Intrathymic adeno-associated virus gene transfer rapidly restores thymic function and long-term persistence of gene-corrected T cells

Marie Pouzolles, Alice Machado, Mickaël Guilbaud, Magali Irla, Sarah Gailhac, Pierre Barenes, Daniela Cesana, Andrea Calabria, Fabrizio Benedicenti, Arnauld Sergé, et al.

To cite this version:
Marie Pouzolles, Alice Machado, Mickaël Guilbaud, Magali Irla, Sarah Gailhac, et al.. Intrathymic adeno-associated virus gene transfer rapidly restores thymic function and long-term persistence of gene-corrected T cells. Journal of Allergy and Clinical Immunology, Elsevier, 2019, 145 (2), pp.679-697.e5. 10.1016/j.jaci.2019.08.029. hal-02350097

HAL Id: hal-02350097
https://hal.archives-ouvertes.fr/hal-02350097
Submitted on 24 Jan 2020

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L’archive ouverte pluridisciplinaire HAL, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d’enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.
Intrathymic AAV gene transfer rapidly restores thymic function and long-term persistence of gene-corrected T cells

Gene delivery:
Intrathymic injection

ZAP-70−/− Immunodeficient thymus

Thymocytes:
- DN: Double Negative
- DP: Double Positive CD4+CD8+
- SP4: Single Positive CD4+
- SP8: Single Positive CD8+
- Treg: regulatory T cells

Functional gene-corrected T cells

Lymph nodes
Intrathymic AAV gene transfer rapidly restores thymic function and long-term persistence of gene-corrected T cells

Marie Pouzolles, PhD¹; Alice Machado, MSc¹#; Mickaël Guilbaud, MSc²#; Magali Irla, PhD³; Sarah Gailhac, MSc¹; Pierre Barennes, MSc⁴; Daniela Cesana, PhD⁵; Andrea Calabria, PhD⁵; Fabrizio Benedicenti, PhD⁵; Arnauld Sergé, PhD³; Indu Raman, MSc⁶; Quan-Zhen Li, MD, PhD⁶,⁷; Eugenio Montini, PhD⁵; David Klatzmann, MD, PhD⁴,⁸; Oumeya Adjali, MD, PhD²; Naomi Taylor, MD, PhD¹,⁹; and Valérie S. Zimmermann, PhD¹

¹Institut de Génétique Moléculaire de Montpellier, University of Montpellier, CNRS, Montpellier, France; ²INSERM UMR1089, Université de Nantes, Centre Hospitalier Universitaire de Nantes, Nantes, France; ³Center of Immunology Marseille-Luminy (CIML), INSERM U1104, CNRS UMR7280, Aix-Marseille Université UM2, Marseille, 13288 cedex 09, France; ⁴Sorbonne Université, INSERM, Immunology-Immunopathology-Immunotherapy (i3), Paris, France; ⁵San Raffaele Telethon Institute for Gene Therapy (SR-Tiget), IRCCS, San Raffaele Scientific Institute, Milan, 20132, Italy; ⁶Microarray Core Facility, University of Texas Southwestern Medical Center, Dallas, TX, USA; ⁷Department of Immunology, University of Texas Southwestern Medical Center, Dallas, TX, USA; ⁸AP-HP, Hôpital Pitié-Salpêtrière, Biotherapy (CIC-BTi) and Inflammation-Immunopathology-Biotherapy Department (i2B), Paris, France; ⁹Present address: Pediatric Oncology Branch, Center for Cancer Research, National Cancer Institute, National Institutes of Health (NIH), Bethesda, Maryland

#equal contribution
Co-corresponding authors: O. Adjali, N. Taylor or V.S. Zimmermann, Institut de Génétique Moléculaire de Montpellier, 1919 Route de Mende, 34293 Montpellier, Cedex 5, France; Tel: 33 4 67 61 36 29, Fax: 33 4 67 04 02 31; emails: oumeya.adjali@univ-nantes.fr, taylorn4@mail.nih.gov, zimmermann@igmm.cnrs.fr

Funding Sources: M.P. was supported by a PhD fellowship from the LABEX EpiGenMed and the FRM. M.I. and O.A. are supported by INSERM, N.T. by INSERM and the NIH, and V.Z. by CNRS. This work was funded by the AFM, grant R01AI059349 from the National Institute of Allergy and Infectious Diseases, the ANR, ARC and INCa.

Disclosure statement: None of the authors have any disclosures to declare.
ABSTRACT

Background: Patients with T cell immunodeficiencies are generally treated by allogeneic hematopoietic stem cell transplantation but alternatives are needed for patients without matched donors. An innovative intrathymic (IT) gene therapy approach, directly targeting the thymus, may improve outcome.

Objective: To determine the efficacy of IT-adeno-associated virus (AAV) serotypes to transduce thymocyte subsets and correct the T cell immunodeficiency in a ZAP-70-deficient murine model.

Methods: AAV serotypes were injected intrathymically into WT mice and gene transfer efficiency was monitored. ZAP-70−/− mice were intrathymically injected with an AAV8 vector harboring the ZAP-70 gene. Thymus structure, immunophenotyping, TCR clonotypes, T cell function, immune responses to transgenes and autoantibodies, vector copy number and integration were evaluated.

Results: AAV 8, 9 and 10 serotypes all transduced thymocyte subsets following in situ gene transfer, with transduction of up to 5% of cells. IT injection of an AAV8-ZAP-70 vector into ZAP-70−/− mice resulted in a rapid thymocyte differentiation, associated with the development of a thymic medulla. Strikingly, medullary thymic epithelial cells expressing the autoimmune regulator AIRE were detected within 10 days of gene transfer, correlating with the presence of functional effector and regulatory T cell subsets with diverse TCR clonotypes in the periphery. While thymocyte reconstitution was transient, gene-corrected peripheral T cells, harboring approximately 1 AAV genome/cell, persisted for >40 weeks and AAV vector integration was detected.

Conclusions: Intrathymic AAV-transduced progenitors promote a rapid restoration of the thymus architecture with a single wave of thymopoiesis generating long-term peripheral T cell function.
Key Messages:

- Intrathymic AAV gene correction of an immunodeficiency promotes the differentiation of a normal thymic architecture within 10 days post gene transfer
- Intrathymic AAV gene transfer results in vector integration with the persistence of gene-corrected peripheral T cells for >40 weeks

Capsule Summary:

Intrathymic AAV-mediated gene therapy presents a novel therapeutic option for immunodeficient patients, promoting a rapid reconstitution of the thymic environment and subsequent T cell reconstitution.

Key Words:

Severe combined immunodeficiency, Gene therapy, ZAP-70, thymus, intrathymic gene transfer, medulla formation, T cell reconstitution, humoral immunity

Abbreviations:

AAV: adeno-associated virus
AIRED: autoimmune regulator
DN: double negative CD4-CD8-
DP: double positive CD4+CD8+
ELISA: enzyme-linked immunosorbent assay
GvHD: graft versus host disease
HLA: human leukocyte antigen
HSC: hematopoietic stem cell
Ig: immunoglobulin
IT: intrathymic
IV: intravenous
LN: lymph node
MFI: mean fluorescence intensity
mTEC: medullary thymic epithelial cell
PCR: polymerase chain reaction
PID: primary immunodeficiency
SCID: severe combined immunodeficiency
SEM: standard error of the mean
SP4: single positive CD4
SP8: single positive CD8
TEC: thymic epithelial cell
TCR: T cell receptor
Vg/Dg: vector genomes/ diploid genomes
ZAP-70: Zeta-associated protein of 70kDa
INTRODUCTION

Primary immunodeficiency diseases (PID) consist of a heterogeneous group of more than 200 genetic disorders\(^1\), with severe combined immunodeficiency (SCID) characterized by defective T and B lymphocyte function. Transplantation of histocompatible hematopoietic stem cells (HSCs) is the optimal treatment for infants with SCID but in the absence of histocompatible donors, these patients typically receive an HSC transplant from HLA-haploidentical donors. In the latter case, T cells are extensively depleted from the graft in an effort to prevent graft versus host disease (GvHD). Although recent modifications of this protocol have resulted in an increased survival rate, significant short-term and long-term complications are still reported and the lag time during which the patient remains susceptible to infections is quite long\(^2\). Thus, although this treatment is generally successful, it remains important to develop new therapeutic approaches.

Significant efforts have gone into developing gene therapy strategies for these patients. Indeed, gene therapy trials for X-SCID and more recently for adenosine deaminase-deficiency and Wiskott-Aldrich demonstrated that this strategy can cure human disease and they have continued to marketing approval\(^3\)-\(^{12}\). The selective advantage of corrected progenitors and the massive expansion of corrected T cells has facilitated this success. Adverse events due to insertional mutagenesis of gammaretroviral vectors\(^{13}\)-\(^{16}\) have resulted in the development of lentiviral-based clinical trials for SCID patients. Notably, mutagenesis has not been reported in the latter\(^5\),\(^{17}\),\(^{18}\) but it is still important to continue to explore and develop new therapeutic strategies.

