Circulating Antibodies and Macrophages as Modulators of Adenovirus Pharmacology

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Running Title: Circulating antibody levels affect adenovirus uptake

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Adenovirus serotype 5 (Ad5) naturally infects the liver after intravenous injection making it a candidate for hepatocyte-directed gene transfer. While Ad5 can be efficient, most of the dose is destroyed by liver Kupffer cells before it can reach hepatocytes. In contrast, Ad5 bearing the hexon from Ad6 (Ad5/6) evades Kupffer cells. While Ad5/6 dramatically increases hepatocyte transduction in BALB/c mice, it surprisingly had little effect in C57BL/6 mice. To determine the source of this strain-specific variation, the roles of Kupffer cells, liver sinusoidal endothelial cells (LSECs), hepatocytes, scavenger receptors, clotting factors, and immunoglobulins were analyzed. Kupffer cell numbers and LSECs, clotting factor X, and hepatocyte infectability did not vary between different strains of mice. In contrast, high levels of immunoglobulins correlated negatively with Ad5 liver transduction in different mouse strains. Removal of immunoglobulins by use of Rag-deficient mice restored Ad5 transduction to maximal levels. Removal of Kupffer cells by predosing or by testing in colony-stimulating factor knock-out mice restored Ad5 transduction in the presence of immunoglobulins. Partial reconstitution of IgM in Rag mice resulted in significant reduction in liver transduction by Ad5, but not Ad5/6. These data suggest a role for IgM-mediated clearance of Ad5 via Kupffer cells and evasion of this clearance mechanism by Ad5/6. These mechanisms may play a vital role in Ad pharmacology in animals and in humans.
Adenovirus serotype 5 (Ad5) is one of the most robust vectors for in vivo liver-directed gene transfer. Ad5 liver transduction is mediated in part by its surprisingly high affinity for vitamin K-dependent blood clotting factors. In particular, factor X (FX) has been shown to bind to the hexon protein of Ad5 with subnanomolar affinity and, in one model, may act as a bridge to the virus retargeting it to heparan sulfate proteoglycans on hepatocytes (12, 18, 22, 25). Up to 98% of systemically delivered Ad5 is eliminated by liver Kupffer cells before reaching hepatocytes (5). Kupffer cells can phagocytose particles as large as 2 μm in diameter, which is appropriate for virus bound to host proteins and cells (4). Uptake into these cells is likely mediated by broadly specific scavenger receptors (27), that can recognize the hypervariable (HVR) loops 1, 2, 5 and 7 of hexon (1). While there is good evidence for interactions between Ad5 and scavenger receptors (10), Kupffer cells in wild-type and scavenger receptor SR-A knock out mice take up similar amounts of Ad5, suggesting that the host may have additional, redundant mechanisms for viral recognition removal from the systemic circulation (8). Kupffer cells are not the only components of the reticuloendothelial system that remove foreign particles from the blood stream. Under normal conditions, liver sinusoidal endothelial cells (LSECs) can pinocytose particles less than 0.23 μm in diameter, also allowing uptake of 90 nm adenoviral virions (4). Since
LSECs also express scavenger receptors SREC-I and SREC-II (2) and also absorb Ad5 from the blood stream. Beyond LSECs and Kupffer cells, there are likely a variety of other non-productive pharmacological elimination routes for intravenously-injected Ad5 (reviewed in (13)).

While Ad5 appears to be effective at mediating liver transduction, there is surprisingly wide variation between it and other highly conserved members of species C adenoviruses (26). For instance, Ad6, a lower seroprevalence species C Ad (19), mediates three times higher liver transduction than Ad5, but both Ad5 and Ad6 mediate higher transduction than Ad1 and 2 (26). These differences were observed in BALB/c mice although surprisingly, when Ad5 and Ad6 were compared in C57BL/6 mice, Ad6 no longer mediated higher transduction than Ad5 (14).

The variability of adenovirus pharmacology in different strains of mice has been noted previously. Tao et al. demonstrated earlier that at moderate doses (1 $\times$ $10^{10}$ virus particles (vp)), Ad5 displays 400-fold variations in transgene expression in immunocompetent C57Bl/6, BALB/c, and C3H mice or in immunodeficient nude or Rag-1 mice (3). Similarly, Snoeys et al. observed that i.v. injection of Ad5 expressing apolipoprotein A-I at high doses of 5 $\times$ $10^{10}$ vp produced 3-fold higher expression in C57BL/6 mice than in BALB/c mice (23). When doses were reduced to 1.5 $\times$ $10^{10}$ vp, expression was 60-fold higher in C57BL/6 mice than in BALB/c mice. This lower transduction coincided with the
observation that BALB/c mice sequestered 20 times more Ad5 DNA in their Kupffer cells and 3 times more Ad5 in their LSECs than C57BL/6 mice. These data indicate there are large differences in how different strains of mice remove adenovirus vectors from their circulation may make extrapolation of vector pharmacology between inbred mouse strains and outbred humans difficult. To better understand adenovirus pharmacology, we have investigated the roles of cells of the reticuloendothelial system and the effects of natural antibodies in different strains of mice.

