

# miRNA-mediated Silencing in Hepatocytes Can Increase Adaptive Immune Responses to Adenovirus Vector-delivered Transgenic Antigens

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Adenovirus vectors based on human serotype 5 can induce potent CD8 T cell responses to vector-encoded transgenic antigens. However, the individual contribution of different cell types expressing antigen upon adenovirus vector injection to the generation of antigen-directed adaptive immune responses is poorly understood so far. We investigated the role of hepatocytes, skeletal muscle, and hematopoietic cells for the induction of cellular and humoral immune responses by miRNA-mediated tissue-specific silencing of antigen expression. Using hepatitis B small surface antigen (HBsAg) as the vector-encoded transgene we show that adenovirus vector dissemination from an intramuscular (i.m.) injection site into the liver followed by HBsAg expression in hepatocytes can limit early priming of CD8 T cells and the generation of anti-HBsAg antibody responses. However, hepatocyte-specific miRNA122a-mediated silencing of HBsAg expression overcame these limitations. Early clonal expansion of K<sup>b</sup>/S<sub>190-197</sub>-specific CD8 T cells was significantly enhanced and improved polyfunctionality of CD8 T cells was found. Furthermore, miRNA122a-mediated antigen silencing induced significantly higher anti-HBsAg antibody titers allowing an up to 100-fold vector dose reduction. These results indicate that miRNA-mediated regulation of antigen expression in the context of adenovirus vectors can significantly improve transgene product-directed immune responses. This finding could be of interest for future adenovirus vaccine vector development.

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## INTRODUCTION

Adenovirus (Ad) vectors based on human adenovirus serotype 5 belong to the most immunogenic genetic vaccine vectors available to date. They are considered highly valuable tools to generate efficacious preventive or therapeutic vaccines against severe life-threatening diseases including HIV/AIDS, malaria, and tuberculosis.<sup>1</sup> However, data from the recently failed STEP study<sup>2</sup> strongly suggest that comprehensive understanding of both transgene

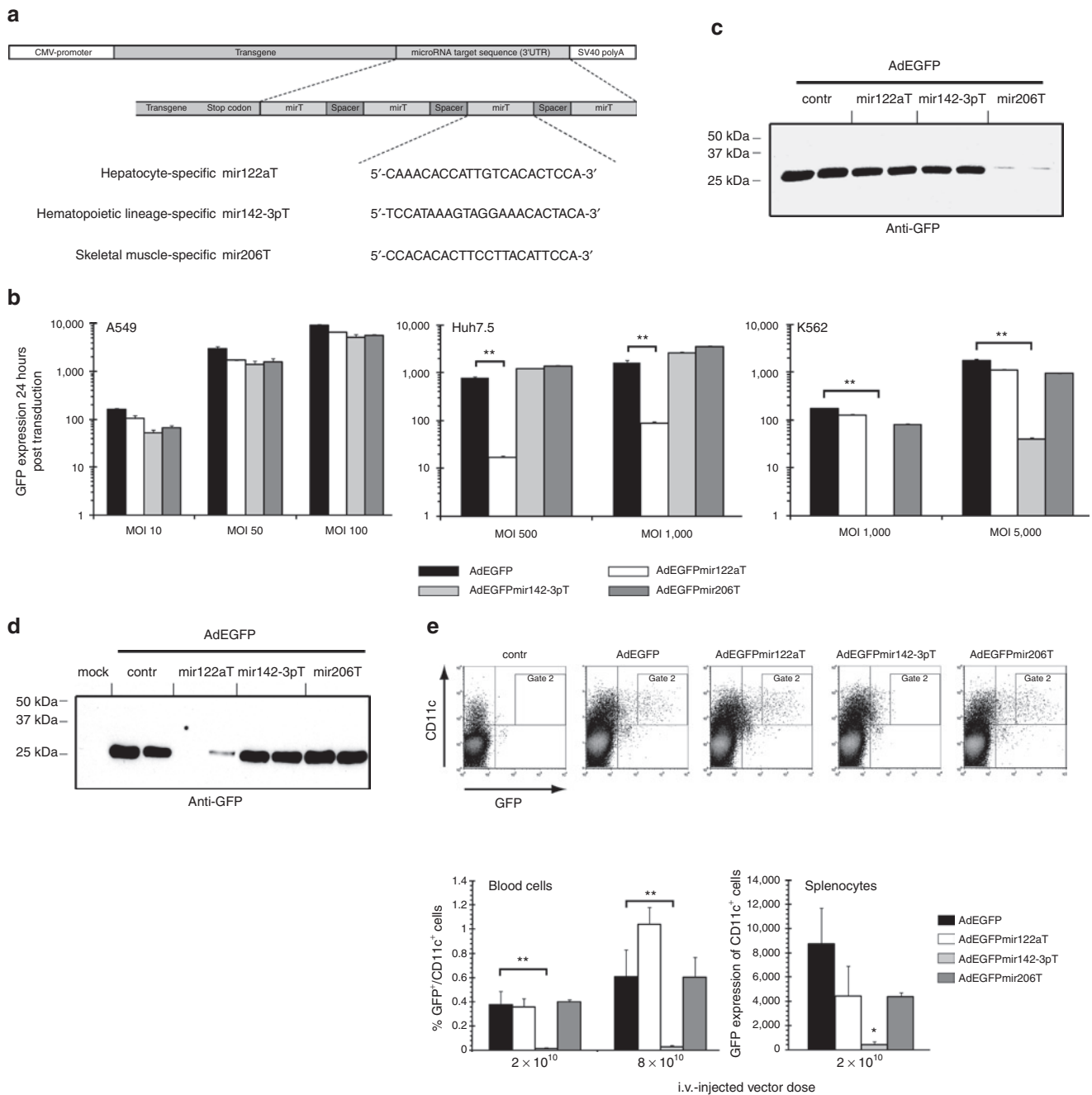
product-directed and, importantly, vector-directed immunity is paramount for the endeavor to develop efficacious genetic vaccines based on adenovirus.<sup>3</sup> It is generally agreed upon that transgene product-directed and vector-directed immunity can only be understood and beneficially modulated when basic questions addressing *in vivo* vaccine vector biology are thoroughly investigated.

Adenovirus-based vectors can transduce a wide variety of different cell types both *in vitro* and *in vivo*. *In vitro* transduction is well investigated and mainly depends on interactions of the vectors with the coxsackie and adenovirus receptor and  $\alpha_v\beta_{3/5}$  integrins.<sup>4,5</sup> However, the mechanisms underlying transduction upon vector injection *in vivo* are much more complex. For example, it has been shown that the strong hepatocyte tropism of Ad5-based vectors depends on a blood coagulation factor-mediated bridging mechanism that tethers the Ad5 capsids to cell surface receptors on hepatocytes.<sup>6</sup> Importantly, vector dissemination to hepatocytes has been observed after local intramuscular (i.m.) vector injection<sup>7,8</sup>—the most common route for vaccine vector delivery.

Presumably owed to the complexity of the transduction patterns *in vivo*, only very little is known about the individual contribution of different Ad-transduced cell types for induction and maintenance of transgene product-directed cellular and humoral immune responses. Even the role of a potential direct transduction of professional hematopoietic antigen-presenting cells by Ad5-based vectors has been a matter of controversy.<sup>9-11</sup> Only very recently, Bassett *et al.* demonstrated that both hematopoietic and nonhematopoietic antigen-presenting cells are required for the maintenance of the typical Ad vector-induced CD8<sup>+</sup> T cell memory phenotype.<sup>12</sup> Finally, approaches to improve humoral transgene product-directed responses have so far been based on the use of different vaccination routes,<sup>13-15</sup> but the precise role of different vector-transduced nonhematopoietic cells in this process has hardly been elucidated.

