Chapter 7

Use of immunosuppressive regimens to reduce humoral immunogenicity generated by primary AAV vector delivery

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Abstract

Adeno-associated virus (AAV) vector-based gene therapy has proven to be effective in both clinical and pre-clinical settings. However, a successful re-administration of the therapy remains a challenge due to development of neutralizing antibodies (NAB) against the AAV capsid after the first administration. Therefore, exploration of strategies to minimize existing anti-AAV NAB to a level that would allow re-administration is of great interest.

In this study we evaluated the potential of a combined therapy of bortezomib and anti-CD20 monoclonal antibody, to reduce the pre-existing humoral immunity to AAV in mice.

The combination of bortezomib and anti-CD20 failed to reduce high anti-AAV1 NAB titer to a level that would permit re-administration of AAV1. In contrast, this immunosuppressive treatment prevented immune response against the immunogenic protein (ovalbumin) delivered as a transgene by AAV1.

Introduction

Recombinant adeno-associated viral vectors (AAVs) show great promise for gene therapy in a variety of different genetic disorders [1- 3]. AAV vectors have an excellent safety profile [4, 5] and were shown to mediate a stable therapeutic transgene expression in several non-clinical studies [1, 6-9] and more recently in clinical studies [10-13]. The majority of the transgenic proteins are expressed from episomal, double stranded DNA [14]. This characteristic raises the possibility that transgene expression will decline overtime with the natural turnover of transduced cells. Therefore, repeated administration may become necessary to maintain expression of the transgenic protein at therapeutic levels. The major challenge in a successful re-administration of AAV vectors, is the presence of neutralizing antibodies (NAB) that develop after the first administration. Those neutralizing serotype-specific antibodies directed towards the viral capsid proteins are reducing efficient gene transfer with rAAV of the same serotype [15, 16]. Therefore, the generation of a humoral immune response is limiting the use of the "vector of choice" more than once which is a potential concern for life–long chronic disorders for which re-administration might have to be considered. Hence, strategies to decrease existing anti-AAV NAB titers need to be explored.

Bortezomib, a selective inhibitor of the 26S proteasome [17, 18] has been shown to significantly decrease AAV specific humoral immune responses after AAV-based gene delivery in mice [19]. However, this inhibition was only partially effective and insufficient to allow subsequent re-administration with a recombinant AAV vector of the same serotype [19]. This limitation was shown to be due to the combination of residual antibody levels and the inability of bortezomib to completely deplete the memory B cells [19].

In this study, we explored the potential of a immune suppression protocol for which bortezomib was combined with an anti-CD20 monoclonal antibody, targeted against pre-B cells, mature B cells and memory B cells [20, 21], to reduce level of AAV specific NAB. The efficacy of the combined immunosuppressive regimen was challenged by injecting mice with AAV1 carrying an expression cassette for ovalbumine (OVA).

Materials & methods

Study Design

C57BL/6 mice (males, age 6-8 weeks) were obtained from Harlan Laboratories, the Netherlands (n=6 per group). The experimental protocol was approved by the ethical committee for animal welfare of the AMC (Academic Medical Center, Amsterdam, the Netherlands). The general procedure is summarized in **Figure 1.** Mice received intramuscular injection of AAV1-CMV-OVA at day 0, at 1 x 10^{13}gc/kg or PBS/5% sucrose. Blood was drawn weekly and at sacrifice. Treatment was initiated with three different experimental regiments. A group receiving mono-therapy with bortezomib (velcade) at day 28, 33 and 36, a group receiving mono-therapy with anti-CD20 antibody (eBioscience, clone: AISB12) at day 22 and 27, a group receiving poly-therapy with both bortezomib (day 28, 33 and 36) and anti-CD20 (Day 22 and 27). Anti-CD20 was administered intraperitoneal at 100 µg/mouse; bortezomib was administered intravenously at 0.75 mg/kg. The control groups were mice injected with PBS and mice injected with AAV1-CMV-OVA only.

AAV vector production and characterization

AAV vector (AAV1-CMV-OVA) was produced in insect cells according to a technology adapted from R. M. Kotin R.M. Kotin [24]. It was purified with an AVB sepharose column using the ÄKTA explorer system (GE Healthcare). Diafiltration and concentration of the AAV elution in PBS-/-, 5% sucrose buffer was performed with the use of hollow fiber membrane (Spectrum labs). The titer of AAV vector genomes copies (gc/ml) in the final product was determined by Taqman QPCR amplification.

