Testing recombinant adeno-associated virus-gene loading of dendritic cells for generating potent cytotoxic T lymphocytes against a prototype self-antigen, multiple myeloma HM1.24

Maurizio Chiriva-Internati, Yong Liu, Jon A. Weidanz, Fabio Grizzi, Hong You, Weiping Zhou, Klaus Bumm, Barthel Barlogie, Jawahar L. Mehta, and Paul L. Hermonat

Recent studies demonstrate that recombinant adeno-associated virus (rAAV)-based antigen loading of dendritic cells (DCs) generates significant and rapid (one stimulation per 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 major histocompatibility complex (MHC) class 1–restricted, anti-HM1.24–specific CTL killing was demonstrated against MM cells. Furthermore, higher expression of interferon-γ (IFN-γ) in T cells and higher expression levels of, in order of significance, CD80 (2.6- to 3.8-fold increase), CD86, and CD40 on DCs were also observed. The use of synthetic HM1.24-positive target cells further demonstrated the antigen specificity of these CTLs. There was also no evidence of natural killer cell involvement. These data extend our earlier studies and suggest that the rAAV-loading of DCs may be a particularly good protocol for generating CTLs against self-antigens, which may not otherwise be considered good targets because of their low immunogenicity. We also show that HM1.24 may be an effective antigen for targeting MM. (Blood. 2003;102:3100-3107)

© 2003 by The American Society of Hematology

Introduction

Multiple myeloma (MM) is a malignancy characterized by clonal proliferation and accumulation of immunoglobulin-producing plasma cells, which are terminally differentiated B cells. 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. 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 autotransplantation. 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 (mAb HM1.24) that appears to be a novel terminal B-cell–restricted antigen expressed on tumor cells and on mature immunoglobulin-secreting B cells (plasma cells and lymphoplasmaicytoid cells) but not on other cells in the peripheral blood, bone marrow, liver, spleen, kidney, or heart of healthy persons or patients with non–plasma-cell–related malignancies. HM1.24 effectiveness against MM has been demonstrated through tumor cell lysis by mAb and complement. The HM1.24 coding sequence is 1 kb long, an ideal size for ligation into any viral vector including adeno-associated virus (AAV).

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

#### Generating HM1.24 cDNA and RT-PCR analysis for HM1.24 expression

Total RNA from 2 human MM cell lines, ARK-B and ARP-1, was used to obtain HM1.24 mRNA by reverse transcription–polymerase chain reaction (RT-PCR). HM1.24 expression of rAAV-loaded DCs was also analyzed by RT-PCR. Total RNA was first isolated from these cells with TRIzol (Gibco BRL/Life Technologies, Rockville, MD) and was treated with 10 U/g RNase-free DNase I (Promega, Madison, WI) for 1 hour at 37°C. Messenger RNA was then separated with the use of the Oligotex mRNA kit (Qiagen, Valencia, CA). First-strand cDNA synthesis was performed at 37°C for 1 hour in a final volume of 25 μL reaction buffer (0.5 μg mRNA; 50 mM Tris-HCl, pH 8.3; 75 mM KCl; 3 mM MgCl2; 10 mM dithiothreitol [DTT]; 0.5 g oligo(dT)15 (Promega); 0.5 mM each of the 4 deoxynucleotide triphosphates; 30 U RNasin ribonuclease inhibitor (Promega); and 50 mM Tris-HCl, pH 8.3; 75 mM KCl; 3 mM MgCl2; 10 mM dithiothreitol [DTT]; 0.5 g oligo(dT)15 (Promega); 0.5 mM each of the 4 deoxynucleotide triphosphates; 30 U RNasin ribonuclease inhibitor (Promega); and 0.5 U TaKaRa Z-Taq polymerase according to the manufacturer’s protocol (TaKaRa Shuzo, Otsu, Japan). The HM1.24 primer set was designed from the HM1.24 sequences described by Ohtomo et al. This primer set (upstream, 5′-TGTTGATCTGGGTGATGCA-3′; downstream, 5′-ATGGTGCGTCTACCAG-3′) refers to the SV40 early enhancer/promoter. Targeted amplification of the HM1.24 cDNA was performed in a 100-μL reaction volume containing 2.5 U TaKaRa Z-Taq polymerase (Promega), 100 μM each of the 4 deoxynucleotide triphosphates; 30 U RNasin ribonuclease inhibitor (Promega); and 0.5 U TaKaRa Z-Taq polymerase according to the manufacturer’s protocol (TaKaRa Shuzo, Otsu, Japan). The HM1.24 primer set was designed from the HM1.24 sequences described by Ohtomo et al. This primer set (upstream, 5′-TGTTGATCTGGGTGATGCA-3′; downstream, 5′-ATGGTGCGTCTACCAG-3′) targeted the amplification of the HM1.24-coding sequences from nucleotides 8 to 557. Control RT-PCR analysis of expression of the housekeeping gene TFIIB was also undertaken with the primer set 5′-CAGAATATTTGCTATGTGAGG-3′ and 5′-ATGCCAATAATTTAGCCTGAA-3′, which amplified nucleotides 356 to 1314 of that mRNA. To ensure that DNA did not contribute to the results, direct PCR (no RT step) was also undertaken.

