Single-dose AAV vector gene immunotherapy to treat food allergy

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Immunotherapies for patients with food allergy have shown some success in limiting allergic responses. However, these approaches require lengthy protocols with repeated allergen dosing and patients can relapse following discontinuation of treatment. The purpose of this study was to test if a single dose of an adeno-associated virus (AAV) vector can safely prevent and treat egg allergy in a mouse model. AAV vectors expressing ovalbumin (OVA) under an ubiquitous or liver-specific promoter were injected prior to or after epicutaneous sensitization with OVA. Mice treated with either AAV8-OVA vector were completely protected from allergy sensitization. These animals had a significant reduction in anaphylaxis mediated by a reduction in OVA-specific IgE titers. In mice with established OVA allergy, allergic responses were mitigated only in mice treated with an AAV8-OVA vector expressing OVA from an ubiquitous promoter. In conclusion, an AAV vector with a liver-specific promoter was more effective for allergy prevention, but higher OVA levels were necessary for reducing symptoms in preexisting allergy. Overall, our AAV gene immunotherapy resulted in an expansion of OVA-specific FoxP3$^+$ CD4$^+$ T cells, an increase in the regulatory cytokine IL-10, and a reduction in the IgE promoting cytokine IL-13.

INTRODUCTION

Food allergy (FA) is an abnormal response of the immune system to innocuous foreign food proteins driven by immunoglobulin E (IgE), with milk, eggs, peanut, tree nuts, soy, wheat, and fish representing the most common allergenic foods.[1] Sensitization to food allergens in humans can occur via the gut, respiratory tract, and the skin,[2–4] and is associated with a loss in allergen-specific regulatory T cell (Tregs) numbers and/or function.[5,6] Subsequent allergen exposures lead to presentation on mature antigen-presenting cells and activation of allergen-specific CD4$^+$ T effector and B cells. Symptoms range from mild rashes to rare life-threatening anaphylactic reactions, with fear of exposure as a source of severe anxiety and stress in patients and their families.[7–10]

Standard of care for FA is strict allergen avoidance and intervention with epinephrine for severe anaphylactic reactions[11,12]; however, emerging allergen-specific immunotherapies now provide expanded treatment options. The landmark LEAP trial demonstrated that regular, repeated exposure to peanuts in infancy prevented peanut allergy.[13] This same approach of feeding other foods during infancy also holds promise for preventing allergy.[14] For existing food allergies, desensitization with immunotherapies based on incremental doses of allergen administration can deviate from Th2 immunity, leading to the generation of allergen-specific IgG4 and Tregs.[15,16] However, these treatments share some limitations, including repeated administration, anaphylactic reactions, patient compliance, and loss of efficacy after treatment is discontinued. Effective antigen-specific immunotherapies are needed for FA patients that provide timely and lasting protection without requiring frequent dosing and associated adverse effects.

The liver plays an important role in maintaining immune homeostasis to ingested dietary antigens arriving from the portal vein,[17,18] where liver resident cells contribute to produce an immune tolerogenic microenvironment.[19,20] Notably, this native tolerance pathway within the liver is preserved with adeno-associated virus (AAV) viral vector-mediated expression of a transgene in hepatocytes.[21] The induction of antigen-specific peripheral FoxP3$^+$ Tregs plays a central role in this process[22] by suppressing the activation of antigen-specific B and T cells. Importantly, AAV liver gene transfer can eradicate existing pathogenic antibodies and lead to the restoration of immune tolerance.[23–25]

Our hypothesis is that expressing an egg allergen, ovalbumin (OVA), in liver using an AAV8-OVA vector could prevent and treat egg FA. To test this, we injected heterozygous female flaky tail mice (FT$^{+/-}$) with an AAV8-OVA vector containing a liver-specific promoter (Transthyretin, TTR) or an ubiquitous promoter (Elongation factor 1α, EF1α) into naive or OVA-sensitized mice.[26] Overall, our data show that high antigen levels are more effective at reducing anaphylaxis after allergen challenge. Moreover, detailed immunological

studies revealed different mechanisms are involved in tolerance induction when AAV gene immunotherapy is administered prophylactically or after FA onset.

RESULTS

Hepatic restricted OVA expression is more effective for preventing FA

Our first goal was to determine if AAV8-OVA liver gene immunotherapy would prevent OVA FA in female heterozygous flaky tail (FT$^{+/-}$) mice. These mice have mutations in two proteins involved in skin barrier defense against pathogens and allergens: filaggrin and mattrin, associated with allergic disease in humans and mice,[27,28] and can be sensitized by applying allergen to the skin.[26] To determine that OVA was required for both sensitization and challenge in order to trigger an allergic response, we set up two control experimental groups. In one group, naive FT$^{+/-}$ mice were challenged with an intraperitoneal (IP) injection of 1 mg OVA and in the second group of FT$^{+/-}$ mice were sensitized with OVA and challenged with PBS. As expected, neither group had systemic allergic responses (Figures S1A and S1B), even though the OVA-sensitized mice had significantly higher levels of OVA-specific IgG1 and IgE compared with the naive group (Figures S1C and S1D). These data show, in this model, that systemic allergic responses are allergen dependent.

Next, female FT$^{+/-}$ mice were intravenously (IV) injected with 1×10^{11} vector genomes (vg) of an AAV8-EF1α-OVA, AAV8-TTR-OVA, or control AAV8-ApoE-hAAT-FIX vector, and 4 weeks later plasma OVA levels were measured by ELISA (Figure 1A). FT$^{+/-}$ mice injected with the EF1α-OVA vector had 4-fold higher OVA levels in plasma compared with mice receiving the hepatocyte-specific TTR-OVA vector (Figure 1B). No OVA was detected in the plasma of mice receiving the control vector. A similar profile of OVA expression was measured in liver lysates (Figure S2). Next, mice underwent epicutaneous sensitization followed by IP challenge (Figure 1A). Only the AAV8-FIX treated group developed severe systemic anaphylaxis measured by a decrease in core body temperature 30 min after challenge ($-4.7 \pm 1.4^{\circ}$C) (Figure 1C) and high symptom score (Table 1) (Figure 1D). The majority of FT$^{+/-}$ mice treated with an OVA-secreting vector (EF1α and TTR) exhibited prophylactic protection from anaphylaxis symptoms (Figures 1C and 1D).

AAV-OVA gene immunotherapy reduces OVA-specific IgG1 and IgE titers and OVA-induced basophil activation

As expected, OVA-specific IgG1 and IgE antibodies were undetectable in any group (Figures S3A and S3B) prior to sensitization. Following sensitization, mice treated with either the EF1α-OVA or TTR-OVA vector had reduced OVA-specific IgG1 and IgE levels compared with the AAV8-FIX control group (Figures 1E and 1F). However, OVA-specific IgG1 and IgE titers were only significantly lower in mice treated with the TTR-OVA vector, in line with their reduced symptom score (Figure 1D). Recent studies in humans and mice support a role for an alternative anaphylaxis pathway mediated by release of platelet activating factor following formation of IgG-allergen immune complexes.[29,30] Here, we set out to determine the

relative contribution of the classical and alternative pathway in driving anaphylaxis in FT$^{+/-}$ mice. OVA-sensitized FT$^{+/-}$ mice were pretreated with 150 mg of triprolidine (H1R antagonist-IgE pathway), 25 mg of ABT-491 (platelet activation factor receptor antagonist-IgG pathway), or both 15 min before challenge with OVA. We observed a reduction in hypothermia and no mortality in both groups pretreated with triprolidine. Notably, there was a dramatic reduction in hypothermia and a more rapid recovery in the group treated with both agents. However, no symptomatic changes were detected in the group pretreated with ABT-491 alone compared with untreated control mice (Figure S4). These data suggest that, in this model, the IgE pathway is primarily driving allergic responses to OVA, but the alternative pathway can modulate the severity and duration of this response.

