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Running title: AAV/HM1.24 induces robust cytotoxic response

Key words: dendritic cells, cytotoxic T lymphocytes, multiple myeloma, adeno-associated virus, HM1.24, CD80, transduction

Abbreviations: adeno-associated virus, AAV; cytotoxic T lymphocytes, CTL; dendritic cell, DC interferon, IFN; interleukin, IL

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ABSTRACT

Recent studies demonstrate that recombinant adeno-associated virus (rAAV)-based antigen-loading of dendritic cells generates significant and rapid (one stimulation/one week) cytotoxic T lymphocyte (CTL) responses in vitro against viral antigens. As a more extensive analysis of the rAAV system we have used a self-antigen, HM1.24, expressed in multiple myeloma (MM). Again, with one stimulation, significant MHC Class I-restricted, anti-HM1.24-specific CTL killing was demonstrated against multiple MM cells. Furthermore, higher expression of interferon-gamma (IFNr) in T cells and higher expression levels of, in order of significance, CD80 (2.6-3.8 fold increase), CD86, and CD40 on DC were also observed. The use of synthetic HM1.24-positive target cells further demonstrated the antigen-specificity of these CTL. There was also no evidence of natural killer cell involvement. These data extend our earlier studies, and suggest that the rAAV-loading of DC may be a particularly good protocol for generating CTL against self-antigens which may not otherwise be considered good targets due to their low immunogenicity. We also show that HM1.24 may be an effective antigen for targeting MM.
INTRODUCTION

Multiple myeloma (MM) is a malignancy characterized by clonal proliferation and accumulation of immunoglobulin-producing plasma cells, which are terminally differentiated B-cells (1). It is important to note that patients who have not responded to high-dose chemotherapy treatment plus autologous stem cell transplantation respond well to allogeneic transplantation and that a graft-versus-myeloma effect can be extremely powerful (2). These studies suggest that immunologic manipulations might be an appropriate treatment avenue for myeloma, especially if a state of minimal residual disease can be achieved after auto transplantation. In the search for an appropriate anti-MM antigenic target for immunotherapy, the self-antigen HM1.24 protein appears plausible. The HM1.24 antigen is defined by a monoclonal antibody (MoAb HM1.24) that appears to be a novel terminal B-cell-restricted antigen (3,4) expressed on tumor cells and on mature immunoglobulin-secreting B cells (plasma cells and lymphoplasmacytoid cells) but not on other cells in the peripheral blood, bone marrow, liver, spleen, kidney, or heart of normal individuals or patients with non-plasma-cell-related malignancies. HM1.24 effectiveness against MM has been demonstrated through tumor cell lysis by MoAb and complement (5,6). The HM1.24 coding sequence is 1 kilobase in length and an ideal size for ligation into any viral vector including adeno-associated virus (AAV)(4).

The manipulation of antigen-presenting cells, such as dendritic cells (DC), is a recognized approach toward developing effective immunotherapeutic protocols. DC are potent, professional antigen-presenting cells that can initiate a primary immune response to antigens by naive T cells (7). Various protocols for generating DC in vitro from peripheral blood have recently been developed. These new technologies permit in vitro manipulation of DC for clinical studies (8,9). These protocols include loading DC with tumor fragments, antigen peptides, defined tumor antigens, or antigen genes by way of retrovirus and adenovirus vectors (10-22). We have recently shown that AAV-based vectors are appropriate for viral antigen and cytokine gene delivery into DC (20,22). In head-to-head comparisons AAV-based viral antigen gene loading of DC was found to be superior to protein-loading in the ability to generate significant CTL in a short period of time (21,22). In this study, we test the ability of recombinant AAV loading of DC to generate CTL specific for a self-antigen, whose responding T cell precursors are usually 100-fold more rare than anti-viral responders.
MATERIALS AND METHODS

