Oral-tolerization prevents immune responses and improves transgene persistence following AAV-mediated gene transfer

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Short title: oral-tolerization improves transgene persistence

Abstract

Gene therapy represents a feasible strategy to treat inherited monogenic diseases and intramuscular (i.m.) injection of recombinant adeno-associated viral (AAV) vector is now recognized as a convenient and safe method of gene transfer. However, this approach is hampered by immune responses directed against the vector and against the transgenic protein. We used here to reproduce this situation a mouse model where robust immune responses are induced following injection of an AAV vector coding for an immunogenic transgenic protein. We show that prophylactic oral administration of the immunogenic protein before AAV-mediated gene transfer completely prevented antibody formation and cytotoxic $CD8^+$ T cell response. Consistently, prophylactic oral-tolerization considerably improved long-term transgene persistence and expression. Mechanistically, inhibition of the cytotoxic immune response involved abortive proliferation of antigen-specific cytotoxic $CD8^+$ T cells, up-regulation of the PD-1 immunoregulatory molecule, down-regulation of the Bcl-2 anti-apoptotic factor, and their deletion in the context of AAV-mediated gene transfer. Hence, gene therapy may represent an ideal situation where oral-tolerization can be adopted before or at the same time as vector injection to efficiently prevent deleterious immune responses directed against the transgenic protein.

Keywords: gene therapy, AAV vectors, immune responses, oral tolerance, transgenic proteins, transgene persistence.

Introduction

The use of viral-derived vectors in gene therapy settings represents a promising strategy to treat monogenic diseases or to induce the expression of a given secreted therapeutic transgenic protein *in vivo* [1, 2]. Recombinant AAV vectors represent a safe and efficient way to achieve *in vivo* gene transfer and, depending on the serotype, can be used to transduce different target tissues [3, 4]. AAV vectors also demonstrated several advantages over other viral-derived vectors, as their lower immunogenicity, their ability to transduce non-dividing cells, and their lower risk to induce insertional mutagenesis [1-5].

AAV-mediated gene transfer and long-term transgene expression has been achieved in several preclinical animal models as well as in clinical trials where it was demonstrated to be safe and effective [6-8]. However, AAV-mediated gene transfer triggers immune responses directed against the viral capsid proteins and/or against the transgenic proteins [5, 7, 9-14]. Activation of cytotoxic $CD8^+$ T cells mediates the destruction of transduced cells and loss of transgene expression [7, 9, 14-16] and humoral immunity generates neutralizing antibodies [5, 16-19]. To date, administration of immunosuppressive drugs and/or careful patients selection to avoid immune responses directed notably against the transgenic protein represent the only approaches to circumvent this limitation [7, 11, 18, 20-24]. Yet, immune responses have been observed in clinical trials despite the use of immunosuppressive drugs [18, 21]. Hence, strategies aiming to circumvent immune responses following AAV vectors injection should greatly enhance long term transgene expression and may broaden the number of patient electable for gene therapy [10, 11]. In our study, we evaluated an alternative approach to promote antigen-specific oral-tolerization instead of systemic immune suppression.

Oral tolerance is characterized by a specific inhibition of the immune responses directed against defined antigens administered by the oral route. Therefore, it has the advantage over drug-based immunosuppression to be antigen-specific and long-lasting. Tolerance induction represents the “default” response of the intestinal immune system. Intestinal dendritic cells (DCs), located in the lamina propria and mesenteric lymph nodes (MLNs), have been implicated in the uptake of orally derived antigens and in their tolerogenic presentation to T cells [25-30]. Several mechanisms have been identified that could account for the induction of oral tolerance as anergy and/or deletion of antigen-specific T cells [31, 32] or induction of regulatory T cells (Tregs) [25, 33].

Encouraging results have been obtained in clinical trials using oral tolerance to treat allergies while partial clinical responses, limited to particular subsets of individual, were observed in the context of autoimmune diseases. This suggests that the efficacy of oral-tolerization might depend on the clinical situation and on the specific immune status of each patient [34, 35]. One explanation may reside in the difficulty to tolerize secondary/memory immune responses suggesting that oral-tolerization may be beneficial in prophylactic rather than curative scenario [36]. Interestingly, gene therapy may offer such clinical situations by providing the possibility to tolerize individuals before vector injection.

We tested in the present study the effects of oral-tolerization in an animal model of AAV-mediated gene transfer. For that, the protein coded by the vector was first orally administrated for 7 days prior to AAV-mediated gene transfer. To stringently evaluate the tolerization capacity of this protocol, we chose here as a protein model the highly immunogenic ovalbumin (Ova) antigen and the i.m. route. We previously showed that i.m. injection of AAV-Ova induces prominent humoral and cellular immune responses that are associated with rapid loss of transgene expression [37, 38]. We provide here the first proof-of principle that oral-tolerization prior to AAV-mediated gene transfer completely abrogates humoral and cellular immune responses directed against a soluble immunogenic transgenic protein. This was associated with long-term transgene expression and with the maintenance of the secreted transgenic protein in the circulation of tolerized animals.

Results

Oral-tolerization prevents immune responses directed against the transgenic protein

Intramuscular administration of AAV vectors can lead to a robust immune response that correlates with the elimination of the transduced cells and with the disappearance of the transgenic protein [37, 39]. We investigated here, in an animal model, the efficiency of oral-tolerization to prevent the immune response directed against the transgenic protein. For that, mice were orally-tolerized with the model protein Ovalbumin (Ova), given at 1% in the drinking water, prior to i.m. administration of AAV-Ova at day 0. Immune responses were then monitored over time from days 14 to 80 (**Fig. 1a**). In untolerized control animals, up to 18% of circulating $CD8^+$ recognized Ova 14 days post AAV-Ova transduction. In contrast, orally-tolerized mice displayed little if any cellular immune responses (**Fig. 1b, c**). Ova-specific $CD4^+$ and $CD8^+$ immune responses were also undetectable by the more sensitive ELISpot assay in orally-tolerized mice at day 80 (**Fig. 1f, g**). Similarly, oral-tolerization significantly prevented the formation of anti-Ova IgG antibodies (**Fig. 1d, e**). Of note, this oral-tolerization protocol completely blocked immune responses irrespectively of the administrated dose of AAV-Ova, i.e. $3.5x10^9$, 5×10^{10} or 10^{11} vg (**supplemental Fig. 1a-c**). Also, oral feeding for only 5 days (**supplemental Fig. 1d-f**), or using a 10-fold lower concentration of Ova (*i.e.* 0.1%) (**Supplemental Fig. 1g**), similarly resulted in complete prevention of immune responses, suggesting that robust tolerization mechanisms are induced following oral feeding.

We then evaluated the capacity of our tolerization protocol to inhibit immune responses in less favorable conditions resulting from the use of incompletely purified AAV-preparations or from the use of self-complementary AAV vectors (scAAV). Indeed, both conditions are associated with the triggering of TLR receptors and activation of innate immune cells. To mimic these situations, we co-injected our AAV-Ova vector with contaminating CpG-ODNs, a potent TLR9 agonist. Our results demonstrate that, even in this more immunogenic situation, oral-tolerization prevented cellular and humoral immune responses directed against Ova and improved its persistence in the circulation (**Supplemental Fig. 2)**.

Next, we investigated the functionality of the cytotoxic T cells generated upon AAV-Ova transduction using a well-characterized $CD8^+$-dependent anti-tumor response *in vivo* [38, 40]. For that, Ova-bearing EG7 cells were injected s.c. in tolerized or control mice 14 days after AAV-Ova injection and tumor growth was monitored overtime. Results showed that virtually all control mice

rejected Ova-bearing tumor cells (**Fig. 1h, i**). In contrast, all tolerized mice developed tumors within the first 30 days after EG7 inoculation in agreement with the absence of functional anti-Ova cytotoxic T cells response in tolerized mice.

$CD8^+$ T cells do not infiltrate muscles in tolerized mice

We next investigated the presence of mononuclear cells infiltration in transduced muscles sections. For that, gastrocnemius were harvested 21, 29 or 46 days after gene transfer for histological analyses. Hematoxylin and eosin staining revealed at day 21 and 29 conspicuous mononuclear cell infiltration and myofiber necrosis/regeneration evidenced by the presence of centronucleated fibers in muscle sections of untolerized mice (**Fig. 2a, b**). Immunofluorescence confirmed the presence of cytotoxic T cells expressing both CD3 and CD8 that surrounded muscle fibers (**Fig. 2g, h**). At the latest time point analyzed, histological muscle structure tended to normalize suggesting termination of the immune response and almost complete muscle regeneration (**Fig. 2c**). In contrast, little if any mononuclear cell infiltration was detected in orally-tolerized mice (**Fig. 2d-f**) and $CD8^+$ cytotoxic T cells were virtually absent at all analyzed time points (**Fig. 2j-l**). Only $CD3^-CD8^+$ cells, possibly corresponding to DCs, were sparsely detected. Thus, oral-tolerization prior to AAV-mediated gene transfer protects transduced muscle fibers from $CD8^+$ cytotoxic T cells.

Oral-tolerization favors transgene long-term expression

We next examined whether the control of immune responses was associated with the long-term persistence of the transgene. For that, the relative abundance of DNA and mRNA coding for Ova were quantify in transduced muscles 80 days after AAV-Ova injection. As anticipated, we observed significant higher copy numbers of the transgene and of its corresponding mRNA, in muscle samples of tolerized mice (**Fig. 3a, b**). Concordantly, quantification of Ova in serum confirmed the stable expression of Ova in tolerized mice while Ova was under the limit of detection in control mice (**Fig. 3c**). Hence, these results show that oral-tolerization sustains transgene persistence and expression, and allows long-term maintenance of the secreted transgenic protein.

Oral-tolerization induces abortive proliferation of adoptively transferred Ova-specific $CD8^+$ T cells

To investigate the mechanisms involved, we first analyzed the cell subsets present in the MLNs. No difference was found in the frequencies nor in the phenotypes of the analyzed subsets, that included Tregs, Bregs and DCs (**Supplemental Fig. 3**). Also, depletion/inactivation of the vast majority of $CD4^+Foxp3^+$ Tregs at the end of the oral-tolerization protocol was not sufficient to

abrogate the induced tolerance (**Supplemental Fig. 4**). We next investigated the possible induction of anergy and/or deletion of antigen-specific T cells. As anergy can be breached by pro-inflammatory infections, we next infected orally-tolerized mice with a replicative *Listeria monocytogenes* strain expressing Ova (Lm-Ova). In contrast to what we observed following injection of the replication-defective AAV-Ova (**Fig. 1**), mice responded well to Lm-Ova (**Fig. 4a, b**), indicating that Ova-specific T cells are still present and are anergized rather than deleted during the initial step of oral-tolerization.

To study more precisely the fate of anti-Ova $CD8^+$ T cells, Ova-specific $CD8^+$ T cells from TCR-Tg OT-I mice harboring the CD45.1 congenic marker (OT-I $CD45.1^+$ cells) were adoptively transferred into CD45.2 recipients. Transferred cells were significantly more abundant in tolerized mice than in control animals and expressed the CD44 activation marker (**Fig. 4c, d**). Labeling with a fluorescent dye revealed that adoptively transferred OT-I $CD45.1^+$ cells indeed proliferated in the MLNs of tolerized mice (**Fig. 4e**), but also acquired expression of the PD-1 exhaustion/anergic marker alongside cell division (**Fig. 4f**). One third of transferred cells also acquired expression of CD73, a regulatory molecule involved in anergy [41] (**Fig. 4g, h**). Finally, as anergic T cell tend to die by apoptosis [42], we further analyzed the intracellular level of the anti-apoptotic factor Bcl-2 and observed that transferred cells displayed reduced levels of Bcl-2 (**Fig. 4g, h**). Taken together, these results suggest that Ova-specific T cells abortively proliferate in the MLNs upon encounter of their nominal antigen brought by the oral route.