IgE antibodies bind to the high-affinity FceRI surface receptor expressed on basophils and mast cells. Increased surface expression of CD200R is associated with interleukin (IL)-4 secretion in activated basophils and was validated as a surface marker for activation of murine basophils.[31] Mice treated with AAV8-EF1α-OVA and AAV8-TTR-OVA vectors showed reduced basophil activation, measured by the change in CD200R expression following OVA stimulation, compared with the AAV-FIX control group (Figure 1G). A representative gating strategy used for the basophil activation test is depicted in Figure S5.

AAV8-OVA prophylaxis reduces total plasma cell and memory B cell numbers

IgG1$^+$ memory cells are generated within the germinal center (GC)[32] by activation of naive B cells that differentiate into memory and plasma cells. IgG$^+$ memory B cells are considered the main reservoir for short-lived IgE$^+$ plasma cells in both mice and humans.[33,34] Thus, we set out to determine the impact of AAV8-OVA gene immunotherapy on different splenic B cell subpopulations (Figure S6). In the two groups receiving the OVA vectors, no differences were observed in total splenic GC B cells (Figure 2A) and plasmablasts (Figure 2B). However, a significant reduction in plasma cells (only TTR-OVA group) (Figure 2C) and memory B cells (Figure 2D) was found compared with the control group. No differences were observed in IgG$^+$ memory B cells between groups (Figure 2E). Overall, the B cell numbers are consistent with the measured OVA-specific plasma IgE and IgG1 titers in the treatment and control groups.

AAV8-OVA gene immunotherapy suppresses Th2 responses

The generation of high-affinity OVA-specific antibodies is dependent on help provided by CD4$^+$ T cell subsets. Among these, follicular helper T cells (TFH) play a central role in inducing both affinity maturation and class switching.[35,36] T follicular regulatory cells (TFR) act as a balance to TFHs and are critical for resolving and preventing aberrant humoral responses.[37,38] Effector memory T cells (TEM) are antigen experienced T cells with the ability to migrate to sites of inflammation (CD44$^+$CD62L$^-$) and rapidly respond to secondary antigen exposures.[39] Importantly, allergen-specific TEM are correlated with FA allergic responses.[40] Thus, we measured the numbers of Th2, Treg,

Figure 1. A single dose of AAV8-OVA prevents allergic sensitization

(A) Experimental timeline followed in prevention studies. (B) OVA expression in plasma by ELISA. (C) Changes in core body temperature and (D) symptom score (see Table 1) following challenge with OVA. (E) OVA-specific levels of IgG1 and (F) IgE. (G) Basophil activation test measuring the difference in CD200R mean fluorescence between OVA-stimulated and non-stimulated blood samples obtained after sensitization of mice pretreated with AAV8-TTR-OVA (n = 8), EF1α-OVA (n = 8), or ApoE-hAAT-FIX (n = 7) vectors. Data are representative of one of three independent experiments and are presented as single data points and means ± standard deviation. Statistical testing was conducted using one-way ANOVA with Tukey's multiple comparison for all datasets excluding panel (F), which was performed using the Kruskal-Wallis test with Dunn's multiple comparison. *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$.

TFH, and TFR TEM subpopulations (Figure S7) following *in vitro* OVA stimulation by flow cytometry to investigate the mechanism of prophylactic protection with AAV-OVA gene immunotherapy. We found a significant decrease in Th2 TEM cells in TTR-OVA and EF1α-OVA injected animals compared with the AAV8-FIX control group (Figure 3A). No differences were observed in the other T cell subpopulations analyzed (Figures 3B–3D). Supernatants from the stimulated splenocytes were collected to measure cytokines associated

with Th1 immune responses (interferon [IFN]γ), regulatory and anti-inflammatory responses (IL-10), and IgE class switching (IL-13). In the AAV8-OVA-secreting groups we observed an overall low reactive profile with increases only observed in IL-10 following OVA stimulation. In contrast, the FIX control treated mice showed significant up-regulation of all cytokines (Figures 3E–3G). Further, we analyzed GATA3 expression in Treg TEM cells, since it was reported that these cells are less suppressive with a Th2-like phenotype.[41–43] Flow

Table 1. Symptom score

Score	Symptoms
0	No symptoms
1	Scratching and rubbing around the nose and head
2	Puffiness around the eyes and mouth, diarrhea, piroerection, reduced activity, and/or decreased activity with increased respiratory rate
3	Wheezing, labored respiration, and cyanosis around the mouth and the tail
4	No activity after prodding, or tremor and convulsion
5	Death

cytometry analysis showed approximately three times the number of GATA3$^+$ TEM Tregs cells in the control group compared with the secreted AAV8-OVA-treated mice (Figure S8).

These data suggest that secreted AAV8-OVA immunotherapy prevents sensitization through blocking the induction of the Th2 response in FT$^{+/-}$ mice. Although we did not observe any changes in total Treg cell numbers between groups, we cannot rule out that peripheral induced OVA-specific Treg may have contributed to this protection.

Higher allergen expression is needed for effective FA treatment
Next we determined if AAV immunotherapy could reverse established OVA FA. FT$^{+/-}$ mice were sensitized and challenged with OVA and blood was collected for IgG1 and IgE determinations prior to challenge (Figure 4A). Two days later, mice were IV injected with 1×10^{11} vg of one of three AAV8 vectors EF1α-OVA, TTR-OVA, or ApoE-hAAT-FIX control. Four weeks later, the mice were challenged with 0.5 mg of OVA protein by IP administration (Figure 4A). In contrast to the prophylaxis study, the EF1α-OVA treated group was more responsive with reduced allergic symptoms (Figure 4B) and hypothermia (Figure 4C), while the TTR-OVA group had only a slight amelioration in these parameters. These data suggest that when treating an established allergic response, higher antigen levels may be needed.

OVA-specific antibody levels and B cell expansion increase despite symptomatic improvement
OVA-specific IgG1 and IgE levels in plasma obtained prior to and 4 weeks after AAV treatment were determined in OVA-sensitized FT$^{+/-}$ mice. OVA-specific immunoglobulins showed that OVA-specific IgG1 titers 4 weeks after AAV8 injection were relatively higher in the EF1α-OVA-treated mice (23,337 ± 16,931 ng/mL) compared with the other groups (14,494 ± 3,800 ng/mL and 11,707 ± 6,466 ng/mL for TTR-OVA and FIX groups, respectively) (Figure 4D). OVA-specific IgE levels were increased 4 weeks after AAV injection only in AAV8-OVA-treated groups (Figures 4D and 4E). These results suggest that the protective effect at this time point may be in part related to increased OVA-specific IgG1 competitive binding and blocking of IgE-mediated activation of basophils and mast cells. AAV8-OVA-

treated groups had an increased frequency of splenic GC B cells, plasmablasts, and plasma cells and a reduction in IgG$^+$ memory B cells, but no changes were observed in memory B cells (Figures 5A–5E) when compared with controls. The frequencies of B cell subpopulations support the theory that OVA-specific IgG1 competitively blocks IgE-mediated activation of basophils and mast cells.