Following transplantation of SCID patients with allogeneic healthy HSCs or gene-corrected autologous HSCs, T lymphocyte differentiation occurs in the
Pouzolles et al. 7

thymus. These HSCs, administered by intravenous (IV) injection, must continuously home to the thymus because under physiological conditions, thymic settling progenitors are only able to give rise to a single round of thymocyte differentiation. Notably though, the intrathymic injection of HSCs as well as pro-T cells improves T cell differentiation in the thymus and increases the expansion of supporting thymic epithelial cells. Additionally, the thymus can be targeted by the direct injection of antigens or vectors expressing genes of interest, with recombinant Adeno-Associated Virus (rAAV) vectors transducing thymocytes with a 10-fold higher efficiency than lentiviral vectors. Indeed, rAAV vectors hold great promise for gene transfer therapies because they are capable of infecting non-dividing cells and particles of high titer and purity can be produced.

rAAV vectors were initially developed as single stranded viral DNA vectors. The transduction efficiency of these “conventional” rAAV vectors, based on the AAV2 serotype, is known to be tissue-dependent with significant gene transfer in various tissues but only low level infection of murine hematopoietic cells. However, several AAV serotypes have exhibited increased ability to transduce HSCs and long-term transgene persistence has been detected.

In an attempt to achieve efficient gene transfer in the thymus and correct T cell deficiency, we evaluated in situ intrathymic gene transfer using rAAV2 vectors cross-packaged into AAV 8, 9 and 10 serotypes. Intrathymic administration of all 3 serotypes led to transgene expression in all thymocyte subsets but AAV8 exhibited the highest gene transfer efficiency, resulting in the transduction of up to 5% of all thymocytes. Using mice that are immunodeficient due to mutations in the ZAP-70 protein tyrosine kinase as a paradigm, we found that the intrathymic injection of an AAV2/8-ZAP-70 vector resulted in the
development of gene-corrected mature thymocytes within 10 days of gene transfer. Concurrently, AAV8-ZAP-70 gene transfer promoted the development of the thymic medulla, containing AIRE-expressing medullary thymic epithelial cells (mTECs) that mediate T-cell tolerance. Furthermore, AAV-transduced T cells were detected in the peripheral circulation by 2 weeks and these T cells exhibited long-term function for greater than 10 months, responding robustly to T cell receptor (TCR) stimulation. Thus, the thymus immune niche can be shaped by an intrathymic AAV-based strategy, accelerating the restoration of its architecture and facilitating a transient thymocyte differentiation with long term peripheral T cell function.
METHODS

AAV vector stocks, harboring the GFP or ZAP-70 genes, were administered by intrathymic injection into WT C57Bl/6 mice and ZAP-70−/− mice as indicated. All animal experiments were performed in accordance with the recommendations of the CNRS Animal Care Committee and were consistent with the guidelines set by the Panel on Euthanasia (AVMA) and the NIH Guide for the Care and Use of Laboratory Animals. T cell reconstitution was monitored by flow cytometry and frozen thymic sections were stained as previously described. Vector genomes were monitored by real time PCR, for integration analyses, we adopted a sonication-based linker-mediated PCR method as previously described. Anti-OVA IgG responses were evaluated following ovalbumin immunization, serum anti-ZAP-70 antibodies as well as anti-dsDNA, anti-RNP and anti-SSA antibodies were evaluated by enzyme-linked immunosorbent assay (ELISA), neutralizing antibodies were monitored as previously described and gene expression (Foxp3, IL-10) was evaluated by qRT-PCR. TCR repertoire was evaluated by deep sequencing and data analyzed using R Studio. Screening for IgM/IgG reactivity against autoantigens was performed using autoantibody arrays.
RESULTS

Intrathymic AAV8 gene therapy reconstitutes the thymus architecture within 1.5 weeks and promotes a transient thymocyte differentiation

We first assessed the potential of self-complementary rAAV2 vectors (scAAV) harboring the GFP transgene and pseudotyped with the 8, 9 and 10 capsid serotypes to transduce the murine thymus. Our analyses revealed thymocyte transduction by all 3 serotypes; efficiencies in non-conditioned immunocompetent mice ranged from 2-5% by three days post injection (Fig 1, A). Transduced cells were found in all subsets, including the most immature CD4^-CD8^- double negative (DN), double positive (DP) and the most mature single positive (SP) CD4 (SP4) and CD8 (SP8) thymocytes (Fig 1, A, B). Mice transduced with AAV9 vectors exhibited lower levels of DP thymocytes, within both the non-transduced and transduced subsets, suggesting a possible toxicity (Fig 1, A, B). However, total thymocyte numbers were not significantly altered, with a mean of 180x10^6/ thymus. Furthermore, in mice undergoing IT administration with the AAV2/8 vector, the repartition of thymocyte subsets remained stable (Fig 1, B).

Following transduction of thymocytes, mature SP4 and SP8 thymocytes migrated to the periphery, strikingly representing up to 1% of peripheral T lymphocytes in lymphoid-replete WT mice at day 10 (Fig 1, C). However, gene-transduced peripheral T cells decreased by 2-fold at day 30, likely due to the emigration of newly differentiated mature thymocytes. Given the high-level transduction of thymocytes by AAV8, including immature DN thymocyte
progenitors, we studied the potential of this serotype to correct thymocyte differentiation in an immunodeficient mouse model.

Using a model of ZAP-70-deficient mice, exhibiting an arrest in thymocyte differentiation at the DP stage (reviewed in\textsuperscript{58}), we assessed whether the IT administration of an AAV8 vector could be used to efficiently achieve gene transfer in defective thymocytes. Importantly, 3 days following IT transfer of the AAV2/8-GFP vector used above (Fig 1, A), the transduction of both DN and DP thymocytes was detected (Fig 1, D). Notably, gene transfer was detected in 2-5% of ZAP-70\textsuperscript{-/-} thymocytes, resulting in the transduction of a mean of 0.8x10\textsuperscript{6} DN thymocytes and 4.6x10\textsuperscript{6} DP thymocytes (n=11, Fig 1, D). Thus, the level of IT AAV8 transduction in thymocytes of ZAP-70\textsuperscript{-/-} immunodeficient mice was similar to that detected in WT mice, supporting the utilization of this strategy for gene transfer.

Defects in the development of SP thymocytes, irrespective of the mutation, result in a lack of a functional thymic medulla and it is likely that these stromal abnormalities contribute to immune dysregulation in the thymus (reviewed in\textsuperscript{65, 66}). Defective medulla formation is classically associated with a lack of mTECs expressing the autoimmune regulator (AIRE). Its absence results in an aberrant T-cell selection\textsuperscript{67} and the subsequent development of autoreactive T cells (reviewed in\textsuperscript{68, 69}). Using a transgenic model, it has been shown that the leaky differentiation of as few as 3.5x10\textsuperscript{5} non-transgenic SP4 thymocytes, bearing TCRs with reactivities against self-antigens, is sufficient for the formation of a functional medulla\textsuperscript{70}. As we detected the transduction of >1x10\textsuperscript{6} DN/DP thymocytes in ZAP-70\textsuperscript{-/-} mice using an AAV8 gene transfer approach (Fig 1, E), we hypothesized that an IT AAV8 gene therapy strategy might promote the generation of sufficient SP4 thymocytes for medulla formation. We therefore assessed whether thymic architecture is modified by the intrathymic
administration of single-stranded (ss) AAV8-ZAP-70 virions (1-3x10^{13} vector genomes (vg)/kg). In the absence of gene transfer, thymi of ZAP-70^{-/-} mice harbored only a minimal medulla with a mean of 115 AIRE^{+} cells/mm^{2} of tissue (Fig 2, A, B). Notably, within 10 days following IT AAV8-ZAP-70 gene transfer, there was a dramatic generation of medullary tissue and the number of AIRE^{+} mTECs significantly increased to 295/mm^{2} of tissue (p<0.001; Fig 2, A, B). While the numbers of AIRE^{+} cells decreased between 3 and 10 weeks post IT AAV8-ZAP-70 gene transfer (154/mm^{2} to 95/mm^{2}), they remained significantly higher than those in age-matched ZAP-70^{-/-} mice treated with control AAV8-GFP vector (p<0.05; Fig 2, B). Moreover, medulla development was robust, increasing from <0.03 mm^{2} to 0.06 mm^{2} by 1.5 weeks post gene transfer (p<0.01, Fig E1, A). It is interesting to note that medullary area remained significantly elevated for the first 3 weeks post gene transfer but then decreased at week 10, suggesting a transient thymic reconstitution (Fig E1, A).

