Available online at www.sciencedirect.com
ScienceDirect
Virology 374 (2008) 453–467
VIROLOGY
www.elsevier.com/locate/yviro

Replication-attenuated Human Adenoviral Type 4 vectors elicit capsid dependent enhanced innate immune responses that are partially dependent upon interactions with the complement system

Zachary C. Hartman [a,b], Daniel M. Appledorn [c,d], Delila Serra [a], Oliver Glass [b], Todd B. Mendelson [a], Timothy M. Clay [b], Andrea Amalfitano [a,c,d,*]

[a] Division of Medical Genetics, Department of Pediatrics, Duke University Medical Center, Durham, North Carolina 27710, USA
[b] Department of Surgery, Duke University Medical Center, Durham, North Carolina 27710, USA
[c] Department of Microbiology and Molecular Genetics, Michigan State University, East Lansing, Michigan 48824, USA
[d] Department of Pediatrics, Michigan State University, East Lansing, Michigan 48824, USA

Received 13 November 2007; returned to author for revision 7 December 2007; accepted 2 January 2008
Available online 15 February 2008

Abstract

Human Adenovirus Type 4 (HAdV-4) is responsible for epidemic outbreaks of Acute Respiratory Disease (especially in military recruits), and is known to cause significant morbidity with several reported cases of mortality. However, we do not understand why this serotype causes such high morbidity, and have little insight into the immunobiology of HAdV-4 infections. We have now developed a replication attenuated HAdV-4 vector system, and through it, demonstrate that HAdV-4 virions have enhanced infectivity of certain cell types and reveal aspects of the serotype-specific heightened innate immunogenicity of infectious HAdV-4 capsids both *in vitro* and *in vivo*. We further found that elements of this serotype-specific immunogenicity were dependent upon interactions with the complement system. These findings provide insights into the mechanisms possibly underlying the known morbidity accompanying wild-type HAdV-4 infections as well as highlight important considerations when considering development of alternative serotype vectors.

Keywords: Adenovirus; Vectors; Serotype 4; Innate immune response; Complement; Inflammation; Adaptive response

Introduction

Human Adenovirus Type 4 (HAdV-4) is known to be one of the predominant serotypes causing Acute Respiratory Disease (ARD), accounting for 60–80% of ARD affecting military camps (Hilleman, 1957; Hilleman et al., 1955). This particular adenoviral serotype is associated with high rates of morbidity and has been associated with recruit mortality, prompting the development of a HAdV-4 vaccine (Levin et al., 1967; Top et al., 1971b,c,a; Russell et al., 2006b; Chanock et al., 1966). While its virulence has long been recognized, recent studies have also demonstrated that the transmission of HAdV-4 is more epidemic than previously appreciated. In one of the few viral transmission studies to date, evidence of HAdV-4 infection was documented to increase from 33% of recruits at the beginning of basic training to ~97% of recruits by the sixth week of basic training (Russell et al., 2006a). While these characteristics are considered detrimental, it is possible that their utilization in a HAdV-4 based vector platform may prove to be clinically useful, for example when utilized in vaccine applications that require heightened immunogenicity. In this study, we characterize the immunogenic properties of a first generation HAdV-4 vector, and evaluate its potential as a tool for viral and clinical research.

Although previous studies have employed partially modified HAdV-4 viruses obtained by recombination based methods in mammalian cells (*in vivo* manipulation), we used a bacterial

Abbreviations: Ad, adenovirus; HAdV-4, Human Adenovirus Type 4; HAdV-5, Human Adenovirus Type 5; dpi, days post-infection; ELISA, Enzyme-linked Immunosorbance Assay; ELISPOT, Enzyme Linked Immuno-Spot; moi, multiplicity of infection; Th1, T-helper type 1; LacZ, beta-galactosidase; wpi, weeks post-infection.

* Corresponding author. 4194 Biomedical and Physical Sciences Bldg, Michigan State University, East Lansing, MI 48823, USA.
E-mail address: amalfit1@msu.edu (A. Amalfitano).

0042-6822/$ - see front matter © 2008 Elsevier Inc. All rights reserved.
doi:10.1016/j.virol.2008.01.017

(*in vitro*) based method to efficiently engineer an [E1−,E3−] HAdV-4 based vector bearing reporter transgenes, as has been done with the more commonly used HAdV-5 serotype (Hsu et al., 1992; Chengalvala et al., 1991, 1997; Lubeck et al., 1989; Hodges et al., 2001). In doing so, we are able to directly compare the infectivity and immunogenicity of these two different viral serotype based vectors.

Our results demonstrate higher infectivity of the HAdV-4 based vector in certain epithelial cell lines *in vitro*, as well as preferential localization to the spleen following intravascular injection *in vivo* relative to identical treatments utilizing HAdV-5 vectors. Our studies also demonstrate an enhanced innate immunogenicity of HAdV-4 based vectors that is capsid dependent. We further determined that the enhanced innate immunogenicity of HAdV-4 is in part, dependent upon a functional complement system *in vivo*, a result paralleling previous findings noted with use of HAdV-5 vectors. Finally, we found that early innate immune responses to HAdV-4 do not substantially impact upon downstream adaptive immune responses to HAdV-4 capsid proteins, but rather, elicit diminished antibody responses to vector encoded transgene products. Besides the pragmatic implications of our results relative to the use of alternative serotype Ad based vectors in general, our results also suggest that the infectious and immunogenic nature of HAdV-4 is significantly attributable to its particular capsid composition.

Results

Development of a plasmid based HAdV-4 system

To explore the nature, and exploit the potential of HAdV-4, we developed a plasmid based system that would allow for the insertion of multiple gene expression cassettes into the E1 and/or E3 regions of HAdV-4, thereby generating a replication incompetent HAdV-4 based vector platform. To achieve this we employed a plasmid based, bacterial homologous recombination-approach that has proved successful for the construction of other Ad serotype vectors (Nilsson et al., 2004). We first constructed the HAdV-4 genomic "Rescue" shuttle vector, HAdV-4-pShuttle, that contained both the 5′ and 3′ terminal ends of the HAdV-4 genome. HAdV-4-pShuttle was BamHI linearized and co-transformed into recombinogenic *Escherichia coli* BJ5183 cells along with purified wild-type HAdV-4 genomic DNA. A kanamycin resistant clone containing the HAdV-4 genomic sequence (pHAdV-4) was obtained and the HAdV-4 RI-67 identity of this clone was confirmed by multiple restriction enzyme digests and a 1× sequence of the entire plasmid (data not shown). The left and right ITR elements of the HAdV-4 genome, present in pHAdV-4, were separated from the bacterial origin and kanamycin resistance genes (contained in pHAdV-4) with I-SceI. Transfection of the I-SceI digested plasmid resulted in onset of typical Ad cytopathic effects in 293 cells, confirming integrity of the total HAdV-4 genomic construct.

We simultaneously constructed a shuttling plasmid, pHAdV-4[E1−], for the rapid mobilization of desired genetic sequences into the E1 region of the HAdV-4 genome, rendering the HAdV-4 vector replication incompetent unless grown on E1 transcomplementing cell lines (see Supplementary Fig. 1). A second shuttle plasmid, pHAdV-4[E3−], was also constructed to facilitate "shuttling" of genes into the E3 region (see Supplementary Fig. 1). [E1−,E3+] and [E1−,E3−]HAdV-4 plasmids with and without transgene insertions were I-SceI digested and transfected into 293 cells, where all viruses were successfully grown to high titer, as has been demonstrated for related Simian Group E Ad vectors (Farina et al., 2001). Integrity of the HAdV-4 genomes contained within the $CsCl_2$ purified vectors was confirmed by subsequent sequencing and restriction enzyme digest based mapping. Of note, all HAdV-4 vector preparations tested negative for any detectable RCA (Replication Competent Adenovirus, or E1+ adenovirus), likely owing to the significant sequence difference between HAdV-4 E1a and E1b gene sequences and the Ad5 derived E1a and E1b sequences present in human 293 cells. Finally, silver staining of $CsCl_2$ purified HAdV-4 vectors revealed them to have equivalent protein content per equivalent numbers of viral capsids (based upon SDS disruption and OD_{260} method for viral particle number determination) in comparison to similarly assessed HAdV-5 based vectors (data not shown).

Differential HAdV-4 infectivity in vitro

Previous investigations using wild-type HAdV-4 in tissue culture systems, as well as high hospitalization rates accompanying natural HAdV-4 infection, suggest fundamental differences exist between these two serotypes that may impact both its infectivity and immunogenicity (Zhang et al., 2003; Mei et al., 2002; Chengalvala et al., 1997; Russell et al., 2006a). To formally test for these differences, we infected several cell lines with equal particle numbers of [E1−,E3−] HAdV-4 or HAdV-5 vectors, both expressing an identical LacZ reporter transgene driven by a CMV enhancer/promoter element (Figs. 1A–F). Using LacZ activity as a determinant of cellular transduction, we found that both vectors transduced human embryonic kidney 293 cells and liver derived Hep3B cells equally well. However, although the differences were modest, these analyses revealed that HAdV-4 more efficiently transduced human lung epithelial A549 cells, HepG2, and HeLa cells than did HAdV-5. Conversely, the HAdV-5 based vector was significantly more efficient at transducing RAW264.7 mouse macrophages. To more accurately assess the infectivity of these viruses, we used real-time PCR to amplify, and quantify CMV promoter sequences (contained in both vectors) present within the infected cells. Using this assay, we confirmed the transduction efficiencies observed in HeLa, HepG2 and Hep3B cells, however, we found comparable numbers of either vector genomes present within RAW264.7 cells (Fig. 1G). A recent study concluded that vector induced interferon responses could repress the CMV promoter, the same promoter used to drive the LacZ transgene in our vector constructs (Hensley et al., 2007). To assess this, we evaluated interferon-beta production following RAW264.7 infection by either HAdV-4 or HAdV-5 vectors (Fig. 2). At 24 hpi, we found that only intact and not heat disrupted HAdV-4 vectors induced significantly greater levels of IFN-β, suggesting that interferon mediated promoter repression may be responsible

