Liver INTERNATIONAL

Liver International ISSN 1478-3223

VIRAL HEPATITIS

Transient depletion of specific immune cell populations to improve adenovirus-mediated transgene expression in the liver

Pilar Alzuguren[1,*], Sandra Hervas-Stubbs[1], Gloria Gonzalez-Aseguinolaza[1], Joanna Poutou[1], Puri Fortes[1], Uxua Mancheno[1], Maria Bunuales[1], Cristina Olagüe[1], Nerea Razquin[1], Nico Van Rooijen[2], Monica Enguita[1] and Ruben Hernandez-Alcoceba[1]

1 Division of Hepatology and Gene Therapy, CIMA, Foundation for Applied Medical Research, University of Navarra, Pamplona, Spain
2 Department of Molecular Cell Biology, Vrije Universiteit Medical Center, Amsterdam, the Netherlands

Keywords
adenovirus – B cells – CD4$^+$ T cells – CD8$^+$ T cells – clodronate liposomes – immunosuppression – Kupffer cells – monoclonal antibodies – neutralizing antibodies – NK cells

Correspondence
Ruben Hernandez-Alcoceba, Gene Therapy Unit, CIMA, Av. Pio XII,
55 E-31008-Pamplona, Spain
Tel: +34-948194700
Fax: +34-948194717
e-mail: rubenh@unav.es

Received 7 June 2013
Accepted 17 April 2014

DOI:10.1111/liv.12571
Liver Int. 2015; 35: 1274–1289

Abstract

Background & Aims: Adenoviral (Ad) vectors are currently one of the most efficient tools for *in vivo* gene transfer to the liver. However, anti-Ad immune responses limit the safety and efficacy of these vectors. The initial inflammatory reaction is a concern in terms of toxicity, and it favours the development of cellular and humoral responses leading to short transgene persistence and inefficient vector re-administrations. Therefore, safe and simple ways to interfere with these processes are needed. Study ways to deplete specific immune cell populations and their impact on liver-directed gene transfer. *Methods:* First-generation Ad vectors encoding reporter genes (luciferase or β-galactosidase) were injected intravenously into Balb/c mice. Kupffer cells and splenic macrophages were depleted by intravenous administration of clodronate liposomes. B lymphocytes, CD4$^+$, CD8$^+$ T lymphocytes or NK cells were depleted by intraperitoneal injection of anti-M plus anti-D, anti-CD4, anti-CD8 or anti-asialo-GM1 antibodies respectively. Long-term evolution of luciferase expression in the liver was monitored by bioluminescence imaging. *Results:* The anti-CD4 monoclonal antibody impaired cellular and humoral immune responses, leading to efficient vector re-administration. Clodronate liposomes had no impact on humoral responses but caused a 100–1000 fold increase in liver transduction, stabilized transgene expression, reduced the concentration of inflammatory cytokines, and inhibited lymphocyte activation. *Conclusions:* Transient CD4$^+$ T-cell depletion using antibodies is a clinically feasible procedure that allows efficient Ad redosing. Systemic administration of clodronate liposomes may further increase the safety and efficacy of vectors.

Viral vectors are currently the most efficient way to deliver genes to the target cells in gene therapy approaches, especially *in vivo* (1). However, viral-induced immune responses limit the applicability of these vectors for several reasons. First, they can compromise their safety, as severe inflammatory responses may occur (2). Second, transduced cells may present viral antigens and therefore they can be recognized and eliminated by cytotoxic cellular responses (3). Third, the generation of neutralizing antibodies (NAbs) against structural components of the viral vectors drastically reduces the efficacy of redosing (4, 5). Adenoviral (Ad) vectors are especially sensitive to these negative effects because of their elevated immunogenicity (6) and the fact that a significant percentage of the adult population has been previously exposed to the most commonly used adenovirus serotypes (7, 8). These vectors are highly efficient *in vivo*, possess a very stable genome which is easy to manipulate in the laboratory, and the production of clinical-grade stocks has been standardized. Chromosomal integration in infected cells is very low and has not been associated with oncogenic transformation (9). Vectors derived from human Ad type 5 show a marked hepatic tropism and are promising tools for liver-directed gene therapy approaches (10). Despite all these properties, the current use of Ad vectors in humans is mainly restricted to vaccination or oncolytic strategies due to the short duration of transgene expression (1). In an attempt to reduce their immunogenicity, progressive removal of viral genes has led to the development of the third-generation ('gutless', helper-dependent or

*Both authors contributed equally to this work.

high-capacity) Ad vectors, which are devoid of all viral coding sequences (11). This eliminates the possibility of cellular responses against residual expression of viral genes in transduced cells. However, immune reactions against capsid proteins remain intact and pose a challenge for the maintenance of transgene expression and the efficacy of redosing (12).

Identification of methods to circumvent anti-adenovirus responses would increase their applicability, especially in gene therapy approaches that require long-term expression. Previous studies indicate that treatment with classical immunosuppressive agents such as glucocorticoids, cyclosporine, FK506 or cyclophosphamide reduce immune responses against Ad vectors (13–15). In a recent study performed in non-human primates, an intensive protocol using five clinically available drugs simultaneously allowed Ad redosing in one of the two animals treated (16). Characterization of mild, yet effective regimes that could be well tolerated by subjects treated with gene therapy is essential. Manipulation of specific components of the immune system may change the response against the virus in different ways. The rapid advance in monoclonal antibody (mAb) therapies offers excellent opportunities for selective interventions readily applicable in the clinic. In principle, abrogation of cytotoxic $CD8^+$ T lymphocyte function should prevent elimination of transduced cells and prolong transgene expression, whereas impairment of B lymphocytes would reduce the generation of NAbs and allow efficient re-administration. Interaction between antigen presenting cells (APCs) and $CD4^+$ T cells plays a crucial role in Ad recognition and rejection. Interference with costimulatory interactions between these cells using CTLA4Ig (17, 18) or blocking antibodies against CD40 ligand (18–22) are promising options. However, cardiovascular side effects observed in patients treated with anti-CD40 ligand mAbs compromise the clinical translation of this strategy (23, 24). Selective and transient depletion of cell populations is an alternative approach. In the case of liver-directed gene therapy, depletion of resident hepatic macrophages (Kupffer cells) is especially appealing. Apart from their functions as APCs, they limit the access of Ad to hepatocytes thanks to their strategic localization in the hepatic sinusoid (25). Therefore, Kupffer cell depletion using systemic administration of dichloromethylene diphosphonate (clodronate) encapsulated in liposomes has been described to enhance liver transduction and stabilize transgene expression (26, 27). Although partial $CD4^+$ T-cell depletion reduces the titre of anti-Ad NAbs and improves the efficacy of vector redosing (28, 29), little is known about the effect of $CD8^+$ T or NK cell depletion. Differences between the Ad vectors and animal models used in these studies complicate the interpretation of results. The aim of this study is to analyse the effect of selective lymphocyte depletions (B, T and NK cells) in comparison with monocyte/macrophage depletion. For the first time, we provide a side-by-side comparison of these leucocyte depletions in mice treated with first-generation Ad vectors expressing the reporter gene luciferase. We describe the impact of these manipulations on toxicity, long-term transgene expression and efficacy of redosing. Our results point to clodronate liposomes and anti-CD4 depleting antibodies as the most effective and clinically compatible ways to improve the performance of Ad vectors. We have studied the mechanisms leading to these effects and discuss the implications for the design of tailored immunosuppression protocols.

Materials and methods

Adenoviral vectors and cells

Vectors used in this study are replication-deficient, first-generation (E1/E3-deleted) adenoviruses expressing transgenes under the control of the CMV promoter. Transgenes are: firefly luciferase in the Ad-CMV-Luc virus (Vector Biolabs, Philadelphia, PA, USA); β-galactosidase (LacZ) in the AdLacZ virus (30); and human interleukin-12 in the Ad.IL-12 virus (31). All Ad vectors were amplified in HEK293 cells (ATTC CRL-1573) and purified by ultracentrifugation in CsCl gradients. Quantification of infectious units (iu) was done using the Adeno-X rapid titre kit (Clontech, Mountain View, CA, USA). The iu:vp (viral particle) ratio ranged from 1:50 to 1:200 among different viral stocks. BNL 1ME A.7R.1 (ATCC TIB-5) is a methylcholantrene-transformed liver cell line. All cells were maintained in DMEM growth medium supplemented with 10% fetal bovine serum, 100 u/ml penicillin, 100 μg/ml streptomycin and 2 mM L-glutamine. Except when noted, all cell culture reagents were from Gibco. All cells were maintained at 37°C with 5% CO_2 in a humidified incubator.

Antibodies for cell depletion

B cell depletion was achieved by a combination of rat igG2a kappa mAbs against μ and δ murine immunoglobulin heavy chains (clones LO-MD-6 and LO-MM-9, respectively, from the University of Louvain) (32). The hybridoma cell lines anti-CD4 (GK 1.5) and anti-CD8β (H3S-17-2) were obtained from ATCC. The mAb produced by these hybridomas were purified from respective culture supernatant by affinity chromatography in sepharose protein G columns according to manufacturer's instructions (GE Healthcare Bio-sciences AB, Uppsala, Sweden). NK cell depletion was achieved by rabbit ploYclonal anti-asialo-GM1 Ab (Wako, Osaka, Japan). IgG from Rat serum was obtained from Sigma-Aldrich and was used as control Ab.

Preparation of clodronate liposomes

Dichloromethylene-biphosphonate (clodronate) was a kind gift of Roche Diagnostics Gmbh, Mannheim,

Germany. Clodronate was encapsulated in liposomes as previously described (33).

Animals and treatment procedures

Female 5–7 weeks-old Balb/c and C57BL/6J mice were purchased from Harlan. μMT knock-out mice (B6.129S2-Ighm^{tm1Cgn}/J) were obtained from Dr Claude Leclerc (Institut Pasteur, Paris, France). Ad vectors (typically 1×10^9 iu) were administered intravenously by tail vein injection. Macrophage depletion was achieved by intravenous injection of clodronate liposomes (2 μl/g mice in 50 μl saline) 24 h before viral infection. Antibodies were injected intraperitoneally following the following doses and schedules: for B cell depletion, a mixture of anti-μ and anti-δ mAbs (500 μg each) were administered on days −3, −1 and 4 (considering 0 the day of virus injection); for $CD4^+$, $CD8^+$ T cell or NK cell depletion, 100 μg of the corresponding antibodies were administered on days −6, −2, 5, 12, 19 and 27. In each case, the same amount of unspecific IgG was given to the animals in the control group. The efficacy of depletions was analysed by flow cytometry at the indicated times. Dexamethasone was administered intraperitoneally using the following protocol: 10 mg/kg 15 and 2 h before virus administration; 5 mg/kg on days 1 and 2, and 1 mg/kg on days 3–7. Partial hepatectomies were performed under inhalatory anaesthesia. The liver was exposed through median laparotomy, and the left and median lobes (approximately 80% liver mass) were removed after ligation of vessels.