The aim of this study was to investigate the influence of different cell types on the induction of transgene product-directed humoral and cellular immune responses after Ad vector vaccination employing cell-type specific silencing of transgene expression. We focused on cells of hematopoietic origin, skeletal muscle cells, and hepatocytes since these are the predominantly transduced

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**Figure 1** *In vitro* and *in vivo* expression profiles of miRNA-regulated adenovirus (Ad) vectors. **(a)** Schematic representation of miRNA-regulated expression cassettes inserted into E1-deleted adenoviral vectors. **(b)** miRNA-target sequences specifically downregulate Ad vector-delivered transgenes *in vitro*. A549, Huh7.5, and K562 cells were transduced with indicated pMOI of EGFP-expressing miRNA-regulated Ad vectors or control vector without mirT (AdEGFP) in a 24-well plate format. EGFP expression was quantified after 24 hours by flow cytometry as  $(\text{GMean}_{\text{transduced}} \times \% \text{GFP}^+ \text{ cells}_{\text{transduced}}) - (\text{GMean}_{\text{untransduced}} \times \% \text{GFP}^+ \text{ cells}_{\text{untransduced}})$ . The mean values ( $\pm$  SD) of one out of three independent experiments are shown. **(c)** Differentiated C2C12 myotubes were transduced with 2,000 pMOIs of EGFP-expressing miRNA-regulated Ad vectors or control vector (AdEGFP). 48 hours post transduction cell lysates were prepared and EGFP expression was analyzed by western transfer (20  $\mu$ g protein per lane). **(d,e)** BALB/c mice (three mice per group) were injected i.v. with reporter gene expressing AdEGFPmirTs. Injected mice were sacrificed 48 hours postinjection and tissues were analyzed for EGFP expression. **(d)** EGFP expression of the different reporter gene expression vectors in livers was analyzed by western transfer of lysates of isolated hepatocytes 48 hours after i.v. delivery of  $2 \times 10^{10}$  Ad VP. EGFP expression was strongly reduced in hepatocytes of mice injected with AdEGFPmir122aT. Lysates of two out of three injected mice per vector are shown. **(e)** EGFP expression in CD11c<sup>+</sup> cells of hematopoietic lineage is downregulated by mir-142-3p. Isolated blood cells and splenocytes of mice injected i.v. with indicated doses of AdEGFPmirTs were stained for CD11c and analyzed for EGFP expression by flow cytometry. Representative dot plots of blood cells are shown (upper panels). The mean percentages ( $\pm$  SD) of GFP<sup>+</sup>/CD11c<sup>+</sup> blood cells in gate 2 for two different vector doses are presented in the lower left panel. The mean GFP expression ( $\pm$  SD) of splenocytes (lower right panel) was calculated as  $(\text{events GFP}^+ \text{CD11c}^+_{\text{injected}} \times \text{Gmean of GFP}^+ \text{CD11c}^+_{\text{injected}}) - (\text{events GFP}^+ \text{CD11c}^+_{\text{uninjected}} \times \text{Gmean of GFP}^+ \text{CD11c}^+_{\text{uninjected}})$ . Significance levels of data were calculated by unpaired Student's *t*-test. \**P* < 0,05; \*\**P* < 0,01. CMV, cytomegalovirus; contr, control; EGFP, enhanced green fluorescence protein; i.v., intravenously; mirT, microRNA target sequence; pMOI, particle multiplicity of infection; SV, simian virus; UTR, untranslated region; VP, vector particles.

cell types after i.m. Ad vaccine vector delivery. In order to selectively silence transgene expression in skeletal muscle, hepatocytes, and cells of hematopoietic origin we relied on miRNA-mediated tissue-specific silencing, a technology that has been described in the context of lentivirus vectors. Brown *et al.* demonstrated that the incorporation of target sequences with perfect complementarity to tissue-specific endogenous miRNAs into the 3'-untranslated region (3'UTR) of a transgene leads to tissue-specific translational repression and degradation of the transgene messenger RNA.<sup>16</sup> In the context of Ad vectors only hepatocyte-specific mir-122a has been used to control liver toxicity of a suicide gene<sup>17</sup> or pathology of wild-type adenovirus<sup>18,19</sup> and so far this technology has not been explored for Ad vector silencing in skeletal muscle cells or cells of hematopoietic origin or ultimately for the modulation of transgene product-directed immune responses.

Our data show that antigen silencing in hepatocytes could significantly enhance early priming of CD8 T cells specific for an epitope generated only by the endogenous processing pathway. Independent of the antigen processing pathways cytokine polyfunctionality of specific CD8 T cells was generally improved by hepatocyte-specific antigen silencing. In contrast to published studies with lentivirus vectors,<sup>20</sup> silencing in cells of hematopoietic origin did not induce immunological tolerance towards the transgene products, presumably due to the strong adjuvant effect of first-generation Ad vectors or cross-priming mechanisms. Silencing of transgene expression in hepatocytes also induced significantly increased anti-transgene product-directed antibody levels in two different mouse strains. This silencing allowed for an up to 100-fold reduction of the vector dose compared to a non-regulated Ad vector to achieve the same specific antibody titer. Our findings thus exemplify the need for precise tissue control of Ad vaccine vectors, contribute to a better understanding of Ad vaccine vector immunobiology and may have direct impact on the development of safe and efficacious Ad vector-based vaccines.

## RESULTS

### miRNA-regulated Ad vectors are specifically and efficiently silenced in various cell types *in vitro* and *in vivo*

To characterize miRNA-mediated tissue-specific silencing of transgene expression *in vitro* and *in vivo* we constructed a set of miRNA-regulated Ad vectors bearing expression cassettes for enhanced green fluorescent protein (AdEGFPmirTs) with insertions in their 3'UTRs of none (control) or four tandem repeats of tissue-specific miRNA target sequences with perfect complementarity (Figure 1a and Table 1).<sup>21-23</sup>

Flow cytometric quantification of EGFP expression after transduction of lung-derived A549 cells, which do not express

any miRNA with complementarity to the employed target sequences revealed equal expression levels for all vectors. In contrast, AdEGFPmir122aT was specifically silenced in hepatoma-derived Huh7.5 cells (up to 80-fold) and AdEGFPmir142-3pT was specifically silenced in myelogenous leukemia-derived K562 cells (up to 200-fold) (Figure 1b). In addition, specific silencing of AdEGFPmir142-3pT was corroborated in primary human macrophages differentiated from peripheral blood mononuclear cells (Supplementary Figure S1a,b). Western blot analysis and fluorescence microscopy revealed specific and nearly quantitative silencing of AdEGFPmir206T in differentiated C2C12 myotubes (Figure 1c, Supplementary Figure S1c) but not in undifferentiated myoblasts (data not shown).

To demonstrate silencing *in vivo*, Ad vectors were intravenously (i.v.) injected into BALB/c mice and 48 hours later EGFP expression was quantified in liver (Figure 1d), blood cells, (Figure 1e, upper panels and lower left panel) and spleen (Figure 1e, lower right panel). Injection of AdEGFP (control without target sequences), AdEGFPmir142-3pT, and AdEGFPmir206T resulted in strong EGFP expression in hepatocytes. In contrast, AdEGFPmir122aT showed no or very little EGFP content in isolated hepatocytes (Figure 1d), indicating efficient hepatocyte-specific silencing of this vector *in vivo*. In agreement with the results obtained in primary human macrophages (Supplementary Figure S1b) analysis of blood cells revealed that injection of AdEGFPmir142-3pT did not result in EGFP expression in CD11c<sup>+</sup> blood cells (Figure 1e, upper panels and lower left panel), even not at a fourfold higher dose of  $8 \times 10^{10}$  VP. This vector was also silenced in splenocytes (Figure 1e, lower right panel).

Analysis of muscle cryosections after i.m. vector injection demonstrated silencing only for AdEGFPmir206T whereas the other vectors induced strong EGFP expression in muscle fibers around the injection site (Supplementary Figure S1d).

Taken together, these results demonstrated the feasibility of miRNA-mediated cell-type specific silencing of Ad vectors in hepatocytes, splenocytes and CD11c<sup>+</sup> cells, and skeletal muscle *in vivo*.