As medulla formation and the generation of AIRE^{+} mTECs are dependent on a cross-talk with mature SP thymocytes (reviewed in^{65, 66, 68, 69}), these data strongly suggested that T cell differentiation proceeded rapidly following IT gene delivery. Furthermore, mTEC turnover is estimated at 2-3 weeks^{71, 72}, suggesting that the decrease in AIRE^{+} cells that we observed at 10 weeks post gene transfer (Fig 2, B) was associated with a single wave of thymocyte differentiation. Indeed, within the WT thymus, the percentages of gene-transduced cells that represented mature SP4 and SP8 thymocytes peaked at day 10 following gene transfer, likely representing the maturation of DP thymocytes^{27, 73, 74}, and decreased by 3 weeks, consistent with SP thymocyte emigration^{75} (Fig 1, C and 2, C, p<0.01). Differentiation of SP4 and SP8 thymocytes was detected in AAV8-ZAP-70-treated ZAP-70^{-/-} mice but was not associated with a significant change in total thymocyte numbers, likely due to similar initial thymocyte numbers in the WT and KO mice (Fig E1, B). Notably,
the maturation of gene-transduced SP8 thymocytes in IT AAV8-ZAP-70-corrected ZAP-70$^{-/-}$ mice followed a similar kinetics to that detected in WT mice (Fig 2, C). SP4 thymocytes peaked even earlier, at 3 days post transduction (Fig 2, C, p<0.01, right panel). These data are in agreement with the kinetic signaling model of thymocyte differentiation wherein all DP thymocytes are first signaled to an intermediate SP4 thymocyte fate, before SP8 differentiation. Mature SP thymocytes were detected at all time points in WT mice, but as expected from the physiological involution of the thymus with age, the absolute numbers of SP thymocytes decreased between 0.5 (3 days) and 3 weeks post gene transfer (note that mice were 3 weeks of age at the time of gene transfer; Fig 2, D). The total numbers of SP thymocytes in ZAP-70$^{-/-}$ mice following AAV8-ZAP-70 gene transfer were greater than 2x10$^6$ at 3 days post gene transfer (Fig 2, D). Consistent with the gradual loss of AIRE$^+$ cells and the decreasing medulla size following IT gene correction (Fig 2, B), SP thymocytes decreased to baseline levels at 3 weeks (Fig 2, D). Together these data strongly suggest that intrathymic AAV gene correction provides for a single wave of thymocyte differentiation.

Intrathymic AAV8-ZAP-70 transduction promotes long term maintenance of peripheral T cells and T lineage-specific gene transfer
Consistent with the kinetics of thymocyte differentiation and emigration, gene-corrected T cells were detected in the blood stream within 3 weeks following IT transfer (Fig 3, A). T cells were monitored in peripheral blood samples between 3 and 40 weeks post transduction. It is notable that a single wave of T cell differentiation allowed for the maintenance of a stable level of peripheral T cells for the 40 week evaluation period (>10 months). AAV8-ZAP70-transduced KO mice exhibited a significantly higher percentage of peripheral T cells relative to KO or IT:AAV8-ZAP-70 transduced KO mice at all
time points evaluated; 3-10 weeks, 11-20 weeks, 21-30 weeks and 31-40 weeks (p<0.0001 for all time points, Fig 3, A).

The thymus is the site of T cell differentiation but B cells and hematopoietic progenitors with myeloid lineage potential are also present in this organ\cite{78,79}. Furthermore, AAV virions can diffuse, potentially resulting in the transduction of other cell types (reviewed in\cite{80,81}). It was therefore important to compare the transduction of T cells and non-T cells by AAV8-ZAP-70 virions following intrathymic gene transfer. As expected, almost 100% of all peripheral T cells, encompassing both CD4 and CD8 subsets, expressed the AAV8-encoded ZAP-70 transgene (Fig 3, B). Notably though, transgene expression was not detected in significant levels in non-T cell hematopoietic lineages, including B cells and myeloid cells, nor in the bone marrow during the entire 40 week follow up period (Fig 3, B). Furthermore, expression levels of ectopic ZAP-70 in gene-transduced peripheral T cells were approximately 2-4-fold higher than endogenous levels, monitored as a function of MFI, and these levels did not significantly change during the 40 week follow up (Fig 3, C).

We next assessed whether the maintenance of transduced T cells in the peripheral circulation was associated with a stable percentage and number of CD3\(^+\) T cells in the lymph nodes of AAV8-ZAP-70-treated mice (Fig 3, D). While T cell levels were significantly lower than those detected in WT mice, both in percentages and absolute numbers, they were induced by 3 weeks post intrathymic transduction, revealing the rapid initial wave of AAV-transgene-mediated thymocyte differentiation. Furthermore, they remained stable for >10 months. The progression of immature DP thymocytes through positive selection requires ZAP-70-dependent TCR signaling\cite{82,83}. Positively selected DP thymocytes then undergo CD4/CD8 lineage choice, a complex process that has been shown to occur through kinetic signaling: Under conditions of persistent
TCR signaling, CD4 T cell differentiation occurs whereas MHC-class I-specific CD8 T cells only differentiate following a disruption of MHC-class II/TCR signals and subsequent cytokine signaling. Interestingly, in the presence of high levels of ectopic ZAP-70 from the AAV8-ZAP-70 vector (Fig 3, C), both the thymic and peripheral CD4/CD8 ratios in AAV8-ZAP-70-treated mice was significantly skewed to a CD4 lineage fate (p<0.05; Fig 2, D and 3, E).

Intrathymic AAV gene transfer results in the development of T cells with integrated AAV8-ZAP-70 vectors and diverse αβ T cell receptor clonotypes

The persistence of gene-transduced T cells for >40 weeks was surprising in light of high level T cell proliferation and the propensity of AAV vectors to remain episomal. We therefore first assessed vector copy numbers in peripheral T cells and determined that they ranged from approximately 0.1 to 1 vector genomes per diploid genome (Vg/Dg), at time points ranging between 3 to 43 weeks post intrathymic gene transfer (Fig 4, A). The low copy number suggested that the vector was integrated but to further assess this point, AAV8-ZAP-70-reconstituted T cells were TCR-stimulated and copy number was evaluated. TCR-stimulated AAV8-ZAP-70-reconstituted T cells proliferated robustly, as monitored by dilution of the CTV proliferation dye, demonstrating a reconstituted TCR signaling cascade (Fig 4, B). Moreover, while the vast majority of reconstituted T cells underwent >4 divisions, AAV copy number (Vg/Dg) was not significantly altered, strongly pointing to vector integration (Fig 4, B).

To directly assess vector integration, genomic DNA was fragmented by sonication (to avoid biases caused by the non-random distribution of restriction sites on the genome), ligated to a barcoded linker cassette prior to PCR amplification, and evaluated via sonication linker-mediated (SLiM) PCR.
Nested PCR with vector and linker cassette specific oligonucleotides allowed detection of PCR products with at least two different primers in LN samples from 3 of 6 IT:AAV8-ZAP-70-transduced mice (and 2 of 2 mice at 43 weeks post gene transfer; Fig 4, C). Thus, integrated AAV is likely to be responsible for driving ZAP-70 expression in peripheral T cells, under conditions of long-term persistence and TCR stimulation.

The potential of differentiated T cells to recognize a large array of foreign proteins is fostered by the unparalleled diversity of the TCR with recombined α and β polypeptide chains allowing for the generation of >$10^{15}$ unique receptors$^{87}$. However, under conditions of limited thymocyte differentiation, repertoire diversity can be reduced$^{34}$. Furthermore, infections and tumors can drive an antigen-mediated bias in αβ TCR repertoires (reviewed in$^{88}$). While only few studies have monitored TCR repertoires in patients or mice treated with rAAV vectors, the robust T cell response against an AAV transgene in one trial was shown to be associated with a biased TCRBV repertoire$^{89}$. It was therefore of important to assess the repertoire diversity in our gene corrected mice. As expected, the numbers of detected TRA sequences in KO as well as AAV8-GFP-transduced mice was minimal. However, they increased rapidly following ZAP-70 gene transfer (Day 10, Fig 4, D). TCR repertoire diversity was lower than that of WT mice but the number of distinct TRAV, TRAJ, TRBV, and TRBJ clonotypes increased until 3 weeks post gene transfer and remained stable until 43 weeks (Fig 4, D). In WT mice, clonotype distribution was not yet fully diverse at day 10 of life, being made up of a few mildly expanded TCRs. By week 3, WT mice exhibited repertoires that were fully diverse, with 1-3 copies per clonotype (Fig 4, D, bottom). The repertoires of AAV8-ZAP-70-reconstituted mice were less diverse than WT mice, with an expansion of some clonotypes, but a diverse population of TCRs was detected (Fig 4, D).
Induction of humoral immunity in IT-AAV8-ZAP-70-treated mice

To determine whether AAV8-ZAP-70-tranduced mice can mount an immune response, mice were immunized with ovalbumin (OVA) at a late time point post gene transfer (46 weeks). At this time point, neither WT nor KO mice harbored OVA-specific antibodies. Notably though, mice that were intrathymically transduced with AAV8-ZAP-70 but not AAV8-GFP generated a significantly higher level of anti-OVA IgG antibodies by 6 weeks post immunization (1:2,000 dilution, p<0.001, Fig 5, A). While antibody generation was lower than that detected in WT mice, AAV8-ZAP-70-transduced mice were already 1 year post gene transfer and the level of IgG generation was highly significant relative to KO mice (p<0.001; Fig 5, A).