Tumours were established by subcutaneous inoculation of 1×10^6 BNL cells in Balb/c mice. Administration of the Ad-CMV-Luc virus was performed by intratumoral injection in a total volume of 50 μl dissolved in saline solution. Transgene expression was analysed by BLI as previously described. Tumour progression was monitored by direct measurement by calliper. Volumes were calculated using the formula $V = 0.5 \times D \times d^2$, where D and d are the maximum and minimum tumour diameters respectively. When necessary, mice were euthanized using a mixture of O_2 and CO_2 gases. All mice were maintained under standard conditions in the animal facilities of the Center for Applied Medical Research, Pamplona, Spain. All procedures were carried out following protocols approved by the local ethical committees in accordance with recommendations for proper care and use of laboratory animals.

Flow cytometry

Cell suspension of the spleen was obtained by mechanically disrupting the tissue with a syringe plunger in cold culture medium (RPMI 1640). After red blood cell removal by ACK treatment, splenocytes were washed in cell culture medium and filtered through a 70 μm nylon cell strainer (BD Biosciences, San Diego, CA, USA). Livers were treated with 400 U/ml collagenase D and 50 μg/ml DNase I (Roche Diagnostics, Indianapolis, IN, USA). After mechanical tissue dissociation, cells were filtered and washed with PBS. To enrich liver cell suspension in leucocytes, hepatocytes were removed using Percoll gradients. The single cell suspension obtained was pretreated with anti-CD16/32 (clone 2.4G2; BD Biosciences-Pharmingen) to reduce non-specific binding to Fc receptors. Then, cells were stained with the following antibodies: CD8a FITC-conjugated (53–6.7; eBioscience, San Diego, CA, USA), CD4 PE-conjugated (GK1.5 Biolegend; San Diego, CA, USA), DX5 APC-conjugated (17A2 Biolegend).

To analyse βGalactosidase (βGal)-specific CD8 T cells in liver infiltrating leucocytes, cells were isolated at day 7 after infection and stained first with H-2Kb-$\beta Gal_{876\text{-}884}$-tetramer-PE (Proimmune, Oxford, UK) and then with anti-CD8-FITC (53-6-7) mAbs. After surface staining, cells were fixed and permeabilized with Cytofix/CytoPerm (BD Biosciences) and then stained with anti-human granzyme B-APC (GB11) mAb in the presence of purified Rat IgG.

For IFNγ intracellular staining, cells (10^5 cells/well) were incubated for 4 h in the presence of Brefeldin A (10 μg/ml) and Monensine (2 μg/ml) (SIGMA) with the following stimuli: $\beta Gal_{876\text{-}884}$ peptide (10 μg/ml) (Proimmune); β-gal protein (10 μg/ml) (Alpha Diagnostic International, San Antonio, TX, USA) or Ad Capsids proteins (10 μg/ml) purified from a CsCl gradient. Then, cells were sequentially labelled with anti-CD4-APC (RM4-5) and anti–CD8-FITC (53-6-7) mAbs, fixed/permeabilized and finally stained with anti–IFN-γ-PE (XMG1.2) in the presence of Rat IgG.

To check B cell depletion, whole blood was stained with anti-CD19-PE (6D5) and anti-B220-FITC (RA3-6B2). T-cell depletion was analysed using anti-CD3-Alexa Fluor 488 (145-2C11), anti-CD4-APC (RM4-5) ($CD4^+$ T cells) and anti–CD8-PercPCy5.5 (53-6-7) ($CD8^+$ T cells); or anti-CD3-Alexa Fluor 488 (145-2C11) and anti-CD335 (NKp46)-PE (29A1.4) (NK cells). After staining, cells were treated with FACSLysing Solution (BD Bioscience). All mAbs were from Biolegend. Appropriate isotype controls were used to verify staining specificity. Cells were acquired on either a FACSCalibur or FACSCANTO cytometer and analysed using FlowJo (Tree Star Inc., Ashland, OR, USA).

Immunohistochemistry

Immunohistochemical staining for F4/80 (rat anti-mouse, 1:400; eBiosciences, 14-4801-82) was performed using the EnVisionTM+ System (Dako, Glostrup, Denmark) according to the manufacture's recommendations. After deparaffinization, antigen retrieval was performed using a solution of 20 μg/ml proteinase K for 30 min at 37°C. Sections were incubated with rabbit anti-rat secondary antibody (1:200; Dako, E0468) for 30 min and then with the labelled polymer.

Thymidine incorporation

To analyse cellular responses against Ad, liver infiltrating leucocytes (5×10^5 cells/ml) were cultured for 72 h in medium alone [X-vivo medium (BioWhittaker, Walkeshville, MD, USA) supplemented with 2 mM glutamax (Invitrogen, Carlsbad, CA, USA), and 1% penicillin/streptomycin (Invitrogen)] or containing Ad Capsids proteins (10 μg/ml). [^{3}H]-thymidine uptake was assessed by filtration on an automatic cell harvester and by measuring nuclear radioactivity (filters) on a scintillation plate reader (Topcount; Perkin Elmer, Waltham, MA, USA).

Quantification of NAbs

Anti-adenovirus type 5 NAbs were determined using a modified luciferase-based virus neutralization assay, as previously described (16).

In vivo bioluminescence detection

Mice received an intraperitoneal injection of D-Luciferin (3 mg dissolved in 100 μl PBS; Sigma, St. Louis, MO, USA) and were anesthetized. Detection took place 5 min later in a dark chamber connected to a cooled charged coupled device (CCD) camera (IVIS; Xenogen, Alameda, CA, USA). Acquisition time ranged from 1 s to 5 min depending on intensity. Light emission (photons/s) was quantified and analysed using Living Image Software (Caliper, Hopkinton, MA, USA). Unless otherwise indicated, evolution of light emission is represented as fold values using as a reference the initial (day 1) luminescence in the control group, which presented absolute values of 1×10^7 to 3×10^8 photons/s, depending on individual experiments. Measurement was discontinued in an experimental group when all animals showed values close to background ($0.5–1 \times 10^5$ photons/s) in two consecutive time points. The absence of transgene expression in these groups was verified at the end of the experiment, but it is not represented in the graphics.

ELISA

Sera were assayed for the human and murine IL-12 (p70), murine IL-6 and murine IFN-γ using OptEIA ELISA Set kits (all from BD Biosciences) according to the manufacturer's protocol.

Quantification of luciferase activity in tissue extracts

Liver samples were lysed in Reporter Lysis Buffer (Promega, Madison, WI, USA) and centrifuged at 17 000*g* in a cooled microfuge for 10 min. The luciferase activity was measured in the supernatant using the Luciferase Reporting Assay System (Promega) in a Berthold microplate luminometer. Activity was expressed in relative lights units/μg protein.

Statistical analysis

The Mann–Whitney test was applied for statistical comparisons. Analysis was performed using the GraphPad Prism program (GraphPad Software, La Jolla, CA, USA).

Results

Depletion of Kupffer cells and splenic macrophages by clodronate liposomes increases and stabilizes liver transduction but does not reduce anti-Ad humoral responses.

Balb/c mice were treated with a single intravenous injection of 2 μl/g mice clodronate liposomes. Twenty-four hours later, they were sacrificed to analyse macrophage and dendritic cell (DC) frequency in liver and spleen. Flow cytometry analysis showed an efficient depletion of hepatic and splenic macrophages (Fig. 1A). Immunohistochemical analysis of liver samples confirmed the absence of Kupffer cells in clodronate liposomes-treated mice (Fig. S1). In contrast, the proportion of DCs was not significantly changed in the liver, and only partially reduced in the spleen (Fig. 1A). This is in agreement with previous studies describing stronger depletion of macrophages than DCs (34). Then, we performed systemic administration of a first-generation Ad vector expressing the reporter gene luciferase (Ad-CMV-Luc) 24 h after treatment with clodronate liposomes. A drastic reduction in the inflammatory response was observed, compared with control animals that received virus without pretreatment. This includes inhibition in serum concentration of the cytokines interleukin-6 (IL-6), IL-12 and interferon gamma (IFN-γ) measured 6, 24 and 24 h after viral infection, respectively (Fig. 1B). Activation of $CD4^+$, $CD8^+$ T cells and NK cells was also reduced in spleen and liver of treated animals, as determined by CD69 staining (Fig. 1C). Next, we studied the consequences of clodronate liposome treatment in the ability of Ad-CMV-Luc to transduce the liver. As expected, the reduction in Kupffer cells led to a strong enhancement of transgene expression, determined by *in vivo* bioluminescence imaging (BLI) (Fig. 2A). In Balb/c mice, the increase ranges between 100 and 1000 times over control animals. In rats and other mouse strains such as C57BL/6, we observed that clodronate liposomes enhance Ad-mediated liver transduction as well, although the increase is approximately 10 times (not shown). This is consistent with differences in the ability of Kupffer cells to sequester Ad in each strain (35). In order to validate the *in vivo* BLI data, we prepared liver extracts from control and clodronate liposome-treated animals in an independent experiment, and determined luciferase activity *in vitro*. The result confirmed the increased

