

AAV2/IL-12 gene delivery into dendritic cells (DC) enhances CTL stimulation above other IL-12 applications

Evidence for IL-12 intracrine activity in DC

Chang-Xuan You,^{2,3,†} Min Shi,^{1,2,†} Yong Liu,^{1,3,†} Maohua Cao,¹ Rongcheng Luo^{2,*} and Paul L. Hermonat^{1,3,*}

¹Department of Internal Medicine; University of Arkansas for Medical Sciences; Little Rock, AR USA; ²Department of Oncology; Nanfang Hospital; Southern Medical University; Guangzhou, China; ³Department of Obstetrics and Gynecology; University of Arkansas for Medical Sciences; Little Rock, AR USA

[†]These authors contributed equally to this work.

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Abbreviations: AAV, adeno-associated virus; CEA, carcinoembryonic antigen; ⁵¹Cr, chromium 51; DC, dendritic cell; eg, encapsidated genomes; IFN, interferon; IL, interleukin; Mo, monocyte; r, recombinant

Adoptive transfer of antigen-specific cytotoxic T lymphocytes (CTL) holds significant promise in treating cancer and Th1 response cytokines are critical for their stimulation. Recently we reported that interleukin 7-(IL-7) and interferongamma-(IFN γ) autocrine/T cell gene delivery resulted in superior ex vivo CTL stimulation over paracrine/DC delivery. IL-12 is yet another important Th1 cytokine which affects both DC and T cells. Here, using adeno-associated virus Type 2 (AAV2) gene delivery, IL-12-paracrine/DC gene delivery gave significantly superior stimulation of carcinoembryonic antigen (CEA)-specific CTL killing over that induced by autocrine gene delivery (or exogenous IL-12 addition). This is surprising as both AAV2/IL-12-treated T cells and DC secreted approximately the same level of IL-12. Paracrine IL-12 gene delivery also resulted in highest IL-12/IL-10 secretion ratio by DC and highest CD40, CD80, CD83 and CD86 expression. Moreover, AAV2/IL-12-DC stimulated the highest T-cell IFN γ production, highest T cell proliferation, highest CD69⁺/CD8⁺ levels, and lowest level of CD25⁺/CD4⁺ Treg. These data strongly suggest that the primary activity of IL-12 during CTL generation is upon the DC. These data are also consistent with there being novel activity for IL-12 within the DC itself, not involving its surface receptor; an “intracrine” activity. Given the plethora of IL-12 studies, these data also suggest that this gene delivery comparison approach could be useful for uncovering new cytokine activities and mechanism(s) of action gone unrecognized by conventional immunologic assays. Finally, these data further suggest AAV2/IL-12 intracrine gene delivery into DC may have utility in immunotherapy protocols involving antigen-specific CTL.

Introduction

One approach in immunotherapy is the genetic manipulation of professional antigen presenting dendritic cells (DC) for the stimulation of cytotoxic T lymphocytes (CTL).¹ Protocols for generating DC from peripheral blood monocytes (and other sources) have been widely used and permit the convenient ex vivo manipulation of DC for laboratory and clinical studies.^{2,3} We have utilized adeno-associated virus (AAV2) to load antigen genes into monocytes and upon induction of their differentiation the resulting DC demonstrate higher capability for CTL stimulation than protein-loaded DC.⁴⁻⁷ AAV2 has been in use since 1984 and of various viral vectors used for clinical gene therapy AAV2 has the superior safety record.⁸⁻¹⁰ While this technique has worked very well for generating CTL ex vivo for laboratory assays, there must

be further improvement for the best hope of overcoming resident tolerance present in tumors in patients.¹¹⁻¹³

An additional immune-gene therapy approach which may have merit is the delivery of Th1 response-associated cytokines. Cytokine gene therapy would seem to offer large advantages over exogenous cytokine application as most cytokines have extremely short half-lives, ranging from a few minutes to a number of hours. The delivery of the cytokine gene allows for the continuous expression of fresh, maximally biologically active protein which may have two logarithms higher biological activity over commercial preparations on a per weight basis.⁴ Additionally, when using ex vivo gene delivery strategies we can pick and choose which specific immune cell type, DC (paracrine) or T cell (autocrine), expresses the cytokine (or receptor). Recently we studied the activities of interleukin (IL) 2, IL-7 and interferongamma (IFN γ)

*Correspondence to: Paul L. Hermonat and Rongcheng Luo; Email: plhermonat@uams.edu and lrc@nfhoc.com
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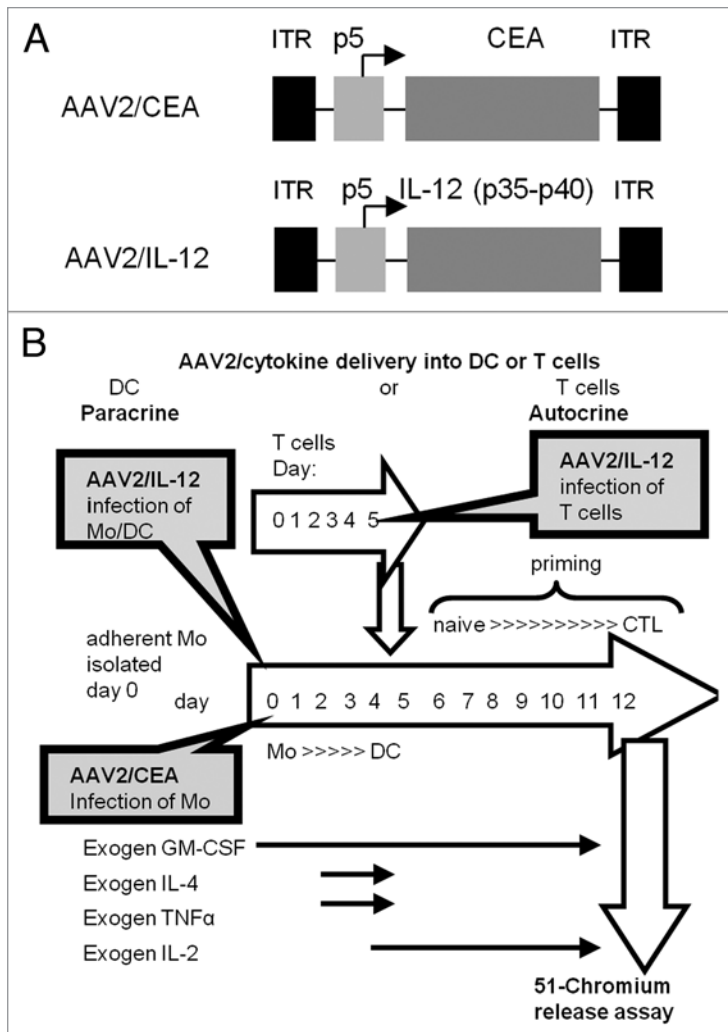


