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Chapter 4

Adeno-associated virus mediated delivery of Tregitope 167 ameliorates experimental colitis


Abstract

Aim

To explore the anti-inflammatory potential of adeno-associated virus-mediated delivery of Tregitope 167 in an experimental colitis model.

Methods

The Trinitrobenzene sulfonate (TNBS) model of induced colitis was used in Balb/c mice. Subsequently after intravenous adeno-associated virus-mediated Tregitope delivery, acute colitis was initiated by intra-rectal administration of 1.5 mg TNBS in 40% ethanol followed by a second treatment with TNBS (0.75 mg in 20% ethanol) 8 days later. Control groups included mice not treated with TNBS (healthy control group) and mice treated by TNBS only (diseased group). At the time of sacrifice colon weight, the disease activity index and histology damage score were determined. Immunohistochemical staining of the colonic tissue was performed to assess the cellular infiltrate and the presence of transcription factor Forkhead Box-P3 (Foxp3). Thymus, mesenteric lymph nodes, liver and spleen tissue were collected and the corresponding lymphocyte populations were further assessed by flow cytometry analysis for the expression of CD4+ T cell and regulatory T cell associated markers.

Results

The Tregitope 167 treated mice gained an average of 4% over their initial body weight at the time of sacrifice. In contrast, the mice treated with TNBS alone (no Tregitope) developed colitis, and lost 4% of their initial body weight at the time of sacrifice ($P$ value <0.01). The body weight increase that had been observed in the mice pre-treated with Tregitope 167 was substantiated by a lower disease activity index and a decreased colon weight as compared to the diseased control group ($P$ value <0.01 and <0.001 respectively). Immunohistochemical staining of the colonic tissues for CD4+ showed that inflammatory cell infiltrates were present in TNBS treated mice with or without administration with Tregitope 167 and that these cellular infiltrates consisted mainly of CD4+ cells. For both TNBS treated groups CD4+ T cell infiltrates were
observed in the sub-epithelial layer and the lamina propria. CD4+ T cell infiltrates were also present in the muscularis mucosa layer of the diseased control mice, but were absent in the Tregitope 167 treated group. Numerous Foxp3 positive cells were detected in the lamina propria and sub-epithelium of the colon sections from mice treated with Tregitope 167. Furthermore, the Foxp3 and glycoprotein A repetitions predominant (GARP) markers were significantly increased in the CD4+ T lymphocyte population in the thymus of the mice pre-treated with adeno-associated virus serotype 5 (cytomegalovirus (CMV) promoter-Tregitope 167), as compared to lymphocyte populations in the thymus of diseased and the healthy control mice (P value <0.05 and <0.001 respectively).

**Conclusion**

This study identifies adeno-associated virus-mediated delivery of regulatory T-cell epitope 167 as a novel anti-inflammatory approach with the capacity to decrease intestinal inflammation and induce long-term remission in IBD.
Introduction

Inflammatory Bowel Diseases (IBD) are inflammatory diseases that affect mostly young adults [1, 2]. Although the precise pathogenesis has not been identified, it is generally accepted that IBD result from inappropriate mucosal immune system responses against intestinal flora and other luminal antigens [3-5]. IBD are associated with a reduction in quality of life [6-8] and no curative treatments are available.

Despite the fact that novel treatment strategies, including tumor necrosis factor (TNF)-neutralizing antibodies, have greatly expanded the therapeutic armamentarium, these therapeutics do not prevent complications in IBD and many patients still have to undergo surgery [9]. New treatment strategies that would prevent the initiation of inflammation and enable long-term remission would improve the lives of millions of individuals who are affected by IBD world-wide [10, 11].