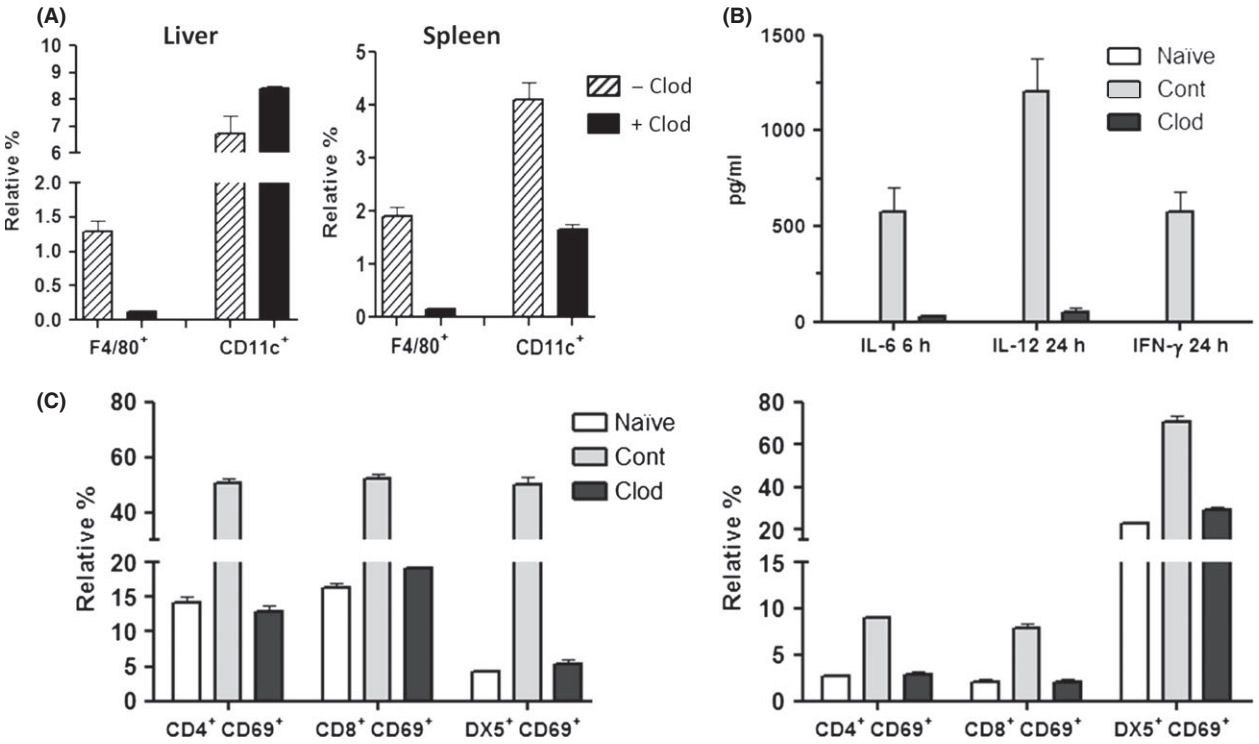

Fig. 1. Clodronate liposomes deplete Kupffer cells and decrease inflammatory responses against Ad.(A) Balb/c mice were treated with intravenous clodronate liposomes (2 μl/g of animal weight) and sacrificed 24 h later. Control mice were left untreated and sacrificed at the same time. The percentage of macrophages (F4/80$^+$) and dendritic cells (CD11c$^+$) cells was determined by flow cytometry and represented in dashed (– Clod) or black (+ Clod) columns. (B, C) Control (light grey bars) or clodronate liposomes-treated mice (dark grey bars) received an intravenous injection of Ad-CMV-Luc (10^9 iu/mouse). The naïve group represents non-infected mice (white bars). (B) The concentration of cytokines IL-6, IL-12 and IFN-γ was measured in serum at the indicated times after infection by ELISA. (C) Twenty-four hours after adenovirus administration, mice were sacrificed and the activation status of CD8 T cells, CD4 T cells and NK (DX5$^+$) cells obtained from the liver (left panel) and spleen (right panel) was analysed by flow cytometry after immunostaining with the activation marker CD69. The bars represent the percentage of CD8, CD4 or DX5-positive cells expressing CD69 (mean ± SD), relative to each cell population.

luciferase content in these animals (Fig. 2B). The non-invasive follow-up of luciferase activity *in vivo* revealed that pretreated animals maintained transgene expression over extended periods of time (Fig. 2A). In contrast, light emission in control animals was barely increased over background 40 days after infection. In this representative experiment, we monitored expression for more than 4 months and then studied the possibility of Ad redosing. Independent assays indicate that transgene expression is maintained in liposome clodronate-treated mice for more than 8 months (Fig. S2), with a slow decline compatible with hepatocyte turnover. Since luciferase expression is greatly stabilized by clodronate liposomes, it would be difficult to assess the efficacy of a second Ad-CMV-Luc administration. Therefore, we treated mice with an intravenous injection of the Ad.hIL12 virus, a first-generation Ad vector carrying the human IL-12 gene (36). Since this human cytokine is not biologically active in mice but it is secreted and can be easily detected in the serum by ELISA, we used it as reporter gene in this experimental setting. As shown in Fig. 2C, the concentration of IL-12 in animals pre-exposed to Ad was drastically reduced, in comparison with naïve animals treated with the same dose of Ad.hIL12. Kupffer cell depletion before the first Ad injection caused only a marginal, non-significant increase in IL-12 expression. This correlates with a high titre of anti-Ad NAbs in the serum of mice before the administration of Ad.hIL12 (Fig. 2D). Thus, although clodronate liposomes can enhance and stabilize Ad-mediated transgene expression, they are not efficient in preventing humoral responses. Since the anti-inflammatory drug dexamethasone has been described to reduce anti-Ad immune responses (15) and it can affect the number and function of Kupffer cells in certain circumstances (37), we included a group of animals pretreated with this drug in the experiment described above. We did not find any inhibitory effect of dexamethasone in the number of Kupffer cells or the activation of hepatic lymphocytes in response to Ad (data not shown). A moderate increase in liver transduction was observed compared with control animals (Fig. 2A), compatible with partial inhibition of macrophage function. However, dexamethasone caused no modification in the kinetics of transgene

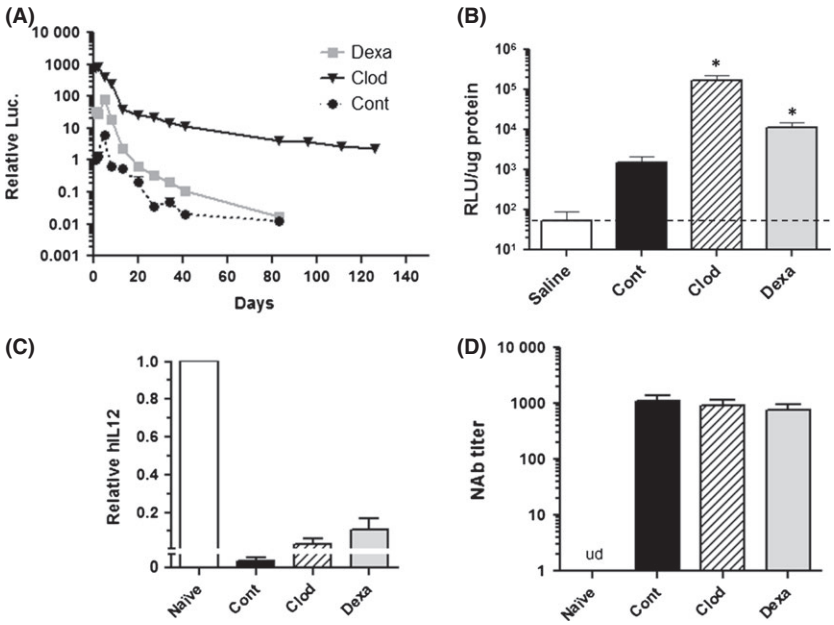

Fig. 2. Short pretreatment with clodronate liposomes increases and prolongs Ad-mediated transgene expression but does not reduce anti-Ad humoral response. Mice received an intravenous injection of Ad-CMV-Luc (10^9 iu/mouse) at day 0 of the experiment. One subset of animals (n = 12) were treated with 2 μl/g mice clodronate liposomes 24 h before infection (Clod group). Other mice received one cycle of dexamethasone treatment spanning days −1 to 7, as described in Methods (Dexa group). The remaining mice were left untreated (Cont group). (A) Evolution of transgene expression analysed by BLI. The graphic shows relative light emission for each group (average fold of photons/s), using as a reference the initial (day 1) luminescence in the control group. Differences between Clod and Cont or Dexa groups was significant (P = 0.02). (B) A subset of animals (n = 5) from the control and Clod groups were sacrificed 48 h after infection, and the luciferase activity was determined in liver extracts (represented in relative luciferase units/μg protein). A group of animals not injected with the vector (saline) were included to assess the background luciferase activity in this assay. (C) At the end of the observation period, all mice received an intravenous injection of the Ad-.hIL12 virus, and the presence of hIL-12 was detected in serum 48 h later by ELISA. The graphic represents the relative concentration of the cytokine, using the naïve group as a reference. (D) Presence of anti-Ad NAbs in the serum of the indicated groups before the second viral administration. The naïve group corresponds to age-matched mice without previous exposure to Ad.*P = 0.02. ud, undetectable.

expression. Regarding humoral responses, only a small, non-significant reduction in NAbs was observed (Fig. 2D), which correlated with a slight increase in the efficacy of redosing (Fig. 2C). Therefore, under our assay conditions, dexamethasone caused no relevant modification in the performance of Ad vectors in the liver.

Reduction of B lymphocytes has no effect on anti-Ad humoral responses

Transient depletion of B lymphocytes before and after vector administration could prevent the stimulation of humoral responses against the virus. In principle, the availability of clinically approved mAbs such as Rituximab with the ability to deplete human B cells makes this approach especially attractive. To investigate this possibility in our animal model, we treated Balb/c mice with a combination of anti-μ and anti-δ monoclonal antibodies (32) at 500 μg/kg each, in repeated doses spanning 3–4 days before and after administration of the Ad-CMV-Luc virus. We obtained a significant (albeit partial) reduction in circulating B lymphocytes that persisted for 10–20 days and then slowly recovered (Fig. 3A). This treatment resulted in an increase in liver transduction (Fig. 3B), but the kinetics of transgene expression was not different from control or unspecific rat IgG-treated mice (Fig. 3B). Moreover, re-administration of the same vector 2 months later caused no further elevation in luciferase activity, suggesting that the virus was neutralized before infecting a relevant number of hepatocytes. This is consistent with the presence of anti-Ad NAbs observed in the serum of animals (Fig. 3C). The maintenance of humoral responses was because of the incomplete B cell depletion obtained with the anti-μ and anti-δ mAbs (as observed with Rituximab in primates and humans) (16). This is further confirmed by the fact that μMT knock-out mice lacking B cells (38) do not produce NAbs in response to Ad, and the vector is able to transduce the liver upon re-administration (Fig. S3). Interestingly, a moderate increase in transgene expression was observed in μMT mice in comparison with the parental C57BL/6 mice, in agreement with other knock-out mice lacking B cells (39). As recently