Increased numbers of OVA-specific Treg and TFR cells are associated with reduced anaphylactic symptoms
Since epicutaneous sensitization is conducted on the skin covering the neck, we selected the cervical lymph nodes (CLNs) as draining and spleen as a non-draining lymphoid tissue to determine changes in TEM subpopulations after stimulation with OVA (Figure S9). In general, we observed a significant increase in Treg TEM cells in AAV8-OVA-treated groups compared with the AAV8-FIX control group in CLN (Figure 6B) and spleen (Figure 6F). Similarly, a significant increase in the number of TFR TEM cells was observed in the EF1α-OVA group in CLN (Figure 6D) and in all AAV8-OVA-treated groups in spleen (Figure 6H) when compared with the AAV8-FIX control group. No differences were observed in Th2 and TFH cells in CLN (Figures 6A and 6C) and spleen (Figures 6E and 6G). Next, we measured changes in cytokine production in OVA-stimulated splenocytes. AAV8-OVA-treated groups showed a trend of increased IFNγ secretion (Figure 7A), although this was not significant. However, both AAV8-OVA-treated groups had significantly higher IL-10 and lower IL-13 secretion compared with the FIX control group (Figures 7B and 7C).

Finally, we analyzed the frequencies of OVA-specific CD4$^+$ T cells from allergic mice treated with AAV8-OVA gene therapy using an OVA MHC-II tetramer (Figure S9). Although CLNs isolated from mice treated with AAV8-OVA vectors did not show any statistical differences in OVA-specific Th2, Treg, and TFH TEM cells (Figures 7D–7F), OVA-specific Treg TEM cells showed increased levels compared with the control group. OVA-specific TFR TEM cells (Figure 7G) were the only cell subset that was significantly increased after treatment with AAV8-OVA vectors. However, in splenocytes we did not see changes in Th2 TEM cells and only observed higher numbers of OVA-specific Treg, TFH, and TFR TEM cells compared with control group (Figures 7D–7G). Interestingly, the EF1α-OVA group showed a higher number of OVA-specific Tregs TEM both in CLN and spleen, suggesting that OVA-specific Treg generation may be related to an improvement in the allergic symptoms.

AAV immunotherapy effectiveness is substantially improved for established OVA allergy in the absence of a primary challenge
Although we had limited success in treating established OVA allergy, we hypothesized that we could improve treatment efficacy by altering the sensitization protocol and timing of AAV immunotherapy. Our initial approach was to verify sensitization by performing a challenge prior to AAV vector administration. However, because this model requires a substantial amount of allergen to trigger systemic anaphylaxis, unlike what is needed in FA patients, this may lead to a substantial increase in anti-OVA IgE and IgG1 levels that may make it more
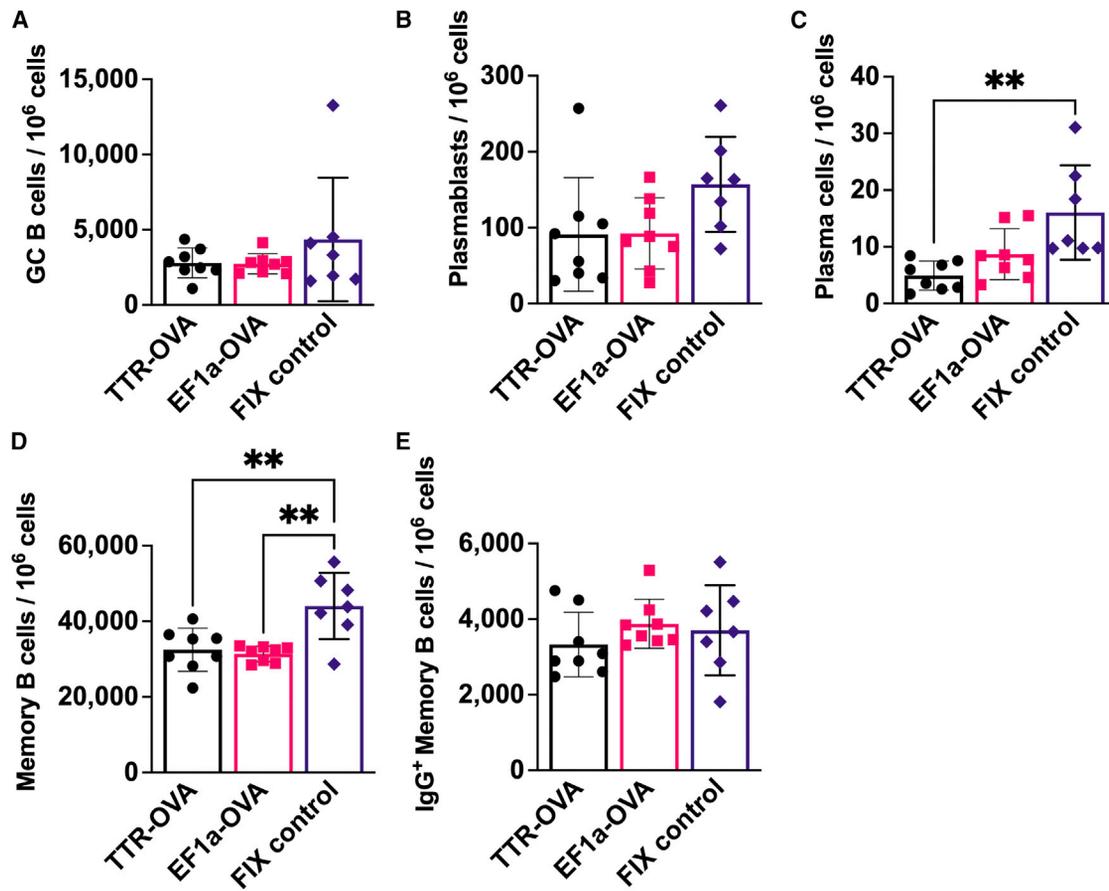
Figure 2. AAV8-OVA prophylactic treatment inhibits pathogenic B cell expansion

(A–E) Flow cytometry determination of the number of splenic (A) germinal center B cells, (B) plasmablasts, (C) plasma cells, (D) total memory B cells, and (E) IgG+ memory B cells per million splenocytes from sensitized mice pretreated with AAV8-TTR-OVA (n = 8), EF1α-OVA (n = 8), or ApoE-hAAT-FIX (n = 7) vectors. Data are presented as single data points and means ± standard deviation. Statistical testing was conducted using one-way ANOVA with Tukey's multiple comparison; **p < 0.01.

challenging to induce tolerance. Because we can measure sensitization by other means, such as anti-OVA IgE and BAT, we elected to test if AAV immunotherapy would be effective in sensitized OVA that had not undergone a primary challenge with OVA. FT^{+/−} mice were sensitized with OVA and blood was collected for IgG1 and IgE determinations prior to AAV administration (Figure S10A). Then, mice were IV injected with 1×10^{11} vg of EF1α-OVA or ApoE-hAAT-FIX control. Four weeks later, the mice were bled and challenged with 0.5 mg of OVA protein by IP administration (Figure S10A). Mice treated with the EF1α-OVA AAV8 vector showed significantly lower response to OVA measured by hypothermia and allergic symptoms (Figures S10B and S10C) and lower production of OVA-specific IgE and IgG1 (Figures S10D and S10E) compared with FIX control mice. With this new protocol, we were able to prevent systemic anaphylaxis (hypothermia) in 85% of the treated mice.