Virus neutralizing assay

HEK293T cells were seeded in 96-well plates (Corning) that were pre-coated with 0.25% poly L-lysine at a density of 1 x 10^5cells/well in 100 µl of DMEM with 10% FBS and 1% Penicillin/Streptomycin. Cells were incubated overnight at 37°C in 5% CO_2 water jacket incubator. Medium was then removed and the following mix was added: AAV5-CMV-GFP for anti-AAV5 NAB

assay or AAV1-CMV-GFP for anti-AAV5 NAB assay with heat-inactivated plasma sample in a total volume of 100 µl of DMEM without phenol red and 1% Penicillin/Streptomycin. The mix was incubated for 1 hour at 4°C prior to addition on the cells. Medium of the Hek293T cells was removed by aspiration, and then the mix was added and incubated for 16-20 h at 37°C. Serial dilutions of test plasma that were prepared were: 1:100, 1:200, 1:400, 1:800, 1:1600, 1:3200, 1:6400, and 1:12800. As a positive control, cells without plasma addition in the mix were analyzed. After 16-20 h, cells were rinsed with PBS, collected after trypsinization and fixed in PBS, 2% Formaldehyde, 1% BSA. GFP expression of the cells was analyzed by flow cytometry (FACScalibur, Becton Dickinson) in channel FL1 at a wavelength of 530 nm. The analysis was performed with the Cellquest Pro software. The percentage of inhibition was calculated related to GFP expression measured in AAV HEK293T infected cells (no inhibition, 100% expression). Plasma dilutions causing a 50% reduction of GFP expression when compared to positive control, were determined (EC50 determination after sigmoidal curve fit in GraphPad Prism software). Plasma samples were considered to have neutralizing activity if the lowest plasma dilution inhibited vector transduction by at least 50%, as described before [25, 26, 27, 28].

Assessment of anti-OVA antibody level

Level of anti-OVA antibody in mouse plasma was measured by anti-OVA specific ELISA. Nunc MaxiSorp® flat-bottom 96-well plates (ThermoScientific) were coated with 0.5 µg/ml OVA protein and anti-OVA antibody level in samples was detected with 1:1000 rabbit-anti-mouse-HRP (DAKO).

Statistical analysis

Results are presented as means (+/-standard error of the mean (SEM). Statistical analyses were performed using Prism 5.0 (GraphPad). Data were analyzed using a 2 way ANOVA, followed by Bonferroni post hoc test for multiple comparisons.

Results & Discussion

Experimental setup

Mice were injected intramuscularly with 1 x 10^{13}gc/kg of AAV1-CMV-OVA or PBS. All the mice were followed for 21 days (3 weeks) for the development of NAB against the AAV1 capsid proteins and antibodies against OVA transgene product. From day 22, immunosuppressive (IS) therapy was initiated for 2 weeks, mono-therapy with bortezomib or anti-CD20, and combination-therapy with bortezomib and anti-CD20 (**Figure 1**). The drugs were administered as follows, three times intravenously at a dose of 0.75 mg/kg for the bortezomib (day 28, 33 and 36 of the experiment) and two times intraperitoneally at a dose of 100 μg for anti-CD20 (day 22 and 27 of the experiment).

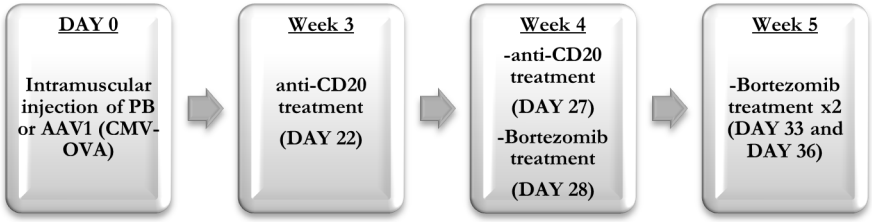

Figure 1. Scheme of the experimental setup. Mice received intramuscular injection of AAV1 (CMV-OVA) at day 0, at 1x10^{13}gc/kg or PBS/5% sucrose. Treatment was initiated with three different experimental regimens (Bortezomib, anti-CD20 antibody and with both Bortezomib and anti-CD20 antibody).