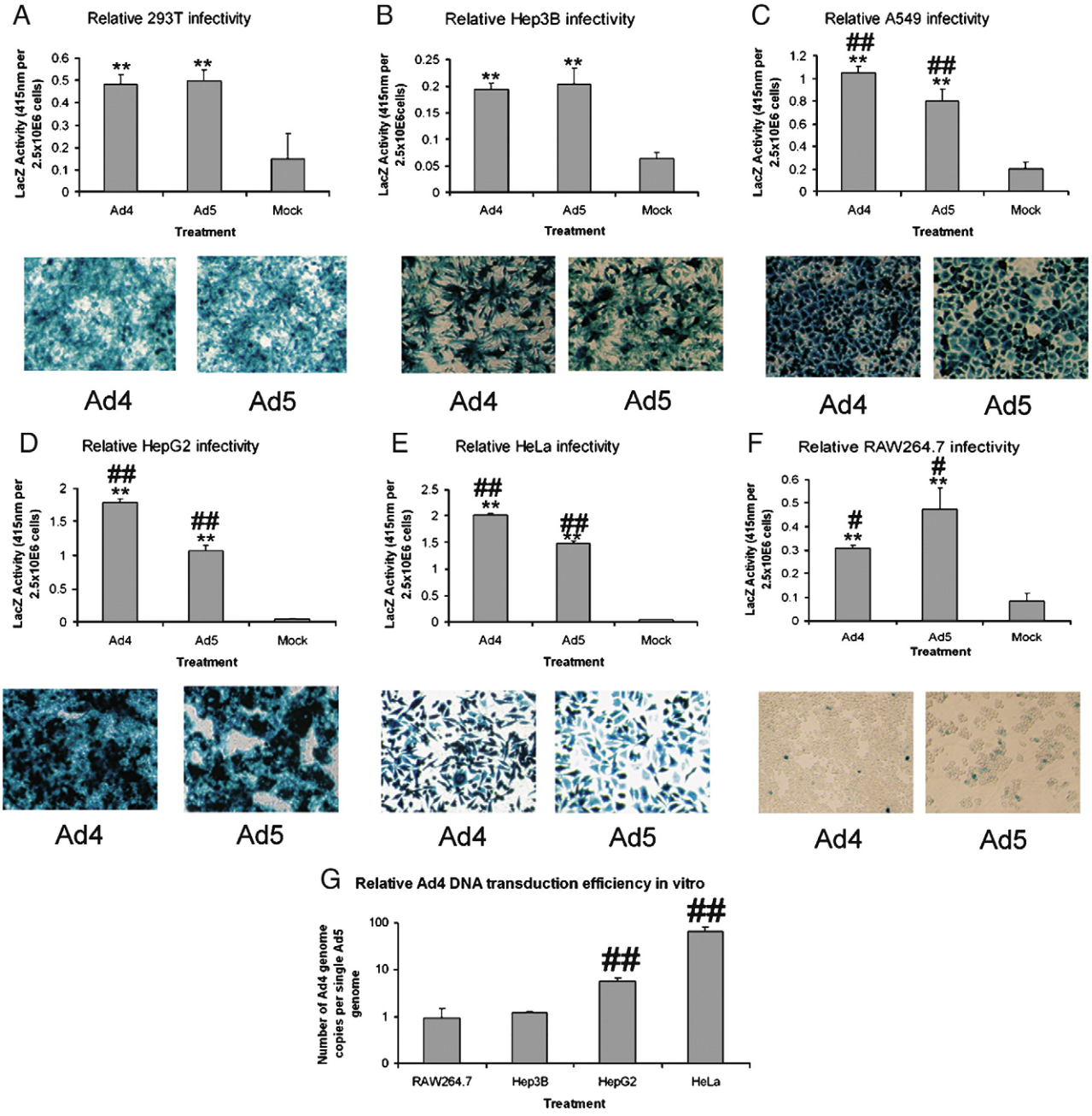

Fig. 1. Comparison of HAdV-5 and HAdV-4 vector infectivity and uptake *in vitro*. (A–F) Cells were plated at equal densities and infected with equivalent particle numbers of LacZ expressing [E1−,E3−]HAdV-4 (left panel) and HAdV-5 (right panel) vectors. Two hundred ninety three cells were infected with 300 vp/cell, Hep3B with 3000 vp/cell, A549 with 3000 vp/cell, HepG2 with 3000 vp/cell, HeLa with 3000 vp/cell, and RAW264.7 with 30,000 vp/cell. At 24 h post-infection (hpi), cells were fixed and stained using X-Gal to visualize the presence of β-galactosidase, as described in Materials and methods. The graphs depict the data obtained from replicate wells that were equivalently infected, but were also lysed and assayed for β-galactosidase activity at 24 hpi as described in Materials and methods. An "*" or "**" indicates β-galactosidase activity levels were significantly different ($p<0.05$ or $p<0.01$ respectively) between Ad infected cells and mock infected cells while "#" or "##" indicates β-galactosidase activity levels were significantly different ($p<0.05$ or $p<0.01$ respectively) between HAdV-4 and HAdV-5 infected cells. (G) Relative quantitative measure of RAW264.7 cells transduced HAdV-4 genomes in comparison to HAdV-5 genomes. HAdV-4 and HAdV-5 were infected with equal particle number of HAdV-4 and HAdV-5 as described in A–F and DNA extracted from cells at 24 hpi and assessed for genome copy number by real-time PCR. Data is shown as HAdV-4 genomes per 1 HAdV-5 genome where "#" or "##" indicates genome copy number differences that were significant different ($p<0.05$ or $p<0.01$ respectively) between HAdV-4 and HAdV-5 infected cells. Error bars are indicative of standard deviations. In all experiments $N=3$ or 4 and error bars are indicative of standard deviations.

for reduced LacZ expression by the HAdV-4 capsid. These results offered initial evidence for the heightened immunogenicity of HAdV-4 vectors compared to HAdV-5 and led us to more fully explore these two vectors in macrophages, a cell type that figures prominently in the innate immune response to adenovirus in multiple contexts.

Enhanced HAdV-4 immunogenicity in vitro

To further evaluate the relative immunogenicities of both viral vectors, we exposed RAW264.7 cells to HAdV-4 or HAdV-5 vectors at a dose of 30,000 viral particles per cell and subsequently assessed the production of multiple cytokines and

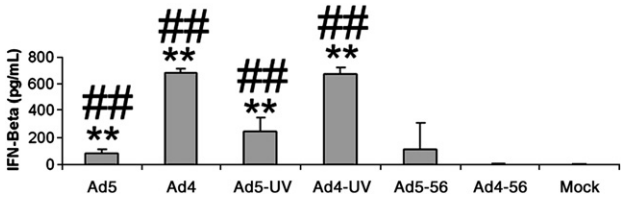

Fig. 2. HAdV-4 vectors induce significantly greater IFN-β responses in comparison to HAdV-5 vectors *in vitro*. Equal numbers of RAW264.7 macrophages were infected with [E1−,E3−]LacZ HAdV-4 and HAdV-5 vectors (either intact, after UV irradiation, or heat treatment at 56 °C) at 3000 vp/cell. Supernatants were collected and measured for IFN-β concentration at 24 hpi. In all experiments $N=3$ and error bars indicate standard deviations. An "*" or "**" indicates those time points when levels of the respective cytokine/chemokine were significantly different ($p<0.05$ or $p<0.01$ respectively) between Ad infected and mock infected cells and "#" or "##" indicates those time points when levels of cytokine/chemokine were significantly different ($p<0.05$ or $p<0.01$ respectively) between HAdV-4 and HAdV-5 counterparts.

chemokines at 6 h post-infection (Fig. 3). At this time point, the HAdV-4 based vector induced significantly higher levels of RANTES, Eotaxin, TNF-α, MCP-1, MIP-1α, and MIP-1β relative to the HAdV-5-based vector. At lower particle titers (3000 vp/cell) only HAdV-4 based vectors demonstrated detectable immunogenicity, inducing significant levels of MIP-1α, MIP-1β, RANTES, and TNF-α. To assess the possibility that these disparate immune responses were due to differences in expression of virally encoded gene products, both serotypes were UV-inactivated (to eliminate both viral replication and gene transcription) before macrophage infection. As before, HAdV-4 UV-inactivated vectors elicited greater cytokine and chemokine responses compared to UV-inactivated HAdV-5 based counterparts (Fig. 3). To test if HAdV-4 DNA was innately more immunostimulatory than HAdV-5 DNA, RAW264.7 macrophages were transfected with vector DNA purified from each virus, and cytokine production was assessed 24 h post-transfection (Supplementary Fig. 2). HAdV-4 DNA proved no more stimulatory than HAdV-5 DNA for any cytokine tested (Fig. 4).

To confirm that the dependence of the differential immunostimulatory nature of HAdV-4 and HAdV-5 vectors was predicated on intact and infectious virions, we again subjected them to

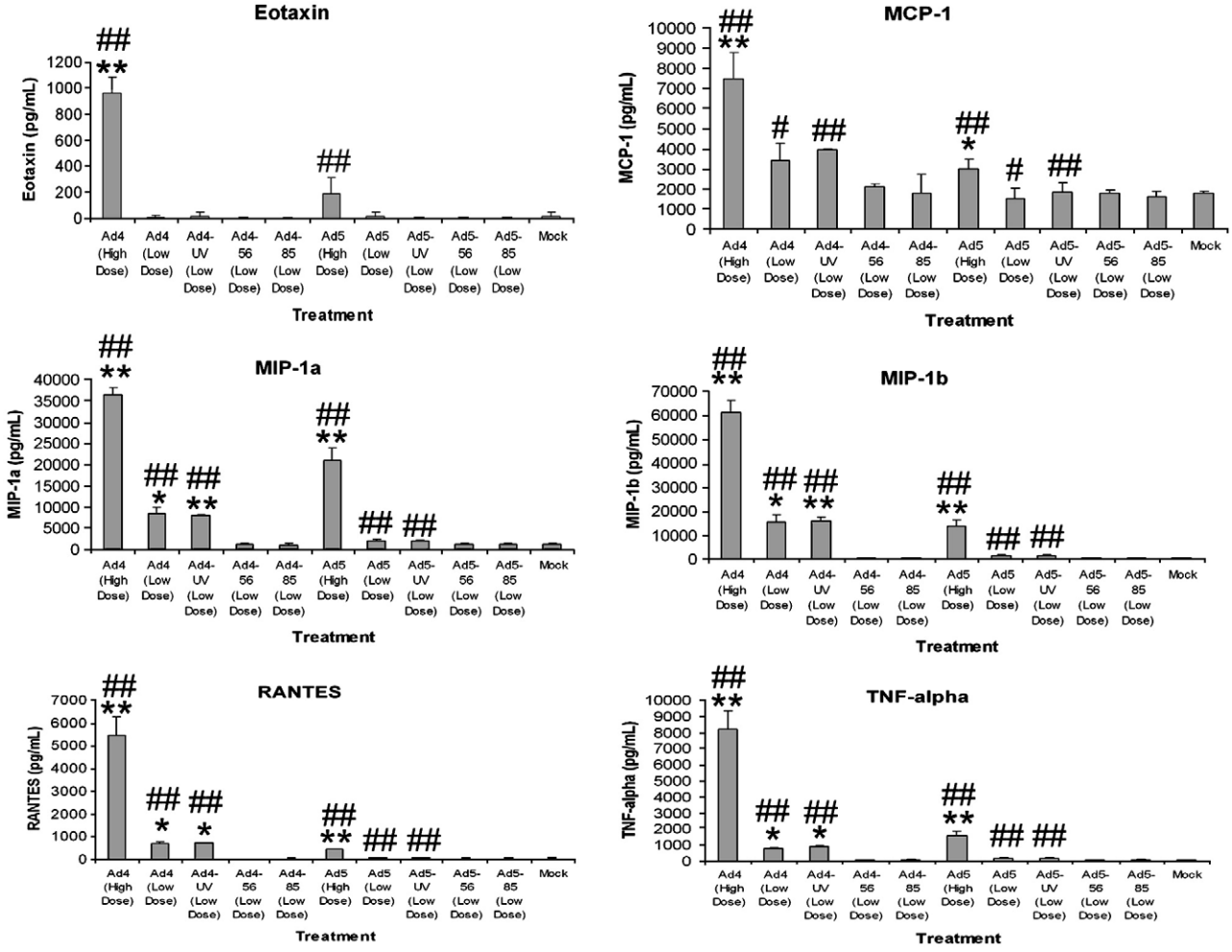

Fig. 3. Significantly elevated cytokine/chemokine response to HAdV-4 vectors in comparison to HAdV-5 vectors by the RAW264.7 murine macrophage line. Equal numbers of macrophages were infected with [E1−,E3−]LacZ HAdV-4 and HAdV-5 vectors at either a high dose (30,000 vp/cell) or a low dose (3000 vp/cell) and supernatant was collected and measured at 6 hpi for all cytokines. Three experimental replicates were performed for all groups ($N=3$). Error bars indicate standard deviations. An "*" or "**" indicates those time points when levels of the respective cytokine/chemokine were significantly different ($p<0.05$ or $p<0.01$ respectively) between Ad injected and mock infected cells and "#" or "##" indicates those time points when levels of cytokine/chemokine were significantly different ($p<0.05$ or $p<0.01$ respectively) between HAdV-4 and HAdV-5 infected cells.