Changes in TCR repertoire distribution can result from immune responses against AAV capsid epitopes as well as the expressed transgene (reviewed in\textsuperscript{90}). Furthermore, different rAAV serotypes have induced T-cell-dependent and –independent anti-capsid humoral responses\textsuperscript{91}, even when injected into an immune-privileged organ such as the brain\textsuperscript{92}. We therefore monitored the presence of anti-capsid antibodies in IT AAV8-treated ZAP-70\textsuperscript{-/-} mice as a function of neutralization; neutralizing factors were defined as serum dilutions that inhibited AAV8 infection by >50%. No control ZAP-70\textsuperscript{-/-} mice harbored neutralizing antibodies and at a 1/100 dilution, antibodies were not detected in IT AAV8-ZAP-70-treated ZAP-70\textsuperscript{-/-} mice. However, at a 1/10 dilution, 10 of 14 IT AAV8-ZAP-70-treated ZAP-70\textsuperscript{-/-} mice were positive between 3 and 17 weeks post transduction (71%). Notably though, at this dilution, anti-AAV8 neutralizing activity was also detected in a high percentage of IT AAV8-GFP-treated ZAP-70\textsuperscript{-/-} mice; mice that do not have peripheral T cells (4 of 5, 80%; Fig 5, B). Thus, the intrathymic injection of the AAV8 serotype induces a low level T cell-independent anti-capsid humoral response.
We next assessed whether the reconstituted mice exhibited an immune response against the ZAP-70 transgene. The intrathymic expression of the injected transgene likely minimizes an immune response, enhancing the deletion of autoreactive thymocytes\textsuperscript{33, 35}, but our findings of a T cell-independent anti-capsid neutralizing activity (Fig 5, B) suggested the possibility of an anti-transgene response. While anti-ZAP-70 antibodies were not detected at 3 weeks post AAV8-ZAP-70 transduction, 5 of 7 mice harbored antibodies at 10 weeks (>25 ng/ml sera; p<0.01). Mean antibody levels then decreased at 17 weeks (from 58 to 34 ng/ml) and by 43 weeks, antibody levels were <30ng/ml (n=5, Fig 5, C) and ZAP-70 protein did not elicit T cell activation (data not shown). In conclusion, anti-transgene antibodies were produced at early time points following intrathymic transduction but levels decreased and were sufficiently low that they did not result in the elimination of gene-transduced T lymphocytes (Fig 2, B).

Patients with primary immunodeficiencies often present autoimmune manifestations and both immunodeficient patients and mice have been shown to produce a diverse range of autoantibodies\textsuperscript{64, 93}. We therefore assessed whether gene-corrected ZAP-70/- mice produced autoantibodies. Using an autoantigen microarray containing 123 different autoantigens, we did not observe increased levels of autoantibody production in age-matched ZAP-70\textsuperscript{+/-} mice as compared to control mice. However, IT injections of both control AAV8-GFP and AAV8-ZAP-70 vectors resulted in the induction of IgM antibodies against a broad panel of autoantigens by 3 weeks post gene transfer (Fig E2). IgG antibodies were significantly higher in IT AAV8-ZAP-70-transduced mice, in accord with the requirement of functional T cells for immunoglobulin class switching. IgG autoantibodies peaked at 10 weeks post transduction\textsuperscript{63, 64} (Fig 6, A). Importantly though, absolute levels of antibodies against 3 tested autoantigens (double-stranded DNA (dsDNA), ribonucleoprotein (RNP), and
Sjögren’s-syndrome-related antigen A (SSA)) were significantly lower in AAV8-ZAP-70-transduced mice at both 10 weeks and 43 weeks post gene transfer than in MRL/lpr mice which spontaneously develop a systemic lupus erythematosus (SLE)-like syndrome. Furthermore, levels in AAV8-ZAP-70-transduced mice were not significantly higher than that detected in control MRL/MpJ mice (Fig 6, B). Thus, intrathymic AAV8 administration induces a rapid but low-level, broad spectrum of autoantibodies that decreases with time. Notably, these low levels of autoantibodies did not result in pathological consequences as mice survived for over 50 weeks without overt autoimmune manifestations.

Intrathymic AAV8-ZAP-70 gene transfer results in the differentiation of effector and regulatory T cell subsets

Under conditions of lymphopenia, naïve T cells proliferate in response to weak TCR interactions with self-peptide/MHC complexes, differentiating into memory-like T cells in the absence of antigenic stimulation (reviewed in⁹⁴). It was therefore important to study the phenotype of peripheral T cells in IT-AAV8-ZAP-70-reconstituted mice. Notably, reconstituted mice exhibited a significant increase in CD62L⁺CD44⁺ effector memory T cells as compared to the majority of CD62L⁺CD44⁻ naïve T cells in WT mice. This biased ratio of memory: naïve T cells was detected throughout the experimental period of 10 months (Fig 7, A). Thus, in our study, a single wave of thymocyte differentiation following IT-AAV8-ZAP-70 gene transfer resulted in the peripheral differentiation of polyclonal T lymphocytes with a memory/effector phenotype.

To determine the potential effector function of AAV8-ZAP-70-transduced T cells, we first assessed in vivo cytokine levels. In vivo, there was a transient increase in TNF-α and IFN-γ levels at 3 weeks post AAV8 transduction (Fig E3, A), correlating with the induction of a low-level humoral response (Fig 6, A and
However, cytokine levels then returned to baseline by 10 weeks (Fig E3, A), consistent with the decrease in autoantibodies. We next examined the ability of peripheral AAV8-ZAP70-reconstituted T cells to secrete cytokines upon anti-CD3/anti-CD28 mAb activation. IL-17, TNF-α and IFN-γ were secreted by T cells from AAV8-ZAP70-reconstituted mice by 3 weeks after gene transfer and secretion of all 3 cytokines was significantly higher than that detected in KO mice by 10 weeks (n=3-10 mice per time point, Fig 7, B; p<0.05, p<0.001). As compared to WT peripheral T cells, IL-17 was the only cytokine that was significantly higher in gene-corrected mice (Fig 7, B; p<0.05). Thus, peripheral AAV8-ZAP70-reconstituted T cells were competent to secrete cytokines following ex vivo TCR stimulation.

Despite high ex vivo TCR-induced cytokine secretion, in vivo cytokine levels were not elevated and AAV-reconstituted mice did not exhibit autoimmune symptoms. It was therefore important to assess whether gene-corrected regulatory T cells (Treg) controlled immune responsiveness in these mice. Notably, the development of Tregs expressing the Foxp3 transcription factor is dependent on a thymic medulla, a structure which was restored by day 10 of gene transfer (Fig 2, A). Accordingly, by 3 weeks post gene transfer, Tregs were detected in the peripheral circulation of AAV8-ZAP-70-treated mice at levels similar to those in WT mice (Fig 7, C). Notably, by 10 weeks post IT gene transfer, the percentages of Tregs were significantly higher in AAV8-ZAP-70-treated mice than in WT mice (Fig 7, C; p<0.05). Importantly, these T cells exhibited an activated phenotype as monitored by significantly higher levels of Treg markers including CTLA4, GITR and CD39 (n=5 per group, Fig E3, B). As regards function, sorted CD4+CD25hi cells from both WT and AAV8-ZAP-70-transduced cells revealed equivalent levels of Foxp3 transcripts (n=12) but IL-10 levels were significantly higher in the latter (p<0.05; Fig 7, D). Furthermore, IL-10 levels were significantly higher in conventional (CD4+CD25− T cells) from
reconstituted mice than WT mice (n=6-9; p<0.005). Thus, a high suppressive environment in reconstituted regulatory T cells likely restrains in vivo immune activation following AAV8-ZAP-70 gene transfer.
DISCUSSION

Thymocyte development is dependent on the thymic stroma, composed of thymic epithelial cells (TECs) and non-epithelial cells that together form an organized 3-dimensional network. The intercellular communications between developing thymocytes and TECs result in a thymus architecture that allows the generation of a diverse and self-tolerant pool of mature T cells. In SCID patients as well as in cancer patients with thymus damage, an abnormal thymic architecture negatively impacts on thymocyte differentiation (reviewed in 19, 65, 66, 97). Here, we show that an intrathymic AAV8 gene correction results in a remarkable generation of mTECs and the development of a medullary architecture in ZAP-70-deficient mice, within 10 days of vector administration. Moreover, the absolute number of mTECs expressing AIRE increased by 3-fold in this period. AIRE is critical for immune tolerance, promoting the deletion of self-reactive thymocytes and the development of regulatory T cells 65, 66, 68, 69. Therefore, to date, the IT AAV gene transfer strategy described here appears to be the most rapid regulator of thymic architecture in immunodeficient mice.

