

Human CD46-transgenic mice in studies involving replication-incompetent adenoviral type 35 vectors

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Wild-type strains of mice do not express CD46, a high-affinity receptor for human group B adenoviruses including type 35. Therefore, studies performed to date in mice using replication-incompetent Ad35 (rAd35) vaccine carriers may underestimate potency or result in altered vector distribution. Here, it is reported that CD46 transgenic mice (MYII-strain) express CD46 in all major organs and that it functions as a receptor for rAd35 vectors. Similar to monkeys and humans, MYII mice highly express CD46 in their lungs and kidneys and demonstrate low expression in muscle. Upon intravenous administration, rAd35 vector genomes as well as expression are detected in lungs of MYII mice, in contrast to wild-type littermates. Expression was predominantly detected in lung epithelial cells. Upon intramuscular administration, the initial level of luciferase expression is higher in MYII mice as compared with wild-type littermates, in spite of the fact that CD46 expression is low in muscle of MYII mice. The higher level of expression in muscle of MYII mice results in prolonged gene expression as assessed by CCD camera imaging for luciferase activity. Finally, a significant dose-sparing effect in MYII mice as compared with wild-type littermates on anti-SIVgag CD8⁺ T-cell induction following intramuscular vaccination with an rA35.SIVgag vaccine was observed. This dose-sparing effect was also observed when reinfusing dendritic cells derived from MYII mice after exposure to rAd35.SIVgag vaccine as compared with rAd35.SIVgag exposed dendritic cells from wild-type littermates. It was concluded that MYII mice represent an interesting preclinical model to evaluate potency and safety of rAd35 vectors.

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INTRODUCTION

It has been demonstrated that members of the human group B adenoviruses and perhaps several serotypes of subgroup D utilize CD46 as a high-affinity receptor (Gaggar *et al.*, 2003; Segerman *et al.*, 2003; Sirena *et al.*, 2004; Wu *et al.*, 2004). Human subgroup B adenoviruses, Ad11 and Ad35, are rare in the human population resulting in low seroprevalence, whereas group C vectors (Ad2 and Ad5), which are commonly used for gene therapy and vaccination approaches, have high seroprevalence (Kostense *et al.*, 2004; Nwanegbo *et al.*, 2004; Vogels *et al.*, 2003). High seroprevalence of Ad2

and Ad5 hampers the utility of these vaccine carriers since high anti-vector immunity effectively blunts vaccine potency (Barouch *et al.*, 2004). Therefore, rAd11 and rAd35 vaccine carriers hold the promise of accurate dose control and consistent induction of immune responses against inserted antigens, which was further strengthened by a study showing strong rAd35-mediated induction of anti-SIVgag T-cell responses in naïve mice or in mice carrying high-level anti-Ad5 neutralizing activity (Barouch *et al.*, 2004; Holterman *et al.*, 2004; Vogels *et al.*, 2003). Interestingly, this study also demonstrates that anti-Ad5 T-cells cross-reacting with rAd35, also known to be present in human PBMC samples (Heemskerk *et al.*, 2003), only marginally affected the potency of the rAd35.SIVgag vaccine, indicating that neutralizing antibodies are predominantly responsible for the diminishing vaccine potency (Barouch *et al.*, 2004). Thus

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far, studies with rAd11 or rAd35 vectors have been performed in inbred mice strains that do not express CD46 (Gaggar *et al.*, 2003). In fact, whereas CD46 protein conservation is high between humans, new-world monkeys (80.2%) and old-world monkeys (87.4%), conservation is poor between humans and pigs, guinea pigs, rats and mice (all <50%). Thus, we hypothesized that perhaps data generated so far on potency and distribution of rAd11 and rAd35 vectors in mice may not be representative of what can be expected of these vectors in monkeys and humans. Therefore, we set out to identify whether distribution and potency of rAd35 vectors differ in mice carrying the CD46 receptor or wild-type (Wt) littermates. Since the human CD46 protein (hCD46) serves as a high-affinity receptor for several important pathogens including *Measles virus* (Dorig *et al.*, 1993; Manchester *et al.*, 1994; Naniche *et al.*, 1993), *Human herpesvirus 6* (Santoro *et al.*, 1999), *Streptococcus pyogenes* (Okada *et al.*, 1995), *Neisseria gonorrhoeae* and *Meningitides* (Kallstrom *et al.*, 1997), different CD46 transgenic mice models have been generated and studied (Horvat *et al.*, 1996; Miyagawa *et al.*, 1997; Rall *et al.*, 1997; Thorley *et al.*, 1997). In choosing among different CD46 transgenic mouse models, we decided on MYII mice, which were obtained by transferring a 420 kilobase yeast artificial chromosome clone carrying the hCD46 gene under its own regulatory sequences (Yannoutsos *et al.*, 1996). These mice express all four major isoforms of CD46 known to be expressed in humans (Kemper *et al.*, 2001; Yannoutsos *et al.*, 1996), i.e. BC1, BC2, C1 and C2, and have been used successfully to establish animal models, for instance for measles virus infection (Oldstone *et al.*, 1999).

Based on the studies described here, showing MYII-specific lung sequestration of rAd35 upon intravenous (i.v.) administration, increased level and longevity of gene marking upon intramuscular (i.m.) administration in muscle and a clear tetramer-positive CD8⁺ T-cell dose-sparing effect upon rAd35.SIVgag vaccination, we conclude that MYII mice provide an interesting preclinical model for testing gene transfer and vaccination regimens involving rAd35 vectors.

METHODS

Adenoviral vectors and *in vitro* transduction. The generation of the Ad35 plasmid system and production, purification and titration of recombinant (r) Ad5 and Ad35 vectors have been described previously (Barouch *et al.*, 2004; Holterman *et al.*, 2004; Vogels *et al.*, 2003). The B16F10 mouse melanoma cell line was obtained from the ATCC and was stably transfected using lipofectamine, with a plasmid expressing the BC1 isoform under the control of the SV40 promoter. Transfectants were selected by co-transfecting a second plasmid, expressing neomycin under the control of the cytomegalovirus promoter, by adding 2.0 mg neomycin ml⁻¹ to cell culture medium. High CD46-expressing cell clones were obtained via FACS sorting (Vantage SE; Becton Dickinson) and cell clone expansion upon limiting dilution. For vector transduction, cells were exposed for 2 h to 1000 or 2500 virus particles (vp) per cell after which virus containing medium was replaced by fresh medium. The percentage of cells expressing green fluorescent protein (GFP) was determined 48 h after vector exposure using FACSCalibur (Becton Dickinson).