Recently, biological therapies that target immune pathways have emerged as a new therapeutic approach for the treatment of immune-mediated diseases. They include administration of monoclonal antibodies against inflammatory cytokines [12] and those that influence immune responses such as certain small molecules, Helminths and stem cells [10, 13, 14]. Since IBD are immune-mediated diseases, these biological therapies are highly promising treatment approaches and have the potential to achieve mucosal tolerance and long-term remission in IBD [10, 12-14]. Here, we introduce regulatory T-cell epitopes (Tregitopes) [15, 16] as novel biological agents that could create new possibilities for the regulation of inflammation and postulate that Tregitopes, delivered by adeno-associated virus (AAV), could be developed as a new therapeutic modality for the treatment of IBD.

Tregitopes are a set of putative regulatory T cell epitopes present in the immunoglobulin G molecule, which have been identified by using computational epitope mapping [15, 16]. Tregitope sequence 167 (Tregitope 167) and an additional sequence (Tregitope 289) were synthesized and shown to bind to multiple Major Histocompatibility complex (MHC) class II molecules and to sup-
press immune response when co-administered with an antigen. Tregitopes 167 and 289 were also shown to expand natural occurring regulatory T (nTreg) cells and to induce a regulatory phenotype and function in peripheral T (iTreg) cells [15, 16]. Harnessing the potential of Treg cells activated or induced by Tregitopes to regulate pathological immune responses in IBD may reduce the requirement for systemic immunosuppressive therapies. However, the use of immunomodulatory peptides in clinical applications for IBD so far have shown that the in vivo delivery of these peptides for therapeutic purposes is hindered by difficulties in obtaining sufficient and stable peptide concentrations [17-19]. Therefore, novel means for stable delivery of regulatory peptides have to be explored. Adeno-associated viruses (AAV) present a good safety profile and have been shown to be effective as gene delivery vectors in the clinic for the treatment of a broad range of diseases [20-22]. Therefore, AAV-mediated delivery represents an attractive approach to deliver the immunomodulatory Tregitope peptides.

In the present study, the potential of AAV-mediated gene therapy for the therapeutic delivery of Tregitope 167 was explored. Systemic AAV-mediated administration of Tregitope 167 was shown to ameliorate the clinical and histopathological severity of Trinitrobenzene sulfonate (TNBS) induced inflammatory colitis in mice. Hence, AAV-mediated delivery of regulatory T-cell epitopes appears to be a promising novel therapeutic approach for the treatment of IBD and could represent an alternative or adjunct to the use of immunosuppressive drugs.

Materials and methods

AAV vector production and characterization

Mouse Tregitope cDNA was synthesized (Integrated DNA Technologies, IDT, Inc) according to the published corresponding sequence [15, 16] and cloned into the plasmid pCH110 [23] under the control of the cytomegalovirus (CMV) promoter. The Woodchuck hepatitis virus post-transcriptional enhancer (WPRE) was incorporated behind the Tregitope 167 cDNA to further optimize gene expression [24]. The AAV vector, AAV5 (CMV-Tregitope 167)
was produced according to a technology adapted from A. Negrete and R.M. Kotin [23]. The AAV batch was purified with an AVB sepharose column using the ÄKTA explorer system (GE Healthcare). After purification, the concentration of AAV vector genomes copies (gc/ml) was determined at 9 x 10^{13} gc/ml by Taqman qPCR amplification. The biological infectivity of AAV5 (CMV-Tregitope 167) was demonstrated in vitro by PCR amplification of the “CMV-Tregitope 167” DNA fragment (product size 402bp) on DNA isolated from HEK293T transduced with AAV5 (CMV-Tregitope 167). Primers designed and synthesized for Tregitope 167 and the CMV promoter were used.

**Induction of colitis and study design**

Balb/c mice (males, age 6-8 weeks) were obtained from Harlan Laboratories, the Netherlands. The experimental protocol was approved by the ethical committee for animal welfare of the AMC (Academic Medical Center, Amsterdam, the Netherlands). Colitis was induced in mice by administration of TNBS (Trinitrobenzene sulfonate, TNBS, Sigma-Aldrich), as described previously [25]. The general procedure is summarized in Figure 1.

