Supplementary Information: Expanded materials and methods

Construction of CMV promoter-driven expression cassettes containing the rabbit IL-10 cDNA and gene

A plasmid containing a rabbit IL-10 cDNA was a kind gift from Dr. Harvey Perkins (Australian National University, Canberra, AU).[1] The IL-10 cDNA was removed and ligated into *Sma*I-digested pCI (Promega, Madison, WI) to yield pCIrbtIL-10cDNA. We cloned the rabbit IL-10 gene by PCR amplification of genomic DNA extracted from the skin of a New Zealand White rabbit. Briefly, two overlapping IL-10 gene fragments (1.4 and 2.9 kb, sharing a common *Bmg*BI site) were amplified by PCR, digested at each end, and ligated together between the *Xho*I and *Xba*I sites of pCI to yield pCIrbtIL-10gene. The cloned IL-10 gene sequence included 5 exons, 4 introns, a 13 bp fragment of the 5´ UTR and a 446 bp fragment of the 3´ UTR, not including the native IL-10 polyadenylation site (GenBank accession DQ437508).

One of our experimental goals was to test the hypothesis that inclusion of the endogenous IL-10 introns in expression cassettes would increase IL-10 expression. Testing of this hypothesis required that we: 1) eliminate all variability—except for the presence of IL-10 introns—between the IL-10 cDNA and genomic expression cassettes; and 2) delete the synthetic intron in the backbone pCI vector. We began by adding the cloned IL-10 genomic 5´ and 3´ UTR sequences to the respective ends of the IL-10 cDNA. To accomplish this, we generated one amplicon from pCIrbtIL-10gene that contained the genomic 5´ sequence and most of exon I, and a second amplicon that contained the genomic 3´ UTR sequence and most of exon 5, respectively. We then used overlap extension and PCR to add these genomic fragments to the ends of a 428 bp *Bsm*I–*Msl*I fragment of pCIrbtIL-10cDNA containing exons 2–4 and parts of exons 1 and 5. Unique 5´ *Psp*XI and 3´ *Xba*I sites were introduced by incorporation into the primers. The final product, a 1001 bp *Psp*XI–*Xba*I fragment, was ligated into *Psp*XI–*Xba*I-cut

pCIrbtIL-10gene, replacing the IL-10 genomic clone with an IL-10 cDNA flanked by endogenous IL-10 genomic 5´ and 3´ UTR sequences. This plasmid was termed "pCIrbtcIL-10-EM+intron" because the synthetic pCI-derived intron was still present (EM = "ends modified").

Comparison of the IL-10 cDNA sequence in pCIrbtcIL-10-EM+intron with the genomic IL-10 sequence we originally obtained (GenBank accession DQ437508) revealed several inconsistencies. Specifically there was a G in exon 5 at a site at which we had found an A previously and there were 4 nucleotides in the 3´ UTR that differed from our original sequence (one T instead of A and 3 bases not present in the original sequence). We were most concerned about the A to G transition in exon 5 because it was non-conservative. By reference to the published rabbit IL-10 cDNA sequence[1] and those of other species, we decided that A was most likely the correct base. We therefore used oligonucleotide-based mutagenesis to change the G to an A in both pCIrbtcIL-10-EM+intron and pCIrbtIL-10gene. At the same time, we also changed the T to an A in the 3´ UTR which made the new sequence consistent with the sequence we originally discovered and published. Because of the lack of a reference rabbit genomic IL-10 sequence we did not change the other 3 variant bases in the 3´ UTR.

We next modified pCI both to eliminate the pCI synthetic intron and to enable excision of expression cassettes that we planned to construct in pCI. We began by introducing a unique *Asc*I site just 5´ of the CMV promoter by digestion with *Bgl*II, blunt-ending with Klenow, ligation of *Asc*I linkers, *Asc*I digestion, and religation to yield pCI-*Asc*I. We next removed the synthetic intron by digestion of pCI-*Asc*I with *Afl*II and *Not*I. After digestion, the linearized, pCI-derived plasmid was blunt-ended and dephosphorylated in preparation for insertion of expression cassettes between the CMV promoter and the SV-40 polyA signal. The IL-10 genomic clone and the modified IL-10 cDNA were both removed from pCIrbtIL-10gene and pCIrbtcIL-10-EM+intron by digestion with *Psp*XI and *Xba*I. The resulting fragments containing the IL-10 cDNA and

genomic sequences were blunt-ended and ligated to the intron-less pCI-*Asc*I plasmid described above, to yield pCMVrbt-cIL-10 and pCMVrbt-gIL-10 (Figure 1).