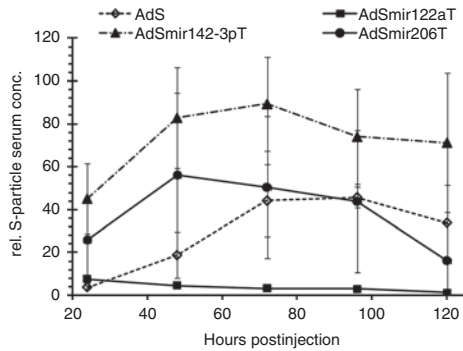
### Secreted systemically circulating serum HBsAg is mainly derived from hepatocytes after i.v. Ad vector administration

To assess the effects of miRNA-mediated tissue-specific silencing on the induction of humoral and cellular immune responses against Ad-encoded transgene products in a vaccination-relevant context we constructed a set of miRNA-regulated Ad vectors expressing the hepatitis B small surface antigen (HBsAg). This antigen which is secreted by transduced cells<sup>24</sup> provides epitopes with well-characterized immunogenicity for T and B cells.<sup>25</sup> These epitopes differ from each other in their MHC class I haplotype restriction and their intracellular processing pathway that generates the antigenic peptide.

HBsAg-encoding Ad vectors bearing no or hepatocyte-irrelevant miRNA target sequences resulted in significant and constant HBsAg serum levels for several days after i.v. injection, whereas the vector AdSmir122aT induced only minimal HBsAg serum levels close or equal to baseline (Figure 2). These data exemplarily demonstrate that miRNA-regulation is functional in the context of HBsAg and

**Table 1** Tissue-specific miRNAs and *in vitro* model cell lines

MicroRNA	Expressed in	Used as model cell line	References
miR-122a	Hepatocytes	Human Huh7.5	21
miR-206	Skeletal muscle	Murine C2C12 (differentiated to myotubes)	23
miR-142-3p	Hematopoietic-lineage cells	Human K562	22



**Figure 2** Hepatocytes are the main source of transgenic HBsAg particles after i.v. delivery of adenovirus (Ad) vectors. BALB/c mice were injected intravenously with  $2 \times 10^{10}$  VP of AdS or AdSmirTs. Blood samples were taken from the tail vein of Ad vector-injected mice at the indicated time-points and S-particle concentrations in sera were measured. The mean serum concentrations ( $\pm$  SD) of three mice per group and time-point are shown. conc., concentration; HBsAg (or: S), hepatitis B small surface antigen; rel., relative; VP, vector particles.

identify hepatocytes as the main source of secreted HBsAg after blood-borne Ad vector-mediated hepatocyte transduction.

### HBsAg-silencing in hepatocytes can enhance early priming of CD8 T cells and improves cytokine polyfunctionality

We vaccinated mice i.m. to investigate the effects of miRNA-mediated tissue-specific silencing on strength and kinetics of HBsAg-specific CD8 T cell responses. In H-2<sup>d</sup>-restricted BALB/c mice the frequencies of L<sup>d</sup>/S<sub>28-39</sub>-specific CD8 T cells were determined by intracellular IFN $\gamma$  staining and, confirming published data,<sup>26</sup> were found to be at about 1% 8 days after vaccination, rising to 4–5% at day 21 without major contraction after 35 days (Figure 3a). Importantly, magnitudes of frequencies were independent of the miRNA target sequence incorporated.

To analyze responses independent of potential immunodominance effects of the L<sup>d</sup>/S<sub>28-39</sub> epitope<sup>25</sup> we vaccinated H-2<sup>b</sup>-restricted C57BL/6 mice with the same set of vectors to determine the frequencies of tetramer<sup>+</sup> CD8 T cells specific for the subdominant K<sup>b</sup>-restricted epitope. In contrast to the L<sup>d</sup>/S<sub>28-39</sub> epitope, this epitope can only be generated by the endogenous processing pathway. Interestingly, when HBsAg expression was silenced in hepatocytes we detected fourfold higher K<sup>b</sup>/S<sub>190-197</sub>-specific CD8 T cell frequencies 8 days after vaccination. Silencing in skeletal muscle showed similar but less pronounced effects. This early clonal expansion completely contracted to equal frequencies between the individual vectors at day 35 (Figure 3b). Subsequent intracellular IFN $\gamma$  staining further corroborated this finding (data not shown). Of note, we detected the same CD8 T cell profiles and differences between AdS and AdSmir122aT locally in the liver (Figure 3c) but observed no relevant differences in L<sup>d</sup>/S<sub>28-39</sub>-specific CD8 T cells in livers of BALB/c mice (Supplementary Figure S2a). This peak response of AdSmir122aT-induced K<sup>b</sup>/S<sub>190-197</sub>-specific CD8 T cells differs markedly from the course of L<sup>d</sup>/S<sub>28-39</sub>-specific CD8 T cells which is characterized by a delayed clonal expansion and a protracted contraction phase. This kinetic was observed for all vectors independent of their miRNA-regulation.

The surprising effect of hepatocyte-specific silencing on priming of K<sup>b</sup>/S<sub>190-197</sub>-specific CD8 T cells suggested significant spreading of the vaccine vector from the i.m. injection site to the liver. Vector spreading was corroborated by fluorescence microscopic analysis of liver cryosections 48–72 hours post i.m. injection of AdEGFP (Figure 3d). Dose-dependent hepatocyte transduction was found for all vector doses tested. Detection of Ad vector genomes by quantitative PCR (qPCR) confirmed presence of vector in the liver after injection of low doses.

We further analyzed polyfunctionality and cytokine phenotype of specific CD8 T cells in spleen after *ex vivo* peptide restimulation. In BALB/c mice we detected an increase in the proportion of IFN $\gamma$ <sup>+</sup>TNF $\alpha$ <sup>+</sup>IL-2<sup>-</sup> CD8 T cells with a concomitant decrease in monofunctional IFN $\gamma$ -secreting cells after 21 days in AdSmir122aT-immunized mice (Figure 4a) suggesting a mature, polyfunctional effector phenotype of these T cells. The same increase was seen in AdSmir122aT-immunized C57BL/6 mice during the peak response after 8 days (Figure 4b). In addition, there were less IFN $\gamma$ <sup>+</sup>TNF $\alpha$ <sup>-</sup>IL-2<sup>+</sup> CD8 T cells detectable which represent a rather immature state. Interestingly, these differences were not yet present after 8 days in BALB/c mice and already disappeared after 21 days in C57BL/6 mice (Supplementary Figure S2b) suggesting the importance of this effect only during the phase of maximal clonal expansion of the CD8 T cells.

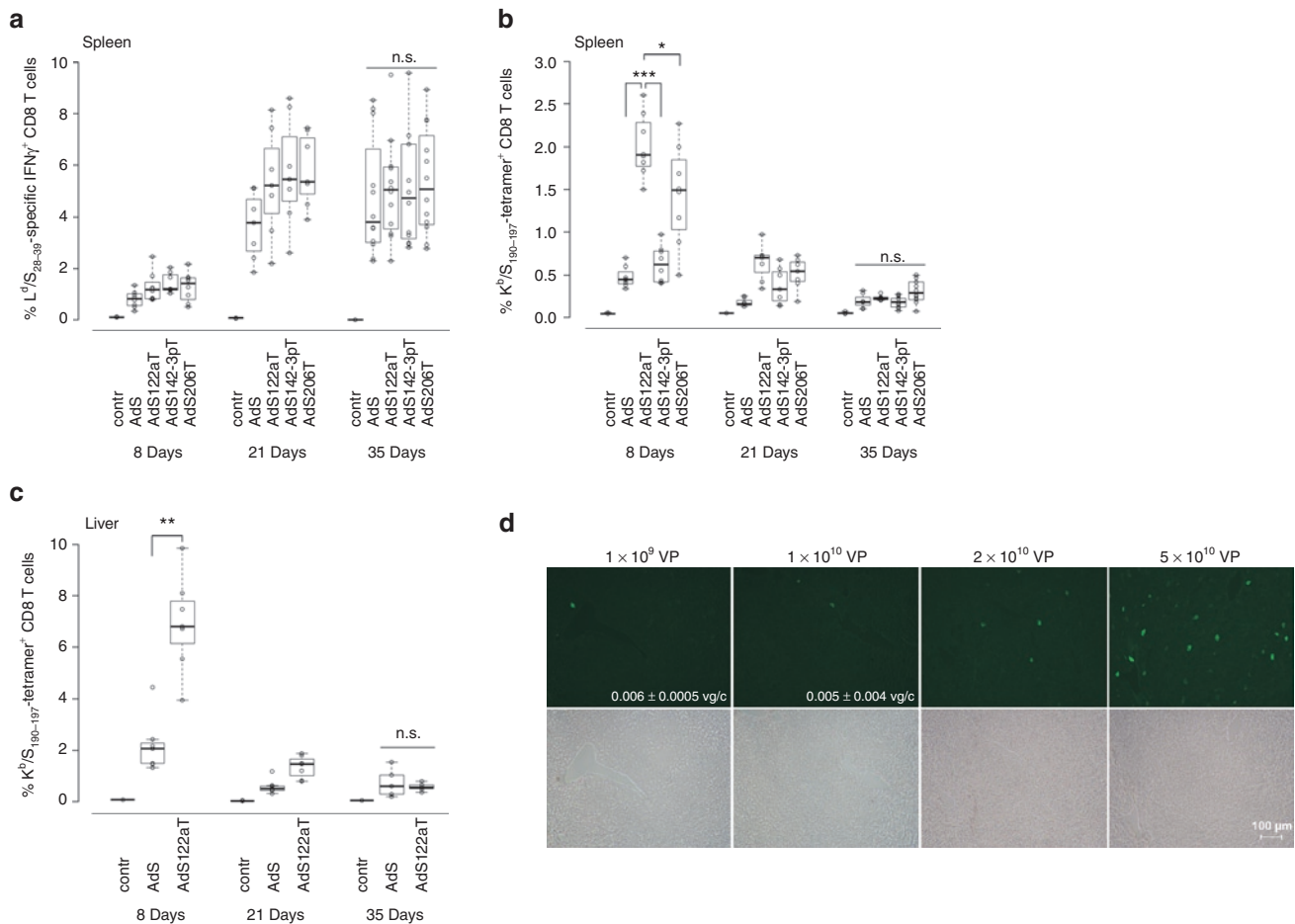
Of note, tissue-specific silencing of HBsAg expression in hematopoietic cells by mir142-3p neither prevented the induction of specific CD8 T cells nor induced an increase in the number of regulatory Foxp3<sup>+</sup>/CD25<sup>+</sup> CD4 T cells (Supplementary Figure S2c) as it has been described in the context of lentivirus vectors.<sup>27</sup>

