

# Intrinsic Transgene Immunogenicity Gears CD8<sup>+</sup> T-cell Priming After rAAV-Mediated Muscle Gene Transfer

Maxime Carpentier<sup>1</sup>, Stéphanie Lorain<sup>2</sup>, Pascal Chappert<sup>1</sup>, Mélanie Lalfer<sup>1</sup>, Romain Harget<sup>3</sup>, Dominique Urbain<sup>1</sup>, Cécile Peccate<sup>2</sup>, Sahil Adriouch<sup>3</sup>, Luis Garcia<sup>4</sup>, Jean Davoust<sup>1</sup> and David-Alexandre Gross<sup>1</sup>

<sup>1</sup>INSERM U1151, Institut Necker Enfants Malades, CNRS, UMR8253, Faculté de Médecine, Université Paris Descartes, Sorbonne Paris Cité, Paris, France; <sup>2</sup>Sorbonne Universités, UPMC Université Paris 06, Myology Research Center, UM76 and INSERM U974 and CNRS FRE 3617 and Institut de Myologie, Paris, France; <sup>3</sup>INSERM U905, Université de Rouen, Institute for Research and Innovation in Biomedicine (IRIB), Rouen, France; <sup>4</sup>UFR des Sciences de la Santé, Université de Versailles Saint-Quentin-en-Yvelines, Montigny-le-Bretonneux, France

Antitransgene CD8<sup>+</sup> T-cell responses are an important hurdle after recombinant adeno-associated virus (rAAV) vector-mediated gene transfer. Indeed, depending on the mutational genotype of the host, transgene amino-acid sequences of foreign origin can elicit deleterious cellular and humoral responses. We compared here two different major histocompatibility complex (MHC) class I epitopes of an engineered ovalbumin transgene delivered in muscle tissue by rAAV1 vector and found very different strength of CD8 responses, muscle destruction being correlated with the course of the immunodominant response. We further demonstrate that robust CD8<sup>+</sup> T-cell priming can occur through the cross-presentation pathway but requires the presence of either a strong MHC class II epitope or antibodies to the transgene product. Finally, manipulating transgene subcellular localization, we found that provided we avoid transgene expression in antigen presenting cells, the poorly accessible cytosolic form of ovalbumin transgene lacking strong MHC II epitope, evades CD8<sup>+</sup> T-cell priming and remains permanently expressed in muscle with no immune cell infiltration. Our results demonstrate that the intrinsic immunogenicity of transgenes delivered with rAAV vector in muscle can be manipulated in a rational manner to avoid adverse immune responses.

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## INTRODUCTION

Gene therapy of monogenic disorders relies on the replacement of a nonfunctional or a missing endogenous protein by a therapeutic gene product. Several clinical developments have been conducted using recombinant adeno-associated virus (rAAV) vectors as vehicles to express therapeutic transgenes in a target tissue, such as factor IX (FIX) in the liver of hemophilia B patients<sup>1</sup> and lipoprotein

lipase (LPL) in the muscle tissue of LPL deficient patients.<sup>2</sup> As evidenced both in animal models and in humans, adverse immune responses against rAAV vector and the therapeutic transgenes themselves represent a major bottleneck, which limits the domain of application of these treatments.<sup>3,4</sup> In addition to preexisting neutralizing antibodies and T-cell responses directed against vector capsids,<sup>1,5-7</sup> immune responses directed towards the transgene have been observed for various transgenes delivered in mice<sup>8-10</sup> and human.<sup>11</sup> rAAV vector serotype, dose and route of administration, immunomodulatory properties and inflammatory status of the targeted tissue and pattern of transgene expression were all shown to influence immune responses directed against transgene of foreign origin.<sup>3,12</sup> Indeed, the presence in the transgene product of amino acids sequences not encoded in the host genome and therefore not previously tolerated by the host immune system also represents a key factor in priming of antitransgene B- and T-cell responses, as shown in both murine and canine model of FIX gene therapy.<sup>13,14</sup> Moreover, among the numerous potential peptides present within a protein, only a fraction of them are correctly processed by the antigen presentation machinery, possess adequate amino-acid sequence to bind given major histocompatibility complex (MHC) haplotype and are therefore presented to a dedicated T-cell repertoire, a general phenomenon known as immunodominance.<sup>15</sup> Thus, few CD8 and CD4 epitopes of real therapeutic transgene have been discovered so far.<sup>8,16-18</sup> In contrast, numerous model transgenes with known epitopes have been extensively studied, but much of them harbored only one known CD8 epitope in a given MHC background (Luciférase,<sup>19</sup> Green Fluorescent Protein (GFP),<sup>20</sup>  $\beta$ -galactosidase<sup>21</sup>), sometime associated with one known CD4 epitope (Ovalbumin (OVA),<sup>9</sup> influenza virus hemagglutinin (HA)<sup>22</sup>), precluding the analysis of differential immunogenicity of multiple epitopes in a given transgene.

Due to the central role of dendritic cells (DC) during the initiation of adaptive immune responses,<sup>23,24</sup> direct transduction of DC was suggested to be a key element driving cellular responses after gene transfer.<sup>25,26</sup> Strategies aiming at preventing expression in

The last two authors contributed equally to this work.

Correspondence: Jean Davoust, INSERM U1151, Hôpital Necker Enfants Malades, 149 rue de Sèvres, 75743 Paris Cedex 15, France. E-mail: [jean.davoust@inserm.fr](mailto:jean.davoust@inserm.fr) or David-Alexandre Gross, INSERM U1151, Hôpital Necker Enfants Malades, 149 rue de Sèvres, 75743 Paris Cedex 15, France. E-mail: [david.gross@inserm.fr](mailto:david.gross@inserm.fr)

