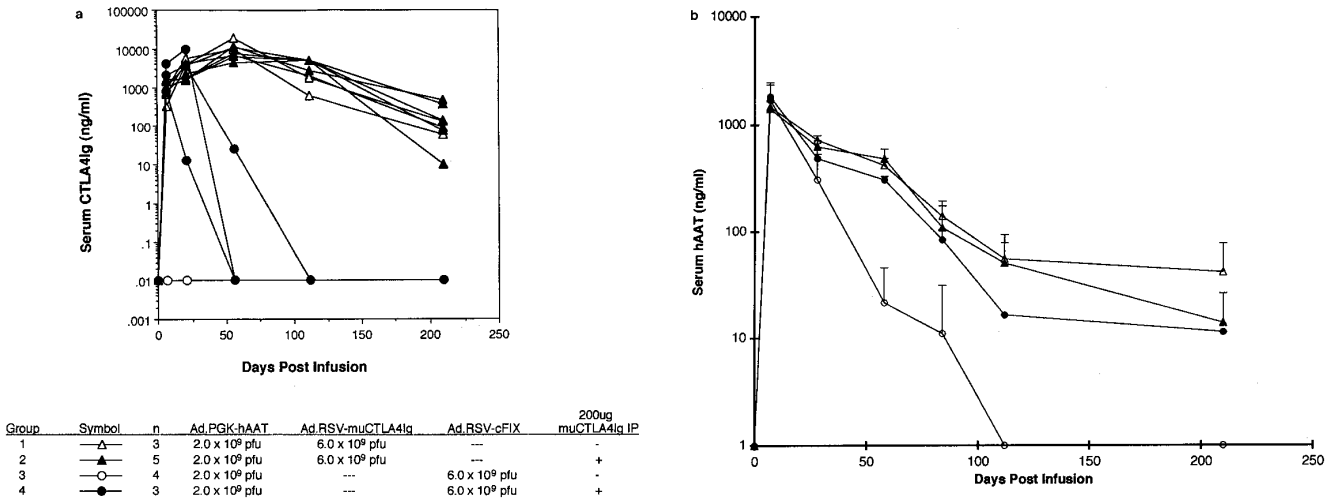
The average serum hAAT levels following transduction for those mice from groups 4 and 5 that have the highest serum levels of muCTLA4Ig (day 21 average level = 1816 ng/ml) , and those mice from groups 2 and 3 receiving the lowest serum levels of muCTLA4Ig (day 21 average level = 77 ng/ml) are shown in Figure 3b. The serum levels of hAAT in the mice from groups 4 and 5 remained elevated longer than for the mice in groups 2 and 3. The two negative control mice that did not receive Ad.RSV-muCTLA4Ig were plotted individually, and showed hAAT expression profiles similar to those seen for the pooled mice from groups 2 and 3, as well as to previously reported data in C3H mice from this laboratory.[1,7] These data suggest that only those mice that obtained serum muCTLA4Ig levels at or near 1 μg/ml had prolonged expression of the hAAT reporter gene.

Antibody titers for neutralizing adenovirus and anti-hAAT antibodies were evaluated in serum samples from each of the animals in this second experiment. As shown in Table 2, neutralizing antibody titers diminished as the amount of transduced Ad.RSV-muCTLA4Ig was increased. Additionally, the number of mice producing detectable anti-hAAT antibody decreased as the amount of transduced Ad.RSV-muCTLA4Ig was increased. Taken together with the expression data (Figure 3), it appears that prolongation of transgene expression and a decrease in neutralizing and anti-hAAT antibody formation occurs only at the highest level of Ad.RSV-muCTLA4Ig transduction (4×10^9 p.f.u.) which results in the highest serum muCTLA4Ig levels.