DISCUSSION

FA is an increasing health problem for adults and children. Although some experimental immunotherapies show promising re-

sults, new treatments are needed to extend therapeutic benefit and to address limitations shared by these therapies, such as daily dosing requirements, unexpected adverse reactions, and loss of protection after treatment is discontinued.[44,45] AAV gene therapy has demonstrated efficacy and safety in the treatment of inherited monogenic disorders like hemophilia B or Pompe disease and has been shown to be effective at eradicating pathogenic immune responses.[23,46] Our hypothesis was that we could use AAV liver gene therapy as an immunotherapy to prevent and treat FA. We tested this hypothesis in a clinically relevant adjuvant-free mouse model with skin sensitization. We demonstrated that a single dosing could protect against OVA sensitization in naive mice and achieve a significant improvement in OVA allergy symptoms in previously sensitized mice.

In prevention studies, hepatic OVA expression significantly reduced OVA skin sensitization and allergic responses. Interestingly, outcomes were comparable with a liver-specific promoter despite an overall lower expression of OVA protein in agreement with what our lab
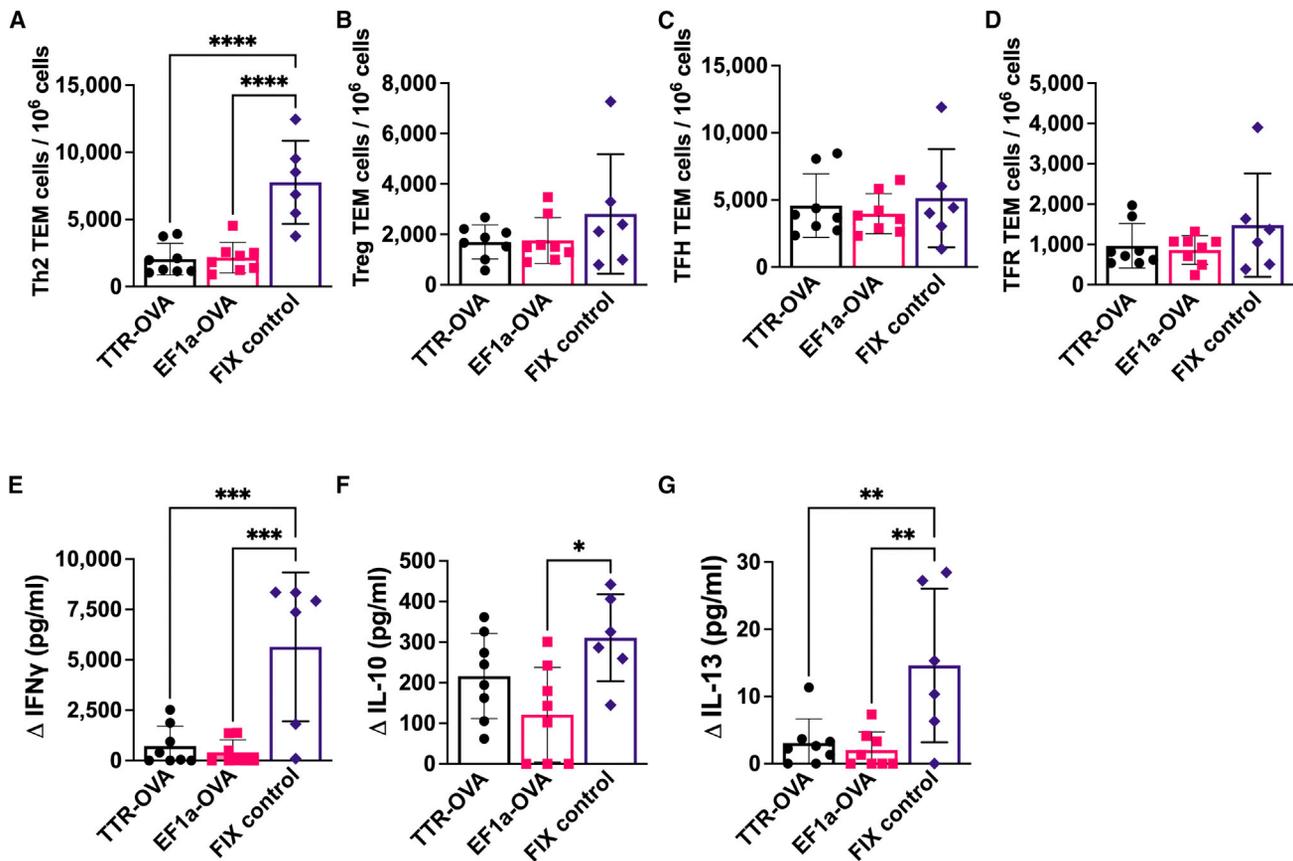
Figure 3. Changes induced in T cells and cytokine secretion by AAV8-OVA gene therapy in prophylaxis studies

(A–G) Flow cytometry determination of the number of splenic (A) Th2, (B) Treg, (C) T follicular helper cells (TFH), and (D) T follicular regulatory cells (TFR) OVA-stimulated T effector memory (TEM) cells per million splenocytes from mice pretreated with AAV8-TTR-OVA, EF1α-OVA, or ApoE-hAAT-FIX vectors. Cytokine expression levels were corrected for background by subtracting basal levels of unstimulated cells from OVA-stimulated cells. Cytokine levels for (E) IFNγ, (F) IL-10, and (G) IL-13 were measured from the supernatants of splenocytes from sensitized mice pretreated with AAV8-TTR-OVA (n = 8), EF1α-OVA (n = 8), or ApoE-hAAT-FIX (n = 6) vectors. Data are presented as single data points and means ± standard deviation. Statistical testing was conducted using one-way ANOVA with Tukey's multiple comparisons. *p < 0.05, ***p < 0.001, and ****p < 0.0001.

and others have reported in other models.[47–49] Importantly, no severe allergic events occurred following AAV8-OVA gene transfer.

Antigen-specific peripheral Tregs are critical for hepatic AAV gene therapy tolerance induction.[50–52] Hepatic AAV-expressed OVA is hypothesized to induce peripheral OVA-specific Tregs through preferential presentation by tolerogenic antigen-presenting cells (APCs).[53] However, the plasticity of peripherally induced Treg may result in an effector phenotype under strong polarizing conditions, such as during allergen sensitization.[42] These Th2-like Tregs can accelerate sensitization through IL-4 cytokine secretion and loss of suppressive function,[41,42] leading to increased activation of TFH cells and B cells and expansion of plasma cells and memory B cells.[54,55] However, the reduction in Th2, plasma cells, and memory B cells suggests that peripheral OVA-specific Tregs induced throughout AAV8-OVA gene therapy maintains their suppressive phenotype during sensitization. In the control group we observed an elevation in both IFNγ and IL-10, which is typically associated with the suppression of Th2 re-

sponses. Here we hypothesize that IFNγ may be acting as an enhancer of mast cell degranulation[56] and IL-10 may be proinflammatory under certain conditions, as it was shown to play a critical role in the development of Th2 responses in an allergic dermatitis mouse model.[57]