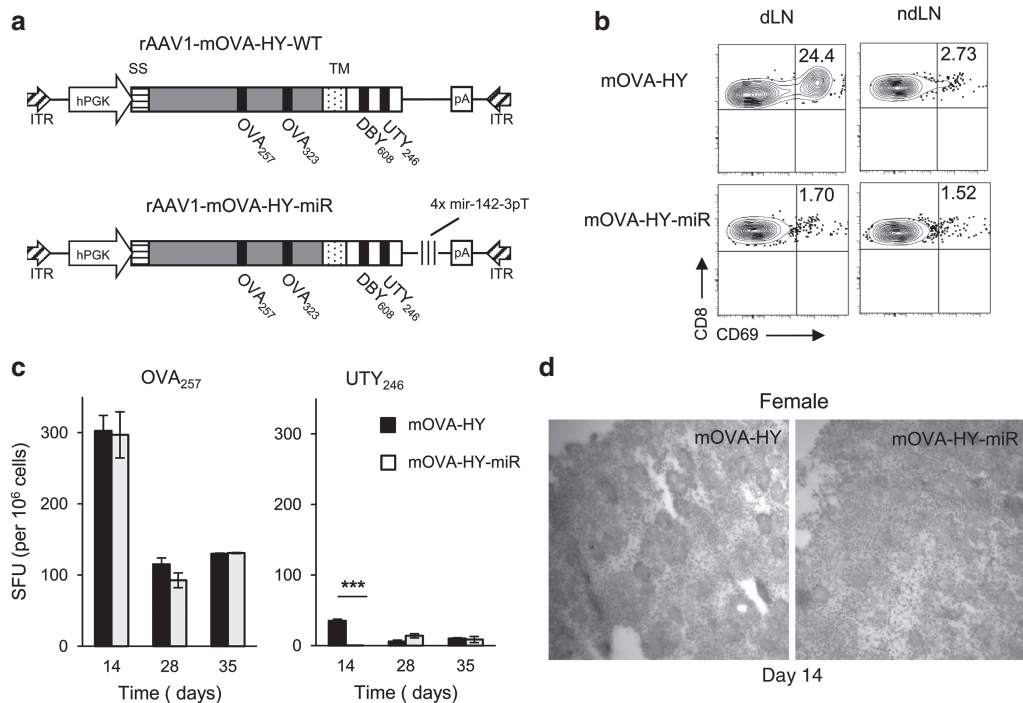
antigen presenting cells (APC) through the use of tissue-specific promoters<sup>27–29</sup> or miRNA-based regulation of transgene expression in the hematopoietic system<sup>30,31</sup> have been used. In this later approach, target sequences of the hematopoietic-specific miRNA142.3p have been added to the transgene coding sequence to destabilize the transgene mRNA and prevent transgene product expression in APC, thus promoting effective immune tolerance for particular transgenes. While efficient at preventing direct presentation of transgene-derived antigens, these strategies cannot prevent the uptake and cross-presentation of transgene products by nontransduced APC patrolling in the target tissue long after gene transfer. In the context of rAAV-mediated gene transfer, cross-presentation of muscle-derived transgene products was shown to be sufficient to prime a functional antitransgene cytotoxic T lymphocyte (CTL) response.<sup>32</sup> Moreover, studies performed in auto-immune disease models have highlighted the importance of helper CD4<sup>+</sup> T-cells activity,<sup>33</sup> antigen forms (soluble versus cell associated)<sup>34</sup> and antigen-specific antibody responses<sup>35</sup> as important determinants of the outcome of cross-presentation events of tissue-specific antigens. Indeed, higher immunogenicity of membrane-bound transgene products over soluble ones was reported in a gene therapy setting,<sup>10</sup> but little attention was devoted to the role of MHC class II and B-cell epitopes in the initiation of adverse antitransgene CD8<sup>+</sup> T-cell responses.

In this study, we explored how the core immunogenic properties of a transgene of foreign origin delivered in muscle with rAAV1 vector regulate CD8<sup>+</sup> T-cell priming against multiple epitopes in a situation where its accessibility to the direct antigen-presentation pathways can be tightly restricted by the adjunction of miR-142-3p target sequences. Analyzing immune responses directed against different cell associated forms of OVA model transgene containing or not well-characterized MHC class I and II epitopes, we identified the immunodominance of individual MHC I epitope, the presence of a strong helper T-cell epitope and the presence of transgene-specific antibodies as three key determinants for the initiation of adverse CD8<sup>+</sup> T-cell responses.

**RESULTS**

**Epitope-dependent CD8<sup>+</sup> T-cell priming in the absence of transgene expression in APC**

To explore if CD8<sup>+</sup> T-cell priming is equally effective against multiple epitopes within a given transgene product, we engineered a recombinant rAAV serotype 1 (rAAV1) vector coding for the full-length membrane bound ovalbumin fused to UTY<sub>246</sub> and DBY<sub>608</sub> male HY antigen epitopes (rAAV1-mOVA-HY) (Figure 1a). In female C57Bl/6 mice (B6), mOVA-HY is a fully foreign antigen containing four well-characterized MHC class I (OVA<sub>257</sub> and UTY<sub>246</sub>) and class II (OVA<sub>323</sub> and DBY<sub>608</sub>) epitopes. Furthermore,



**Figure 1** Restriction of transgene expression to nonhematopoietic lineages has a minor influence on CD8<sup>+</sup> T-cell priming. **(a)** The mOVA-HY construct comprises the leader peptide from the H-2K<sup>b</sup> gene (SS), the full-length OVA cDNA, the H-2D<sup>b</sup> transmembrane sequence (TM) and, fused at the C-terminal, the DBY<sub>608</sub> and UTY<sub>246</sub> epitopes encompassed by 4–15 amino acids of their original protein sequence to ensure normal processing. In the mOVA-HY-miR construct, an additional sequence comprising four repeats of the miR142.3p target sequence were inserted in the 3' untranslated region. **(b)** *Rag2*<sup>-/-</sup> female mice were injected i.m. at day 0 with rAAV1-mOVA-HY or rAAV1-mOVA-HYmiR. On day 3, mice were sacrificed and 2 × 10<sup>5</sup> cells from draining or nondraining lymph nodes were cocultured with 4.10<sup>4</sup> OT-1 cells. After 24 hours, CD69 activation was tested by flow cytometry. One representative experiment out of three is shown. **(c–d)** C57Bl/6 female mice were injected i.m. at day 0 with rAAV1-mOVA-HY (rAAV-WT) or rAAV1-mOVA-HYmir (rAAV-miR). **(c)** On day 14, 28, or 35 mice were sacrificed and their splenocytes were tested by IFN-γ ELISPOT assay against the MHC I-restricted OVA<sub>257</sub> or UTY<sub>246</sub> peptides. Data represent at least six to nine mice per group and per time point pooled from two independent experiments. **(d)** On day 14, tibialis anterior muscles from sacrificed mice were frozen and 7 μm thick transversal cryosections were stained with hematoxylin and eosin. Field size is 720 × 720 micron. One representative muscle out of three is shown.

to inhibit direct transgene expression in antigen presenting cells (APC), we additionally generated a second rAAV1 construct bearing four repeats of the miR-142-3p target (miR-142-3pT) sequence<sup>30</sup> inserted in the 3' untranslated region of the hPGK-driven mOVA-HY expression cassette.

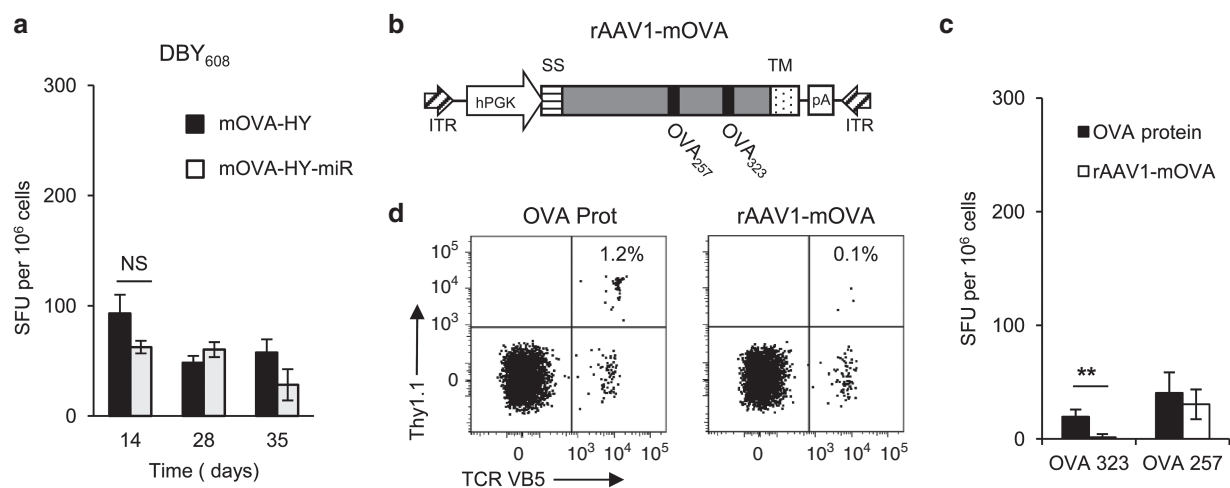
To assess here whether miR-142-3pT sequence controls transgene expression and presentation by APC, *Rag2*<sup>-/-</sup> female mice were injected intramuscularly (i.m.) with either mOVA-HY or mOVA-HY-miR constructs. Their draining inguinal and popliteal lymph nodes or non-draining contralateral lymph nodes cells were isolated 3 days later and used *in vitro* as stimulating APC for anti-OVA<sub>257</sub> OT-I TCR transgenic CD8<sup>+</sup> T cells (OT-I). While draining lymph node cells from mOVA-HY immunized mice were able to present OVA<sub>257</sub> epitope, as measured by overnight CD69 upregulation in tester OT-I cells (Figure 1b), no detectable OVA<sub>257</sub> presentation was evidenced in mOVA-HY-miR immunized mice. This demonstrated that the miR-142-3pT sequence efficiently impaired early presentation of transgene-derived MHC class I epitopes *in vivo*, in line with its known regulation of transgene expression in cells of hematopoietic origin.<sup>30</sup>