Oral-tolerization induces deletion of Ova-specific $CD8^+$ T cells in the context of AAV-mediated gene therapy

We next addressed the fate of antigen-specific cells, not only after oral-tolerization, but also following AAV-mediated gene transfer. For that, OT-I $CD45.1^+$ cells were again injected in congenic CD45.2 recipient mice and followed after AAV-Ova i.m. injection. While previous experiments demonstrated very few transferred $CD45.1^+$ cells in control animals before injection of AAV-Ova (**Fig. 4c, d**), these cells were clearly visible after gene transfer in spleen, draining lymph nodes, and circulation suggesting cell rebound following antigenic stimulation (**Fig. 5a, b**). In contrast, $CD45.1^+$ that were more abundant in tolerized animals before gene transfer (**Fig. 4c, d**), were almost undetectable later in spleen, draining lymph nodes, and circulation of these animals (**Fig. 5a, b**). Lastly, we enumerated transferred cells to ascertain that $CD45.1^+$ cell percentage faithfully reflected their absolute number. The results confirmed that the absolute number of antigen-specific $CD8^+CD45.1^+$ cells gradually increased in the peripheral lymphoid organs of

untolerized mice while, in striking contrast, they completely disappeared from the lymphoid organs of tolerized mice (**Fig. 5c**). Altogether, these data suggest that although antigen-specific $CD8^+$ T cells divided during oral-tolerization, they acquired PD-1, down-regulated Bcl-2 survival factor, and are finally deleted from the repertoire of tolerized mice upon antigen reencounter in the context of AAV-mediated muscle gene transfer.

Prophylactic oral-feeding, but not interventional oral-feeding, prevents immune responses elicited by AAV vectors

As oral-tolerization has yielded limited success in the treatment of autoimmune diseases, we next wondered whether oral-tolerization would be more efficient to prevent, rather than to suppress, immune responses. To examine this question, we applied the oral-tolerization protocol to animals pre-immunized with CFA/Ova before initiation of oral-tolerization. Results showed that oral-tolerization was not efficient to prevent immune responses elicited by AAV-Ova transduction in the pre-immunized group of mice (**Fig. 6a-d**). Similar results were obtained with animals pre-immunized with Lm-Ova to elicit a memory anti-Ova immune response 35 days before the administration of the AAV-Ova vector (data not shown). Thus, oral-tolerization is very efficient to prevent primary immune responses elicited by AAV-vectors but not secondary/memory immune responses. We next directly compared prophylactic and interventional protocols by initiating antigen feeding either before, at the same time, or after vector transduction. Results showed that initiation of oral-tolerization at the same time as vector transduction was as effective as its initiation 7 days before to completely prevent the emergence of the cytotoxic $CD8^+$ T cells response (**Fig. 6e**). However, expression of transgenic protein from single-stranded DNA AAV-vectors is known to require at least 7 days *in vivo*, suggesting that oral-tolerization may still have been initiated in this particular situation before the priming of the immune response. Concordantly, when antigen feeding was initiated 10 days after vector transduction (i.e., at a time point where immune responses become detectable), oral feeding was inefficient to suppress cytotoxic $CD8^+$ T cells response and to improve long-term transgene persistence (**Fig. 6f, g**). Thus, oral-tolerization by antigen feeding is only effective when applied before or at the same time as vector transduction, two situations that are still fully compatible with the specific clinical context of gene therapy.

Discussion

Significant progress has been made in the field of AAV gene therapy to improve efficacy and safety of the vectors. One of the main persisting challenges is the better control of adverse immune responses to improve long-term expression of the transgene. Indeed, immune responses directed against the vector not only compromise transduction efficiency during vector re-injections, but also lead to the elimination of transduced cells by capsid-specific cytotoxic $CD8^+$ T cells [9, 12, 43]. The transgenic protein itself, coded by the vector, also induces immune responses that can impair gene transfer efficacy [5, 16]. Actual strategies used in clinic to overcome this limitation include administration of immunosuppressive drugs and careful selection of the patients presenting the lower risk of developing such immune responses notably against the transgenic protein. For instance, patients enrolled in hemophilia gene therapy trials were elected based on the nature of their genetic mutation (i.e., missense rather that nonsense mutations) and based on their absence of immune responses (i.e., inhibitors formation) to protein replacement therapy [20, 22-24]. Developing strategies to inhibit these immune responses is therefore of major importance, not only to promote long-term transgenic protein expression, but also to extend gene therapy to other patients. We evaluated here an oral-tolerization strategy to specifically dampen the humoral and cellular immune responses directed against the transgenic protein coded by the AAV vector and demonstrated its efficacy to improve long-term transgene expression (**Fig. 1 and 3**).

We used here as a model an AAV-Ova vector to elicit muscle secretion of Ova following its intra-muscular administration. This experimental condition mimics clinical situations where the transgenic protein is meant to be expressed systemically such as gene therapy of hemophilic patients for instance. We chose here a highly immunogenic protein model to generate a stringent model where protocols designed to inhibit immune responses can be faithfully evaluated. We used this experimental setting to show that prophylactic oral administration of the protein of interest is able to completely prevent both humoral (**Fig. 1d, e)** and cellular immune responses (**Fig. 1b, c and f, g**) induced by AAV-mediated muscle gene transfer. Concordantly, the transgenic Ova protein was persistent in the serum until the latest time-points studied (**Fig. 3c**) as was the corresponding Ova mRNA in transduced muscle (**Fig. 3a, b**). This was in agreement with the absence of cytotoxic $CD8^+$ T cells infiltration in the transduced muscles of orally-tolerized mice (**Fig. 2d-f and j-l**).

Regarding the mechanism, we evaluated the potential role of $Foxp3^+$ Tregs in the induction/maintenance of tolerance in our model. Using a robust protocol that we developed to deplete and inactivate $Foxp3^+$ Tregs, we showed that their depletion/inactivation at the end of the oral-tolerization phase was not sufficient to abrogate orally-induced tolerance and to restore cellular and humoral immune responses (**Supplemental Fig. 4).** This suggested, at best, a minor role of these cells in our model. However, the role of other described subsets of regulatory T cells cannot be excluded. Among the subset that have been previously implicated in oral tolerance, we analyzed the proportion $CD4^+LAG\text{-}3^+Foxp3^-$ Tr1 cells [44]. Indeed, this subset is known to secrete IL-10, a well-known immunoregulatory cytokine, and has been previously involved in the induction of oral tolerance [45]. Our results did not reveal substantial difference in the percentages of $CD4^+LAG\text{-}3^+Foxp3^-$ found in the mesenteric lymph nodes of tolerized animals as compared to control mice nor in their levels of LAG-3 expression (**Supplemental Fig. 3i and data not shown**). Yet, further functional investigations, as well as the study of their antigen-specificity, and their numbers in lamina propria or Peyer's patches would be required to formally exclude their implication in our model. Th3 cells, characterized by their $CD4^+LAP^+Foxp3^-$ phenotype and by the production of TGF-β, represent another regulatory T cell subset that has been associated with oral tolerization. Indeed, Th3 cells have been previously demonstrated to be elicited upon oral administration of myelin basic protein (MBP) and to prevent the development of autoimmune encephalomyelitis in a mouse model [46]. As we did not specifically studied this subset, their role cannot be excluded in our model. This may possibly be of importance as immunoregulatory cytokines akin to IL-10 and/or TGF-β produced by Tr1 and Th3 subsets are known to considerably affect the differentiation, survival and functions of $CD8^+$ cytotoxic T cells.

Even if the precise mechanism involved in the regulation of the $CD4^+$ T cell compartment still remain to be precisely studied, we provide herein interesting data on the mechanisms involved in the regulation of $CD8^+$ T cells compartment by oral tolerization. We show here that adoptively transferred antigen-specific $CD8^+$ T cells gradually acquired high levels of PD-1 expression as they proliferated following initial encounter with the orally-derived antigen in MLNs (**Fig. 4e, f**). Abortive T cells proliferation prior to tolerization has already been documented following oral-tolerization [47, 48]. We further followed the fate of Ova-specific $CD8^+$ T cells upon Ova re-encounter in the context of gene therapy. We then showed that Ova-specific $CD8^+$ T which expanded significantly in tolerized animals during the 7 days of Ova-feeding (**Fig. 4e, f and 5c at day 0**), declined rapidly after injection of AAV-Ova while they expanded dramatically in control animals (**Fig. 5**). Hence, $CD8^+$ T cells that have encountered orally-derived antigen during the tolerization

phase are not only incapable to respond to the same antigen in the context of AAV-mediated gene transfer but are also rapidly induced to die.

The data reported herein provide the first proof-of-principle study indicating that oral-tolerization represents a candidate approach to circumvent the bottleneck of the immune response directed toward the transgenic protein. Interestingly, protein of interest can be given prophylactically before AAV vector injection in patients who are immunologically naive to the transgene product, provided that they have never been sensitized by protein replacement therapy. Yet, production of large quantities of proteins that would be compatible with oral administration in humans may represent a challenge. Several production systems and/or method of vectorization can however be envisioned. For instance, transgenic probiotics can be administrated orally in mice and humans without any conspicuous side effect and can possibly be used to directly deliver proteins of interest in the gastrointestinal tract [49-51]. Beside probiotics, transgenic plants represent an alternative low cost method for the production of proteins suitable for oral ingestion. Bioproduction in comestible plants simplify the production steps, eliminate the need of extensive purification, and allow the production of glycosylated proteins that are structurally and functionally closely related to the native protein [52, 53]. Moreover, protein synthetized in plants are naturally bioencapsulated, ensuring their protection from gastric and proteolytic degradation and facilitate their delivery in the gut where plants are digested by the action of commensal bacteria. In addition, protein of interest can be fused to transmucosal carriers that can facilitate their delivery across intestinal epithelium. Plant-based oral tolerance has been recently illustrated in a murine model of hemophilia where prophylactic oral administration of the transplastomic plant expressing recombinant factor IX (F.IX) protein fused to the cholera toxin carrier B was demonstrated to inhibit antibodies formation in the context of protein replacement therapy [54-56]. In the same line, the present results further illustrate the efficacy of a prophylactic oral-tolerization protocol in the context of AAV-based gene therapy to promote specific and long-term tolerance to the transgenic protein coded by the vector.

Prevention of immune sensitization, rather than control of already primed immune responses, may arguably be more efficient and may partly explain the limited success of oral-tolerization strategies in autoimmune diseases. Consistently, we showed that oral-tolerization is efficient to prevent primary immune responses elicited by AAV vectors but not secondary/memory immune responses (**Fig. 6a-d**). Interestingly, oral-tolerization was still effective when initiated at the same time as AAV-vectors injections, possibly in relation with the known delay necessary for transgene expression when using single-stranded DNA AAV vectors. We thus believe that the present study

pave the way for further investigations in larger animals of prophylactic, rather than interventional, oral-tolerization protocols to prevent immune responses against transgenic proteins and/or against the proteins of the vector. Such oral-tolerization protocol could be used in combination with oral rapamycin and/or low-dose i.v. injection of the protein of interest, that have been shown to prevent and reverse inhibitory antibodies formation elicited by i.m. injection of AAV-F.IX in an animal model of hemophilia [57]. Also, given the recently characterized immunoregulatory properties of probiotics, oral administration of modified probiotics expressing the proteins of interest may further improve oral-tolerization protocols by facilitating the tolerization against multiple proteins at the same time and may thus deserve further investigation in the field of gene therapy.