Figure 1. Virus structures and experimental scheme. (A) Shows the structures of the AAV2/CEA and AAV2/IL12 vectors (not to scale). (B) Shows the temporal experimental protocol for transducing DC and T cells, and stimulating CTL. Note that AAV/cytokine vectors either infected Mo/DC at day zero, or naive T cells just prior to co-incubation with AAV/antigen-loaded DC.

under both autocrine and paracrine gene delivery approaches for generating CD8⁺ CTL. While these cytokines are known to affect the phenotypes of both DC and T cells improved CTL stimulation was only observed when IL-7 and IFN γ were delivered by the autocrine approach.^{14,15} In sharp contrast, IL-2 gene delivery did not improve CTL generation by either the autocrine or paracrine strategy over exogenous addition.¹⁴

IL-12 is yet another likely useful Th1 cytokine for immunotherapy protocols. While IL-12 is normally produced by DC and macrophages,¹⁶ it affects the phenotypes of both DC and T cells. Kuge et al. found that IL-12 induced rapid proliferation of CTL, peaking at days 4–5 post-addition.¹⁷ Mehrotra et al. found that IL-12 augmented killing efficiency of CTL cells by 10 to 20-fold on a per cell basis.¹⁸ They also reported IL-12 resulted in higher CTL numbers and that this effect was independent of IL-2. The affect of IL-12 on DC is less studied, but it has been observed that DC maturation/activation and IL-12 production

may be tied events.¹⁹ Recently this view has been refined by Sugiura et al. reporting that IL-12 has a direct effect on DC maturation.²⁰ Here it is demonstrated that IL-12 paracrine/DC gene delivery induces those DC to stimulate a more robust CTL response in vitro. These data suggest that even though both DC and T cells are affected by IL-12, its main role is to enhance the antigen-presenting phenotype of DC. These data are also consistent with there being a “intra-crine” effect by IL-12 on DC.

Results

AAV2/IL-12 ex vivo delivery, state and expression in DC and T cells. Potentially many Th1 response-associated cytokines could be useful in immuno-gene therapy protocols against cancer and viruses. However, much remains unknown about cytokine action and how best to interface cytokine gene delivery with immunotherapeutic protocols. Here we assayed AAV2/IL-12 gene delivery into DC (paracrine) and T cells (autocrine) to improve CTL stimulation against carcinoembryonic antigen (CEA) as depicted in Figure 1. It is important that rAAV2 provirus express their transgenes. Figure 2A and B demonstrate that at least some rAAV provirus are chromosomally integrated by the analysis of vector-chromosomal (*Alu I* repetitive element) junction PCR amplification. Figure 2C–E show that the resulting rAAV2 provirus express their respective transgenes by RT-PCR analysis, in both DC and T cells. To observe both the transduction efficiency and protein expression of the CEA and IL-12 proteins we performed an intracellular staining analysis of transduced and untransduced DC and T cells. The transduction efficiency of DC by AAV2/CEA and AAV2/IL-12 (MOI of 2000), as shown in Figure 2F and G was 87–92%. AAV2 is known to transduce primary T cells and DC even at relatively low multiplicities of infection.^{4–8,14,15,21} The transduction efficiency of CD3⁺ T cells (MOI 200), as shown in Figure 2H, was 79%. Thus transduction efficiency using AAV2 was reasonably high for both DC and T cells.

Characterization of transduced DC. We examined the DC on day 6, as shown in Table 1, for surface expression of CD14, CD40, CD80, CD83 and CD86 by FACS and found that CD80, CD86 and CD83 were upregulated by rAAV2 infection, consistent with previous studies.^{4–7} The addition of exogenous IL-12 or AAV2/IL-12 further upregulated these markers, but the use of AAV2/IL-12 had a more profound effect. Most importantly CD40, CD80 and CD86 were expressed at very high levels, consistent with very professional antigen presenting cells. Moreover, DC maturity was documented to be the highest in the AAV2/IL-12-treated DC as CD83 was expressed at the highest level. We further observed the resulting expression level of IL-12 and IL-10 by DC by these various treatments. Higher IL-12/IL-10 ratios reflect a Th1 response promoting DC, stimulating a more robust Th1 CTL response. As shown in Figure 3A the simple delivery of the CEA antigen by rAAV2 was enough to dramatically increase the IL-12/IL-10 secretion ratio (measured