Immune tolerance in the context of hepatic AAV gene delivery is predicated on a number of variables including, but not limited to, the AAV capsid, promoter, and transgene product expression levels. In mice presensitized to OVA, we found that the AAV8-EF1α-OVA vector was more effective in suppressing allergic symptoms, which is contradictory to previous studies demonstrating that hepatic restricted expression of an antigen is more effective at inducing immune tolerance.[48] However, these studies used an AAV2 capsid that is less efficient at transducing mouse hepatocytes[47] and can transduce antigen-presenting cells.[58] The hepatic tropism of an AAV8 capsid in mice is sufficient to restrict antigen expression to hepatocytes even with an ubiquitous EF1α promoter.[59] Thus, our data suggest that OVA protein levels are the main determinant of successful
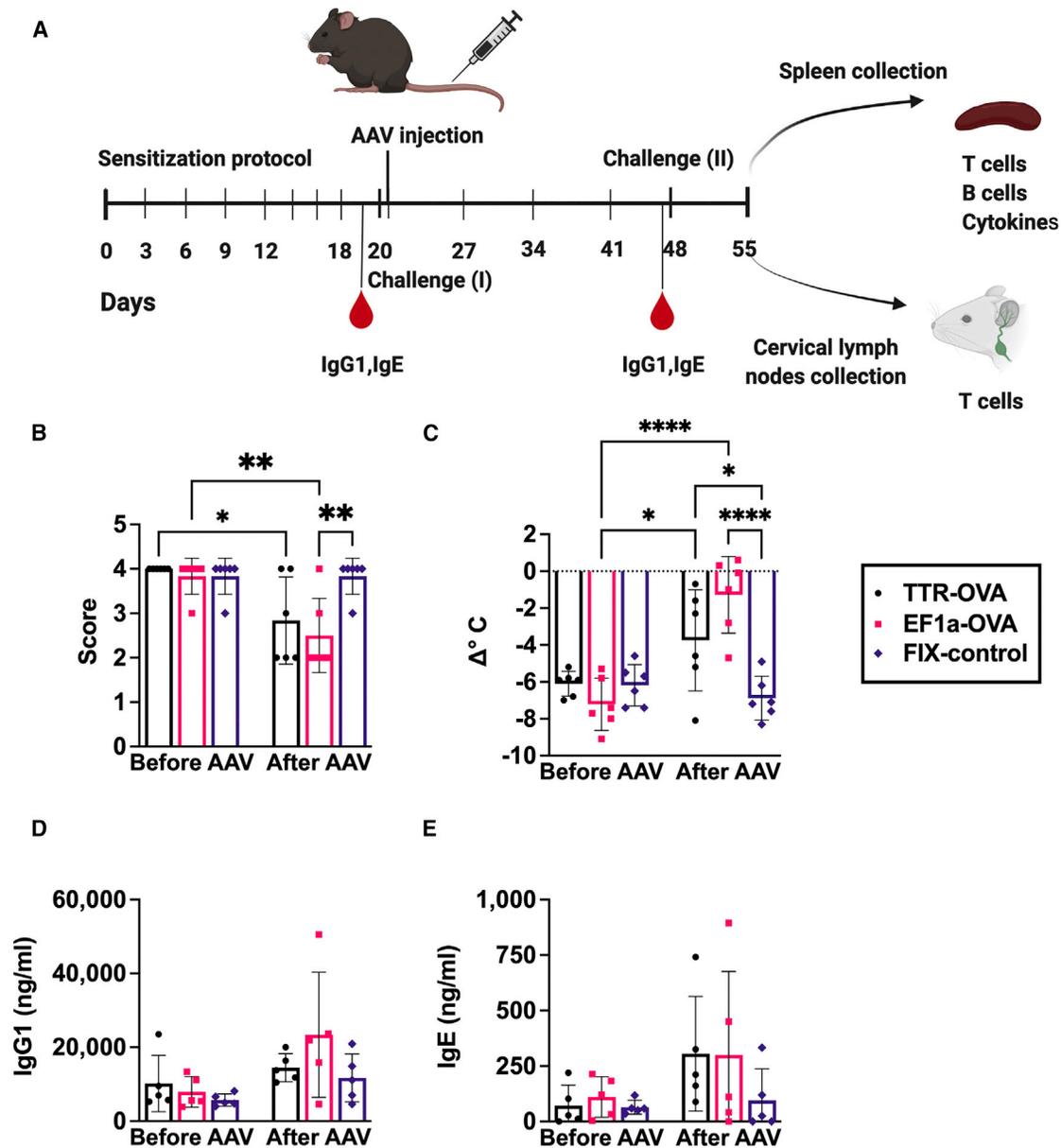
Figure 4. Single-dose treatment with the EF1α-OVA AAV8 vector reduced allergy symptoms

(A) Experimental timeline showing the treatment of OVA-sensitized mice. (B) Changes in symptom score (see Table 1) and (C) core body temperature during the challenge before and after the AAV8 injection. (D) OVA-specific levels of IgG1 and (E) IgE before and after the injection of OVA-sensitized mice with AAV8-TTR-OVA, EF1α-OVA, or ApoE-hAAT-FIX vectors (n = 6 for B and C and n = 5 for D and E). Data are representative of one of three independent experiments. Data are presented as single data points and means ± standard deviation. Statistical testing was conducted using two-way ANOVA with Tukey's multiple comparison; *p < 0.05 and **p < 0.01.

immune tolerance induction in allergic mice. This is in agreement with our previous studies demonstrating that a threshold level of hepatic AAV-expressed FIX protein was necessary to eradicate pathogenic IgE and IgG antibodies in hemophilia B mice. In this model, higher FIX levels (vector doses) correlated with a more rapid eradication of pathogenic antibodies.[23] Similarly, work by Kumar et al. showed that low doses (1×10^9 vg) of the same AAV8-EF1α-OVA vector used in our study on the same C57BL/6 background resulted

in CD8^{+} T cell responses directed against the transgene product (OVA), which was absent at higher vector doses.[59] However, because there are no comparable capsids with strict hepatic tropism in other animal models and humans, translational studies would likely need a strong hepatic promoter for similar outcomes.