### miRNA-mediated suppression of HBsAg expression in hepatocytes induced higher antibody levels after i.m. vaccination

We further investigated humoral immune responses in AdHBsAgmirT-immunized mice and measured anti-HBsAg antibodies in the serum (Figure 5a). Starting from day 21 post-vaccination AdSmir122aT induced higher HBsAg-specific antibody responses compared to AdS (control vector without target sequence) and AdSmir142-3pT with a significant increase of the titers at days 28 and 35. In contrast, the antibody titers after vaccination with AdS and AdSmir142-3pT stayed close to zero or increased only marginally at later time-points. Similar to the CD8 T cell responses in C57BL/6 mice we detected slightly enhanced antibody titers after vaccination also with the skeletal muscle-specific regulated vector AdSmir206T.

The same effect for AdSmir122aT was confirmed in vaccinated BALB/c mice (Figure 5b) excluding a mouse strain-dependent phenomenon. Of note, the induction of increased antibody titers by AdSmir206T was only observed at day 28 postvaccination in this mouse strain and titers did not increase further.

Based on our observations we speculated that antigen expression in hepatocytes as it is induced by dissemination of a non-regulated vector from the i.m. injection site (see Figure 3d) may suppress the generation of anti-HBsAg-directed antibody responses. To validate this hypothesis we performed a coinjection experiment in C57BL/6 mice. We injected  $10^9$  VP of AdSmir122aT into one limb and injected the other limb of the same mouse with



**Figure 3** miRNA-mediated antigen silencing in hepatocytes can increase early priming of HBsAg-specific CD8 T cells. **(a)** Kinetics of L<sup>d</sup>/S<sub>28-39</sub>-specific CD8 T cell frequencies. H-2<sup>d</sup>-restricted BALB/c mice were immunized intramuscularly with 10<sup>8</sup> VP of AdSmirTs. At the indicated time points (8d n = 8, 21d n = 7, 35d n = 12) frequencies of specific IFN $\gamma$ <sup>+</sup> CD8 T cells were determined after *ex vivo* stimulation of splenocytes with L<sup>d</sup>/S<sub>28-39</sub> peptide in the presence of brefeldin A. Combined data of two independent experiments at time-point day 8/35 days are shown. Boxplots show the median percentages of IFN $\gamma$ <sup>+</sup> CD8 T cells. **(b)** Kinetics of K<sup>b</sup>/S<sub>190-197</sub>-specific CD8 T cell frequencies. H-2<sup>b</sup>-restricted C57BL/6 mice were immunized intramuscularly with 10<sup>9</sup> VP of AdSmirTs. At the indicated time points (8d n = 8, 21d/35d n = 7) K<sup>b</sup>/S<sub>190-197</sub>-specific CD8 T cell numbers in spleen of vaccinated C57BL/6 mice were determined by tetramer staining. Combined data of two out of three independent experiments at time point day 8 are shown. Boxplots show the median percentage of tetramer<sup>+</sup> CD8 T cells. **(c)** K<sup>b</sup>/S<sub>190-197</sub>-specific CD8 T cell frequencies in liver. C57BL/6 mice were immunized intramuscularly with 10<sup>9</sup> vector particles of AdS or AdSmir122aT. At the indicated time points nonparenchymal liver cells were analyzed for specific CD8 T cells by tetramer staining (seven mice per vector and time point). **(d)** Liver transduction after intramuscular vector injection. BALB/c mice were injected intramuscularly with 10<sup>9</sup>, 10<sup>10</sup>, 2 × 10<sup>10</sup>, or 5 × 10<sup>10</sup> VP of AdEGFP in a total volume of 100 μl with 50 μl per limb. 48–72 hours postinjection liver cryosections were analyzed by fluorescence microscopy. Annotated numbers indicate vector genome copies per cell (vg/cell) determined by quantitative PCR. P values are based on two-sided Wilcoxon test. \*P < 0.05; \*\*P < 0.01; \*\*\*P < 0.001. HBsAg (or: S), hepatitis B small surface antigen; IFN, interferon; n.s., not significant; VP, vector particles.

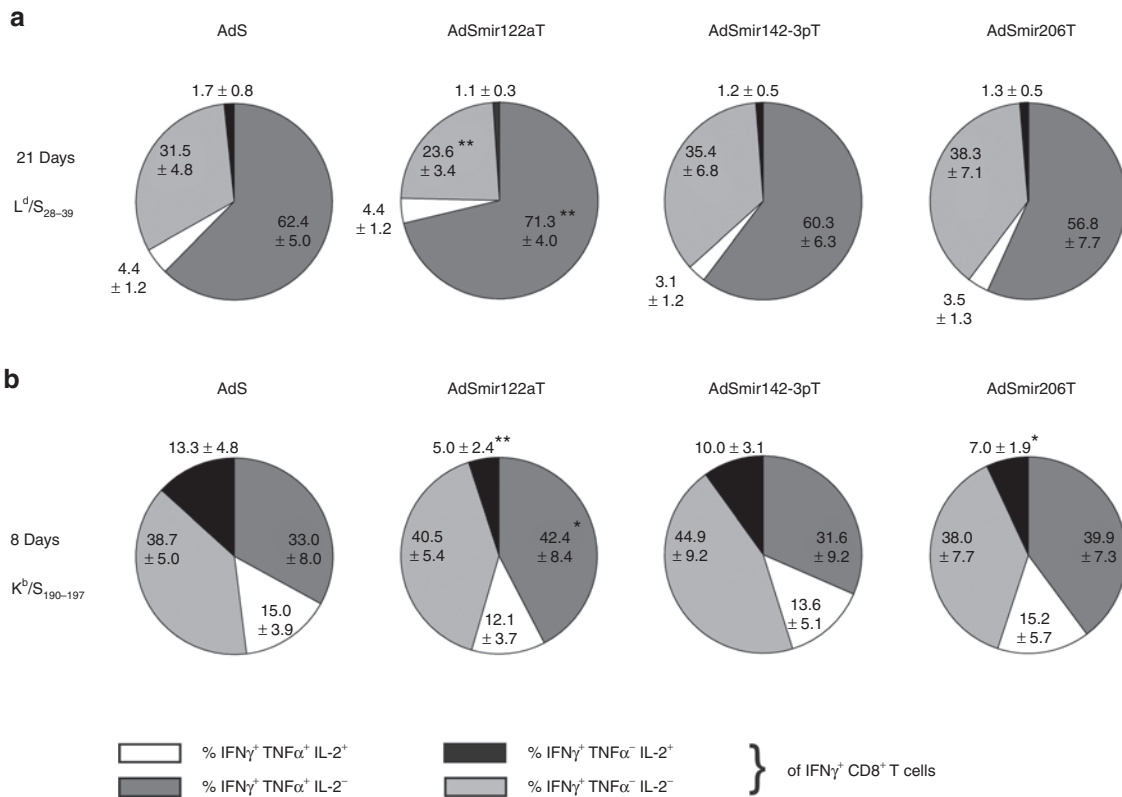
10<sup>8</sup> or 10<sup>9</sup> VP of AdS without any target sequence. Confirming our hypothesis anti-HBsAg antibody titers were significantly reduced after coinjection of both vectors AdS (10<sup>9</sup> particles) and AdSmir122aT (10<sup>9</sup> particles) when compared to those in mice that only received AdSmir122aT (Figure 6). We observed a similar picture after coinjection of only 10<sup>8</sup> AdS vector particles although differences in antibody titers were not statistically significant for this low dose. These data further suggest that the suppressive effects of nonregulated AdS on antibody responses act locally separated from the i.m. injection site and appear to be caused by systemic vector spreading.