MATERIALS AND METHODS

Viruses
Ad5 and Ad5/6 vectors were generated as described previously (14). Briefly, Ad5 was produced using the AdEasy system (Qbiogene/MP Biomedicals) in HEK293 cells and Ad5/6 was created by recombining the hypervariable regions (HVR) 1-7 of Ad6 into the Ad5 backbone. Both vectors are replication defective and contain an eGFP-Luciferase fusion transgene.

Animals
Female BALB/c, C57BL/6, aC57BL/6 (albino), CB6F1 or FVB mice between 6-8 weeks old were obtained from Harlan Sprague-Dawley. Rag -/- BALB/c and C57BL/6 mice between 5-6 weeks old were obtained from Jackson Laboratories.
One male and one female (B6;C3Fe a/a-Csf1op/J) were purchased from Jackson Laboratories and bred in house with aC57BL/6 mice. Homozygote knock-out offspring were selected by the absence of teeth, and were fed a soft dough diet (BioServ). Only female mice were used in luciferase imaging experiments. All animals were housed and bred in the Mayo Clinic Animal Facility and experiments were conducted under the policies and procedures of Mayo Clinic.

**Monitoring luciferase transgene expression**

Mice were injected intravascularly (i.v.) with $1 \times 10^{10}$ vp of Ad vectors via the tail vein. For *in vivo* bioluminescence imaging, mice were anesthetized with ketamine/zylaxine and injected intraperitoneally (i.p.) with 100 μl of D-Luciferin (20mg/ml; Molecular Imaging Products). Albino mice were used or else black/brown fur was shaved from the mouse midsection. Light output was measured with the Lumazone Imagine System (Photometrics, Roper Scientific) for 3 min 1x1 binning using no filters or photomultiplication. Lumazone imaging software was used to determine total light intensity per mouse (photons) for data analysis (14). For *in vitro* luciferase assays, cells were plated into a 96-well plate and infected with either 1,000 or 10,000 vp/cell of Ad5. After incubation at 37°C for 24 h, 25 μl of 5X cold passive lysis buffer and 50 μl of luciferase assay reagent was added to each well. Relative Luminescence Units (RLU) was measured with the Beckman Coulter DTX 880 Multimode Detector system.
Immunohistochemistry

The large liver lobe was harvested from mice and incubated o/n at RT in formalin. The tissue was embedded in paraffin and cut to 4 µm sections. To stain for F4/80+ cells, slides were probed with rat anti-F4/80 primary (AbD Serotec, MCA497EL) at a 1:200 dilution, and an hrp-conjugated anti-rat secondary, with DAB substrate. To stain for CD31+ cells, slides were probed with rabbit anti-CD31 at a 1:200 dilution and an hrp-conjugated anti-rabbit secondary, with DAB substrate. To quantify positively stained cells, images of 5 fields of view for each mouse section were taken. ImageJ software was used to convert them to binary images, and the “Threshold” feature was used to separate out positively stained cells from background nuclei. “Analyze particles” was used to count numbers of stained regions (25-infinity). Normal human liver biopsy tissue was received from Mayo Clinic, and was sectioned, stained with CD68 primary.

Uptake in mouse peritoneal macrophages.

Mice were euthanized and mouse peritoneal macrophages (MPMs) were immediately harvested by peritoneal lavage with 5 ml of endotoxin free PBS (Gibco). Cells were pooled from 3 mice, counted, plated to confluency onto 24-well plates, and incubated at 37°C for 1 h. The adherent cells were washed twice with PBS, infected with 10,000 or 100,000 vp of Ad5 or Ad5/6, and incubated in Dulbecco’s Modified Eagle Medium (DMEM) containing 10% heat-inactivated fetal calf serum (Gibco) and penicillin/streptomycin at 100 U/ml (Gibco). After 24
h at 37°C, the cells were washed twice to remove unbound virus and then harvested for DNA extraction by DNEasy (Qiagen). Real-time PCR was performed with primers against cellular GAPDH and viral CMV (14) and presented as a ratio to determine uptake per cell.