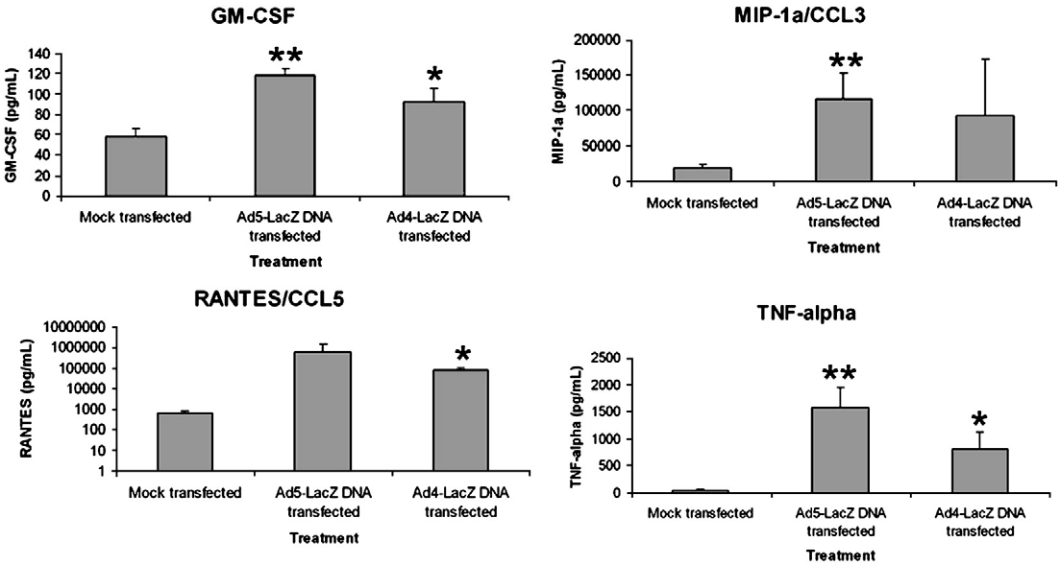

Fig. 4. Equivalent immunogenic response to HAdV-4 and HAdV-5 vector genomes after transfection into RAW264.7 cells. Equal numbers of macrophages were transfected with equal quantities of HAdV-4 or HAdV-5 vector DNA and supernatants harvested at 24 hpi and assessed for cytokine and chemokine production as described in Materials and methods. Three experimental replicates were performed for all groups ($N=3$). Error bars indicate standard deviations. An "*" or "**" indicates that levels of the respective cytokine/chemokine were significantly different ($p<0.05$ or $p<0.01$ respectively) between Ad transfected and mock transfected cells. No significant differences were observed between HAdV-4 and HAdV-5 transfected cells.

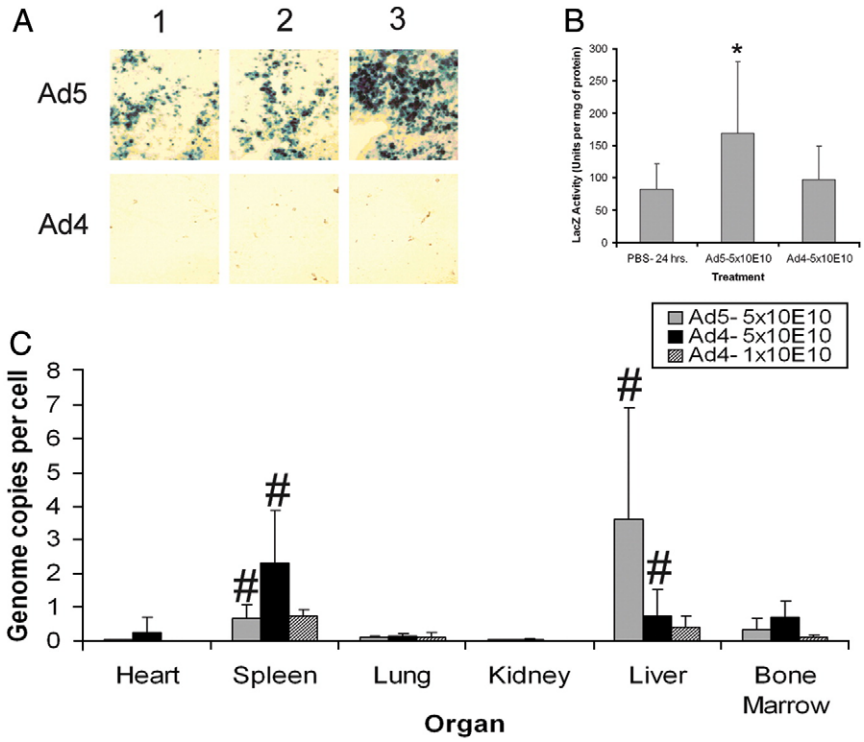

Fig. 5. Biodistribution of intravenously injected HAdV-4 vectors in comparison to HAdV-5 vectors in mice. (A) *In situ* staining of β-galactosidase activity in liver sections of mice injected with 5×10^{10} particles of HAdV-5LacZ or HAdV-4LacZ. Three representative experimental animals are shown for each vector. (B) Quantitative measure of β-galactosidase activity in liver after i.v. injection of 5×10^{10} particles of HAdV-5LacZ or HAdV-4LacZ vector. $N=11$ for HAdV-5 injected mice and $N=3$ for HAdV-4 and mock injected mice. An "*" or "**" indicates that liver LacZ activity was significantly greater ($p<0.05$ or $p<0.01$ respectively) than activity levels observed in mock infected counterparts (C) Real-Time PCR assessment of Ad genome copies per GAPDH copies in multiple organs. $N=3$ for both vector for all organs assayed with the exception of liver where $N=11$ for HAdV-5 injected mice and $N=6$ for HAdV-4 injected mice at both titers. A "#" or "##" indicates significant differences in Ad DNA copies per cell in comparison of mice identically treated with HAdV-4 or HAdV-5 vectors ($p<0.05$ or $p<0.01$ respectively). Error bars in all figures indicate standard deviations.

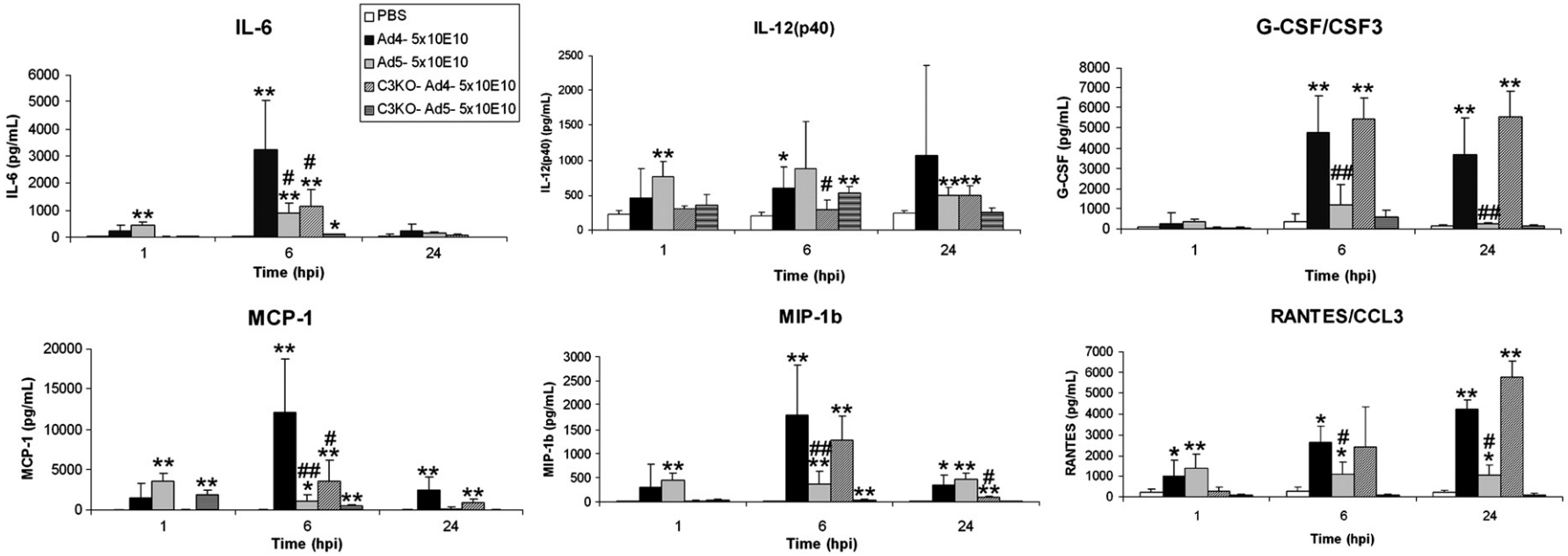

Fig. 6. Significantly elevated plasma cytokine/chemokine concentrations in HAdV-4 infected mice compared to HAdV-5 infected mice. Plasma levels of each of the indicated cytokines or chemokines were measured in the plasma derived from the respectively treated groups of mice as described in Materials and methods. Error bars indicate standard deviations. An "*" or "**" indicates those time points when levels of the respective cytokine/chemokine were significantly different ($p<0.05$ or $p<0.01$ respectively) between Ad injected and mock injected mice and "#" or "##" indicates those time points when levels of cytokine/chemokine were significantly different ($p<0.05$ or $p<0.01$ respectively) between HAdV-4 injected C57BL/6 mice and either HAdV-5 injected C57BL/6 or HAdV-4 injected C3KO mice counterparts. $N=4$, 3, and 4 for mock injected mice at 1, 6, and 24 hpi time points respectively; $N=7$, 9, and 6 at the respective 1, 6, and 24 hpi time points for HAdV-4 injected C57BL/6 mice (all time points). $N=4$, 5, and 3 at 1, 6, and 24 hpi respectively for HAdV-5 injected C57BL/6; $N=4$ for all time points for HAdV-5 injected C3KO mice; $N=4$, 5, and 5 at 1, 6, and 24 hpi for HAdV-4 injected C3KO mice.

various heat treatments prior to macrophage infection (Fig. 3). In this case, cytokine and chemokine inductions were almost completely eliminated for both serotypes. In summary, these results offer indirect evidence that the virion itself, as a three dimensional structure, is the primary determinant of the differential immunogenicity between these two vectors.

Biodistribution of HAdV-4 serotype in vivo

Since HAdV-4 possesses a shorter fiber compared to HAdV-5 (12 and 22 β-repeats respectively), and lack Heparan Sulfate Proteoglycan (HSPG) motifs in their fibers (yet do bind to CAR *in vitro*), we assessed whether these vectors could successfully transduce the murine liver (Shayakhmetov and Lieber, 2000; Ni et al., 2005). While injection of 5×10^{10} adenoviral particles did not result in complete liver transduction, both quantitative and qualitative assessments of liver derived LacZ expression revealed that HAdV-5 LacZ transduced the liver more efficiently than did the HAdV-4 based vectors (Figs. 5A and B). Using a quantitative PCR approach, we corroborated transduction findings in the liver, and further assessed differences in overall vector biodistribution (Figs. 5A and B). This analysis revealed baseline uptake of either virus in the heart, lung, kidney and bone marrow. However, levels of HAdV-4 genomes in the spleen were significantly higher than levels of HAdV-5 genomes. In fact, injection of 5-fold less HAdV-4 virus (1×10^{10} vp/mouse) still resulted in splenic HAdV-4 vector genome levels equivalent to mice injected with HAdV-5 virus at a higher dose of 5×10^{10} vp/mouse (Fig. 5C).