Taken together our data indicate (i) that dose-dependent Ad vector dissemination from the injection site into liver and subsequent antigen expression in hepatocytes can counteract the generation of high level anti-HBsAg antibody immune responses, and

(ii) that miRNA-mediated hepatocyte-specific silencing of transgene expression can overcome this effect.

### Hepatocyte-specific silencing of transgenic antigen expression allowed for up to 100-fold vector dose reduction

Transgene product-specific antibody responses exhibit a clear dose–response correlation.<sup>28</sup> In order to assess whether the effects of silencing in hepatocytes allow for vector dose reduction we performed dose escalation experiments in BALB/c mice with 10<sup>8</sup>, 10<sup>9</sup>, and 10<sup>10</sup> VP of AdS (control vector without target sequence) and AdSmir122aT and determined anti-HBsAg antibody levels 35 days postvaccination (Figure 7a). Injection of 10<sup>8</sup> VP of AdSmir122aT induced higher antibody titers than injection of 10<sup>9</sup> VP of AdS (control without target sequences). Furthermore, the antibody



**Figure 4** Immunization with mir-122a-regulated adenovirus (Ad) vector results in a different cytokine phenotype of HBsAg-specific CD8 T cells. **(a)** Groups of BALB/c mice ( $n = 7$ ) were vaccinated intramuscularly with  $10^8$  VP of AdSmirTs. Twenty-one days postvaccination splenocytes were restimulated *ex vivo* overnight with L<sup>d</sup>/S<sub>28-39</sub> peptide in the presence of brefeldin A with subsequent intracellular cytokine staining of CD8 T cells for IFN $\gamma$ , TNF $\alpha$ , and IL-2. **(b)** Groups of C57BL/6 mice ( $n = 8$ , two independent experiments) were vaccinated intramuscularly with  $10^9$  VP of AdSmirTs. Eight days postvaccination splenocytes were stimulated *ex vivo* overnight with K<sup>b</sup>/S<sub>190-197</sub> peptide in the presence of brefeldin A with subsequent intracellular cytokine staining of CD8 T cells for IFN $\gamma$ , TNF $\alpha$ , and IL-2. Pie charts show the mean percentages  $\pm$  SD of multicytokine-secreting CD8 T cells of total HBsAg-specific CD8 T cells (see **Figure 3a,b**). Significance levels of data were calculated by unpaired Student's *t*-test. \* $P < 0.05$ ; \*\* $P < 0.01$ . IFN, interferon; IL, interleukin; TNF, tumor necrosis factor.

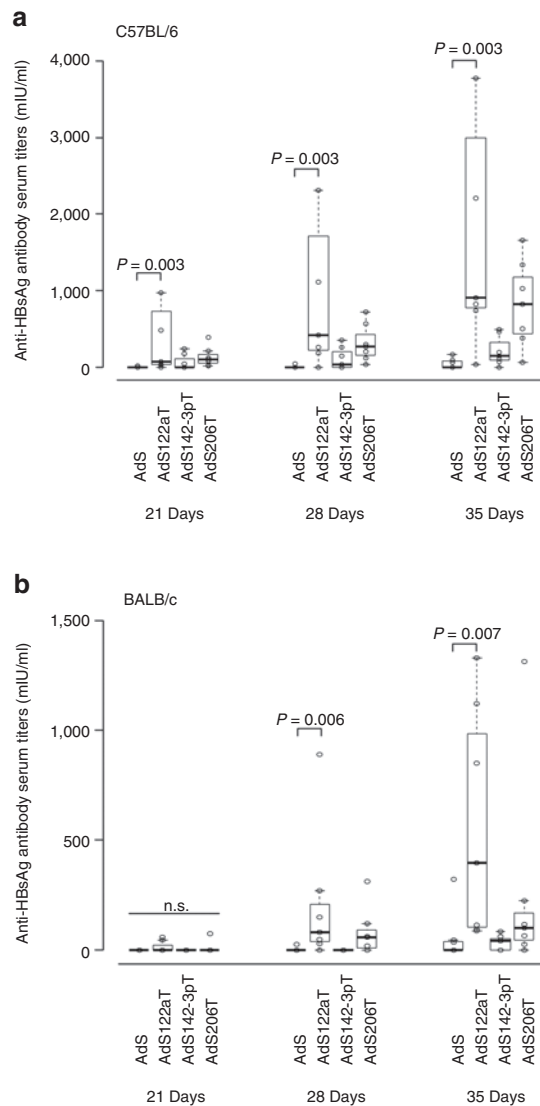
titers obtained by injection of  $10^8$  VP of AdSmir122aT were statistically not different from those obtained after injection of  $10^{10}$  VP of AdS. As a control and to verify that the mice received equal amounts of vector particles for each group we measured the anti-adenovirus antibody levels by enzyme-linked immunosorbent assay (ELISA) (**Figure 7b**). Anti-adenovirus antibody titers were equal in all groups, showed a clear dose-response correlation and thus confirmed delivery of equal vector particle numbers. These observations revealed that miRNA-mediated, hepatocyte-specific silencing of transgene expression allows for an up to 100-fold reduction of the vector dose when transgene product-directed humoral immune responses are of interest.

## DISCUSSION

In this study we exploited a miRNA-mediated silencing strategy to investigate the role of different antigen-expressing tissues for the generation of HBsAg-directed humoral and cellular immune responses in the context of adenovirus gene transfer vectors. Our data suggest that antigen expression in hepatocytes after Ad vector spreading from an i.m. injection site can hinder effective early priming of antigen-specific CD8 T cells and antagonizes the generation of humoral immune responses to the antigen. Importantly, we demonstrated that miRNA-mediated hepatocyte-specific

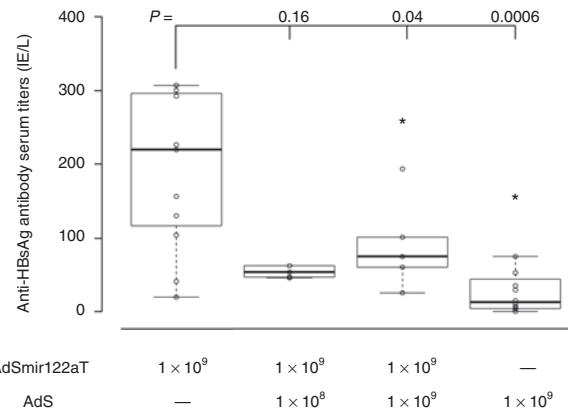
silencing of transgene expression could significantly improve the generation of antigen-specific antibody responses in two mouse strains. In addition, hepatocyte-specific silencing enhanced CD8 T cell priming with concomitant shifting of the cytokine profile to a matured, polyfunctional effector phenotype. Furthermore, our data revealed that miRNA-mediated silencing of transgene expression in cells of the hematopoietic origin did not significantly alter the frequencies of transgene product-specific CD8 T cells.