To compare CD8<sup>+</sup> T-cell priming against each MHC class I epitope, immunocompetent female B6 mice were injected i.m. with either mOVA-HY or mOVA-HY-miR constructs. Surprisingly, we observed similarly potent anti-OVA<sub>257</sub> CD8<sup>+</sup> T-cell responses after rAAV1-mediated gene transfer with both constructs (Figure 1c). In contrast, a tenfold lower CD8<sup>+</sup> T-cell response was observed against anti-UTY<sub>246</sub> epitope with mOVA-HY construct and this weak response was efficiently inhibited at day 14 by the adjunction of miR-142-3pT sequence (Figure 1c). Finally, local inflammation was further confirmed by histological inspection of muscle sections with both vectors (Figure 1d). As early as day 14, large

areas of infiltration and muscle fibers destruction could be evidenced, in line with the strong anti-OVA<sub>257</sub> CD8<sup>+</sup> T-cell response detected by ELISPOT at that time point (Figure 1c). Altogether, these results indicate that strength of CD8<sup>+</sup> T-cell priming is epitope-dependent and that miR-142-3pT-mediated destabilization of mOVA-HY mRNA in cells of hematopoietic origin affects CD8<sup>+</sup> T-cell priming only against weakly immunogenic epitopes.

### CD4<sup>+</sup> T cell help controls CD8<sup>+</sup> T-cell priming against tissue-restricted transgene

In addition to the core immunogenic properties of individual transgene-derived MHC I-restricted epitopes, the establishment of an antitransgene CD4<sup>+</sup> helper T-cell response could influence the strength of CD8<sup>+</sup> T-cell responses<sup>36,37</sup>, prompting us to analyze the CD4<sup>+</sup> T-cell response induced by mOVA-HY and mOVA-HY-miR constructs. Similarly to the anti-OVA<sub>257</sub> CD8<sup>+</sup> T-cell response, the CD4<sup>+</sup> T-cell response against the MHC class II-restricted DBY<sub>608</sub> epitope derived from mOVA-HY-miR vector was not reduced nor delayed compared to the one triggered by mOVA-HY (Figure 2a). Surprisingly, neither of these two vectors was able to induce a detectable response against the MHC class II-restricted OVA<sub>323</sub> epitope (data not shown). As the DBY<sub>608</sub> epitope could behave as a dominant epitope masking the initiation of OVA<sub>323</sub>-specific immune response, we engineered a separate rAAV1 vector coding for the sole full-length membrane bound ovalbumin (mOVA) (Figure 2b). As compared with OVA protein emulsified in incomplete Freund's adjuvant (IFA), this later construct, however, elicited no detectable anti-OVA<sub>323</sub> CD4<sup>+</sup> T-cell responses, as measured by IFN- $\gamma$  ELISPOT assay (Figure 2c). Of note, in female mice, the mOVA vector triggered a seven times weaker CD8<sup>+</sup> T-cell response against the dominant OVA<sub>257</sub>

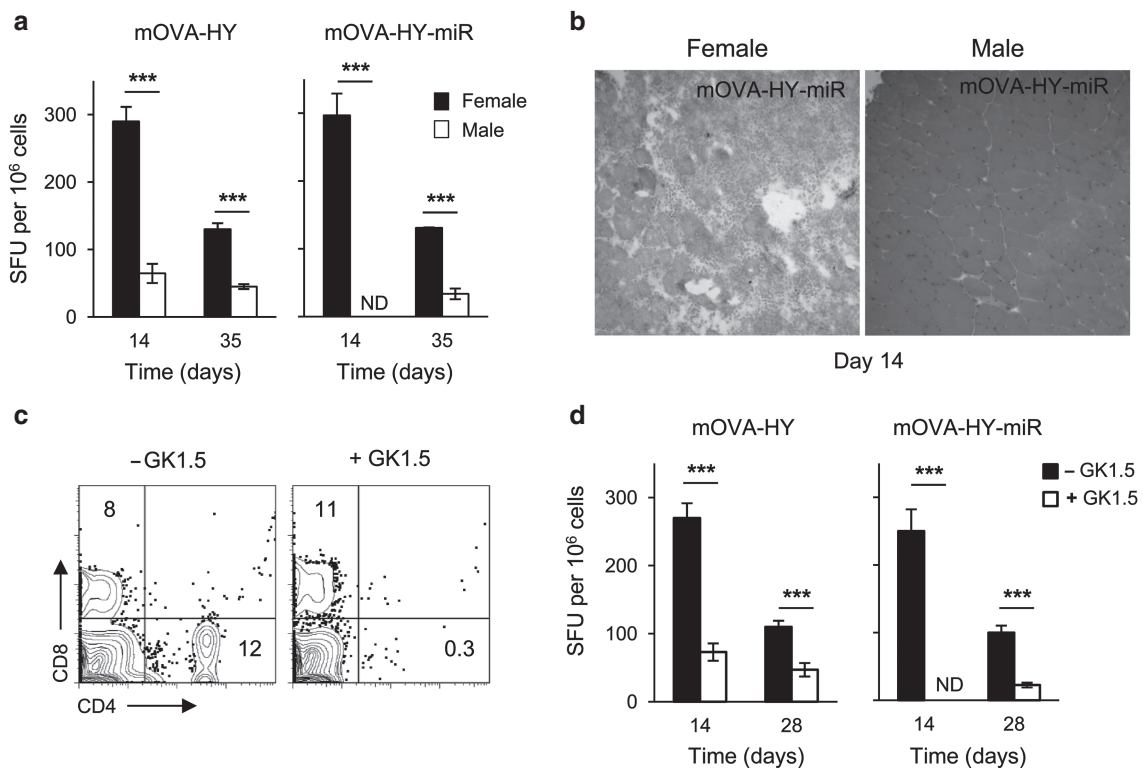


**Figure 2** DBY<sub>608</sub> but not OVA<sub>323</sub> appears as a strong MHC II transgene-derived epitope. **(a)** C57Bl/6 female mice were injected i.m. at day 0 with rAAV1-mOVA-HY or rAAV1-mOVA-HY-miR. On day 14, 28, or 35 mice were sacrificed and their splenocytes were tested by IFN- $\gamma$  ELISPOT assay against the MHC II-restricted DBY<sub>608</sub> peptide. Data represent six to nine mice per group and per time point pooled from two independent experiments. **(b)** The mOVA construct comprises the leader peptide from the H-2K<sup>b</sup> gene (SS), the full-length OVA cDNA and the H-2D<sup>b</sup> transmembrane sequence (TM). **(c-d)** C57Bl/6 female mice were injected s.c. with 100  $\mu$ g of OVA protein emulsified in IFA or i.m. with rAAV1-mOVA. **(c)** On day 14, mice were sacrificed and their splenocytes were tested by IFN- $\gamma$  ELISPOT assay against the MHC II-restricted OVA<sub>323</sub> or MHC I-restricted OVA<sub>257</sub> peptides. Data represent three mice per group. **(d)** Alternatively, mice were transferred at day 0 with 10<sup>6</sup> congenic Thy1.1 TCR transgenic OT-II CD4<sup>+</sup> T cells. On day 8, their PBL were stained to assess OT-II T cells expansion by flow cytometry analysis. Dot plots representative of three and gated on CD4<sup>+</sup> 7-AAD<sup>-</sup> cells are shown.