Materials and methods

Mice

Female C57BL/6Rj (B6) mice were obtained from Janvier Labs. TCR-transgenic OT-I mice, and congenic B6 mice expressing the CD45.1 (Ly5.1) allelic marker were obtained from Charles River Laboratories. [OT-IxLy5.1]F1 mice were used as a source of anti-Ova TCR-transgenic $CD8^+$ T cells expressing the CD45.1 marker. Mice were housed in a specific pathogen-free barrier facility and were between 8–12 weeks of age at beginning of the experiments.

Plasmidic constructs, preparation of recombinant AAV vectors and muscles transduction

The plasmidic construct used for the generation of a recombinant AAV2/1 vector, coding for soluble Ova (AAV-Ova), was kindly provided by Roland W. Herzog [5, 16]. Recombinant AAV2/1-Ova vectors were generated using a standard helper-virus free transient transfection method and pseudotyped with AAV1 capsid proteins as described before [37, 38]. Genome titers, expressed by equivalent vector genomes (vg), were evaluated by dot-blot hybridization and by qPCR. For muscle transduction, mice hind legs were shaved under anesthesia and titrated amounts of AAV-Ova vectors, (i.e., $3.5x10^9$, 5×10^{10} or 10^{11} vg) were injected in each gastrocnemius muscles. In some experiments, mice received together with AAV-Ova ($3.5x10^9$ vg/mice), 10 µg of CpG ODNs 1826 (Sigma-Aldrich) to mimic situations where vector administrations are associated with TLRs activation.

Oral Ova protein administration

Oral-tolerization was induced by offering to mice, *ad libitum*, for 7 consecutive days, a solution of 1% Ova (Sigma-Aldrich, grade II) dissolved in their drinking water. This solution was freshly prepared each day. In some experiments, variation of this protocol were evaluated consisting of oral administration of the protein only for 5 days, or for 7 days at the reduced concentration of 0.1%. All these protocols similarly resulted in the complete prevention of cellular and humoral immune responses directed against Ova following i.m. injections of AAV-Ova vectors (data not shown).

Mice immunization

In some experiments, mice were pre-immunized 21 days before oral-tolerization. For that, 100 µg of Ova was emulsified in CFA and s.c. injected in their shaved back. In other experiments, mice were immunized after oral-tolerization with a highly immunogenic vaccine consisting of a live strain of *Listeria monocytogenes* expressing Ova (Lm-Ova), kindly provided by G. Lauvau [58]. For that,

mice were injected i.v. with 10^6 cfu of titrated Lm-Ova preparations diluted in 100 µl of PBS as described previously [38, 40].

Tumor model

Mice injected 14 days before with AAV-Ova, were subcutaneously injected in their shaved flanks with 10^6 EL4-Ova (EG-7) tumor cells. Mice were then examined every other days and tumor development was monitored for 31 days using a digital caliper as described previously [38, 40].

Adoptive T cell transfer

Ova specific $CD8^+$ T cells were obtained from the spleens of [OT-I x Ly5.1]F1 mice and purified by magnetic sorting using a CD8 negative isolation kit (Invitrogen). More than 93% of the purified cells displayed a $CD3^+CD8^+V\alpha2^+V\beta5^+CD45.1^+CD4^-CD19^-$ phenotype as assessed by flow cytometry. For *in vivo* proliferation assay, cells were stained with 20 µM Cell Proliferation Dye eFluor® 450 (eBiosciences) for 10 minutes and 10^5 washed cells were then adoptively transferred i.v. into each B6 recipient mice.

Antibodies and flow cytometry

Fluorochrome-conjugated antibodies were all obtained from eBioscience except for antibodies to Bcl-2, CD24, CD73, CD80, CD86 and IFNγ obtained from BD Biosciences, and anti-Nrp-1 from Biolegend. Intracellular Foxp3, Helios, Bcl-2, IL-10 staining were done using the Intracellular Fixation & Permeabilization kit (eBiosciences) [38, 40]. PE-conjugated $H\text{-}2K^b/Ova_{257\text{-}264}$ dextramers were used to detect $CD8^+$ T cells that specifically recognize the immunodominant $Ova_{257\text{-}264}$ peptide using the manufacturer's protocol (Immudex). Single-cell suspensions derived from spleen, lymph nodes or peripheral blood (PBL) were analyzed by flow cytometry using a FACSCanto-I or an LSRFortessa (BD Biosciences), and using FlowJo software (Tree Star).

ELISA and ELISpot assays

Quantification of soluble Ova concentration in serum and titration of anti-Ova IgG antibodies were performed by ELISA as previously described [37] . Anti-Ova IgG titers were defined as the dilution yielding the half-maximum optical density obtained with a positive control serum that was used throughout the all study. Titers were calculated using sigmoid curve fitting performed in Prism software (Graphpad). Enzyme-linked immunospot (ELISpot) assays were used to quantify the numbers of Ova-specific $CD8^+$ or $CD4^+$ T cells secreting IFNγ upon *in vitro* restimulation as previously described [37]. For that, 2.5×10^5 to 10^5 splenocytes per well were cultured overnight in

RPMI medium in the presence of 10 µg/ml $Ova_{257-264}$ or $Ova_{323-339}$ peptides for detection of, respectively, MHC I or MHC II restricted cellular immune responses, or, with peptides SNYAKSANV, MIPQYGYL and PQYGYLTL for detection of anti-capsid AAV1 cellular immune responses. Cultures were stopped and treated according to manufacturer's instructions (Diaclone). Number of spots in each well were analyzed with an ELISpot plate reader and a dedicated ImmunoSpots software (C.T.L.).

Histology and immunofluorescence microscopy

Whole gastrocnemius were snap-freeze in isopentane pre-cooled in liquid nitrogen. Transversally cryosectioned slices were either stained with hematoxylin and eosin (H&E) for histological evaluations or prepared for immunofluorescence. Briefly, muscles slices were fixed with 1% paraformaldehyde and incubated with flurochrome-conjugated anti-mouse CD8 (53-6.7) and anti-CD3 (500A2) antibodies, obtained from eBiosciences. Nuclei were counterstained with DAPI. Slides were viewed with a Zeiss Axioplan 2 microscope, and data collected using Zeiss Apotome and Axiovision 4.1 software.

Quantification of Ova cDNA and mRNA in transduced muscles

Ova DNA and corresponding Ova mRNA were quantified from transduced muscles by real-time PCR using SYBR green Mastermix (Roche) in a final volume of 10 µl as described previously [37, 38]. All qPCRs were performed with a LightCycler 480 (Roche). The relative amount of Ova DNA or Ova mRNA were determined using a standard curve obtained after serial dilutions of a plasmidic construct coding for Ova, and normalized for each sample by the amount of Eef2.

Statistical analysis

All data are shown as mean values and error bars represent SEM. Non-parametric tests were used for statistical comparison between experimental groups using one-way ANOVA (Kruskall-Wallis tests). Unless otherwise stated, all the experiments have been repeated at least 3 times and illustrations shown here are representative of all experiments. Differences were considered statistically significant when *p* values were less than 0.05 (*), 0.01 (**) or 0.001 (***). All calculations were performed using the Prism software (Graphpad).

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Authorship Contributions

R.H., B.C., Y.N. and G.R. performed the experiments; L.D. and L.J. provided expert technical helps and animal care; A.S. prepared the AAV vectors; R.H., O.B. and S.A. designed the experiments; R.H. and S.A. analyzed and interpreted the data; R.H., A.S., O.B. and S.A. drafted the manuscript; R.H. and S.A revised and finalized the manuscript.

Disclosure of Conflicts of Interest

The authors declare no competing financial interests

References

1. Wang, L, and Herzog, RW (2005). AAV-mediated gene transfer for treatment of hemophilia. *Curr Gene Ther* **5**: 349-360.
2. Mingozzi, F, and High, KA (2011). Therapeutic in vivo gene transfer for genetic disease using AAV: progress and challenges. *Nature reviews Genetics* **12**: 341-355.
3. Zincarelli, C, Soltys, S, Rengo, G, and Rabinowitz, JE (2008). Analysis of AAV serotypes 1-9 mediated gene expression and tropism in mice after systemic injection. *Mol Ther* **16**: 1073-1080.
4. Petrs-Silva, H, and Linden, R (2013). Advances in recombinant adeno-associated viral vectors for gene delivery. *Curr Gene Ther* **13**: 335-345.
5. Mays, LE, and Wilson, JM (2011). The complex and evolving story of T cell activation to AAV vector-encoded transgene products. *Mol Ther* **19**: 16-27.
6. Maguire, AM, Simonelli, F, Pierce, EA, Pugh, EN, Jr., Mingozzi, F, Bennicelli, J, *et al.* (2008). Safety and efficacy of gene transfer for Leber's congenital amaurosis. *N Engl J Med* **358**: 2240-2248.
7. Manno, CS, Pierce, GF, Arruda, VR, Glader, B, Ragni, M, Rasko, JJ, *et al.* (2006). Successful transduction of liver in hemophilia by AAV-Factor IX and limitations imposed by the host immune response. *Nat Med* **12**: 342-347.
8. Gaudet, D, Methot, J, Dery, S, Brisson, D, Essiembre, C, Tremblay, G, *et al.* (2013). Efficacy and long-term safety of alipogene tiparvovec (AAV1-LPLS447X) gene therapy for lipoprotein lipase deficiency: an open-label trial. *Gene therapy* **20**: 361-369.
9. Mingozzi, F, Maus, MV, Hui, DJ, Sabatino, DE, Murphy, SL, Rasko, JE, *et al.* (2007). CD8(+) T-cell responses to adeno-associated virus capsid in humans. *Nat Med* **13**: 419-422.
10. Nayak, S, and Herzog, RW (2010). Progress and prospects: immune responses to viral vectors. *Gene Ther* **17**: 295-304.
11. Mingozzi, F, and High, KA (2011). Immune Responses to AAV in Clinical Trials. *Curr Gene Ther* **11**: 321-330.
12. Manning, WC, Zhou, S, Bland, MP, Escobedo, JA, and Dwarki, V (1998). Transient immunosuppression allows transgene expression following readministration of adeno-associated viral vectors. *Hum Gene Ther* **9**: 477-485.
13. Fields, PA, Kowalczyk, DW, Arruda, VR, Armstrong, E, McCleland, ML, Hagstrom, JN, *et al.* (2000). Role of vector in activation of T cell subsets in immune responses against the secreted transgene product factor IX. *Mol Ther* **1**: 225-235.
14. Yuasa, K, Yoshimura, M, Urasawa, N, Ohshima, S, Howell, JM, Nakamura, A, *et al.* (2007). Injection of a recombinant AAV serotype 2 into canine skeletal muscles evokes strong immune responses against transgene products. *Gene Ther* **14**: 1249-1260.
15. Gao, G, Wang, Q, Calcedo, R, Mays, L, Bell, P, Wang, L, *et al.* (2009). Adeno-associated virus-mediated gene transfer to nonhuman primate liver can elicit destructive transgene-specific T cell responses. *Hum Gene Ther* **20**: 930-942.
16. Wang, L, Dobrzynski, E, Schlachterman, A, Cao, O, and Herzog, RW (2005). Systemic protein delivery by muscle-gene transfer is limited by a local immune response. *Blood* **105**: 4226-4234.
17. Wang, Z, Tapscott, SJ, Chamberlain, JS, and Storb, R (2011). Immunity and AAV-Mediated Gene Therapy for Muscular Dystrophies in Large Animal Models and Human Trials. *Front Microbiol* **2**: 201.
18. Ferreira, V, Twisk, J, Kwikkers, K, Aronica, E, Brisson, D, Methot, J, *et al.* (2014). Immune responses to intramuscular administration of alipogene tiparvovec (AAV1-LPL(S447X)) in a phase II clinical trial of lipoprotein lipase deficiency gene therapy. *Hum Gene Ther* **25**: 180-188.