Previous studies with lentivirus vectors demonstrated that silencing of factor IX expression specifically in hematopoietic professional antigen-presenting cells by the mir142-3p target sequence effectively prevented immune-mediated transgene rejection and stably corrected hemophilia B mice.<sup>29</sup> However, Ad vectors appear to induce immunity against transgene products to a significant degree by cross priming<sup>30,31</sup> what may serve as an explanation for the ineffectiveness of mir-142-3p-mediated silencing to suppress adaptive immune responses. Dendritic cells are certainly key players for CD8 T cell responses after Ad vector immunization<sup>11,32</sup> and various approaches employing transcriptional<sup>9</sup> or transductional Ad vector targeting<sup>33-35</sup> to dendritic cells can improve adaptive immune responses to the transgene product. However, none of these studies clearly show the absolute necessity of endogenous antigen production in pAPCs to



**Figure 5** Suppression of antigen expression in hepatocytes enhances anti-HBsAg antibody responses of adenovirus (Ad) vector-based genetic vaccines. Female C57BL/6 (**a**) or BALB/c (**b**) mice were immunized intramuscularly with  $10^9$  (**a**) or  $10^8$  (**b**) AdSmirT VP (seven mice per group). Blood samples were taken at days 21, 28, and 35 after vaccination. Serum was prepared and analyzed for anti-HBsAg antibody titers. Boxplots show the median of HBsAg-specific antibody titers. *P* values are based on two-sided Wilcoxon test. For day 35 one out of two (**b**) or three (**a**) independent experiments are shown. HBsAg (or: S), hepatitis B small surface antigen; VP, vector particles.

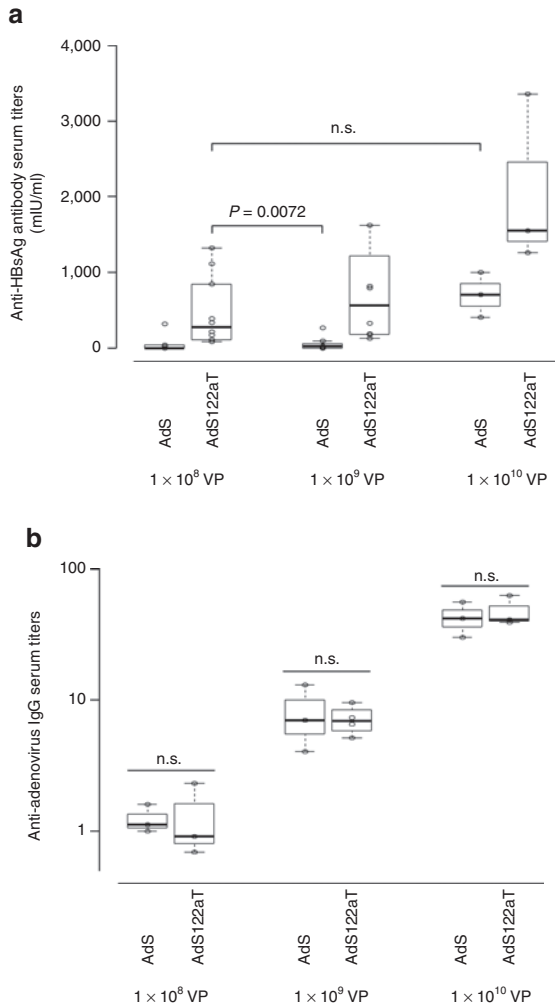
generate an adaptive immune response. Our data rather suggest that endogenous antigen synthesis in pAPCs is not per se required. Instead, other cell types might be able to present antigen and prime CD8 T cells. It has been reported for Ad vectors that endothelial cells, myoblasts and even muscle stem cells can act as APCs.<sup>10,36</sup> Regarding mir-142-3p-based silencing strategies studies based on plasmid-mediated gene transfer<sup>37</sup> and our data show that the immunological outcome is highly dependent on the gene transfer vector system and other variables. Incorporation of mir-142-3p does therefore not provide an all-purpose-tool in the development of gene transfer vector systems to prevent immune responses *e.g.*, when long term gene transfer is desired.



**Figure 6** Coinjection of ubiquitously expressing adenovirus (Ad) vector partly counteracts the effects of hepatocyte-silenced Ad vector on antibody titers. Groups of C57BL/6 mice were vaccinated intramuscularly with  $10^9$  VP of AdSmir122aT in the right limb and coinjected with  $10^8$  VP ( $n = 5$ ) or  $10^9$  VP ( $n = 5$ ) of AdS in the left limb. Control groups received a single injection of  $10^9$  VP of AdSmir122aT ( $n = 11$ ) or AdS ( $n = 12$ ). Five weeks postvaccination serum was analyzed for anti-HBsAg antibody titers by enzyme linked immunosorbent assay. Boxplots show the median with 25% and 75% percentiles. *P* values are based on one-sided Wilcoxon test. HBsAg (or: S), hepatitis B small surface antigen; VP, vector particles.

The hepatic environment as a site of antigen presentation has been associated with immunological tolerance in many reports<sup>38</sup> and active targeting of transgene expression to hepatocytes has been used to induce immunological tolerance in the context of adeno-associated virus- and lentivirus vector-mediated gene transfer.<sup>39–41</sup> Annoni *et al.* further showed that miRNA-mediated suppression of antigen expression in hematopoietic cells promotes tolerance to the transgene only with concomitant antigen expression in hepatocytes.<sup>27</sup> Gene transfer approaches using Ad vector-based systems with reduced immunogenicity (E1/E3-deleted and helper-dependent vectors) revealed that active tolerance to human factor IX and human  $\alpha 1$  antitrypsin can be achieved after *i.v.* delivery to immunocompetent mice suggesting a predominant role for hepatocyte transduction in this process.<sup>42,43</sup>

In line with this, our data indicate that also in a vaccination context hepatocyte transduction after *i.m.* delivery limits the generation of an adaptive immune response. It has been shown that *i.v.* immunization with Ad vectors results in a functional exhausted phenotype that directly correlated with persistent antigen expression in liver.<sup>44</sup> This supports our finding that HBsAg-silencing in hepatocytes improves the polyfunctionality of CD8 T cells and favors a mature effector phenotype. In the same study peripherally expressed antigen was associated with specific deletion of CTLs. This fits to our observations that also muscle-specific antigen silencing conferred some beneficial effects for CD8 T cell priming and antibody responses. A detailed analysis of vaccination route and dose by Holst *et al.* further demonstrated that the reduced quality of Ad vector-induced CD8 T cells is directly connected to excessive Ad vector dissemination from a peripheral site to the liver or systemic vector administration.<sup>45</sup> We show here that already minimal vector dissemination from muscle to the liver negatively impacts on both cellular and humoral immune responses and this can be overcome by miRNA-regulation. In



**Figure 7** miRNA-regulated adenovirus (Ad) vector-induced humoral immune responses at low doses are superior to those of nonregulated Ad vector at higher doses. **(a)** anti-HBsAg antibody responses to increasing doses of Ad vector. BALB/c mice were immunized intramuscularly with  $10^8$  ( $n = 10$ ),  $10^9$  ( $n = 7-8$ ), or  $10^{10}$  ( $n = 3$ ) VP of AdS or AdSmir122aT, respectively. Thirty-five days postinjection mice were killed and sera were analyzed for anti-HBsAg antibody titers. Combined data of two independent experiments are shown for vector doses of  $10^8$  and  $10^9$ . Boxplots show the median with 25% and 75% percentiles. **(b)** A subset of each group ( $n = 3$ ) of the sera in **a** was analyzed for anti-adenovirus IgG by enzyme-linked immunosorbent assay. Boxplots show the median antibody titers calculated by the reciprocal dilution where a fixed threshold of absorbance is reached. *P* values are based on two-sided Wilcoxon test. HBsAg (or: S), hepatitis B small surface antigen; n.s., not significant; VP, vector particles.

fact, the peak-contraction kinetic of  $K^b/S_{190-197}$ -specific CD8 T cells induced by AdSmir122aT resembles that of Holst *et al.* after footpad-vaccination which resulted in enhanced quality and functionality of these CD8 T cells. We have observed enhanced early CD8 T cell expansion with AdSmir122aT for an epitope that can exclusively be generated by the endogenous processing pathway, but not for an epitope from the same antigen that can be generated by both the endogenous and exogenous processing pathway.<sup>25</sup> Due to the need for the endogenous processing pathway this finding indicates that direct MHC class I presentation of this epitope by hepatocytes is responsible for repression of CD8

T cell expansion. However, the underlying mechanisms remain unclear. Hepatocyte-specific silencing might not have an impact on CD8 T cell expansion in case of an epitope predominantly processed by the exogenous pathway after uptake of secreted antigen. Blocking this pathway or using an intracellular version of HBsAg could answer this question. However, improved polyfunctionality was also observed in the CD8 T cell population specific for this epitope.