We also constructed a "Null" expression cassette, without a transgene, for use as a negative control. To construct this cassette, pCI was digested with *Bgl*II and *Cla*I to release a 1341-bp fragment including the CMV promoter, synthetic intron, and SV40 poly A signal. We used this empty or "Null" expression cassette (Figure 1) to generate the HDAdNull vector described below.

Construction of murine endothelin-1 promoter-driven expression cassettes

To enable construction of vectors in which IL-10 expression is driven by the murine endothelin-1 (mET-1) promoter, we obtained plasmid pqET-1 from Dr. T. Quertermous (Stanford University, Palo Alto, CA). pqET-1 contains 1.36 kb of the mET-1 promoter, the first (untranslated) exon, and part of the first intron of the mouse ET-1 gene.[2] pqET-1 was digested with *Apa*I and *Eco*RI to release a 1078 bp fragment containing the 3´ portion of ET-1 exon 1 and the 5´ end of ET-1 intron 1. pqET-1 was also digested with *Spe*I and *Kpn*I to release a 1504 bp fragment containing 1.36 kb of ET-1 promoter and the 5´ portion of exon 1. These two fragments of the ET-1 gene were blunt-ended with Klenow and T4 polymerase, respectively, and ligated into a pCI-derived plasmid from which the CMV promoter and artificial intron had been removed. These ligations placed the two ET-1 genomic sequences downstream and upstream (respectively) of a genomic clone of rabbit apolipoprotein A-1 (cloning of the rabbit apoA-1 gene will be reported separately). This plasmid, pmET1-gApoA1, was digested with *Sal*I and *Not*I, removing the apoA-1 genomic sequence but preserving the SV-40 poly A signal and the ET-1 genomic sequences. The plasmid was then blunt-ended with Klenow and ligated to either the modified IL-10 cDNA or IL-10 genomic clones described above. The ligation products, termed pmET1rbt-cIL-10 and pmET1rbt-gIL-10, respectively, included 1.36 kb

of mET-1 promoter, the complete mET-1 exon 1 (in 2 pieces) and 866 bp of mET-1 intron 1 (Figure 1).

Construction of expression cassettes containing mET-1-derived enhancer elements

To increase expression from cassettes containing the mET-1 promoter, we inserted additional copies of a 45-bp endothelium-specific enhancer element ("ETE")[2] upstream of the native ETE in the mET-1 promoter. One copy of the ETE is present in the native ET-1 promoter (at position -364 to -319), and was included in the mET-1 promoter fragment used to construct all of the mET-1 vectors. To begin to construct cassettes that included multiple copies of the ETE, we used the ETE in the native mET-1 promoter as a template for PCR-based synthesis of ETE concatamers. This approach eventually yielded ETE concatamers containing 1–4 copies of the ETE (termed "1X, 2X, 3X and 4XETE"). When ligated 5´ of the native ETE in the mET-1 promoter, these 1–4 copies yielded expression cassettes containing 1–5 direct repeats of the ETE (Supplementary Figure 1). Extensive efforts by us and two contract laboratories to synthesize the "3XETE" inverted repeat construct reported by Bu *et al.*[2] were unsuccessful. Transfection of plasmids containing 1– 5 copies of the ETE (see Results) identified the 4XETE as the best-performing synthetic enhancer-promoter. Therefore, only construction of the 4XETE is described here.

The 4XETE element was constructed by PCR amplification using the native 1XETE-containing mET-1 promoter as a template. The forward (5´) primer for this PCR included a copy of the ETE, adding this second copy upstream of the native ETE. The reverse primer covered the ET-1 promoter at approximately –149 bp, including the *Xba*I site. The amplification product (amplicon "A") included 2XETE and the mET-1 promoter between the native ETE and -149. A second amplicon (amplicon "B") containing 2XETE was generated by PCR amplification of the 1XETE using the same forward primer used for amplicon A (containing 1XETE) and a reverse primer covering