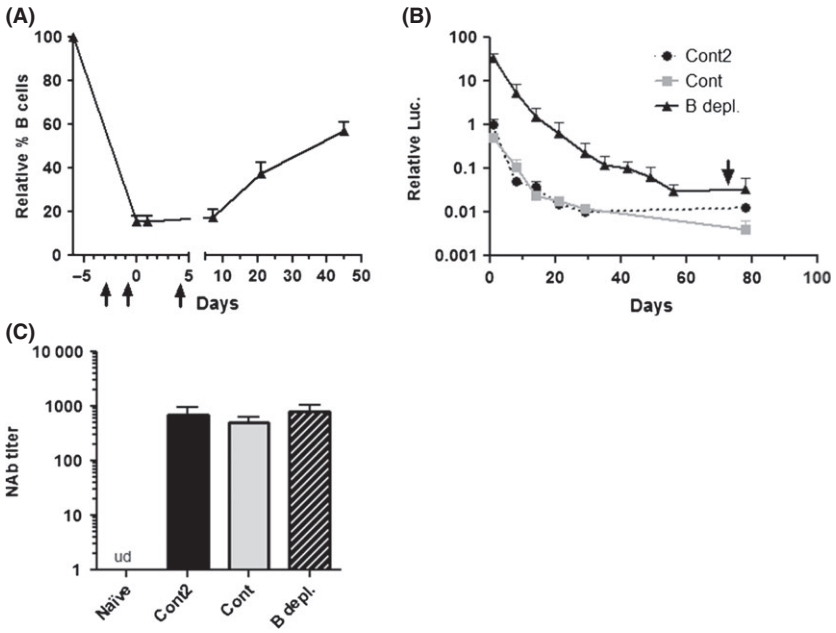

Fig. 3. Partial depletion of B lymphocytes does not reduce anti-Ad humoral response. Balb/c mice received an intravenous injection of Ad-CMV-Luc (10^9 iu/mouse) at day 0 of the experiment. One subset of animals ($n = 5$) were treated with 500 μg anti-D plus anti-M antibodies by intraperitoneal injection at days −3, −1 and 4 (B-depl. group). Other groups received an equivalent dose of unspecific rat IgG (Cont) or were left untreated (Cont2). (A) Efficacy of depletion (percentage of B cells), using as a reference the values prior to mAb administration. PBLs were stained with anti-CD19 and anti-B220 mAbs and analysed by flow cytometry. (B) Evolution of transgene expression analysed by BLI. All mice received a second injection of Ad-CMV-Luc at day 77 (indicated with an arrow), and BLI continued until the next day. The graphic represents the relative light emission, using as a reference the initial luminescence in the control group. (C) Presence of anti-Ad NAbs in serum before virus re-administration in the indicated groups. ud, undetectable.

described, this effect can be caused by the absence of IgM in these animals (39).

Depletion of NK cells does not improve Ad-mediated liver transduction

In order to analyse the influence of NK cells on Ad-mediated liver transduction, this cell population was depleted by intraperitoneal injection of a standard anti-asialo-GM1 Ab (100 μg/mouse) on days −6, −2, 5, 12, 19 and 27 relative to Ad-CMV-Luc administration. Control animals received vehicle alone, since no effect of unspecific rabbit IgG was observed in previous experiments. This protocol achieved a drastic reduction of NK cells at the time of Ad injection (Fig. 4A), although their number progressively recovered in subsequent weeks. However, analysis of transgene expression over time revealed no differences between NK cell-depleted and control animals (Fig. 4B). Once luciferase activity declined below 1% of initial values, a second administration of Ad-CMV-Luc was performed. Compared with the first viral dose, an equivalent loss of efficacy was observed in both groups, which is consistent with the amount of NAbs generated (Fig. 4C). Therefore, depletion of NK cells during Ad administration neither stabilizes transgene expression nor allows efficient redosing of the virus.

Depletion of $CD4^+$ T lymphocytes reduces anti-Ad humoral response and allows efficient re-infection

Next, we investigated the effect of selective $CD4^+$ or $CD8^+$ T lymphocyte depletions on the performance of the Ad-CMV-Luc vector. Mice were treated with several doses of anti-CD4 or anti-CD8 antibodies (100 μg/mouse) during 1 month, starting 1 week before infection, as described in previous section. Double depletions were not pursued, since pilot experiments showed interference between both antibodies and only partial depletions were achieved, especially in the case of $CD4^+$ T cells (not shown). Regarding single depletions, $CD4^+$ cell population was efficiently suppressed, and started to recover soon after antibody withdrawal (Fig. 5A). Treatment with the anti-CD8 antibody caused a parallel decline in $CD8^+$ T cells during the first 2 weeks, and then a very slow recovery, with counts still below 50% of normal values 1 month after the last dose of antibody. A relative increase in $CD4^+$ T cells was observed in these animals. Under these circumstances, transgene expression was partially stabilized compared with non-depleted animals, with no significant differences between $CD4^+$ and $CD8^+$ T-cell depletions. This stabilization correlated with a slower decline in the vector copy numbers in the liver of mice, analysed in an independent study in which

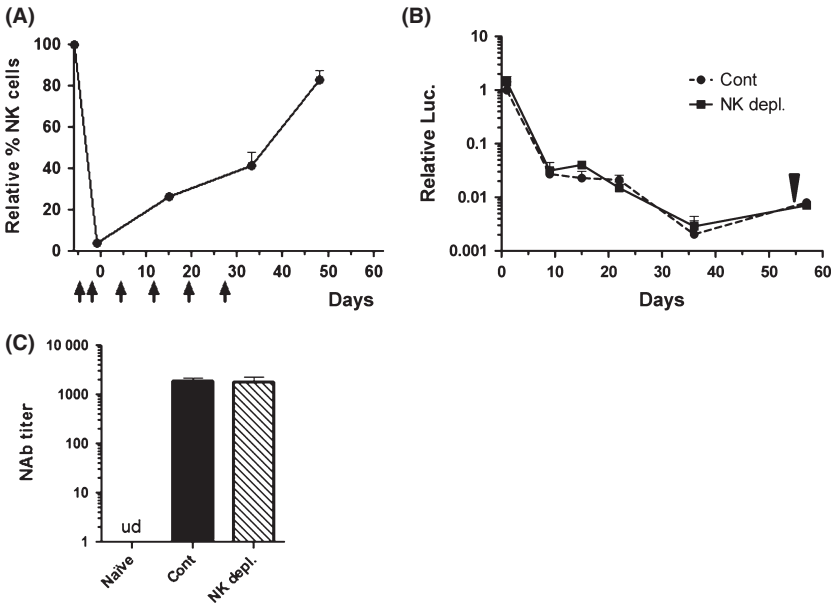

Fig. 4. Depletion of NK cells does not improve Ad-mediated liver transduction. Balb/c mice received an intravenous injection of Ad-CMV-Luc (10^9 iu/mouse) at day 0 of the experiment. Different subsets of animals ($n = 6$) were treated with 100 μg of rabbit ploYclonal anti-asialo-GM1 Ab by intraperitoneal injection at days −6, −2, 5, 12, 19 and 27, or were left untreated (Cont). (A) Efficacy of depletion (percentage of NK cells), using as reference the values prior to Ad administration. PBLs were stained with anti- CD335 (NKp46) and anti-CD3 mAbs and analysed by flow cytometry. (B) Evolution of transgene expression analysed by BLI. All mice received a second injection of Ad-CMV-Luc at day 56 (indicated with an arrow), and BLI continued until the next day. The graphic represents the relative light emission, using as a reference the initial luminescence in the control group. (C) Presence of anti-Ad NAbs in serum before virus re-administration in the indicated groups. ud, undetectable.

subsets of animals were sacrificed at 5 or 30 days after infection (Fig. S4). Luciferase activity was still detectable 4 months after infection (Fig. 5B), and it took approximately 9 months to become indistinguishable from control mice, close to background luminescence (Fig. 5C). Interestingly, we found that anti-Ad NAbs were virtually undetectable in $CD4^+$ T cell-depleted animals, whereas the titre was not reduced by $CD8^+$ T depletion (Fig. 5D). At this point, we performed re-administration of the Ad-CMV-Luc vector. In agreement with the titre of NAbs, we observed efficient liver transduction only in animals devoid of $CD4^+$ T cells at the time of initial infection (Fig. 5C). The persistence of luciferase expression over extended periods of time after an initial decay could be as a result of genome integration of the transgene in a small population of hepatocytes (9). In order to rule out this possibility, we performed partial hepatectomies in a subgroup of animals that received an intravenous injection of the Ad-CMV-Luc vector in the context of $CD4^+$ or $CD8^+$ T-cell depletion, as described above. Two thirds of liver parenchyma (left and medial lobes) was surgically removed 4 months after infection, when luciferase expression was in a stable period. A proportional reduction in light emission was typically observed in all mice (see one representative example of each group in Fig. 6). Subsequent BLI monitoring revealed that light emission did not recover after hepatectomy, and remained localized in the preserved liver sections (right and caudate lobes), whereas newly regenerated tissue was negative. This result indicates that any potential vector integration cannot play a major role in the stabilization of transgene expression observed here. Animals were followed until luminescence was close to background, and then a second dose of the Ad-CMV-Luc vector revealed that only the liver of animals that were originally depleted of $CD4^+$ T cells could be efficiently transduced, in correlation with the absence of NAbs. Regarding potential virus-mediated toxicity in mice partially immunosuppressed by $CD4^+$ T-cell depletion, no relevant elevation of liver enzymes were observed compared with non-depleted animals receiving the same dose of vector (Fig. S5).