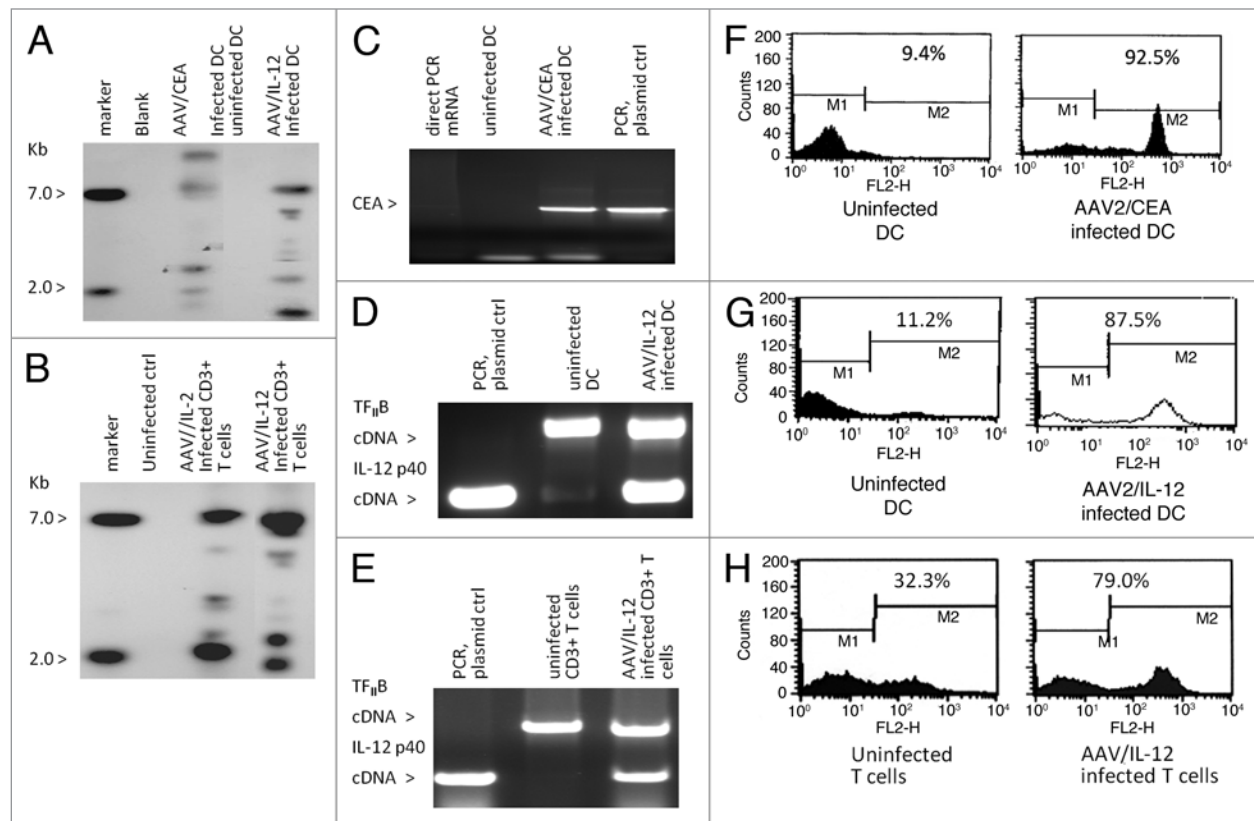


Figure 2. Provirus integration, transgene RNA and protein expression. Shown is the AAV proviral DNA chromosomal integration into DC (A) and T cells (B) by PCR amplification of vector-chromosomal (AluI element) junctions. Also shown is the transcriptional expression of the rAAV provirus and their respective transgenes by RT-PCR analysis for CEA in transduced DC (C), IL-12 in transduced DC (D) and IL-12 in transduced T cells (E). (F–H) show transduction efficiency of AAV2/CEA and AAV2/IL-12 in DC and T cells. Transduction efficiency in DC was about 90% (F and G). The transduction efficiency of CD3⁺ T cells was approximately 79% (H).

in conditioned medium in pg/ml), as analyzed by ELISA, over mock-treated DC. The addition of exogenous IL-12 in addition to the AAV2/CEA transduced DC gave no significant effect on improving this ratio. In contrast, the transduction of the IL-12 gene into DC dramatically increased the IL-12:IL-10 secretion ratio above all other treatments. We further analyzed these DC to investigate the percentage of cells involved in the secretion of these cytokines by observing cytokine by intracellular staining. **Figure 3B** shows that, consistent with the levels of secreted IL-12 shown A, the AAV2/IL-12 treated DC had the highest percentage of cells actively producing IL-12 and the lowest producing IL-10, as analyzed by intracellular staining. These data suggest that the AAV2/IL-12 treatment resulted in the most Th1 response-promoting DC. These data also suggest that IL-12 levels were inversely associated IL-10 expression in DC.

Characterization of transduced and stimulated T cells. The ability of AAV2/IL-12 transduced T cells to produce and secrete IL-12 was analyzed (**Fig. 4A**) and was shown to be comparable to that of transduced DC (**Fig. 3A**) A robust Th1 CTL response is usually consistent with a high CD8:CD4 ratio. The resulting cell population stimulated by the various DC treatments was analyzed by FACS and the results listed in **Table 2** upper part. Note that all T-cell populations generated with all AAV2-transduced DC had a higher CD8/CD4 ratios than mock treated

DC, indicating a robust Th1 CD8⁺ T-cell response, and consistent with high levels of CD80 and CD86 expressed by AAV2-infected DC. However T cells derived from AAV2/IL-12-treated DC had the highest CD8/CD4 ratios, consistent these cells having high CD80, CD83 and CD86 expression, and lowest IL-10 expression.

CD3 cells were next isolated from T cells generated by various treatments and analyzed for IFN γ expression, another Th1 response cytokine expressed by T cells and a predictor of CTL killing capability.²² **Figure 4B** shows exogenous IL-12 treatment of DC or T cells did not enhance IFN γ expression compared with control T cells (CTL) generated by AAV2/CEA-loaded DC alone. AAV2/IL-12 treatment of T cells also did not enhance IFN γ expression compared with these control T cells. Only the paracrine treatment of DC with AAV2/IL-12 resulted in an increase in the percent of T cells that expressed IFN γ . These data suggest that the gene delivery of IL-12 only by a paracrine approach, into DC, can significantly enhance IFN γ expression in the resulting CTL population. To our knowledge, such paracrine “favoritism” for IL-12 has not been reported previously and suggests an intracrine activity for IL-12.