To determine if cFIX produced from the control adenovirus Ad.RSV-cFIX altered the rate of clearance of serum hAAT, the serum cFIX levels were determined for three time-points in the mice from groups 1 to 3 in the titration experiment and are shown in Table 3. As evident in Table 3, all mice had undetectable serum cFIX levels by day 140, and four of eight mice had undetectable cFIX levels by day 84. This rate of clearance of canine factor IX is quite similar to the rate of clearance of hAAT in the same mice (Figure 3b). Additionally, the clearance of hAAT seen in Figure 3b for the control mice is similar to that in the mice receiving cFIX (groups 2 and 3), and is similar to that previously reported.[1,7] Together, these findings suggest that the control vector used in these experiments, Ad.RSV-cFIX, does not significantly interfere with the clearance of serum hAAT.

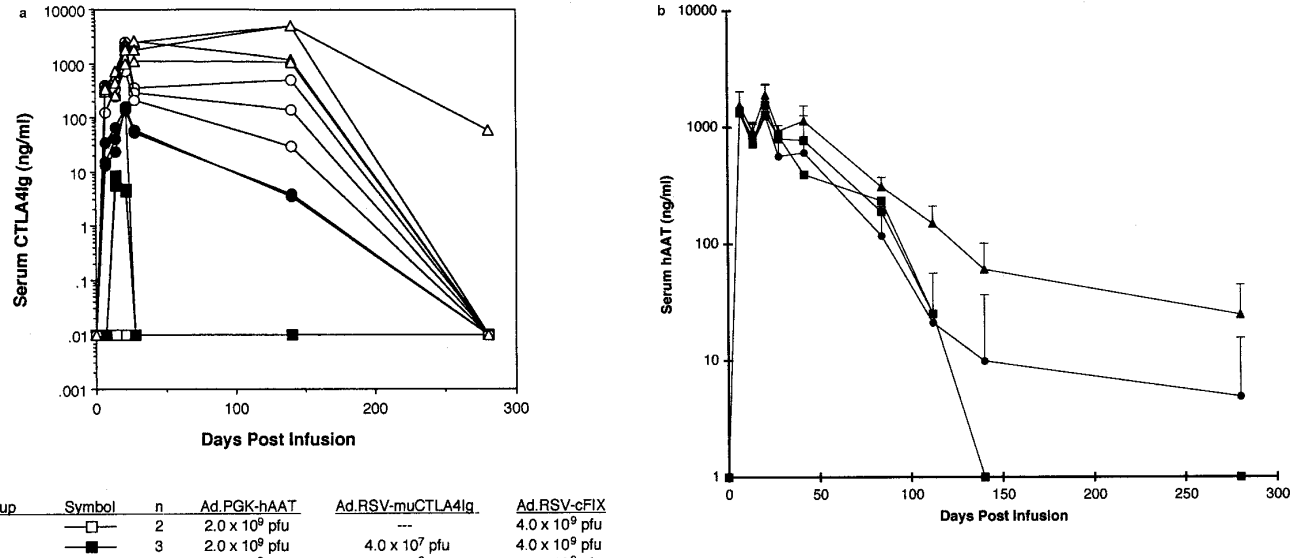
Effect of Ad.RSV-muCTLA4Ig on T cell proliferative responses and IFN-γ secretion

Previous studies with soluble muCTLA4Ig have demonstrated an attenuation of both the T cell proliferative response and IFN-γ secretion following stimulation with adenovirus *in vitro*.[7] The latter secretion of IFN-γ from splenocytes of animals following treatment with recombinant adenovirus vectors signifies a type 1 (TH1) lymphokine response which includes the secretion of Il-2 that in concert with IFN-γ, stimulates the production of IgG2a class anti-adenovirus neutralizing antibodies.[7,20] To determine if these responses were also suppressed following administration of Ad.RSV-muCTLA4Ig, splenocytes were prepared from mice treated with 2×10^9 p.f.u. of Ad.PGK-hAAT and 6×10^9 p.f.u. of either Ad.RSV-muCTLA4Ig or a control adenovirus, Ad.RSV-cFIX, as well as a naive mouse, and then analyzed *in vitro* for T cell proliferative responses and IFN-γ secretion. Shown in Figure 4, the T cell proliferative responses 15 days after the initial transduction of adenovirus were robust in response to both Ad/RSV-hAAT and Ad.RSV-βgal in the mouse treated with the relevant control virus, Ad.RSV-cFIX, as we have previously reported.[7] In contrast, both