epitope compared to the one triggered with the mOVA-HY vector (Figure 2c). The impaired functional presentation of the OVA<sub>323</sub> class II-restricted epitope in this setting was further confirmed by analyzing the *in vivo* activation of anti-OVA<sub>323</sub> OT-II TCR transgenic CD4<sup>+</sup> T cells (OT-II). While OT-II were readily expanded in recipient mice immunized with OVA protein emulsified in IFA, the rAAV1-mediated expression of the mOVA construct in muscle fibers failed to induce a detectable OT-II expansion (Figure 2d). This lack of anti-OVA<sub>323</sub> response was observed at all time points and was also confirmed using a rAAV1 vector encoding for a secreted OVA protein (sOVA); in the same conditions, anti-DBY<sub>608</sub> Marilyn T cells were expanded by mOVA-HY vector (Supplementary Figure S1). These results indicate that the DBY<sub>608</sub> peptide is the major MHC class II-restricted epitope present in our mOVA-HY transgene.

This unique responsiveness to the DBY<sub>608</sub> epitope provided us with an interesting model system to evaluate the impact of CD4<sup>+</sup> T-cell help on the antitransgene CD8<sup>+</sup> T-cell responses. Indeed, antitransgene CD4<sup>+</sup> T-cell responses are not expected in male B6 mice since (i) male mice are naturally tolerant to male antigens such as DBY<sub>608</sub> and UTY<sub>246</sub> epitopes and (ii) the OVA<sub>323</sub> epitope does not induce detectable CD4<sup>+</sup> T cells. Comparing anti-OVA<sub>257</sub> CD8<sup>+</sup> T-cell responses in male and female B6 mice,

we noted a reproducible fivefold reduction in the response against the mOVA-HY transgene in the absence of anti-DBY<sub>608</sub> responses (Figure 3a). This lower anti-OVA<sub>257</sub> T-cell response in male mice was similar to the one generated against the mOVA vector in female mice (Figure 2c), excluding a sex-related effect. More strikingly, in male recipients, the anti-OVA<sub>257</sub> CD8<sup>+</sup> T-cell response was delayed with the mOVA-HY-miR construct, with no detectable response by day 14 and a low albeit functional response by day 35 (Figure 3a). In line with the delayed CD8<sup>+</sup> T-cell response, muscles from rAAV1-mOVA-HY-miR injected male mice presented no noticeable inflammation by day 14, while numerous muscle fibers from female mice appeared already surrounded by infiltrating cells (Figure 3b). To control for the presence of a potentially uncharacterized MHC II-restricted epitope in the mOVA-HY construct, we repeated these experiments in female mice after depletion of CD4<sup>+</sup> T cells. Anti-CD4 GK1.5 antibody-mediated treatment was started 3 days before the injection of rAAV1 vectors and maintained until the end of the experiment, leading to profound and sustained depletion of CD4<sup>+</sup> T cells (Figure 3c) and a lack of DBY<sub>608</sub>-specific immune responses as assessed by IFN-γ ELISPOT assay (data not shown). The anti-OVA<sub>257</sub> immune response generated here in the absence CD4<sup>+</sup> T cells (Figure 3d) was comparable to that obtained after



**Figure 3** Antitransgene CD4<sup>+</sup> helper T-cell response dictates the magnitude of antitransgene CD8<sup>+</sup> T-cell responses and early efficacy of miR142-3pT-mediated regulation. (a–b) C57Bl/6 female or male mice were injected i.m. at day 0 with rAAV1-mOVA-HY or rAAV1-mOVA-HY-miR. (a) On day 14 or 35, mice were sacrificed and their splenocytes were tested by IFN-γ ELISPOT assay against OVA<sub>257</sub> peptide. Data represent 6–10 mice per group and per time point pooled from two to four independent experiments. (b) On day 14, tibialis anterior muscles were frozen and 7 μm thick transversal cryosections were stained with hematoxylin and eosin. Field size is 720 × 720 micron. One representative muscle out of three is shown. (c–d) A group of C57Bl/6 female mice were injected by 60 μg of GK1.5 depleting antibody at day -3, 0, 7, 1, and 21. GK1.5-treated and control C57Bl/6 female mice were injected i.m. at day 0 with rAAV1-mOVA-HY or rAAV1-mOVA-HY-miR. (c) Their PBL were stained to assess the efficiency of CD4 depletion by flow cytometry. Representative dot plots for CD4 and CD8 expression on live PBL at day 14 are shown. (d) On day 14 or 28, mice were sacrificed and their splenocytes tested by IFN-γ ELISPOT assay against OVA<sub>257</sub> peptide. Data represent 6–10 mice per group and per time point pooled from 2–4 independent experiments. ND, not detected.

injection of rAAV1-mOVA-HY into male mice or rAAV1-mOVA into female mice (Figures 2c and 3a), confirming the predominant role of the DBY<sub>608</sub> epitope in the adaptive immune response directed against the mOVA-HY transgene. Interestingly, the emergence of a delayed anti-OVA<sub>257</sub> CD8<sup>+</sup> T-cell responses previously observed against the OVA-HY-miR transgene in male mice was also observed at day 28 in CD4-depleted female mice (Figure 3d).