The generation of responder CTL involves both the proliferation of CD4⁺ helper T cells as well as proliferation of the CD8⁺ T cells themselves. To test the level of T-cell proliferation we

Table 1. Characterization of DC on day 6

DC treatment	CD14	CD40	CD80	CD83	CD86	HLA-DR
Ctrl (mock)	20.5	24.8	38.7	28.55	65.3	92.3
AAV2/CEA	14.5	42.45	58.3	43.7	87.4	98.1
AAV2/CEA ⁺ exogen IL-12	10.7	50.8	61.8	53.9	87.8	97.1
AAV2/CEA ⁺ AAV2/IL-12	6.5	66.9	84.6	65.9	95.7	97.2

Surface expression of CD14, CD40, CD80, CD83, and CD86 were analyzed for percent positivity by FACS.

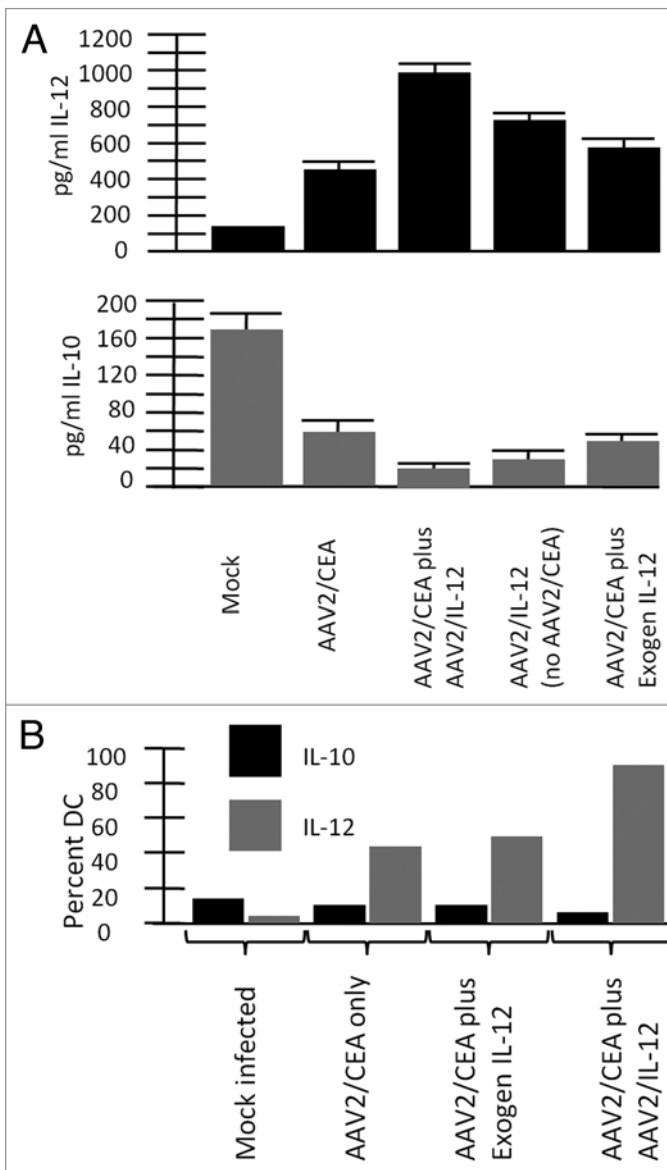


Figure 3. Transduction efficiency and effect on DC. (A) Shows IL-12 and IL-10 secretion by DC under various treatments as analyzed by ELISA. Note that AAV/IL-12 treated DC secreted the highest level of IL-12 and the lowest producing IL-10. (B) Shows an analysis of IL-12 and IL-10 percent expression in DC under various treatments as analyzed by intracellular staining. Note that AAV/IL-12 treated DC had the highest percentage of cells actively producing IL-12 and the lowest producing IL-10.

performed the standard protocol for the generation of antigen-specific CTL. However, in addition to loading the DC with the antigen (AAV2/CEA) we included the delivery of AAV2/IL-12 into DC or T cells. Proliferation of CD3⁺ T cells was measured by the incorporation of ³H-TdR, and the results are shown in **Figure 4C**. In sharp contrast, the paracrine delivery of AAV2/IL-12 into DC resulted in very high T-cell proliferation. T-cell proliferation resulting from AAV2/IL-12 into DC was much higher than the use of exogenous IL-12 cytokine. We expected that these levels of T-cell proliferation would predict the level of target cell killing by these proliferating T cells (CTL).

There are only limited studies on the delivery of cytokine genes into T cells so we further analyzed these cells for changes in known important markers by autocrine IL-2 and IL-12 gene delivery. The CD8/CD4 ratio is one important attribute already discussed and this ratio was not so different as shown in **Table 2** lower part, between autocrine IL-12 gene delivery and exogenous IL-12 treatment. As also shown in this part both autocrine IL-12 gene delivery and exogenous IL-12 treatments gave T-cell populations with high levels of CD69⁺ cells, with AAV2/IL-12 delivery resulting in the highest level. CD4⁺/CD25⁺ T regulator (Treg) cells are also critical as they are involved in the suppression of Th1 response. As shown in **Table 2** lower part both autocrine IL-12 gene delivery and exogenous IL-12 treatments gave T-cell populations with low levels of Tregs, with AAV2/IL-12 delivery resulting in the lowest level. All these data are consistent with the T-cell populations resulting from AAV2/IL-12 transduced DC as having higher IFN expression, and should be consistent with higher killing ability.