**Predosing**

For the Ad5 predose, $3 \times 10^{10}$ vp of Ad5-dsred in 100 μl of PBS was injected i.v.. After 4 h, $1 \times 10^{10}$ vp of Ad5 or Ad5/6 was injected i.v.. For the poly (I) predose, 100 μg of polyniosinic acid in 100 μl PBS was injected 5 min before Ad vector injection. Luciferase expression was measured as light output 24 h later by *in vivo* imaging.

**IgM Complementation**

BALB/c mice were exsanguinated and serum was used for IgM purification using CaptureSelect IgM Affinity Matrix (BAC BV, the Netherlands) yielding approximately 0.3 mg IgM per mouse. IgM purity was verified by SDS-PAGE. Endotoxin levels were less than 0.6 EU/ml. Pathogen testing by Impact III PCR profile by IDEXX-RADIL did not detect any mouse pathogens. PBS or a maximal feasible injection amount of 445 μg of purified IgM was injected i.v. into each mouse followed 1 h later with $1 \times 10^{10}$ vp of Ad5 or Ad5/6 by the i.v. route. Luciferase expression was measured at 24 h later by *in vivo* imaging.
Enzyme Linked Immunosorbent Assays

To determine antibody levels, serum was harvested from different mouse strains by submandibular bleed into serum separator tubes (BD Microtainer) and allowed to clot for 30 min. The serum was separated by spinning at 13,000 x g for 2 min. The mouse hybridoma subisotyping kit (EMD Millipore) was used to assess circulating antibody isotype. For antibody binding to Ad vectors, 120 ng of Ad5 or Ad5/6 was coated onto 96-well ultra-high binding ELISA plates for 1 h at 37°C (Immunolon 4HBX). The wells were washed 3 times with PBS and then blocked for 2 h at RT with 3% BSA in PBS. The wells were washed 3 times with PBS and then serum (1:50 dilution in PBS) was added. After 2 h at RT, the wells were washed 3 times with PBS and the procedure for the hybridoma subisotyping kit was used to determine specific isotype binding.

To determine FX levels, mice were anesthetized, and whole blood was collected by retroorbital bleed into 3.2% sodium citrate. The Mouse Factor X total antigen ELISA kit (Molecular Innovations) was used to determine FX levels.

Isolating and infecting primary hepatocytes

Mice were anesthetized. The inferior vena cava was cannulated and the portal vein was cut. The blood in the liver was replaced with 24 g/L HEPES buffer with 9.5 g/L EGTA. The liver and catheter were cut away and placed into an ex vivo system that maintains a steady temperature of 37°C. The liver was perfused with 300 mg/L of collagenase and 4 g/L albumin solution for 10 min. After perfusion,
the liver cells were gently teased apart and hepatocytes were separated out with two gentle spins at 30 x g. Cells counted by hemacytometer, were plated into a 96-well plate and infected with 1,000 or 10,000 vp/cell of Ad5. After 24 h, transduction was assessed by luciferase assay.

Data analysis

Graphs and statistical analyses were performed using Prism Graphical software. Analysis between two and three groups was determined by Unpaired t-test, and one-way ANOVA (Bonferroni post-test), respectively. To determine the R-squared correlation coefficient between relative amounts of antibody isotypes and expression, a semi-log (X is log, Y is linear) trend-line was fitted.

RESULTS

Mouse strains show dramatic differences in liver expression by Ad5 and Ad5/6. To test differences in adenoviral pharmacology, we compared replication defective Ad5 and Ad5/6 (14) viruses, both expressing luciferase. Ad5/6 is an Ad5 vector whose hexon hypervariable regions (HVRs) were replaced with those from species C family member Ad6. Previous work in BALB/c mice demonstrated that Ad5/6 mediates up to 10-fold higher liver transduction than Ad5 in BALB/c mice (14). This effect correlated to reduced recognition of Ad5/6
scavenger receptors (1). Therefore, Ad5/6 was used in these studies as a vector with reduced tropism to Kupffer cells, at least in BALB/c mice. Ad5 and Ad5/6 were injected intravenously (i.v.) by tail vein into BALB/c and C57BL/6 mice. Mice were injected with $1 \times 10^{10}$ vp, a dose that is susceptible to Kupffer cell absorption (3, 7), and light output from luciferase transgene expression was measured 24 h later. Consistent with previous results (14), Ad5/6 mediated 50-fold higher transduction in the livers of BALB/c mice than Ad5 (Fig. 1A). Ad5 transduction was similar in both strains of mice, with expression being only 1.5-fold higher in C57BL/6 mice. In contrast to the results in BALB/c, Ad5/6 mediated no increase in transduction in C57BL/6 mice. These results were consistent whether C57BL/6 mice were imaged with their normal black fur, were shaved, or were on an albino background (Fig. 1B). To determine if this effect was dominant or recessive, Ad5 and Ad5/6 were compared in F1 progeny of C57BL/6 and BALB/c mice (CB6F1) (Fig. 1A). In this case, transduction by both Ad5 and Ad5/6 was intermediate between BALB/c and C57BL/6 mice suggesting a more subtle genotype-phenotype relationship.