An alternative explanation for the reduction in the severity of systemic anaphylaxis is competition of OVA-specific IgG1 antibodies
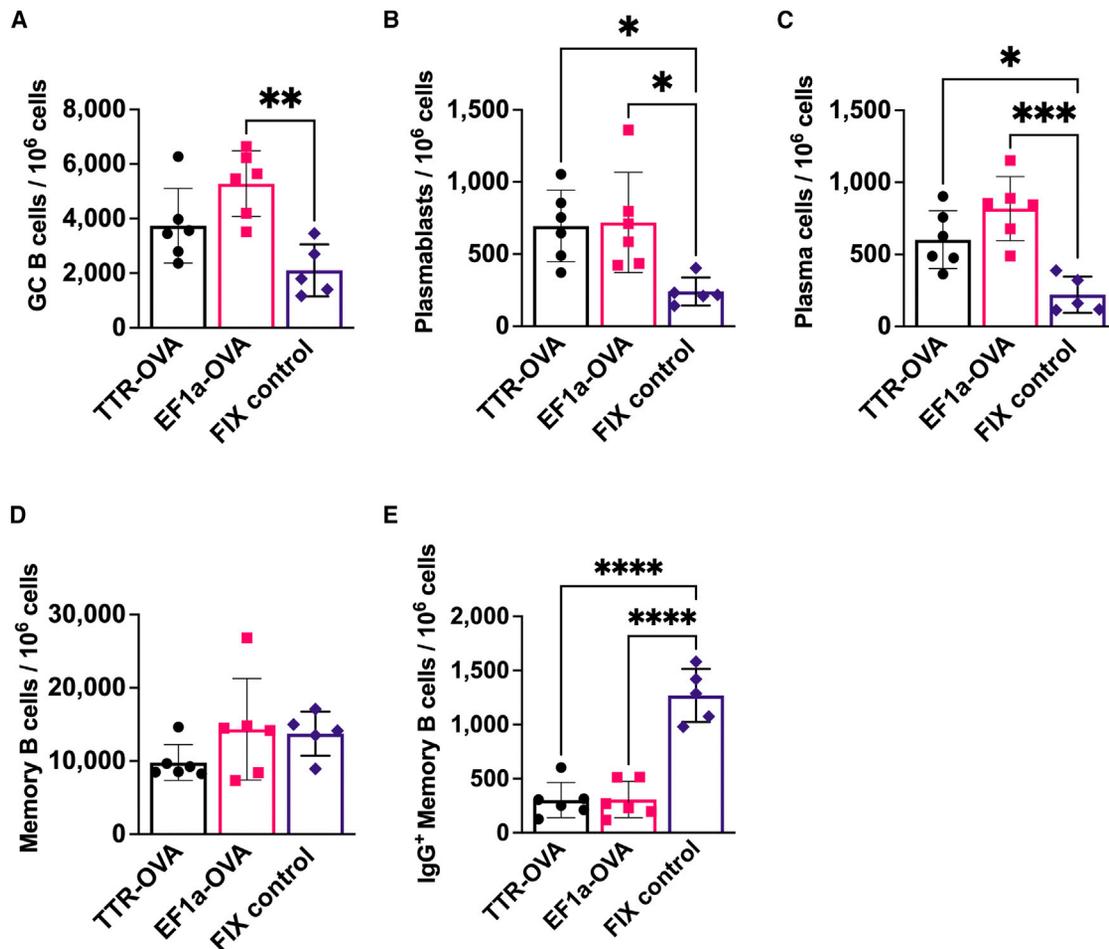
Figure 5. Changes induced by AAV8-OVA treatment in B cell subpopulations in OVA-sensitized mice

Determination of the number of splenic (A) germinal center B cells, (B) plasmablasts, (C) plasma cells, (D) memory B cells, and (E) IgG[+] memory B cells per million of splenocytes of sensitized mice treated with AAV8-TTR-OVA (n = 6), EF1α-OVA (n = 6), or ApoE-hAAT-FIX (n = 5) vectors. Data are presented as single data points and means ± standard deviation. Statistical testing was conducted using one-way ANOVA with Tukey's multiple comparison. *p < 0.05, **p < 0.01, ***p < 0.001, and ****p < 0.0001.

with IgE binding and crosslinking and direct suppression of mast cells and basophils through the inhibitory FcγRIIb receptor, as reported in humans[60] and mice.[61] This mechanism has been described previously in other successful FA immunotherapies.[62–64] It is unclear why anti-OVA IgE and IgG1 antibody titers persisted and even increased following AAV8-OVA gene therapy, despite previous eradication in hemophilia B mice.[23] One cause may be the timing between OVA challenge and AAV8-OVA vector administration (1–2 days post challenge) may result in impaired tolerance because of the spike in anti-OVA antibodies immediately following OVA challenge. This is supported by our modified treatment protocol where we observed a significant reduction in anti-OVA IgE and IgG1 titers (Figure S10). OVA-IgG1 immune complexes may redirect OVA from tolerogenic dendritic cells[65] and drive plasma cell differentiation from existing memory B cells.[66,67] Alternatively, OVA sensitization in FT^{+/−} mice may result in the generation of long-lived plasma cells, which may be refractory to Treg inhibition. Thus, successful elimination of

anti-OVA IgE and IgG1 antibodies in this model may require longer follow-up.

The reduction in anaphylaxis severity is associated with an increase in OVA-specific regulatory T cells (Tregs and TFR) in AAV8-OVA-treated animals. We hypothesize that skin sensitization results in the trafficking of OVA into the CLNs[68] promoting the differentiation and expansion of OVA-specific B and T cells. In the spleen and other non-draining lymphoid organs, exposure to OVA from sensitization requires the migration of APCs, B cells, and T cells from the CLN.[69,70] After AAV8-OVA administration, OVA protein is hypothesized to be taken up and presented by tolerogenic APCs in the liver, generating OVA-specific CD4^{+}FoxP3^{+} TEM cells (Treg and TFR) that can migrate to other tissues, such as lymph nodes.[71] The activation of these cells with OVA protein would lead to an increase of IL-10 cytokine expression that would counteract the Th2 type responses toward an immune tolerance
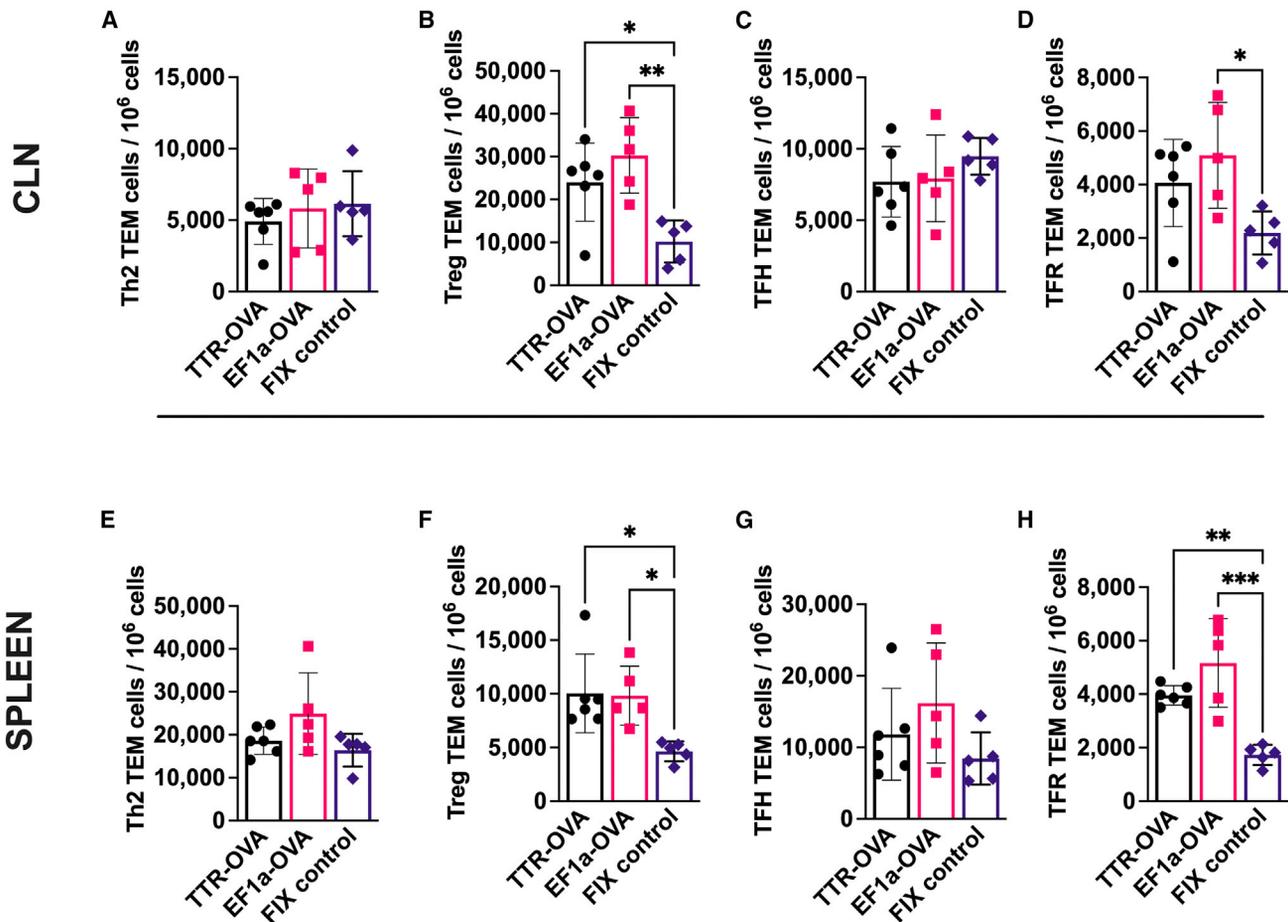
Figure 6. AAV8-OVA gene therapy induces Treg and TFR cells in allergic mice