19. Wang, L, Cao, O, Swalm, B, Dobrzynski, E, Mingozzi, F, and Herzog, RW (2005). Major role of local immune responses in antibody formation to factor IX in AAV gene transfer. *Gene Ther* **12**: 1453-1464.
20. Manno, CS, Chew, AJ, Hutchison, S, Larson, PJ, Herzog, RW, Arruda, VR, *et al.* (2003). AAV-mediated factor IX gene transfer to skeletal muscle in patients with severe hemophilia B. *Blood* **101**: 2963-2972.
21. Ferreira, V, Petry, H, and Salmon, F (2014). Immune Responses to AAV-Vectors, the Glybera Example from Bench to Bedside. *Front Immunol* **5**: 82.
22. Mingozzi, F, and High, KA (2013). Immune responses to AAV vectors: overcoming barriers to successful gene therapy. *Blood* **122**: 23-36.
23. Nathwani, AC, Tuddenham, EG, Rangarajan, S, Rosales, C, McIntosh, J, Linch, DC, *et al.* (2011). Adenovirus-associated virus vector-mediated gene transfer in hemophilia B. *N Engl J Med* **365**: 2357-2365.
24. Nathwani, AC, Reiss, UM, Tuddenham, EG, Rosales, C, Chowdary, P, McIntosh, J, *et al.* (2014). Long-term safety and efficacy of factor IX gene therapy in hemophilia B. *N Engl J Med* **371**: 1994-2004.
25. Coombes, JL, Siddiqui, KR, Arancibia-Cárcamo, CV, Hall, J, Sun, CM, Belkaid, Y, *et al.* (2007). A functionally specialized population of mucosal CD103+ DCs induces Foxp3+ regulatory T cells via a TGF-beta and retinoic acid-dependent mechanism. *J Exp Med* **204**: 1757-1764.
26. Schulz, O, Jaensson, E, Persson, EK, Liu, X, Worbs, T, Agace, WW, *et al.* (2009). Intestinal CD103+, but not CX3CR1+, antigen sampling cells migrate in lymph and serve classical dendritic cell functions. *J Exp Med* **206**: 3101-3114.
27. Weiner, HL, da Cunha, AP, Quintana, F, and Wu, H (2011). Oral tolerance. *Immunol Rev* **241**: 241-259.
28. Groux, H, O'Garra, A, Bigler, M, Rouleau, M, Antonenko, S, de Vries, JE, *et al.* (1997). A CD4+ T-cell subset inhibits antigen-specific T-cell responses and prevents colitis. *Nature* **389**: 737-742.
29. Bilsborough, J, George, TC, Norment, A, and Viney, JL (2003). Mucosal CD8alpha+ DC, with a plasmacytoid phenotype, induce differentiation and support function of T cells with regulatory properties. *Immunology* **108**: 481-492.
30. Goubier, A, Dubois, B, Gheit, H, Joubert, G, Villard-Truc, F, Asselin-Paturel, C, *et al.* (2008). Plasmacytoid dendritic cells mediate oral tolerance. *Immunity* **29**: 464-475.
31. Chen, Y, Inobe, J, Marks, R, Gonnella, P, Kuchroo, VK, and Weiner, HL (1995). Peripheral deletion of antigen-reactive T cells in oral tolerance. *Nature* **376**: 177-180.
32. Friedman, A, and Weiner, HL (1994). Induction of anergy or active suppression following oral tolerance is determined by antigen dosage. *Proc Natl Acad Sci U S A* **91**: 6688-6692.
33. Zhang, X, Izikson, L, Liu, L, and Weiner, HL (2001). Activation of CD25(+)CD4(+) regulatory T cells by oral antigen administration. *J Immunol* **167**: 4245-4253.
34. Wherrett, DK, Bundy, B, Becker, DJ, DiMeglio, LA, Gitelman, SE, Goland, R, *et al.* (2011). Antigen-based therapy with glutamic acid decarboxylase (GAD) vaccine in patients with recent-onset type 1 diabetes: a randomised double-blind trial. *Lancet* **378**: 319-327.
35. Dello Iacono, I, Tripodi, S, Calvani, M, Panetta, V, Verga, MC, and Miceli Sopo, S (2013). Specific oral tolerance induction with raw hen's egg in children with very severe egg allergy: a randomized controlled trial. *Pediatr Allergy Immunol* **24**: 66-74.
36. Chung, Y, Chang, SY, and Kang, CY (1999). Kinetic analysis of oral tolerance: memory lymphocytes are refractory to oral tolerance. *J Immunol* **163**: 3692-3698.
37. Adriouch, S, Franck, E, Drouot, L, Bonneau, C, Jolinon, N, Salvetti, A, *et al.* (2011). Improved Immunological Tolerance Following Combination Therapy with CTLA-4/Ig and AAV-Mediated PD-L1/2 Muscle Gene Transfer. *Front Microbiol* **2**: 199.

38. Franck, E, Bonneau, C, Jean, L, Henry, JP, Lacoume, Y, Salvetti, A, *et al.* (2012). Immunological tolerance to muscle autoantigens involves peripheral deletion of autoreactive CD8+ T cells. *PloS one* **7**: e36444.
39. Dobrzynski, E, Mingozzi, F, Liu, YL, Bendo, E, Cao, O, Wang, L, *et al.* (2004). Induction of antigen-specific CD4+ T-cell anergy and deletion by in vivo viral gene transfer. *Blood* **104**: 969-977.
40. Hubert, S, Rissiek, B, Klages, K, Huehn, J, Sparwasser, T, Haag, F, *et al.* (2010). Extracellular NAD+ shapes the Foxp3+ regulatory T cell compartment through the ART2-P2X7 pathway. *J Exp Med* **207**: 2561-2568.
41. Resta, R, Yamashita, Y, and Thompson, LF (1998). Ecto-enzyme and signaling functions of lymphocyte CD73. *Immunol Rev* **161**: 95-109.
42. Parish, IA, Rao, S, Smyth, GK, Juelich, T, Denyer, GS, Davey, GM, *et al.* (2009). The molecular signature of CD8+ T cells undergoing deletional tolerance. *Blood* **113**: 4575-4585.
43. Manning, WC, Paliard, X, Zhou, S, Pat Bland, M, Lee, AY, Hong, K, *et al.* (1997). Genetic immunization with adeno-associated virus vectors expressing herpes simplex virus type 2 glycoproteins B and D. *J Virol* **71**: 7960-7962.
44. Gagliani, N, Magnani, CF, Huber, S, Gianolini, ME, Pala, M, Licona-Limon, P, *et al.* (2013). Coexpression of CD49b and LAG-3 identifies human and mouse T regulatory type 1 cells. *Nat Med* **19**: 739-746.
45. Tsuji, NM, Mizumachi, K, and Kurisaki, J (2001). Interleukin-10-secreting Peyer's patch cells are responsible for active suppression in low-dose oral tolerance. *Immunology* **103**: 458-464.
46. Chen, Y, Inobe, J, Kuchroo, VK, Baron, JL, Janeway, CA, Jr., and Weiner, HL (1996). Oral tolerance in myelin basic protein T-cell receptor transgenic mice: suppression of autoimmune encephalomyelitis and dose-dependent induction of regulatory cells. *Proc Natl Acad Sci U S A* **93**: 388-391.
47. Castro-Junior, AB, Horta, BC, Gomes-Santos, AC, Cunha, AP, Silva Steinberg, R, Nascimento, DS, *et al.* (2012). Oral tolerance correlates with high levels of lymphocyte activity. *Cellular immunology* **280**: 171-181.
48. Sun, J, Dirden-Kramer, B, Ito, K, Ernst, PB, and Van Houten, N (1999). Antigen-specific T cell activation and proliferation during oral tolerance induction. *J Immunol* **162**: 5868-5875.
49. Robert, S, Gysemans, C, Takiishi, T, Korf, H, Spagnuolo, I, Sebastiani, G, *et al.* (2014). Oral delivery of glutamic acid decarboxylase (GAD)-65 and IL10 by Lactococcus lactis reverses diabetes in recent-onset NOD mice. *Diabetes* **63**: 2876-2887.
50. Takiishi, T, Korf, H, Van Belle, TL, Robert, S, Grieco, FA, Caluwaerts, S, *et al.* (2012). Reversal of autoimmune diabetes by restoration of antigen-specific tolerance using genetically modified Lactococcus lactis in mice. *J Clin Invest* **122**: 1717-1725.
51. Rottiers, P, De Smedt, T, and Steidler, L (2009). Modulation of gut-associated lymphoid tissue functions with genetically modified Lactococcus lactis. *Int Rev Immunol* **28**: 465-486.
52. Wang, X, Sherman, A, Liao, G, Leong, KW, Daniell, H, Terhorst, C, *et al.* (2013). Mechanism of oral tolerance induction to therapeutic proteins. *Adv Drug Deliv Rev* **65**: 759-773.
53. Kwon, KC, Verma, D, Singh, ND, Herzog, R, and Daniell, H (2013). Oral delivery of human biopharmaceuticals, autoantigens and vaccine antigens bioencapsulated in plant cells. *Adv Drug Deliv Rev* **65**: 782-799.
54. Verma, D, Moghimi, B, LoDuca, PA, Singh, HD, Hoffman, BE, Herzog, RW, *et al.* (2010). Oral delivery of bioencapsulated coagulation factor IX prevents inhibitor formation and fatal anaphylaxis in hemophilia B mice. *Proc Natl Acad Sci U S A* **107**: 7101-7106.

55. Sherman, A, Su, J, Lin, S, Wang, X, Herzog, RW, and Daniell, H (2014). Suppression of inhibitor formation against FVIII in a murine model of hemophilia A by oral delivery of antigens bioencapsulated in plant cells. *Blood* **124**: 1659-1668.
56. Wang, X, Su, J, Sherman, A, Rogers, GL, Liao, G, Hoffman, BE, *et al.* (2015). Plant-based oral tolerance to hemophilia therapy employs a complex immune regulatory response including LAP+CD4+ T cells. *Blood* **125**: 2418-2427.
57. Nayak, S, Sarkar, D, Perrin, GQ, Moghimi, B, Hoffman, BE, Zhou, S, *et al.* (2011). Prevention and Reversal of Antibody Responses Against Factor IX in Gene Therapy for Hemophilia B. *Front Microbiol* **2**: 244.
58. Lauvau, G, Vijh, S, Kong, P, Horng, T, Kerksiek, K, Serbina, N, *et al.* (2001). Priming of memory but not effector CD8 T cells by a killed bacterial vaccine. *Science* **294**: 1735-1739.
59. Kohm, AP, McMahon, JS, Podojil, JR, Begolka, WS, DeGutes, M, Kasprowicz, DJ, *et al.* (2006). Cutting Edge: Anti-CD25 monoclonal antibody injection results in the functional inactivation, not depletion, of CD4+CD25+ T regulatory cells. *J Immunol* **176**: 3301-3305.

Figure Legends

Figure 1. Oral-tolerization prevents immune responses directed against the transgenic protein.