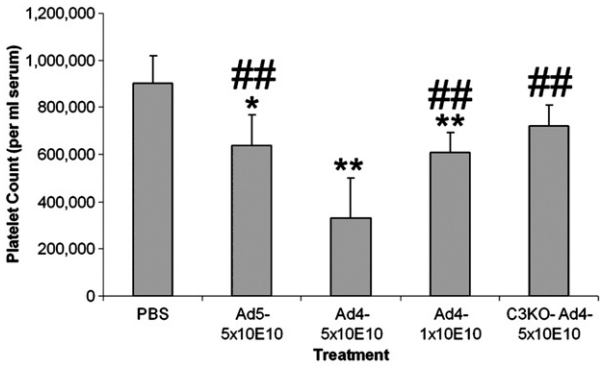

Fig. 7. HAdV-4 vectors induce a more significant thrombocytopenia upon intravenous administration in comparison to HAdV-5 counterparts. Platelet enumerations were performed in the indicated groups of control or virus injected mice as described in Materials and methods. All mice were on the C57BL/6 background, including the C3KO group of mice. Error bars indicate standard deviations. An "*" or "**" indicates those time points when platelet levels are significantly different ($p<0.05$ or $p<0.01$ respectively) between Ad injected and mock injected mice and "#" or "##" indicates those time points when platelet concentrations were significantly different ($p<0.05$ or $p<0.01$ respectively) between HAdV-4 injected C57BL/6 mice and either HAdV-5 injected C57BL/6 or HAdV-4 injected C3KO mice counterparts. $N=3$ for mock injected mice and $N=5$ for HAdV-5 injected mice. For HAdV-4 injected mice, $N=7$ for mice injected with 5×10^{10} particles, $N=5$ for mice injected with 1×10^{10} particles and $N=7$ for C3KO mice injected with 5×10^{10} particles.

HAdV-4 vectors promote increased pro-inflammatory cytokine and chemokine responses in vivo

To determine differences in innate immunogenicity *in vivo*, we initially injected mice with either HAdV-4 or HAdV-5 vectors at a dose of 1.5×10^{11} particles per animal, a dose we have previously used to evaluate innate responses to HAdV-5 based vectors (Hartman et al., 2007; Kiang et al., 2006b). However, at this dose HAdV-4 injected animals rapidly progressed to a moribund state (within 20 min of injection) and could not be fully evaluated. Thus we proceeded with experiments at vector doses of 5×10^{10} particles per mouse, and collected serum at 1, 6, and 24 hpi to assess the resulting induction of plasma cytokines and chemokines (Fig. 6). Although both vectors induced relatively similar levels of IL-12(p40) at all tested time points, we found that the HAdV-4 vector induced significantly higher levels of several pro-inflammatory cytokines and chemokines relative to the HAdV-5 vector, inclusive of IL-6, G-CSF, MIP-1β, MCP-1, and RANTES at 6 hpi and G-CSF and RANTES at 24 hpi. Thus, relative to HAdV-5 vectors, HAdV-4 vectors elicited a more pronounced inflammatory cytokine and chemokine profile *in vivo*, mirroring many of the *in vitro* differences observed after exposure of macrophages to either vector.

HAdV-4 vector enhanced induction of thrombocytopenia in vivo

HAdV-5 vectors induce thrombocytopenia in a variety of species including humans (Morral et al., 2002; Toietta et al., 2005; O'Neal et al., 1998; Cichon et al., 1999; Raper et al., 2002). Based on the cytokine/chemokine profile differences we noted between HAdV-5 and HAdV-4 vectors *in vivo*, we sought to determine if similar, serotype-specific differences in Ad-initiated thrombocytopenia also exist (Fig. 7). Measures of thrombocytopenia from Ad injected animals at 24 hpi revealed a significantly greater level of thrombocytopenia in HAdV-4 injected animals relative to HAdV-5 injected animals at identical doses. Furthermore, injection of a half-log lower dose of HAdV-4 LacZ virus (1×10^{10} particles) resulted in thrombocytopenia similar to that observed in mice injected with the higher dose of HAdV-5 LacZ.

The role of complement in HAdV-4 thrombocytopenia and inflammatory response

We previously determined that HAdV5-based vector induced thrombocytopenia is dependent on the presence of an intact complement system (Kiang et al., 2006b). Furthermore, our previous *in vitro* investigations revealed that both HAdV-5 and HAdV-4 particles bound to complement and activated the alternative complement pathway *in vitro* in a capsid-dependent manner (Jiang et al., 2004). Thus, we sought to determine whether complement was involved in HAdV-4 mediated thrombocytopenia and inflammatory cytokine responses. To assess this, we injected complement deficient mice (C3-KO) with 5×10^{10} particles of HAdV-4 LacZ virus and measured thrombocytopenia at 24 hpi. Blood platelet levels in C3KO HAdV-4 injected mice were significantly higher than levels observed in

their HAdV-4 injected C57BL/6 counterparts and were also not significantly different from levels in mock injected mice (Fig. 7). Thus, in accord with previously published observations made following HAdV-5 LacZ injection, an intact complement system mediates Ad-induced thrombocytopenia in response to intravenous injection of HAdV-4.

We have previously shown that the full elaboration of cytokines induced following systemic HAdV-5 injection is dependent on a functional complement system (Kiang et al., 2006a). To evaluate whether complement was similarly required for the induction of inflammatory cytokines in response to HAdV-4, we intravenously injected either HAdV-4 or HAdV-5 based vectors at a dose of 5×10^{10} vp/mouse into complement deficient animals (Fig. 6). At this viral dose, the requirement of complement in the elaboration of HAdV-5 induced cytokines was largely recapitulated in comparison to previously published data. In a similar fashion, we also found that the induction of IL-6, MCP-1, and MIP-1β following HAdV-4 injection was, in part, dependent on a functional complement system. However, in contrast to HAdV-5 injected complement deficient animals, the HAdV-4 mediated induction of IL-12(p40), G-CSF, and RANTES was not complement dependent (Fig. 6). Thus, while complement plays a significant role in enhancing the HAdV-5 and HAdV-4 induction of certain inflammatory cytokines/chemokines, the levels and or type of dependence is serotype-specific.

Adaptive immune responses to HAdV-4 and HAdV-5 vectors

The innate response to a pathogen can significantly impact upon the resulting adaptive immune response to the same pathogen. Therefore, we next sought to determine whether differences in the innate immune responses that we noted between the two Ad serotype vectors would translate into altered adaptive immune responses. To address this question, mice were injected either intravenously or in the footpad with 5×10^{10} particles of HAdV-4 or HAdV-5 vectors expressing LacZ, and were tested at 2 weeks post-injection (wpi) for transgene-specific IgG antibody responses (Figs. 8A and B). Interestingly, we found that HAdV-5 vectors induced significantly more IgG antibodies to the encoded LacZ transgene compared to HAdV-4 injected counterparts, a result that was replicated independent of the route of virus administration. Since IL-6 and other cytokines can skew Th1/Th2 differentiation, we sought to determine whether these serotype-specific differences in antibody induction were simply limited to the Th1 IgG class (Diehl et al., 2000; Diehl and Rincon, 2002). To ascertain Th2 responses, we investigated the induction of Th2 IgE transgene-specific antibodies at 2 wpi (Supplementary Fig. 3). As with IgG, we found that the levels of LacZ-specific IgE were significantly greater in HAdV-5-injected animals as compared to HAdV-4 injected animals. We also confirmed that this response was not due to a generalized alteration of the murine immune

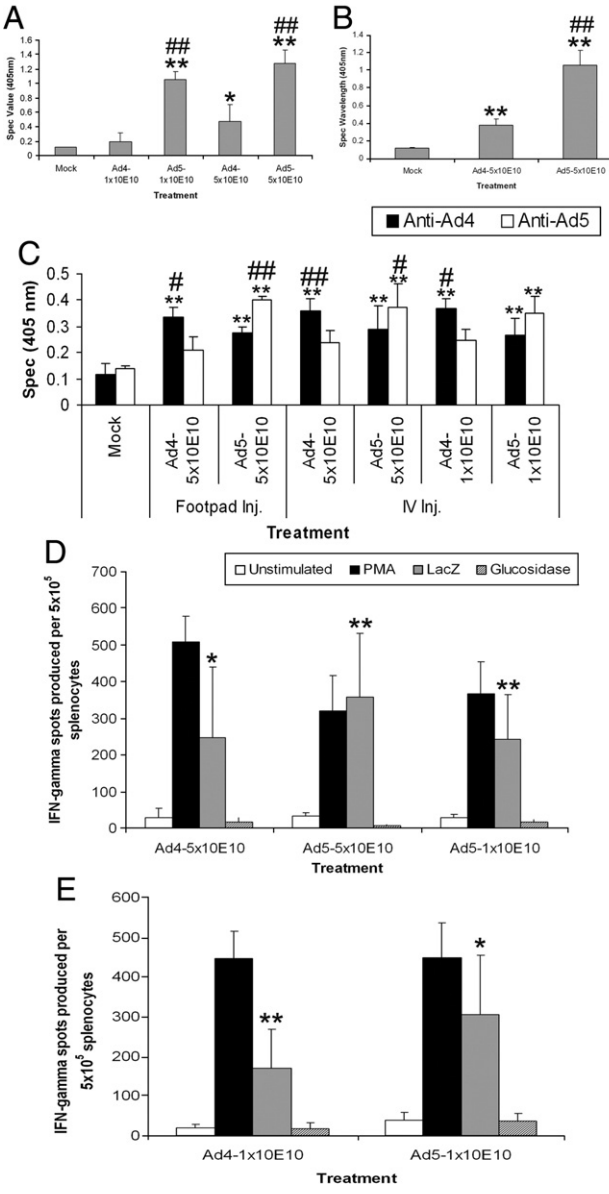

Fig. 8. Diminished transgene-specific IgG responses (but unaffected CTL transgene-specific responses) in HAdV-4 vector injected mice in comparison to HAdV-5 vector injected mice. Serum from immunized mice (all 14 days post respective Ad vector injection) was quantified for anti-LacZ or anti-Ad antibodies using LacZ and Ad specific ELISA assays as described in Materials and methods. Data are shown as absorbance values at appropriate serum dilutions, see Materials and methods for full details. (A) Anti-LacZ IgG antibody ELISA after intravenous administration of Ad vectors. For HAdV-5 injected mice, $N=5$ and 4 for mice treated with 1×10^{10} and 5×10^{10} particles respectively. For HAdV-4 injected mice, $N=5$ and 3 for mice treated with 1×10^{10} and 5×10^{10} particles respectively. $N=4$ for mock injected mice. (B) Anti-LacZ IgG antibody ELISA after footpad injection of Ad vectors. $N=4$, 5, and 4 for the respective HAdV-4 injected group, HAdV-5 injected group and mock injected group. (C) Anti-Ad serotype-specific IgG antibody ELISA. IgG antibodies were assessed to serotype-specific capsids using conditions and replicates described in (A) and (B). (D and E) Day-14 LacZ specific ELISpot-IFN-γ responses of C57BL/6 mice after footpad injection (D) or intravenous injection (E) of HAdV-4LacZ, HAdV-5LacZ, or control vehicle. Splenocytes derived from the respectively treated mice (14 days post Ad injection) were cultured and exposed to the respective antigens (antigen-specific LacZ protein or non-specific glucosidase) as described in Materials and methods. For all D and E groups, $N=5$ and error bars are indicative of standard deviation. An "*" or "**" indicates the levels of the respective IgG antibody was significantly different ($p<0.05$ or $p<0.01$ respectively) between Ad injected and mock injected mice and "#" or "##" indicates those time points when the IgG antibody concentration was significantly different ($p<0.05$ or $p<0.01$ respectively) between Ad injected between HAdV-4 or HAdV-5 injected groups.