Depletion of $CD4^+$ T lymphocytes reduces cellular responses against vectors and transgenes

The fact that $CD4^+$ T-cell depletion not only reduced anti-Ad humoral responses, but also stabilized transgene expression, led us to investigate the importance of this population in activation of cellular responses against the vector and the transgene. To this end, we treated mice with a first-generation Ad vector encoding

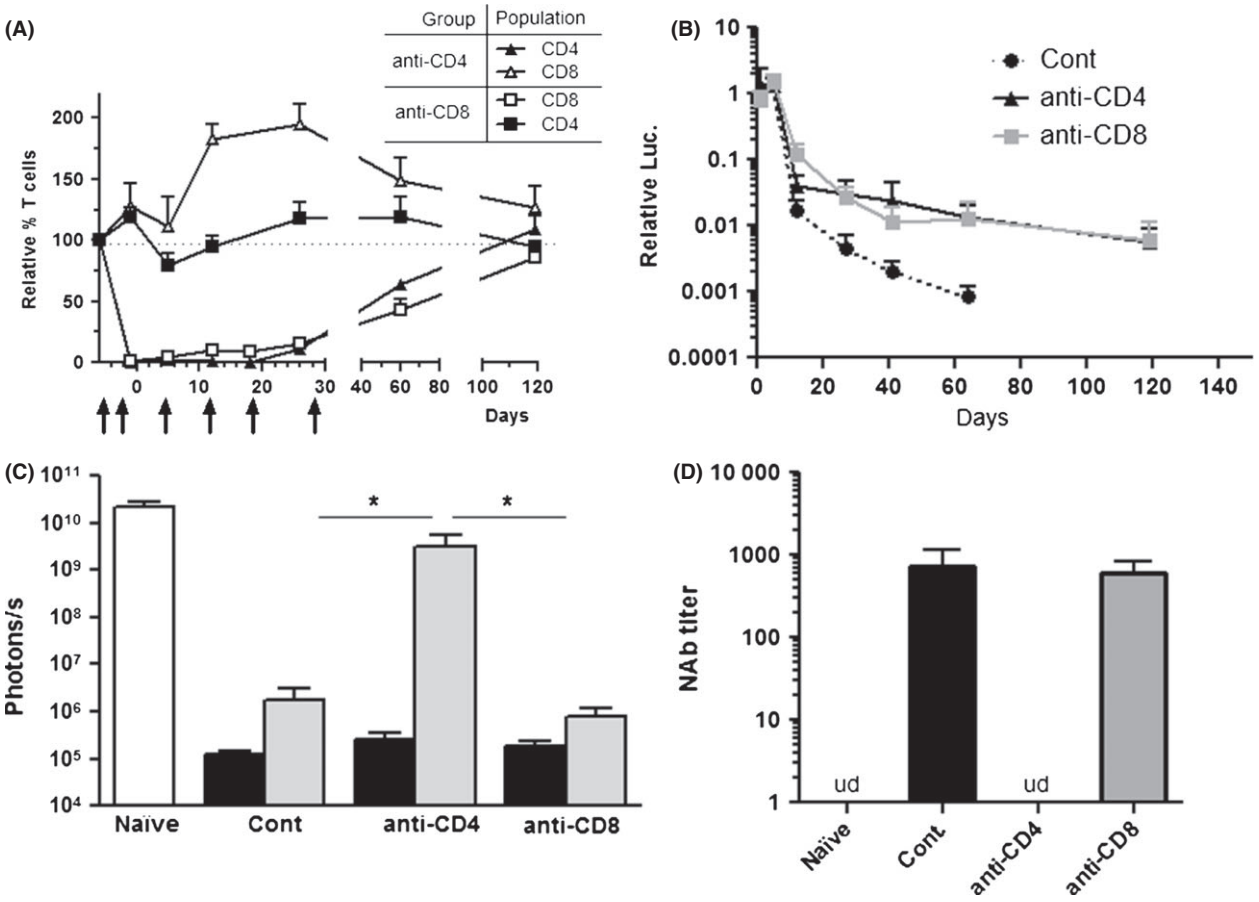

Fig. 5. Depletion of CD4$^+$ T lymphocytes reduces anti-Ad humoral response and allows efficient re-infection. Balb/c mice received an intravenous injection of Ad-CMV-Luc (10^9 iu/mouse) at day 0 of the experiment. Different subsets of animals ($n = 7$) were treated with 100 μg of anti-CD4 or anti-CD8 mAbs by intraperitoneal injection at days −6, −2, 5, 12, 19 and 27. Other group received the same total dose of unspecific rat IgG (Cont). (A) Efficacy of depletion (percentage of CD4 or CD8 cells), using as a reference the values prior to mAb administration. PBLs were stained with anti-CD4 and anti-CD8 mAbs and analysed by flow cytometry. (B) Evolution of transgene expression analysed by BLI. Differences between the anti-CD4 or anti-CD8 and the Cont groups were significant ($P = 0.03$). (C) Animals were kept until luminescence decreased below 10^6 photons/s in all groups (10 months after infection). At this moment, the same dose of Ad-CMV-Luc was administered to all groups. The graphic shows light emission (in photons/s) before (black columns) or 24 h after re-infection (grey columns). Naïve animals were used as control (white column). (D) Presence of anti-Ad NAbs in serum before virus re-administration in the indicated groups. *$P < 0.05$. ud, undetectable.

the model antigen β-galactosidase (AdLacZ) at day 0 of the experiment. Some animals received the anti-CD4 antibody on days −6, −2 and 5, whereas others were left untreated. Two days later (1 week after virus administration) they were sacrificed, together with a group of naïve (mock-infected) mice. The frequency of specific CD8$^+$ T lymphocytes against the transgene was determined by pentamer staining among leucocytes infiltrating the liver. The result shown in Fig. 7A indicates a significant reduction in this cell population in the absence of CD4$^+$ T cells. In addition, their cytotoxic potential was diminished, as indicated by the granzyme expression of βGal-specific CD8 T cells (Fig. 7B). Furthermore, when liver infiltrating leucocytes were exposed to the dominant MHC-I restricted peptide from βGal protein ($\beta Gal_{876\text{-}884}$ peptide), activation of IFNγ expression was suppressed (Fig. 7C). Interestingly, these differences were evident in the liver but not in peripheral blood since the frequency of circulating βGal-specific CD8 T cells was very low (not shown). To evaluate cellular responses against the vector, liver infiltrating leucocytes from the different experimental groups were exposed to Ad capsids *in vitro*, and stimulation of cell proliferation was measured by thymidine incorporation. As shown in Fig. 7D, a significant reduction was observed in CD4$^+$ T cell-depleted animals. Together, this set of results indicates that these cells are crucial not only for humoral, but also cellular immune responses against Ad vectors and transgenes.

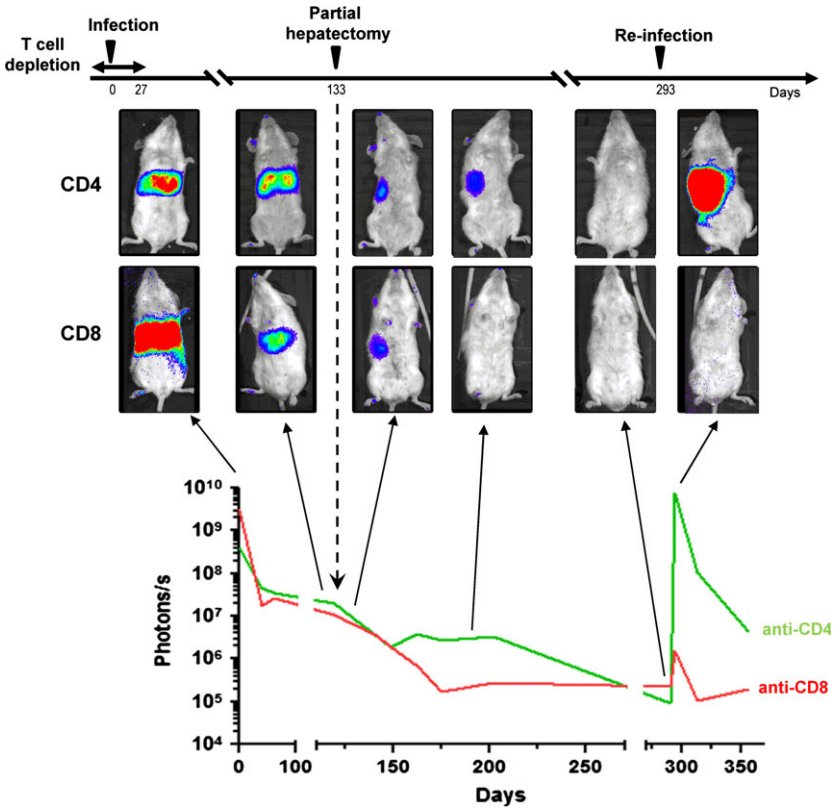

Fig. 6. Stabilization of transgene expression mediated by T-cell depletion does not involve vector integration**.** Balb/c mice received an intravenous injection of Ad-CMV-Luc (10^9 iu/mouse) at day 0 of the experiment. Different subsets of animals ($n = 5$) were treated with 100 μg of anti-CD4 or anti-CD8 mAbs by intraperitoneal injection at days −6, −2, 5, 12, 19 and 27. Four months after infection, mice underwent partial hepatectomy, as indicated in the schematic representation of the protocol. Transgene expression was analysed by BLI at different times until the signal virtually disappeared (10 months after infection). At this point, mice received a second administration of the Ad-CMV-Luc. The graphic represents luciferase activity (photons/s) of one representative animal in each group. Pictures were taken at the times indicated by the arrows, including before and after hepatectomy and re-infection. In the artificial colour code, red and blue represent maximal and minimal light emission respectively.

Clodronate liposomes impair antibody-mediated depletion of $CD4^+$ T lymphocytes and preclude efficient redosing of Ad

Taking into account the results described so far, the combination of clodronate liposomes and anti-CD4 antibody seemed a promising pharmacological intervention to improve the performance of Ad vectors. In principle, this would increase liver transduction, stabilize expression and inhibit the appearance of NAbs, thus allowing efficient re-administrations. When we applied this protocol to our liver transduction model in Balb/c mice, the first observation was that the extent of $CD4^+$ T-cell depletion at the time of virus infection was lower in animals receiving both clodronate liposomes and anti-CD4 antibody (Fig. 8A). Data from BLI in this group was compatible with full effect of clodronate liposomes, i.e. increased and sustained liver transduction, compared with the control animals (Fig. 8B). However, when we analysed anti-Ad NAbs 2 months after infection, we found that the combination treatment only produced a small reduction in the titres, as opposed to complete protection in the anti-CD4 only group (Fig. 8D). Consistently, the efficacy of subsequent Ad.hIL12 administration was not fully preserved, as determined by human IL-12 quantification in serum (Fig. 8C).

Transient depletion of $CD4^+$ T lymphocytes improves the efficacy of intratumoral Ad redosing

Ad vectors (either defective or replicative) have been extensively tested for the treatment of cancer patients following repeated intratumoral administrations. However, the rapid appearance of NAbs drastically reduces the efficacy of this approach (40). In order to assess if a brief depletion of $CD4^+$ T lymphocytes could improve Ad redosing in tumours, we used an immunocompetent (syngeneic) tumour model based on subcutaneous injection of liver cancer cells in Balb/c mice. The Ad-CMV-Luc vector was administered by intratumoral injection in animals depleted or not of $CD4^+$ T cells.