![Figure 1. Schematic overview of the Trinitrobenzene sulfonate (TNBS) induced colitis model.](image)

Mice were injected intravenously with either PBS or AAV5 (cytomegalovirus (CMV) promoter-Tregitope 167). 10 d after AAV-mediated Tregitope delivery acute colitis was initiated by intra-rectal administration of 1.5 mg TNBS in 40% ethanol followed by a second TNBS treatment (0.75 mg in 20% ethanol) 8 days later. Control groups consisted of mice not treated by TNBS (healthy control group) and mice treated by TNBS only (diseased control group).

Mice were injected intravenously with either PBS or AAV5 (CMV-Tregitope) 10 days before acute colitis was initiated by intra-rectal administration of 1.5 mg TNBS in 40% ethanol. Consecutively, a second TNBS treatment (0.75 mg in 20% ethanol) was done 8 days after the first TNBS treatment as described
precedently [25]. Mice not treated with TNBS (healthy control group) and mice treated with TNBS only (diseased control group) were used as references to monitor colitis development. A concomitant sham AAV control vector was not used in this study as this control has been shown to be equivalent to saline control [26, 27]. Even though AAV-mediated gene transfer leads to the development of neutralizing antibodies against the vector capsid [28], preventing vector re-administration, no inflammatory responses against the AAV capsid were documented in *in vivo* gene transfer mice models using AAV vectors [27, 29].

**Assessment of Inflammation**

The body weights of the mice were recorded daily, and wasting disease progression was expressed by the percentage of weight loss as compared to body weight at the day of initiation of TNBS treatment (Figure 2). Animals were withdrawn from the study when their weight loss was >25% of their original body weight. At the time of sacrifice, colons were collected and presence of loose stool and visible fecal blood was assessed.

At the time of sacrifice, a composite score (disease activity index, DAI) was established as described previously [25]. Body weight loss was scored on a scale of 0–4 (0, <1%; 1, 1%-5%; 2, 5%-10%; 3, 10%-15%; 4, >15%). Loose stool was scored on a scale of 0–4 (0, normal; 1, loose droppings; 2, loose stools, colon filled with feces; 3, loose stool, feces only near cecum; 4, empty bowel). Visible fecal blood was scored on a scale of 0–4 (0, negative; 2, positive; 4, gross bleeding). The DAI consists of a combination of body weight loss, loose stool and visible fecal blood scores divided by 3 as described previously [25].

Colon tissue weights were recorded and used as an indicator of disease-related intestinal wall thickening. Increased colon weight has been shown to correlate with increased colon inflammation [25]. Colons were first divided longitudinally into two parts: one part was immediately frozen in liquid nitrogen for protein extraction and cytokine level determination, while the second part was stored in formalin and embedded in paraffin for immunohistological evaluation. Blood was collected by orbital puncture immediately following sacrifice.
and plasma was separated by centrifugation (5000 rpm for 5 min). Plasma samples were stored at −80°C until analysis.

**Histological analysis**

Colonic segments were fixed in 10% formalin overnight and thereafter stored in 70% ethanol before embedding in paraffin. Tissue sections (7 μm thick) were stained with haematoxylin for histology scoring. The histology damage score was calculated using the following criteria: percentage of area involved, number of follicle aggregates, edema, fibrosis, erosion/ulceration, crypt loss, and infiltration of mononuclear and polymorphonuclear cells as described previously [30]. The percentage of area involved and crypt loss were scored on a scale of 0–4 (0, normal; 1, <10%; 2, 10%; 3, 10%–50%; and 4, >50%). Erosions were defined as 0 if the epithelium was intact, 1 if the lamina propria was involved, 2 if ulcerations involved the submucosa, and 3 when ulcerations were transmural. The severity of the other parameters was scored on a scale of 0–3 (0, absent; 1, weak; 2, moderate; and 3, severe) [30]. A certified pathologist scored all the tissue sections (blinded analysis).