the 3´ end of the ETE. Amplicon B therefore was designed to have 2 ETE repeats. The pmET1rbt-cIL-10 plasmid was then digested with *Xho*I and *Xba*I, removing all mET-1 promoter sequence 5´ of the *Xba*I site, and blunt-ended. Amplicons A and B were also blunt-ended and ligated together. The ligation product of amplicons A + B was then ligated to the *Xho*I – *Xba*I-digested, blunt-ended pmET1rbt-cIL-10 plasmid. The plasmid generated by this ligation (later termed 2XETE-pmET1rbt-cIL-10) was sequenced and found to contain only 2 (not 4) direct repeats of the ETE: the native ETE plus an additional copy 5´ of the native copy, with an *Xho*I site 5´ of the upstream ETE. To add additional copies of the ETE to 2XETE-pmET1rbt-cIL-10, amplicon B was self-ligated and digested with *Xho*I. This was intended to yield a 2XETE fragment, comprising 2 ETE inverted repeats flanked by *Xho*I sites. The 2XETE-pmET1rbt-cIL-10 plasmid was then digested with *Xho*I, opening it upstream of the 2XETE, and the *Xho*I-digested amplicon B-self-ligation product was inserted by ligation. The plasmid product was sequenced and—surprisingly—found to containing 4 direct repeats of the ETE including the native ETE and 3 additional ETE copies immediately 5´ of the native ETE. This plasmid was named p4XETErbt-cIL-10 (Figure 1). Because initial *in vitro* studies suggested that the IL-10 cDNA expressed at a higher level than the genomic clone (Figure 2a), the 4XETE enhancer-containing promoter was tested only with the IL-10 cDNA, not with the IL-10 gene.

Addition of the woodchuck hepatitis virus post-transcriptional regulatory element (WPRE) We used the WPRE (woodchuck hepatitis virus post-transcriptional regulatory element) to attempt to further improve the IL-10 expression cassettes. The WPRE was obtained from Dr. David Russell (University of Washington, Seattle) and includes nucleotides 1093 to 1684 of the WHV genome (WHV8; Accession no. J04514).[3] We also used a truncated version of the WPRE, termed “oPRE” or “optimal Post-transcriptional Regulatory Element”, obtained from Dr. Axel Schambach (Hannover

Medical School, Germany).[4] The oPRE lacks the X Protein coding sequence and promoter, which are associated with oncogenic activity.[5] The oPRE includes nucleotides 901 to 1481 of the WHV genome (WHV8; Accession no. J02442).[4] The WPRE and oPRE were removed from plasmids pSK+WPRE-B11 and Sin11 CMV GFP oPRE, respectively by restriction enzyme digestion followed by blunt-ending with Klenow. Both PRE fragments were ligated into the unique *Pac*I site of plasmids pBshuttle-CMVrbt-cIL-10 and pBshuttle-4XETErbt-cIL-10 after blunt-ending of the *Pac*I-digested plasmids with T4 polymerase and dephosphorylation. Plasmids were obtained with both orientations of the PRE fragments in both of the pBshuttle-derived plasmids: pBshuttle-CMVrbt-cIL-10-WPRE/oPRE and pBshuttle-4XETErbt-cIL-10-WPRE/oPRE.

Generation of HDAd-IL-10 vectors

To enable efficient transfer of the IL-10 expression cassettes into the HDAd backbone plasmid pC4HSU (Microbix Biosystems, Toronto, ON, Canada) we constructed a shuttle plasmid that could be use to insert cloned sequences into pC4HSU by homologous recombination (Supplementary Figure 4). To generate this plasmid ("pBshuttle"), we removed a 2.6 kb *Eco*RI fragment containing the unique *Asc*I site from pC4HSU and ligated it to *Eco*RI-digested pBSK (-) (Stratagene, La Jolla, CA). The ligation product, pBshuttle, contains a single *Asc*I site, flanked on each side by over 1 kb of sequence from pC4HSU. pBshuttle was then used to transfer expression cassettes into pC4HSU according to the following general protocol (Supplementary Figure 4): 1) Removal of an expression cassette from a source plasmid by restriction enzyme digestion, blunt-ending, ligation of *Asc*I linkers, and digestion with *Asc*I; 2) Ligation of the cassette into *Asc*I-digested pBshuttle; 3) Excision of the expression cassette from pBshuttle along with the flanking sequences derived from pC4HSU, using restriction sites not present in the cassette; 4) *Asc*I digestion and phosphatase treatment of pC4HSU; 5) Electroporation of the excised expression cassette and *Asc*I-cut pC4HSU into recombination-permissive

BJ5183 cells (Stratagene);[6] 6) Screening of ampicillin-resistant BJ5183 colonies for presence of the expression cassette by PCR; 7) Transformation of plasmid DNA from PCR-positive colonies into recombination-resistant DH510B cells (Invitrogen, Carlsbad, CA); 8) Screening for recombinant clones by colony PCR and transforming the plasmid DNA isolated from positive clones into DH5α cells (suitable for amplification of large plasmids); 9) Screening of plasmid DNA from ampicillin-resistant DH5α colonies by restriction digestion, to detect presence of the recombinant pC4HSU-derived plasmid; and 10) Preparation of large amounts of plasmid DNA from a colony that contains the desired recombinant plasmid.