Mouse strains have similar levels of liver Kupffer cells and LSECs. Adenoviruses can be absorbed a variety of cells in the body (reviewed in (13)). Liver Kupffer cells have become the focus of interest, since they may play the largest role in Ad5 absorption after intravenous injection. More recently LSECs have been recognized as another cell type that can absorb Ad after i.v. injection.
(8). A simple explanation for variations in virus pharmacology in mouse strains could be that they have large differences in raw numbers of Kupffer cells and/or LSECs. To test this, livers from the mice were immunohistochemically stained for Kupffer cells using anti-F4/80 antibody and for LSECs using CD31 antibody. Gross histological stains and counts failed to find any obvious numerical differences in either cell type in any of the tested mouse strains analyzed (Fig. 2A and Fig. 3A, B). Staining of human liver sections revealed a Kupffer cell density similar to that of mice (Fig. 2B and 3E).

Host factor X (FX) levels and hepatocyte susceptibility to infection do not correlate with murine liver expression. FX facilitates liver transduction by both Ad5 and Ad5/6 (14). To determine if FX levels could explain differences in liver transduction between strains, plasma was assayed for FX by ELISA (Fig. 3C). FX levels were insignificantly different between the strains. To test whether Ad vectors might transduce hepatocytes from different strains to different degrees at the cellular level, hepatocytes from various strains of mice were purified and infected with 1,000 vp/cell of Ad5 (Fig. 3D). Transduction was assessed by luciferase assay after 24 h and showed no significant difference between in hepatocytes expression from the different mice.

Ad5/6 evades macrophages and Kupffer cells regardless of mouse strain. Phagocytosis of Ad5 by Kupffer cells not only destroys the virus, but also kills the
Previous work in BALB/c mice showed that Ad5/6 causes less Kupffer cell depletion than Ad5 at the same dose (14). To compare this in the two mouse strains, both were injected with $3 \times 10^{10}$ vp of PBS, Ad5, or Ad5/6. After 6 hours, their livers were harvested for immunohistochemistry with F4/80 antibody (Fig. 2A). In both strains of mice, Ad5 injection markedly reduced F4/80-positive staining whereas Ad5/6 showed considerably lower clearance of these cells. Variations in Ad tropism in the strains could be due to differences in the phagocytic activities of their cells. To compare the phagocytic functions of macrophages between the two mouse strains, mouse peritoneal macrophages (MPMs) were harvested from BALB/c and C57BL/6 mice. MPMs were collected and Ad5 or Ad5/6 were incubated with the cells at 1000 vp/cells for 24 hours. DNA was harvested from the cells and real-time PCR used to determine uptake of Ad genomes in the cells. C57BL/6 macrophages took up approximately 25% less Ad5 and 75% less Ad5/6 virions than BALB/c cells, which accords with observations that C57BL/6 Kupffer cells take up less Ad5 after i.v. injection (Fig. 3F). Consistent with previous results (14), Ad5/6 was taken up less efficiently by macrophages from both BALB/c and C57BL/6 compared to Ad5.

Predosing in BALB/c and C57BL/6 mice. Negatively-charged polyinosinic acid, or poly (I), can be used to predose mice and block scavenger receptors on both Kupffer cells and LSECs. As a result, a subsequent dose of Ad evades uptake and more effectively transduces the liver (27). In BALB/c mice, poly (I)
boosted both Ad5 and Ad5/6 transduction in the liver to peak levels (Fig. 4A).

Poly (I) also boosted Ad5 and Ad5/6 in C57BL/6 mice, although overall levels of transduction were nearly 10-fold lower (Fig. 4B).

The lethal effects of Ad5 on Kupffer cells can also be used to predose mice (20). Previous work in BALB/c mice confirmed that predosing with Ad5 enhances Ad5, but not Ad5/6, transduction (14). To test whether this effect was consistent between the different mouse strains, C57BL/6 mice were predosed with Ad5 and then dosed four hours later with either Ad5 or Ad5/6 (Fig. 4C). As before, Ad5 predosed Ad5, but not Ad5/6 in the C57BL/6 mice. Although statistically significant, Ad5 predosing was considerably weaker in the C57BL/6 mice, remaining 10-times below the peak photon level achieved in BALB/c mice.