Generation of the HM1.24 cDNA and RT-PCR analysis for HM1.24 expression

Total RNA from two human MM cell lines, ARK-B and ARP-1, were used to obtain HM1.24 mRNA by reverse transcription-polymerase chain reaction (RT-PCR). HM1.24 expression of rAAV-loaded DC was also analyzed by RT-PCR. Total RNA was first isolated from these cells with TRIzol total RNA (Gibco BRL/Life Technologies Inc., Rockville, MD) and treated with 10 U/g of RNase-free DNase I (Promega Corp., Madison, WI) for 1 hour at 37°C. Messenger RNA was then separated with the use of the Oligotex mRNA kit (QIAGEN Inc., Valencia, CA). The first-strand cDNA synthesis was performed at 37°C for 1 hour in a final volume of 25 ul of reaction buffer (0.5 ug mRNA; 50 mM Tris-HCl, pH 8.3; 75 mM KCl; 3 mM MgCl₂; 10 mM DTT; 0.5 g oligo(dT)15 (Promega Corp.); 0.5 mM of each of the four deoxynucleotide triphosphates; 30 U of RNasin ribonuclease inhibitor (Promega Corp.); and 200 U of M-MLV reverse transcriptase (Promega Corp.). PCR amplification of the cDNA was performed in a 100 ul reaction volume containing 2.5 U of TaKaRa Z - Taq polymerase according to the manufacturer's protocol (TaKaRa Shuzo Co. Ltd, Otsu, Japan). The HM1.24 primer set was designed from the HM1.24 sequences described by Ohtomo et al. (4). This primer set (upstream 5'-TCATGGCATCTACTT CGTATGAC-3' and downstream 5'-GGATCTCACTGCAGCAGAGC-3') targeted the amplification of the HM1.24-coding sequences from nucleotides 8 to 557 (23). A control RT-PCR analysis of expression of the housekeeping gene TFIIB was also undertaken with the primer set 5'-GTGAAGATGGCGTCTACCAG-3' and 5'-GCCTCAATTTATAGCTGTGG-3', which amplified nucleotides 356 to 1314 of that mRNA. To ensure that DNA did not contribute to the results, a direct PCR (no RT step) was also undertaken.

Construction of the AAV/HM1.24/Neo genome and generation and titering of virus stocks

The HM1.24 cDNA was sequenced before it was ligated into an AAV vector, dl6-95. The AAV/HM1.24/Neo genome was constructed as a plasmid in a similar manner to that previously described for other AAV vectors (20, 22). AAV/HM1.24/Neo virus stocks were generated, using either complementor plasmids ins96-0.8 or pSH3 using 293 cells as described previously (20,22,23). To generate purified recombinant AAV virus, the technique described by Auricchio et al. was used (24). Purity of the viral preparation was assessed on SDS-PAGE, and AAV capsid proteins were detected by Comassie blue staining (data not shown). The titer of purified virus, in encapsidated genomes per milliliter (eg/ml), was calculated by dot-blot hybridization, as previously described (20,22). Lysates of 293 cells were used as a virus-negative control for mock infection.

Cellular Materials

Two myeloma cell lines, ARP-1 and ARK-B, gifts from J. Epstein, University of Arkansas for Medical Sciences, Arkansas) were used. The ARP-1 and ARK-B cell lines were established from bone marrow aspirate of patients with multiple myeloma. Peripheral blood mononuclear cells (PBMC) were obtained from three healthy male and female donors. Anti-Syndecan-1 (CD138) antibody-sorted tumor cells were obtained from four patients with multiple myeloma. The HLA phenotypes of these patient's cells, cell lines, and donor's cells are shown in Table 1. All clinical materials were obtained with the patients' consents and approval from the local ethics committee.
Generation and infection of monocyte-derived DC
DC (2 X 10^5 adherent monocytes [Mo]) were generated and infected (0.5 ml of virus [10^9 eg/ml]) as previously described (20,22). Recombinant GM-CSF (sargramostim; Leukine, Immunex Corp., Seattle, WA) at a final concentration of 800 IU/ml was included in the medium throughout the culture. To induce the maturation of monocytes into DC, human interleukin-4 (IL-4) (R&D Systems Inc., Minneapolis, MN) at 1000 IU/ml was added on day 3.

Generation of autologous HM1.24-positive target cells
Non-adherent peripheral blood mononuclear cells (PBMC) isolated from normal donors were infected with AAV/HM1.24 virus (no Neo), at a multiplicity of infection of 100, four days before 51Cr labeling and the 51-chromium-release assay. All clinical materials were obtained with patient consent and approval from the local ethics committee.