Group	Symbol	n	Ad.PGK-hAAT	Ad.RSV-muCTLA4Ig	Ad.RSV-cFIX	200ug muCTLA4Ig IP
1	—△—	3	2.0 x 10^9 pfu	6.0 x 10^9 pfu	---	-
2	—▲—	5	2.0 x 10^9 pfu	6.0 x 10^9 pfu	---	+
3	—○—	4	2.0 x 10^9 pfu	---	6.0 x 10^9 pfu	-
4	—●—	3	2.0 x 10^9 pfu	---	6.0 x 10^9 pfu	+

Figure 2 *Serum muCTLA4Ig and hAAT levels following administration of both Ad.RSV-muCTLA4Ig and soluble muCTLA4Ig. Shown are the serum muCTLA4Ig levels for each mouse (a), and the group average serum hAAT levels (b) following transduction with the recombinant adenoviruses as denoted in the key below (a). The error bars in (b) are standard deviations.*



Group	Symbol	n	Ad.PGK-hAAT	Ad.RSV-muCTLA4Ig	Ad.RSV-cFIX
1	—□—	2	2.0 x 10^9 pfu	---	4.0 x 10^9 pfu
2	—■—	3	2.0 x 10^9 pfu	4.0 x 10^7 pfu	4.0 x 10^9 pfu
3	—●—	3	2.0 x 10^9 pfu	4.0 x 10^8 pfu	3.6 x 10^9 pfu
4	—○—	3	2.0 x 10^9 pfu	2.0 x 10^9 pfu	2.0 x 10^9 pfu
5	—△—	4	2.0 x 10^9 pfu	4.0 x 10^9 pfu	---

Figure 3 *Serum muCTLA4Ig and hAAT levels following transduction with increasing amounts of Ad.RSV-muCTLA4Ig. Shown are the serum muCTLA4Ig levels for each mouse (a) following transduction with the recombinant adenoviruses as denoted in the key. The serum hAAT levels for both control mice (■), and the average serum hAAT for all mice in groups 2 and 3 (●) as well as groups 4 and 5 (▲) are shown in (b). The average day 21 muCTLA4Ig concentration for the combined animals from groups 2 and 3 was 77 ng/ml, and for groups 4 and 5 was 1816 ng/ml. The error bars in (b) are standard deviations.*

animals receiving Ad.RSV-muCTLA4Ig showed diminished, yet detectable proliferative responses. Secretion of IFN-γ from these same animals following stimulation with Ad/RSV-hAAT and Ad.RSV-βgal was determined (Figure 5), and shows an attenuated level of IFN-γ secretion following administration of Ad.RSV-muCTLA4Ig compared with control adenovirus. Together, these two studies demonstrate that muCTLA4Ig produced from Ad.RSV-muCTLA4Ig has a detectable but attenuated TH1 response, an effect similar to that seen following administration of soluble muCTLA4Ig alone.[7]

Discussion

It is our hypothesis that constitutive expression of muCTLA4Ig in transduced cells may result in prolonged transgene expression at serum muCTLA4Ig levels which do not cause systemic immunosuppression. We demonstrate that a range of serum muCTLA4Ig levels can be achieved by administering varying amounts of a recombinant adenovirus constitutively expressing muCTLA4Ig, but that only those amounts of Ad.RSV-muCTLA4Ig which result in high serum levels of muCTLA4Ig (>1 μg/ml) result in the prolongation of transgene