Vector expression in macrophage colony-stimulating factor (M-CSF) knock-out mice. Predosing with Ad5 can eliminate Kupffer cells; however, this first dose of virus could also block many other receptors and cell types. Similarly, anionic poly (I) likely blocks the interactions of many charged surfaces in the mice. To probe Ad5 and Ad5/6 interactions with Kupffer cells by alternate means, macrophage colony-stimulating factor (M-CSF) mutant C57BL/6 mice were used (28). M-CSF knock-out mice have a 77% reduction in the numbers of Kupffer cells and 84% reduction in phagocytosis (6). When M-CSF knock-out mice were injected with Ad5 and Ad5/6, both vectors mediated similar levels of liver transduction (Fig. 4D). Unlike pharmacologic predosing with either Ad5 or
poly (I), genetic knock-down of liver Kupffer cells was able to raise liver transduction in C57BL/6 mice to the peak photon level observed under optimal conditions in BALB/c mice.

Antibody levels correlate inversely with Ad5 liver expression. These data suggest that the Ad5 and Ad5/6 differ primarily in interactions with Kupffer cells and/or macrophages in different mouse strains. Antibody opsonization can play a key role in macrophage-dependent clearance of foreign particles. Pathogen recognition can be mediated by antibodies generated by adaptive immune responses or by natural antibodies that are encoded in the germline. Prior data suggests that Ad5 may be targeted to Kupffer cells by natural antibodies (27).

To test whether antibody levels vary in different mouse strains, fresh serum was harvested from naïve mice and analyzed for isotype distribution by ELISA. BALB/c mice had higher ELISA levels of antibodies than C57BL/6 mice (Fig. 5A and B). F1 progeny of BALB/c and C57BL/6 mice (CB6F1) had total antibody OD450 levels that were nearly as high as BALB/c mice with most isotypes being slightly lower than the BALB/c mice. On the other hand, FVB mice had total levels that were actually lower than in C57BL/6 mice. Interestingly, FVB mice were unique in mediating very robust Ad5 liver transduction up to the peak photon level with no manipulation (Fig. 6A). When the observed isotype profiles and transduction data were taken together, liver transduction by Ad5 was inversely correlated with IgM, IgG2b, IgG3, and IgA antibodies (Fig. 6C and...
Table 1). /6 expression did not negatively correlate with IgM, IgG2b, IgG3, and IgA antibody OD levels, but instead correlated positively with higher IgG2a isotypes observed in BALB/c and CB6F1 mice (Fig 6D and Table 1).

Liver transduction in mice with no antibodies. Previous work showed that C57BL/6 Rag -/- mice that lack any immunoglobulins have markedly reduced Kupffer cell uptake of Ad5 implicating natural antibodies in this sequestration (24). To further confirm the link between circulating antibodies and Ad expression in different strains of mice, Ad5 and Ad5/6 were tested in Rag -/- mice on either a BALB/c or a C57BL/6 genetic background (Fig. 6B). In these mice lacking immunoglobulins, transduction by both vectors was similar on both strain backgrounds. Ad5 transduction reached the $10^{12}$ photon level in the absence of immunoglobulins in a manner similar to that observed in M-CSF knock-out mice. Interestingly, like FVB mice that have low levels of circulating natural antibodies, Ad5/6 transduction in Rag -/- mice was also lower than Ad5.

Natural antibodies can bind directly to Ad capsid. Direct binding of immunoglobulins to virions would be the simplest means to affect Ad pharmacology. To test this, fresh serum from naïve mice was tested for isotype-specific binding to Ad5 and Ad5/6 by ELISA (Fig. 7). All isotypes bound directly to both Ad vectors, although IgM from all murine strains and IgG2b from C57BL/6 mice mediating the highest binding.
Administration of IgM antibodies to Ig-deficient BALB/c Rag-/- mice reduces liver transduction by Ad5. BALB/c mice have circulating levels of IgM of approximately 1,000 µg/ml. To test if IgM can mediate effects on Ad liver transduction, IgM antibodies were injected into Ig-deficient Rag knock-out mouse on the BALB/c background. Bulk IgM was purified from untreated BALB/c mice. 445 µg of this IgM was injected into 8 Rag mice and 8 mice received PBS as a control. One hour later, the animals were injected i.v. with 1 x 10^{10} vp of Ad5 or Ad5/6 and luciferase expression was measured at 24 hours later (Fig. 8). Under these conditions, IgM complementation reduced liver transduction by Ad5 by more than 5-fold relative to mice that received PBS (p < 0.05 by one way ANOVA). In contrast, IgM reduced Ad5/6 transduction slightly, but this was not statistically significant. These data suggest that bulk IgM antibodies can mediate reduced liver transduction observed different strains mice.