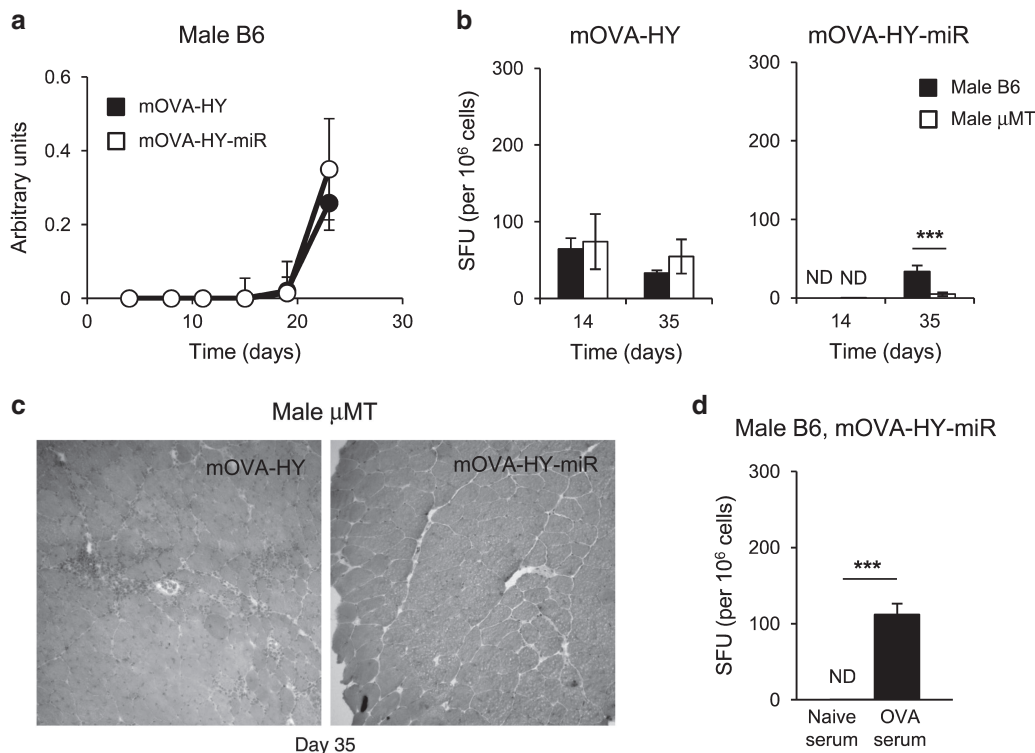
Overall, these results indicate that the presence of a strong helper epitope, as exemplified here with the male DBY<sub>608</sub> epitope, can dictate both the intensity and the kinetic of antitransgene CTL responses. In the context of miR142-3pT-regulated vector to avoid direct transgene presentation by APCs, the absence of transgene-specific CD4<sup>+</sup> T-cell responses was sufficient to delay for several weeks the induction of a functional antitransgene CD8<sup>+</sup> T-cell response, albeit insufficient to prevent late CD8 priming.

### Antitransgene antibodies requirement for CD8<sup>+</sup> T-cell cross-priming

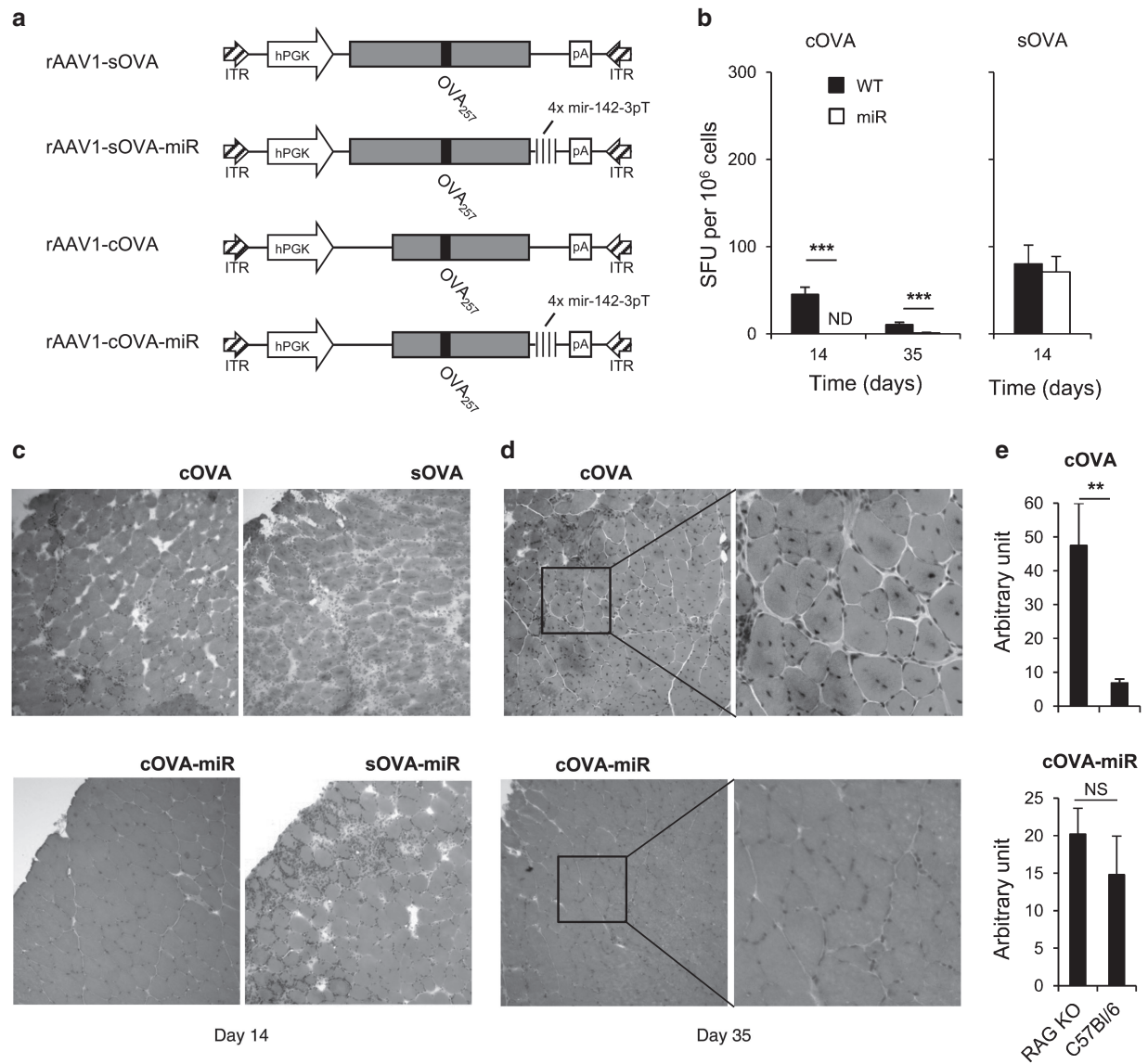
As antitransgene CD8<sup>+</sup> T-cell responses preclude stable transgene expression and establishment of long-term transgene tolerance, we wished to delineate the cellular events leading to the delayed priming of a CD8<sup>+</sup> T-cell response in the absence of both CD4<sup>+</sup> T-cell help and direct presentation of transgene-derived

epitopes. As auto-antibodies in an autoimmunity setting were shown to enhance cross-presentation of tissue specific antigens,<sup>35</sup> we assessed whether anti-OVA antibodies would similarly be responsible for the priming of the delayed CD8<sup>+</sup> T-cell response observed against the mOVA-HY-miR transgene in the absence of CD4<sup>+</sup> T-cell help. In male mice, we found no differences in the kinetics and intensities of anti-OVA antibody responses against mOVA-HY or mOVA-HY-miR transgenes (Figure 4a). This indicates that restriction of antigen expression to nonhematopoietic lineages does not interfere with the initiation of antitransgene B-cell response. Interestingly, this also showed that antitransgene humoral response precedes anti-CD8<sup>+</sup> T-cell response in rAAV1-mOVA-HY-miR injected mice by 7–10 days.