The absolute vector dose applied for an effective vaccination with Ad vectors has to be considered as an especially critical parameter as it is directly associated with the extent of vector dissemination and hepatocyte transduction. Data from our experiments demonstrate the effectiveness of mir-122a to control hepatic antigen expression over a dose range from  $10^8$  to  $10^{10}$  VP resulting in higher antibody levels compared to a nonregulated vector. However, a coinjection of  $10^8$  VP of this nonregulated vector already started to partially counteract this effect. As vector toxicity is a major concern of Ad vector-based vaccines maximal effectiveness at low doses is a desirable feature of future developments. Our observation that vector dose of AdSmir122aT could be lowered 100-fold without decreasing antibody titers might therefore be promising.

The beneficial effects for the induction of cellular and humoral immune responses by mir-122a-mediated antigen suppression in this study have been shown for a defined model antigen with clinical relevance in hepatitis B virus vaccination. However, it has to be evaluated if these effects are HBsAg-specific or also hold true for other secreted or intracellular antigens. Taken together, the results presented here provide evidence that minimizing antigen expression in hepatocytes through miRNA-regulation might be a useful strategy to enhance immune responses to Ad vector-delivered antigens. Combining other approaches to reduce liver transduction might further include physical detargeting of Ad vectors from liver<sup>46</sup> or the use of serotypes with decreased liver tropism and will contribute to a more rational design for effective Ad vector-based genetic vaccines.

## MATERIALS AND METHODS

**Plasmid construction.** Coding sequences of EGFP (from pEGFP-N1; Clontech, Heidelberg, Germany) or HBsAg (from pCI/S small<sup>47</sup>) were cloned into a cytomegalovirus (CMV) promoter-driven expression cassette derived from pCMV $\beta$  (Clontech) that was subcloned into pGS70 plasmid backbone.<sup>48</sup> To insert miRNA target sequences into the 3'UTR of EGFP or HBsAg plasmid constructs were digested by *NotI* and ligated with annealed oligonucleotides. The following oligonucleotides were used.

mir122aT: 5'-GGCCGCAGTCGACCAAAACACCATTGTCACACTCCATTTCGAAACAAACACCATTGTCACACTCCAACCGGTACAACACCATTGTCACACTCCAATGCATACAAACACCATTGTCACACTCCACGCGCAC-3', 5'-GGCCGTGCGCGTGGAGTGTGACAATGGTGTGTTTGTATGCATTGGAGTGTGACAATGTGTTTGTACGCGTTGGAGTGTGACAATGGTGTGTTTGTGTTTGAATGGAGTGTGACAATGGTGTGTTTGGTTCGACTGC-3', mir142-3pT: 5'-GGCCGCAGTCGACTCCATAAAGTAGGAAACACTACATCACTTCCATAAAGTAGGAAACACTACAACCGGTCCATAAAGTAGGAAACACTACACGATTCCTAAAGTAGGAAACACTACACGCGCGCAC-3', 5'-GGCCGTGCGCGTGTAGTGTTCCTACTTTATGGAATCGTGTAGTGTTCCTACTTTATGGAACCGGTTGTAGTGTTCCTACTTTATGGAAGTGTAGTGTTCCTACTTTATGGAGTGCAGTGC-3', mir206T: 5'-GGCCGCAGTCGACCCACACACTTCCTTACATTCCATCACTCCACACT



TCCTTACATTCCAACCGGTCCACACACTTCCTTACATTC  
CACGATCCACACACTTCCTTACATTCACGCGCGCAC-3',  
 5'GGCCGTGCGCGCTGGAATGTAAGGAAGTGTGTGGA  
 TCGTGGAAATGTAAGGAAGTGTGTGGACCGGTTGGAA  
 TGTAAGGAAGTGTGTGGAGTGATGGAATGTAAGGAAG  
 TGTGTGGGTCGACTGC-3'. The four tandem repeats of perfectly  
 complementary miRNA target sequences in the sense oligonucleotides  
 are underlined. CMV-EGFPmirTs and CMV-HBsAgmirTs expression  
 cassettes were excised by *PacI* and cloned in place of the E1-deleted region  
 of infectious plasmid pGS66.<sup>48</sup>

**Ad vectors.** Ad5-based E1-deleted first generation vectors were generated by transfection of *SwaI*-linearized infectious plasmids into E1-transcomplementing cell line N52.E6 followed by subsequent vector amplification. Vector purification was performed by CsCl density gradient and vector titers were determined by a DNA-based slot-blot procedure as described previously.<sup>49</sup>

**Cell lines.** N52.E6 cell line<sup>48</sup> was cultured in  $\alpha$ -minimal essential medium (Gibco, Eggenstein, Germany) with passaging twice weekly. Hepatocarcinoma cell line Huh7.5 was obtained from Apath, Brooklyn, NY and maintained in Dulbecco's modified Eagle's medium (Gibco) and subcultured twice weekly. A549 cell line (ATCC #CCL-185; ATCC, Manassas, VA) was kept in minimal essential medium (Gibco) and passaged twice weekly. Myelogenous leukemia cell line K562 (ATCC #CCL 243; ATCC) was cultured in RPMI-1640 (Gibco) and subcultured twice weekly. C2C12 myoblasts (ATCC #CRL 1772; ATCC) were cultured in Dulbecco's modified Eagle's medium and subcultured three times a week. All media were supplemented with 10% fetal calf serum (FCS) and 1% penicillin/streptomycin/glutamine. For differentiation of C2C12 myoblasts into myotubes  $8 \times 10^4$  cells were seeded on a 24 well plate and kept in skeletal muscle differentiation medium (PromoCell, Heidelberg, Germany) supplemented with 3% FCS and Supplement-Mix (C-39366; PromoCell) for 48 hours. Medium was then replaced by growth medium (Dulbecco's modified Eagle's medium, 10% FCS, 1% P/S/G) and cells were cultured to complete differentiation without passaging for 30 days.

**Mice.** Female C57BL/6 and BALB/c mice were obtained from Charles River (Sulzfeld, Germany) and kept in standard pathogen-free environment in the animal facility of the University of Ulm. For all immunization experiments mice were used at an age of 6–8 weeks. All animal experiments were approved by the Animal Care Commission of the Government of Baden-Württemberg and were in accordance with institutional guidelines.

**Vector injection and immunization.** For transgene expression analysis indicated amounts of Ad vector particles ( $2 \times 10^{10}$ – $8 \times 10^{10}$ ) were diluted in a total volume of 200  $\mu$ l phosphate-buffered saline (PBS) and injected into the tail vein. For i.m. injection mice were anaesthetized by intraperitoneal injection with ketamine/xylazine and  $5 \times 10^{10}$  vector particles in a total volume of 100  $\mu$ l were injected into the gastrocnemius muscle at two sites on each limb. Immunizations were performed by injection of either  $1 \times 10^8$  or  $1 \times 10^9$  vector particles in 50  $\mu$ l of PBS (25  $\mu$ l per gastrocnemius muscle) with an Omnican 20 syringe (Braun, Melsungen, Germany).