DISCUSSION

Ad5 is a hepatotropic virus that has been studied extensively as a potential gene therapy vector for liver-directed gene therapy. Mice have been used as the preferred small animal model in the vast majority of preclinical investigations, although dramatic differences in Ad5 liver expression have been documented in different murine strains (3, 27).
Transduction of the liver by Ad5 depends on interactions with blood components such as circulating cells, blood factors, antibodies, and complement upon i.v. administration (reviewed in (13)). These interactions increase Ad5 delivery to the liver, where resident liver macrophages called Kupffer cells can deplete over 98% of an injected dose. There is also evidence to suggest that other cells in the reticuloendothelial system of the liver, such as liver sinusoidal endothelial cells (LSECs) also sequester some proportion of virus particles. To enter the space of Disse and transduce hepatocytes, virions must evade phagocytosis by Kupffer cells or LSECs. Those that escape the liver can distribute to other organs like the spleen and lungs.

While it is certainly known that mice differ from humans in many aspects, more recent data has suggested strong variations in Ad5 pharmacology between inbred mouse strains. After we observed profoundly better liver transduction by Ad5/6 virus than Ad5 in BALB/c, we were surprised to find essentially no improvement in C57BL/6 mice. This study aimed to resolve these differences with the hope of ultimately extrapolating the underlying biological differences in these inbred models to humans.

Differences in the transduction by Ad5 and Ad5/6 in BALB/c, C57BL/6, CB6F1, FVB, Rag-/-, and M-CSF-/- strains support the hypothesis that different strains have host-specific factors that influence Ad5 and Ad5/6 transduction. Several experiments in this work confirm that Ad5/6 evades macrophages regardless of mouse strain. For instance, Ad5/6 was taken up less efficiently in
both BALB/c and C57BL/6 peritoneal macrophages in vitro. Furthermore, mice that were predosed with Ad5 to destroy Kupffer cells showed an increase in Ad5 expression and no increase in Ad5/6 expression. Finally, genetic reduction in Kupffer cells in M-CSF mutant mice ablated the difference between the two vectors on the C57BL/6 mouse background. Predosing with poly (I) boosted transduction by both Ad5 and Ad5/6. This difference in predosing between Ad5 and poly (I) suggested that non-Kupffer cells may also be involved in virus sequestration. Kupffer cells and LSECs both express several classes of scavenger receptors and poly (I) can disrupt uptake of Ad into either of these cell types. Therefore, one hypothesis is that Ad5 is absorbed by both Kupffer cells and LSECs and Ad5/6 is absorbed primarily by LSECs particularly in C57BL/6 mice. This hypothesis is consistent with previously published data showing that C57BL/6 mice take up 3 times more Ad DNA in their LSECs than in their Kupffer cells whereas uptake in BALB/c mouse cells is reversed (23). While this hypothesis may be accurate at some level, the observed equalization of Ad5 and Ad5/6 transduction in C57BL/6 mice with M-CSF knocked-out suggests that Kupffer cells, and perhaps other macrophages in the body, are the predominant actors on Ad5 and Ad5/6 even in C57BL/6 mice. To our knowledge, M-CSF is not directly involved in LSEC biology, so its knock-out most likely does not affect the function of these cells. However, indirect effects may occur. Whether poly (I) inhibits uptake of virus into another location remains to be determined.
Pathogens can be neutralized by a number of pathways (Fig. 8).

Antibodies can trigger uptake directly through Fc receptors or indirectly by activating complement and binding complement receptors (reviewed in (9, 16)).

Earlier work with Ad5 demonstrated binding of IgM antibodies to Ad5 capsid and weak binding of IgG to the virus (27). This work also showed strong complement factor C3 activation during elimination of Ad5 by Kupffer cells.

We show here that addition of IgM to Ig-deficient mice reduces liver transduction by Ad5. This supports the observed correlation between IgM levels in different mice and liver transduction by Ad5. It should be noted that we were only able to introduce 570 µg of IgM to each of the Rag mice due to constraints in i.v. injection volumes. Considering that BALB/c mice have approximately 1,000 µg/ml of IgM and approximately to 2 mg of total IgM, this antibody reconstitution would result in only 25% of normal IgM levels. Therefore, the 5-fold effect on Ad5 transduction observed may under-represent the possible effects of natural antibodies on the virus. Natural antibody effects are also likely affected by variations in other important factors like complement factor C3 in the Rag mice (27).