Human and rhesus monkey monocyte-derived dendritic cells (DC) as well as mouse bone marrow-derived DC of different strains were cultured and characterized essentially as described previously (Ophorst *et al.*, 2004).

CD46 expression and haemagglutination. To analyse CD46 surface expression by FACS, cells were stained for 30 min at 4 °C with 1:20 diluted (PBS/0.5% BSA) anti-human CD46 coupled to fluorescein isothiocyanate (FITC; Leinco). For Western blot analysis, frozen tissue of human (*n*=1), monkey (*n*=1) or mouse origin (*n*=3) was homogenized with a blender in appropriate volumes of RIPA (0.5 g deoxycholic acid, 1% NP40, 10% SDS and PBS) buffer containing protease inhibitors (mini complete; Roche). Protein lysates were clarified by centrifugation and total protein content was determined using the Pierce BCA kit. Of each tissue, 10 µg total protein was loaded onto a 4–12% Bis/Tris gel (Invitrogen) and transferred onto an Immobulon-P transfer membrane (Millipore). After blocking with TBST (0.1% Tween 20 in Tris-buffered saline) containing 5% (w/v) non-fat dry milk (Bio-Rad), membranes were incubated for 1 h with a 1:200 diluted goat polyclonal anti-CD46 antibody (Santa Cruz Biotechnology). Subsequently, a 1:15 000 diluted horseradish peroxidase-coupled rabbit anti-goat IgG polyclonal antibody (Santa Cruz Biotechnology) was used to visualize signals by enhanced chemiluminescence detection kit (ECL; Amersham Biosciences). For haemagglutination assays, mouse blood was diluted to 3% erythrocyte density in preservation medium (Sanquin). Adenovirus stock solutions of rAd5 and rAd35 were diluted from 2 × 10⁹ to 9.8 × 10⁵ vp per 100 µl PBS containing 5% sucrose, 0.7 mM CaCl₂ and 0.7 mM MgCl₂. As negative control only PBS containing 5% sucrose, 0.7 mM CaCl₂ and 0.7 mM MgCl₂ was used. The 3% erythrocyte solution was further diluted to 0.4% erythrocyte density in PBS. Subsequently, 50 µl 0.4% erythrocyte solution was added and plates were incubated for 1 h at 37 °C at 10% CO₂. Before read-out, plates were centrifuged for 1 min at 201 relative centrifugal force.

Breeding of MYII mice and Wt littermates. For breeding purposes, Wt C57BL/6 mice were obtained through a registered breeding facility (Harlan, The Netherlands). MYII mice were obtained from Dr Grosveld (Erasmus University of Rotterdam, The Netherlands). Animal studies were performed according to Dutch law (Dutch Animal Experimentation Act) and the guidelines from the council of the European Committee (EU Dir. 86/609) on the protection of experimental animals. MYII mice and offspring were housed under quarantine conditions in individual ventilated type II cages (Techniplast), since their microbial status did not meet the FELASA standard. MYII mice and Wt littermates were obtained via breeding of MYII males with Wt C57BL/6 females after more than six rounds of backcrossing to C57BL/6. Genetic screening of offspring was performed through DNA analysis of tail tissue. Mouse DNA was purified using the Wizard SV 96 genomic DNA purification system (Promega). To discriminate between Wt littermates and MYII mice, oligonucleotides: MCPExon6F (5'-CAGTACTTG GGATCCCCCAGTTC-3') and MCPExon7R (5'-GGTTTTGTAC-TAGATGGA GGCAGCA-3') were used in a PCR assay on purified mouse DNA. Male and female offspring between 6 and 12 weeks of age were used for all the mice experiments described. Experiments described here were all performed with mice housed under quarantine conditions given their health status, whereas MYII mice adhering to the FELASA standard have in the meantime been successfully obtained for future studies through embryo transfer at Harlan.

Distribution experiments. Wt littermates and MYII mice (*n*=3 per group) were injected i.v. via the tail vein or i.m. via the musculus quadriceps with 10¹¹ rAd5.Luc or rAd35.Luc vp in 50 µl PBS containing 5% sucrose, 0.7 mM CaCl₂ and 0.7 mM MgCl₂. Transgene expression in these mice was assessed 24, 48 and 72 h after vector administration and directly after intraperitoneal (i.p.)

injection of 2 mg per mouse luciferin in 30 μ l PBS. A peltier cooled CCD camera system NightOWL (Berthold Technologies) coupled to the WinLight32 software was used for this purpose. For Q-PCR analysis, MYII mice and Wt littermates ($n=10$ per group) were injected intravenously (i.v.) with 10^{11} rAd35.Luc vp. Three untreated mice were included as controls. Mice were sacrificed after 48 h and organs were isolated. For Q-PCR analysis, DNA was isolated from organs using the DNeasy tissue kit (Qiagen). DNA was analysed for the presence of Ad35 genome using forward primer 5'-GTTTCAGG-GCCAGGTAGACTTTG-3' and reverse primer 5'-CGCGGAAATTC-AGGTAATAAAC-3', both primers recognizing sequences present within the cytomegalovirus promoter present in rAd35, and VIC-CCCATTACGTGGAGTTTCGATTACCG-TAMRA was used as a probe. To determine the number of copies per cell, Q-PCR analysis was performed on the same samples using 18S rDNA sequences as targets for primers as described previously (Klein *et al.*, 1999, 2000). Protein extracts derived from isolated organs were obtained as described previously (Havenga *et al.*, 2002; Ophorst *et al.*, 2004) and luciferase reporter gene expression levels were determined using the Promega luciferase detection system. For immunohistochemical analysis of lung tissue, mice were injected i.v. with 10^{11} vp of rAd35.lacZ ($n=5$ per group) and were sacrificed 48 h after vector administration. Lung tissue was fixed for 30 min in 4% formaldehyde. After overnight staining with 1 mg X-Gal ml^{-1} , lung tissue was again exposed to 4% formaldehyde for 6 h. After dehydration, 5 μ m slides were generated that were stained using a standard haematoxylin-phloxine-saffron protocol.