**Immunohistochemistry**

Colon tissues sections of 7 μm were aceton-fixed and stained with rat anti-mouse (rm) CD4 (1:100, BD550278), rm CD8a (1:50, BD550281), rm CD19 (1:50, BD550284), rm Foxp3 (1:100, eBiosciences14-5773-82) and rm F4/80 (1:500). Prior to anti-rat biotin conjugated secondary antibody (1:50, BD51-7605kc) and streptavidin-HRP (BD) incubations, endogenous peroxidases were blocked by incubation with 0.3% H₂O₂ for 20 minutes. After 5 min of DAB staining (BD), the sections were counter-stained with haematoxylin, dehydrated and mounted in entallan.

**Flow cytometry**

Thymus, mesenteric lymphoid nodes, liver and spleen tissue were collected upon sacrifice. Cell suspensions obtained from each of the tissue samples were prepared using 40 μm cell strainers (BD Biosciences) and stained for T cell surface markers CD4 (clone RM4-5, eBioscience), CD8 (Clone 53-6.7, Miltenyi) and Treg cell surface markers GARP and CD25 (clone YGIC86 and
clone PC61.5 respectively, both eBioscience) as well as for the intracellular Treg cell marker Foxp3 (clone FJK16, eBioscience). The analysis was performed by flow cytometry (FACSCalibur, BD Biosciences).

**Statistical analysis**

The results are presented as means (+/- standard deviation (SD) or standard error of the mean (SEM), where appropriate). Statistical analyses were performed using Prism 5.0 (GraphPad). Data were analyzed using a 1 way ANOVA, followed by Dunn’s post hoc test for multiple comparisons.

**Results**

We investigated the potential for AAV5-mediated delivery of regulatory T-cell epitopes to prevent the development of TNBS induced colitis. Mice treated intra-rectally with TNBS in ethanol developed a severe illness as reflected in the progressive body weight loss over time and an increase in disease activity index, histology damage score and mucosal inflammatory parameters at the time of sacrifice.

**Tregitope 167 delivery protects against TNBS colitis development**

Development of colitis in the TNBS mice model is strongly associated with wasting disease [31]. Daily weight determination is therefore important to determine colitis severity and is indicative of differences in colitis development between experimental groups [31]. Animals were withdrawn from the study when their weight loss was >25% of their original body weight.

The body weight of the mice was monitored daily after the first TNBS treatment as an indication of the severity in the colitis development between experimental groups (Figure 2). TNBS treated mice that were pre-administered with Tregitope 167, showed a body weight that increased over time and was comparable to the weigh gain of untreated healthy control mice (Figure 2). The Tregitope 167 treated mice gained an average of 4% over their initial body weight at the time of sacrifice (Figure 3.A). In contrast, the mice treated with TNBS alone (no Tregitope) developed colitis, and lost 4% of their initial body weight at the time of sacrifice (Figure 3.A).
Figure 2. Adeno-associated virus serotype 5 mediated delivery of regulatory T-cell epitope 167 ameliorates Trinitrobenzene sulfonate induced colitis development over time. Mice were injected intravenously with either PBS or AAV5 (cytomegalovirus (CMV) promoter-Tregitope 167). 10 d after AAV-mediated Tregitope delivery, acute colitis was initiated by intra-rectal administration of 1.5 mg TNBS in 40% ethanol followed by a second TNBS treatment (0.75 mg in 20% ethanol) 8 days later. Control groups consisted of mice not treated by TNBS (healthy control group) and mice treated by TNBS only (diseased control group). Disease progression was assessed by changes in daily body weight. Animals were withdrawn from the study when their weight loss was >25% of their original body weight. Overall for the healthy controls, \( n = 9 \); AAV5 (CMV-Tregitope 167) treated, \( n = 7 \); diseased controls, \( n = 6 \), were included in the analysis. The data were analyzed using a 1 way ANOVA, followed by Dunn’s post hoc test for multiple comparisons. Data are presented as means +/- SEM of all the mice. \( ^d \)P value <0.0001 between PBS (diseased control group) and both AAV5 (CMV-Tregitope 167) treated and the healthy control group. PBS: Phosphate-buffered saline; TNBS: Trinitrobenzene sulfonate; AAV: Adeno-associated virus.
Increases in colon weight, as well as in the disease activity index are both indicative of colonic inflammation and were determined at the time of sacrifice. The body weight increase that had been observed in the mice pre-treated with Tregitope 167 was substantiated by a lower disease activity index (Figure 3.B) and a decreased colon weight (Figure 3.C) as compared to the diseased control group ($P$ value <0.01 and <0.001 respectively).