As the crosstalk between mTECs and developing thymocytes is required for T cell maturation, extensive research has focused on the identification of factors, conditions and cell types that enhance mTEC reconstitution (reviewed in 68). The differentiation of autoreactive mature CD4 thymocytes regulates the formation and organization of the medulla in an antigen-dependent manner as shown by the importance of the CD28–CD80/86 and CD40–CD40L costimulatory pathways as well as RANK-RANKL and LTαβ-LTβR interactions (reviewed in 68). Furthermore, sex steroid ablation was found to enhance thymocyte differentiation by increasing Notch ligand expression on TECs 99-101 while keratinocyte growth factor (KGF), IL-22, Bmp4 and FoxN1 directly promote TEC proliferation and survival 102-104. Specific cell types such as pro-T
cells facilitate thymus reconstruction\textsuperscript{32, 105, 106} while innate lymphoid cells induce an IL-22–mediated TEC survival\textsuperscript{107}. Chemokines can also facilitate thymic reconstitution; under conditions of thymic damage, CCL21 treatment of hematopoietic progenitors rescues their migration while a combined inhibition of p53 and KGF increases intrathymic CCL21 expression and TEC recovery\textsuperscript{108, 109}. Notably though, the success of these approaches often requires a significant lag time whereas an IT AAV gene strategy corrected the medullary microenvironment within <10 days, concomitant with a correction of the T cell deficiency and the differentiation of SP4 thymocytes. It will be of interest to determine whether a combined approach, concomitantly correcting a T cell deficiency and the medullary microenvironment\textsuperscript{59}, further promotes reconstitution. Furthermore, AAV can be used to deliver TEC-inducing molecules to patients suffering from suboptimal T cell differentiation—for example, boosting thymocyte differentiation in HSC-transplanted cancer patients by IT AAV administration of the IL-22 cytokine. This type of intrathymic targeting approach, by ultrasound-mediated guidance, has been shown to be minimally invasive and safe in both mice and macaques\textsuperscript{28, 31, 37, 110}. Further studies, focused on the intrathymic administration of vectors and hematopoietic progenitor cells into non-human primates by interventional radiologists, will help to establish the clinical framework in which this type of therapeutic strategy can be developed.

AAV vectors have successfully been used to achieve gene transfer in numerous tissues\textsuperscript{81}. Nevertheless, studies of AAV gene transfer in the hematopoietic system have been more sparse, likely due to the extensive division of these cells. However, AAV1, modified AAV6 and AAV7 vectors have been found to efficiently transduce HSCs\textsuperscript{54, 57, 111, 112}. Furthermore, we show here that AAV8, 9 and 10 serotypes promote efficient thymocyte transduction. While the property of AAV vector genomes to exist primarily as episomes would
be expected to negatively affect their ability to be used for a stable gene therapy in dividing cells, long-term persistence of transgene expression has been detected in HSCs\textsuperscript{54, 55, 57}. Moreover, it has recently been shown that T lymphocytes represent the in vivo site of AAV persistence following natural infection\textsuperscript{113}. This is likely linked to the AAV vector-mediated long-term transgene maintenance (>10 months) that we detected in gene-corrected T lymphocytes. Indeed, our findings that AAV vector copy number in reconstituted T cells was approximately 1 and that long-term reconstituted T cells harbored integrated vector is likely to broaden the scope of immunological deficiencies for which this type of approach can be envisioned. As persistence of intrathymic-injected progenitors with a specific T cell receptor gene provided for life-long immunity\textsuperscript{31}, it will be of interest to determine whether the direct targeting of thymocytes with an AAV8-encoded tumor-specific TCR or chimeric antigen receptor (CAR) will promote an anti-tumor response that is superior to that currently achieved by ex vivo manipulation of peripheral T cells.

Immune responses against AAV capsid serotypes as well as expressed transgenes have hampered the success of AAV gene therapy trials (reviewed in\textsuperscript{114}). Even following AAV injections into regions that are considered to be immune-privileged such as the brain\textsuperscript{92} or the intravitreal space\textsuperscript{115, 116}, anti-capsid antibodies have been detected. Nevertheless, the development of immunosuppressive treatments has protected against AAV capsid immunity\textsuperscript{117, 118, 119}. Indeed, long-term transgene expression has been observed following the systemic AAV administration of genes such as myotubularin\textsuperscript{120, 121} and microdystrophin\textsuperscript{122}, promoting AAV-based treatments for patients with X-linked myotubular myopathy and Duchenne muscular dystrophy, respectively.

While T cell immunity can provoke anti-AAV reactivity, it can also limit pathological responses. The recruitment of suppressive regulatory T cells as
well as organ-specific T cell exhaustion can result in extended transgene expression\textsuperscript{123,124}. While we initially hypothesized that the direct administration of AAV into the thymus would result in the deletion of autoreactive thymocytes and the subsequent absence of an immune response\textsuperscript{33, 35}, we detected low level humoral responses to both the AAV8 capsid and the ZAP-70 transgene. Interestingly, anti-capsid responses were induced at similar levels in ZAP-70\textsuperscript{-/-} mice treated by IT administration of the control AAV8-GFP vector. As T cell differentiation did not proceed in these latter mice, these data indicate the development of a T cell-independent immune response. In contrast, IgG autoantibodies were generated in AAV gene-corrected ZAP-70\textsuperscript{-/-} mice, pointing to the importance of T cells in immunoglobulin class switching. Moreover, similarly to patients where AAV transduction correlated with increases in specific TCRBV families\textsuperscript{123}, the humoral immune response in IT AAV8-ZAP-70-treated mice was associated with a diverse but biased TCR repertoire. Notably though, the levels of generated autoantibodies were significantly lower than that detected in SLE-prone MRL/lpr mice and none of the IT gene therapy-treated mice developed symptoms of autoimmune disease during the 10 month follow up period. This is likely due to 1) the low and transient nature of the autoantibody response and 2) the extensive differentiation of AAV-corrected thymocytes to a Treg fate. Notably, AAV gene transfer resulted in a T effector/Treg ratio that allowed for a balanced immune homeostasis.

Combination therapies will likely optimize life-long T cell differentiation in SCID patients and T cell reconstitution in cancer patients. An ideal treatment will promote 1) HSC expansion, 2) the migration and entry of hematopoietic progenitors into the thymus 3) a thymus architecture that is conducive to thymocyte maturation and 4) the selection of a broad-based repertoire of self-tolerant effector and regulatory T cells. One bottleneck is progenitor entry as the thymus is not continually receptive to the import of hematopoietic
progenitors, at least in mice\textsuperscript{125, 126}. Furthermore, irradiation reduces progenitor homing by >10-fold\textsuperscript{109}. While this bottleneck can be partially overcome by injecting HSCs or hematopoietic progenitors directly into the thymus\textsuperscript{24, 27, 28, 31, 110, 127, 128}, histocompatible donors are not always available. Our study reveals the potential of an IT AAV8 gene therapy strategy to promote an extremely rapid reconstitution of the thymus microenvironment and the differentiation of mTEC-dependent regulatory T cells within a 7-10 day period.

In our studies, a single wave of thymocyte differentiation promoted the long-term maintenance of peripheral gene-corrected T cells. Indeed, in several “experiments” of nature”, patients with mutations that generally result in a SCID phenotype were found to be relatively healthy with significant T cell numbers, due to a reversion mutation. As this type of event is statistically improbable, it is likely that the peripheral T cell reconstitution detected in these patients (with mutations in WAS, RAG1, LAD, NEMO, CD3zeta, γc and ADA) is due to a reversion mutation in a single hematopoietic progenitor\textsuperscript{129-141}. While these data strongly support the hypothesis that gene correction of a very limited number of thymic progenitors can give rise to a large number of peripheral T cells, it remains to be determined whether T cells in these patients are due to a single wave of thymocyte differentiation. Moreover, in the context of an intrathymic gene transfer approach, it will be important to assess whether the remnant thymus present in many patients with SCID as well as the natural process of thymic involution would negatively impact on the type of therapeutic strategy described here. Importantly, we found that intrathymic gene transfer into Rag2-/- mice with a remnant thymus is feasible by a surgical approach but the success of intrathymic HSC transfer into older mice with an involuted thymus is significantly reduced (our unpublished observations). Thus, a combination therapy of intrathymic AAV gene transfer, promoting a restoration of the thymic architecture and medulla formation, followed by an intravenous HSC
transplantation, allowing long term T cell differentiation in a reconstituted thymus, may presents a novel approach for the treatment of immunodeficient patients requiring a rapid T cell reconstitution.

Acknowledgements
We thank all members of our lab for their scientific critique and support. The authors thank the Center for Production of Vector (CPV- vector core from University Hospital of Nantes / French Institute of Health (INSERM) / University of Nantes) http://umr1089.univ-nantes.fr/plateaux-technologiques/cpv/centre-de-production-de-vecteurs-2194757.kjsp?RH=1518531897125 and are grateful to Myriam Boyer of Montpellier Rio Imaging for support in cytometry experiments, Léo Garcia for his assistance in bioinformatic analyses, Luz Blanco and Mariana Kaplan for generously providing serum from autoimmune MRL/lpr mice and providing advice on ELISAs and the ZEFI staff for their support of animal experiments.