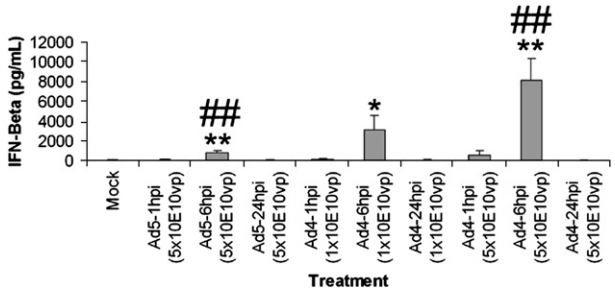

Fig. 9. Significantly elevated IFN-β response to HAdV-4 vectors in comparison to HAdV-5 vectors after intravenous injection *in vivo*. Plasma levels of injected mice were measured for IFN-β by ELISA in the respectively treated groups of mice as described in Materials and methods. For HAdV-5 injected mice, $N=5$ and 4 for mice treated with 1×10^{10} and 5×10^{10} particles respectively. For HAdV-4 injected mice, $N=5$ and 3 for mice treated with 1×10^{10} and 5×10^{10} particles respectively. The $N=4$ for mock injected mice. Error bars indicate standard deviations. An "*" or "**" indicates those time points when levels of the respective cytokine/chemokine were significantly different ($p<0.05$ or $p<0.01$ respectively) between Ad injected and mock injected mice and "#" or "##" indicates those time points when levels of cytokine/chemokine were significantly different ($p<0.05$ or $p<0.01$ respectively) between HAdV-4 and HAdV-5 counterparts.

system, since both vectors were still capable of eliciting significant IgG responses to their respective capsids (Fig. 8C).

It is possible that HAdV-4 enhanced type I interferon responses observed *in vitro* also occur *in vivo* leading to the repression of LacZ transgene expression and thus serotype-specific, diminished anti-LacZ antibody responses. As we found *in vitro*, the HAdV-4 based vector elicited significantly greater plasma levels of IFN-β relative to the HAdV-5 vector (Fig. 9), potentially explaining the diminished IgG and IgE antibody responses to LacZ observed in HAdV-4 vector treated mice.

We next sought to assess if T-cell responses to the encoded transgene (LacZ) also differed between vectors. Two weeks after footpad injection of 5×10^{10} vp of HAdV-4 or HAdV-5 LacZ particles, LacZ specific T-cell responses were measured using an ELISPOT assay. As shown in Fig. 8D, the number of transgene-specific IFNγ producing T-cells was similar between the two vector platforms. Similar results were also obtained when lower doses of vector were injected intravenously (Fig. 8E). Thus, despite diminished anti-LacZ antibody responses relative to HAdV-5, HAdV-4 based vectors were capable of inducing significant transgene-specific T-cell responses equivalent to that of HAdV-5 based vectors.

HAdV-4 vectors elicit enhanced pro-inflammatory cytokines/chemokines compared to HAdV-5 vector in human primary dendritic cells

We wished to determine if the differences between the HAdV-4 and HAdV-5 based vectors noted in murine systems could be detected in human derived immune cells. To address this, we infected human PBMC derived dendritic cells with 50,000 vp/cell of either HAdV-4 or HAdV-5 LacZ vectors. At 24 hpi, supernatants were harvested and cytokine/chemokine secretion was assessed (Fig. 10). At this time point, the HAdV-4 based vector induced significantly higher levels of IP-10 (CXCL10), MCP-1, and RANTES relative to the HAdV-5-based vector. These results mirror our previous findings using murine cells, and suggest that the significantly altered immunogenicity of HAdV-4 relative to HAdV-5 can also be demonstrated in human cells.

Discussion

Human Ad Type 4 is the sole member of the human Group E adenoviruses, and possesses several interesting etiologic features; being responsible for periodic epidemic outbreaks of infection in military populations, causing high rates of morbidity, and occasional cases of mortality (Russell et al., 2006a,b; Hilleman, 1957; Gray et al., 2000). These features suggest that HAdV-4 virions may possess differential infective abilities and higher immunogenicity relative to other related Ad serotypes.

In this study, we created an [E1−,E3−] replication defective HAdV-4 platform to investigate its properties relative to the commonly used [E1−,E3−] replication defective HAdV-5 based vector. It is becoming increasingly clear that use of alternative serotype vectors may be useful not only in the context of avoiding pre-existing immunity to a respective vector serotype (e.g. HAdV-5) but that altered serotypes of vectors may also offer improved efficacy or safety for particular usages. HAdV-4 vectors are being evaluated for use as a vector in multiple vaccine efforts including HIV. *In vitro*, we found that HAdV-4 vectors displayed enhanced infectivity in several cell types and elicited greater immunogenic responses in murine macrophages, the latter result being dependent upon exposure to intact HAdV-4 capsids. In a

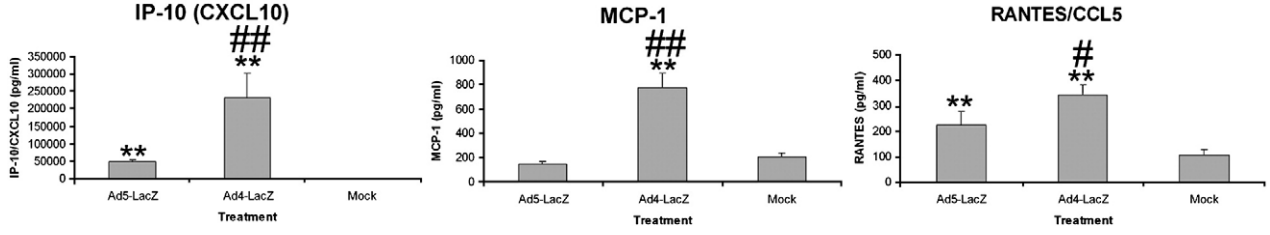

Fig. 10. HAdV-4 vectors induce significantly elevated cytokine secretion compared to HAdV-5 vectors in human dendritic cells. Primary dendritic cells (DCs) from healthy human donors were prepared as described in Materials and methods. DCs were infected with 50,000 vp/cell of [E1−,E3−]LacZ HAdV-4 and HAdV-5 vectors. Supernatants were collected and measured at 24 hpi for all cytokines. Three experimental replicates were performed for all groups ($N=3$). Error bars indicate standard deviations. An "*" or "**" indicates those time points when levels of the respective cytokine/chemokine were significantly different ($p<0.05$ or $p<0.01$ respectively) between Ad injected and mock treated cells and "#" or "##" indicates those time points when levels of cytokine/chemokine were significantly different ($p<0.05$ or $p<0.01$ respectively) between HAdV-4 and HAdV-5 infected cells.

direct comparison to HAdV-5 based vectors *in vivo*, we found that a HAdV-4 vector displayed altered tissue biodistributions, and induced a heightened, partially complement dependent, innate immune response characterized by a higher induction of inflammatory cytokines and a more dramatic stimulation of thrombocytopenia on a per particle basis. However, we found that the heightened innate inflammatory nature of the HAdV-4 vector had only minor effects on subsequent adaptive responses, primarily limited to antibody responses to the transgene encoded by the HAdV-4 vector.

Specifically, we found that HAdV-4 LacZ more efficiently infected HeLa and HepG2 cells compared to HAdV-5. These results corroborate and expand upon previous studies that have detailed an enhanced ability of wild-type HAdV-4 to bind and infect certain cell types in comparison to wild-type HAdV-5 (Zhang et al., 2003; Mei et al., 2002). However, we noted that exposure of RAW264.7 murine macrophages to HAdV-4 or HAdV-5 revealed comparable levels of vector genomic DNA content but reduced capabilities of the HAdV-4 vector to drive expression of a CMV enhancer dependent transgene (LacZ). We also found that HAdV-4 vectors induced significantly greater secretion of multiple cytokines, including IFN-β, when directly compared to HAdV-5 vectors. This may offer a possible explanation for the decreased CMV-driven gene expression noted from the HAdV-4 vector in murine macrophages, since similar results have been confirmed in IFN-receptor KO mice infected with a similar immunostimulatory Ad serotype(Hensley et al., 2007).

Our studies also revealed that cytokine and chemokine inductions by the Ads were dependent on intact/infectious virions and were independent of viral genome transcription (for example, when UV-inactivated vectors were utilized). We also found that transfection of DNA derived from either HAdV-4 or HAdV-5 based vectors resulted in equivalent immune responses, suggesting that DNA-dependent effects were not primarily responsible for the differences in immunogenicity we noted between these serotypes *in vitro*. While some of the HAdV-4 induced cytokines (IL-6 and TNF-α) were broad spectrum inflammatory cytokines, other cytokines induced by HAdV-4, such as MIP-1α, MIP-1β, and RANTES, have been grouped and classified as 'type 1' cytokines, which function together in the effector phase of macrophage activation (Dorner et al., 2002). This cytokine profile suggests that the HAdV-4 vector may enable more vigorous macrophage activation, possibly contributing to the enhanced morbidity noted during HAdV-4 infections.

In vivo assessments of immunogenicity revealed HAdV-4 vectors to be more immunogenic compared to HAdV-5 at early time points. For example, high dose HAdV-4 injection resulted in significant morbidity and thus did not allow for complete evaluation. However, lower dose injections of HAdV-4 vectors were tolerated and could be compared to lower dose HAdV-5 vector injections. We also observed decreased hepatocyte transduction by HAdV-4-based vectors, possibly due to its short fiber and lack of a HSPG binding motif (Shayakhmetov and Lieber, 2000; Ni et al., 2005). Interestingly, the spleen was found to contain more HAdV-4 genomic content then HAdV-5 at 24 hpi, which may be indicative of more active phagocytosis by different types of sentinel immune cells *in vivo*.

In agreement with results obtained *in vitro*, we also noted that HAdV-4 vectors triggered a profound inflammatory response *in vivo*, characterized by significantly higher cytokine, chemokine, and IFN-β secretion compared to HAdV-5 vectors. This HAdV-4 inflammatory response was also accompanied by a higher propensity to initiate thrombocytopenia relative to HAdV-5 based vectors.

We have previously shown that HAdV-4 capsids can bind and activate complement *in vitro* (Jiang et al., 2004); in this report we show that several HAdV-4 induced immune responses are also complement dependent *in vivo*. For example, complement deficient mice injected with HAdV-4 vectors had diminished inflammatory cytokine plasma levels as well as a significantly diminished ability to induce thrombocytopenia as compared to HAdV-4 injected wild-type mice. These results were similar to those previously noted with HAdV-5 vectors, thus demonstrating the importance of the complement system in the initiation of several serotype independent Ad-induced inflammatory responses (Kiang et al., 2006b). These observations should heighten scrutiny when one considers utilization of alternative serotype Ad vectors in general, as we have recently shown that each serotype has a unique, innate immune response profile that must be fully delineated prior to clinical use (Appledorn et al., in press).