The histology damage score was performed on HE stained tissue sections. The score was calculated using the following criteria: percentage of area involved, number of follicle aggregates, edema, fibrosis, erosion/ulceration, crypt loss, and infiltration of mononuclear and polymorphonuclear cells. The histological scoring showed that the AAV5 (CMV-Tregitope 167) pre-treated mice presented a decreased severity of colitis as compared to the diseased control group (Figure 3.D) as a result of lower levels of inflammation, namely decreased cellular infiltrations, little crypt loss and the absence of erosions and ulceration (Figure 4).

The TNBS induced colitis model is characterized by the local infiltration of CD4$^+$ T cells in the intestinal mucosa [32]. Immunohistochemical staining of the colonic tissues for CD4$^+$ showed that, at the day of sacrifice, inflammatory cell infiltrates were present in TNBS treated mice with or without administration with Tregitope 167 and that these cellular infiltrates consisted mainly of CD4$^+$ cells. For both TNBS treated groups CD4$^+$ T cell infiltrates were observed in the sub-epithelial layer and the lamina propria. CD4$^+$ T cell infiltrates were also present in the muscularis mucosa layer of the diseased control mice, but were absent in the AAV5 (CMV-Tregitope 167) treated group (Figure 5).

**Increase of regulatory markers expression in the intestinal mucosa and thymus of mice administered with Tregitope 167**