IL-12 paracrine gene delivery enhances CTL stimulation. Having characterized the AAV2/cytokine transduced DC and T cells we then assayed the resulting CTL for their ability to kill a genetically altered CEA-positive lymphoblastoid cell line (LCL) which was HLA A2-matched with blood donors. To do this, we performed the experiment depicted in **Figure 1** and tested for target killing using the standard ⁵¹Cr release assay. We first assayed the CEA-specific T cell killing of CEA-positive SW480 cells in **Figure 5A**. The results show CTL stimulated by AAV2/IL-12 paracrine delivery into DC gave 43% increase in killing over AAV2/CEA-only stimulated CTL control (54.3% vs. 37.9%, respectively, $p < 0.05$) killing. AAV2/IL-12 paracrine delivery also stimulated CTL with 30% higher killing ability than autocrine delivery (54.3% vs. 41.8%, respectively, $p < 0.05$). Exogenous addition of also IL-12 gave no advantage. The killing was also shown to be MHC/HLA Class I restricted using polymorphic anti-Class I antibody, indicating killing by CD8⁺ T cells. The high CTL killing induced by the AAV2/CEA-plus-AAV2/IL-12-transduced DC is fully consistent with the highest IL-12 secretion/production and lowest IL10 secretion/production by DC (**Fig. 3A and B**), the highest IFN γ production by T cells in **Figure 4B**, and the stimulation of the highest level of proliferation of T cells in **Figure 4C** shown by this same treatment. Next, these same set of CEA-specific CTL were tested for killing of CEA⁺ lymphoblastoid cell line (LCL) targets, as shown in **Figure 5B**, and very similar killing resulted (HLA A2 positive). Again the paracrine IL-12 delivery resulted in CTL which

could kill at high levels than no IL-12 addition, exogenous IL-12 addition or autocrine IL-12 addition. Finally, in **Figure 5C** these most effective CTL killers, those produced from dual AAV2/CEA-plus-AAV2/IL-12-treated DC, were tested against a variety of targets and demonstrated significant killing only against CEA-positive SW480, fully consistent with the resulting CTL being CEA-antigen specific killing.

Discussion

Here it is demonstrated that IL-12-paracrine gene delivery into DC by AAV2 results in the stimulation of a significantly more robust CD8⁺ CTL response than IL-12-autocrine gene delivery into T cells or exogenous IL-12 addition. This is surprising as AAV2/IL-12-transduced T cells secreted approximately the same level of IL-12 as similarly treated DC (**Figs. 2D and 3A**). Moreover, IL-12 autocrine delivery into T cells also gave no advantage in CTL generation over exogenous IL-12 or over AAV2/CEA-only transduced DC. Supporting the enhanced activity of AAV2/IL-12 paracrine gene delivery, this approach also resulted in highest IL-12/IL-10 secretion ratio by DC, consistent with a high Th1 response-promoting environment, as well as high expression of CD40, CD80, CD83 and CD86. AAV2/IL-12 paracrine gene delivery also induced alterations in stimulated T cells, resulting in the highest T-cell IFN γ production, the highest T-cell proliferation, the highest CD69⁺/CD8⁺ levels, and a low level of CD25⁺/CD4⁺ Treg. While all blood donors were considered to be AAV2-seropositive, target cell killing was shown to be MHC/HLA Class I-restricted, further indicating killing by CD8⁺ CTL. Again, all these findings are consistent with the highest Th1 T-cell stimulation by AAV2/IL-12-transduced DC.

During T-cell stimulation and priming by antigen presenting cells, either in the lymph node or in tissue culture, the DC and T cell are in direct physical contact. Such close proximity results in there being very little difference as to which cell type is specifically secreting IL-12 (or any other cytokine for that matter) as all would receive a very similar cytokine concentration. Yet, here we have identified a tropism of effect for IL-12 expression specifically within DC. While both T cells and DC are affected by IL-12, the data presented here strongly suggest that the primary activity of IL-12 is on the DC in regards to the generation of antigen-specific, MHC/HLA Class I-restricted CD8⁺ CTL response. Moreover, in this study we compare 20 ng/ml commercial IL-12 to 800 pg/ml freshly secreted IL-12. Thus, in the data presented, the commercial product already has a greater than 20-fold advantage over that produced by the delivered gene. Yet the delivered IL-12 gene into DC gives a significantly enhanced effect. Thus, these multiple high activities of AAV2/IL-12-transduced DC are most consistent with this effect being an “intracrine” activity for IL-12 within the DC, and not present in T cells. The activity of intracellular IL-12 appears different, and more potent, in affecting DC phenotype than extracellular IL-12. That external IL-12, either exogenous IL-12 or IL-12 provided by adjacent AAV2/IL-12-transduced T cells, was insufficient to induce DC to stimulate such robust CEA-specific CTL provides evidence of