Vaccination and immunological read-out. To vaccinate animals via different routes, all mice received a dose of 10^8 vp of rAd35.SIVgag ($n=5$ per group) in PBS containing 5% sucrose, 0.7 mM CaCl_2 and 0.7 mM MgCl_2 . For i.m. immunization, animals received 2×50 μ l via the musculus quadriceps, 2×10 μ l intranasally (i.n.), 50 μ l for both intradermal (i.d.) and i.v. routes, and 100 μ l for i.p., subcutaneous (sc) and oral (po) routes. Four weeks after vaccination presence of SIVgag-specific T cells was determined in spleen via interferon (IFN) γ ELISPOT, whereas SIVgag-specific antibodies were determined by ELISA as previously described (Barouch *et al.*, 2004; Sprangers *et al.*, 2003). Induction of anti-SIVgag CD8⁺ T cells present in blood was determined over time using AL11 tetramer staining as described previously (Barouch *et al.*, 2004). Adenovirus neutralizing antibodies were determined using an assay previously published (Barouch *et al.*, 2004; Sprangers *et al.*, 2003). Subsequent i.m. vaccination experiments were performed with high doses (10^9 vp) or low doses (10^7 vp) injected in 2×50 μ l PBS containing 5% sucrose, 0.7 mM CaCl_2 and 0.7 mM MgCl_2 .

DC transfer experiments. Isolation and characterization of DC derived from bone marrow of both mice strains was performed as described previously (Ophorst *et al.*, 2004). DC were transduced with 20 or 2000 vp per cell of rAd35.SIVgag for 2 h, after which cells were seeded in fresh medium for another 48 h. Subsequently, 5×10^5 DC, corresponding to 10^7 and 10^9 vp per mouse, were transferred to homotypic mice via i.m. (2×50 μ l PBS) administration ($n=5$ per group). Immunological read-out for the presence of anti-SIVgag-specific T cells after injection of genetically modified DC in mice was performed as described above.

RESULTS

Expression of hCD46 in transgenic mice and its role as an rAd35 receptor

While pioneering on the concept of using alternative adenoviral vectors as compared with the commonly used type 5 vector (Goossens *et al.*, 2001; Havenga *et al.*, 2001,

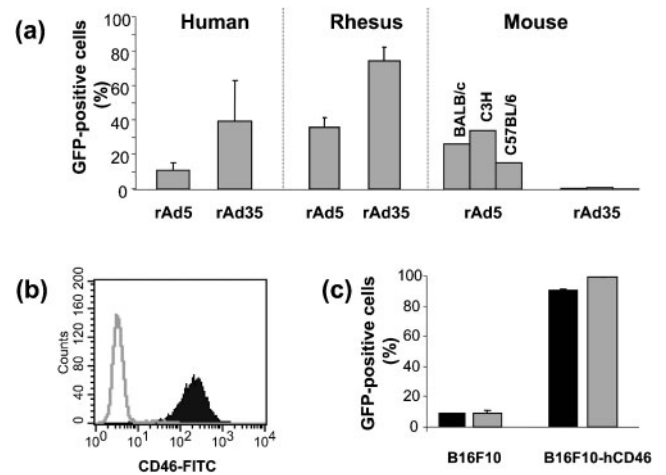


Fig. 1. (a) Primary DC derived from human PBMC ($n=3$), rhesus macaques PBMC ($n=3$) or mice PBMC (three strains, pooled DC of three mice per strain) were exposed for 2 h to 2500 vp per cell of either rAd5.GFP or rAd35.GFP. Shown is mean values \pm standard deviation (SD) of GFP expression 48 h after vector exposure. (b) Mouse melanoma B16F10 cells were stably transfected with a plasmid encoding hCD46 thereby creating B16F10-hCD46. Expression of hCD46 in B16F10-hCD46 cells (dark grey plot) is shown as compared with parental B16F10 cells (light grey overlay). (c) Mouse B16F10-hCD46 and parental cells were exposed for 2 h to 1000 vp (black bar) or 2500 vp (grey bar) of rAd35 vector carrying GFP. The percentage of GFP-expressing cells was determined 48 h after infection, whereby non-infected cells served to set a background value at 1% GFP-positive cells. Shown are mean values \pm SD of one representative experiment performed in triplicate.

2002; Holterman *et al.*, 2004; Vogels *et al.*, 2003), we observed that the receptor for human type 35, at least, was not conserved between species. This observation is exemplified in Fig. 1(a), demonstrating that rAd35 vector efficiently transduces monocyte-derived DC from human and rhesus monkeys, while DC derived from diverse mouse strains are refractory to rAd35 transduction. With the knowledge that subgroup B viruses can utilize different cellular attachment molecules (Mei *et al.*, 1998, 2002) including CD46 (Gaggar *et al.*, 2003), we tested whether our rAd35 vector also utilizes CD46 as a receptor. Hereto, we generated mouse B16F10 melanoma cells that stably express the BC1 isoform of hCD46 on the membrane (Fig. 1b) and observed that rAd35-mediated gene transfer of GFP improved from 10% GFP-positive cells in B16F10 cells towards >90% in B16F10-hCD46 cells (Fig. 1c). Thus, hCD46 expression dramatically augments rAd35 vector cell transduction of these otherwise refractory mouse cells.