Detection of viral integration by PCR/Southern blot analysis
Chromosomal integration of the AAV/HM1.24/Neo genome was undertaken by vector-chromosome junction PCR amplification and Southern blot analysis, as previously described (20-22).

Generation and testing of HM1.24-specific CTLs
CTL experiments were done in quadruplicate using cells from three donors. For each experiment the non-adherent PBMCs from the same healthy donor (A1) were washed and resuspended in AIM-V at 10-20x10^6 cells per well in six-well culture plates with AAV/HM1.24/Neo-loaded DC (ratios of responders to DC from 20:1). The cultures were supplemented with GM-CSF (800 U/ml) and recombinant human IL-2 (10 U/ml). After 7 days of co-culture, the cells were used for cytotoxicity assays in a 6-hour chromium-release assay, as previously described (21,22). To determine the HLA-dependency of the CTL activity the 50 ul of antibodies against HLA Class I (W6/32) and Class II (L243), at the concentration of 50 ug/ml, were preincubated with the target cells for 30 minutes before addition of the stimulated T cells. K562 cells were used as targets to observe natural killer (NK) cell activity. In all these CTL killing assays spontaneous release of chromium never exceeded 25% of maximum release.

Flow cytometry analysis of intracellular cytokines
This protocol was adapted from that described by Pala et al and modified as published previously (21,22,25). Cell surface marker analysis of T cells and DC by fluorescence-activated cell scanning. Cells were analyzed by flow cytometry (FACScan; BD Biosciences), as described previously (21,22).

Statistics
All results are expressed as mean +/- SD. The data were analyzed by nonparametric ANOVA test. If differences were detected between means, Newman-Keuls test was used for multiple comparison. Differences were considered as significant if P<0.05.
RESULTS
AAV/HM1.24/Neo-transduced DC express HM1.24
The goal of this study was to determine whether recombinant adeno-associated virus (rAAV)-based gene loading of the multiple myeloma auto-antigen HM1.24 gene into DC could elicit a significant CTL response against HM1.24-positive targets and MM. The myeloma cell lines ARK-B and ARP-1 were each used to generate an HM1.24 cDNA. Figure 1 shows the RT-PCR amplification of the HM1.24-coding sequences from both the ARK-B and the ARP-1 cell lines. The PCR-only control (no RT step), lacking an HM1.24 amplified product, indicating that no DNA was present in our RNA samples. The HM1.24 cDNA from ARK-B was inserted into a gutted AAV vector (dl6-95) to generate an AAV/HM1.24/Neo vector as described in Materials and Methods. Figure 2A shows a structural map of the resulting AAV/HM1.24/Neo vector. In this vector, the HM1.24 gene was expressed from the AAV p5 promoter, which is known to be active in DC (20-22). Before use the AAV/HM1.24/Neo vector was sequenced and found to contain only the HM1.24 and Neo genes. No extraneous genes or DNA sequences were found. A two-step process was used to generate an AAV/HM1.24/Neo virus stock, as previously described (20). Two clones were isolated and used to generate AAV/HM1.24/Neo viral stocks. Figure 2B shows that the titer of the virus stocks was approximately 10^9 eg (encapsidated genomes)/ml.

Protocols for the generation of DC by differentiation of PBMCs usually involve treating adherent monocytes with GM-CSF and IL-4. We modified this protocol to promote AAV vector transduction in DC precursor monocytes by treating adherent monocytes just after AAV infection with GM-CSF alone for several days before the addition of IL-4 on day 3 (21). This allows for a brief period of monocyte proliferation which promotes higher levels of AAV transduction (20). A schematic diagram of the experimental protocol is shown in Figure 2C. The transduction of the Mo/DC population was confirmed by measuring polyadenylated RNA expression of the HM1.24 transgene. At day 10, polyadenylated RNA was isolated from AAV/HM1.24/Neo-infected and mock-infected DC culture. This mRNA was then analyzed by RT-PCR for HM1.24 expression. A cellular gene, TF II B, was included as a control. As shown Figure 3, HM1.24 mRNA expression only took place in virally infected DC. A PCR-only control (no RT step) failed to generate a product, indicating that there was no DNA contamination in our samples.