#### Constructing the AAV/HM1.24/Neo genome and generation of the titer of virus stocks

The HM1.24 cDNA was sequenced before it was ligated into an AAV vector, d6-95. The AAV/HM1.24/Neo genome was constructed as a plasmid in a manner similar to that previously described for other AAV vectors. AAHV/HM1.24/Neo virus stocks were generated using either complementary plasmids ins96-0.8 or pSH3 using 293 cells as described previously. To generate purified recombinant AAV virus, the technique described by Auricchio et al was used. Purity of the viral preparation was assessed on sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE), and AAV capsid proteins were detected by Coomassie 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. Lysses of 293T cells were used as a virus-negative control for mock infection.

### Cellular materials

Two myeloma cell lines, ARK-1 and ARK-B (gifts from J. Epstein, University of Arkansas for Medical Sciences), were used. The ARK-1 and ARK-B cell lines were established from bone marrow aspirate of patients with multiple myeloma. Peripheral blood mononuclear cells (PBMCs) were obtained from healthy male and female donors. Anti–syndecan-1 (CD138) antibody-sorted tumor cells were obtained from 4 patients with multiple myeloma. The HLA phenotypes of these patients’ cells and cell lines and from donor cells are shown in Table 1. All clinical materials were obtained with each patient’s consent and with approval from the local ethics committee.

### Generation and infection of monocyte-derived DCs

DCs (2×10⁵ adherent monocytes) were generated and infected (0.5 mL virus [10⁸ eg/mL]) as previously described. Recombinant granulocyte macrophage–colony-stimulating factor (GM-CSF) (sargramostim [Leukine]; Immunex, Seattle, WA) at a final concentration of 800 IU/mL was included in the medium throughout the culture. To induce the maturation of
Chromosomal integration of the AAV/HM1.24/Neo genome was under- taken by vector-chromosome junction PCR amplification and Southern blot analysis using primers targeting the SV40 early promoter of the vector and the cellular repetitive Alu element. The products underwent Southern blotting and were probed with 32P-Neo labeled DNA. The positive control lane contained 100 ng HI-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.

Differences were considered significant if \( P < .05 \).
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 $10^6$ e.g/mL.

Protocols for generating DCs by differentiating 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 adding IL-4 on day 3. This allows for a brief period of monocyte proliferation which promotes higher levels of AAV transduction. A schematic diagram of the experimental protocol is shown in Figure 2C. The transduction of the monocyte/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, TFR2R, was included as a control. As shown in Figure 3, HM1.24 mRNA expression took place only 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 DCs. Chromosomal integration, though not essential for gene expression by AAV vectors, signifies a permanent genetic alteration of the DC and is often desirable for viral transduction.

Analysis was carried out by PCR amplification of vector-chromosome junctions with primers complementary to the SV40 promoter within the vector and the Alu repetitive chromosomal element. rAAV-cell junction products were analyzed by PCR amplification and Southern blot analysis, with probing for the Neo gene sequences. Multiple vector-chromosome junction products were observed in the AAV/HM1.24/Neo-infected DCs, but not in mock-infected (uninfected) DCs (Figure 4), indicating vector-chromosomal integration in the DC population.