Flow cytometry determination of the number of Th2, Treg, T follicular helper cells (TFH), and T follicular regulatory cells (TFR) OVA-stimulated T effector memory (TEM) cells per million lymphocytes from cervical lymph nodes (CLNs) (A–D) or splenocytes (E and F) from mice pretreated with AAV8-TTR-OVA (n = 6), EF1α-OVA (n = 5), or ApoE-hAAT-FIX (n = 5) vectors. Data are presented as single data points and means ± standard deviation. Statistical testing was conducted using one-way ANOVA with Tukey's multiple comparisons. $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$.

state. As a result, the severity of the allergic response during contact with allergen would be reduced.

In summary, this work has characterized for the first time the dynamics of B and T cell subsets during AAV liver-directed gene immunotherapy in an FA model. We have demonstrated that a single dose of an AAV-OVA vector can safely treat FA. Induction of allergen-specific Treg and TFR cells was associated with reduced incidence and severity of allergic responses. However, further studies are needed to optimize AAV gene immunotherapy, identify adjunct therapies, and extend to FAs with multiple allergens.

METHODS

AAV production

TTR-OVA, EF1α-OVA, and ApoE-hAAT-FIX AAV8 vectors were produced by transfection of HEK-293 cells and purified by iodixanol gradient as published.[72]

Mice

C57BL/6J mice were purchased from Jackson Laboratories (Bar Harbor, ME, USA). Flaky tail mice with homozygous mutations in $FLg^{ft/ft}/Tmem79^{ma/ma}$ (FT$^{-/-}$) were kindly donated by Dr. Joan Cook-Mills. Female FT$^{+/-}$ mice were generated by breeding FT$^{-/-}$ male mice with C57BL/6J females as described.[73,74] These studies were approved by the Indiana University Institutional Animal Care and Use Committee.

Animal treatment

For the FA prevention studies, 6-week-old female FT$^{+/-}$ mice were IV injected with 1×10^{11} vg per mouse of TTR-OVA, EF1α-OVA, or ApoE-hAAT-FIX vectors in a total volume of 200 μL in sterile PBS. Mice were bled from the retro-orbital plexus 4 weeks after AAV injection under anesthesia using heparinized capillary tubes. Then, mice were sensitized based on Walker et al.[26] with 20 μg of *Alternaria alternata* extract (Greer Laboratories, Lenoir, NC, EEUU) followed
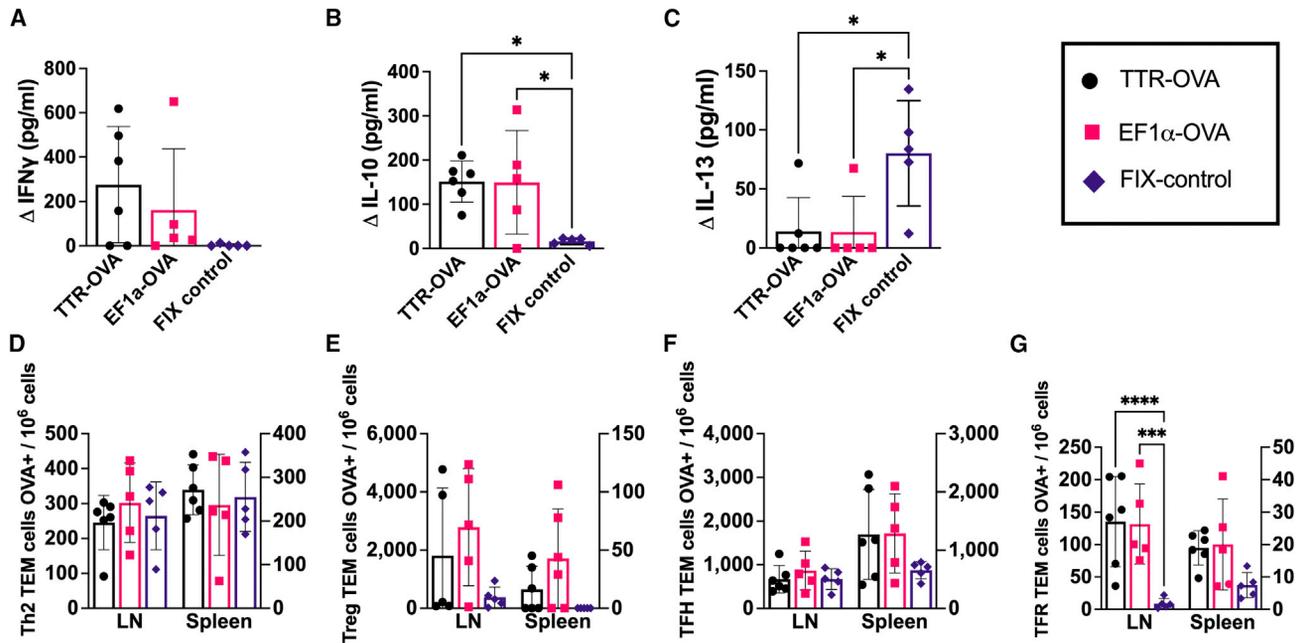
Figure 7. An increase in OVA-specific Treg and TFR TEM cells is associated with a reduction in systemic anaphylaxis in OVA-sensitized mice

Cytokine expression levels were corrected for background by subtracting basal levels of unstimulated cells from OVA-stimulated cells. Cytokine levels for (A) IFNγ, (B) IL-10, and (C) IL-13 were measured from the supernatants of splenocytes from mice treated with AAV8-TTR-OVA (n = 6), EF1α-OVA (n = 6), or ApoE-hAAT-FIX (n = 5) vectors. Flow cytometry determination of OVA-specific (D) Th2, (E) Treg, (F) T follicular helper (TFH), and (G) T follicular helper regulatory (TFR) effector memory cells (TEM) from cervical lymph nodes (CLNs) (left y axis) and spleen (right y axis) from mice treated with AAV8-TTR-OVA, EF1α-OVA, or ApoE-hAAT-FIX vectors. Data are presented as single data points and means ± standard deviation. Statistical testing was conducted using one-way ANOVA with Tukey's multiple comparison for datasets excluding (E) and (G), which was performed using the Kruskal-Wallis test with Dunn's multiple comparison; *p < 0.05, and ****p < 0.0001.

by 200 μg of OVA protein (Sigma, San Luis, MI, EEUU). This procedure was repeated on days 0, 3, 6, 9, 12, 14, 16, and 18. FT$^{+/-}$ mice were bled as previously described on day 19 and challenged through IP injection of 1 mg of OVA.