AAV/HM1.24/Neo infection results in chromosomal integration
We next investigated chromosomal integration of the AAV/HM1.24/Neo vector in DC. Chromosomal integration, while not essential for gene expression by AAV vectors, signifies a permanent genetic alteration of the DC and is often desirable for viral transduction. The analysis was carried out by PCR amplification of vector-chromosome junctions with primers complementary to the SV40 promoter within the vector and the AluI repetitive chromosomal element (20). rAAV-cell junction products were analyzed by PCR amplification and Southern blot analysis, with probing for the Neo gene sequences. Multiple vector-chromosomal junction products were observed in the AAV/HM1.24/Neo-infected DC, but not in mock-infected (uninfected) DC (Figure 4), indicating vector-chromosomal integration in the DC population.

AAV/HM1.24/Neo-transduced DC stimulated HM1.24-specific CTLs
Next, the ability of the AAV/HM1.24/Neo vector to generate effective CTL was analyzed. DC were loaded by one of two techniques, lysate or vector (Figure 2C). In our chromium-release
In assays, Figures 5-10, we utilize a total of eight target cell types. One type consisted of autologous PBMC. As late B cells are only a small percentage of PBMC, PBMC serve as an autologous, antigen-negative control as verified by RT-PCR. PBMC were transfected with an HM1.24 expression plasmid to yield an autologous, antigen-positive control. Two additional targets were the HM1.24 positive myeloma cell lines ARK-B and ARP-1 (22,26). A final target type was primary multiple myeloma cells taken from four patients (PT1, PT2, PT3, and PT4). To determine the ability of AAV/HM1.24/Neo-transduced DC to stimulate HM1.24-specific CTLs, we carried out a standard 6-hour chromium-release assay (^{51}Cr ) on day 7, using the T-cell populations primed in co-culture with the rAAV-transduced DC.

Experiments shown in Figure 5 were designed to test the antigen specificity of AAV-based DC loading. In this experimental type four different DC loading treatments were carried out, each using one of four different AAV vectors (each containing a different transgene or wild type [wt] AAV). Only one vector, AAV/HM1.24/Neo, contained the HM1.24 gene. The four different DC treatments by these vectors were then compared for the ability to stimulate CTL killing of HM1.24-positive cells. As can be seen only those T cells incubated with AAV/HM1.24/Neo-loaded DC were able to kill the HM1.24-positive autologous target cells. The other three AAV viruses, wt AAV, AAV/Neo, and AAV/E7/Neo, all lacking HM1.24, when used to load DC failed to stimulate killing of the HM1.24-positive targets. All three donors gave similar results. These data are fully consistent with strong antigen-specific CTL response.

Experiments shown in Figure 6 were designed to test the specificity of killing by T cells stimulated by AAV/HM1.24 /Neo-loaded DC in a fully autologous system. Autologous targets were generated by infecting donor PBMC with AAV/HM1.24 virus (no Neo) four days before the CTL assay. These HM1.24-infected PBMC were found to express HM1.24 by RT-PCR analysis, while unaltered PBMC did not express HM1.24 (data not shown). The results show that only HM1.24-expressing autologous cells were targeted for killing, consistent with strong antigen specificity.

Experiments shown in Figure 7 were also designed to test the specificity of killing by T cells stimulated by AAV/HM1.24 /Neo-loaded DC. This time many different HLA-matched antigen-positive and antigen-negative targets. While unaltered PBMC and K562 cells do not express HM1.24, the MM cells from patients 1-4 do express (data not shown), as do the two MM cell lines (Figure 1). The results show that only HM1.24 expressing cells were targeted for killing. These data, and those of Figure 6 are fully consistent with the resulting CTL being highly HM1.24-specific.

Next, Class I-restriction was examined using the four different PT-14 cells as targets, as seen in Figure 8. Where indicated the targets were pre-incubated with anti-isotype, anti-Class I or anti-Class II antibody. As can be seen the CTL killing was blocked by anti-Class I antibody, but not by anti-isotype or anti-Class II antibody, strongly suggesting Class I-restriction.