To evaluate the role of antitransgene B-cell responses in driving the delayed antitransgene CTL responses against the mOVA-HY-miR transgene in male mice, we injected B cells deficient male  $\mu$ MT mice with either rAAV1-mOVA-HY or rAAV1-mOVA-HY-miR vectors. While no differences in CD8<sup>+</sup> T-cell responses against the mOVA-HY construct occurred between WT and  $\mu$ MT male mice, these responses were profoundly impaired in  $\mu$ MT male mice injected with rAAV1-mOVA-HY-miR vector, both at early (day 14) and late (day 35) time points (Figure 4b). At this later time point, muscles of  $\mu$ MT male mice expressing the mOVA-HY-miR



**Figure 4** Antitransgene B-cell response is required for CD8<sup>+</sup> T-cell cross-priming in the absence of CD4<sup>+</sup> T-cell responses. **(a)** C57Bl/6 male mice were injected i.m. at day 0 with rAAV1-mOVA-HY or rAAV1-mOVA-HY-miR. Every 4 days, blood sample were collected and sera were analyzed by ELISA assay for the presence of anti-OVA IgG. Data represent six mice per group pooled from two independent experiments. **(b–c)** C57Bl/6 or  $\mu$ MT male mice were injected i.m. at day 0 with rAAV1-mOVA-HY or rAAV1-mOVA-HY-miR. **(b)** On day 14 or 35, mice were sacrificed and their splenocytes tested by IFN- $\gamma$  ELISPOT assay against OVA<sub>257</sub> peptide. Data represent six mice per group per time point pooled from two independent experiments. **(c)** On day 35, tibialis anterior muscles were frozen and 7  $\mu$ m thick transversal cryosections were stained with hematoxylin and eosin. Field size is 720  $\times$  720 micron. One representative muscle out of three is shown. **(d)** C57Bl/6 male mice were injected i.m. with rAAV1-mOVA-HY-miR and coinjected i.v. with 100  $\mu$ l of serum from naive or OVA-immunized mice. On day 16, mice were sacrificed and their splenocytes tested by IFN- $\gamma$  ELISPOT assay the OVA<sub>257</sub> peptide. Data represent six mice per group pooled from two independent experiments. ND, not detected.



**Figure 5** Inhibition of direct presentation prevents CD8<sup>+</sup> T-cell priming to cytosolic transgene devoid of functional MHC class II epitope. **(a)** Secreted sOVA construct comprises the full-length OVA cDNA while cytoplasmic cOVA construct is deleted from 126 amino acids at the Cter OVA cDNA. For both constructs, additional constructs bearing four repeats of the miR142.3p target sequence in the 3' untranslated region were generated. **(b-d)** C57Bl/6 female mice were injected i.m. at day 0 with rAAV1-cOVA, rAAV1-cOVA-miR, rAAV1-sOVA, or rAAV1-sOVA-miR. **(b)** At day 14 or 35, mice were sacrificed and their splenocytes tested by IFN- $\gamma$  ELISPOT against the OVA<sub>257</sub> peptide. Data represent six mice per group per time point pooled from two independent experiments. ND, not detected. **(c)** At day 14 or **(d)** day 35, tibialis anterior muscles from mice injected with rAAV1-cOVA or rAAV1-cOVA-miR were frozen and 7  $\mu$ m thick transversal cryosections were stained with hematoxylin and eosin. Field size is 720  $\times$  720 micron and inserts size is 300  $\times$  300 micron. One representative muscle out of three is shown. **(e)** Rag2<sup>-/-</sup> or C57Bl/6 female mice were injected i.m. at day 0 with rAAV1-cOVA or rAAV1-cOVA-miR. On day 35, tibialis anterior muscles from sacrificed mice were removed and OVA mRNA was quantified by Q-RT-PCR. Data represent four mice per group. One of two different experiments is shown.

transgene appeared spared from cellular infiltration, in contrast with those expressing the mOVA-HY transgene (Figure 4c). It is also interesting to note that the impact of CD4<sup>+</sup> T-cell help on the magnitude of antitransgene CTL response in this model was mostly independent from B-cell responses, as shown by the strong CD8<sup>+</sup> T-cell responses observed in  $\mu$ MT female mice against both transgenes from day 14 (data not shown).

Next, to assess whether antitransgene antibodies could indeed enhance the priming of CD8<sup>+</sup> T-cell against cross-presented epitopes in the absence of helper T-cell responses, we performed passive antitransgene antibodies transfer

experiments. Male B6 mice were thus coinjected i.m. with the rAAV1-mOVA-HY-miR vector and i.v. with serum from either naïve (naïve serum) or OVA/IFA-immunized (OVA serum) mice. As expected, male mice injected with naïve serum did not elicit any detectable anti-OVA<sub>257</sub> CD8<sup>+</sup> T-cell response by day 16 (Figure 4d), whereas a strong anti-OVA<sub>257</sub> CD8<sup>+</sup> T-cell response was observed in OVA serum-injected male mice. Altogether, these data demonstrate that antitransgene B-cell responses trigger the initiation of delayed CD8<sup>+</sup> T-cell responses whenever the transgene is not expressed in APC and lacks functional MHC II epitopes.

## Impact of transgene subcellular localization on CD8<sup>+</sup> T-cell response

Overall, our results indicate that both CD4<sup>+</sup> T-cell help and anti-transgene antibodies are sufficient to trigger functional CD8<sup>+</sup> T-cell responses against miR142-3pT-regulated transgenes. Thus, miR142-3pT-regulated transgene devoid of major MHC class II epitope and poorly accessible to antibodies should not trigger functional CD8<sup>+</sup> T-cell responses in immunocompetent mice. To test this hypothesis, we produced four additional rAAV1 constructs encoding for cytoplasmic (cOVA) and secreted (sOVA) forms of the full-length ovalbumin, with or without miR142-3pT sequences (Figure 5a). Based on our above results with the membrane form of OVA (Figure 2), these two new OVA constructs are devoid of functional MHC class II epitope. Additionally, while the secreted form of OVA is readily accessible for antibody binding, the cytoplasmic OVA transgene should avoid antibody-mediated detection. Accordingly, we detected no OVA-specific antibodies in rAAV1-cOVA-miR injected mice by day 14, in contrast to rAAV1-cOVA, -sOVA, and -sOVA-miR injected mice (data not shown). Next, we analyzed anti-OVA<sub>257</sub> CD8<sup>+</sup> T-cell responses in these mice. While the cOVA construct generated a CD8<sup>+</sup> T-cell response similar in strength and kinetics to that observed against mOVA transgene devoid of functional MHC II epitope, no response occurred after injection of rAAV1-cOVA-miR vector at all time points considered (Figure 5b). This was correlated with an absence of detectable infiltrates in transduced muscles at day 14 (Figure 5c) and, at day 35, muscles were still intact and spared from any infiltration whereas muscles injected with rAAV1-cOVA harbored numerous centro-nucleated muscle fibers indicative of an active repair and regeneration process (Figure 5d). Importantly, quantifying OVA mRNA by Q-RT-PCR, we found that cOVA mRNA expression was identical at day 35 in immunocompetent and Rag2<sup>-/-</sup> mice injected with rAAV1-cOVA-miR vector in contrast to significant difference between immunocompetent and Rag2<sup>-/-</sup> B6 mice receiving rAAV1-cOVA (Figure 5e). Finally, rAAV1-sOVA-miR vector generated a rapid CD8<sup>+</sup> T-cell response consistent, with the presence of anti-OVA antibodies (Figure 5b) leading to muscle destruction (Figure 5c).

## DISCUSSION

Combining the use of different cell-associated forms of a model transgene encoding for multiple MHC class I and II epitopes and the careful dissection of transgene-specific CD8<sup>+</sup> T-cell priming after rAAV-mediated gene transfer in muscle, we delineated three key features regulating the immunogenicity of a transgene: (i) the presence of immunodominant MHC I epitope, (ii) the presence of MHC II epitope and (iii) the accessibility of the transgene to antibody-mediated recognition. Moreover, we found that cross-priming depends on the intrinsic immunogenicity of the transgene and this can alter the efficiency of the miR142-3pT immunoregulation strategy used to avoid antitransgene response.