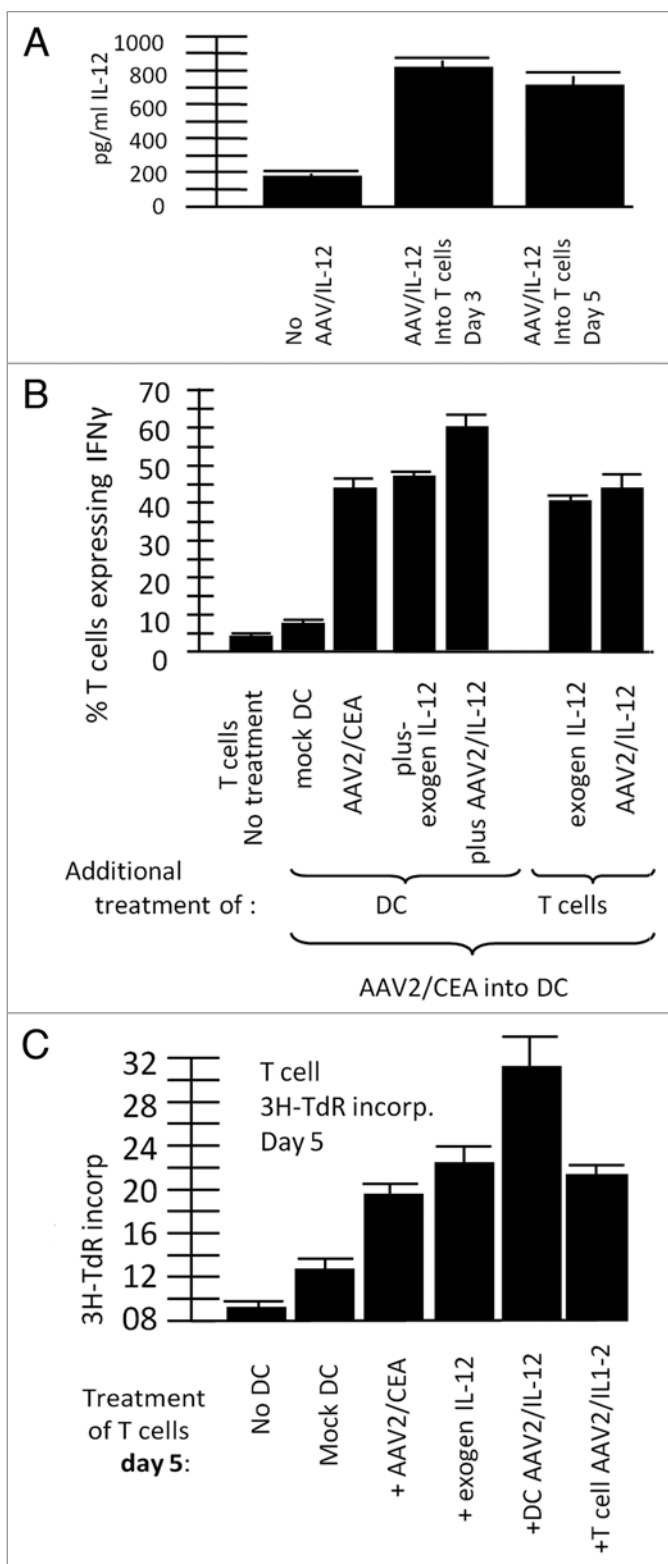


Figure 4. Characterization of T cells under indicated treatments. **(A)** Shows the amount of IL-12 secreted by T cells at 80 h after AAV/IL-12 transduction under the indicated treatment. Note that secretion levels of T cells were similar to those of DC shown in Figure 3A. **(B)** Shows the percentage of T cells which express IFN γ , after indicated treatment, as determined by intracellular staining. **(C)** Shows the proliferation of CD3⁺ T cells, after the indicated treatment, as measured by the incorporation of 3H-TdR.

Table 2. Characterization of resulting T-cell populations stimulated by the various DC (top) or T-cell (bottom) treatments

DC treatment	CD8 ⁺ /CD4 ⁺	CD69 ⁺ , CD8 ⁺	CD25 ⁺ , CD4 ⁺
Ctrl (mock)	19.4/17.9	13.6	18.2
AAV2/CEA	38.6/20.9	49.4	8.4
AAV2/CEA ⁺ exogen IL-12	42.2/11.7	52.7	8.1
AAV2/CEA ⁺ AAV2/IL-12	67.0/17.5	74.9	3.2
T-cell treatment	D8 ⁺ /CD4 ⁺	CD69 ⁺ , CD8 ⁺	CD25 ⁺ , CD4 ⁺
exogen IL-12	41.2/7.7	62.6	7.3
AAV2/IL-12	44.2/10.8	57.3	8.1

Resulting T-cell population stimulated by the indicated DC treatment were analyzed for percent positivity by FACS. Resulting T-cell population stimulated by the indicated T-cell treatment on Day 5, in conjunction with AAV2/CEA treatment of DC, were analyzed for percent positivity of CD8 and CD4; CD69 or D25 expression by FACS.

a novel activity for IL-12 cytokine produced within the DC itself, not involving its cell surface receptor.

We report here the first evidence for IL-12 intracrine activity. Intracrine activity has been described for some other interleukins such as IL-1 α and IL-6.^{23,24} Our study on IL-12, an important cytokine, suggests there has been inadequate study of complex issues of cytokine phenotype and expression, in defining their constitutive, autocrine, paracrine or intracrine (and other) actions. Some studies have been undertaken in which a specific cell type is incubated with a cytokine and the study is described as an “autocrine” study; but this is inaccurate. The half-life of extracellular cytokines is usually very short and the addition of exogenous cytokine results in only a short pulse of activity. Moreover the expression of many cytokines, and their receptors, are often complex and transient. Many cytokines and cytokine receptors have negative feedback loops preventing runaway immune response, either stimulation or tolerance. But one example would be IL-3’s downregulation of its own receptor.²⁵

Given the high number of IL-12 studies already undertaken, including those on AAV delivery of IL-12,^{26,27} the finding of a new activity for IL-12, resulting from only modest IL-12 expression, suggests that the gene delivery approach, for studying cytokine function, is likely to uncover new activities and mechanism(s) of action previously unrecognized by conventional immunologic approaches. IL-12 “intracrine” activity has never been suggested previously. As IL-12 is a heterodimer, we do not know if it is the p35, p40 or if both subunits are needed for intracrine activity. Moreover, our recent studies on IL-7 and IFN γ gene delivery “tropism” is also consistent with intracrine activity for these cytokines, but within the T cells (not DC).^{14,15} This suggests that cytokine tropism is a more common activity than previously recognized. In fact, the issue of intracrine activity of any cytokine/lymphokine would seem best studied by the technique of gene delivery as we have utilized here. Finally, these data further suggest AAV2/IL-12 cytokine gene delivery into DC may have utility in immunotherapy protocols involving antigen-specific CTL.