To generate HDAd virions from the HDAd backbone plasmids, we linearized the recombinant pC4HSU-derived HDAd plasmids by *Pme*I digestion and transfected the plasmid DNA into 293Cre4 cells[7] (Microbix) using Lipofectamine 2000 (Invitrogen). HDAd genomes were then rescued and amplified by addition of the "H14" helper virus (Microbix), at an MOI of 100.[8,9] After six serial passages and amplifications, HDAd vectors were purified by CsCl density gradient ultracentrifugation.

References

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Supplementary Figure Legends

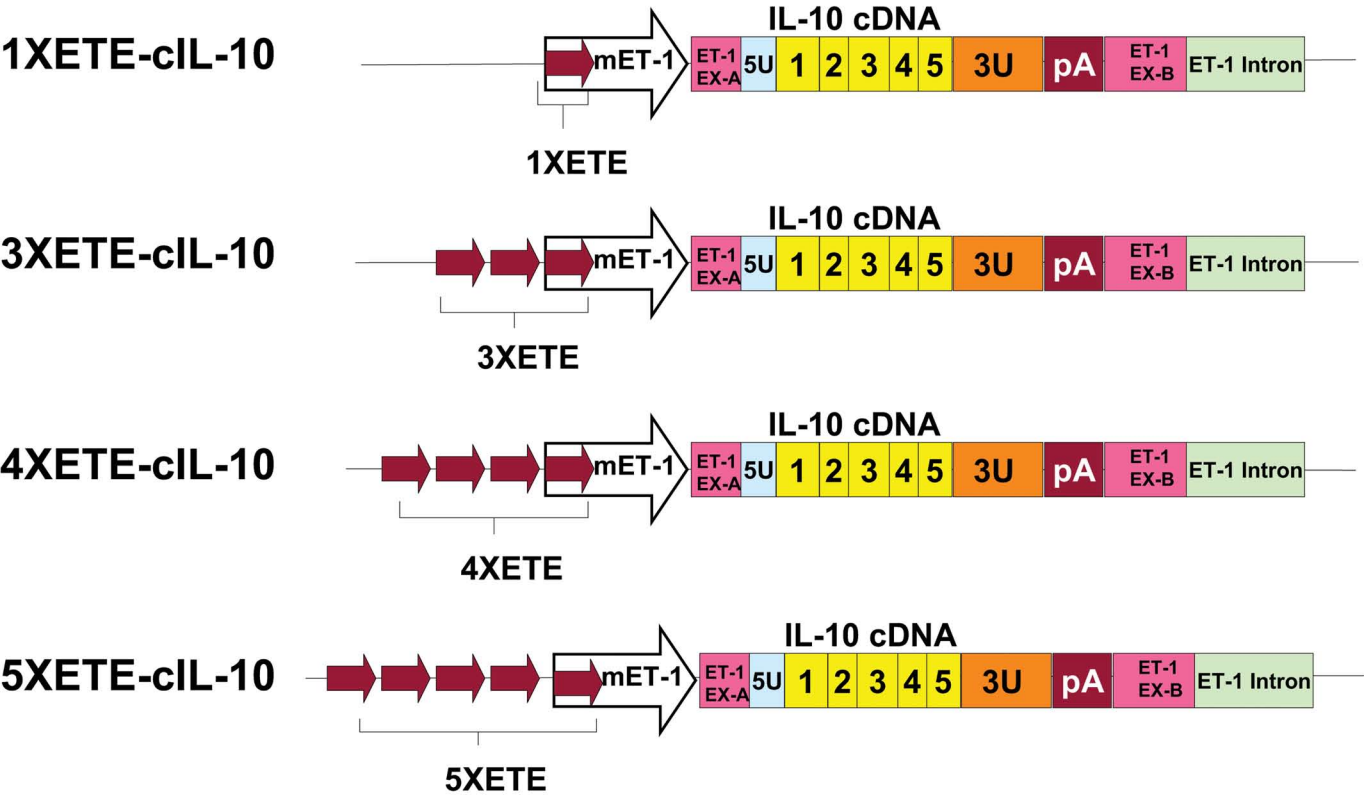
Supplementary Figure 1. IL-10 expression cassettes with ETE enhancer copies upstream of a truncated mET-1 promoter. Addition of 2, 3, or 4 direct repeats upstream of the endogenous ETE in the native ET-1 promoter yields "3X", "4X", and "5X" ETE direct repeat-containing vectors, respectively. Other symbols and abbreviations are as in Figure 1.