(**a**) Protocol outline. B6 mice were given 1% of Ova, *ad libitum*, in drinking water during 7 days and then injected with AAV-Ova ($5x10^{10}$ vg/mouse) in both gastrocnemius (n=5 per group). Immune responses were monitored between days 14 and 80 after injections. (**b**) Blood samples were collected 14 days post AAV-Ova transduction and percentage of Ova-specific $CD8^+$ T cells was determined using H-$2K^b$/$Ova_{257-264}$ dextramers staining and flow cytometry in control (ctrl) and tolerized (tol) mice. The representative flow cytometric panels shown here represent percentages of Ova-specific $CD8^+$ T cells in the gated $CD8^+$ population (**c**) Percentages of Ova-specific $CD8^+$ T cells in the blood were determined at the indicated times points post AAV-Ova transduction. (**d-e**) Sera were collected at 80 days (**d**) or at the indicated time points (**e**) post AAV-Ova transduction and anti-Ova IgG titers were determined by ELISA. Curves represent optical density plotted against dilutions factors from data obtained at day 80 (**d**). The IgG titers correspond to the dilution yielding the half-maximum optical density obtained with a positive control serum used in all experiments (**e**). (**f-g**) At day 80, splenocytes were harvested and analyzed by *ex vivo* ELISpot assays for their capability to secrete IFNγ after restimulation with the $Ova_{257-264}$ peptide. (**f**) Representative ELISpot data obtained with control and tolerized mice. (**g**) Average number of spot forming cell (SFC) per 10^6 splenocytes obtained in control and tolerized group of mice (n=5/group). (**h-i**) 10^6 EG7 Ova-bearing tumor cells were injected 14 days after AAV-Ova or PBS injections (n=5/group). Mice were monitored for tumor development (**h**) and tumor volume (**i**).

Figure 2. Oral-tolerization prevents muscle infiltration by $CD8^+$ T cells.

Mice were tolerized or not during 7 days with 1% Ova in drinking water and injected as before with AAV-Ova ($5x10^{10}$ vg/mouse) in both gastrocnemius at day 0 (n=6 per group). Muscles were collected at the indicated times points post AAV-Ova injection. Muscle sections from control (a-c) and tolerized (**d-f**) mice were stained with hematoxylin and eosin to evaluate muscle histology and the overall level of mononuclear cell infiltration. Muscle sections from control (**g-i**) and tolerized (**j-l**) mice were analyzed by immunofluorescence after immunostaining with anti-CD3 (green) and anti-CD8 (red) antibodies and nucleus counterstaining with DAPI (blue). Scale bars represent 50 μm and arrows in (**a)** and (**b)** show area containing infiltrating cells.

Figure 3. Oral-tolerization favors transgene long-term expression.

AAV-Ova injected muscles were harvested at day 80 from tolerized or control mice and transgene persistence and expression were evaluated using qPCR and qRT-PCR. (**a**) Transgene persistence in transduced muscle was evaluated by quantification of Ova DNA by qPCR. (**b**) Transgene expression was evaluated by quantification of the corresponding Ova mRNA by qRT-PCR. Data were normalized against the level of Eef2 mRNA (arbitrary units). (**c**) Mean concentrations of Ova in the sera of tolerized and control mice were determined 18, 35 and 80 days after AAV-Ova injection by ELISA.

Figure 4. Oral-tolerization induces abortive proliferation, expression of PD-1 and downregulation of Bcl-2 in adoptively transferred Ova-specific CD8$^+$ T cells.

(**a**-**b**) Mice were orally-tolerized during 7 days with 1% Ova in drinking water and then injected i.v. with 10^6 pfu of a live strain of Listeria monocytogenes expressing Ova (Lm-Ova) (n=5/group). Blood samples were collected after infection to monitor the cellular levels of Ova-specific CD8$^+$ T cells by flow cytometry. (**a**) Representative cytometric profiles obtained at day 10 post-infection were gated on the CD8$^+$ population. (**b**) Percentage of anti-Ova CD8$^+$ T cells 10 and 22 days post-infection are shown. (**c**-**h**) 10^5 Ova-specific CD8$^+$ T cells (OT-I CD45.1$^+$), stained with a proliferation tracking dye, were adoptively transferred into CD45.2 congenic mice. Recipient mice were then orally-tolerized during 7 days and MLNs were harvested and analyzed by flow cytometry (n=5/group). (**c**) Representative cytometric profiles, gated on the CD8$^+$ population, and (**d**) percentage of CD45.1$^+$ cells in MLNs are shown. (**e**) Cytometric profiles representing the level of cell proliferation (dilution of the proliferation dye) and PD-1 expression on gated CD45.1$^+$ cells. (**f**) Percentage of CD45.1$^+$PD-1$^+$ cells in the gated CD8$^+$ cells and their proliferation index. (**g**) Cytometric profiles showing the expression of CD73 (top) and Bcl-2 (bottom) on the gated CD45.1$^+$ cells. (**h**) Percentage of CD45.1$^+$CD73$^+$ cells (left) and mean fluorescence intensity of Bcl-2 staining (right).

Figure 5. Oral-tolerization induces deletion of Ova-specific CD8$^+$ T cells in the context of gene therapy.

As in figure 4, 10^5 OT-I CD45.1$^+$ cells were adoptively transferred into CD45.2 congenic recipient mice, which were then orally-tolerized during 7 days. At the end of the oral-tolerization protocol, mice were injected with AAV-Ova in both gastrocnemius ($3.5x10^9$ vg). Spleen, MLNs and draining lymph nodes (dLNs) were harvested for flow cytometry analysis (n=5/group). (**a**) Percentage of transferred CD45.1$^+$ cells in the gated CD8$^+$ population was evaluated in spleen and dLNs 10 days

post transduction. (**b**) In other groups of mice, percentage of transferred $CD8^+CD45.1^+$ cells was also evaluated in the blood at day 14 post-injection. (**c**) Total number of $CD45.1^+$ transferred $CD8^+$ cells was enumerated from the harvested pooled lymphoid organs (spleen, dLNs and MLNs) before AAV-Ova injection (at day 0) and 10 and 17 days thereafter.

Figure 6. Oral-tolerization is very efficient to prophylactically prevent immune responses, but not to suppress already primed immune responses.

(**a**-**c**) Mice were pre-immunized or not with CFA/Ova. Three weeks later, mice were orally-tolerized during 7 days with 1% Ova solubilized in their drinking water. At the end of the tolerization protocol, mice were transduced using $3.5x10^9$ vg of AAV-Ova injected in both gastrocnemius. (**a**) $CD8^+$ cellular immune responses were monitored by flow cytometry in pre-immunized and non-pre-immunized groups by collecting blood samples 15 days after AAV-Ova injections. Bar graph represents the percentage of Ova-specific $CD8^+$ T cells in each indicated groups. (**b**-**c**) 80 days after AAV-Ova injections, splenocytes were harvested and Ova-specific $CD8^+$ (**b**) and $CD4^+$ (**c**) immune responses were evaluated by ELISpot assays. (**d)** 80 days after AAV-Ova injections, sera were collected and anti-Ova IgG were titrated by ELISA. **(e**-**g**) Mice were orally-tolerized during 7 to 10 days with 1% Ova in their drinking water either before (day -7 to 0), at the same time (day 0 to 10), or after (day 10 to 20) AAV-Ova injection. (**e**-**f**) Blood samples were collected 14 days (**e**) or 21 days (**f**) post AAV-Ova transduction and the percentage of Ova-specific $CD8^+$ T cells was determined using H-$2K^b$/$Ova_{257-264}$ dextramers staining and flow cytometry in the indicated group of mice. (**g**) Transgene persistence and expression was evaluated by quantification of Ova mRNA by qRT-PCR in the indicated group of mice. Data are normalized against the level of Eef2 mRNA (arbitrary units).

Supplemental Figure 1. Evaluation of oral-tolerization protocols differing in duration and antigen dose.

(a-c) Mice (n=5 per group) were given 1% Ova in their drinking water for 7 days and were then injected with different vector doses: $1x10^{11}$ vg/mouse (**a**), $5x10^{10}$ vg/mouse (**b**) or $3.5x10^9$ vg/mouse (**c**). Percentages of Ova-specific $CD8^+$ T cells were determined by flow cytometry in control and tolerized mice at day 14 (**a**-**c**). (**d**-**f**) Mice (n=6 per group) were given 1% Ova in their drinking water for 5 days and were then injected with $1x10^{11}$ vg/mouse. (**d**) Blood samples were collected and Ova-specific $CD8^+$ T cells were determined by flow cytometry at day 14. (**e**-**f**) Sera were collected 80 days after AAV-injection and anti-Ova IgG titers (**e**) and mean concentrations of Ova in sera (**f)** were determined by ELISA. (**g**) Mice (n=5 per group) were given with 0.1% Ova in their

drinking water for 7 days and were then injected with $3.5x10^9$ vg/mouse. Ova-specific $CD8^+$ T cells in blood were determined 14 days after AAV-injection by flow cytometry.

Supplemental Figure 2. Oral-tolerization inhibits immune responses even in situations where AAV vector administration is associated with concomitant TLR9 activation.

B6 mice (n=7 per group) were given 1% Ova in their drinking water for 7 days and were then injected with AAV-Ova ($3.5x10^9$ vg/mouse) contaminated with CpG-ODNs 1826 (10 µg/mouse) in their gastrocnemius muscles. Immune responses were monitored between days 15 and 40 post vector injections. (**a**) Blood samples were collected at different time points and percentages of Ova-specific $CD8^+$ T cells were determined by flow cytometry in untolerized control mice (CpG) and in tolerized mice (CpG tol). (**b**) Sera were collected at days 21 and 39 post AAV-injections and anti-Ova IgG titers were determined by ELISA. (**c**) Splenocytes were harvested at day 40 and analyzed for IFNγ secretion after *ex vivo* restimulation with MHC I restricted $Ova_{257-264}$ peptide, MHC II restricted $Ova_{323-339}$ peptide, or with MHC I restricted AAV1 capsid peptides. (**d**) Transduced muscles were harvested at day 40 and Ova mRNA were quantified by qRT-PCR. (**e**) Mean Ova concentrations in sera were analyzed by ELISA 39 days post AAV-injections.

Supplemental Figure 3. Oral-tolerization is not associated with obvious modification of immune cell subsets in mesenteric lymph nodes.

Mesenteric lymph nodes (MLNs) were harvested at the end of the oral-tolerization protocol, (i.e. at day 0), and cellular subsets were analyzed by flow cytometry. (**a**) Percentage of each indicated leukocyte population was determined in control and tolerized mice by flow cytometry. (**b**) Percentage of gated $CD11c^+$ DCs subsets expressing $CD103^+$ or expressing both $CD103^+$ and $CD24^+$. (**c**) Percentage of $CD19^{high}CD86^+IL\text{-}10^+$ (considered as regulatory B10 cells) and level of expression of IL-10 were assessed by intracellular staining. Percentage of B10 cells were found to be equivalent in the two groups of mice (12.48% ±2.3 in control mice and 10.57% ±3.8 in tolerized animal) as were their levels of IL-10 expression, represented here by the mean fluorescence intensity (MFI) of IL-10 intracellular staining. (**d**-**h**) $CD4^+Foxp3^+$ Tregs from MLNs were analyzed for expression of Helios and Nrp-1 markers as well as for CTLA-4, Foxp3 and CD25 contents. (**d**) Representative cytometric profiles and (**e**) percentage of Tregs subsets according to the expression of Helios and Nrp-1 are shown. (**f**-**h**) Mean fluorescence intensity (MFI) of CTLA-4 (**f**), Foxp3 (**g**) and CD25 (**h)** staining in the gated $CD4^+Foxp3^+$ Tregs population is shown. (**i**) No significant differences were found neither on the proportion of $CD4^+CD25^-LAG\text{-}3^+$ cells (3.6% ±0.5 in control vs. 2.4% ±0.2 in tolerized animals) nor in the mean expression level of LAG-3.