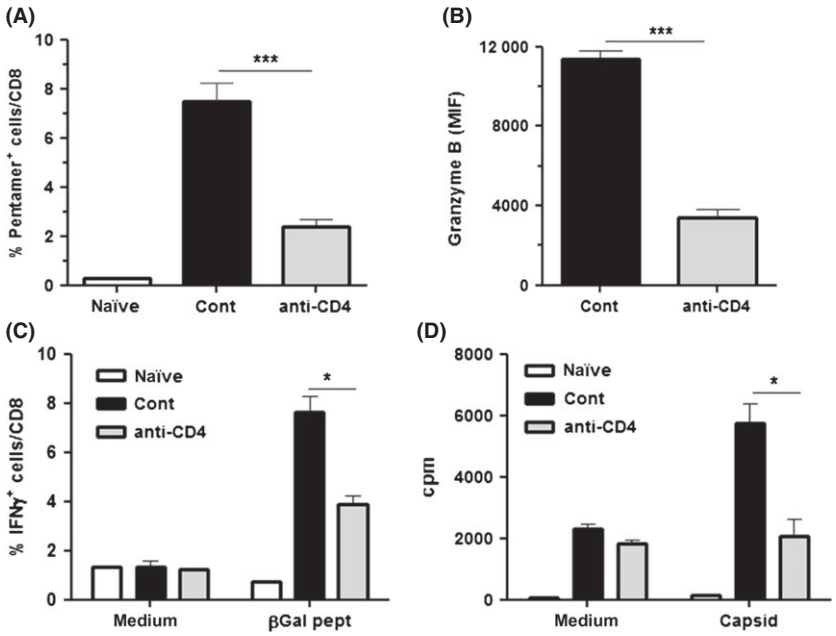

Fig. 7. Depletion of $CD4^+$ T lymphocytes inhibits $CD8^+$ T cell responses against transgene and vector. Balb/c mice received an intravenous injection of AdLacZ (10^9 iu/mouse) at day 0 of the experiment. Different subsets of animals ($n = 7$) were treated with 100 μg of anti-CD4 by intraperitoneal injection at days −6, −2 and 5. Mice were sacrificed at day 7 and liver infiltrating leucocytes were isolated by Percoll gradient. (A) Frequency of βGal-specific CD8 T cells among total liver infiltrating CD8 T cells determined by pentamer staining. (B) Granzyme B expression levels of βGal-specific CD8 T cells. Expression levels of Granzyme B are depicted as Median Intensity fluorescence (MIF). Data shown correspond to cells gated as βGal-pentamer$^+$$CD8^+$. (C) Percentage of $IFN\gamma^+CD8^+$ T cells upon re-stimulation with βGal-peptide. (D) *In vitro* responses to adenoviral capsids measured by ^{3}H-Thy incorporation of liver infiltrating leucocytes. Results are expressed as the mean ± SEM and are representative of two independent experiments. $^*P < 0.05$; $^{***}P < 0.001$.

Our results show that mice with an intact immune system mount a humoral response against the virus that decreases transgene expression of a second Ad dose given 2 weeks later (50-fold reduction, Fig. 9A). In contrast, NAb formation was abolished by the anti-CD4 mAb (Fig. 9B), and the efficacy of the second intratumoral Ad injection was preserved. Depletion of $CD4^+$ T cells did not favour the progression of tumours (not shown).

Discussion

The activation of the host innate and adaptive immune response following systemic administration of Ad vectors constitutes a principal impediment to successful clinical gene replacement therapies. Taking into account the defective nature of the Ad vectors used for this indication, the immune system is no longer a safeguard against potential deleterious effects. Instead, innate responses account for most of the acute, potentially life-threatening toxicity observed with high doses of Ad vectors (2). This inflammatory reaction against viral capsids is mainly mediated by macrophage activation and production of IL-6 and IL-12, and it cannot be avoided by genome modifications in the vector. Thus, mitigation of this process is crucial to increase the safety of any kind of Ad vector. Consistent with previous observations (26, 27), we found that transient depletion of Kupffer cells and splenic macrophages by a single dose of clodronate liposomes exerts a strong anti-inflammatory effect in animals receiving high doses of first-generation Ad vectors. Apart from the reduction in cytokine release, we provide new data supporting that clodronate liposomes drastically inhibit activation of crucial components of the immune system such as $CD4^+$, $CD8^+$ and NK cells in spleen and liver. These observations offer a logical explanation for the long-term stability of transgene expression achieved with this reagent. Thus, a significant amount of evidence from different groups has been accumulated over more than a decade, indicating the need for clinical testing of systemic administration of clodronate liposomes. If this substance (now approved for loco-regional applications in rheumatoid diseases) was well-tolerated upon intravenous injection, it would open new possibilities for Ad-based liver gene therapy. In the meantime, some substitutes with potential interference with macrophage function have been proposed, such as the contrast agent gadolinium (41) or high fat emulsions for parenteral nutrition (35). However, it is not clear if these substances will have the same properties as clodronate liposomes. Intravenous administration of gadolinium or the bisphosphonate zoledronic acid, which is toxic for macrophages (42), had no positive effect on Ad-mediated liver transduction in our experimental conditions (not

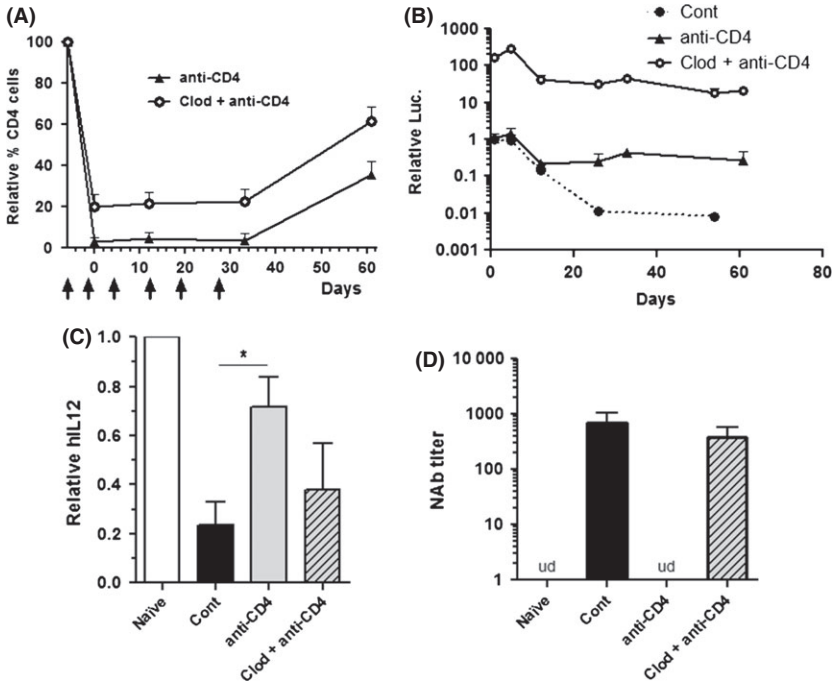

Fig. 8. Clodronate liposomes impair antibody-mediated CD4+ T-cell depletion and efficient vector re-administration. Balb/c mice received an intravenous injection of Ad-CMV-Luc (10^9 iu/mouse) at day 0 of the experiment. Different subsets of animals ($n = 7$) were treated with 100 μg of anti-CD4 by intraperitoneal injection at days −6, −2, 5, 12, 19 and 27, in the absence (anti-CD4 group) or presence (Clod + anti-CD4 group) of clodronate liposome treatment (2 μl/g intravenously at day −1). Other group received the same total dose of unspecific rat IgG (Cont). (A) Efficacy of depletion (percentage of CD4 cells), using as a reference the values prior to mAb administration. PBLs were stained with anti-CD4 mAbs and analysed by flow cytometry. (B) Evolution of transgene expression analysed by BLI. Differences between the Cont group and the anti-CD4 or Clod + anti-CD4 groups were significant ($P = 0.005$ and $P = 0.001$ respectively). (C) All mice received an intravenous injection of the Ad-CMV-hIL12 virus, and the presence of hIL-12 was detected in serum 48 h later by ELISA. The graphic represents the relative concentration of the cytokine, using the naïve group as a reference. (D) Presence of anti-Ad NAbs in serum before virus re-administration in the indicated groups. *$P = 0.05$. ud, undetectable.

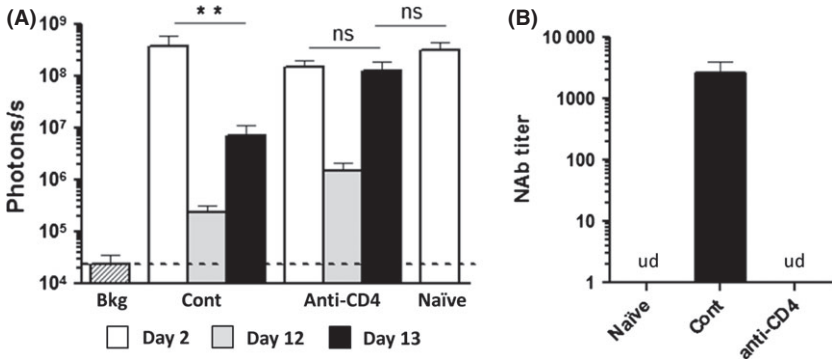

Fig. 9. Transient depletion of CD4+ T lymphocytes improves the efficacy of intratumoral Ad redosing. Tumours were established by subcutaneous inoculation of BNL cells in Balb/c mice. Two doses of Ad-CMV-Luc (5×10^8 iu) were administered intratumorally at days 0 and 12. One group of animals ($n = 5$) received 100 μg of the anti-CD4 mAb on days −6, −2, 5 and 12, whereas the other group (Cont) was left untreated. The naïve group corresponds to animals that received only the second dose of vector. (A) Transgene expression determined by BLI in the tumour area at the indicated times. Background (Bkg) corresponds to light emission from mice before virus administration (B) Presence of anti-Ad NAbs in serum before vector redosing. *$P = 0.03$; **$P = 0.006$; ns: non-significant difference.