Higher levels of IgM, IgG2b, IgG3, and IgA antibodies in mice correlated negatively with liver transduction by Ad5. What was surprising was the observation that none of these antibody levels correlated with Ad5/6 transduction. This was consistent with the relatively weak effect of IgM reconstitution in Rag mice on Ad5/6 transduction. In contrast, IgG2a antibodies
positively correlated with Ad5/6 activity. Given that antibodies can mediate opsonization and phagocytosis through a number of mechanisms (Fig. 8), exactly how, or if, IgG2a antibody binding has direct effects or more complicated effects remains to be determined.

IgG2a and IgG2b and their human homologs IgG1 and IgG3 are described as strongly opsonizing (11). Comparison of the ability of different isotypes of murine antibodies to opsonize *E. coli* showed that anti-bacterial effects were strongest by IgM with IgG2a being next most potent (17). In this case, antibacterial effects were directly correlated to the ability to deposit complement factor consistent with previous work on Ad5 clearance (27). In contrast, comparison of anticancer effector functions between the same idiotype-bearing antibody with different Fc regions from IgG1, IgG2a, IgG2b, and IgG3 demonstrated that IgG2a was by far the most potent at facilitating Fc receptor-mediated anticancer functions (16). Therefore, it is possible that Ad5 is indeed cleared by IgM and complement as well as by IgG antibodies via Fc receptors (Fig. 8). In the case of Ad5/6, most of its biology appears inverted from Ad5 perhaps suggesting disruption at some step between Ig binding, complement binding, and phagocytic uptake. Ad5/6 or Ad6 may bind IgM or other Ig's in such a way that the virus can avoid phagocytosis as has been shown in both RAW264.7 cells and here in BALB/c and C57BL/6 macrophages. Ad5/6 is able to avoid scavenger receptors *in vitro*, but it remains to be determined if it also avoids complement receptors and/or Fc receptors.
Previous work implicated the binding of natural antibodies to Ad5 capsid, combined with complement activation, as a primary opsonin mechanism for targeting the virus to Kupffer cells (27). This prior work is seminal in describing a clearance pathway for Ad5. We would stipulate that this clearance mechanism may be particular to Ad5, rather than all Ad serotypes as evidenced by variations in tropism for Ad1, 2, 5, and 6 of species C Ads (26), marked variations between Ad5 and Ad5/6 (14), and likely wider ranging systemic and liver pharmacology for other Ad species (21). Despite being highly conserved, Ad5 and Ad6 (and Ad5/6) differ greatly in \textit{in vivo} pharmacology and even in direct recognition by scavenger receptors SRA-II and SREC. Species B and species D viruses with smaller HVRs in their hexons appear less likely to bind FX and at least a subset (Ad11, 35, 26, and 48) do not appear to infect mouse liver hepatocytes (as evidenced by minimal release of liver enzymes (21) and unpublished observations). Predosing mice with Ad11, 35, 26, and 48 does not enhance subsequent transduction by Ad5 ((21) and unpublished observations) suggesting that these other serotypes may not be absorbed by the same cells or locations as Ad5.
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REFERENCES


Table 1. R-squared values for the correlation between Ad vector expression and relative amounts of natural antibody isotypes.

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Figure Legends

Figure 1. Ad vector expression is highly variable between mouse strains. A) BALB/c, C57BL/6, and their F1 progeny (CB6F1) mice were injected i.v. with 1 x $10^{10}$ vp of Ad5 or Ad5/6. After 24 h, light output was quantified as a measure of luciferase expression (n = 10-18). B) BALB/c and unshaved and shaved C57BL/6, and C57BL/6 albino mice were injected i.v. with 1 x $10^{10}$ vp of Ad5 or Ad5/6. *, p<0.05; **, p<0.005; ns, not significant.

Figure 2. Kupffer cell numbers in mice and humans. A) BALB/c and C57BL/6 mice were injected with 3 x $10^{10}$ vp of PBS, Ad5GL, or Ad5/6GL. After 6 h, the large liver lobe was harvested, formalin fixed, and paraffin embedded. Immunohistochemistry for F4/80+ cells was performed on 4 µM sections (dark brown staining). B) Kupffer cell density in human liver. Normal human liver biopsy tissue was harvested, paraffin embedded and sectioned (4 um thick). Immunohistochemical staining for CD68 was used to visualize Kupffer cells.