Given the availability of CD46 transgenic mice, we assessed the expression level of hCD46 protein in MYII mice. Hereto, Western blot analysis was performed using protein samples derived from diverse tissues of either MYII mice or Wt littermates. As shown in Fig. 2(a), hCD46 could not be

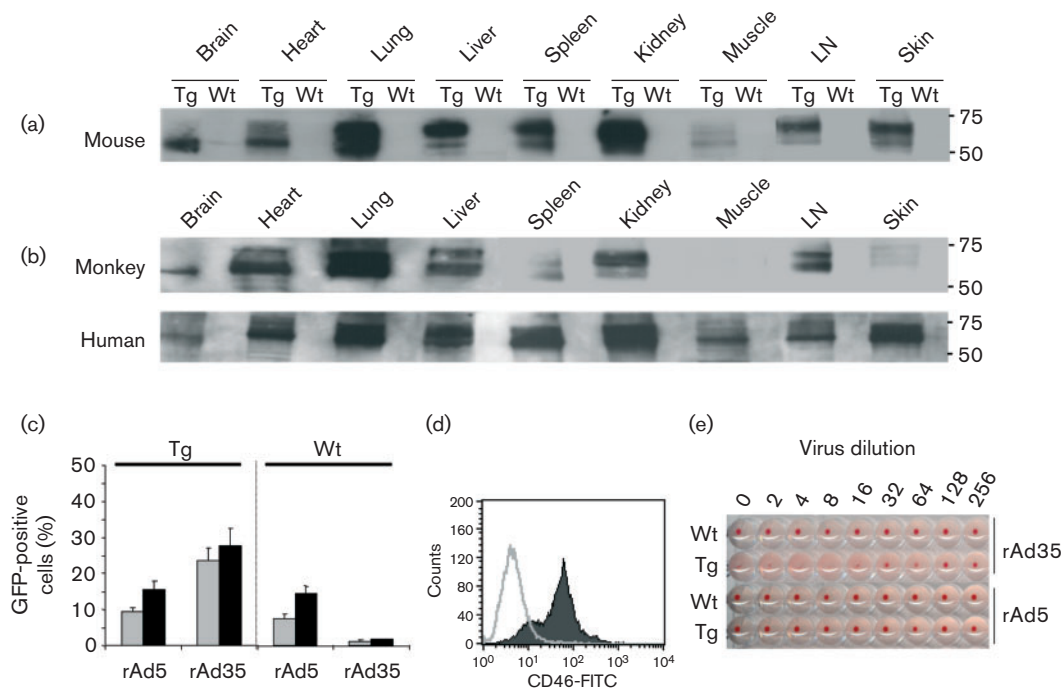


Fig. 2. (a) Western blot analysis using 10 µg of total protein extracted from a panel of organs derived from MYII mice (Tg, $n=3$) or Wt littermates ($n=3$). The position of the protein marker for 75 and 50 kDa size are shown. LN, Lymph node. (b) Western blot analysis using 10 µg of total protein extracted from a panel of organs derived from either rhesus macaque ($n=1$) or human ($n=1$). The positions of the protein markers for 75 and 50 kDa size are shown. LN, Lymph node. (c) DC of Tg mice and Wt littermates were exposed for 2 h to 1000 vp per cell (grey bars) or 2500 vp per cell (black bars) using rAd5.GFP or rAd35.GFP. Shown is mean value \pm standard error of the mean (SEM) of one representative experiment performed on three mice. (d) Flow cytometric analysis of hCD46 expression on the DC membranes of Tg mice (dark grey plot) or Wt littermates (light grey overlay). (e) Haemagglutination pattern of rAd35 and rAd5 viruses with erythrocytes derived from Wt littermates or Tg mice. Virus preparations used were either undiluted (0) or diluted up to 1 : 256 using PBS. Positive haemagglutination is scored when upon centrifugation, virus-erythrocyte interaction prevents formation of an erythrocyte pellet at the bottom of the wells of 96-well plates.

detected in Wt littermates, whereas MYII mice displayed expression in all major organs with most pronounced expression in the lungs and kidneys and least expression in muscle. Similar Western blot analysis on protein samples derived from organs of non-human primate or human origin (Fig. 2b) indicated that in man and monkey, expression of CD46 is also most pronounced in the lungs and kidneys and low in muscle. To identify whether the hCD46 present in MYII mice functions as rAd35 receptor, we exposed primary bone marrow-derived DC of MYII mice and Wt littermates to rAd35.GFP and rAd5.GFP as a control. The efficient infection of DC derived from MYII mice with rAd35.GFP (Fig. 2c), coupled to detection of hCD46 on the membrane of these cells (Fig. 2d), indicates that hCD46 functions as rAd35 receptor in these mice.

Subsequent haemagglutination studies using erythrocytes derived from MYII mice and Wt littermates demonstrate that the hCD46 protein was expressed on erythrocytes derived from MYII and functionally binds rAd35 vector (Fig. 2e).

Collectively, these data show that: (i) Wt inbred strains of mice do not express a high-affinity receptor for rAd35; (ii) hCD46 expressed in MYII mice functions as a high-affinity receptor for rAd35; (iii) the low hCD46 protein expression in muscle and the high level in the lungs and kidneys of MYII mice is similar to the hCD46 profile in non-human primates and human, in spite of the fact that MYII mice contain 10 hCD46 gene copies (Yannoutsos *et al.*, 1996); and (iv) erythrocytes of MYII mice express hCD46 capable of binding to the rAd35 vector.

Vector distribution of rAd35 vector in hCD46-transgenic mice

We have previously reported that i.v. administration of rAd35.Luc vector did not result in vector accumulation in any organ of different Wt inbred mice strains as demonstrated by the lack of luciferase activity (Vogels *et al.*, 2003). Here, we demonstrate that high dose, i.v. delivery of rAd35.Luc vector into MYII mice also does not result in high-level luciferase expression in this organ or any other