shown). Functional inhibition of macrophages with dexamethasone led to a moderate increase in the initial transgene expression, but not stabilization. Regarding specific lymphocyte depletions using mAbs, we found that prolongation of transgene expression in the liver was equivalent when $CD8^+$ or $CD4^+$ cells were transiently removed. This is in agreement with the inhibition of $CD8^+$ activation observed in the liver in the

absence of $CD4^+$ cells. Other factors such as direct vector toxicity and transcriptional silencing can contribute to the decline in transgene expression in the liver (43). The relative importance of these factors may depend on viral dose, route of administration, properties of the transgene, and the characteristics of the host. Targeting of $CD4^+$ cells has the important added advantage of drastically inhibiting anti-Ad humoral responses and allowing efficient redosing of the vector. This effect was highly reproducible and was not observed with any of the other treatments, including clodronate liposomes, dexamethasone, B cell, NK cell or $CD8^+$ T-cell depletions. Our data provide new arguments in favour of transient $CD4^+$ T-cell depletion for efficient Ad redosing. Previous works had found only partial preservation of vector efficacy upon re-administration (28, 29), probably because their depletion was less intense. In fact we found that any interference with the ability of the anti-CD4 antibody to deplete the target cells (such as concomitant anti-CD8 administration or macrophage depletion) impaired the protection from humoral responses. The need for efficient depletion should be taken into consideration for potential application of clinically compatible anti-CD4 mAbs such as Zanolimumab (44) in gene therapy approaches. Current clinical trials using this drug for the treatment of inflammatory diseases and T-cell lymphoma have demonstrated specific and efficient $CD4^+$-specific antibody-mediated cell-dependent cytotoxicity (45). Importantly, $CD4^+$ cell depletion for more than 2 months was well tolerated. In our Balb/c model, six doses of the anti-mouse antibody spanning 1 month were highly efficient in preventing anti-Ad humoral responses, and recovery of $CD4^+$ cells was relatively fast upon discontinuation, in contrast to B or $CD8^+$ T-cell depletions. If these observations were recapitulated in humans, this protocol would be safe and fully compatible with long-term gene replacement strategies. In contrast, targeting B cells seems more challenging, since we and others (16) have shown that the extent of depletion obtained with mAbs is not sufficient to abrogate anti-Ad humoral responses. Using a knock-out animal (μMT mice) we have verified that a complete lack of B cells abolishes anti-Ad NAb production and allows efficient re-administration of Ad vectors. Interestingly, these mice show an initial increase in transgene expression upon Ad administration compared with wild type mice, as shown here in animals treated with anti-B cell antibodies. This recapitulates recent observations in another knock-out model lacking B cells (J_HD mice). The effect has been attributed to the lack of IgM in the serum of these mice, since natural antibodies and complement are able to neutralize Ad5 (39). Regarding NK cells, depletion in Balb/c mice using the standard anti-asialo-GM1 Ab was efficient at the time of virus administration. This allows us to conclude that at least in our assay conditions, these cells are not a major barrier for Ad-mediated liver transduction. The progressive recovery of NK cells after the first 3 doses of Ab may be because of anti-asialo GM1 Ab neutralization by endogenous anti-rabbit immunoglobulins elicited in mice.

There are conflicting results in the literature regarding the effect of NK cell depletion on Ad transduction. Some works report an increase in liver transduction early after administration of high viral doses (46). However, other authors (47) describe no differences during the first week and then only a transient increase in NK cell-depleted animals. Only simultaneous inhibition of CD8 functions achieved a robust and sustained improvement in liver transduction. Differences may be because of the viral dose and the transgene expressed by the vector. Together with our data, these results suggest that NK cells are not the main barrier to Ad transduction in the liver when moderate, clinically relevant doses are used. Other method proposed for efficient redosing of Ad vectors is the blockade of CD40 ligand (18, 21). However, there is not general agreement on the efficacy of anti-CD40 ligand mAbs as single agents for liver transduction (21, 28). It has been reported that CD8 T cell responses induced after infection by Ad are dependent on CD4 help (48) but not on CD40 (49) and we show here that CD8 T cell response impairment is important to guarantee a prolonged transgene expression. Interestingly, combination of CD40 blockade with clodronate liposomes reduces acute Ad-mediated inflammation, increases liver transduction, stabilizes transgene expression and allows redosing in mice (19, 20). However, taking into account the increased risk of thromboembolism associated with anti-CD40 ligand mAbs in patients (23), transient depletion of $CD4^+$ T cells with Zanolimumab seems a better option. In order to obtain a similar cooperation between clodronate liposomes and anti-CD4 mAbs, optimization of the protocol is needed to preserve the efficacy of $CD4^+$ cell depletion.

We believe the improvements described here are mainly applicable to the treatment of monogenic diseases. The case of cancer patients is more complex, and the convenience of selective cell depletions will depend on the tumour and the type of gene therapy being applied. We have shown here that transient $CD4^+$ T-cell depletion improves the efficacy of intratumoral Ad redosing. There is some controversy about the impact of anti-viral humoral responses on the efficacy of intratumoral injections. The importance of this phenomenon may be different depending on the type and dose of virus and the characteristics of the tumour. Strong inhibition has been observed both in animal models (50) and in humans (40). In the Balb/c mice syngeneic model used here the blockade was not absolute, but still there was a 50-fold drop in transgene expression in the second virus injection. In principle, stabilization of expression and the possibility of multiple vector administrations should increase the efficacy of many cancer gene therapy approaches such as suicide gene therapy, oncogene inhibition or expression of anti-angiogenic genes.

Although the lack of $CD4^+$ T helper cells may impair tumour control in some circumstances, concomitant reduction of T regulatory cells may be beneficial. In preclinical models, the efficacy of immunogene therapy based on expression of cytokines such as interleukin-12 was largely dependent on $CD8^+$, but not $CD4^+$ T cells (51, 52). Therefore, transient $CD4^+$ T-cell depletion may result in a positive net balance also in adenovirus-mediated cancer gene therapy.

In summary, our data indicate that $CD4^+$ T lymphocytes are a clinically feasible target to circumvent humoral and cellular anti-Ad immune responses. Considering single agent interventions, anti-CD4 depleting antibodies showed the most favourable effect on liver transduction, although concomitant inhibition of innate immune responses would be desirable to increase the safety of Ad vectors.

Acknowledgements

We thank Dr Claude Lecler (Institut Pasteur, France) for the μMT mice and Paul Miller for English editing. *Financial support:* This study has been funded by grants BIO2009/09295, SAF2009-08524, SAF2012-39578, SAF2012-40003, SAF2012-39504, CONSOLIDER INGENIO 2010 from the Spanish Department of Science, RNAREG [CSD2009-00080], Fondo de Investigación Sanitaria (PI11/02327), Fundación Barrié de la Maza, Condesa de Fenosa and the UTE project CIMA. S.H.-S. was supported by MEC (RYC-2007-00928).

Conflict of interest: The authors do not have any disclosures to report.

References

1. Ginn SL, Alexander IE, Edelstein ML, Abedi MR, Wixon J. Gene therapy clinical trials worldwide to 2012 – an update. *J Gene Med* 2013; **15**: 65–77.
2. Raper SE, Chirmule N, Lee FS, *et al.* Fatal systemic inflammatory response syndrome in a ornithine transcarbamylase deficient patient following adenoviral gene transfer. *Mol Genet Metab* 2003; **80**: 148–58.
3. Yang Y, Jooss KU, Su Q, Ertl HC, Wilson JM. Immune responses to viral antigens versus transgene product in the elimination of recombinant adenovirus-infected hepatocytes in vivo. *Gene Ther* 1996; **3**: 137–44.
4. Morral N, O'Neal W, Rice K, *et al.* Administration of helper-dependent adenoviral vectors and sequential delivery of different vector serotype for long-term liver-directed gene transfer in baboons. *Proc Natl Acad Sci USA* 1999; **96**: 12816–21.
5. Tomita K, Sakurai F, Tachibana M, Mizuguchi H. Correlation between adenovirus-neutralizing antibody titer and adenovirus vector-mediated transduction efficiency following intratumoral injection. *Anticancer Res* 2012; **32**: 1145–52.
6. Zaiss AK, Machado HB, Herschman HR. The influence of innate and pre-existing immunity on adenovirus therapy. *J Cell Biochem* 2009; **108**: 778–90.
7. Sun C, Zhang Y, Feng L, *et al.* Epidemiology of adenovirus type 5 neutralizing antibodies in healthy people and AIDS patients in Guangzhou, southern China. *Vaccine* 2011; **29**: 3837–41.
8. Thorner AR, Vogels R, Kaspers J, *et al.* Age dependence of adenovirus-specific neutralizing antibody titers in individuals from sub-Saharan Africa. *J Clin Microbiol* 2006; **44**: 3781–3.
9. Stephen SL, Montini E, Sivanandam VG, *et al.* Chromosomal integration of adenoviral vector DNA in vivo. *J Virol* 2010; **84**: 9987–94.
10. Haisma HJ, Bellu AR. Pharmacological interventions for improving adenovirus usage in gene therapy. *Mol Pharm* 2011; **8**: 50–5.
11. Alba R, Bosch A, Chillon M. Gutless adenovirus: last-generation adenovirus for gene therapy. *Gene Ther* 2005; **12** (Suppl. 1): S18–27.
12. Brunetti-Pierri N, Ng P. Helper-dependent adenoviral vectors for liver-directed gene therapy. *Hum Mol Genet* 2011; **20**: R7–13.
13. Bangari DS, Mittal SK. Current strategies and future directions for eluding adenoviral vector immunity. *Curr Gene Ther* 2006; **6**: 215–26.
14. Arruda VR, Favaro P, Finn JD. Strategies to modulate immune responses: a new frontier for gene therapy. *Mol Ther* 2009; **17**: 1492–503.
15. Seregin SS, Appledorn DM, McBride AJ, *et al.* Transient pretreatment with glucocorticoid ablates innate toxicity of systemically delivered adenoviral vectors without reducing efficacy. *Mol Ther* 2009; **17**: 685–96.
16. Fontanellas A, Hervas-Stubbs S, Mauleon I, *et al.* Intensive pharmacological immunosuppression allows for repetitive liver gene transfer with recombinant adenovirus in nonhuman primates. *Mol Ther* 2010; **18**: 754–65.
17. Jooss K, Turka LA, Wilson JM. Blunting of immune responses to adenoviral vectors in mouse liver and lung with CTLA4Ig. *Gene Ther* 1998; **5**: 309–19.
18. Kay MA, Meuse L, Gown AM, *et al.* Transient immunomodulation with anti-CD40 ligand antibody and CTLA4Ig enhances persistence and secondary adenovirus-mediated gene transfer into mouse liver. *Proc Natl Acad Sci USA* 1997; **94**: 4686–91.
19. Stein CS, Pemberton JL, van Rooijen N, Davidson BL. Effects of macrophage depletion and anti-CD40 ligand on transgene expression and redosing with recombinant adenovirus. *Gene Ther* 1998; **5**: 431–9.
20. Kuzmin AI, Galenko O, Eisensmith RC. An immunomodulatory procedure that stabilizes transgene expression and permits readministration of E1-deleted adenovirus vectors. *Mol Ther* 2001; **3**: 293–301.
21. Yang Y, Su Q, Grewal IS, *et al.* Transient subversion of CD40 ligand function diminishes immune responses to adenovirus vectors in mouse liver and lung tissues. *J Virol* 1996; **70**: 6370–7.
22. Haegel-Kronenberger H, Haanstra K, Ziller-Remy C, *et al.* Inhibition of costimulation allows for repeated systemic administration of adenoviral vector in rhesus monkeys. *Gene Ther* 2004; **11**: 241–52.
23. Boumpas DT, Furie R, Manzi S, *et al.* A short course of BG9588 (anti-CD40 ligand antibody) improves serologic activity and decreases hematuria in patients with proliferative lupus glomerulonephritis. *Arthritis Rheum* 2003; **48**: 719–27.