Figure 3. Cellular and functional measurements in mice and humans. Immunohistochemistry in the livers of different mice for A) F4/80+ Kupffer cells and B) CD31+ endothelial cells performed on 4 µM tissue sections. Numbers of positively stained cells from 5 fields of view were quantified with ImageJ. C) FX levels in different strains of mice. Whole blood was collected into sodium citrate.
and an ELISA was used to determine the amount of mouse FX in the plasma (n = 4 wells) D) Infectivity of purified mouse hepatocytes from different strains. Mice (pools of 3) were perfused ex vivo with collagenase and hepatocytes were separated by gentle centrifugation. The cells were infected in suspension with 1,000 vp/cell of Ad5 and measured for luciferase expression after 24 h (n = 8 wells). E) Kupffer cell numbers in human liver. Normal human liver biopsy tissue was harvested, paraffin embedded and sectioned (4 um thick). Immunohistochemical staining for CD68 was used to visualize Kupffer cells (Fig. 2B), and positively stained cells were counted from 5 fields of view with ImageJ. F) Uptake of Ad5 or Ad5/6 by mouse peritoneal macrophages (MPM). MPM were collected from 3 BALB/c or 3 C57BL/6 mice and pooled. These samples were infected in vitro with 1000 vp/cell of the indicated vector. After 24 h, DNA was harvested from the cells and real-time PCR was used to determine the number of viral genomes taken up in relation to cellular genomes (CMV:GAPDH) (n = 3 wells).

Figure 4. Predosing effects in different mouse strains. A) BALB/c or B) C57BL/6 mice were predosed i.v. with PBS or 100 μg of polyinosinic acid, poly (I) and injected 5 min later with 1 x 10^{10} vp of Ad5 or Ad5/6. C) C57BL/6 mice were predosed i.v. with PBS or 3 x 10^{10} vp of in irrelevant Ad5-dsRed vector, and then injected i.v. 4 h later with 1 x 10^{10} vp of Ad5 or Ad5/6. D) M-CSF knockout mice
were injected i.v. with $1 \times 10^{10}$ vp of Ad5 or Ad5/6. All injected mice were imaged for luciferase expression after 24 h. ($n = 2-5$). *, $p<0.05$; ns, not significant.

**Figure 5. Antibody levels vary distinctly between murine strains.** Serum was harvested from mice (pool of two mice per strain) and analyzed for circulating antibody isotypes by ELISA. Shown are A) total antibody levels or B) antibody levels divided by isotype ($n = 6$ wells). *, $p<0.05$; **, $p<0.005$; ns, not significant.

**Figure 6. Ad expression is related to circulating antibody levels**
A) CB6F1 (F1 progeny of BALB/c and C57BL/6), FVB, B) Rag -/- BALB/c and Rag -/- C57BL/6 mice were injected i.v. with $1 \times 10^{10}$ vp of Ad5 or Ad5/6. Luciferase expression as measured by light output was quantified ($n = 2-10$). *, $p<0.05$; **, $p<0.01$; ns, not significant. C) Ad5 and D) Ad5/6 expression levels from BALB/c, CB6F1, C57BL/6, and FVB mouse strains were plotted against relative levels of antibody isotypes titers.

**Figure 7. Antibodies can bind directly to the Ad capsid.** A) Ad5 or B) Ad5/6 was coated onto ELISA plates, bound with serum from BALB/c C57BL/6 and FVB mice, and specifically probed with isotype specific antibodies. Relative levels of natural antibody binding was determined with an hrp-conjugated secondary ($n = 6$ wells).
Figure 8. Partial restoration of IgM in Ig-deficient rag/- mice reduces liver transduction by Ad5. Groups of 4 BALB/c rag/- mice were injected i.v. with either PBS or 445 µg of purified IgM from BALB/c mice. One hour later, the mice were injected i.v. with 1 x 10^{10} vp of Ad5 or Ad5/6. All injected mice were imaged for luciferase expression after 24 h. (n = 4). *, p<0.05 by one way ANOVA.

Figure 9. Representation of multiple receptors systems on Kupffer cells for Ad recognition and uptake. Adenovirus type 5 can bind scavenger receptors (such as SR-AI, SR-AII, SREC) based on negative charge interactions. Natural or pre-existing neutralizing antibodies can bind directly to capsid proteins, or indirectly via C3 complement binding. Monomeric IgG or pentameric IgM antibodies are recognized by different Fc receptors. Complement receptors can attach to complement proteins bound either directly or indirectly to the adenoviral surface.
Khare et al. Figure 2

**A**

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**B**

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Khare et al. Figure 3

A. Mouse Kupffer cells

B. Mouse LSECs

C. Amount of FX (μg/ml)

D. Luminescence (RLU)

E. Human Kupffer cells

F. Mouse macrophages

Balb/c C57BL/6 FVB

Balb/c C57BL/6 FVB

BALB/c C57BL/6 FVB

Females Males

Ad5 Ad5/6

BALB/c C57BL/6
Khare et al. Figure 4
Khare et al. Figure 7

A

**Ad5**

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Absorbance @ 620nm

B

**Ad5/6**

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Absorbance @ 620nm
Khare et al. Figure 9