Supplemental Figure 4. Tregs depletion/inactivation is not sufficient to abrogate oral tolerance.

As treatment with the anti-CD25 antibody (PC-61) is known to inactivate Tregs suppressive functions without necessarily depleting them [59], three strategies were evaluated to inactivate and deplete Tregs *in vivo*: two injections of PC-61 anti-CD25 antibody (days - 3 and - 1 before AAV injection) [40], injection of 60 mg of NAD alone [40], or the combination of both treatments. (**a**) Cytometric profiles gated on $CD4^+$ cells showing percentage of $Foxp3^+$ and $CD25^+$ cells following these treatments. (**b**) Total number of $CD4^+Foxp3^+$ cells in blood after treatment with PC-61 and/or NAD. Experiments were performed three times, except for NAD treatment that has been extensively studied in our previous study [40] and was tested here only once. (**c**-**e**) Tregs were inactivated/depleted in vivo by co-treatment with two injections of PC-61 (days - 3 and - 1 before AAV injection) and one injection of NAD at the end of the oral-tolerization protocol (day - 1) before injection with AAV-Ova ($5x10^{10}$ vg) at day 0 (n=5-7). Blood was collected at the indicated time points to monitor the cellular Ova-specific cytotoxic T cell response by flow cytometry. Representative flow cytometry profiles at day 14 on $CD8^+$ gated cells (**c**) and bar graph (**d**) representing the percentage of Ova-specific $CD8^+$ T cells 14 to 42 days after vector injection on the different groups of mice are shown. (**e**) Sera were collected at day 49 to assess the humoral anti-Ova antibody response by ELISA.

Figure 1

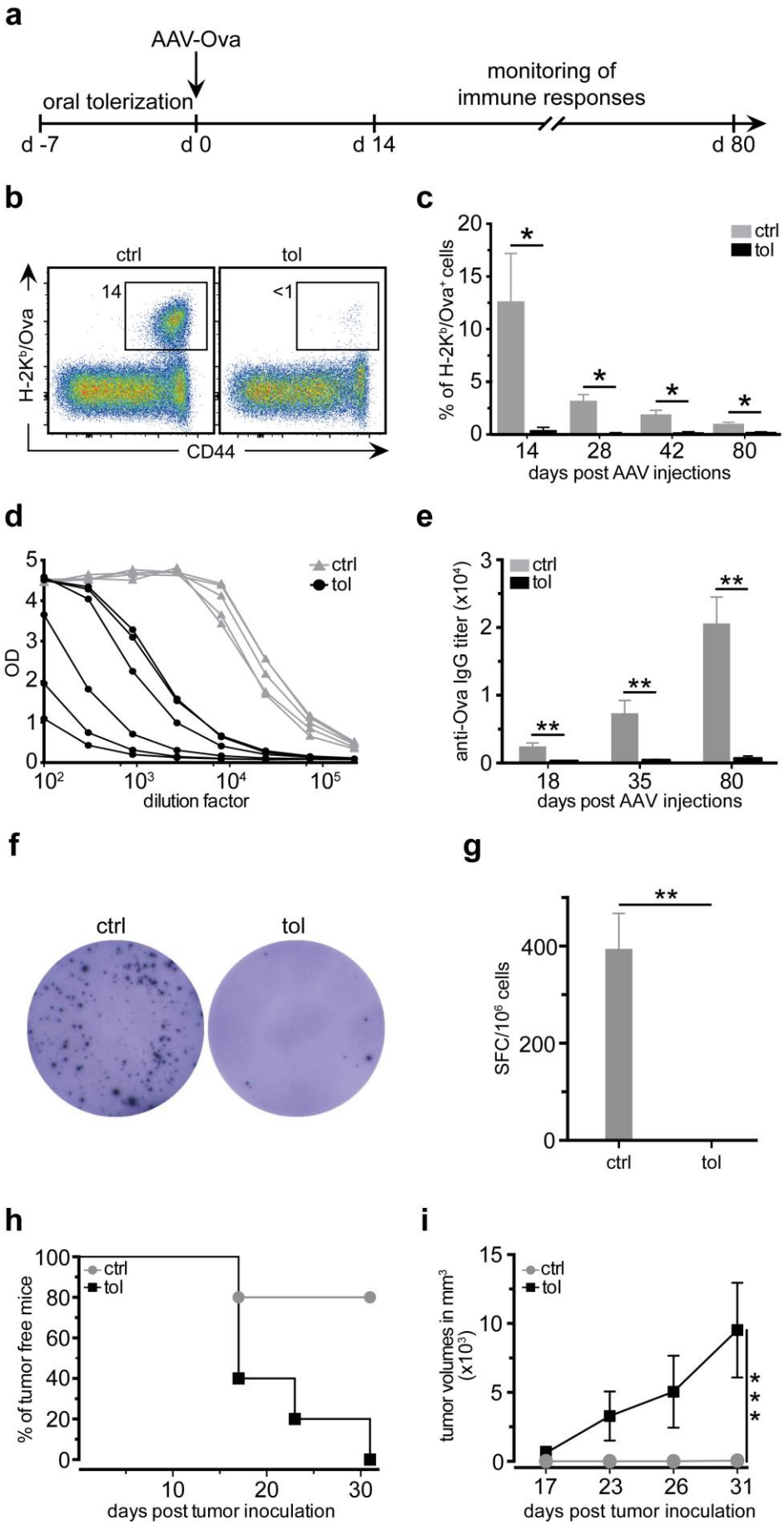

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Figure 2

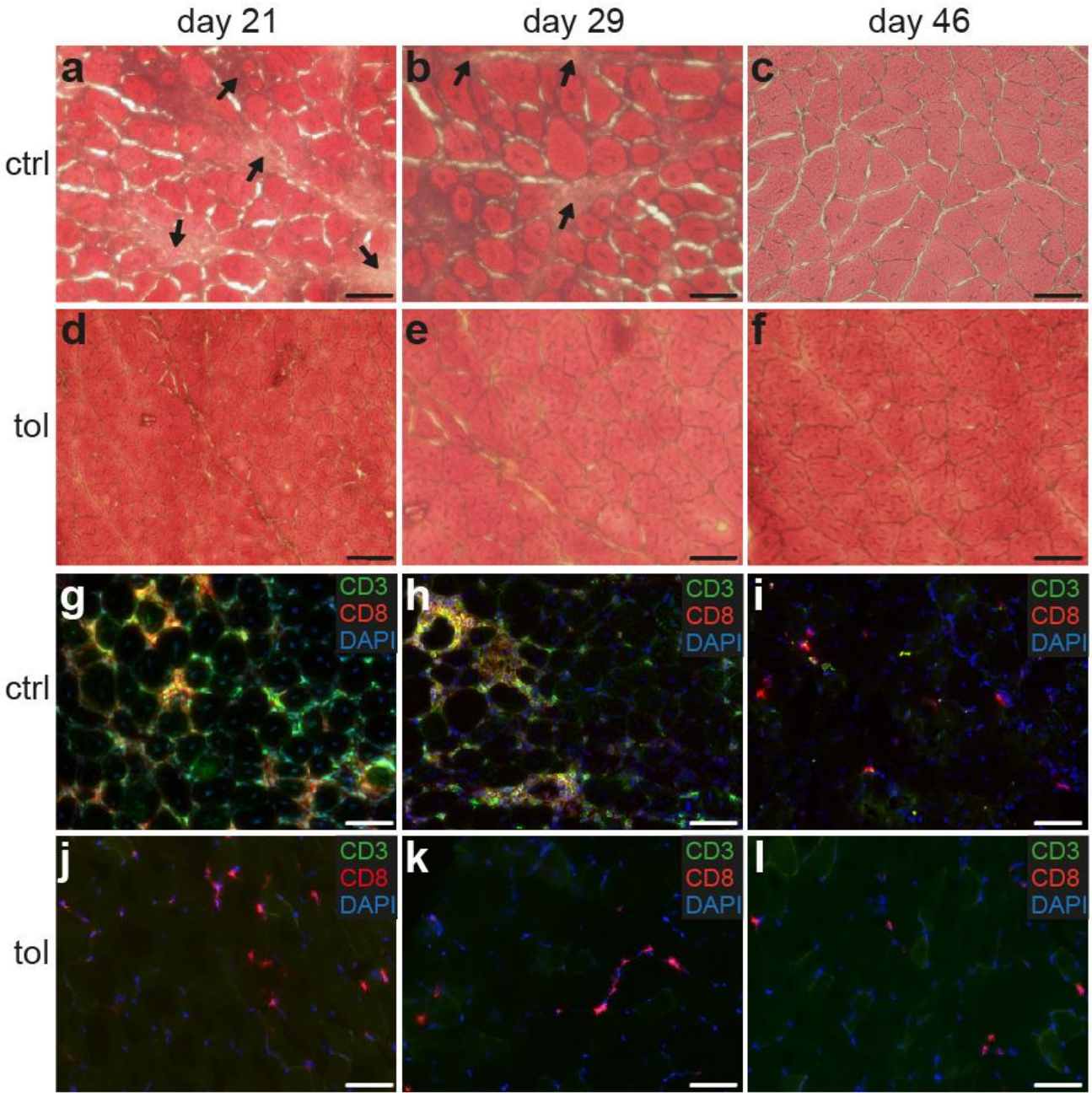

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k
l
CD3
CD8
DAPI

Figure 3

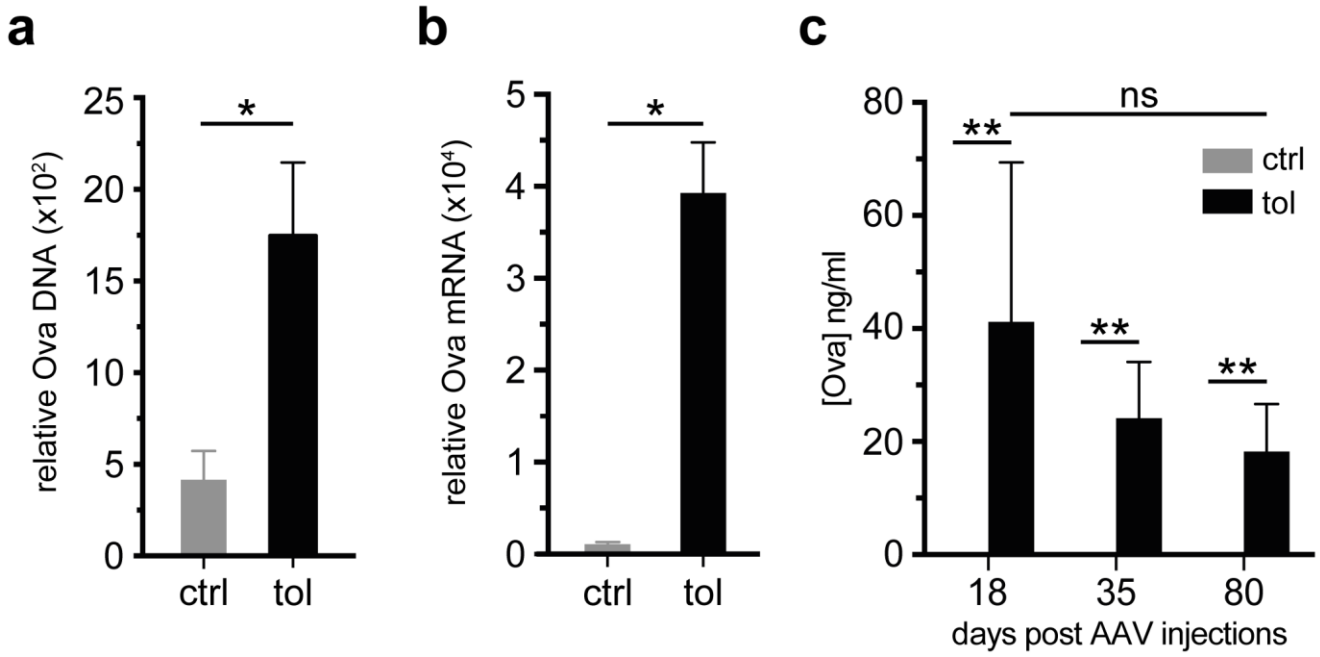

a
b
c
relative Ova DNA (x10²)
relative Ova mRNA (x10⁴)
[Ova] ng/ml
ctrl
tol
*
**
ns
18
35
80
days post AAV injections