An CTL assay type, shown in Figure 9, similar to Figure 5, was designed to test the dose-dependent nature of the AAV-based DC loading on CTL killing. In this experiment three different dosages of AAV/HM1.24/Neo vector was used for DC loading, as well as a zero virus control. As can be seen the percentage of target killing effected by the stimulated T cells directly correlated with the
amount of AAV/HM1.24/Neo used to load the DC at day 0. Finally in Figure 10, different effector:target ratios were used to test how robust the CTL killing efficiency was. As can be seen the killing of the targets was indeed dependent upon the effector:target ratio, with a higher ratio resulting in higher killing.

**Immunophenotypes and cytokine profile of T cells**

Immunophenotyping of the AAV/HM1.24/Neo-loaded DC-primed T-cell populations showed that they expressed predominantly CD8 (80%), in contrast to lysate-loaded, mock-infected cells (45%) (Figure 11). Furthermore, the CD8-to-CD56 ratio was substantially higher in the AAV-loaded situation, increasing from a ratio of 1.9 to 8. To determine the cytokine profile of the T cells generated from co-culture with AAV/HM1.24/Neo-loaded DC or lysate-loaded DC, we carried out intracellular staining of these T cells for IFN-γ and IL-4. Figure 11 demonstrates that most of the AAV/HM1.24-loaded or -primed T cells expressed IFN-γ (64%) and very little IL-4 (4%), suggesting that these cells are of the Th1 and Tc1 phenotypes. A smaller proportion of IFN-γ-producing T cells (30%) were observed in the T-cell populations primed by lysate (mock)-loaded DC (zero virus control).

**Characterization of DC by various manipulations**

Finally, we phenotyped the DC generated from the lysate-loaded, wild-type AAV-loaded, and AAV/HM1.24/Neo-loaded DC populations from four individuals (each done four times) by flow cytometry. The results demonstrate that DC generated by all three techniques share common DC markers (Figure 12). As can be seen in Figure 12A, the results show that the mean fluorescence index (MFI) for all three co-stimulatory CD80, CD86, and CD40 was significantly increased ($P = 0.000$ to 0.042, see Figure 12B) by both AAV/HM1.24/Neo and wild type AAV compared to the lysate control. Of the three CD80 was upregulated the most (2.6-3.8 fold increase), followed by CD86 and CD40 (both about 1.5 fold). The percentage of cells expressing these markers was also higher in both the wt AAV and AAV/HM1.24 situations. Thus, in addition to the ability of AAV vectors to transduce high percentages of DC (21,22), the upregulation of these multiple co-stimulatory molecules could help to explain the rapid CTL expansion observed.

**Discussion**

We and others have hypothesized that antigen gene delivery into DC may be more efficient for the generation of CTL than delivery of the antigen as a lipofected, exogenous protein. Although there is some controversy as to AAV’s effectiveness at transducing DC and some other hematopoietic cells, we have not yet found a donor whose Mo/DC are unable to be transduced by AAV-2 (20-22). Furthermore in various studies, AAV has been shown to be an effective gene-delivery vector for both immortalized tissue culture cells and primary hematopoietic cells (20-22,27-35). We recently showed that it is possible to successfully transduce, with chromosomal integration, the GM-CSF cytokine and the human papillomavirus (HPV) E6 and E7 antigen genes into monocytes and derived DC via rAAV (20-22). In fact, this DC loading technique was found to be highly effective, generating significant CTL with only one DC-T cell co-incubation and in only one week. To our knowledge no other group is using our technique of transducing Mo-derived DC with rAAV. This is by first treating the rAAV-infected Mo with only
GM-CSF and then adding IL-4 after several days to induce differentiation into DC. This technique converts >90% of DC (22).