Author Contributions
M.P., O.A., N.T. and V.S.Z. conceived the study. M.P., A.M., M.G., S.G. and V.S.Z. designed, performed and analyzed the experiments. M.I. and A.S. performed and analyzed histological analyses; TCR repertoire analysis was performed by P.B. and D.K.; D.C., F.B., A.S. and E.M. designed and performed the integration analyses; and I.R. and P-Z.L. performed the autoantibody microarray analyses. M.P, N.T. and V.S.Z. wrote the manuscript and A.M., M.I. and O.A. provided important critical review.
REFERENCES


FIGURE LEGENDS

FIG 1. AAV 8, 9 and 10 serotypes efficiently transduce all thymocyte subsets. A, scAAV 8, 9 and 10 serotypes harboring GFP were administered intrathymically (IT) into WT mice and GFP expression was assessed at day 3. Representative CD4/CD8 profiles from non-transduced (grey) and transduced (green) GFP+ thymocytes are presented. B, Quantification of the percentages of GFP+ double negative (DN), double positive (DP), single positive CD4 (SP4) and single positive CD8 (SP8) thymocytes are shown and compared to non-transduced WT mice (upper panel). Thymocyte subset repartition within non-transduced (GFP-) and transduced (GFP+) compartments of AAV8-GFP-transduced mice are presented (lower panel; means±SEM). C, The percentages of GFP+ thymocytes and lymph node (LN) T cells are presented as a function of time (n=3-5 mice per time point). D, Representative plots of GFP- and GFP+ thymocytes at day 3 following IT injection of the scAAV-GFP vector into ZAP-70-/- (KO) mice (left). Quantification of GFP+ cells in KO and AAV-transduced KO mice (n=11) are presented (top). The mean percentages of DN and DP thymocytes within the non-transduced (GFP-, grey) and transduced (GFP+, green) subsets (left bottom panel) as well as the absolute numbers of transduced GFP+ thymocytes are shown (right bottom panel).

FIG 2. Intrathymic AAV8-ZAP-70 gene transfer results in the rapid reconstitution of the thymic architecture in ZAP-70-/- mice. A, ssAAV8-ZAP-70 or scAAV8-GFP virions were IT-injected into ZAP-70-/- (KO) mice and thymi from WT, KO and gene-transduced mice were evaluated for the presence of a thymic medulla by keratin14 (K14) staining and AIRE+ cells. Representative confocal images of thymic tissue sections at 1.5 (10 days), 3 and 10 weeks (w) post gene transfer are shown. B, The number of AIRE+ cells/mm² of thymus were quantified in the indicated conditions. Each point
represents the quantification of individual medulla derived from 3 distinct mice. **C**, Mean percentages of mature SP4 and SP8 thymocytes within the transduced GFP+ and ZAP-70+ subsets are shown in WT (left panel) and ZAP-70−/− (right panel) mice at the indicated time points following intrathymic AAV8-GFP and AAV8-ZAP-70 gene transfer, respectively. **D**, Absolute numbers of SP4 and SP8 thymocytes in WT mice (left panel) and AAV8-ZAP-70-transduced mice (right panel) were determined from the SP4 and SP8 gates at the indicated time points (n=5). Statistical significance was determined using an unpaired 2-tailed t-test or a one-way ANOVA with a Tukey’s multiple comparison test; *, p<0.05; **, p<0.01; ***, p<0.001; ****, p<0.0001.

**FIG 3. Intrathymic AAV8 transduction results in long-term maintenance of peripheral T cells and T lineage-specific transgene expression.** **A**, The presence of peripheral blood T cells was monitored by flow cytometry following IT administration of AAV8-GFP or AAV8-ZAP-70 vectors and data from individual mice are presented at 3-43 weeks. Statistical differences between mice treated by IT AAV8-ZAP-70 gene transfer as compared to IT AAV8-GFP transfer or control KO mice were evaluated by a one-way ANOVA with a Tukey’s multiple comparison test for the following time groups; 3-10 weeks, 11-20 weeks, 21-30 weeks and 31-40 weeks (***, p<0.001, for all groups). **B**, The percentages of peripheral CD4+, CD8+, CD19+ and CD11b+ hematopoietic cells expressing ectopic ZAP-70 following IT AAV8-ZAP-70 transduction are presented. **C**, ZAP-70 protein expression in transduced T cells was evaluated by intracellular staining and MFI relative to WT T cells are presented at the indicated time points. **D**, Representative plots of CD3+ lymph node T cells are presented. Total T cell numbers (means±SEM) are shown (right). **E**, The repartition of CD4/CD8 lymphocytes and mean CD4/CD8 ratios are shown at the indicated time points. Statistical significance was determined by a 2-tailed unpaired t-test *, p<0.05.
FIG 4. AAV8-ZAP-70-transduced peripheral T lymphocytes exhibit stable vector genomes following TCR-induced proliferation and diverse TCR repertoires. A, AAV genome copy number in lymph node samples was assessed by qPCR in IT AAV8-ZAP-70-transduced mice at the indicated time points. Vector genomes per diploid genome (Vg/Dg) were quantified relative to the albumin gene and normalized to the percentage of T cells. B, Proliferation was assessed following TCR stimulation by CTV fluorescence and representative histograms at day 0 (light grey) and day 4 (dark grey) are shown. AAV genome copy was monitored as above and quantifications (n=9) are shown (p, non-significant (ns)). C, Schematic representation of the AAV-ZAP-70 vector are shown. PCR products, representing integrated vector, are shown at the indicated time points and T cells from WT mice are presented as a negative control. D, The number of distinct TRA V, TRA J, TRA VJ, TRB V, TRB J, TRB VJ, TRA and TRB clonotypes are presented (top). Clonotype frequencies are presented with each clonotype represented by a dot whose size is proportional to its frequency. Violin plots represent the density of the distribution and black dots show the median frequency per repertoire (bottom).

FIG 5. Induction of a T cell-independent humoral response to AAV8 capsid epitopes following intrathymic vector administration. A, Total IgG titers against OVA were monitored in non-immunized WT, KO and IT-AAV8-ZAP-70-treated KO mice (46w post gene transfer) and 6 weeks post immunization by ELISA. Reactivity was monitored as a function of dilution, as indicated (n=4). Statistical significance was evaluated at each dilution by a one-way ANOVA with a Tukey’s multiple comparison test for each dilution and is noted for significant differences; ***, p<0.001; **, p<0.01. B, Neutralizing antibodies (Nab) against the AAV8 serotype was monitored in WT and ZAP-70<sup>−/−</sup> mice following intrathymic AAV8-GFP and AAV8-ZAP-70 transduction. Serum
dilutions of 1:10, 1:100 and 1:1000 are shown by the decreasing slope of the triangle, respectively, and Nab activity is defined as a decrease in AAV transduction of >50% compared to AAV incubation with PBS alone (red line). Each point represents serum from an individual mouse at 3, 10 and 17 weeks post transduction and mean levels are indicated by a horizontal line. C, Antibodies against ZAP-70 protein were measured by ELISA and data are presented as ng of antibody/ ml of serum. Antibody levels of >25ng/ml are considered significant.

**FIG 6.** Transient induction of a broad spectrum of antibodies in ZAP-70^+^ mice following intrathymic AAV8 gene transfer remains significantly lower than that detected in autoimmune mice. A, The presence of IgG autoantibodies in serum samples of ZAP-70^+^ (KO) mice and following IT administration of either AAV8-GFP or AAV8-ZAP-70 vectors was evaluated at the indicated time points. IgG autoantibodies against 123 antigens were assessed by protein microarray and heat maps showing autoantibody reactivity relative to levels in age-matched WT mice are presented. B, The levels of autoantibodies against RNP, dsDNA and SSA were evaluated by ELISA in control MRL/MpJ mice, autoimmune-prone MRL/lpr mice and IT:AAV8-ZAP-70-treated mice at 10 and 43 weeks post gene transfer. Each point represents serum from an individual mouse and statistical significance was determined by a one-way ANOVA with a Tukey’s multiple comparison test and significance is indicated; **, p<0.01; ***, p<0.001 ****, p<0.0001, ns-non significant.

**FIG 7.** AAV8-ZAP-70-transduced thymocytes differentiate into effector and regulatory T cells. A, The presence of naïve (CD62L^+^CD44^−^, N), central memory (CD62L^+^ CD44^+, CM), and effector memory (CD62L^−^CD44^+, EM) cells within the CD4 and CD8 subsets were monitored at 3, 10, 17 and 43 weeks post IT-AAV8-ZAP-70 gene transfer. The percentages of cells in each
subset were quantified at each time point and mean levels ± SEM (n=4-5 mice per time point) are presented. **B**, LN cells from the indicated mice were stimulated ex vivo with anti-CD3/anti-CD28 mAbs for 3 days and cytokine secretion was measured. Each point represents data from a single mouse and horizontal lines represent mean levels (pg/ml). **C**, The percentages of Foxp3+CD25+ peripheral Tregs was evaluated and representative dot plots are presented (left) together with mean percentages ± SEM at each time point (n=5-14 mice per time point, right). **D**, Conventional and regulatory CD4 T cell subsets were FACS-sorted on the basis of CD25 expression. Foxp3 and IL-10 transcripts in each subset were monitored by qRT-PCR (n=2 to 4 mice in 2 independent experiments). Statistical significance was determined by a 2-tailed unpaired t-test and significance is indicated; *, p<0.05; **, p<0.01; ***, p<0.001 ****, p<0.0001.
MATERIALS AND METHODS

rAAV constructs and vector production. The human ZAP-70 gene was cloned downstream of the murine phosphoglycerate kinase (PGK) promoter with the bovine growth hormone (BGH) polyadenylation signal sequences flanked by two AAV2-ITRs sequences. For the green fluorescent protein (GFP) coding vector plasmid, the PGK promoter is upstream of the GFP sequence and the simian virus 40 (SV40) polyadenylation signal.