The reported ability of Tregitopes to both activate and induce Treg cells led us to further assess the presence of Treg-cell associated markers in the colonic tissues.
Figure 3. Adeno-associated virus serotype 5 mediated delivery of regulatory T-cell epitope 167 ameliorates colonic inflammation as determined at the day of sacrifice. Mice were injected intravenously with either PBS or AAV5 (cytomegalovirus (CMV) promoter-Tregitope 167). 10 d after AAV-mediated Tregitope delivery acute colitis was initiated by intra-rectal administration of 1.5 mg TNBS in 40% ethanol followed by a second treatment with TNBS (0.75 mg in 20% ethanol) 8 days later. Control groups consisted of mice not treated by TNBS (healthy control group) and mice treated by TNBS only (diseased control group). Disease progression was assessed by changes in daily body weight as well as macroscopic and microscopic scores on the day of sacrifice. A. Animal body weight change on day 20 upon sacrifice. The values of body weight are expressed as a percentage of body weight on the day of the first TNBS treatment; B. Macroscopic disease score. The disease activity index (DAI) consist of a combination of body weight loss, loose stool and visible fecal blood scores divided by 3 at the day of sacrifice; C. Assessment of colonic weight upon sacrifice as an index of disease-related intestinal wall thickening; D. Histological grading of colonic colitis scores. The histology damage score was calculated using the following criteria: percentage of area involved, number of follicle aggregates, edema, fibrosis, erosion/ulceration, crypt loss, and infiltration of mononuclear and polymorphonuclear cells. Individual mice are depicted; from the AAV5 (CMV-Tregitope 167) pre-treated group an outlier was removed. Animals were withdrawn from the study when their weight loss was >25% of their original body weight. Overall for the healthy controls, n = 9; AAV5 (CMV-Tregitope 167) treated, n=6; diseased controls, n=6, were included in the analysis. The data were analyzed using a 1 way ANOVA, followed by Dunn’s post hoc test for multiple comparisons. Data are presented as means +/- SD of all the mice. P value <0.05 (a), P value, <0.01 (b), P value <0.001 (c) vs PBS (diseased control group).
Figure 4. Hematoxylin and eosin-stained paraffin section from colon tissue.
A, B: Healthy control mice; C, D: Diseased control mice; E, F: AAV5 (CMV-Tregitope 167) pre-treated mice. Histological evidence that AAV5 (cytomegalovirus (CMV) promoter-Tregitope 167) ameliorates TNBS-induced pathology. Images depicted of an HE-stained paraffin section of a representative mouse colon from each group at the moment of sacrifice. The images of the diseased control (C, D) demonstrated acute inflammation: elongated villi, abundant transmural cellular infiltrate, erosions and crypt loss as compared to both the AAV5 (CMV-Tregitope 167) pre-treated and healthy control mice. TNBS: Trinitrobenzene sulfonate; AAV: Adeno-associated virus.
Figure 5. Immunohistochemistry pictures depicting CD4 staining colon tissue. 
A, B: Healthy control mice; C, D: Diseased control mice; E, F: AAV5 (cytomegalovirus (CMV) promoter-Tregitope 167) pre-treated mice. Specific immunohistochemical staining showed inflammatory cell infiltrates present in TNBS treated mice with or without administration with Tregitope 167 consisted of CD4 positive cells, localized in the subepithelial layer, in the lamina propria (C-F) and for the diseased control also in the muscular layer (C, D). Depicted are representative data from a single mouse. TNBS: Trinitrobenzene sulfonate; AAV: Adeno-associated virus.
Figure 6. Immunohistochemistry pictures depicting Forkhead Box-P3 staining colon tissue. A, B: Healthy control mice; C, D: Diseased control mice; E, F: AAV5 (cytomegalovirus (CMV) promoter-Tregitope 167) pre-treated mice. Colon tissue were prepared and assessed by immunohistochemistry for the expression of the transcription factor Foxp3 as a marker for regulatory T cells. Numerous Foxp3 positive cells were detected in the lamina propria and sub-epithelium of the colon sections from mice treated with Tregitope 167 (E, F). Foxp3 positive cells were rarely present in the colon of healthy and diseased control mice (A-D). Depicted are representative data from a single mouse. Foxp3: Forkhead Box-P3
Colon tissues were prepared and the presence of transcription factor Foxp3 was assessed by immunohistochemistry, so as to determine whether regulatory T cells were present in the peri-colonic infiltrates [33]. Numerous Foxp3 positive cells were detected in the lamina propria and sub-epithelium of the colon sections from mice treated with Tregitope 167 (Figure 6). Foxp3 positive cells were absent or sporadic in the colon of healthy and diseased control mice (Figure 6).