Humoral response against OVA

To assess the influence of bortezomib and anti-CD20 on the humoral immune response against the OVA transgene, the levels of specific anti-OVA antibodies generated, were assessed over time (**Figure 2.A-C**). The data obtained show a significant inhibitory effect of bortezomib (67.19 % on day 28 and 71.18 % on day 35) (**Figure 3.A, B**) and anti-CD20 (61.51% on day 28 and 44. 38 % on day 35) (**Figure 3.A, B**) on the humoral response raised against the OVA protein when compared to the control without IS. No signif-

icant additive inhibitory effect on anti-OVA antibody level was observed when anti-CD20 and bortezomib treatment were combined (**Figure 2.C**). The inhibitory effect of the IS treatment on the humoral response was transient as the antibodies level increased at day 42 of the experiment (**Figure 2.A-C**).

Humoral response against AAV1- Neutralizing antibodies (NAB) against the AAV1 vector capsid

To determine the effect of bortezomib and anti-CD20 on the levels of neutralizing antibodies against the AAV1 viral capsid proteins, a neutralizing antibody assay was performed on mice plasma samples obtained at days 0, 14, 28, 42, 56 and 84 (**Figure 4**). After bortezomib administration (days 28, 33 and 36), a decrease was observed in the levels of the NAB titers against the AAV1 capsid at days 42 (13.6%), 56 (26.3%) and 84 (38.7%) when compared to the control without IS (**Figure 4.A**). After anti-CD20 administration (days 22, and 27), a decrease was observed in the levels of the NAB titers against the AAV1 capsid (day 84) when compared to the control without IS (35.5%) (**Figure 4.B**). The delayed response to the anti-CD20 antibody treatment is consistent with the mechanism of action of this antibody [22]. The combination of both IS drugs did not show an additive effect on the reduction of anti-AAV1 NAB levels (**Figure 4.C**). The effect of the IS treatment on the humoral response was sustained until the end of the observation period as the neutralizing antibodies levels remained low until day 84 for both bortezomib and anti-CD-20. However, the anti-AAV NAB titer obtained after treatment (a titer of 2592) was not low enough to perform re-administration, as previously reported [19].

We conclude therefore that a combined treatment with bortezomib and anti-CD20 antibody when a high titer (~3550) of anti-AAV1 NAB has already been established cannot reduce the level of those antibodies sufficiently to permit re-administration. Therefore, other combination of treatments that include additional cellular targets will be explored in the future.

Safety

No weight loss or signs of illness were observed in the mice receiving the immunosuppressive drugs alone or in combination.