Table 2 Antibody titers

Group	*Ad. RSV-muCTLA4Ig (p.f.u.)*[a]	*Anti-hAAT IgG (12 weeks)*	*Ad/Neutralizing (4 weeks)*	*Ad/Neutralizing (12 weeks)*
I	0	+, ++	256, 256	64, 256
II	4×10^7	–, +, +++	1024, 256, 256	256, 256, 64
III	4×10^8	–, +++, +++	256, 256, 64	256, 256, 64
IV	2×10^9	++, –, –	16, 16, 16	16, 16, 64
V	4×10^9	–, –, –, –	<16, 16, ⩽16, 16	<16, 16, ⩽16, 16

Anti-human alpha-1-antitrypsin (hAAT) and adenovirus neutralizing antibodies for each of the mice in Figure 3.
Scoring is as noted in the text of Table 1. The order of the data are the same within each group for all three determinations.
[a]Additional viruses are as noted in the legend for Figure 3.

Table 3 Serum cFIX levels

Group	*Ad. RSV-cFIX (p.f.u.)*[a]	*Average serum cFIX (ng/ml)*		
		Day 7	*Day 84*	*Day 140*
I	4×10^9	368 (334, 402)	60 (135, 25)	<1 (<1, <1)
II	4×10^9	555 (412, 703)	<1 (<1, <1)	<1 (<1, <1)
II	3.6×10^9	621 (540, 716)	107 (25, 162)	<1 (<1, <1)

Serum canine factor IX (cFIX) levels were determined for all the animals in this experiment receiving near 4×10^9 p.f.u. of Ad.RSV-cFIX. Shown is the average value and range in parenthesis. Serum levels below the sensitivity of the assay were listed as <1.
[a]Additional viruses administered are as noted in the key for Figure 3.

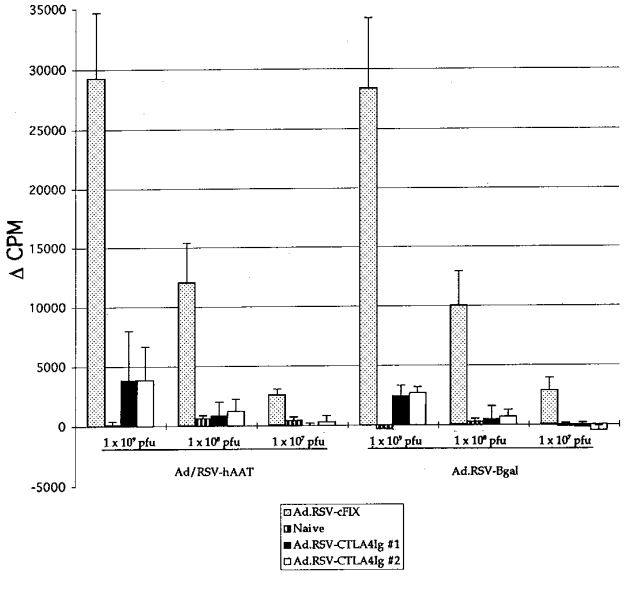


Figure 4 *Splenocyte proliferation following treatment with Ad.RSV-muCTLA4Ig. Splenocytes were obtained from mice 15 days after the administration of 2×10^9 p.f.u. Ad.PGK-hAAT in combination with 6×10^9 p.f.u. of Ad.RSV-muCTLA4Ig or Ad.RSV-cFIX, or mice not previously exposed to adenovirus (naive). These cells were cultured in replicates of four with medium alone, anti-murine CD3, or with the indicated amounts of UV-inactivated Ad/RSV-hAAT or Ad.RSV-βgal for 72 h, then ^{3}H-thymidine was added. After 24 h, cells were harvested and ^{3}H-thymidine uptake determined. The error bars represent two standard deviations. Results for unstimulated and anti-CD3-stimulated cells were similar and are not shown.*