Figure 4

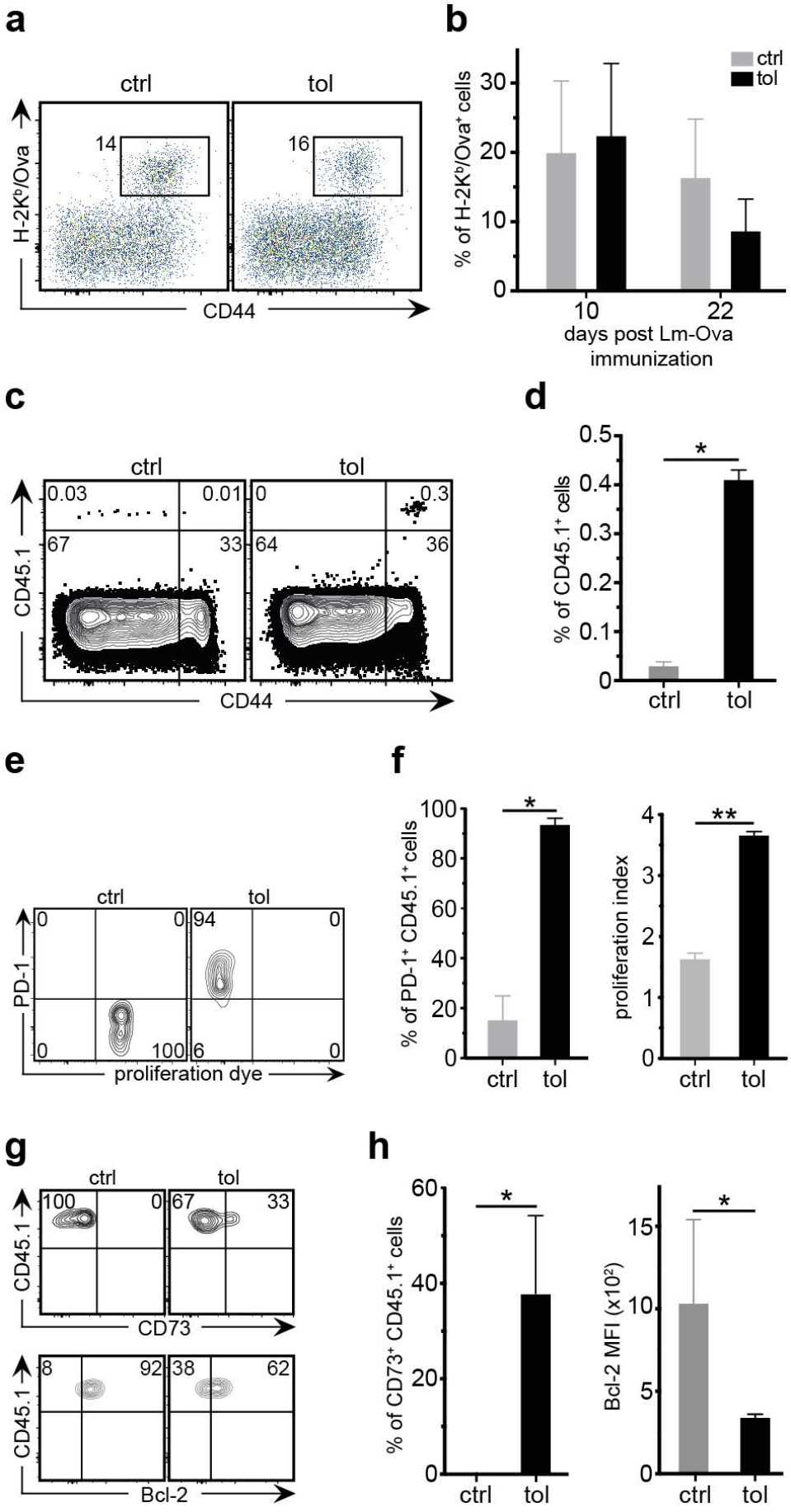

a
ctrl
tol
14
16
H-2Kb/Ova
CD44
b
ctrl
tol
% of H-2Kb/Ova+ cells
30
20
10
0
10
22
days post Lm-Ova immunization
c
ctrl
tol
0.03
0.01
0
0.3
67
33
64
36
CD45.1
CD44
d
% of CD45.1+ cells
0.5
0.4
0.3
0.2
0.1
0
*
ctrl
tol
e
ctrl
tol
0
0
94
0
0
100
6
0
PD-1
proliferation dye
f
% of PD-1+ CD45.1+ cells
100
80
60
40
20
0
*
ctrl
tol
proliferation index
4
3
2
1
0
**
ctrl
tol
g
ctrl
tol
100
0
67
33
CD45.1
CD73
8
92
38
62
CD45.1
Bcl-2
h
% of CD73+ CD45.1+ cells
60
40
20
0
*
ctrl
tol
Bcl-2 MFI (x10²)
15
10
5
0
*
ctrl
tol

Figure 5

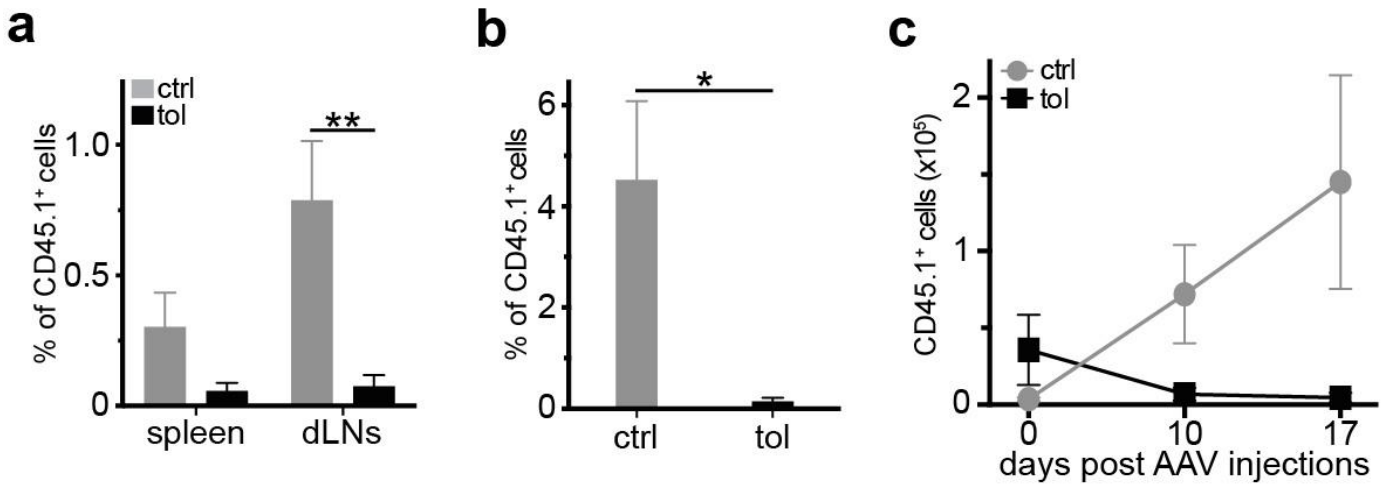
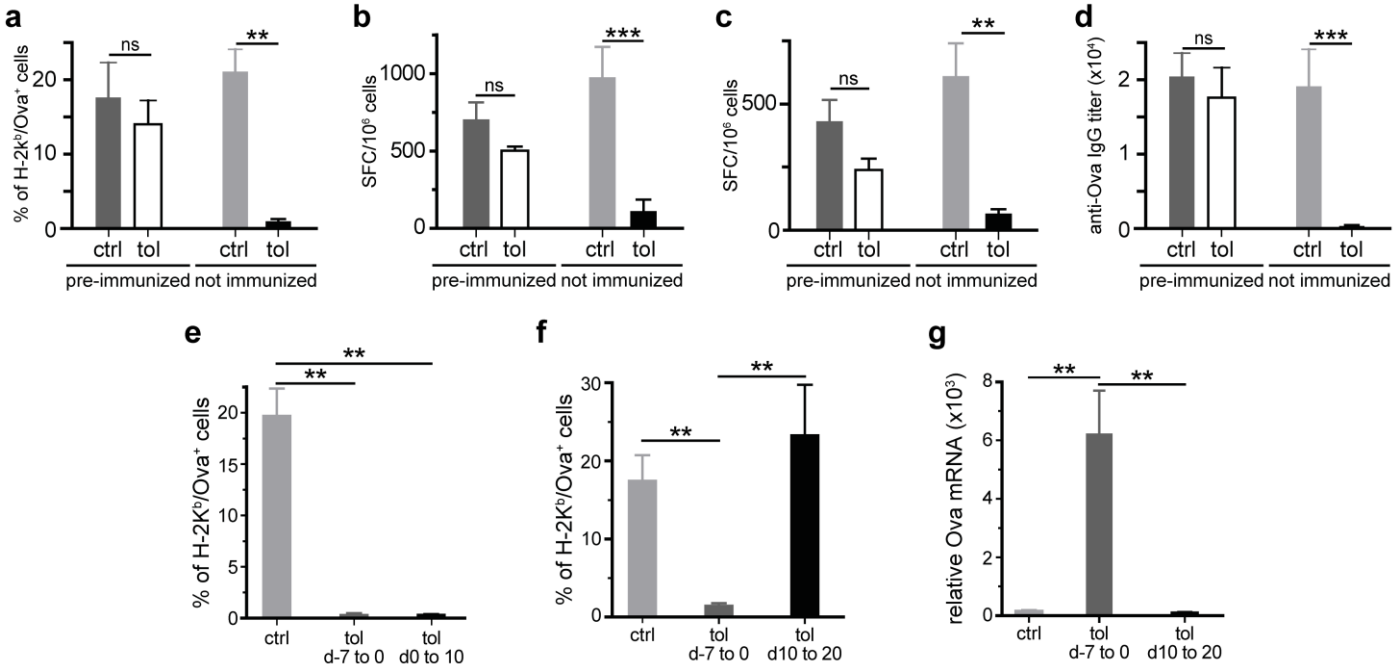
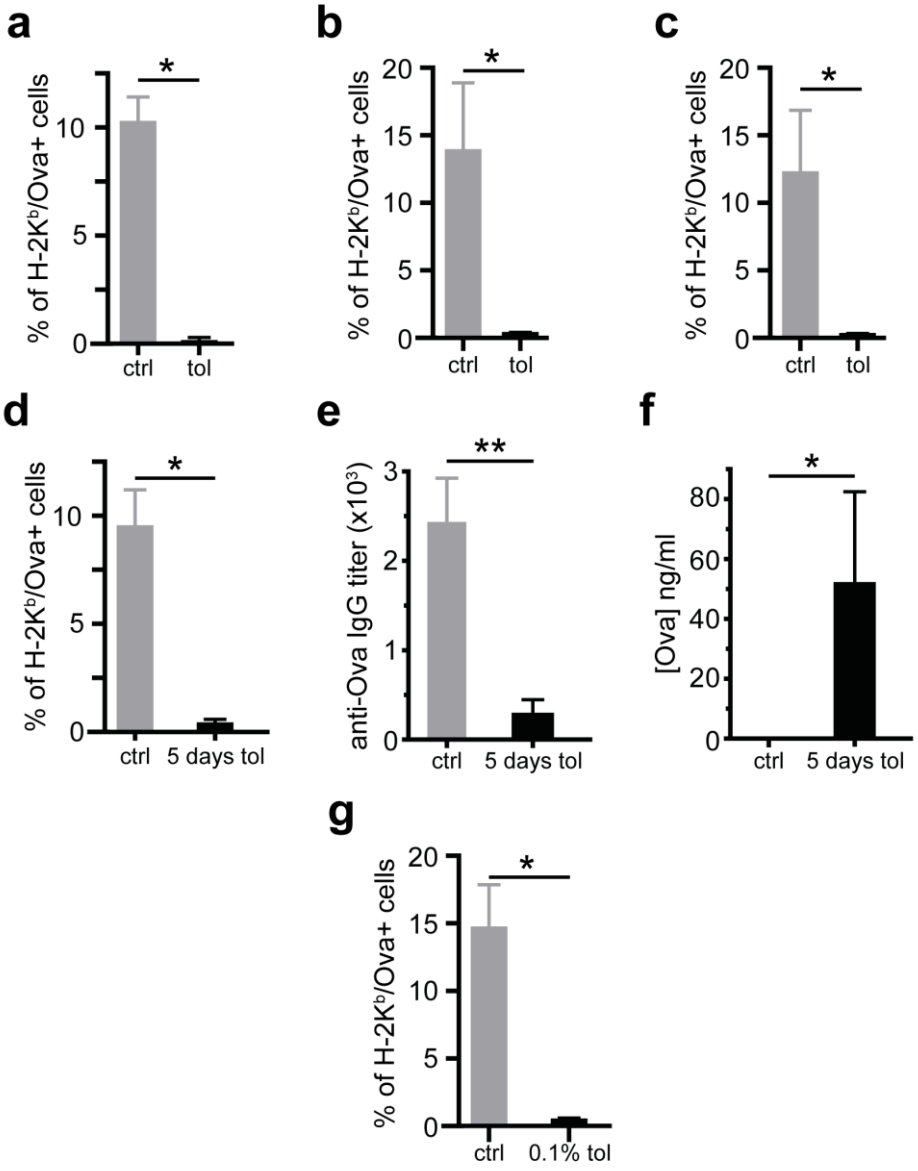