Supplementary Figure 2. Effect of post-transcriptional regulatory elements on transgene mRNA levels. (**a**) Bovine aortic EC were transfected with plasmids containing the CMV-cIL-10 (CMV) or the 4XETE-cIL-10 (4XETE) cassettes, with the woodchuck hepatitis virus post-transcriptional regulatory element (WPRE) inserted in a forward (F) or reverse (R) orientation. IL-10 mRNA was measured in cell extracts by qRT-PCR. Mean IL-10 expression for the CMV-cIL-10 group without the WPRE was assigned a value of 100%. IL-10 expression in individual wells was calculated as a percentage of this value. (**b**) Similar experiment to (**a**) except that the optimized woodchuck hepatitis virus posttranscriptional regulatory element (oPRE) was used instead of the WPRE. Results from a negative control transfection (with pBShuttle, which lacks an IL-10 expression cassette) are included (left-most group). Bars are group means.

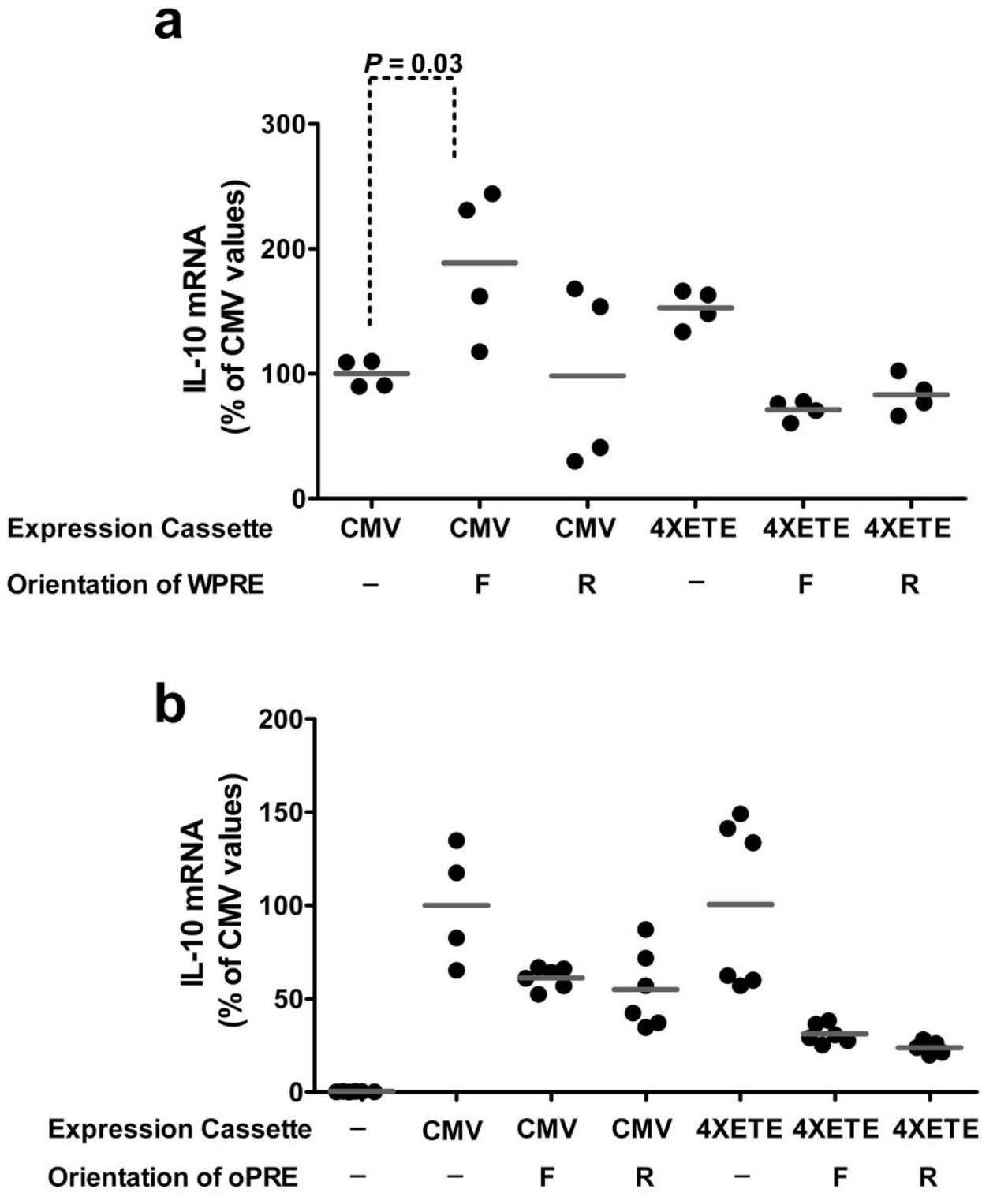
Supplementary Figure 3. Western analysis of IL-10 protein. (**a**) BAEC were transduced with HDAdNull, HDAd-CMV-cIL-10, or HDAd4XETE-cIL-10-oPRE. Conditioned medium was collected and 50 μl/lane analyzed to detect IL-10 protein. Each lane is from cells in a separate well. Size markers are in kDa. (**b**) Quantitation of IL-10 signal on western blot in (**a**), using densitometry; mean ± SD.