Materials and Methods

Cells. HEK293, K562, SW480 (colorectal adenocarcinoma), LNCap-FGC (prostate cancer), Hs578T (breast cancer) and H2126 (lung cancer) cell lines were obtained from The American Culture Collection (ATCC). EBV-transformed B cells (LCL) derived from five healthy donors were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS). PT3 cervical cancer cells have been described previously in reference 28. The peripheral blood mononuclear cells (PBMC) from five healthy donors were separated by routine Ficoll gradient method. Blood donors gave informed consent in writing, and the study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki as reflected in a priori approval by our Human Research Internal Review Board. The HLA haplotype of all donors were HLA A2, as are SW480 and LCL cells.

Recombinant AAV2 vectors. Human CEA (GenBank M29540.1), respectively was amplified by reverse-transcription polymerase chain reaction (RT-PCR). Trizol reagent (Invitrogen) was used to isolate total mRNA from SW480 cells. Total mRNA was then separated using Oligotex mRNA isolation kit (Qiagen). After first-strand cDNA synthesis, PCR amplification for each of the cDNA was performed using the following primer pairs: CEA; 5'-ACC ATG GAG TCT CCC TCG-3' and 5'-CTA TAT CAG AGC AAC CCC-3' that amplify the sequence from nucleotides 112 to 2,223.²⁹ The fused IL-12 p35-p40 cDNA (GenBank AF180562.1 and AF180563.1)³⁰ was purchased from InvivoGen (porf-hil12 g2). Both the CEA and IL-12 cDNAs were then individually cloned behind AAV p5 promoter to generate AAV/CEA and AAV/IL-12, respectively. While, the p5 promoter is not as strong as the CMV promoter it is adequate for the effective expression of cytokines and antigens as demonstrated previously in reference 4, 14 and 15.

Generation of recombinant AAV2 virus. The recombinant AAV (rAAV) plasmids were co-lipofected into HK 293 cells with AAV Type 2 capsid complementor plasmid pSH3,³¹ virus purified from cell lysates by heparin sulfate column chromatography,³² and titered by dot blot hybridization as described previously in references 4–7.

Generation of DC infected by recombinant AAV2. PBMC were isolated and cultured (5×10^6) for two hours with AIM-V medium, then non-adherent cells were removed. The remaining adherent monocytes (Mo) were infected immediately with 1×10^9 encapsidated genomes (eg) of AAV2/CEA virus or AAV2/CEA plus AAV2/IL-12. After four hours the medium/virus solution was removed and the cells were fed with the medium containing recombinant human GM-CSF (Immunex, 800 IU/ml). At day 2 and 4, to induce the maturation of Mo into DC, recombinant human IL-4 and TNF α (R and D SYSTEMS) at 1,000 IU/ml and 20 ng/ml were added to the medium, respectively. The AAV2/CEA-infected Mo were also treated with or without exogenous IL-12 (R and D SYSTEMS, 20 ng/ml). The medium and cytokines were replaced every two days. Finally, at day 5 the DC were mixed with CD3⁺ T cells.

Infection of CD3⁺ T cells with rAAV2. The Pan T-cell Isolation Kit II (Miltenyi Biotec) was employed to isolate CD3⁺

T cells from the non-adherent cells from the PBMC according to the kit instruction. Cells were cultured in AIM-V medium. Just before addition to the day 5 Mo/DC culture the CD3⁺ T cells (5×10^6) were infected with 1×10^9 eg/ml of AAV2/IL-12 (a multiplicity of infection of 200) in Aim-V medium for 2 h. CD3⁺ T control cells were cultured using exogenous IL-12 (20 ng/ml).

Analysis of rAAV chromosomal integration. The total cellular DNA was isolated from the rAAV-infected or uninfected DC or T cells using DNAzol reagent (Invitrogen) according to supplier's protocol. Chromosomal integration of the AAV/CEA genome was studied by vector-chromosome junction PCR amplification and Southern blot analysis, as previously described in reference 15.

RT-PCR expression analysis of transduced DC or T cells for CEA and IL-12 expression. CEA and IL-12 mRNA expression was assessed by RT-PCR. Isolation and amplification of generated cDNA, generated as described earlier. CEA was designed following primer pair: 5'-CTC CTG CTC ACA CCC TCA CT-3' and nt 5'-CGT TGG AGT TGT TGC TGG TG-3' that amplify the sequence from nucleotides 166 to 1,114.

Analysis of transduction and expression of CEA antigen and the cytokines by intracellular staining. At day 5 of Mo/DC culture and CD3⁺ T cells at 80 h post-AAV2/IL-12 infection on day 5, the intracellular staining assay was employed to analyze the expression and efficient transduction by rAAV2.³³

Analysis of IL-2, IL-10 and IL-12 protein expression in the DC. Expression level of IL-10 and IL-12 in DC were analyzed by intracellular staining. From 68 h to 92 h of rAAV2 transfection, IL-10 and IL-12 p70 were measured in the AAV2/CEA plus AAV2/IL-2 or AAV2/IL-12-transfected DC supernatants by enzyme-linked immunosorbent assay (ELISA) using commercially available kits (Biosource International). The sensitivity of the ELISA was < 0.5 pg/ml. Their levels were also measured in AAV2/CEA-only transduced and mock DC.

Cell surface marker analysis of DC. For the analysis of DC a part of FITC- or PE-labeled monoclonal antibodies recognizing the following antigens was used: CD14, CD40 (Chemicon International), HLA-DR, CD80, CD83, CD86 and isotype-matched antibodies (BD PharMingen). After 6 d the non-adherent DC were harvested (> 95% viable as assessed by Trypan blue exclusion) the cells counted and distributed. Stained cells were assayed for surface markers according to the routine method.

Analysis of IL-12 protein secretion by T cells. At 80 h after AAV2/IL-12 infection IL-12 p70 was measured in the different treated CD3⁺ T-cell supernatants by ELISA as described above.