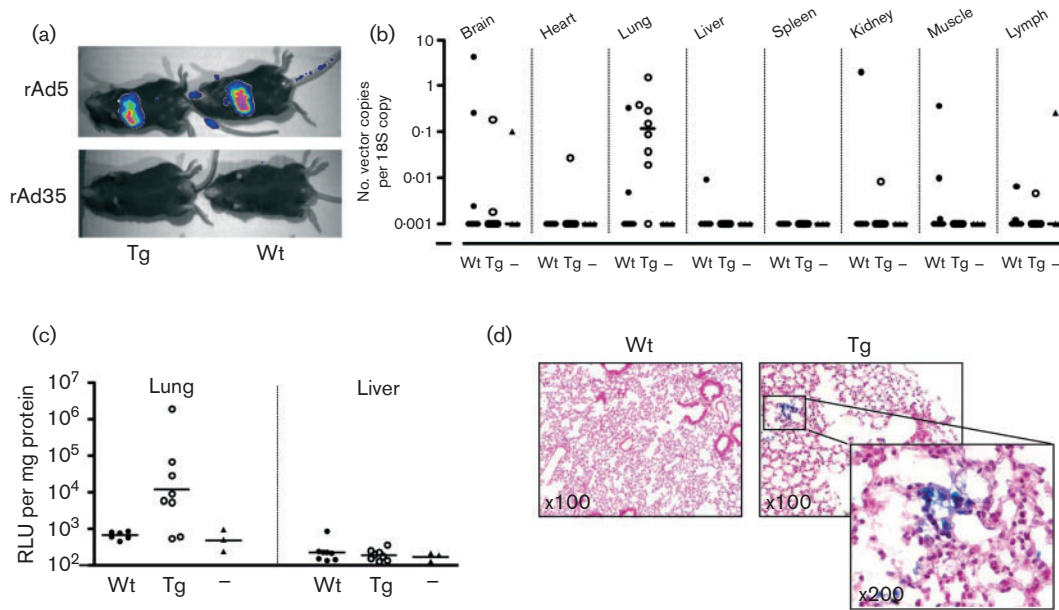


Fig. 3. (a) Expression of luciferase in MYII mice (Tg) or Wt littermates as visualized by CCD camera technology measured 24 h after i.v. administration of 1×10^{11} vp rAd5.Luc or rAd35.Luc vector and directly after i.p. administration of luciferin substrate. Colouring patterns indicate luciferase expression intensity with the red colour representing highest expression and the blue colour representing lowest detectable luciferase expression. (b) Number of rAd35 genome copies per cellular 18S rDNA copy as detected in organs of Wt littermates (black circles) or Tg mice (open circles) as determined by Q-PCR ($n=10$ mice per strain). Whereas circles represent results of individual mice the black lines represent mean values. As negative controls (-, triangle), organs from three untreated mice were analysed in parallel using Q-PCR. (c) Luciferase expression in lung and liver tissue of Wt littermates (black circles) or Tg mice (open circles) 48 h after i.v. administration of 10^{11} vp of rAd35.Luc vector using protein lysate extracted from these tissues. Each circle represents value obtained for individual mice, whereas black lines represent mean values. Lung and liver derived from untreated Wt mice ($n=3$) served as negative controls (-, triangle) for the *in vitro* luciferase assay. Data are expressed in relative light units (RLU) obtained per mg of total protein. (d) Representative haematoxylin-phloxine-saffron stained 5 µm slides from lung tissue of either Wt littermates or Tg mice 48 h after i.v. injection of 10^{11} vp rAd35.lacZ.

organ (Fig. 3a). As expected, administration of rAd5.Luc vector resulted in clear luciferase activity in liver of either MYII mice or Wt littermates. Next, we performed Q-PCR analysis on major organs of MYII mice and Wt littermates 48 h after systemic administration of 10^{11} vp of rAd35.Luc vector. With the exception of lung tissue, none of the tested organs derived from MYII mice sequestered rAd35. In addition, none of the organs derived from Wt littermates sequestered rAd35 (Fig. 3b). In lungs of MYII mice, low copy numbers of rAd35 genomes were consistently detected, which correlated with low but clearly detectable luciferase expression upon rAd35.Luc gene transfer using a sensitive *ex vivo* luciferase detection assay (Fig. 3c, lung: $P=0.02$, ANOVA). A subsequent identical experiment using rAd35.lacZ showed the presence of lacZ-positive cells in lungs that appeared to be predominantly epithelial cells (Fig. 3d).

Since i.m. delivery of adenoviral vectors is the most commonly used route of administration, particularly for vaccination purposes, we next determined the ability of rAd35 to transduce muscle of either MYII mice or Wt

littermates. Upon i.m. administration of rAd35.Luc vector, we observed that the initial level of luciferase activity was higher in muscle of MYII mice as compared with Wt littermates, which probably resulted in the prolonged luciferase activity as observed using real-time *in vivo* imaging for luciferase expression via CCD camera technology (Fig. 4). Together, the data demonstrate that (i) rAd35 sequesters in the lungs of MYII mice in contrast with Wt littermates upon i.v. administration; (ii) rAd35 does not sequester in the liver of MYII mice in contrast to rAd5; and (iii) upon high-dose i.m. administration a higher level of luciferase expression is obtained in MYII mice as compared with Wt littermates, resulting in prolonged gene expression in spite of the fact that these mice express low-level hCD46 in muscle.

rAd35-mediated insert-specific immune responses

Wild-type littermates and MYII mice were vaccinated via different routes using an intermediate dose (10^8 vp) of rAd35.SIVgag vaccine. Four weeks after vaccination, animals were sacrificed and T-cell responses against SIVgag were

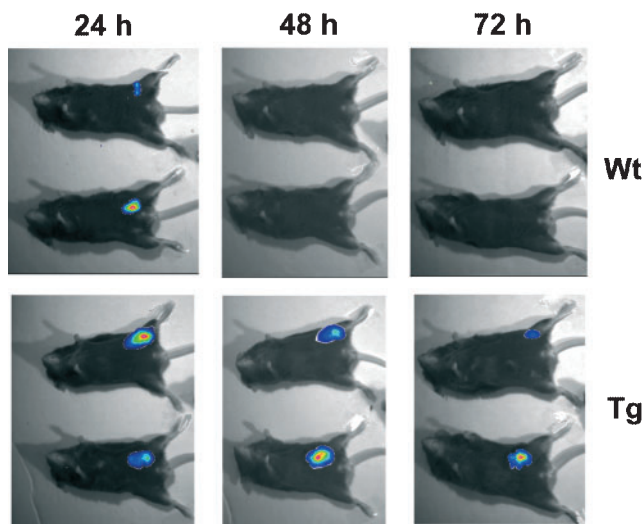


Fig. 4. Expression of luciferase in MYII mice (Tg) or Wt littermates as visualized by CCD camera technology measured 24, 48 and 72 h after i.m. administration of 1×10^{11} vp rAd35.Luc vector and directly after i.p. administration of luciferin substrate. Colouring patterns indicate luciferase expression intensity with the red colour representing highest expression and the blue colour representing lowest detectable luciferase expression.