Indeed, the most surprising result in our study came from the initial observation that very efficient antitransgene CD8<sup>+</sup> T-cell responses could be primed in the absence of direct presentation by APC. Early comparison of antitransgene immune responses triggered by rAd- and rAAV-mediated gene transfers suggested that transgene expression in APC was a necessary step for the

induction of transgene-specific immune responses.<sup>25</sup> On the other hand, suboptimal activation of CD8<sup>+</sup> T cells directly on non-hematopoietic cells after rAAV2-OVA delivery in the liver,<sup>38</sup> or cross-priming of CD8<sup>+</sup> T cells after irradiation and bone-marrow reconstitution of mice injected in the muscle 9 weeks earlier<sup>32</sup> have been shown. In line with the initial view, the use of tissue-specific promoters<sup>27-29</sup> and more recently the addition of 3' targets for endogenous microRNA<sup>30,39</sup> confirmed the impact of antigen-expression in APC on antitransgene responses. Aside from the transgene itself, two other parameters could explain our results: the target tissue and the vector used. First, we choose the tibialis anterior muscle as a target tissue, an organ notably less tolerogenic than the liver<sup>13,40</sup> used in the original miR142.3pT studies by Naldini and coll.<sup>30,39</sup> The use of a rAAV1 vector instead of a lentiviral vector<sup>30,39</sup> introduce a second potential variable, notably due to the known B-cell and CD8<sup>+</sup> T-cell responses directed against the rAAV capsids.<sup>12</sup> A prohibitive effect of either variable, however, is unlikely as miR-142-3p-mediated regulation was shown to promote tolerance to a muscle-targeted rAAV1-encoded transgene bearing little mutational differences with the host,<sup>31</sup> a result that we reproduced in our study with the cytosolic OVA construct.

In agreement with our results, the sole use of microRNA target sequences was recently proven insufficient to promote long-term tolerance of two clinically relevant neo-antigens, muscle-targeted human  $\alpha$ -sarcoglycan (SGCA) in *sgca*<sup>-/-</sup> dystrophic mice<sup>31</sup> and liver-targeted  $\alpha$ -L-iduronidase.<sup>41</sup> Tissue-specific promoters have similarly demonstrated mixed results on the regulation of transgene-specific antibody<sup>42,43</sup> and cellular responses,<sup>39</sup> attributed at that time to the potential leakiness of such tissue-restricted promoters.<sup>44</sup> These studies, as well as our present results, clearly suggest that efficient cross-presentation of transgene-derived epitopes acquired from transduced tissues is sufficient to prime a functional antitransgene CTL response, as previously observed for muscle-targeted  $\beta$ -galactosidase.<sup>32</sup> The broad spectrum of impact of miR-142-3p-mediated regulation on antitransgene CTL responses described in this study, from no detectable impact in female immunized with a mOVA-HY construct, partial effect in male injected with a mOVA-HY construct, to lack of detectable immune response in mice injected with a cOVA construct, demonstrate that the immunogenicity of the transgene itself is a major parameter aside from the vector used and the choice of target tissue.

Our results demonstrated that the presence of a single foreign MHC class II epitope in the transgene itself, DBY<sub>608</sub> here, controls the outcome of CD8<sup>+</sup> T-cell priming following gene transfer even in the absence of direct APC transduction. It is well known that CD4<sup>+</sup> T-cell help can greatly influence the outcome of the CD8<sup>+</sup> T-cell priming, affecting both the intensity and quality of the CD8<sup>+</sup> T-cell response being generated<sup>36,37,45</sup> and the importance of this given MHC class II epitope for CD8<sup>+</sup> T-cell priming has previously been discussed.<sup>46</sup> Consistently, strong immune responses after rAAV-mediated gene-transfer were mostly detected against model transgene harboring identified MHC class II epitopes, such as OVA,<sup>9</sup> HA,<sup>22</sup> or FIX<sup>16</sup> and not against transgene such as Luciferase, GFP, or  $\beta$ -galactosidase devoid of known MHC class II epitopes.

Another key result from our work is the demonstration that antitransgene antibodies can efficiently enhance CD8<sup>+</sup> T-cell priming against membrane-bound transgene. This is reminiscent

of the key role of autoantibodies in the regulation of self-antigen presentation<sup>35</sup> and could result from a modified clearance of antigen-Ig complexes and altered targeting of DC subtypes in muscle-draining lymph nodes and/or a direct enhancement of antigen uptake by APCs through FcγR-mediated pathways. Unlike antitransgene CD4<sup>+</sup> T-cell response, antitransgene antibodies formation was not as a prerequisite to mount an efficient immune antitransgene CTL response, as comparable CD8<sup>+</sup> T-cell priming were observed with OVA-HY constructs in both μMT and WT C57BL/6 female mice (data not shown). However, antitransgene B-cell response are of crucial importance in absence of strong CD4<sup>+</sup> T-cell help, as evidenced by the absence of detectable CD8<sup>+</sup> T-cell priming against the membrane-associated form of OVA encoded by the mOVA-HY-miR construct in B-cell deficient μMT male mice or against the cytosolic-associated form of OVA encoded by the cOVA-miR construct in WT mice. The idea that cytoplasmic transgene could evade such antibody-mediated cross-presentation is compatible with previous findings by Sarukhan *et al.* showing that β-gal, a protein normally cytoplasmic and nonimmunogenic after rAAV2-mediated intramuscular immunization, can trigger an functional CTL response when retargeted at the cell membrane.<sup>10</sup>

Finally, it is important to note that immune responses generated against each defined MHC class I epitope, and their sensitivity to miR-142-3p-mediated regulation, can be extremely different. The magnitude of the CD8<sup>+</sup> T-cell response against the OVA<sub>257</sub> epitope appeared ten times higher than the one against UTY<sub>246</sub>, a phenomenon referred to as immunodominance.<sup>15</sup> This hierarchy was maintained independently of the nature of the antigen presentation pathway and miR-142-3p-mediated regulation only inhibited responses against the weaker of the two epitopes. In this latter case, we suggest that differences in MHC class I antigen processing and/or in the stability of subdominant epitope association with MHC class I molecules lead to a lower production of MHC-peptide complexes by the cross-presentation pathway. It is likely that the ordinarily weak response to the UTY<sub>246</sub> epitope requires an above threshold amount of peptide-MHC<sub>246</sub> complexes, which is not reached with miRNA142.3p-regulated AAV vector at day 14. The outcome in term of tolerance to the transgene itself serves as a reminder that lack of immunogenicity to a given epitope is not necessarily predictive of the global immune outcome. Given the currently limited knowledge on transgene-derived MHC I, but also MHC II, epitopes in current human clinical trials, this epitope-dependent immunogenicity suggests that T-cell assays to peptides banks are required to adequately follow antitransgene immune responses.<sup>1,2</sup>

Our study highlights a previously unappreciated importance of MHC class II epitopes and B-cell responses during the initiation of antitransgene CD8<sup>+</sup> T-cell responses resulting either from the direct or the cross presentation pathways. Our results also delineate settings in which the addition of target sequences for miR142-3p mediates long-term expression of an immunogenic transgene. The miR142-3p sequence used here is fully conserved and highly expressed in hematopoietic cells from mice to humans,<sup>47</sup> allowing translational applications in humans. All over, our study demonstrates that in addition to immune responses against rAAV vector capsids, the intrinsic immunogenicity of the

transgene is worth considering for the design of rAAV gene therapy trials and may advocate for an appropriate immunomodulatory treatment.