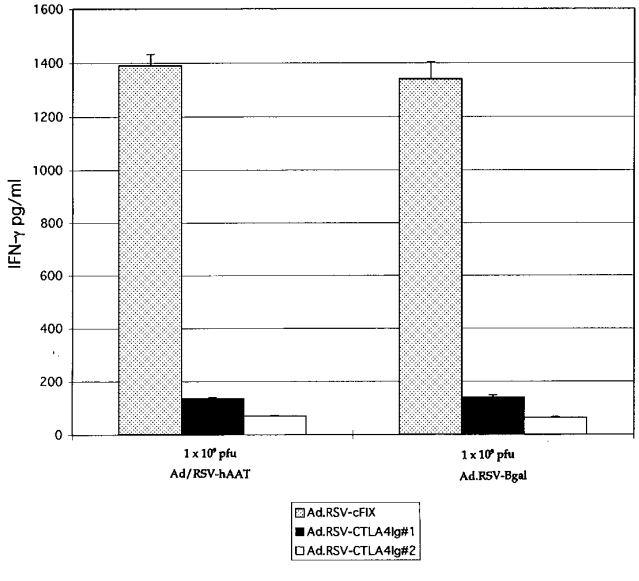


Figure 5 *IFN-γ production in splenocytes of mice following administration of Ad.RSV-muCTLA4Ig. Splenocytes were obtained from mice 15 days after the administration of 2×10^9 p.f.u. Ad.PGK-hAAT in combination with 6×10^9 p.f.u. of Ad.RSV-muCTLA4Ig or Ad.RSV-cFIX, or mice not previously exposed to adenovirus (naive). These cells were cultured in replicates of four with medium alone, anti-murine CD3, or with the indicated amounts of UV-inactivated Ad/RSV-hAAT or Ad.RSV-βgal for 72 h. At this time, the cell supernatants were removed, pooled for each replicate, and the amount of IFN-β was determined in triplicate by ELISA. The error bars represent the standard error of the mean. No INF-γ secretion was detected in the naive mice. Results for unstimulated and anti-CD3-stimulated cells were similar and are not shown.*

expression. The addition of a single dose of soluble muCTLA4Ig (200 μg) at the time of virus administration did not significantly alter the duration of transgene expression over the administration of Ad.RSV-muCTLA4Ig alone. One outcome unique to those mice receiving Ad.RSV-muCTLA4Ig is that neutralizing antibody titers 13 weeks after virus administration were decreased further than those titers seen following administration of soluble muCTLA4Ig alone. This is not unexpected since the serum muCTLA4Ig levels in these same mice persisted, whereas levels declined rapidly in those mice receiving soluble muCTLAIg alone.

Serum CTLA4Ig levels as low as 1 μg/ml were previously associated with continued inhibition of T cell-dependent immune responses to the neoantigen TNP-KLH.[21] This appears to be in good accord with our *in vivo* data showing the titer of neutralizing antibody and formation of anti-hAAT antibodies are diminished only in those animals receiving the highest amounts of Ad.RSV-muCTLA4Ig and corresponding highest serum levels of muCTLA4Ig (in the range of 1 μg/ml). This suggests that local production of muCTLA4Ig in the target organ is not sufficient to block an effective immune response. Whether the local production of muCTLA4Ig from hepatocytes transduced with Ad.RSV-muCTLA4Ig results in higher local levels of muCTLA4Ig in the mouse liver when compared with the administration of 200 μg of soluble muCTLA4Ig remains unclear. The clarification of this issue is further complicated by the lack of knowledge as to the specific tissue of adenoviral antigen presentation following i.v. administration which may include such extrahepatic tissues as the spleen or lymph nodes.