24. Robles-Carrillo L, Meyer T, Hatfield M, *et al.* Anti-CD40L immune complexes potently activate platelets in vitro and cause thrombosis in FCGR2A transgenic mice. *J Immunol* 2010; **185**: 1577–83.
25. Khare R, Chen CY, Weaver EA, Barry MA. Advances and future challenges in adenoviral vector pharmacology and targeting. *Curr Gene Ther* 2011; **11**: 241–58.
26. Kuzmin AI, Finegold MJ, Eisensmith RC. Macrophage depletion increases the safety, efficacy and persistence of adenovirus-mediated gene transfer in vivo. *Gene Ther* 1997; **4**: 309–16.
27. Schiedner G, Hertel S, Johnston M, *et al.* Selective depletion or blockade of Kupffer cells leads to enhanced and prolonged hepatic transgene expression using high-capacity adenoviral vectors. *Mol Ther* 2003; **7**: 35–43.
28. McClane SJ, Chirmule N, Burke CV, Raper SE. Characterization of the immune response after local delivery of recombinant adenovirus in murine pancreas and successful strategies for readministration. *Hum Gene Ther* 1997; **8**: 2207–16.
29. Ye X, Robinson MB, Pabin C, Batshaw ML, Wilson JM. Transient depletion of CD4 lymphocyte improves efficacy of repeated administration of recombinant adenovirus in the ornithine transcarbamylase deficient sparse fur mouse. *Gene Ther* 2000; **7**: 1761–7.
30. Mazzolini G, Qian C, Narvaiza I, *et al.* Adenoviral gene transfer of interleukin 12 into tumors synergizes with adoptive T cell therapy both at the induction and effector level. *Hum Gene Ther* 2000; **11**: 113–25.
31. Sangro B, Mazzolini G, Ruiz J, *et al.* Phase I trial of intratumoral injection of an adenovirus encoding interleukin-12 for advanced digestive tumors. *J Clin Oncol* 2004; **22**: 1389–97.
32. Chentoufi AA, Nizet Y, Havaux X, *et al.* Differential effects of injections of anti-mu and anti-delta monoclonal antibodies on B-cell populations in adult mice: regulation of xenoreactive natural antibody-producing cells. *Transplantation* 1999; **68**: 1728–36.
33. Van Rooijen N, Sanders A. Liposome mediated depletion of macrophages: mechanism of action, preparation of liposomes and applications. *J Immunol Methods* 1994; **174**: 83–93.
34. Perruche S, Zhang P, Liu Y, *et al.* CD3-specific antibody-induced immune tolerance involves transforming growth factor-beta from phagocytes digesting apoptotic T cells. *Nat Med* 2008; **14**: 528–35.
35. Snoeys J, Mertens G, Lievens J, *et al.* Lipid emulsions potently increase transgene expression in hepatocytes after adenoviral transfer. *Mol Ther* 2006; **13**: 98–107.
36. Sangro B, Melero I, Qian C, Prieto J. Gene therapy of cancer based on interleukin 12. *Curr Gene Ther* 2005; **5**: 573–81.
37. Muratore CS, Harty MW, Papa EF, Tracy TF Jr. Dexamethasone alters the hepatic inflammatory cellular profile without changes in matrix degradation during liver repair following biliary decompression. *J Surg Res* 2009; **156**: 231–9.
38. Berglof A, Sandstedt K, Feinstein R, Bolske G, Smith CI. B cell-deficient muMT mice as an experimental model for Mycoplasma infections in X-linked agammaglobulinemia. *Eur J Immunol* 1997; **27**: 2118–21.
39. Xu Z, Qiu Q, Tian J, *et al.* Coagulation factor X shields adenovirus type 5 from attack by natural antibodies and complement. *Nat Med* 2013; **19**: 452–7.
40. Penuelas I, Mazzolini G, Boan JF, *et al.* Positron emission tomography imaging of adenoviral-mediated transgene expression in liver cancer patients. *Gastroenterology* 2005; **128**: 1787–95.
41. Lieber A, He CY, Meuse L, *et al.* The role of Kupffer cell activation and viral gene expression in early liver toxicity after infusion of recombinant adenovirus vectors. *J Virol* 1997; **71**: 8798–807.
42. Moreau MF, Guillet C, Massin P, *et al.* Comparative effects of five bisphosphonates on apoptosis of macrophage cells in vitro. *Biochem Pharmacol* 2007; **73**: 718–23.
43. Mian A, Guenther M, Finegold M, *et al.* Toxicity and adaptive immune response to intracellular transgenes delivered by helper-dependent vs. first generation adenoviral vectors. *Mol Genet Metab* 2005; **84**: 278–88.
44. Reichert JM. Antibody-based therapeutics to watch in 2011. *MAbs* 2011; **3**: 76–99.
45. Rider DA, Havenith CE, de Ridder R, *et al.* A human CD4 monoclonal antibody for the treatment of T-cell lymphoma combines inhibition of T-cell signaling by a dual mechanism with potent Fc-dependent effector activity. *Cancer Res* 2007; **67**: 9945–53.
46. Geng J, Wang X, Wei H, Sun R, Tian Z. Efficient attenuation of NK cell-mediated liver injury through genetically manipulating multiple immunogenes by using a liver-directed vector. *J Immunol* 2013; **190**: 4821–9.
47. Peng Y, Falck-Pedersen E, Elkon KB. Variation in adenovirus transgene expression between BALB/c and C57BL/6 mice is associated with differences in interleukin-12 and gamma interferon production and NK cell activation. *J Virol* 2001; **75**: 4540–50.
48. Yang Y, Xiang Z, Ertl HC, Wilson JM. Upregulation of class I major histocompatibility complex antigens by interferon gamma is necessary for T-cell-mediated elimination of recombinant adenovirus-infected hepatocytes in vivo. *Proc Natl Acad Sci USA* 1995; **92**: 7257–61.
49. Van Deusen KE, Rajapakse R, Bullock TN. CD70 expression by dendritic cells plays a critical role in the immunogenicity of CD40-independent, CD4+ T cell-dependent, licensed CD8+ T cell responses. *J Leukoc Biol* 2010; **87**: 477–85.
50. Bunuales M, Garcia-Aragoncillo E, Casado R, *et al.* Evaluation of monocytes as carriers for armed oncolytic adenoviruses in murine and Syrian hamster models of cancer. *Hum Gene Ther* 2012; **23**: 1258–68.
51. Mazzolini G, Qian C, Xie X, *et al.* Regression of colon cancer and induction of antitumor immunity by intratumoral injection of adenovirus expressing interleukin-12. *Cancer Gene Ther* 1999; **6**: 514–22.
52. Rodriguez-Madoz JR, Prieto J, Smerdou C. Semliki forest virus vectors engineered to express higher IL-12 levels induce efficient elimination of murine colon adenocarcinomas. *Mol Ther* 2005; **12**: 153–63.

Supporting information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Depletion of Kupffer cells by clodronate liposomes. Balb/c mice were treated or not with intravenous clodronate liposomes (2 μl/g of animal weight) and sacrificed 24 h later. Immunohistochemical detection of Kupffer cells in liver sections using anti-F4/80 antibody. Microphotographs show representative fields at 200× (left) and 400× (right) magnification.

Fig. S2. Long-term stabilization of hepatic transgene expression by clodronate liposomes. Mice received an intravenous injection of Ad-CMV-Luc (10^9 iu/mouse) at day 0 of the experiment. One subset of animals ($n = 5$) was treated with clodronate liposomes 24 h before infection (Clod group), whereas other was left untreated (Cont. group). Evolution of transgene expression analysed by BLI. The graph shows relative light emission for each group (average fold of photons/s), using as a reference the initial (day 1) luminescence in the control group. Differences between groups were significant ($P = 0.006$).

Fig. S3. Lack of anti-Ad humoral response in μMT mice. Wild type C57BL/6J mice or μMT knock-out mice lacking B cells ($n = 6$) received an intravenous injection of Ad-CMV-Luc (10^8 iu/mouse) at day 0 of the experiment. (A) Evolution of transgene expression analysed by BLI. The graph shows relative light emission for each group, using as a reference the initial (day 1) luminescence in the wild type group (in this case, 1.87×10^7 photons/s). (B) At the end of the observation period, all mice received an intravenous injection of the Ad.hIL12 virus, and the presence of hIL-12 was detected in serum 48 h later by ELISA. The graph represents the relative concentration of the cytokine, using an age-matched group of wild type or mutant naïve animals as a reference. (C) Presence of anti-Ad NAbs in the serum of the indicated groups before the second viral administration.

Fig. S4. Transient $CD4^+$ T-cell depletion stabilizes vector genome copy numbers in the liver. Balb/c mice received an intravenous injection of Ad-CMV-Luc (10^9 iu/mouse) at day 0 of the experiment. One group of animals ($n = 10$) was assigned to the CD4-depleted group, whereas other group received the same total dose of unspecific rat IgG (Cont). The complete depletion protocol consisted on intraperitoneal injections of 100 μg anti-CD4 mAb at days −6, −2, 5, 12, 19 and 27. A subset of mice from each group ($n = 5$) was sacrificed 5 or 30 days after vector administration, and viral copy numbers were quantified in liver samples by qPCR. The graph represents copy numbers per milligram of liver tissue.

Fig. S5. Transient $CD4^+$ T-cell depletion does not increase adenovirus-mediated liver damage. Balb/c mice received an intravenous injection of Ad-CMV-Luc (10^9 iu/mouse) at day 0 of the experiment. Different subsets of animals ($n = 7$) were treated with 100 μg of anti-CD4 or anti-CD8 mAbs by intraperitoneal injection at days −6, −2, 5, 12, 19 and 27. Other group received the same total dose of unspecific rat IgG (Cont). Blood collection was performed at the indicated days for determination of liver enzymes aspartate and alanine aminotransferases (AST and ALT, panels A and B respectively). Average normal values for each parameter are indicated with a dotted line.