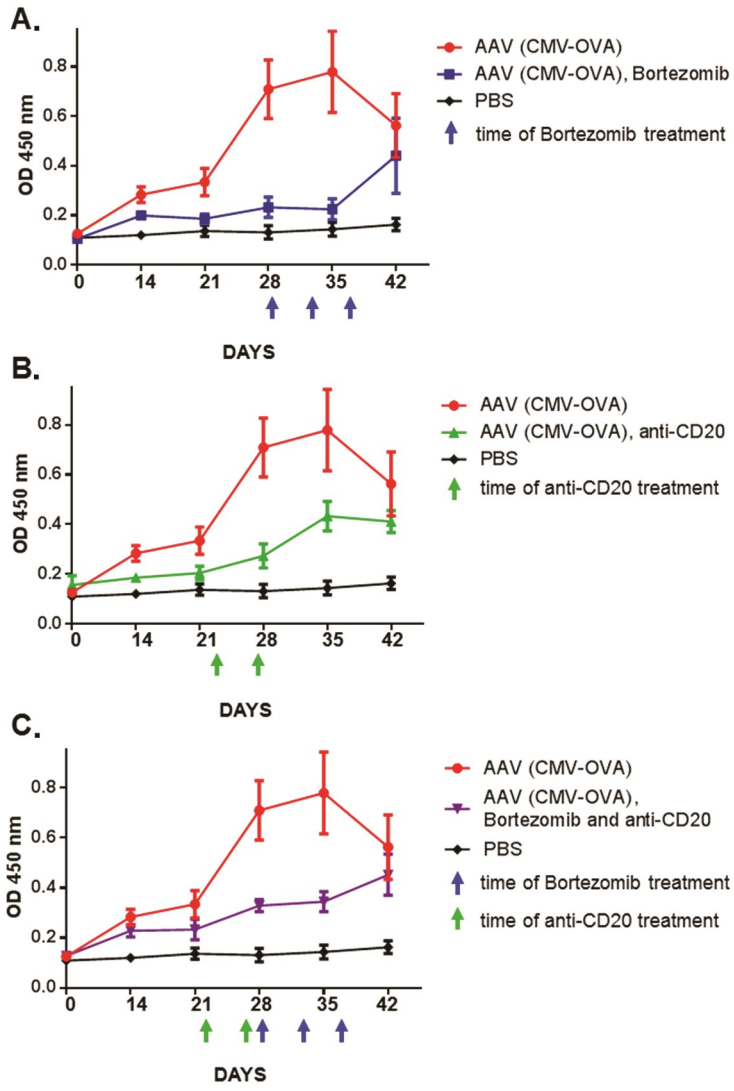

Figure 2. Immune suppressive regimens reduce specific humoral immune responses against OVA protein following AAV1 intramuscular delivery

Mice received intramuscular injection of AAV1 (CMV-OVA) at day 0, at $1x10^{13}$gc/kg or PBS/5% sucrose. Treatment was initiated with three different experimental regimens (Bortezomib, anti-CD20 antibody and with both Bortezomib and anti-CD20 antibody). Development of anti-OVA antibodies was monitored overtime in plasma of mice treated with Bortezomib (**A**), anti-CD20 (**B**) or Bortezomib and anti-CD20 (**C**).

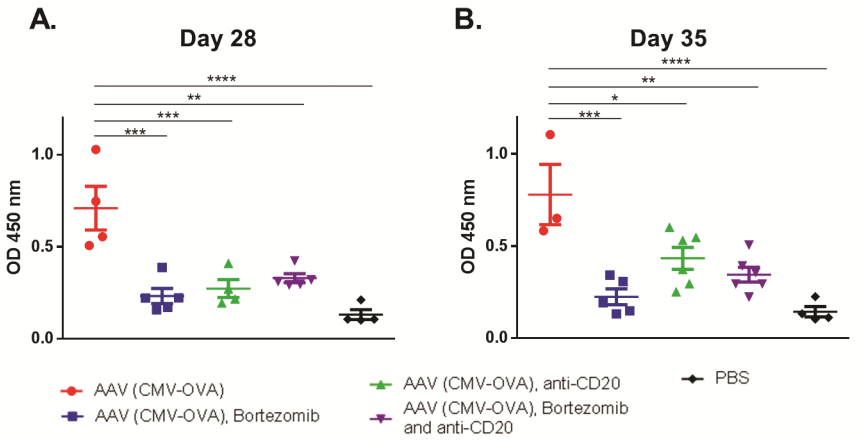

Figure 3. Immune suppressive regimens reduce specific humoral immune responses against OVA protein following AAV1 intramuscular delivery.
Mice received intramuscular injection of AAV1 (CMV-OVA) at day 0 or PBS/5% sucrose. Treatment was initiated with three different experimental regimens (Bortezomib, anti-CD20 antibody and with both Bortezomib and anti-CD20 antibody). Development of anti-OVA antibodies was monitored overtime after treatment with Bortezomib, anti-CD20 or both on day 28 (A) and 35 (B) of experiment. The data were analysed using one-way ANOVA, followed by Bonferroni's Multiple Comparison Test. *P<0.05, **P<0.01, ***P,0.001, ****P<0.0001.