The precise mechanism by which muCTLA4Ig prolongs transgene expression from recombinant adenovirus vectors remains unknown. A previous report looking for the induction of 'tolerance' following administration of CTLA4Ig shows that multiple 200 μg i.p. doses of soluble muCTLA4Ig (with resultant serum CTLA4Ig concentrations near 10 μg/ml) led to 'tolerance' towards the specific primary neoantigen following a second administration.[21] Following a third presentation of the same antigen, this 'tolerance' was lost. We have similarly reported that following administration of soluble CTLA4Ig at or near the time of adenovirus administration, we did not observe tolerance to adenovirus antigens.[7,8] Thus, it is our interpretation that the mechanism leading to prolonged transgene expression following coadministration of Ad.RSV-muCTLA4Ig and a reporter adenovirus is related to suppression of T cell activation, or possibly tolerance to some but not all viral antigens. Nevertheless, there is no evidence for the development of tolerance to the vector. The production of some late, albeit low-level, anti-adenovirus antibodies and splenocyte proliferation in response to the vector following administration of Ad.RSV-muCTLA4Ig supports this hypothesis. Additionally, the role that constant adenovirus antigen synthesis has on the duration of transgene expression remains to be determined, but could account for the eventual loss of reporter gene expression.

The formation of anti-hAAT antibodies of IgG class as well as the formation of neutralizing anti-adenovirus antibodies suggests that the immune system of these mice following treatment with Ad.RSV-muCTLA4Ig is not completely inhibited. We have also determined that these animals are able to mount T cell-dependent humoral immune responses to a neoantigen once serum muCTLA4Ig levels have returned to undetectable levels (data not shown). Further characterization of these immune responses following Ad.RSV-muCTLA4Ig administration is in progress.

It remains unclear what role the transgene product itself plays in the ultimate elimination of transgene expression.[3,22] The experiments in this study (Tables 1 and 2) suggest that most animals obtaining sufficient muCTLA4Ig to prolong transgene expression, whether by administration of soluble muCTLA4Ig or by administration of a recombinant adenovirus expressing muCTLA4Ig, also have diminished levels of anti-hAAT antibody formed. This finding, although not conclusive, suggests a relationship between the formation of anti-hAAT antibody and the elimination of transgene expression. Additional studies beyond the scope of this work are necessary to determine whether anti-hAAT antibodies directly mediate loss of serum hAAT or are merely a marker for this.

A recent study looking at the effects of coadministration of a monoclonal antibody (MR1) to CD40 ligand, another cell-surface molecule involved in APC as well as T cell activation, has been shown to prolong adenovirus-mediated transgene expression and decrease anti-adenovirus neutralizing antibody formation so as to allow a low amount of secondary virus transduction.[5] In this study, the low level of secondary gene transfer likely occurs because secondary transduction was performed before the MR1 antibody has completely cleared. In contrast, we have found administration of MR1 alone is less efficient at prolonging adenovirus-mediated gene expression and inhibiting humoral immunity compared with muCTLA4Ig.[8] However, administration of both muCTLA4Ig and MR1 together at the time of recombinant adenovirus administration shows a similar prolongation of transgene expression and blockage of immune responses, but also allows for successful administration and persistent gene expression from a second recombinant adenovirus in 50% of the treated animals.[8] Perhaps the constitutive co-expression of muCTLA4Ig and MR1 from a recombinant adenovirus vector would allow prolonged gene expression in the absence of high, immunosuppressive, systemic levels of both muCTLA4Ig and MR1.

While it appears Ad.RSV-muCTLA4Ig does not provide a significant advantage in the duration of transgene expression *in vivo* when compared with administration of soluble muCTLA4Ig, this virus construct could prove useful in the treatment of autoimmune medical conditions which require continuous immunosuppression.[23] Currently, phase I clinical trials are in progress on the efficacy of soluble CTLA4Ig in the treatment of these types of conditions (PS Linsley, unpublished data).

Materials and methods

Animal studies

Animal studies were performed in accordance with the institutional guidelines set forth by the University of Washington. Female C3H/Hej mice were obtained from Jackson Laboratories (Bar Harbor, ME, USA) at 5–6 weeks of age and housed in SPF facilities. Mice were injected with recombinant adenovirus diluted to 200 μl in DME

media from GIBCO BRL (Gaithersburg, MD, USA) by tail vein injection as previously described.[24] Blood samples for serum protein analysis were obtained by the retro-orbital technique. Mice found to have serum hAAT levels less than 500 ng/ml in serum samples taken 3–7 days after injection, and thought to have received poor injections, were removed from the study. The animals were killed by cervical dislocation.