Generation of cytotoxic T lymphocytes (CTL). At day 5 of DC culture the mature DC were harvested and mixed with CD3⁺ T lymphocytes (ratio of 20:1, T:DC) in AIM-V containing recombinant human IL-2 (20 IU/ml) and IL-7 (20 ng/ml). The exogenous IL-12-treated CD3⁺ T-cell-DC mixtures were cultured with 20 ng/ml of IL-12. The medium and cytokines were replaced every two days. After 7–8 d post-priming the cells were harvested and characterized for markers and ⁵¹Cr-target killing.

Analysis of T-cell proliferation stimulated by rAAV2-infected DC. After the CD3⁺ T cells were mixed with the DC at day 5, each

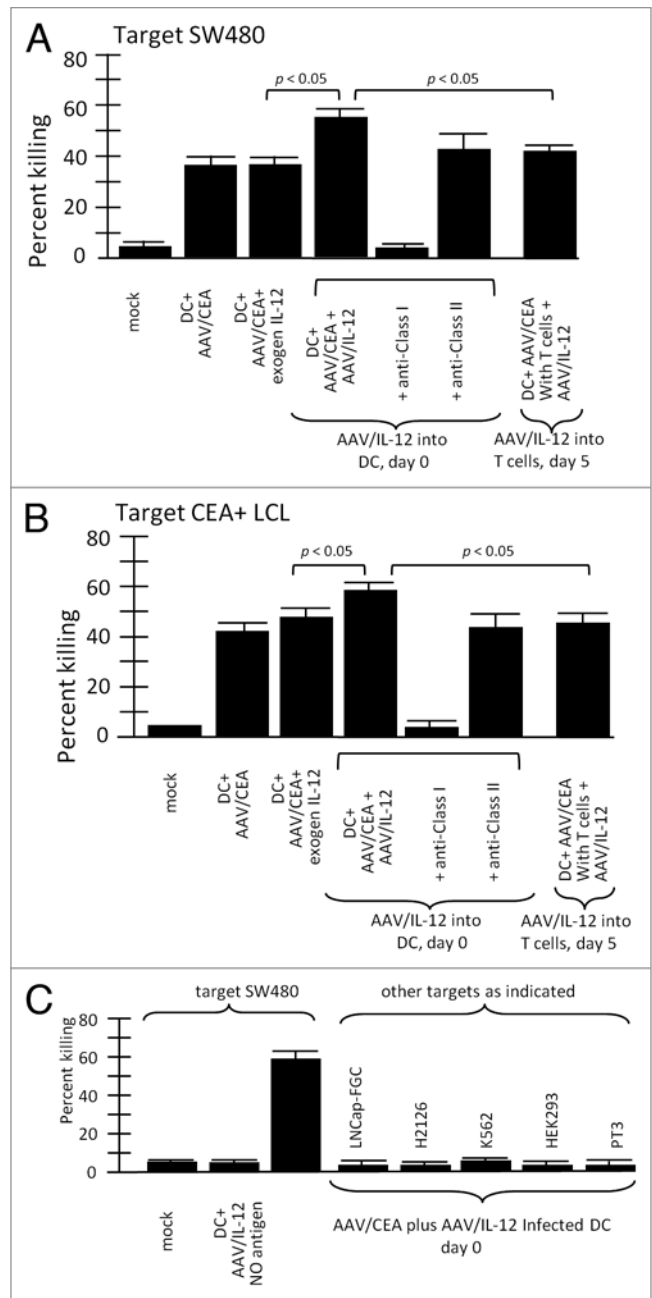


Figure 5. CEA-specific CTL killing resulting from indicated treatment by standard ⁵¹Cr release assay. All parts show anti-CEA CTL killing stimulated from the indicated treatments. (A) Utilizes SW480 (CEA⁺, HLA A2⁺) as targets. Note that the CTL stimulated by AAV/IL-12 transduced DC cells was significantly higher than when the T cells, themselves, were AAV/IL-12 transduced or controls. Also note killing was MHC/HLA Class I-restricted. (B) Utilizes genetically altered CEA⁺/LCL (HLA A2⁺) cells as targets and gives similar results to (A). (C) Shows CEA-specific CTL are tested against a variety of cancer cells demonstrating antigen-specific killing. K562 cells, commonly used to demonstrate NK killing, are not significant targets of anti-CEA CTL.

group of the mixed cells were inoculated into 5 wells of 96-well cell culture plates, respectively, 5×10^5 cells/well. After the mixed cells were cultured for 8 h in 37°C, 5% CO₂, ³H-TDR incorporation test was performed according to the routine method.

CD marker analysis of activated T-cell populations. For the analysis of activated T cells, at the end of day 12 of the mixed cell culture the primed T-cell populations were analyzed for their surface markers with immunofluorescence staining by FACS. A part of FITC- or PE-labeled monoclonal antibodies recognizing the following antigens was used: CD4, CD8, CD25 and CD69 (BD PharMingen).

Analysis for level of IFN γ in the activated T-cell populations. At day 14 post-priming T cells were harvested. The intracellular staining assay was performed to analyze the expression of IFN γ in the T cells. FITC-labeled anti-IFN γ monoclonal antibody (BD PharMingen) was used.

Analysis of CEA-specific and MHC Class I-restricted CTL killing activity. At day 12, 6-h chromium-51 (^{51}Cr) release assay was used to analyze the killing activity of CTL elicited by AAV2/CEA-infected and control DC against the target cells.^{2-7,14,15} Effector and target cells were mixed at a 20:1 ratio. To determine HLA-restriction involved in target lysis, anti-HLA Class I monoclonal antibodies were used to block cytotoxicity. K562 cells were

used as targets to observe natural killer (NK) cell activity and a series of cells (without CEA expression) were used as negative controls. LCL derived from different donors were infected by AAV2/CEA/Neo virus, and cultured in the medium plus 50 $\mu\text{g}/\text{ml}$ of G418 for more than 15 d to generate CEA expressing LCL cells.

Disclosure of Potential Conflicts of Interest

The authors declare no financial or commercial conflict of interest.

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