For the FA treatment studies, the same AAV vectors were injected in mice previously sensitized as described above and after 4 weeks, mice were bled and challenged.

Challenge procedure

FT$^{+/-}$ female mice were challenged by IP injection of 1 mg (for allergy prophylaxis studies) or 0.5 mg of OVA (for allergy treatment studies) diluted in 200 μL of sterile PBS. Core body temperature was recorded before and 15, 30, and 45 min after OVA administration with a rectal thermometer (Physitemp Instruments, Clifton, NJ, EEUU). Phenotype following challenge was recorded using a symptom scale (Table 1). For anaphylaxis pathway determination, sensitized FT$^{+/-}$ female mice were pretreated with 150 mg of triprolidine (Sigma), 25 mg of ABT-491 (Sigma), or both 15 min before challenge with OVA. Then, mice were challenged as described above.

Liver lysates preparation

Liver was collected from FT$^{+/-}$ mice 4 weeks after AAV administration. Livers were homogenized in lysis buffer using a Bead Mill 24

(Thermo Fisher Scientific, Waltham, MA, EEUU) and protein lysates were obtained using the Qproteome Mammalian Protein Prep Kit (Qiagen, Germantown, MD, EEUU) following the manufacturer's instructions. Protein content was determined using a Bradford assay (Bio-Rad, Hercules, CA, EEUU).

Analysis of plasma samples

OVA-specific IgG1 titers were determined in plasma by ELISA based on Biswas et al.[75] IgE levels were determined using an Anti-Ovalbumin IgE ELISA Kit (Cayman Chemical, Ann Arbor, MI, EEUU) following the manufacturer's instructions. Levels of OVA in mouse plasma and liver lysates were quantified using an in-house OVA-specific ELISA previously described by Dobrzynski et al.[76] OVA levels under 30 ng/mL were considered negative and adjusted to zero.

Flow cytometry
Basophil activation test

This assay was performed using heparinized blood samples. Briefly, whole blood was 1:1 diluted with RPMI 1640 cell culture media (Life Technologies, Carlsbad, CA, EEUU) containing 2 μg/mL of OVA, 7.5 ng/mL of anti-IgE (R35-92) (Becton Dickinson, Franklin Lakes, NJ, EEUU) for positive control, or no protein for negative control. After a 2-h incubation at 37°C, samples were stained with surface antibodies anti-CD3 (145-2C11), B220 (RA3-6B2), CD49b (DX5),

IgE (RME-1), and CD200R (OX-110) (Biolegend, San Diego, CA, EEUU). Red blood cells were lysed using Versalyse (Beckman Coulter, Brea, CA, EEUU) and samples were analyzed using an Attune NxT (Thermo Fisher Scientific) flow cytometer. Relative expression levels of the CD200R basophil activation marker were determined with FCS express software (De novo software, Pasadena, CA, EEUU) from mean fluorescence intensities (Figure S4).

B cell staining

One week after IP challenge, splenocytes were isolated and single cell suspensions were stained for B cell subpopulations. Surface characterization was carried with monoclonal antibodies from Biolegend (anti-CD19, CD138, CD267, CD38, GL7, IgM, IgD, and IgG) and anti-CD95 from Miltenyi (Bergisch Gladbach, Germany) as described in Table S1. B cells were phenotyped using an Attune NxT flow cytometer (Thermo Fisher Scientific) (Figure S5) and analyzed with FCS express software (De Novo Software).

T cell staining

For prevention studies, splenocytes obtained 1 week after IP challenge were cultured for 72 h in RPMI 1640 media enriched with glutamine (Gibco, Amarillo, TX, EEUU), penicillin, streptomycin (Gibco), and 10% fetal bovine serum (Corning, New York, NY, EEUU) and stimulated with OVA (100 μg/mL). Cells were stained for flow cytometry analysis and culture supernatants were stored at −80°C for IL-10, IL-13, and IFNγ cytokine determination by ELISA. Surface characterization was carried with monoclonal antibodies from Biolegend (anti-CD3, CD4, CD25, CXCR5, PD1, CD44, and CD62L) as described in Table S2. For intracellular staining, cells were fixed using the eBioscience FOXP3/Transcription Factor Staining Buffer Set (Invitrogen, MA, EEUU) following the manufacturer's instructions and stained with intracellular antibodies anti-GATA3 (Biolegend) and anti-FoxP3 (eBioscience). Dead cells were excluded using Fixable Aqua LIVE/DEAD Fixable Dead Cell dyes (Thermo Fisher Scientific) (Figure S6).

CLN lymphocytes and splenocytes obtained 1 week after IP challenge from allergy reversion studies were cultured as described above. For OVA-specific T cell determination, cells were stained with 18 μg/mL of an OVA MHC-II tetramer (sequence HAAHAEINEA) PE labeled at 37°C for 90 min. Tetramer was kindly provided by NIH Tetramer Core Facility (contract number 75N93020D00005). Later, surface and intracellular characterization was carried out following the same procedure and using the monoclonal antibodies described above in the prevention studies, but substituting anti-GATA3 with anti-IL-4 antibody (Biolegend) (Table S2) in the intracellular staining step for Th2 phenotyping. (Figure S8).

All cells were phenotyped using an Attune NxT flow cytometer (Thermo Fisher Scientific).

Cytokine determination

Cytokine levels of IL-10, IL-13, and IFNγ in culture supernatants were measured using Mouse DuoSet ELISA Kits (R&D, Minneapolis, MN, EEUU) following the manufacturer's instructions.

Statistical analysis

Statistical tests were selected based on testing for normal distribution using Anderson-Darling, D'Agostino & Pearson, Shapiro-Wilk, and Kolmogorov-Smirnov normality tests in GraphPad Prism 9 software. Experiments with two groups with non-normal distribution were analyzed with the Mann-Whitney statistic test. All experiments with three or more groups were analyzed with either one-way or two-way ANOVA to compare means. If all groups passed the normality test they were analyzed using either one-way ANOVA with Tukey's multiple comparison test to test for significant differences in means with a single independent variable (treatment) or two-way ANOVA with Tukey's multiple comparison test to test for significant differences in means with two independent variables (time and treatment). If any group failed the normality test, we used the Kruskal-Wallis test with Dunn's multiple comparison test.

DATA AVAILABILITY

Data are available upon reasonable request.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.omtm.2022.07.008.

AUTHOR CONTRIBUTIONS

M.G.V. conducted the experiments, analyzed the data, and helped with the manuscript writing. X.L. produced the AAVs used in this study. M.M.M. helped with the experiments. M.K. and H.D. helped with the manuscript writing. D.M.M. designed and helped with the experiments and manuscript writing.

DECLARATION OF INTERESTS

M.K. has received grant funding from NIH and the Department of Defense, and has received consulting fees from Ukko.

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