Recombinant adenoviruses

Construction of the recombinant E1-deficient Ad5 vectors Ad.PGK-hAAT,[25] Ad.RSV-cFIX[26] and Ad.RSV-βgal[1] have been previously described. Ad.PGK-hAAT expresses human α-1 antitrypsin serum protein under the control of the PGK promoter. Ad.RSV-cFIX is a recombinant adenovirus which expresses canine factor IX under the control of the RSV-LTR promoter. The muCTLA4Ig cDNA clone containing a murine Ig tail was graciously provided by Dr PS Linsley from Bristol Meyer Squibb (Seattle, WA, USA). The recombinant adenovirus constitutively expressing muCTLA4Ig (Ad.RSV-muCTLA4Ig) was prepared by cloning the muCTLA4Ig cDNA as a *Hin*dIII/*Xba*I fragment into pSP72 vector from Promega (Madison, WI, USA) containing the RSV-LTR promoter and bovine polyadenylation signal (BPA). The promoter, muCTLA4Ig cDNA, and bovine polyadenylation signal were then excised intact as a *Xho*I fragment, and cloned into the left-end adenoviral vector pXCJL.1.[24] Orientation was ascertained by restriction digestion. Recombinant adenovirus was rescued following cotransfection of this construct with pBHG10[27] in 293 cells.[28,29] Recovered plaques were expanded and characterized by restriction digestion.[28] Desired recombinants were grown in large scale and then purified and concentrated on two cesium chloride gradients.[28] Each virus preparation was assayed for wild-type contamination[1] and quantified both spectrophotometrically (OD 260) and by plaque assay.[28]

Analysis of murine serum

Serum hAAT,[7] muCTLA4Ig[7] and cFIX[26] levels were determined by ELISA on duplicate samples using multiple dilutions to ensure that readings were made on the linear portion of the standard curve. Those values below the sensitivity of the test were recorded as 1 μg/ml for hAAT, 0.01 μg/ml for serum muCTLA4Ig and <1 for cFIX. Serum neutralizing antibody analysis was performed as previously described.[2,7]

Anti-hAAT antibody assay

The anti-hAAT antibody levels were determined by ELISA assay modified from that previously described for the detection of hAAT.[7] Briefly, ELISA plates coated with immune-specific anti-hAAT antibody were blocked in TBST (10 mM Tris, 100 mM NaCl and 0.05% Tween 20, pH 7.5) + 5% milk and 1% BSA, and then 'loaded' with hAAT protein by incubation for 2 h at room temperature (RT) with human calibrator serum No. 4 (Atlantic Antibodies, Stillwater, MN, USA) diluted 1:50 in TBST. For each two-sample well, two wells were mock-loaded with blocking buffer to determine if individual high-serum hAAT levels would sufficiently interfere with the assay. After washing three times with TBST, the samples diluted 1:1000 in TBST were loaded on to the plate along with a similarly diluted naive serum as a negative control. Murine IgG2b anti-hAAT monoclonal antibody No. 178260 (Calbiochem, La Jolla, CA, USA) serially diluted in blocking buffer from 10^{-2} to 10^{-6} was also included on each plate as a positive control. Following another 2 h incubation at RT, the plates were washed four times with TBST and incubated with HRP-labeled sheep anti-murine IgG whole molecule antibody A-6782 (Sigma, St Louis, MO, USA) for another 2 h. Following a final five washes, detection was as previously described.[7]

Administration of soluble muCTLA4Ig

Soluble muCTLA4Ig was graciously provided by the Bioprocess Research Group of Bristol Meyer Squibb (Seattle, WA, USA). It was administered as a single 200 μg i.p. bolus just before the tail-vein administration of the adenovirus preparation.

Biological assays

Splenocytes were prepared as previously described,[7] and determined to be >90% viable by Trypan blue staining before the start of the experiments. The details of the proliferation and IFN-γ secretion assays were as previously described.[7]

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