determined using the IFN- γ ELISPOT assay. As shown in Fig. 5(a), marked differences were observed in vaccination responses using different routes of immunization with rAd35 vector (Kruskal–Wallis test for differences between

route of administration $P < 0.0001$). Clearly, T-cell responses obtained via i.m., i.n. or i.d. administration proved highly consistent with little variation between animals. In contrast, i.v., sc or i.p. delivery resulted in substantial variation between animals, whereas oral administration (po) resulted in absence of T-cell response. The high variation between animals after i.v., i.p. and sc administration does not seem to reflect technical failure, since anti-Ad35 vector antibody responses (Fig. 5b) proved to be very well conserved within groups of animals, in particular upon i.v. and i.p. delivery. No significant differences could be observed between MYII mice and Wt littermates in anti-SIVgag T-cell responses for any of the vaccination routes tested using a vaccine dose of 10^8 vp (Wilcoxon test: P -values i.m. 0.46, i.p. 0.70, sc 0.25, i.n. 0.62, po 0.32, i.d. 0.92 and i.v. 0.45). Antibody responses against the SIV-gag protein were low using Ad35.SIVgag vaccine, irrespective of the route or mouse strain immunized (data not shown). To investigate the possibility of vaccine threshold effects in relation to presence or absence of the hCD46 receptor, MYII mice and Wt littermates were vaccinated with either a high (10^9 vp) or low (10^7 vp) rAd35.SIVgag vaccine dose. For this experiment animals were vaccinated using the i.m. route, based on the consistent anti-SIVgag T-cell responses obtained using the 10^8 vp vaccine dose (Fig. 5a, i.m.). As shown in Fig. 6(a), vaccination at 10^9 vp did not result in significant differences in anti-SIVgag T-cell responses between MYII mice and Wt littermates (mixed models with fixed effects, $P = 0.12$). In contrast, strong SIV-gag-specific tetramer-positive CD8 $^+$ response ($> 4\%$) could be detected in MYII mice starting 14 days after immunization

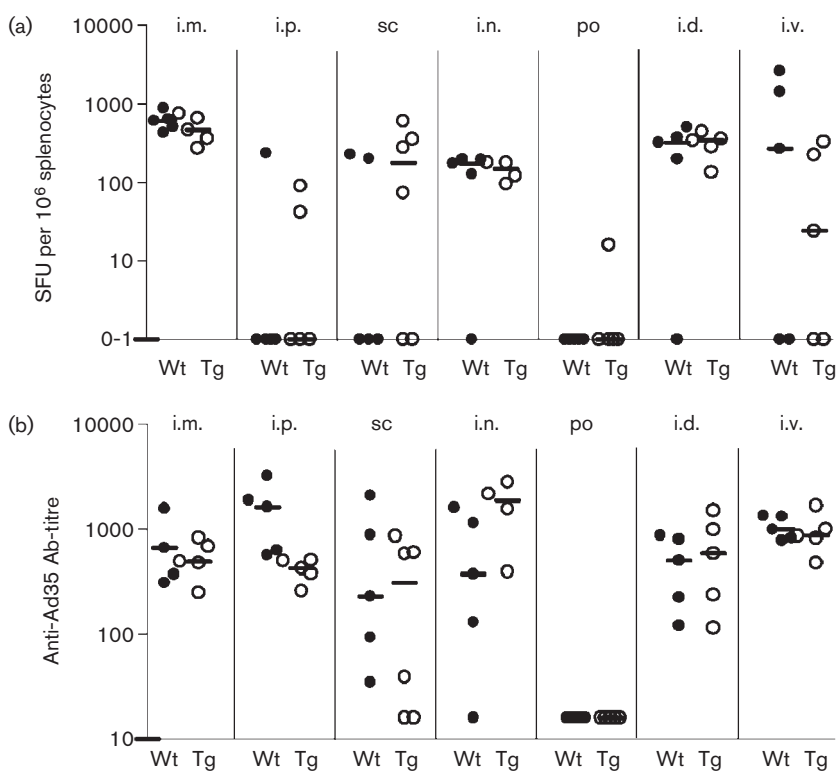


Fig. 5. Wt littermates (black circles) or MYII mice (Tg, open circles) were immunized via different routes using a dose of 10^8 vp of rAd35.SIVgag. Specific T-cell responses (a) as well anti-rAd35-neutralizing antibodies (b) were measured 4 weeks after immunization. Routes tested included: i.m., i.p., sc, i.n., po, i.d. and i.v. vaccine delivery. Each circle represents the value obtained for individual mice, whereas black lines represent mean values. T-cell responses are expressed as the number of IFN- γ producing units (SFU) per 10^6 splenocytes tested using ELISPOT assay. The neutralizing antibody titre against Ad35 was expressed as a dilution of the serum that inhibited $> 90\%$ of the luciferase activity in the absence of serum.