Our previous studies show that rAAV-loading DC can rapidly generate antigen-specific CTL against viral antigens (21,22). Here we have increased the difficulty of the CTL generation, by studying a self-antigen, the late B cell marker HM1.24. Generating a rapid CTL response against a self-antigen would likely be a more difficult task, as putatively there would be a much lower (about 1% that for viral antigens) precursor T cell frequency against such auto-antigens. We have demonstrated here that rAAV-loading of DC with HM1.24 generated antigen-specific CTL in substantial numbers, in only one week and only one stimulation as we have found for generating CTL against viral antigens (21,22). Another group has also recently demonstrated that AAV is effective at stimulating T cell response against self antigens (36). Based on this and our previous studies, we hypothesize that the AAV vector is causing a fundamental change in DC performance, perhaps by modifying co-stimulatory ligand expression on DC that result in more efficient generation of antigen-specific CTL (21,22). In fact, in Figure 12 we observe high CD80, CD86, and CD40 upregulation stimulated by either rAAV infection or wild type AAV. The similarity of upregulation of these co-stimulatory molecules by wt AAV and AAV/HM1.24/Neo suggest that most likely something present within the virus particle itself is causing this increase in expression. Possibly it is the AAV viral capsid proteins that are causing this effect. This hypothesis can be tested. For CD80, with the marker most strongly upregulated, both AAV and the HM1.24 transgene may be contributing to this upregulation.

Some may argue that the CTL expansion we observe is simply too rapid to be antigen-specific, and that what we are observing is possibly non-specific killing, possibly NK activity. Yet our controls show strong antigen specificity and MHC Class I-restriction. For example, in Figure 5B second lane, it is demonstrated that autologous PBL are not targeted for killing unless these target cells have been pre-loaded with antigen (lanes 3-5). Without loading the antigen there is no significant killing. Furthermore, the lack of involvement of NK cells in the killing that we observe is demonstrated in two different assay systems. First, K562 cells are shown not to be significant targets in Figures 5B and C. Second, our stimulated T cell populations contain only low levels of NK cells as shown in Figure 6. We can find no evidence of significant non-specific killing activity.

This issue can be further addressed on a mathematical level knowing the frequency of self-antigen recognizing T cells and the speed of T cell replication. It has been reported that once activated, T cells are capable of dividing 2-6 times per 24 hours (37,38). Also it has been reported that self-antigen specific T cells, for any particular protein, are usually present at frequencies of 10^5, approximately 100 fold more rare than virus-specific T cells (39, 40). Therefore, when we stimulate using approximately 10^7 T cells in our assays, it is mathematically possible to generate anywhere from 10^5-10^9 antigen specific T cells over a one week expansion. In any case, our data strongly support that AAV-loading derived CTLs are antigen/HM1.24 specific.

We believe it likely that there are multiple reasons why AAV-loading of DC is effective. One reason is the high transduction frequency which we have observed (>90% DC transduction, viral genetic alteration). The increased expression of CD80, CD86, and CD40 may also contribute to the generation of the robust CTL response. Ultimately we would like to uncover all of the mechanisms of action which make AAV-based loading so effective. This study also suggests that HM1.24 may be a useful target for anti-myeloma immunotherapy. ARK-B myeloma cell line and the three patient MM cells were shown to be excellent targets for anti-HM1.24-sensitized T cells.
 (>70% killing), while the other cell line (ARP-1) showed lower target killing (30%). Our findings suggest that rAAV/antigen gene loading of DC may be a particularly good protocol for CTL generation against self-antigens which may not otherwise be considered good targets due to low immunogenicity, as for example MM idiotype.

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Competing Interests statement
The authors declare they have no competing financial interests.
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Table 1: HLA typing of donors and cell lines.