Surprisingly, the more pro-inflammatory HAdV-4 capsid vector did not result in improved adaptive immune responses to the LacZ transgene, as compared to a "less inflammatory" HAdV-5 based vector expressing LacZ, a result previously noted and demonstrated to be mediated by heightened interferon responses (Hensley et al., 2007). It is possible that the low level of liver transduction observed following HAdV-4 LacZ injection resulted in reduced expression of the LacZ transgene and a subsequently lower anti-LacZ IgG response. However, it was also noted that HAdV-4 elicited diminished LacZ specific IgG responses compared to HAdV-5 after footpad injection. Since footpad injections do not result in high levels of liver transduction, it is unlikely that the differences in LacZ expression in the liver contributed to the reduced LacZ specific IgG responses, and that this response may be more reflective of inherent differences in the innate immune responses elicited by the serotypes specific capsids themselves.

Finally, this study also demonstrates enhanced secretion of multiple inflammatory cytokines following HAdV-4 infection of human dendritic cells, in a manner qualitatively similar to findings we noted in murine systems. This result suggests that intrinsic differences in the capsid structure of HAdV-4 can impact significantly upon numerous pro-inflammatory pathways in human cells, and may thus partially explain the high morbidity noted in humans upon infection with wild-type HAdV-4 and potentially other adenoviral serotypes.

In summary, our study provides a new tool for basic and clinical researchers, and reveals important insights into the infectious nature and immunogenicity of HAdV-4 relative to HAdV-5 based vectors. More importantly, the utilization of alternative serotype gene transfer vectors can alter important aspects of a respective vector class' biology in ways that may not always be predictable. Thus, utilization of alternative serotype vectors to

overcome a specific limitation (i.e.: pre-existing immunity to a respective serotype vector) or to improve efficacy (i.e.: improved usage as a vaccine platform) may also introduce new limitations.

Materials and methods

Construction of HAdV-4 genomic plasmid

Wild-type Adenovirus Type 4 was purchased from ATCC (strain RI-67, ATCC catalog #1081) and grown on 293 cells using standard infectious procedures. Virus was purified using a CsCl double-banding procedure and Viral DNA extracted using phenol chloroform based procedures. A primer designed to the Adenovirus Type 4 fiber was generated (AACCCAAGCTTCTTACTATAACTCTT) and was used to initially confirm the sequence identity of the resulting virus. Restriction digest mapping analysis using BstXI, XbaI, XhoI, and BamHI, confirmed the serotype identity of the resulting virus based on portions of the known HAdV-4 sequence present in the literature at that time.

Since, much of the HAdV-4 sequence was unknown at this point, large scale directional sequencing using directional primers was conducted to span the unknown portions of the Adenovirus Type 4 genome and was completed with 1× coverage (Duke HAdV-4 sequence available upon request). When a published HAdV-4 sequence became available in February of 2005 (Genbank # AY594253) comparison between the two genomes revealed no detectable difference and thus provided additional verification of serotype identity.

To obtain an HAdV-4 genomic plasmid an "HAdV-4 rescue shuttle" was built by cloning the terminal "left arm" of the linear Adenoviral Type 4 genome (HAdV-4 sequence nt# 1-1878) using primers 5′-CCTCGAGGTCGACATTACCCTGTTATCCCTACATCATCAATAATATACCTTATTTT-3′ and 5′-GCAAGCTTACTGCTGCAGTGGTTCTGCCAGGAG-3′) and the "right arm" (HAdV-4 sequence nt# 33708-35994) using primers 5′-CTAATCCGTACTAGTATTACCCTGTATCCCTACATCATCAATAATATACCTTATTTT-3′ and 5′-GTGGATCCGAACATGCAGCCCTGGATAATT-3′. These PCR fragments were cloned into pCR2.1-TOPO cloning vectors, then cut with BamHI and I-SceI and these two fragments were directionally ligated with a kanamycin resistance-bacterial origin containing DNA fragment also flanked with I-SceI sites. Restriction digests and sequencing confirm the sequence identity and correct orientation of the resulting clones, denoted as pHAdV-4[E1+] shuttle. This plasmid was linearized by digesting with BamHI and co-transformed along with HAdV-4 genomic DNA into BJ5183 bacteria cells. Kanamycin-resistant clones were tested by PCR screen and resultant "Wild-type HAdV-4" clones were grown in Stbl2 cells (Gibco, La Jolla, CA).

Construction of HAdV-4 vector plasmid shuttle system

To create a HAdV-4 based, [E1−] shuttle plasmid (for rapid mobilization of genes into the larger HAdV-4 genome), the portion of the Ad Type 4 genome spanning HAdV-4-nucleotides #3085–#5978 nucleotides was additionally inserted into HAdV-4-pShuttle. This region was PCR amplified from HAdV-4 genomic DNA using the following tailed primer sequences: 5′-CAGGTGATCACCGCGGCGGCCGCTCTAGAACTAGTGCTAGCGATATCCCCAGGTGCAATATGCATCTGG-3′ and 5′-TAGCAAGCTTAACTGACAACCTGAGTGCG-3′. These specifically tailed primers incorporate a 5′-BclI and 3′-HindIII recognition sites into the 5′ and 3′ ends of the PCR products derived from use of these primers. The resultant ~2900 bp fragment was digested using BclI and HindIII and directionally ligated into the HAdV-4 pShuttle (previously digested with BglII and HindIII). Restriction digests as well as multiple sequencing reactions confirmed the sequence identity and correct orientation of the resulting clones, denoted as pHAdV-4[E1−] Shuttle (see Supplementary Fig. 1). Once pHAdV-4[E1−] shuttle was generated, it was linearized by digestion with NruI, and co-transformed into electro-competent BJ5183 bacterial cells along with the I-SceI digested pHAdV-4 plasmid to generate p[E1−] HAdV-4 genomic plasmids, a HAdV-4 containing plasmid deleted for the E1 genes of HAdV-4, specifically nucleotides #475–#3085.

To produce HAdV-4 based vectors that were also E3 deleted (to effectively increase carrying capacity of the HAdV-4 vector system further) we next created a HAdV-4 E3 shuttling system. To generate this, we PCR amplified the two HAdV-4 E3 "adjacent" regions of HAdV-4 flanked with specifically indicated restriction enzyme sites (nt. 25,810-28,366; and nt 30,575-34,536) using the following primer sets: 5′-CATCGTCTCCTGCTTGCAAGCTTG-3′ (HindIII); 5′-TCGTCGACTCTAGAGGATCCGCTCAGGCACACGATCGCGGTG-3′ (SalI); 5′-GTCGACGATATCCTCGAGATGCTTCTACTATTGCTAGCTC-3′ (SalI); and 5′-GCGAATTCCCGTGTTACCTTTGACCTG-3′ (EcoRI). These fragments were then respectively digested using EcoRI and HindIII, and ligated into a pUC19 plasmid that had been similarly digested. Restriction digests and sequencing confirmed the correct sequence and orientation of the resulting clone, pHAdV-4-E3. The pHAdV-4-E3 shuttle was co-transformed with NdeI linearized p[E1−] HAdV-4 genomic plasmids into BJ5183 cells, and kanamycin-resistant clones were isolated and restriction enzyme mapped to confirm generation of p[E1−,E3−]HAdV-4 genomic plasmids (full cloning and mapping details available upon request). After successful recombination, all [E1−,±E3−] HAdV-4vector plasmids were transferred to Stbl2 *E. coli* cells to permit high levels of plasmid growth without vector recombination.

HAdV-4 vector construction and Ad vector purification

Briefly, a CMV-driven bacterial β-galactosidase gene (LacZ) was subcloned into the pHAdV-4[E3−]shuttle plasmid (via XhoI restriction site) and the pHAdV-4[E3−] CMV-LacZ shuttle plasmid was co-transformed into BJ5183 cells with NdeI linearized pHAdV-4[E1−] to generate p[E1−,E3−]HAdV-4-CMV-LacZ. The p[E1−,E3−]HAdV-4-CMV-LacZ plasmid was linearized with I-SceI to release the HAdV-4-ITR's from the bacterial origin and kanamycin resistance portions of the plasmid, and $CaPO_4$ transfected into human 293 cells (containing Ad5 derived E1 region). Viral CPE was noted, and the resultant [E1−,E3−]HAdV-

4-LacZ vector was amplified by repeated passaging on greater numbers of 293 cells. After the respective vectors were grown to high titers, concentration and purification of the vectors was performed using a previously described cesium chloride double-banding method (Amalfitano et al., 1998). All resultant Ad vector stocks were evaluated for the presence of replication-competent Ad (RCA) via a polymerase chain reaction (PCR)-based RCA assay, essentially as described by Lochmuller et al. (1994). Briefly, DNA isolated from the respective Ad vector preparations was subjected to PCR using primers located within the E1 region of both serotypes. Known quantities of Ad2 wild-type genome (Invitrogen, Carlsbad, CA) or purified HAdV-4 DNA (RI-67 strain, ATCC) were used as positive controls for the respective vector preparations. The RCA assay confirmed that the viral preparations lacked significant amounts of replication- competent (E1+) viral genomes, based on the sensitivity of the RCA assay being able to detect at least 7.8×10^4 [E1+] Ad genomes in a sample containing $2.75\text{-}3\times10^{10}$ vector particles (data not shown). To ensure that samples were not LPS-contaminated, purified vectors were tested using an E-Toxate kit (Sigma, St. Louis, Missouri) per manufacturers' recommendations with all preps containing <0.01 EU per viral injection dose. Finally, vector preparations were evaluated for potential differences in total protein content using a Pierce (Rockford, IL) Bicinchoninic Acid (BCA) Assay using the manufacturer's recommendations to evaluate the total viral protein content for the respective vectors using published literature. Both HAdV-4 and HAdV-5 vectors (and wild-type counterparts) were found to have similar protein content per equivalent numbers of viral particles.

In vitro assays

All cell lines were obtained from the American Tissue Culture Collection (ATCC) and cultured according to suggested guidelines. Viral infectivity was assessed by plating cells in 6 well dishes at concentrations of 4×10^5 cells per well. LacZ transducing units were quantified on all cell types 24 h post-infection using 2–6 fold log dilutions of vector in medium, as previously described (Amalfitano et al., 1998). Additionally, a quantitative assessment of LacZ transduction capability was also obtained through a whole cell harvest of replicate wells through use of a Beta-Galactosidase Activity kit (Stratagene, La Jolla, CA) as per manufacturer's recommendations. UV inactivation was performed using a ~400 mW/cm^2 UV dose, shown to yield more than a 6-$\log_{10}$ inactivation of Ads (Gerba et al., 2002; Hartman et al., 2006), and confirmed by separate infectivity assays (data not shown). Heat treatment consisted of placing Ad samples in a 56 °C water bath for 10 min to dissociate the penton-fiber from the capsid as has been previously shown (Russell et al., 1967; Rexroad et al., 2003). All vectors undergoing heat treatment were found to have lost over ~5 logs of infectivity on 293 cells, assessed by visualization of LacZ transduction. Equal OD_{260} particle numbers (determined as previously described, (Maizel et al., 1968a,b)) of [E1−, E3, LacZ +] purified HAdV-4 or HAdV-5 based vectors were used to infect wells containing indicated cell types. Transfection experiments were carried out using 1.5 μg of vector DNA extracted from viral preparations per 1 million RAW264.7 cells using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer's recommendations. Overlying medium from the infected cells was harvested at indicated time points and assessed using ELISA.

Culturing of human dendritic cells

Human dendritic cells were prepared essentially as described in Morse et al. Briefly, peripheral blood monocytes from healthy donors were thawed and allowed to adhere to a plate in serum-free AIM-V media (Invitrogen, Carlsbad, CA). After 2 h, the plate was gently washed with PBS and adherent monocytes were cultured in AIM-V supplemented with GM-CSF (800 U/ml) and IL-4 (500 U/ml; R&D Systems, Minneapolis, MN). After culturing in this media for 5 days, cells were assessed by FACS for HLA-DR and CD45 expression then infected using 50,000 vp/cell of HAdV-4 or HAdV-5 based vectors or mock infected.