AAV/HM1.24/Neo-transduced DCs stimulated HM1.24-specific CTLs

Next, the ability of the AAV/HM1.24/Neo vector to generate effective CTLs was analyzed. DCs were loaded by 1 of 2 techniques—lysat or vector (Figure 2C). In our 51Cr assays (Figures 5, 6, 7, 8, 9, 10), we used 8 target cell types. One type consisted of autologous PBMCs. Because late B cells are only a small percentage of PBMCs, PBMCs serve as an autologous, antigen-negative control as verified by RT-PCR. PBMCs 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. A final target type was primary multiple myeloma cells taken from 4 patients (patients 1–4). To determine the ability of AAV/HM1.24/Neo-transduced DCs to stimulate HM1.24-specific CTLs, we carried out a standard 6-hour 51Cr assay on day 7 using the T-cell populations primed in coculture with the rAAV-transduced DCs.
Experiments shown in Figure 5 were designed to test the antigen specificity of AAV-based DC loading. In this experimental type, 4 different DC loading treatments were carried out, each using 1 of 4 different AAV vectors (each containing a different transgene or wild-type AAV). Only one vector, AAV/HM1.24/Neo, contained the HM1.24 gene. The 4 different DC treatments by these vectors were then compared for their ability to stimulate CTL killing of HM1.24-positive cells. As can be seen, only T cells incubated with AAV/HM1.24/Neo-loaded DC were able to kill the HM1.24-positive autologous target cells. When used to load DCs, the other 3 AAV viruses—wt AAV, AAV/Neo, and AAV/E7/Neo, all lacking HM1.24—failed to stimulate killing of the HM1.24-positive targets. All 3 donors gave similar results. These data are fully consistent with a 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 DCs in a fully autologous system. We generated autologous targets by infecting donor PBMCs with AAV/HM1.24 virus (no Neo) 4 days before the CTL assay. These HM1.24-infected PBMCs were found to express HM1.24 by RT-PCR analysis, whereas unaltered PBMCs 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 DCs. This time many different HLA-matched antigen-positive and antigen-negative targets were used. Although unaltered PBMCs and K562 cells did not express HM1.24, the MM cells from patients 1 to 4 did (data not shown), as did the 2 MM cell lines (Figure 1). The results showed that only HM1.24-expressing cells were targeted for killing. These data and those in Figure 6 are fully consistent with the resultant CTLs being highly HM1.24 specific.

Next class 1 restriction was examined using the 4 different PT1-4 cells as targets, as seen in Figure 8. Where indicated, the targets were preincubated with anti-isotype, anti-class 1, or anti-class 2 antibody. Anti-class 1 antibody, but not anti-isotype or anti-class 2 antibody, blocked CTL killing, strongly suggesting class 1 restriction.

A CTL assay type, shown in Figure 9 and similar to that in Figure 5, was designed to test the dose-dependent nature of AAV-based DC loading on CTL killing. In this experiment, 3 different dosages of AAV/HM1.24/Neo vector were used for DC loading and a zero virus control (lysat). 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 DCs at day 0. Finally, in Figure 10, different effector-target ratios were used to test how robust CTL killing efficiency was. Killing of the targets was indeed dependent on the effector-target ratio, with a higher ratio resulting in higher killing.

### Immunophenotypes and cytokine profiles 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 lystate-loaded, mock-infected cells (45%) (Figure 11). Furthermore, the CD8/CD56 ratio was substantially higher in AAV loading, increasing from a ratio of 1.9:8. To determine the cytokine profile of the T cells generated from coculture with AAV/HM1.24/Neo-loaded DCs or lystate-loaded DCs, we carried out intracellular staining of these T cells for IFN-γ and IL-4. Figure 11 demonstrates that most (64%) of the AAV/HM1.24-loaded or -primed T cells...
expressed IFN-γ and that few (4%) expressed IL-4, suggesting that these cells are of the Th1 (helper) and Th1 (cytotoxic) phenotypes. A smaller proportion (30%) of IFN-γ-producing T cells were observed in the T-cell populations primed by lysate (mock)-loaded DCs (zero virus control).

Characterization of DCs by various manipulations

Finally, we phenotyped the DCs generated from the lysate-loaded, wild-type, AAV-loaded, and AAV/HM1.24/Neo-loaded DC populations from 4 patients (4 times each) using flow cytometry. DCs generated by all 3 techniques share common DC markers (Table 2). As can be further seen in Table 3, the mean fluorescence intensity (MFI) for all 3 markers—costimulatory CD80, CD86, and CD40—was significantly increased \((P = .000 to P = .042)\) by AAV/HM1.24/Neo and wild-type AAV compared with the lysate control. Of the 3 markers, CD80 was up-regulated the most (2.6- to 3.8-fold increase), followed by CD86 and CD40 (both approximately 1.5-fold). The percentage of cells expressing these markers was also higher in wild-type AAV and AAV/HM1.24. Thus, in addition to the ability of AAV vectors to transduce high percentages of DCs, the up-regulation of these multiple costimulatory molecules could help to explain the rapid CTL expansion observed.

Discussion

Other investigators and we have hypothesized that antigen gene delivery into DCs may be more efficient for generating CTLs than delivering the antigen as a lipoprotein, exogenous protein. Although there is some controversy as to AAV effectiveness at transducing DCs and some other hematopoietic cells, we have not yet found a donor whose monocytes/DCs are unable to be transduced by AAV-2. Furthermore, in various studies, AAV has been shown to be an effective gene-delivery vector for immortalized tissue culture cells and primary hematopoietic cells. 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 DCs by rAAV. In fact, this DC-loading technique was found to be highly effective, generating significant CTLs with only one DC–T-cell coinubation and in only 1 week. To our knowledge no other group is using our technique of transducing monocyte-derived DCs with rAAV. This occurs by first treating the rAAV-infected monocytes with only GM-CSF and then adding IL-4 after several days to induce differentiation into DCs. This technique converts more than 90% of DCs.