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Figure 1. Analysis and generation of HM1.24 cDNA in myeloma cell lines. RT-PCR was performed on polyA-selected RNA to generate HM1.24 cDNA, as described in Materials and Methods. Note that both ARK-B and ARP-1 cell lines expressed HM1.24, as indicated by the appropriately sized band. All the MM cell lines and MM primary cells used in this study were found to express HM1.24 by RT-PCR (data not shown).
Figure 2. AAV/HM1.24/Neo vector, producer cell lines, and experimental scheme. (A) A structural map of the AAV/HM1.24/Neo virus (also known as dl6-95/HM1.24p5/NeoSV40) shows the names of the components at the top. TR (black box) refers to the AAV terminal repeats. P5 (bent arrow) refers to the AAV p5 promoter. SV40 (bent arrow) refers to the SV40 early enhancer/promoter. The boxes labeled HM1.24 and Neo represent the indicated open reading frames. (B) Titer analysis of the AAV/HM1.24/Neo stock used in these experiments. (C) A graphic depiction of the experimental protocol.
Figure 3. HM1.24 mRNA expression in infected DC. Total RNA was isolated from three cell populations: mock-infected and AAV/HM1.24/Neo-infected adherent monocytes at 72 hours postinfection. These samples were analyzed by RT-PCR and PCR, as indicated, for the presence of HM1.24 RNA, as described in Materials and Methods. The positive control was the PCR product resulting from using the AAV/HM1.24/Neo vector plasmid as a template. Another control was RT-PCR analysis for the cellular TFIIB mRNA. Note that only RNA from cells infected with AAV/HM1.24/Neo virus resulted in an appropriate RT-PCR-sized product, whereas mock-infected cells did not.
Figure 4. Chromosomal integration by AAV/HM1.24/Neo in DC. Cells, treated as indicated and as described in Materials and Methods and sorted for CD83 expression, were further analyzed for chromosomally integrated AAV/HM1.24/Neo viral genomes. Total cellular DNA (0.1 ug) from infected, CD83+-selected, and uninfected cells served as a template in PCR amplification assays using primers targeting of the SV40 early promoter of the vector and the cellular repetitive AluI element. The products underwent Southern blotting and were probed with 32P-Neo DNA. The positive control lane contained 100 ng of BamHI-digested AAV/HM1.24/Neo plasmid (6.5 kb and 2 kb). Note that multiple Neo-positive bands resulted from the infected cell population, indicating chromosomal integration by the vector, and that multiple vector-positive cell clones were present in the population.
Figure 5. Loading specificity: CTL response generated by various AAV vectors against an HM1.24-positive autologous target. Shown are CTL response by three different donors (each done in quadruplicate) at the top and a compilation of the results from all three donors just below. These data demonstrate the cytotoxic response resulting from the indicated AAV vector loading techniques in a fully autologous system. Target cells were generated by introduction of the HM1.24 gene into autologous PBL four days before the CTL assay as described in the Materials and Methods section. Equivalent encapsidated genomes of the indicated vectors were used to load the DC. Note that T cells stimulated by lysate (mock-infected, no virus) loaded DC, wild type (wt) AAV loaded DC, AAV/Neo loaded DC, or AAV/E7/Neo loaded DC did not kill HM1.24-positive targets. However T cells stimulated by AAV/HM1.24/Neo loaded DC did kill HM1.24-positive target cells. These data strongly suggest high antigen loading specificity of the CTL generated by AAV/HM1.24/Neo infection of DC.
Figure 6. Target specificity: AAV/HM1.24 generated CTL response against HM1.24-positive and -negative autologous targets. Shown are CTL response by three different donors (each done in quadruplicate) at the top and a compilation of the results from all three donors at the bottom. These data demonstrate the cytotoxic response resulting from AAV/HM1.24/Neo vector loading of DC is antigen specific in a fully autologous system. CTL were generated by AAV/HM1.24/Neo-loading (1 ml of virus or approximately 2X10⁹ EG) DC and T cells stimulated as described in Figure 2. Two target types are used: autologous PBMC (no HM1.24 antigen) and PBMC which had been pre-loaded with M1.24 antigen four days before the chromium release assay. Not that only the HM1.24-positive PBMC targets were killed by the CTL.
Figure 7. Target specificity: AAV/HM1.24 generated CTL response against various HM1.24-positive and -negative HLA-matched targets. (A) A key shows the eight different target cells used in the experiments displayed in panels B and C. (B) Shows a stack of CTL experiments from each of three donors (each done in quadruplicate) and a compilation of the three experiments, as indicated. Of the target cells used only autologous PBMC and K562 cells were HM1.24-negative. All others displayed some level of HM1.24 expression by RT-PCR analysis (data not shown). Note that all four primary MM (HM1.24 positive) and both MM cell lines were killed by the HM1.24-stimulated CTL. However also note that HM1.24-negative PBMC and K562 cells were not killed, indicating strong antigen specificity of the CTL generated by AAV/HM1.24 loading.