Animal procedures

Adult C57BL/6 mice and B6.129S4-C3^{tmlCrr} mice back-crossed with C57BL/6 mice were purchased from Jackson Laboratory (Bar Harbor, ME) and bred as previously described (Kiang et al., 2006b; Wessels et al., 1995). Virus treatment of animals (3–4 months in age) consisted of intravenous injection (via the retro-orbital sinus) of 200 μl of a phosphate-buffered saline solution (PBS, pH 7.4) containing virus particles of the respective AdV-LacZ serotype 4 or 5 based vectors. Plasma and liver samples were subsequently obtained and processed at the indicated times post-injection using procedures approved by the Duke University (Durham, NC) Institutional Animal Care and Use Committee.

Assessment of viral transduction in vivo

Sections of snap frozen liver tissue embedded in Optimal Cutting Temperature (OCT) fluid was sectioned at 7 μm and then stained *in situ* for LacZ expression using X-Gal substrate as previously described (Everett et al., 2004; Hartman et al., 2007; Kiang et al., 2006b,a). To also quantitatively assess overall LacZ activity, snap frozen samples were homogenized and LacZ activity quantified using a β-Galactosidase Activity kit (Stratagene, La Jolla, CA) as previously described (Kiang et al., 2006b).

Ad genome copy number per cell

To assess the number of Ad genome copies per cell, tissues were snap frozen in liquid nitrogen and then homogenized using a mortar and pestle. After homogenization, DNA was extracted using a Gentra DNA Extraction kit (Gentra Systems, Minneapolis, MN) and copy numbers assessed using Real-Time PCR based quantification. PCR reactions were performed on an ABI 7900HT Fast Real-Time PCR System using the SYBR Green PCR Mastermix (Applied Biosystems, Foster City, CA).

Primers used to detect HAdV-5 were forward, 5′- CGCTCAGTACCAACAGAGGA-3′, and reverse, 5′-AGATGCTTCAACAGCACGTC -3′ and primers used to detect HAdV-4 were 5′-GACTCGTTCCTGGTCCAGAT-3′ and 5′- GTTAACCTCGTCCACCACCT-3′. Both vectors were also assessed using CMV promoter specific primers: 5′- ACGCCAATAGGGACTTTCCATTGAC-3′ and 5′- AGGGGGCGTACTTGGCATATGATAC-3′. As an internal control for insuring adequate DNA amplification, cellular DNA was quantified using primers spanning a region of GAPDH sequence (forward, 5′-AGAACATCATCCCTGCATCC-3′, and reverse, 5′-CACATTGGGGGTAGGAACAC-3′). All PCRs were subjected to the following procedure: 95.0 °C for 10 min followed by 40 cycles of 95.0 °C for 15 s followed by 60.0 °C for 1 min. Standard curves were run in duplicate and consisted of 6 half-log dilutions using total genomic DNA or either HAdV-5- or HAdV-4-[E1−]AdLacZ extracted DNA. These standard curves were used to determine the number of viral genomes present per cell from each sample. Melting curve analysis confirmed the quality and specificity of the PCR.

ELISA and platelet measurements

To characterize Ad impact on systemic inflammation, a panel of cytokines was investigated using a multi-plexed Enzyme Linked Absorbance Assay (ELISA) based system, as previously described (Hartman et al., 2007). Serum samples harvested at 0, 1, 6, and 24 h post-infection as well as tissue culture supernatants were analyzed using a Luminex Array reader and the Bio-Rad, BioPlex Murine Cytokine 23-plex kit or BioPlex Human 27-plex kit according to the manufacturer's recommendations. Interferon-β measurements were performed using a PBL Interferon-β ELISA kit (Piscataway, NJ) according to the manufacturer's recommendations. Statistically significant differences for ELISA were determined using a two-tailed homoscedastic Student's *t*-test.

Platelet enumeration

o assess Ad vector induced thrombocytopenia, platelets were measured at the given time points by retro-orbital blood draws using the Unopette (Fisher-Scientific) system as previously described (Kiang et al., 2006b). As per manufacture's recommendations, platelets were counted using a Neubauer hemocytometer and platelet levels were ascertained from these counts. Statistically significant differences for platelet measurements were determined using a two-tailed homoscedastic Student's *t*-test.

Adenovirus antibody ELISA and LacZ ELISpot

To assess differential vector effects on the adaptive immune response, mice were injected with 5×10^{10} particles of either HAdV-4 or HAdV-5 LacZ bearing adenovirus via the footpad and sacrificed 14 days after injection. To quantify murine anti-adenovirus antibodies, a previously described ELISA method was used (Michou et al., 1997). Briefly, 1×10^{9} adenovirus particles per well were bound to a 96 well plate in a bicarbonate solution (200 mm $NaHCO_3$, 81 mm Na_2CO_3, pH 9.5) at 4 °C overnight. Wells were washed three times (PBS with .05% Tween 20) and blocked for an hour at room temperature using a 2% bovine serum albumin (BSA) in PBS solution, rinsed in PBS, and incubated with serum (at indicated dilution) for 60 min at 37 °C. Wells were repeatedly washed with 400 μl of wash buffer after which, 100 μl per well of a 1:2500 dilution of sheep anti-mouse IgG or IgE H+L antibody (Jackson Immunoresearch Laboratories) was added and allowed to incubate for 60 min at 37 °C. Finally, 100 μl per well of OPD substrate solution was added to each well and allowed to incubate for 20 min. Absorbance was measured at 405 nm.

To quantify alloantigen-primed IFN-γ-producing T-cells, an Enzyme Linked Immuno-Spot (ELISpot) assay was used, essentially as described previously (Hobeika et al., 2005; Clay et al., 2001; Maecker et al., 2005; Morse et al., 2005). Multiscreen-HA 96-well plates (Millipore, Bedford, MA) were coated overnight with capture anti-IFN-γ antibody (AN 18, at 1 mg/ml), washed five times with PBS, and then blocked for 2 h with RPMI1640-25mM HEPES-10%FBS at 37 °C. Capture and detection anti-IFN-γ monoclonal antibodies were purchased from MABtech USA (Mariemont, OH). Donor splenocytes (5×10^{5}) were added to each well followed by addition of PMA+Ionomicin (0.263 μg and 2.1 μg per well respectively), LacZ protein (100 μg/ml, Sigma-Aldrich, St. Louis, MO), Amyloglucosidase protein (100 μg/ml, Sigma-Aldrich, St. Louis, MO), or media alone. Plates were incubated for 24 h at 37 °C, then washed five times with PBS. Biotinylated detection anti-IFN-γ antibody (R4-6A2; at 1 mg/ml) was then added at a 1:1000 concentration in PBS +1%BSA and allowed to incubate for 2 h at room temperature. Plates were then washed five times with PBS, after which, 100 μl of HRP-conjugated strepavidin (1/1000 dilution; MABtech USA, Mariemont, OH) was added to each well for 60 min at room temperature. Plates were again washed five times with PBS, developed with 3-amino-9-ethyl-carbazole [AEC] (Sigma-Aldrich, St. Louis, MO), reconstituted in acetate buffer for 4 min in the dark, washed with H_2O, and air dried. Membranes were attached to sealing tape (Millipore, Bedford, MA) and the number of spots per well was determined using a KS ELISpot Automated Reader System with KS ELISpot 4.2 Software (Carl Zeiss, Inc., Thornwood, NY).

Acknowledgments

We would like to thank Dr. Greg Sempowski for his expertise and support with bead array based assays. AA and ZH were supported in part by a grant from the Children's Miracle Network (Durham, NC), AA was further supported by grants from the NIH (RO1DK-069884 and P01 CA078673) and the Osteopathic Heritage Foundation.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.virol.2008.01.017.

References

Amalfitano, A., Hauser, M.A., Hu, H., Serra, D., Begy, C.R., Chamberlain, J.S., 1998. Production and characterization of improved adenovirus vectors with the E1, E2b, and E3 genes deleted. J. Virol. 72, 926–933 (JID — 0113724).

Appledorn, D.M., Kiang, A., McBride, A., Jiang, H., Seregin, S., Scott, J.M., Stringer, R., Kousa, Y., Hoban, D., Frank, M.M., Amalifitano, A., in press. Wild-type Adenoviruses from groups A-F evoke unique innate immune responses, of which HAd3 and SAd23 are partially complement dependent. Gene Ther.

Chanock, R.M., Ludwig, W., Heubner, R.J., Cate, T.R., Chu, L.W., 1966. Immunization by selective infection with Type 4 Adenovirus grown in human diploid tissue cultures. I. Safety and lack of oncogenicity and tests for potency in volunteers. JAMA 195, 445–452.

Chengalvala, M., Lubeck, M.D., Davis, A.R., Mizutani, S., Molnar-Kimber, K., Morin, J., Hung, P.P., 1991. Evaluation of Adenovirus Type 4 and Type 7 recombinant hepatitis B vaccines in dogs. Vaccine 9, 485–490.

Chengalvala, M.V., Bhat, B.M., Bhat, R.A., Dheer, S.K., Lubeck, M.D., Purcell, R.H., Murthy, K.K., 1997. Replication and immunogenicity of Ad7-, Ad4-, and Ad5-hepatitis B virus surface antigen recombinants, with or without a portion of E3 region, in chimpanzees. Vaccine 15, 335–339.

Cichon, G., Schmidt, H.H., Benhidjeb, T., Loser, P., Ziemer, S., Haas, R., Grewe, N., Schnieders, F., Heeren, J., Manns, M.P., Schlag, P.M., Strauss, M., 1999. Intravenous administration of recombinant adenoviruses causes thrombocytopenia, anemia and erythroblastosis in rabbits. J. Gene Med. 1, 360–371.

Clay, T.M., Hobeika, A.C., Mosca, P.J., Lyerly, H.K., Morse, M.A., 2001. Assays for monitoring cellular immune responses to active immunotherapy of cancer. Clin. Cancer Res. 7, 1127–1135.

Diehl, S., Rincon, M., 2002. The two faces of IL-6 on Th1/Th2 differentiation. Mol. Immunol. 39, 531–536.

Diehl, S., Anguita, J., Hoffmeyer, A., Zapton, T., Ihle, J.N., Fikrig, E., Rincon, M., 2000. Inhibition of Th1 differentiation by IL-6 is mediated by SOCS1. Immunity 13, 805–815.

Dorner, B.G., Scheffold, A., Rolph, M.S., Huser, M.B., Kaufmann, S.H., Radbruch, A., Flesch, I.E., Kroczek, R.A., 2002. MIP-1alpha, MIP-1beta, RANTES, and ATAC/lymphotactin function together with IFN-gamma as type 1 cytokines. Proc. Natl. Acad. Sci. U. S. A. 99, 6181–6186.

Everett, R.S., Evans, H.K., Hodges, B.L., Ding, E.Y., Serra, D.M., Amalfitano, A., 2004. Strain-specific rate of shutdown of CMV enhancer activity in murine liver confirmed by use of persistent [E1(−), E2b(−)] adenoviral vectors. Virology 325, 96–105.

Farina, S.F., Gao, G.P., Xiang, Z.Q., Rux, J.J., Burnett, R.M., Alvira, M.R., Marsh, J., Ertl, H.C., Wilson, J.M., 2001. Replication-defective vector based on a chimpanzee adenovirus. J. Virol. 75, 11603–11613.