Our previous studies show that rAAV-loading DCs can rapidly generate antigen-specific CTLs against viral antigens. Here we...
have increased the difficulty of 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 because putatively there would be a lower (approximately 1%) that for viral antigens) precursor T-cell frequency against such autoantigens. We have demonstrated here that rAAV-loading of DCs with HM1.24 generated antigen-specific CTLs in substantial numbers, in only 1 week and 1 stimulation, as we have found for generating CTLs 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 causes a fundamental change in DC performance, perhaps by modifying costimulatory ligand expression on DCs that results in more efficient generation of antigen-specific CTLs.21,22 In fact, Tables 2 and 3 illustrate high CD80, CD86, and CD40 up-regulation stimulated by either rAAV infection or wild-type AAV. The similarity of up-regulation of these costimulatory molecules by wild-type AAV and AAV/HM1.24/Neo suggests that most likely something within the virus particle itself causes this increase in expression, possibly the AAV viral capsid proteins. This hypothesis can be tested. For CD80, with the marker most strongly up-regulated, AAV and the HM1.24 transgene may contribute to this up-regulation.

Some may argue that the CTL expansion we observe is simply too rapid to be antigen specific and that what we observe is possibly nonspecific killing or NK activity. Yet our controls show strong antigen specificity and MHC class 1 restriction. For example, the second lane of Figure 5B demonstrates that autologous PBLs are not targeted for killing unless these target cells have been preloaded 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 we observe is demonstrated in 2 different assay systems. First, K562 cells are shown in Figures 5B and C not to be significant targets. 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, nonspecific killing activity.

This issue can be further addressed on a mathematical level if the frequency of self-antigen–recognizing T cells and the speed of T-cell replication are known. It has been reported that once activated, T cells are capable of dividing 2 to 6 times in 24 hours.37,38 It has also been reported that self-antigen–specific T cells, for any particular protein, are usually present at frequencies of 10−5 cells—approximately 100-fold more rare than virus-specific T cells.39,40 Therefore, when we use approximately 105 T cells for stimulation in our assays, it is mathematically possible to generate anywhere from 103 to 109 antigen-specific T cells in a 1-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 DCs is effective. One reason is the high transduction frequency we have observed (more than 90% DC transduction, viral genetic alteration). The increased expression of CD80, CD86, and CD40 may also contribute to generating the robust CTL response. Ultimately, we would like to uncover all the mechanisms of action that make AAV-based loading so effective. This study also suggests that HM1.24 may be a useful target for antimyeloma immunotherapy. The ARK-B myeloma cell line and the 3 patient MM cells were shown to be excellent targets for anti-HM1.24–sensitized T cells (more than 70% killing), whereas the other cell line (ARP-1) showed lower target killing (30%). Our findings suggest that rAAV/antigen gene loading of DCs may be a particularly good protocol for CTL generation against self-antigens that may not otherwise be considered good targets because of low immunogenicity (for example, the MM idiomtype).

Acknowledgments

We thank Drs Carlo Croce and Soldano Ferrone for reviewing this manuscript and for many helpful discussions. We also thank the University of Arkansas for Medical Sciences Office of Grants and Scientific Publications for editorial assistance during the preparation of this manuscript.

References


Table 3. P values for indicated comparisons of DCs on day 7

<table>
<thead>
<tr>
<th>Marker</th>
<th>Lysate vs wt AVV</th>
<th>Lysate vs AAV/HM1.24/Neo</th>
<th>AAV/HM1.24/Neo vs wt AVV</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD14</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>CD80</td>
<td>.016</td>
<td>.000</td>
<td>NS</td>
</tr>
<tr>
<td>CD86</td>
<td>.001</td>
<td>.010</td>
<td>NS</td>
</tr>
<tr>
<td>CD40</td>
<td>.033</td>
<td>.042</td>
<td>NS</td>
</tr>
</tbody>
</table>

Data represent 4 independent experiments performed in quadruplicate. NS indicates not significant.


Testing recombinant adeno-associated virus-gene loading of dendritic cells for generating potent cytotoxic T lymphocytes against a prototype self-antigen, multiple myeloma HM1.24

Maurizio Chiriva-Internati, Yong Liu, Jon A. Weidanz, Fabio Grizzi, Hong You, Weiping Zhou, Klaus Bumm, Barthel Barlogie, Jawahar L. Mehta and Paul L. Hermonat