**Autopsy and cell preparation.** Mice were killed by inhalation of isoflurane (Forene; Abbott, Ludwigshafen, Germany). Blood samples were taken by puncture of the heart. For isolation of blood cells 10  $\mu$ l of heparin was immediately added and erythrocytes were lysed with a 0.8%  $\text{NH}_4\text{Cl}$  0.1%  $\text{KHCO}_3$ -containing buffer. For isolation of hepatocytes and nonparenchymal liver cells livers were perfused through the portal vein with 10 ml of liver perfusion medium (Gibco) and subsequently perfused with 5 ml of liver digestion medium (Gibco). Livers were excised, cut into small pieces, and digested for 30 minutes at 37°C. Crude homogenates were passaged through a nylon mesh and the cell suspension was washed with PBS. Hepatocytes were separated by low-speed centrifugation at 50g with subsequent washing in PBS

and subjected to further analysis. The supernatant containing the nonparenchymal liver cells fraction was subjected to Percoll (EasyColl; Biochrom, Berlin, Germany) density gradient centrifugation (70% Percoll in 1% FCS/PBS and 45% Percoll in 1% FCS/PBS) at 2,000 rpm for 20 minutes. Cells were collected at the interface, washed in PBS, and erythrocytes were lysed as described above. Splenocytes were isolated as previously described.<sup>46</sup> For subsequent culturing cells were resuspended in BioWhittaker-UltraCulture medium (Cambrex Bioscience, Verviers, Belgium) supplemented with 1% penicillin/streptomycin/glutamine.

**Histology and fluorescence microscopy.** Forty-eight hours post vector injection gastrocnemius muscle or perfused liver were excised and fixed in 2% paraformaldehyde in PBS at 4°C over night. Organs were incubated in 30% sucrose and embedded in Tissue-Tek (Sakura, Zoeterwoude, NL) for preparing cryosections of 8  $\mu$ m. Cryosections were mounted with fluorescence mounting medium (Dako, Copenhagen, Denmark) and analyzed for GFP fluorescence on a Zeiss Axioskop2 plus fluorescence microscope.

*In vitro* transduced cell lines were overlaid with PBS and directly analyzed on a Leica DMIL fluorescence microscope without fixing.

**SDS-PAGE and western blot.** Isolated hepatocytes of Ad vector-injected mice were lysed with 1% TritonX-100, insoluble material was cleared by centrifugation and protein concentrations of the supernatants were determined by BCA protein assay reagent (Pierce, Bonn, Germany). 10  $\mu$ g of protein per lane was subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), transferred on a nitrocellulose membrane by western blot for detection of EGFP protein with a monoclonal anti-GFP antibody (Invitrogen, Darmstadt, Germany) and ECL detection kit (Pierce, Bonn, Germany).

**Quantification of HBsAg-particles.** HBsAg-particle serum concentrations were measured by a microparticle enzyme immunoassay (HBsAg (V2); Abbott Diagnostics, Wiesbaden, Germany) on an IMx system according to manufacturer's instruction. Relative S-particle serum concentrations are based on the S/N ratio = (value of sample/MODE 1 calibrator value). MODE 1 calibrator is nonreactive for HBsAg. Samples with a S/N value  $\geq 2$  are considered as reactive. Each reagent pack of a different batch is calibrated by a negative and a positive control containing 4–15 ng/ml of HBsAg.

**Quantification of Ad vector genomes by qPCR.** Total DNA was isolated from liver tissue and analyzed for Ad vector genomes by qPCR with Brilliant II SYBR Green QRT-PCR Master Mix Kit from Stratagene as described earlier.<sup>50</sup> Details can be obtained upon request.

**Determination of antibody titers.** Serum was obtained by centrifugation of blood samples at 14,000 rpm in a microcentrifuge. HBsAg-specific antibody titers were determined by microparticle enzyme immunoassay (AxSYM AUSAB; Abbott Diagnostics) on an IMx system or alternatively by a noncompetitive sandwich ELISA Kit (ETI-AB-AUK-3; Diasorin, Dietzenbach, Germany) according to manufacturer's instructions. Anti-adenovirus-specific IgG titers were quantified by ELISA. In brief, 96-well plates (NUNC-Maxisorp, #267245; NUNC, Rochester, NY) were coated with heat-inactivated (10 minutes, 60°C) adenovirus vector particles ( $6 \times 10^8$  VP/well) at 4°C overnight. Plates were blocked with PBS/3% bovine serum albumin for 1 hour, washed five times with PBS/0.05% Tween20 and incubated with serial dilutions of serum for 2 hours at room temperature. Plates were washed and incubated with horseradish peroxidase-labeled anti-mouse IgG (BD Biosciences, Heidelberg, Germany) for 1 hour at 37°C and developed with o-phenyldiamine dihydrochloride (Sigma, Taufkirchen, Germany) / $\text{H}_2\text{O}_2$  substrate and stopped with 1 mol/l sulfuric acid. Absorbance was measured at 492 nm/620 nm on an ELISA reader.

**Specific CD8<sup>+</sup> T cell frequencies.** IFN $\gamma^+$ /CD8<sup>+</sup> T cells were determined on a FACSCalibur as previously described.<sup>46</sup> In brief, spleen cells or

nonparenchymal liver cells were stimulated overnight with 2.5 µg/ml of anti-genic peptide or control peptide in the presence of 5 µg/ml of brefeldin A. After stimulation Fc-receptors were blocked by anti-CD16/32 mAb (clone 2.4G2) and cells were surface-stained with phycoerythrin-conjugated anti-CD8 mAb (BD Pharmingen, Heidelberg, Germany) or biotin-labeled anti-CD8 mAb (BD Pharmingen) and peridinin-chlorophyll protein-conjugated streptavidin (BD Pharmingen). Cells were fixed with 2% paraformaldehyde and permeabilized with 0.5% saponin containing buffer. Permeabilized cells were stained for intracellular cytokines for 30 minutes at room temperature with fluorescein isothiocyanate-conjugated anti-IFN $\gamma$  mAb (BD Pharmingen) and when indicated with phycoerythrin-conjugated anti-IL-2 mAb (BD Pharmingen) and allophycocyanin-conjugated anti-TNF $\alpha$  mAb (BD Pharmingen). For direct detection of specific T cell receptors cells were stained with allophycocyanin-conjugated anti-CD8 mAb (eBiosciences, Frankfurt, Germany) and phycoerythrin-labeled specific K<sup>b</sup>/S<sub>190-197</sub> MHC/peptide tetramers (#T04001; Beckman-Coulter, Marseille, France) for 30 minutes at 4°C. Cells were washed and analyzed by flow cytometry.

**Regulatory T cells.** The total pool of regulatory T cells was determined by surface staining with allophycocyanin-conjugated anti-CD4 mAb (eBiosciences) and phycoerythrin-conjugated anti-CD25 mAb (eBiosciences). Cells were fixed, permeabilized, and stained for Foxp3 by using the Foxp3 Staining Buffer Set (eBiosciences) and fluorescein isothiocyanate-conjugated anti-Foxp3 mAb (eBiosciences) according to the manufacturer's one-step protocol.

**Synthetic peptides.** Peptides were purchased from JPT Peptide Technologies (Berlin, Germany). L<sup>d</sup>-restricted HBsAg-specific peptide corresponds to residues 28–39 (L<sup>d</sup>/S, IPQLDSWWTSL). K<sup>b</sup>-restricted HBsAg-specific peptide is corresponding to antigen residues 190–197 (K<sup>b</sup>/S, VWLSVWIM). As controls K<sup>d</sup>-restricted CSP-specific peptide from plasmodium berghei (K<sup>d</sup>/CSP<sub>252-260</sub>, SYIPSAEKI) or K<sup>b</sup>-restricted ovalbumin-specific peptide (OVA<sub>257-264</sub>, SIINFEKL) were used. Peptide purity was greater than 70%. Peptide stocks were prepared at 10 mg/ml in DMSO and stored at –20°C. Immediately prior to use peptides were diluted with culture medium.

**Statistics.** The significance of data was determined by unpaired Student's t-test. For CD8 T cell frequencies and antibody titers boxplots show the median (horizontal thick band) with 25% and 75% percentiles (bottom and top of box). P values are based on two-sided Wilcoxon test calculated with R software.

## SUPPLEMENTARY MATERIAL

**Figure S1.** Transgene silencing of miRNA-regulated Ad vectors in skeletal muscle (mir-206) and primary human macrophages (mir-142-3p).

**Figure S2.** L<sup>d</sup>/S<sub>28-39</sub>-specific CD8 T cells in liver, multifunctionality of HBsAg-specific CD8 T cells and total regulatory CD4 T cells.

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