a
ctrl
tol
% of CD45.1+ cells
1.0
0.5
0
**
spleen
dLNs
b
% of CD45.1+ cells
6
4
2
0
*
ctrl
tol
c
ctrl
tol
CD45.1+ cells (x10⁵)
2
1
0
0
10
17
days post AAV injections

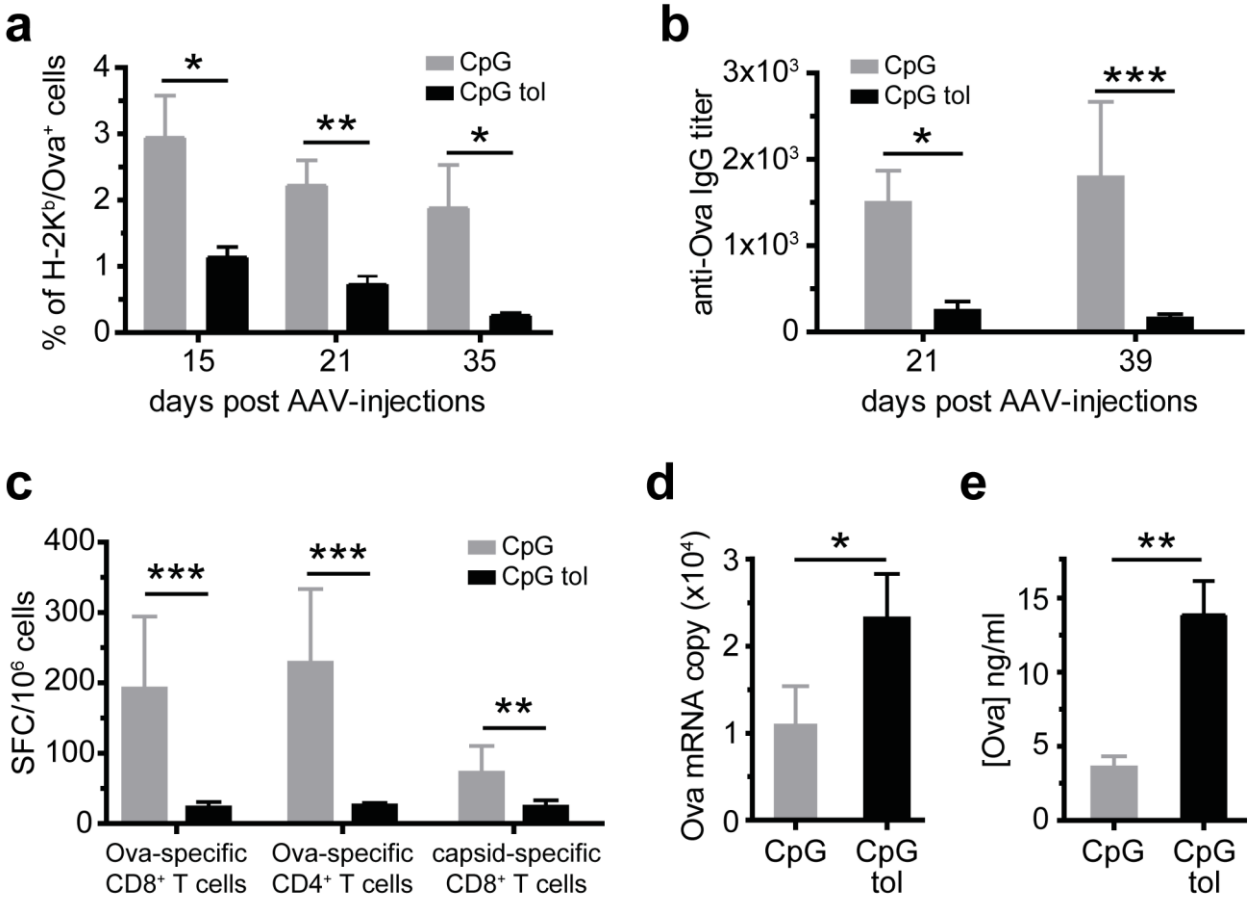
Figure 6


a
% of H-2Kb/Ova+ cells
ns
**
ctrl tol ctrl tol
pre-immunized not immunized
b
SFC/10^6 cells
ns
***
ctrl tol ctrl tol
pre-immunized not immunized
c
SFC/10^6 cells
ns
**
ctrl tol ctrl tol
pre-immunized not immunized
d
anti-Ova IgG titer (x10^4)
ns
***
ctrl tol ctrl tol
pre-immunized not immunized
e
% of H-2Kb/Ova+ cells
**
**
ctrl
tol d-7 to 0
tol d0 to 10
f
% of H-2Kb/Ova+ cells
**
**
ctrl
tol d-7 to 0
tol d10 to 20
g
relative Ova mRNA (x10^3)
**
**
ctrl
tol d-7 to 0
tol d10 to 20

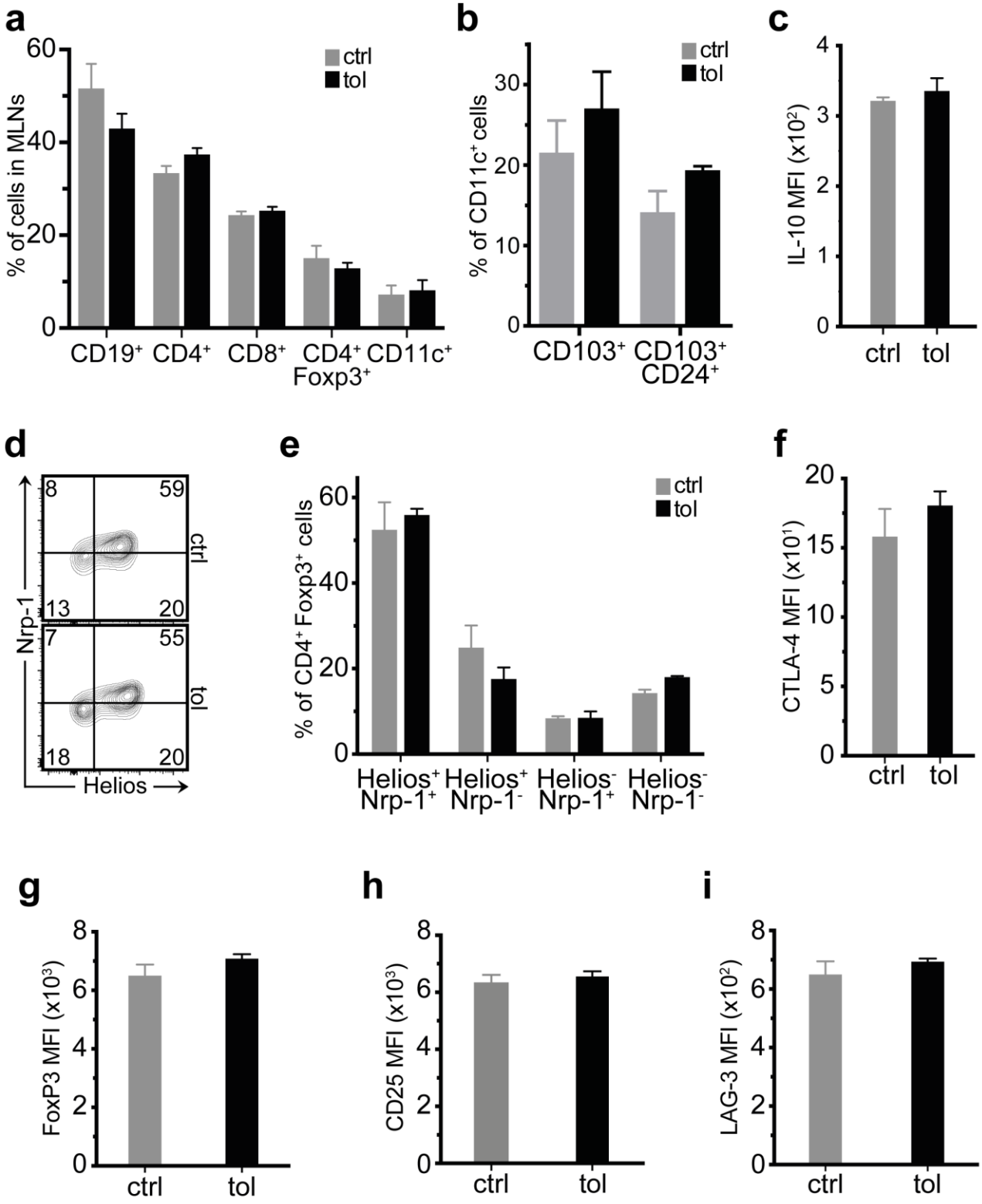
Supplemental figure 1



Supplemental figure 2



Supplemental figure 3



Supplemental figure 4