## MATERIALS AND METHODS

**Mice and animal experiments.** Six to eight week old C57BL/6 mice were from Janvier (Le Genest Saint Isle, France). B6.129S2-Igh-6 tm1Cgn (μMT) and *Rag2*<sup>-/-</sup> Thy1.1 OT-II mice carrying a TCR specific for OVA<sub>323</sub> bound to IA<sup>b</sup> were kind gift of Antonio Freitas and Olivier Lantz respectively. *Rag1*<sup>-/-</sup> OT-I mice carrying a TCR specific for OVA<sub>257</sub> bound to K<sup>b</sup> and CD45.1 *Rag2*<sup>-/-</sup> mice were bred in our animal facility. Mice were housed under specific pathogen-free conditions and handled in accordance with French and European directives. For intramuscular injection, mice were anesthetized and injected into the tibialis anterior with 25 μl of indicated rAAV vector (1.2 × 10<sup>10</sup> vg per mice) diluted in phosphate-buffered saline (PBS). For OT-II cell transfer, 10<sup>6</sup> cells were injected into the retro-orbital venous sinus in a final volume of 200 μl of PBS 1×. For Ovalbumin immunization, mice were challenged subcutaneously at the base of the tail with 50 μg of Ovalbumin protein emulsified in IFA (BD-Difco, Le Pont de Claix, France).

**Plasmid construction and recombinant AAV vector production.** mOVA,<sup>48</sup> cOVA,<sup>49</sup> and sOVA<sup>50</sup> cDNA were inserted by PCR in pSMD2 rAAV2 plasmid between the hPGK promoter and a polyA signal to create respectively pSMD2-mOVA, pSMD2-cOVA and pSMD2-sOVA. The pSMD2-mOVA-HY construct was generated by fusing the last coding codon of mOVA in pSMD2-mOVA to DBY<sub>608</sub> and UTY<sub>246</sub> epitopes encoding sequences encompassed respectively by 5 Nter and 15 Cter amino acids, and 4 Nter and 4 Cter amino acids of their original protein sequence to ensure normal processing. For pSMD2-mOVA-HY-mir, pSMD2-cOVA-mir, and pSMD2-sOVA-mir constructs, four repeats of the mir142.3p target sequence<sup>30</sup> were inserted in the 3' untranslated region of pSMD2-mOVA-HY, pSMD2-cOVA and pSMD2-sOVA constructs respectively. All expression cassettes were verified by sequencing.

For rAAV vector production, rAAV1-pseudotyped vectors were prepared by cotransfection in 293 cells of pSMD2 previous plasmids, pXX6 encoding adenovirus helper functions and pAAV9pITRCO2 that contains the rep and cap genes. Vector particles were purified on iodixanol gradients from cell lysates obtained 48 hours after transfection and titers were measured by quantitative real-time PCR.<sup>6</sup>

**IFNγ-ELISPOT assay.** Multiscreen nitrocellulose microplates (Millipore, Molsheim, France) were coated with antimouse IFNγ Ab (clone R4-6A2). Freshly isolated splenocytes (1.10<sup>6</sup>/well and serial dilutions) were cultured in complete RPMI 1640 medium supplemented with 10% fetal calf serum (CM) with or without 1 μmol/l of indicated peptides. OVA<sub>257</sub> (SIINFEKL), OVA<sub>323</sub> (ISQAVHAAHAEINEAGR), UTY<sub>246</sub> (WMHHNMDLI), and DBY<sub>608</sub> (NAGFNSNRANSSRSS) peptides were synthesized by Proteogenix (Oberhausbergen, France). For each assay, Concanavalin A was added (5 μg/ml) as a positive control. After 20 hours, plates were incubated 2 hours with biotinylated antimouse IFNγ (clone XMG1.2) and 1.5 hours with alkaline phosphatase-conjugated streptavidin (Roche, Mannheim, Germany). Spots were developed by adding peroxidase substrates, 5-bromo-4,3-indolyl phosphate and nitroblue tetrazolium (Promega, Madison, WI) and counted using an AID reader (Autoimmun Diagnostika). Spot forming units (SFU) are represented after subtraction of background values obtained with unpulsed splenocytes (always below 10 spots/well).

**Quantification of OVA mRNA in transduced muscles.** For quantification of OVA mRNA, 100 ng of total RNA were reverse transcribed using iScript DNA Synthesis Kit (Biorad, Marnes-la-Coquette, France). Then, 2 μl of cDNA were subjected to real-time PCR amplification using Ova-F (5'-AAGCAGGCAGAGAGGTGGTA-3'), Ova-R (5'-GAA TGGATGGTCAGCCCTAA-3'), β-actin-F (5'-AAGATCTGGCACCACA CCTTCT-3'), and β-actin-R (5'-TTTTCACGGTTGGCCTTAGG-3')



primers. For all reaction mixtures, 10 µl of Fast Start Universal SYBR green mastermix (Roche, Boulogne-Billancourt, France) was used in a final volume of 20 µl, OVA primers were used at 500 nmol/l and β-actin primers at 400 nmol/l. The absolute amount of OVA mRNA for each sample was then normalized against the β-actin mRNA amount (arbitrary units) and determined using serial dilutions of pAAV-CMV-OVA plasmid and β-actin purified PCR product.

**Fluorescence-activated cell sorting analysis.** All reagents used for flow cytometry were purchased from BD-Biosciences (Le Pont de Claix, France). For PBL staining, erythrocytes were eliminated by hypotonic shock with PharmLysis buffer. Cell suspensions were first incubated with anti-FcγRIII/II (2.4G2) mAb for 15 minutes at 4 °C and then stained for 30 minutes at 4 °C in PBS 1× BSA 0.1% with saturating amounts of combinations of the following mAbs: FITC anti-CD4, PE anti-CD69, Pacific Blue anti-CD8, and APC-Cy7 anti-CD45.2. Dead cells were excluded using 7-actinomycin D (Sigma-Aldrich, Lyon, France) or LIVE/DEAD Fixable Near-IR Dead Cell Stain Kit (Invitrogen, Saint-Aubin, France). Flow cytometric analysis was performed on a BD FACScalibur or FACSCanto II flow cytometer (BD Biosciences). Data were analyzed using FlowJo (Tree Star) software.

**Anti-OVA IgG ELISA.** ELISA microtiter plates (Nunc.) were coated overnight with 50 µl per well of a 10 µg/ml dilution of OVA protein (Sigma-Aldrich) in carbonate buffer pH 9.5. Plates were then extensively washed and then blocked for 2 hours with PBS 1×-BSA 1%-milk 5%. Serial dilutions of experimental sera as well as of a reference serum from an OVA protein-immunized mice, were diluted in PBS 1×-BSA 1% and incubated for 2 hours at room temperature. Bound anti-OVA IgG were detected with HRP-conjugated goat antimouse IgG (Vector Laboratories, Eurobio, Les Ulis, France) and revealed with TMB substrate reagent set (BD Biosciences). The reaction was stopped after 3–5 minutes with 2 mol/l H<sub>2</sub>SO<sub>4</sub> and the absorbance at 450 nm was determined. All sera were tested in duplicate and antibodies levels are represented as a ratio of the reference serum (arbitrary units).

**Statistical analysis.** All data are shown as mean ± SEM. For all statistical analyses, an unpaired *t*-test was performed. Data were considered significant when *P* values were <0.05. (NS) nonsignificant, \**P* < 0.05 and \*\*\**P* < 0.001.

## SUPPLEMENTARY MATERIAL

**Figure S1.** Response of OT-II and Marilyn transgenic T cells to mOVA-HY transgene.

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