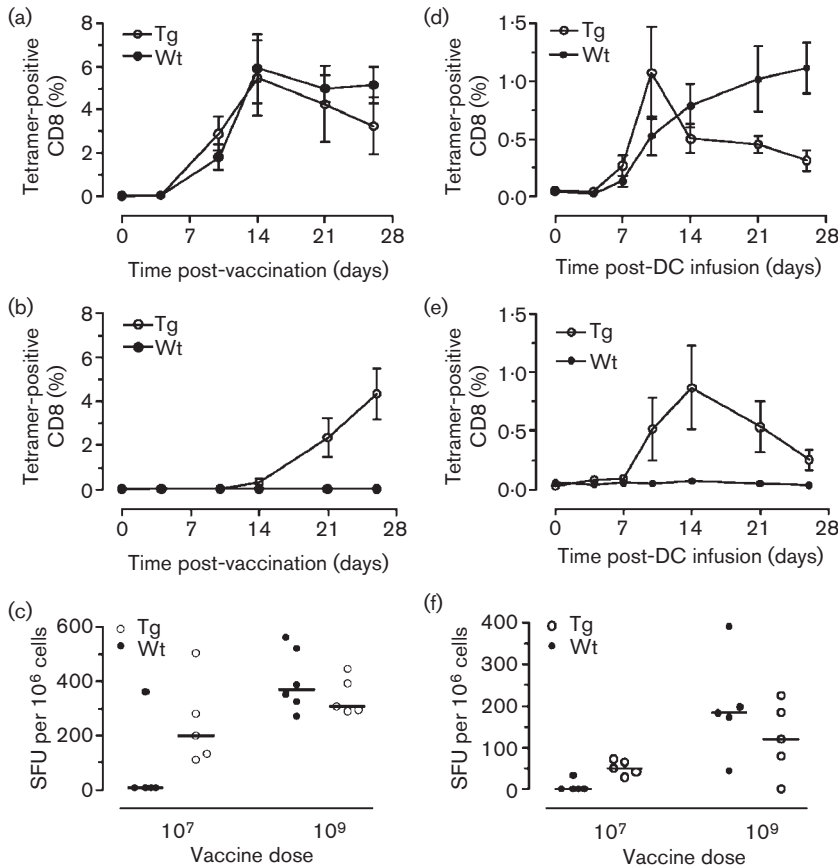


Fig. 6. Wt littermates ($n=5$, black circles) and MYII mice (Tg, $n=5$, open circles) were immunized i.m. with a dose of 10^9 vp (a) or 10^7 vp (b) of rAd35.SIVgag. Animals were subsequently monitored in time for development of anti-SIVgag CD8⁺ responses via tetramer staining. Shown is mean value \pm SEM. (c) Twenty-eight days after immunization, animals ($n=5$) were sacrificed and splenocytes were isolated to determine IFN- γ -positive T cells in ELISPOT assay. Each circle represents the value of individual mice, whereas black lines represent mean values. Wt littermates ($n=5$, black circles) and Tg mice ($n=5$, open circles) received 5×10^5 DC exposed for 2 h to either 10^9 vp (d) or 10^7 vp (e) of rAd35.SIVgag. Animals were monitored in time for development of anti-SIVgag CD8⁺ responses via tetramer staining. Shown is mean value \pm SEM. (f) Twenty-eight days after immunization, the animals ($n=5$) that received rAd35.SIVgag modified DC were sacrificed and splenocytes were isolated to determine IFN- γ -positive T cells in ELISPOT assay. Each circle represents the value of individual mice, whereas black lines represent mean values.

using a 10^7 vp vaccine dose, whereas no tetramer-positive CD8⁺ T-cell response could be detected in Wt littermates (Fig. 6b, $P < 0.001$). The tetramer-positive CD8⁺ responses detected correlated with day 28 IFN- γ ELISPOT results, which also demonstrated clearly detectable IFN- γ producing T-cells in all vaccinated MYII mice as compared with no T-cell response in 4 of 5 Wt littermates ($P = 0.07$). At a vaccine dose of 10^9 vp no difference in ELISPOT results was observed (Fig. 6c, $P = 0.47$). In an effort to link the observed differences in the induction of anti-SIVgag CD8⁺ responses directly to the presence of hCD46, DC derived from MYII mice or Wt littermates were isolated and exposed *ex vivo* to 2000 or 20 vp per cell of rAd35.SIVgag vector. Forty-eight hours after a 2 h vector exposure, 5×10^5 DC were administered i.m. to homotypic mice. As shown in Fig. 6(d), clear tetramer-positive CD8⁺ T-cell responses could be observed in both mice strains after infusion of 5×10^5 DC exposed for 2 h to 2000 vp per cell. In contrast, clear, albeit low, tetramer-positive responses were observed only in MYII mice after receiving 5×10^5 DC exposed for 2 h to 20 vp per cell (Fig. 6e, $P = 0.03$ using mixed models for difference between Wt and Tg over time). Again, day 28 IFN- γ ELISPOT results correlated with CD8⁺ tetramer staining, demonstrating clear detection of IFN- γ producing T cells in MYII mice (Fig. 6f, $P = 0.01$). At 10^9 vp per 5×10^5 DC, no difference in the T-cell response was observed between MYII mice and Wt littermates ($P = 0.46$).

Collectively, these results demonstrate that direct i.m. vaccination of MYII mice with rAd35 vaccine results in a significant ($> 4\%$) *in vivo* rAd35 vaccine dose-sparing effect as compared with Wt littermates. Also, transduction of cultured DC derived from MYII mice with rAd35 results in higher anti-SIVgag CD8⁺ responses *in vivo* upon reinfusion of modified DC as compared with Wt littermates. The latter is most likely related to the increased susceptibility of DC for rAd35 due to expression of hCD46.

DISCUSSION

Many reports have demonstrated that the presence of coxsackie and adenovirus receptor, CAR (Bergelson *et al.*, 1997), on cell lines and primary cells, is important to achieve efficient *in vitro* transduction with Ad5 vectors (Hutchin *et al.*, 2000; Li *et al.*, 1999; Nalbantoglu *et al.*, 1999; Orlicky *et al.*, 2001; Stockwin *et al.*, 2002). Likewise, it has been reported, and confirmed here, that expression of CD46 on cells significantly augments *in vitro* gene transfer using group B vectors (Holterman *et al.*, 2004; Vogels *et al.*, 2003). By exploiting the natural diversity in receptor usage among adenoviruses (Havenga *et al.*, 2002; Von Seggern *et al.*, 1998, 2000), we have identified vectors that significantly increase levels of gene expression in DC and fibroblasts (Ophorst *et al.*, 2004; Rea *et al.*, 2001b), human bone marrow stroma cells (Havenga *et al.*, 2002), haematopoietic stem cells

(Knaan-Shanzer *et al.*, 2001), synoviocytes (Goossens *et al.*, 2001) and smooth muscle cells (Havenga *et al.*, 2001, 2002). Such vectors are in demand within the field of regenerative medicine (de Bruijn *et al.*, 1999; Lamme *et al.*, 1998, 2000; Moutsatsos *et al.*, 2001; Riesle *et al.*, 1998) and *ex vivo* DC-based vaccination approaches (Rea *et al.*, 2001a). Thus, the *in vitro* correlation between cellular receptor expression and vector transduction efficiency is relatively clear.