Thymus, mesenteric lymph nodes, liver and spleen tissue were collected at the time of sacrifice and the corresponding lymphocyte populations were further assessed by flow cytometry analysis for the expression of the CD4 T cell surface marker and the Treg cell associated markers Foxp3 [34], CD25 [35] and GARP [36, 37]. The Foxp3 and GARP markers were significantly increased in the CD4+ T lymphocyte population in the thymus of the mice pre-treated with AAV5 (CMV-Tregitope 167), as compared to lymphocyte populations in the thymus of diseased and the healthy control mice (P value <0.05 and <0.001 respectively, Figure 8). CD4+ thymic lymphocyte population (mean ± SD, 11% ± 2%, n=6) co-expressed Foxp3 and GARP in the thymus of AAV5 (CMV-Tregitope 167) pre-treated mice as compared to the thymic lymphocyte population of diseased (mean ± SD, 7% ± 3%, n=6) and the healthy control groups (mean ± SD, 6% ± 2%, n=9), respectively (Figures 7). Both the relative and absolute number of Foxp3 expressing T cells were expanded in the thymus after AAV5 (CMV-Tregitope 167) pre-treatment (Figure 9). No significant differences in the expression of Foxp3 and GARP in the lymphocyte populations of the mesenteric lymph nodes, liver and spleen were identified.
Figure 7. Adeno associated virus serotype 5 mediated delivery of regulatory T-cell epitope 167 induces Foxp3 and GARP expression in the CD4 positive thymic lymphocyte population. Thymus tissue was collected upon sacrifice. Cells suspensions were prepared and stained for the following markers: CD4, GARP and Foxp3 before analysis by flow cytometry (FACSCalibur, BD Biosciences). Depicted are percentages of CD4 positive, Foxp3 positive and GARP positive thymic lymphocytes. Individual mice are depicted; for the AAV5 (CMV-Tregitope 167) pre-treated group one mouse did not have a thymus and therefore $n=6$ mice were included in this analysis. The data were analyzed using a 1 way ANOVA, followed by Dunn’s post hoc test for multiple comparisons. Data are presented as means +/- SD of all the mice. $P$ value <0.05 (a), $P$ value <0.01 (b), $P$ value <0.001 (c) vs PBS (diseased control group) or healthy control.
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Figure 8. Adeno associated virus serotype 5 mediated delivery of regulatory T-cell epitope 167 induces Forkhead Box-P3 and GARP expression in the CD4 positive thymic lymphocyte population. Thymus tissue was collected upon sacrifice. Cells suspensions were prepared and stained for the following markers: CD4, GARP and Foxp3 before analysis by flow cytometry (FACSCalibur, BD Biosciences). A: Histogram Foxp3 cell count: healthy control; C: Histogram Foxp3 cell count: diseased control; E: Histogram Foxp3 cell count: AAV5 (CMV-Tregitope 167) pre-treated group (grey, filled in) versus unstained control (black continuous line). Gating was done on the CD4 positive thymic lymphocyte population. Depicted are representative data from a single mouse; B: Histogram GARP cell count: healthy control; D: Histogram GARP cell count: diseased control; F: Histogram GARP cell count: AAV5 (CMV-Tregitope 167) pre-treated group (grey, filled in) versus unstained control (black continuous line). Gating was done on the CD4 positive thymic lymphocyte population. Depicted are representative data from a single mouse.
Discussion

Curative treatment approaches for Crohn’s disease and ulcerative colitis represent a significant unmet medical need. Regulatory T (Treg) cells are key players in maintaining peripheral tolerance, preventing autoimmune diseases and limiting chronic inflammation [33, 38]. Therefore, novel strategies that aim for therapeutic tolerance induction and leverage Treg cells are currently being explored [39]. In the present study, the potential for AAV-mediated delivery of an immunomodulatory peptide (Tregitope 167) was investigated.

In this study, we demonstrate that the systemic AAV-based delivery of Tregitope 167 has the potential to prevent the development of fulminant colitis in a TNBS-induced model of IBD. Tregitope 167 was used in our study as its binding affinity for the MHC molecule in Balb/c mice is superior to Tregitope 289 (De Groot, manuscript submitted for publication). The significant de-
crease of colonic inflammation in the Tregitope 167 pre-treated mice was reflected in an overall weight gain and substantiated by a decreased disease activity index, colon weight and histology damage score at the time of sacrifice. Additionally, there was less mucosal inflammation in the AAV (CMV-Tregitope 167) pre-treated mice. This therapeutic benefit corresponded with increases in the relative number of T cells expressing regulatory T cell markers in the colon tissues and among thymic lymphocytes of the Tregitope 167-treated mice.