Gerba, C.P., Gramos, D.M., Nwachuku, N., 2002. Comparative inactivation of enteroviruses and adenovirus 2 by UV light. Appl. Environ. Microbiol. 68, 5167–5169.

Gray, G.C., Goswami, P.R., Malasig, M.D., Hawksworth, A.W., Trump, D.H., Ryan, M.A., Schnurr, D.P., 2000. Adult adenovirus infections: loss of orphaned vaccines precipitates military respiratory disease epidemics. For the Adenovirus Surveillance Group. Clin. Infect. Dis. 31, 663–670.

Hartman, Z.C., Black, E.P., Amalfitano, A., 2006. Adenoviral infection induces a multi-faceted innate cellular immune response that is mediated by the toll-like receptor pathway in A549 cells. Virology 358, 357–372.

Hartman, Z.C., Kiang, A., Everett, R.S., Serra, D., Yang, X.Y., Clay, T.M., Amalfitano, A., 2007. Adenovirus infection triggers a rapid, MyD88-regulated transcriptome response critical to acute-phase and adaptive immune responses in vivo. J. Virol. 81, 1796–1812.

Hensley, S.E., Cun, A.S., Giles-Davis, W., Li, Y., Xiang, Z., Lasaro, M.O., Williams, B.R., Silverman, R.H., Ertl, H.C., 2007. Type I interferon inhibits antibody responses induced by a chimpanzee adenovirus vector. Molec. Ther. 15, 393–403.

Hilleman, M.R., 1957. Epidemiology of adenovirus respiratory infections in military recruit populations. Ann. N.Y. Acad. Sci. 67, 262–272.

Hilleman, M.R., Werner, J.H., Dascomb, H.E., Butler, R.L., Stewart, M.T., 1955. Epidemiology of RI(RI-67) group respiratory virus infections in recruit populations. Am. J. Hyg. 62, 29–42.

Hobeika, A.C., Morse, M.A., Osada, T., Ghanayem, M., Niedzwiecki, D., Barrier, R., Lyerly, H.K., Clay, T.M., 2005. Enumerating antigen-specific T-cell responses in peripheral blood: a comparison of peptide MHC Tetramer, ELISpot, and intracellular cytokine analysis. J. Immunother. 28, 63–72.

Hodges, B.L., Evans, H.K., Everett, R.S., Ding, E.Y., Serra, D., Amalfitano, A., 2001. Adenovirus vectors with the 100K gene deleted and their potential for multiple gene therapy applications. J. Virol. 75, 5913–5920 (JID — 0113724).

Hsu, K.H., Lubeck, M.D., Davis, A.R., Bhat, R.A., Selling, B.H., Bhat, B.M., Mizutani, S., Murphy, B.R., Collins, P.L., Chanock, R.M., 1992. Immunogenicity of recombinant adenovirus-respiratory syncytial virus vaccines with adenovirus types 4, 5, and 7 vectors in dogs and a chimpanzee. J. Infect. Dis. 166, 769–775.

Jiang, H., Wang, Z., Serra, D., Frank, M.M., Amalfitano, A., 2004. Recombinant adenovirus vectors activate the alternative complement pathway, leading to the binding of human complement protein C3 independent of anti-ad antibodies. Molec. Ther. 10, 1140–1142.

Kiang, A., Hartman, Z.C., Everett, R.S., Serra, D., Jiang, H., Frank, M.M., Amalfitano, A., 2006a. Multiple innate inflammatory responses induced after systemic adenovirus vector delivery depend on a functional complement system. Molec. Ther. 14, 588–598.

Kiang, A., Hartman, Z.C., Liao, S., Xu, F., Serra, D., Palmer, D.J., Ng, P., Amalfitano, A., 2006b. Fully deleted adenovirus persistently expressing GAA accomplishes long-term skeletal muscle glycogen correction in tolerant and nontolerant GSD-II mice. Molec. Ther. 13, 127–134.

Levin, S., Dietrich, J., Guillory, J., 1967. Fatal nonbacterial pneumonia associated with Adenovirus Type 4. Occurrence in an adult. JAMA 201, 975–977.

Lochmuller, H., Jani, A., Huard, J., Prescott, S., Simoneau, M., Massie, B., Karpati, G., Acsadi, G., 1994. Emergence of early region 1-containing replication-competent adenovirus in stocks of replication-defective adenovirus recombinants (delta E1 + delta E3) during multiple passages in 293 cells. Hum. Gene Ther. 5, 1485–1491.

Lubeck, M.D., Davis, A.R., Chengalvala, M., Natuk, R.J., Morin, J.E., Molnar-Kimber, K., Mason, B.B., Bhat, B.M., Mizutani, S., Hung, P.P., 1989. Immunogenicity and efficacy testing in chimpanzees of an oral hepatitis B vaccine based on live recombinant adenovirus. Proc. Natl. Acad. Sci. U. S. A. 86, 6763–6767.

Maecker, H.T., Moon, J., Bhatia, S., Ghanekar, S.A., Maino, V.C., Payne, J.K., Kuus-Reichel, K., Chang, J.C., Summers, A., Clay, T.M., Morse, M.A., Lyerly, H.K., DeLaRosa, C., Ankerst, D.P., Disis, M.L., 2005. Impact of cryopreservation on tetramer, cytokine flow cytometry, and ELISPOT. BMC Immunol. 6, 17.

Maizel Jr., J.V., White, D.O., Scharff, M.D., 1968a. The polypeptides of adenovirus. I. Evidence for multiple protein components in the virion and a comparison of types 2, 7A, and 12. Virology 36, 115–125.

Maizel Jr., J.V., White, D.O., Scharff, M.D., 1968b. The polypeptides of adenovirus. II. Soluble proteins, cores, top components and the structure of the virion. Virology 36, 126–136.

Mei, Y.F., Lindman, K., Wadell, G., 2002. Human adenoviruses of subgenera B, C, and E with various tropisms differ in both binding to and replication in the epithelial A549 and 293 cells. Virology 295, 30–43.

Michou, A.I., Santoro, L., Christ, M., Julliard, V., Pavirani, A., Mehtali, M., 1997. Adenovirus-mediated gene transfer: influence of transgene, mouse strain and type of immune response on persistence of transgene expression. Gene Ther. 4, 473–482.

Morral, N., O'Neal, W.K., Rice, K., Leland, M.M., Piedra, P.A., Aguilar-Cordova, E., Carey, K.D., Beaudet, A.L., Langston, C., 2002. Lethal toxicity, severe endothelial injury, and a threshold effect with high doses of an adenoviral vector in baboons. Hum. Gene Ther. 13, 143–154.

Morse, M.A., Clay, T.M., Hobeika, A.C., Osada, T., Khan, S., Chui, S., Niedzwiecki, D., Panicali, D., Schlom, J., Lyerly, H.K., 2005. Phase I study of immunization with dendritic cells modified with fowlpox encoding carcino-embryonic antigen and costimulatory molecules. Clin. Cancer Res. 11, 3017–3024.

Ni, S., Bernt, K., Gaggar, A., Li, Z.Y., Kiem, H.P., Lieber, A., 2005. Evaluation of biodistribution and safety of adenovirus vectors containing group B fibers after intravenous injection into baboons. Hum. Gene Ther. 16, 664–677.

Nilsson, M., Ljungberg, J., Richter, J., Kiefer, T., Magnusson, M., Lieber, A., Widegren, B., Karlsson, S., Fan, X., 2004. Development of an adenoviral vector system with adenovirus serotype 35 tropism; efficient transient gene transfer into primary malignant hematopoietic cells. J. Gene Med. 6, 631–641.

O'Neal, W.K., Zhou, H., Morral, N., Aguilar-Cordova, E., Pestaner, J., Langston, C., Mull, B., Wang, Y., Beaudet, A.L., Lee, B., 1998. Toxicological comparison of E2a-deleted and first-generation adenoviral vectors expressing alpha1-antitrypsin after systemic delivery 2. Hum. Gene Ther. 9, 1587–1598.

Raper, S.E., Yudkoff, M., Chirmule, N., Gao, G.P., Nunes, F., Haskal, Z.J., Furth, E.E., Propert, K.J., Robinson, M.B., Magosin, S., Simoes, H., Speicher, L., Hughes, J., Tazelaar, J., Wivel, N.A., Wilson, J.M., Batshaw, M.L., 2002. A pilot study of in vivo liver-directed gene transfer with an adenoviral vector in partial ornithine transcarbamylase deficiency. Hum. Gene Ther. 13, 163–175.

Rexroad, J., Wiethoff, C.M., Green, A.P., Kierstead, T.D., Scott, M.O., Middaugh, C.R., 2003. Structural stability of Adenovirus Type 5. J. Pharm. Sci. 92, 665–678 (JID — 2985195R).

Russell, W.C., Valentine, R.C., Pereira, H.G., 1967. The effect of heat on the anatomy of the adenovirus. J. Gen. Virol. 1, 509–522 (JID — 0077340).

Russell, K.L., Broderick, M.P., Franklin, S.E., Blyn, L.B., Freed, N.E., Moradi, E., Ecker, D.J., Kammerer, P.E., Osuna, M.A., Kajon, A.E., Morn, C.B., Ryan, M.A., 2006a. Transmission dynamics and prospective environmental sampling of adenovirus in a military recruit setting. J. Infect. Dis. 194, 877–885.

Russell, K.L., Hawksworth, A.W., Ryan, M.A., Strickler, J., Irvine, M., Hansen, C.J., Gray, G.C., Gaydos, J.C., 2006b. Vaccine-preventable adenoviral respiratory illness in US military recruits, 1999–2004. Vaccine 24, 2835–2842.

Shayakhmetov, D.M., Lieber, A., 2000. Dependence of adenovirus infectivity on length of the fiber shaft domain. J. Virol. 74, 10274–10286.

Toietta, G., Mane, V.P., Norona, W.S., Finegold, M.J., Ng, P., McDonagh, A.F., Beaudet, A.L., Lee, B., 2005. Lifelong elimination of hyperbilirubinemia in the Gunn rat with a single injection of helper-dependent adenoviral vector 2. Proc. Natl. Acad. Sci. U. S. A. 102, 3930–3935.

Top Jr., F.H., Buescher, E.L., Bancroft, W.H., Russell, P.K., 1971a. Immunization with live types 7 and 4 adenovirus vaccines. II. Antibody response and protective effect against acute respiratory disease due to Adenovirus Type 7. J. Infect. Dis. 124, 155–160.

Top Jr., F.H., Dudding, B.A., Russell, P.K., Buescher, E.L., 1971b. Control of respiratory disease in recruits with types 4 and 7 adenovirus vaccines. Am. J. Epidemiol. 94, 142–146.

Top Jr., F.H., Grossman, R.A., Bartelloni, P.J., Segal, H.E., Dudding, B.A., Russell, P.K., Buescher, E.L., 1971c. Immunization with live types 7 and 4 adenovirus vaccines. I. Safety, infectivity, antigenicity, and potency of Adenovirus Type 7 vaccine in humans. J. Infect. Dis. 124, 148–154.

Wessels, M.R., Butko, P., Ma, M., Warren, H.B., Lage, A.L., Carroll, M.C., 1995. Studies of group B streptococcal infection in mice deficient in complement component C3 or C4 demonstrate an essential role for complement in both innate and acquired immunity. Proc. Natl. Acad. Sci. U. S. A. 92, 11490–11494.

Zhang, L.Q., Mei, Y.F., Wadell, G., 2003. Human adenovirus serotypes 4 and 11 show higher binding affinity and infectivity for endothelial and carcinoma cell lines than serotype 5. J. Gen. Virol. 84, 687–695.