In contrast, the *in vivo* need for a high-affinity receptor to obtain efficient gene transfer has not been completely elucidated. For instance, it has been suggested by some that viral receptors are not major determinants in adenoviral transduction of muscle (Cao *et al.*, 2001), whereas others have shown that transduction of skeletal muscle in a human CAR transgenic mouse model (Tallone *et al.*, 2001) resulted in an approximately 10-fold improved gene transfer as compared with Wt mice (reviewed by Thirion *et al.*, 2002). Also, a clear discrepancy between *in vivo* rAd5 distribution and CAR expression in mice upon systemic delivery has been reported, demonstrating that anatomical barriers impact on vector distribution (Fechner *et al.*, 1999). Such anatomical barriers challenge the concept of adenoviral vector targeting since any vector, be it of human or non-human origin (Holterman *et al.*, 2004; Kremer *et al.*, 2000; Vogels *et al.*, 2003; Xiang *et al.*, 2002; Zakhartchouk *et al.*, 2003), will need to circumvent such anatomical hurdles.

We have recently shown that in spite of huge differences in the ability of a panel of different fibre-chimeric adenoviral vectors to infect primary mouse muscle cells, DC and fibroblasts *in vitro*, all these vectors elicited similar anti-insert-specific immune responses in mice upon i.m. vaccination (Mercier *et al.*, 2004; Ophorst *et al.*, 2004). Also, clear antigen-specific cellular and humoral immune responses are consistently obtained after i.m. immunization with rAd11 and rAd35 vectors in Wt inbred mouse strains lacking a high-affinity receptor (Barouch *et al.*, 2004; Holterman *et al.*, 2004; Vogels *et al.*, 2003). Such results suggest that in the absence of a high-affinity receptor skeletal muscle, transduction occurs in mice.

Although we observed induction of immune responses in Wt inbred strains, the absence of a high-affinity receptor may result in an altered potency or distribution of these vectors. For these reasons, and knowing that both monkeys and humans widely express CD46, we wanted to investigate the role of the CD46 receptor in *in vivo* rAd35 vector transduction efficiency, rAd35 distribution and rAd35 vaccine potential. We chose the MYII strain, since these mice express all four major hCD46 isoforms (Kemper *et al.*, 2001; Yannoutsos *et al.*, 1996). The MYII mice are further known to contain 10 hCD46 gene copies in their genome and earlier studies using Northern blot analysis have demonstrated that hCD46 expression was similar between humans and MYII mice in the liver and lungs, whereas in the kidneys, heart and spleen higher levels of hCD46 mRNA were detected (Yannoutsos *et al.*, 1996). Liver tropism was assessed by CCD camera imaging and revealed that rAd35

vector does not sequester in liver upon i.v. administration of either MYII mice or Wt littermates. These data corroborate our earlier data in Wt mice (Vogels *et al.*, 2003) and also data of Seshidhar Reddy *et al.* (2003), who demonstrated that an E1B-containing Ad35 vector displays lack of mouse liver tropism. It should be taken into account that MYII mice, in contrast to humans, express hCD46 on their erythrocytes, which may lead to capture of rAd35 vector upon i.v. administration obscuring liver infection in these animals (Yannoutsos *et al.*, 1996). However, the data are also in line with a recent report by Ni *et al.* (2005), showing that rAd5 vectors carrying fibres derived from Ad11 or Ad35 do not target the liver of baboons and do not show endothelial damage and inflammation typically observed with Ad5-based vectors. Thus, the strong liver tropism observed with rAd5 vector, known to cause dose-dependent hepatotoxicity limiting rAd5 dosing, can perhaps be circumvented using rAd35 vectors. Our Q-PCR analysis revealed that rAd35, when given at high dose, consistently accumulates at low rAd35 genome copy numbers in the lungs of MYII mice, correlating with expression detected in this organ. These data, as well as lack of liver infection in hCD46 transgenic mice, are in further agreement with a recent report showing that an rAd11 vector does not accumulate in the liver and can be detected for a short period of time (<72 h) in the lungs of hCD46 mice (Stone *et al.*, 2005).

Upon i.m. administration of rAd35 higher initial marker gene expression was detected in MYII mice as compared with Wt littermates, which most likely translated in the prolonged expression observed in these MYII mice. The clear difference in level and longevity in luciferase expression observed in muscle did not correlate with the vaccination data, showing no difference at either 10^9 or 10^8 vp of rAd35.SIVgag dose in T-cell induction levels. However, a clear and significant tetramer-positive CD8⁺ T-cell response was observed in MYII mice at the low 10^7 vp rAd35 vaccine dose in contrast to no response in Wt littermates. We hypothesize that only limited antigen expression is already sufficient to trigger full blown immune responses and, thus, that a difference between MYII mice or Wt littermates could be observed only at lower dosing.

To provide further evidence that the presence of the high-affinity receptor for rAd35 vector contributes to the antigen-specific immune response, we genetically modified *ex vivo* cultured DC derived from Wt littermates and MYII mice and reinfused cells into naïve recipients. The observed difference in the anti-SIVgag-specific CD8⁺ T-cell response in MYII mice as compared with Wt littermates directly stems from the *ex vivo* modified DC, suggesting that DC derived from MYII mice were more susceptible to rAd35, resulting in higher SIVgag antigen expression and processing.

Future immunohistochemical analysis on muscle sections after direct i.m. immunization with rAd35 vector are needed to formally prove that the presence of CD46 in the muscle of MYII mice results in increased uptake of rAd35 vector by

professional antigen presenting cells as compared with Wt littermates. However, this experiment demonstrates that DC from MYII mice are able to become infected with rAd35 vector, resulting in antigen expression and processing at sufficient level to induce *in vivo* anti-SIVgag CD8⁺ responses upon reinfusion into naïve host.

In summary, the data obtained have demonstrated that in MYII mice the rAd35 vector displays increased targeting and expression of transgenes in lungs after i.v. administration, increased level and longevity of local expression upon i.m. administration, and a significant dose-sparing effect in tetramer-positive CD8⁺ T cell induction as compared with Wt littermates. As such, we conclude that MYII mice provide an interesting preclinical model in studies involving rAd35 vectors.

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