Figure 8. Class I-restriction by the AAV/HM1.24 generated CTL response. The cell key is the same as in Figure 7. These experiments are similar to Figure 5 except here the target cells were preincubated with either anti-isotype, anti-Class I anti-body (W6/32), anti-Class II antibody (L243), or no antibody. Shown at the top are the CTL response (as percent killing) by three different donors (each done in quadruplicate) against the MM cells from four different patients. The experimental labels at the bottom of the figure identify these CTL responses. At the bottom are four sub-figures, each being a compilation of the results from all three donors against one of the patients. Note that the addition of anti-Class I anti-body significantly inhibited killing, while all others did not. Also note that the CTL generated from wt AAV-loading DC did not kill the HM1.24-positive targets. All donors HLA-matched the primary MM cells, as indicated by the key of Figure 6, are used in place of the HM1.24-positive autologous PBL targets. Note that, again, only the anti-Class I antibody inhibited killing, strongly suggesting Class I-restricted killing by these CTL.
Figure 9. Dosage dependent CTL killing. Shown are resulting CTL from AAV-loading-dose responses by three different donors (each done in quadruplicate) at the top and a compilation of the results from all three donors at the bottom. These experiments were done similar to Figure 5 except that the DC where initially loaded (infected at day 0) with different amounts of the AAV/HM1.24/Neo virus as indicated. “Lysate” refers to 293 cell lysates. This cell lysate contains no virus, and is thus a mock infection. Target cells were generated by introduction of the HM1.24 gene into autologous PBL four days before the CTL assay as described in the Materials and Methods section. Note that the resulting CTL gave a level of killing which directly correlated with the amount of virus originally used for loading DC at day 0.
Figure 10: CTL killing at various effector to target ratios. The cell key is the same as in Figure 7. These experiments are similar to Figure 5 except here the target cells were incubated with different amounts of effector T cells (giving different effector:target [E:T] ratios). Like Figure 5, here the target cells were also preincubated with either anti-Class I anti-body (W6/32), anti-Class II antibody (L243), or no antibody. Shown is a two layer panel, and each panel includes the results using two different MM targets (four PT total). At the top of each panel are the CTL response (as percent killing with standard deviation) by three different donors (each done in quadruplicate) against the MM cells from two different PT as indicated. The experimental labels at the bottom of the figure and the target cell key identify these CTL responses, with the E:T ratios indicated at the bottom of each panel. Note that all donor's resulting CTL gave a level of killing which directly correlated with the effector cell: target cell ratio, with increasing ratios (increasing addition of CTL) resulting in increasing target killing. Also note, as in Figure 8, the addition of anti-Class I antibody inhibited killing, while anti-Class II antibody did not.
Figure 11. Two-color flow cytometric characterization and intracellular cytokine expression in primed T-cell populations. Shown is a representative experiment of three such independent experiments (each using a different donor). Shown is the FACS analysis, giving CD8 and CD56 prevalence within the primed population resulting from lysate- or AAV/HM1.24/Neo-loading techniques. In addition, the intracellular prevalence of IFN-γ and IL-4 within primed and stimulated mixed T-cell populations is shown resulting from lysate- and AAV/HM1.24/Neo-loading techniques. Note that the use of AAV/HM1.24/Neo-loading resulted in a higher IFNγ:IL-4 ratio and a lower CD56:CD8 ratio.
Figure 12. Characterization of DC on day 7 under different conditions. Monocytes from four different donors were generated by the three different loading techniques and stimulated to differentiate into DC by GM-CSF and IL-4 as indicated in the Materials and Methods section and shown in Figure 2. The three DC populations were analyzed by FACS for mean fluorescent intensity (MFI) and percent positivity. For the analysis of DC, a panel of MoAbs recognizing the following antigens was used: anti-CD40 (Immunotech, Marseille, France), anti-CD14, anti-CD80 (BD Biosciences), and anti-CD86 (BD Biosciences-PharMingen, San Diego, CA). A shows a quantification of the data from all four donors (each done four times, four independent experiments). In table A both the percent positivity and MFI are given. B shows the $P$ values for the indicated comparisons. NS means not significant.
Testing of recombinant adeno-associated virus-gene loading of dendritic cells for generating potent cytotoxic T lymphocytes against a prototype self-antigen, multiple myeloma HM1.24

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