IBD patients do not present defects in regulatory T cell function or phenotype [40-42] and by consequence are more likely to benefit from therapies aiming at inducing and expanding Treg cells than patients affected by other autoimmune diseases. Tregitope 167 has the potential to both activate nTreg cells and induce iTreg cells [15, 16] and may be suitable as a novel therapeutic agent for IBD. However, the use of immunomodulatory peptides in clinical applications for IBD has been hindered by difficulties associated with the systemic delivery of the therapeutic peptides in sufficient quantity and concentration to the target tissues [17-19]. AAV has proven to be both effective and safe as a gene therapy delivery vector in the clinic [20-22]. Therefore, AAV-mediated delivery of Tregitopes was explored in this study. The AAV serotype 5 (AAV5) was used since pre-existing immunity to AAV5 in humans has been shown to be low [28, 43]. Our data demonstrate that AAV5-mediated delivery may be an efficient approach for stable administration of Tregitopes in vivo. Further studies will need to be performed to determine the duration of the immunological tolerance that is evoked by induction and activation of Treg cells.

Regulatory T (Treg) cells are considered to be essential in the counter-regulation of inflammatory reactions and Foxp3 is considered as a marker of the regulatory phenotype [34, 44, 45]. Staining for Foxp3 in mice pre-treated with Tregitope 167 revealed the presence of Foxp3 positive cells in the lamina propria and sub-epithelium of the colon sections. Additionally, expression of both Foxp3 and GARP were increased in the thymic CD4+ T lymphocyte population in mice pre-treated with AAV5 (CMV-Tregitope 167), indicating an increase in activated regulatory T-cells [34, 36, 37, 44, 45]. The presence of
higher numbers of activated regulatory T cells corresponded with the prevention of fulminant intestinal inflammation \textit{in vivo} in this TNBS model of IBD.

No increase in the Treg cell populations was observed in the mesenteric lymph nodes, liver and spleen in the current study. We hypothesize that this could be due to the duration of the experiment and the timing of the Treg cell evaluation. In other mouse models such as the model of spontaneous encephalomyelitis, the \textit{de novo} generation of Treg cells was initiated intrathymically, and was followed by Foxp3 induction in peripheral tissue at later time points [46].

Tregitopes are T cell epitopes naturally located in immunoglobulins that bind to multiple MHC Class II alleles and induce Treg cell responses. We have demonstrated that antigen presenting cells (APCs) present Tregitopes to nTreg cells, engage feedback mechanisms promoting a tolerogenic APC phenotype, induce Treg cell expansion, and modulate antigen-specific effector T cell responses (Cousens and De Groot, manuscript submitted for publication). Proportions of APC expressing MHC II, CD80, and CD86 are suppressed, consistent with reported effects of Intravenous immunoglobulin (IVIG) [47] and of the immunoglobulin (Ig) G-derived peptide hCDR1 [48]. The basic mechanism of Tregitope tolerance induction is currently proposed to be as follows: 1) APC present Tregitopes to nTreg cells, 2) nTreg cells are activated to proliferate, 3) nTreg cells provide tolerogenic feedback signals to APC, modulating the APC phenotype, and 4) nTreg cells and tolerogenic APC together suppress antigen-specific T cell responses (Cousens and De Groot, manuscript submitted for publication).

A limitation of the colitis model used in this study was the acute necrotizing enterocolitis, occurring in the first 3 days after the first TNBS treatment, a presentation of disease which is unrelated to IBD [25]. Therefore the surviving number of mice, included in the analysis was lower than anticipated, which, for some analysis, conflicted with statistical analysis of the data. As a consequence, a large variability in the colon mucosa cytokines levels was observed after TNBS induced experimental colitis and prevented an accurate analysis of those parameters. Therefore, further development of AAV mediat-
ed delivery of Tregitope 167 in different experimental models of inflammatory disease will be necessary to confirm the obtained results.

In summary, our data provide preliminary evidence supporting the potential use of AAV-based Tregitope delivery as a therapy for the treatment of IBD. Further investigations will permit to define the mechanism by which Tregitope exert their immune regulatory properties, the duration of the effect, the ability of Tregitopes to reduce disease that has already been established and their eventual impact on systemic immunity.

Overall, this study identifies AAV-mediated delivery of regulatory T cell epitope 167 as a novel anti-inflammatory approach with the capacity to decrease intestinal inflammation and induce long-term remission in IBD.
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