Self-complementary (sc) AAV-8, AAV-9 and AAV-10 harboring GFP and single-stranded (ss) AAV-8 harboring ZAP-70 vector stocks were produced by cotransfection of HEK293 cells with the vector plasmid and the pDG plasmid\(^1\), an adenoviral plasmid providing helper functions needed for rAAV assembly, as previously described\(^2\). Supernatants were precipitated with polyethylene glycol (PEG, Sigma-Aldrich) and resuspended in TBS before benzonase digestion. Vector-containing supernatants were purified on a double CsCl gradient with the first gradient centrifuged at 28,000 rpm for 24 hours at 15°C and the band harboring full particles was centrifuged at 38,000 rpm for 48 hours. Viral suspensions were then subjected to 4 successive rounds of dialysis against DPBS in a Slide-a Lyzer cassette (Thermo Scientific). The number of infectious particles/ml in the purified vector prep was determined using a stable rep-cap HeLa cell line in a modified Replication Center Assay (RCA)\(^3\). Vectors were stored at <-70°C in polypropylene low-binding cryovials.

Mice and intrathymic AAV gene therapy. C57Bl/6 mice ZAP-70\(^+/-\) mice were maintained under specific pathogen-free conditions in the IGMM animal facility (Montpellier, France). Serum from female MRL/lpr and sex-matched control
MRL/MpJ mice were generously provided by Luz Blanco and Mariana Kaplan (NIAMS, NIH). Intrathymic injections were performed in 2.5-3 week old non-conditioned mice with rAAV vectors (1-3x10^{13} vg/kg) as previously described\textsuperscript{4}. Briefly, non-conditioned mice were anesthetized with isoflurane and vectors were directly injected into the thymus (20 µl total volume) by insertion of a 0.3 ml 28 gauge 8 mm insulin syringe through the skin into the thoracic cavity above the sternum. All experiments were approved by the local animal facility institutional review board in accordance with national guidelines.

**Immunophenotyping, T cell activations and flow cytometry analyses.**

Cells, isolated from thymi, lymph nodes or spleen, were stained with the appropriate conjugated \(\alpha\)CD3, \(\alpha\)CD4, \(\alpha\)CD8, \(\alpha\)CD11b, \(\alpha\)CD19, \(\alpha\)CD25, \(\alpha\)CD62L, \(\alpha\)CD39, \(\alpha\)CD44 \(\alpha\)CTLA4 and \(\alpha\)GITR mAbs (Becton Dickinson, San Diego, CA). Intracellular staining for Foxp3 and ZAP-70 was performed following fixation/permeabilization (eBioscience) and using the BD Biosciences kit, respectively. Cell activations were performed using plate-bound anti-CD3 (clone 17A2; 1 µg/ml) and anti-CD28 (clone PV-1; 1 µg/ml) mAbs in RPMI 1640 media (Life Technologies) supplemented with 10% FCS. Cell proliferation was monitored by labelling with CTV (Life Technologies; 5 µM) for 3 min at RT. Cytokine production was monitored using a Cytometric Bead Array (CBA) Kit (BD Biosciences). Stained cells were analyzed by flow cytometry (FACS-Canto II or LSR II-Fortessa, Becton Dickinson, San Jose, CA). Data analyses were performed using Diva (BD Biosciences), FlowJo Mac v.10.4.2 software (Tree Star) and FCAP Array Software (CBA analysis).

**Immunohistochemistry.** Frozen thymic sections were stained as previously described\textsuperscript{5}. Sections were stained with anti-keratin 14 (1:800; AF64, Covance Research) and a secondary Cy3-conjugated anti-rabbit (1:500; Invitrogen) Ab
together with an Alexa Fluor 488-conjugated anti-AIRE mAb (1:200; 5H12, eBioscience). They were then counterstained with 1 ug/ml DAPI as reported. Images were acquired on a LSM 780 Zeiss and SP5 Leica confocal microscopes and quantified using ImageJ and Matlab software.

Quantitative real-time PCR for evaluation of vector genomes and qRT-PCR for cellular genes. Total DNA was extracted using the Gentra Puregene kit (Qiagen) and Tissue Lyser II (Qiagen) according to the manufacturer’s instructions. Vector genome DNAs were measured through quantification of BGH-pA by quantitative PCR with Premix Ex Taq kit (Takara). Primers were: 5’-TCTAGTTGCCAGCCATCTGTTGT-3’ (forward) and 5’-TGGGAGTGGCACCTTCCA-3’ (reverse) and the BGH-pA TaqMan probe was: 5’-(6 FAM)-TCCCCCGTGCCCTTCTTGACC-(TAMRA)-3’. Primers for endogenous albumin were: 5’-ACATAGCTTGCTTACAGAGTGAAGGT-3’ (forward) and 5’-AGTGTCTTACATCCTGCCCCTAAA-3’ (reverse). Quantitative PCR for BGH-pA was performed using the following program: initial denaturation 20 sec at 95°C followed by 45 cycles of 1 sec at 95°C and 20 sec at 60°C. qPCR for albumin was performed as follows: initial denaturation for 20 sec at 95°C followed by 45 cycles of 3 sec at 90°C and 30 sec at 60°C. For each qPCR, cycle threshold values (Ct) were compared with those obtained using dilutions of plasmids harboring either the BGH-pA or albumin genes and results are expressed as vector genome per diploid genome (vg/dg).

For assessment of IL-10 and Foxp3 mRNA levels, CD4+CD25− and CD4+CD25Hi cells from WT and IT:AAV8-ZAP-70 mice (15 weeks post-injection) were sorted on a FACSaria and RNA extraction was performed using the RNeasy Mini kit (Qiagen). RNA was reverse-transcribed into cDNA by oligonucleotide priming using the QuantiTect Reverse Transcription Kit (Qiagen). Each sample was amplified in triplicate (LightCycler 480, Roche) and
normalized to HPRT levels using the following specific primers: Foxp3-sense: 5'-GGCCCTTCTCCAGGACAGA-3', Foxp3-antisense: 5'-GCTGATCATGGCTGGTTGT-3', IL10-sense: 5'-AACTGCTCCACTGCCTTGCT-3', IL10-antisense: 5'-GGTTGCCAAGCTATCGGA-3', Hprt-sense: 5'-Hprt-antisense: 5'-

**Vector integration analysis.** To assess AAV integration, we adopted a sonication-based linker-mediated PCR method, as previously described. 7, 8 Briefly, genomic DNA was sheared using a Covaris E220 Ultrasonicator (Covaris Inc.), generating fragments with a target size of 1,000 bp. The fragmented DNA was subjected to end repair, 3' adenylation and ligation (NEBNext® Ultra™ DNA Library Prep Kit for Illumina®, New England Biolabs) to custom linker cassettes (LC) (Integrated DNA Technologies). LC sequences contain: i) 8 nucleotides barcode, used for sample identification and ii) 12 random nucleotides, used for quantification purposes. Ligation products were subjected to 35 cycles of exponential PCR with primers complementary to 8 different regions of the AAV genome (see Fig 4, C and primers available upon request), and the LC. For each set of AAV-specific primers, the procedure was performed in technical replicates (n=2-3) starting from 5 to 120 ng of sheared DNA. All final PCR products were quantified by qPCR using the Kapa Biosystems Library Quantification Kit for Illumina, following the manufacturer’s instructions. qPCR was performed in triplicate on each PCR product diluted 10^-3, and the concentrations were calculated by plotting the average Ct values against the provided standard curve, using absolute quantification.

**TCR Deep Sequencing and Data Processing.** Cells from lymph nodes were pooled, lysed and RNAs extracted. T cell receptor (TCR) alpha/beta libraries
were prepared with 100 ng of total RNA from each sample using the SMARTer Mouse TCR α/β Profiling Kit from Takarabio® and sequenced by MiSeq V3 2x300bp (Illumina®). Raw data were aligned and curated using the MiXCR software (v2.1.10)9. Alpha and Beta chains were analyzed together. Each sample was defined as a combination of TRV-CDR3aa-TRJ sequences and their associated counts. For representation and analyses, datasets were normalized by a sampling step. The sampling size was equal to the smallest sequence number observed in the analyzed samples (n=687). The sampling was performed for each dataset by 100 random draws of sequences and unique TCRs were listed and counted. Analyses and figures were executed and created using R Studio (version 3.5.0).

**Evaluation of humoral immune responses.** For evaluation of in vivo T cell responses, WT and AAV8-reconstituted mice (46w post gene transfer, 49 weeks of age) were immunized with ovalbumin (3x200 μg complexed with complete Freund’s adjuvant at Day 0, incomplete Freund’s adjuvant at day 14 and LPS at day 28). Serum IgG levels were evaluated at time 0 and 6 weeks post immunization by enzyme-linked immunosorbent assay (ELISA). Serum anti-ZAP-70 antibodies were evaluated by ELISA at different time points post gene transfer. Briefly, 96 well plates (Nunc MaxiSorp, Thermo Scientific) were coated with recombinant ZAP-70 protein (0.5 μg/mL, Thermo Scientific), sera were added at 1:100 and 1:500 dilutions and a standard scale was generated by serial dilutions of an anti-ZAP-70 mAb (clone 1E7.2, Thermo Scientific). Responsiveness was monitored by horseradish peroxidase-conjugated goat anti-mouse IgG (Dako) and revealed using 2.2-3,3’5,5’-Tetramethylbenzidine (TMB, BD OptEIA, BD Biosciences). The threshold of positivity was determined by averaging the signal of 23 negative sera obtained from naive or AAV GFP injected mice ± 2*SD. Serum anti-double stranded DNA (dsDNA), anti-ribonucleoprotein (RNP) and SSA (Sjögren’s-syndrome-related antigen A)
were quantified by ELISA following the manufacturer’s instructions (Alpha Diagnostic International, San Antonio, TX).