Supplementary Figure 4. Construction of a shuttle vector for inserting expression cassettes into HDAd by homologous recombination. The HDAd backbone plasmid pC4HSU contains a unique *Asc*I site, flanked by *Eco*RI sites. This *Eco*RI fragment is removed from pC4HSU and ligated to *Eco*RI-digested pBSK+ (Stratagene), to yield pBshuttle. Expression cassettes (green) are constructed separately, ligated to *Asc*I linkers, digested with *Asc*I, then ligated to *Asc*I-digested pBshuttle. The ligation products are screened by restriction enzyme digestion to identify the desired orientation of the expression cassette. The expression cassette and its flanking regions derived from pC4HSU (HR-1 and HR-2) are then removed from the "pBshuttle + cassette" plasmid by digestion with restriction enzymes that cut outside HR-1 and HR-2. The fragment is then electroporated into recombination-permissive bacteria along with *Asc*I-digested pC4HSU. Homologous recombination restores a circular pC4HSU-derived plasmid, which now contains the expression cassette.

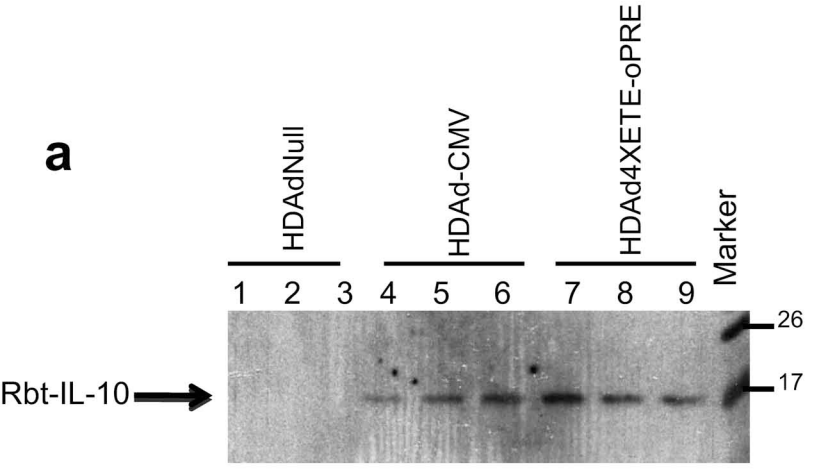
Supplementary Figure S1

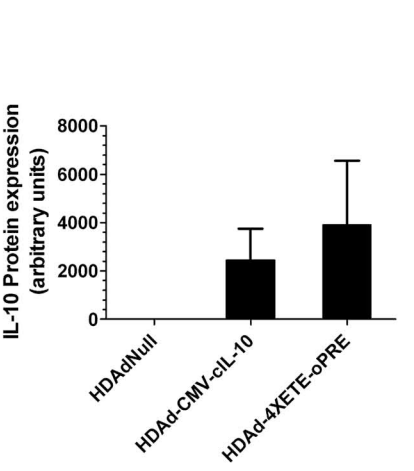


Supplementary Figure S2



Supplementary Figure S3





Supplementary Figure S4