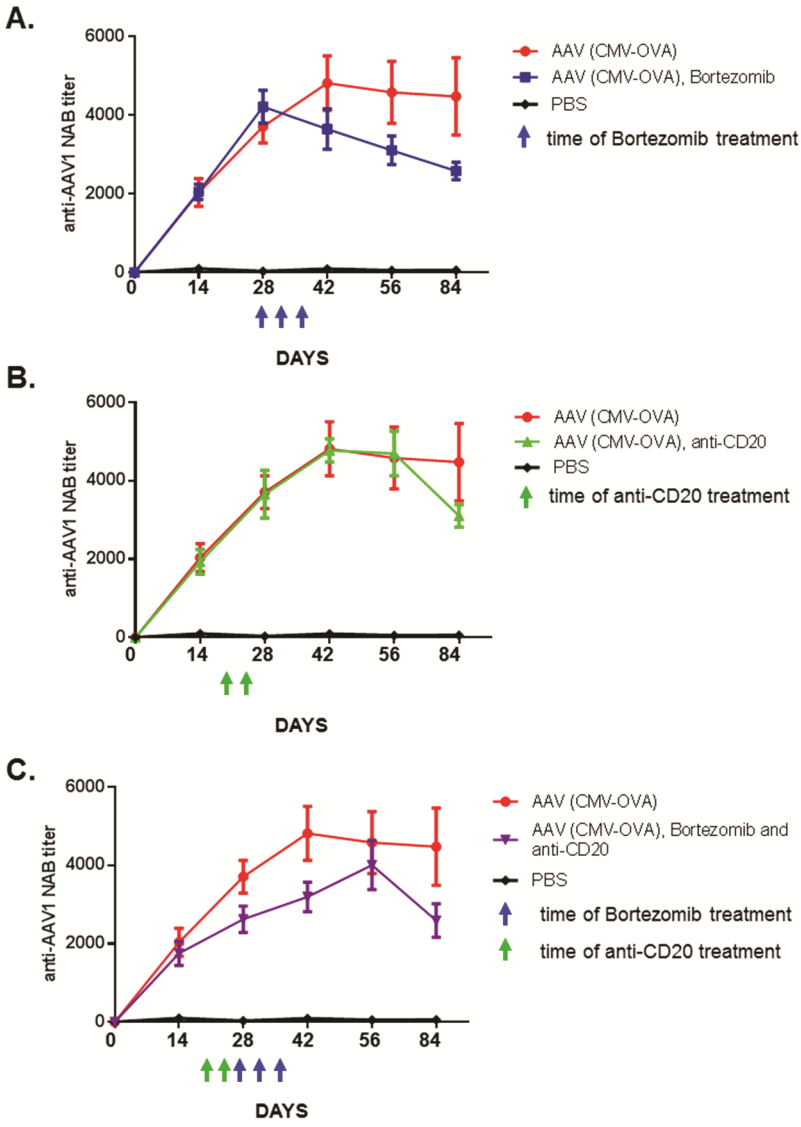

Figure 4. Immune suppressive regimens reduce neutralizing antibody (NAB) titers against AAV1 raised after intramuscular delivery of AAV1.
Mice received intramuscular injection of AAV1 (CMV-OVA) at day 0 or PBS/5% sucrose. Treatment was initiated with three different experimental regimens (Bortezomib, anti-CD20 antibody and with both Bortezomib and anti-CD20 antibody). Development of nAb titer agaist AAV1 was monitored overtime in plasma of mice treated with Bortezomib (**A**), anti-CD20 (**B**) or combined Bortezomib and anti-CD20 (**C**).

It has been described previously that a dose of 1 mg/kg of bortezomib was associated with toxicity in 15% of the animals treated [19] suggesting a narrow therapeutic window of this drug. We report that administration of a 0.75 mg/kg dose of bortezomib either combined with anti-CD20 (100 μg per injection) or not did not appear to induce toxicity in mice.

Additionally thymus, spleen, bone marrow and PBMCs were collected at sacrifice, stained for B and T cell markers and analyzed with the use of flow cytometry. We did not observe any significant differences in expression of CD19, CD25 or CD138 markers (*data not shown*) which demonstrate the absence of long term effects on the adaptive immune system.

Conclusions and future prospects

Overall, this study shows that a combination-therapy of bortezomib with an anti-CD20 monoclonal antibody was not sufficient to lower a high titer of pre-existing antibodies against the AAV1 capsid to a level that will allow an efficient AAV1 re-administration. The same drugs alone or in combination were, in contrast, efficient in preventing immune response against the immunogenic protein (OVA) delivered as a transgene by AAV1. This observation can be related to the differences in the kinetic and mechanism of development of the humoral immune response against the AAV capsid and the transgene product delivered by AAV [23].

The two immunosuppressive molecules used in this study, bortezomib and anti-CD20, are mainly targeting pre-B cells, mature B cells and memory B cells [20, 21]. Additional cellular inhibitors, as T cell inhibitors, may be required in order to bring down the humoral response to the AAV1 capsid. Further studies are needed in order to evaluate the efficacy of combination treatment that targets both B and T cells population as well as the therapeutic benefit in relation to different NAB levels.

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