The presence of neutralizing antibody in serum samples was monitored as previously described\(^\text{10}\). Two hours prior to rAAV infection, a permissive cell line was infected with wild-type Adenovirus serotype 5. During this incubation time, a rAAV2/8 expressing the reporter gene LacZ (encoding beta-galactosidase) was incubated with serial dilutions of serum and the mix was added to the cell line. Infection was assessed using the luminescent β-galactosidase substrate (Tropix Galacto-Star kit, Thermo Scientific) at 48h. The neutralizing capacity of a given serum is expressed as the titer corresponding to the highest serum dilution at which more than 50% of maximal infection is inhibited.

Screening for IgM and IgG reactivity against 123 autoantigens was performed using autoantibody arrays (UT Southwestern Medical Center, Genomic and Microarray Core Facility, Dallas, Texas) as previously described\(^\text{11, 12}\) and heatmaps were generated using R software.

**Statistical analyses**

Data were analyzed using GraphPad software version 5 and 8 (Graph Pad Prism, La Jolla, CA) and p values were calculated using unpaired t-tests, one-way ANOVA (Tukey’s multiple comparison test), and Mann-Whitney tests, as indicated. P values for comparisons of all conditions in the different figure panels are presented in the figure legends.

**Extended Figures**

**Fig E1. Intrathymic AAV8-ZAP-70 gene transfer results in alterations in medulla area in ZAP-70\(^{-/-}\) mice.** A, Thymic medulla area was evaluated by keratin14 (K14) staining. Representative confocal images of thymic tissue...
sections are presented in Figure 2. Quantification of the medullary area in mm² of thymus was quantified in the indicated conditions. Each point represents the quantification of individual medulla derived from 2-3 mice at the indicated time points and significance was determined by a 2-tailed unpaired t-test. ns, non-significant; **, p<0.01; ****, p<0.0001. B, The absolute numbers of thymocytes in IT-AAV8-ZAP-70 transduced KO mice (n=11) were determined at 3 weeks post gene transfer as well as in age-matched WT and KO control mice (n=3-10). Each point represents an individual mouse with horizontal lines showing means. Data between groups were not significantly different.

**Fig E2. Transient induction of a broad spectrum of IgM antibodies in ZAP-70⁻/⁻ mice following intrathymic AAV8 gene transfer.** IgM autoantibody levels were evaluated in sera of ZAP-70⁻/⁻ (KO) mice and following IT administration of either AAV8-GFP or AAV8-ZAP-70 vectors at the indicated time points. IgM autoantibodies against 123 antigens were assessed by protein microarray. Heat maps showing autoantibody reactivity relative to levels in age-matched WT mice are presented.

**Fig E3. High expression of Treg markers in IT: AAV8-ZAP-70-transduced mice.** A, Cytokine levels in sera of WT and ZAP-70⁻/⁻ mice treated by IT AAV8-ZAP-70 gene transfer was evaluated at 3, 10, and 43 weeks post injection by cytokine bead array. Each point represents data from a single mouse and horizontal lines represent mean levels (pg/ml). Statistical significance was determined by a 2-tailed Mann-Whitney test and no groups exhibited significant differences. B, Surface levels of CTLA4, GITR and CD39 on CD4⁺CD25⁺ T cells from WT and IT:AAV8-ZAP-70-transduced KO mice were evaluated by flow cytometry and representative histograms are shown (top). Quantification of the mean fluorescence intensity (MFI) in T cells from individual mice are
presented and significance was assessed by an unpaired t-test (bottom). **, p<0.01; ****, p<0.0001

References
Fig 2

A

WT KO
Transduced thymocytes (%)

WT KO
Transduced thymocytes (%)

B

WT KO
Transduced thymocytes (%)

C

WT: AAV8-GFP
KO: AAV8-ZAP-70

D

WT
KO: AAV8-ZAP-70

Transduced thymocytes (%)

Transduced thymocytes (%)

Thymocytes (x10⁶)

Thymocytes (x10⁶)
**Fig 3**

**A**

Peripheral T cells (%)

![Graph showing weeks post gene transfer vs. peripheral T cells percent](image)

**B**

ZAP-70+ cells (%)

![Graph showing weeks post gene transfer vs. ZAP-70+ cells percent](image)

**C**

ZAP-70 expression (relative to wt)

![Graph showing weeks post gene transfer vs. ZAP-70 expression relative to wt](image)

**D**

![Histogram showing T cells (x10^7)](image)

**E**

![Histogram showing CD4/CD8 ratio](image)
**Fig 5**

**A**

![Graph showing arbitrary unit (log2) against time points (D0 and D43 post-immunization)]

**B**

![Graph showing Nab activity (%) against time points (WT, KO, IT: AAV8-GFP, IT: AAV8-ZAP-70)]

**C**

![Graph showing anti-ZAP-70 (ng/mL sera) against time points (WT, KO, IT: AAV8-GFP, IT: AAV8-ZAP-70)]

Legends:
- WT
- IT: AAV8-GFP
- IT: AAV8-ZAP-70

Time points:
- D0
- D43 post-immunization

Nab activity levels are indicated with different symbols: 
- **: p < 0.001
- ***: p < 0.0001
- *: p < 0.05

Time points include:
- 3W-ZAP
- 10W-ZAP
- 19W-ZAP
- 43W-ZAP

Nab activity and anti-ZAP-70 levels are presented in arbitrary units and ng/mL sera, respectively.
**Fig 7**

**A**

<table>
<thead>
<tr>
<th></th>
<th>IT: AAV8-GFP</th>
<th>IT: AAV8-ZAP-70 (weeks)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>CD4</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>WT</td>
<td>10</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>18</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>64</td>
<td>43</td>
</tr>
<tr>
<td></td>
<td>11</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>0.1</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>1</td>
</tr>
</tbody>
</table>

| **CD8**  |              |                          |
| CD4      |              |                          |
|          | 2            | 17                       |
|          | 19           | 17                       |
|          | 45           | 17                       |
|          | 22           | 78                       |
|          | 46           | 19                       |
|          | 25           | 57                       |
|          | 25           | 25                       |

**B**

<table>
<thead>
<tr>
<th></th>
<th>IL-2</th>
<th>IL-17</th>
<th>TNF-α</th>
<th>IFN-γ</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cytokines (pg/ml)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>WT</td>
<td>3</td>
<td>10</td>
<td>17</td>
<td>43</td>
</tr>
<tr>
<td>KO</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th></th>
<th>IL-10</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cytokines (pg/ml)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>WT</td>
<td>1.5</td>
<td></td>
</tr>
<tr>
<td>KO</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td></td>
<td>10</td>
<td></td>
</tr>
<tr>
<td></td>
<td>17</td>
<td></td>
</tr>
<tr>
<td></td>
<td>43</td>
<td></td>
</tr>
</tbody>
</table>

**C**

<table>
<thead>
<tr>
<th></th>
<th>Foxp3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cytokines (pg/ml)</td>
<td></td>
</tr>
<tr>
<td>WT</td>
<td>10</td>
</tr>
<tr>
<td>KO</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>14</td>
</tr>
<tr>
<td></td>
<td>34</td>
</tr>
<tr>
<td></td>
<td>30</td>
</tr>
</tbody>
</table>

**D**

<table>
<thead>
<tr>
<th></th>
<th>Foxp3</th>
<th>IL-10</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cytokines (pg/ml)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>WT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IT: AAV8-ZAP-70</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD4+ CD25+</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD4+ CD25-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD4+ CD25+</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD4+ CD25-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD4+ CD25+</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD4+ CD25-</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Relative expression**

<table>
<thead>
<tr>
<th></th>
<th>Foxp3</th>
<th>IL-10</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cytokines (pg/ml)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>WT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IT: AAV8-ZAP-70</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD4+ CD25+</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD4+ CD25-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD4+ CD25+</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD4+ CD25-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD4+ CD25+</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD4+ CD25-</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Fig E3**

**A**

<table>
<thead>
<tr>
<th></th>
<th>IL-2</th>
<th>IL-17</th>
<th>TNF-α</th>
<th>IFN-γ</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>KO</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IT: AAV8-ZAP-70 (w)</td>
<td>50</td>
<td>100</td>
<td>200</td>
<td>300</td>
</tr>
</tbody>
</table>

**B**

- **CTLA4**
- **GITR**
- **CD39**

- **Mean ± SD**
  - WT
  - IT: AAV8-ZAP-70
  - WT
  - IT: AAV8-ZAP-70
  - WT
  - IT: AAV8-ZAP-70
  - WT
  - IT: AAV8-ZAP-70

Cytokines (pg/ml)

- IL-2
- IL-17
- TNF-α
- IFN-γ

WT KO

3 10 43

IT: AAV8-ZAP-70 (w)