LETTERS

Hexon-chimaeric adenovirus serotype 5 vectors circumvent pre-existing anti-vector immunity

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A common viral immune evasion strategy involves mutating viral surface proteins in order to evade host neutralizing antibodies. Such immune evasion tactics have not previously been intentionally applied to the development of novel viral gene delivery vectors that overcome the critical problem of anti-vector immunity. Recombinant, replication-incompetent adenovirus serotype 5 (rAd5) vector-based vaccines for human immunodeficiency virus type 1 and other pathogens have proved highly immunogenic in preclinical studies[1,2] but will probably be limited by the high prevalence of pre-existing anti-Ad5 immunity in human populations, particularly in the developing world[3–6]. Here we show that rAd5 vectors can be engineered to circumvent anti-Ad5 immunity. We constructed novel chimaeric rAd5 vectors in which the seven short hypervariable regions (HVRs) on the surface of the Ad5 hexon protein were replaced with the corresponding HVRs from the rare adenovirus serotype Ad48. These HVR-chimaeric rAd5 vectors were produced at high titres and were stable through serial passages *in vitro*. HVR-chimaeric rAd5 vectors expressing simian immunodeficiency virus Gag proved comparably immunogenic to parental rAd5 vectors in naive mice and rhesus monkeys. In the presence of high levels of pre-existing anti-Ad5 immunity, the immunogenicity of HVR-chimaeric rAd5 vectors was not detectably suppressed, whereas the immunogenicity of parental rAd5 vectors was abrogated. These data demonstrate that functionally relevant Ad5-specific neutralizing antibodies are focused on epitopes located within the hexon HVRs. Moreover, these studies show that recombinant viral vectors can be engineered to circumvent pre-existing anti-vector immunity by removing key neutralizing epitopes on the surface of viral capsid proteins. Such chimaeric viral vectors may have important practical implications for vaccination and gene therapy.

Anti-vector immunity represents a key limitation of current recombinant viral gene delivery vectors. Pre-existing anti-vector immunity has been shown to suppress the immunogenicity of rAd5 vector-based vaccines, and the generation of anti-vector immunity after immune priming has been demonstrated to limit the efficiency of homologous boost immunizations[3,7,8]. Our laboratory and others have recently reported that dominant Ad5-specific neutralizing antibodies are directed primarily against the Ad5 hexon major capsid protein[6,9], suggesting the potential utility of hexon modification strategies to construct modified rAd5 vectors that circumvent anti-Ad5 immunity. Hexon exchanges among adenoviruses from different virus subgroups, however, have proven limited by viral structural constraints[9,10]. We sought to alter the antigenicity of the Ad5 hexon without perturbing its core structure by exchanging only the seven short HVRs on the surface of the Ad5 hexon protein, as these HVRs contain the majority of sequence variability among adenovirus serotypes[11]. The crystal structure of the hexon protein reveals a homotrimer of subunits each consisting of a double barrel core, although the HVRs are not fully resolved in the refined structure[12,13]. We therefore modelled the HVRs with reasonable geometry with respect to the rest of the molecule in O[14] and produced ribbon diagrams and space-filling models using MolScript[15]. This model suggests that the HVRs in fact define most of the solvent-exposed surface of the hexon trimer and thus may contain key Ad5-specific neutralizing antibody epitopes (Fig. 1a).

We constructed chimaeric, replication-incompetent, E1/E3-deleted rAd5 vectors in which either the first and most diverse hexon HVR, or alternatively all seven hexon HVRs, were specifically replaced with the corresponding HVRs from Ad48, which has particularly low seroprevalence in humans[4] (Fig. 1b). We termed these vectors rAd5HVR48(1) and rAd5HVR48(1–7), respectively. We did not alter the sequences of the hexon regions between the HVRs, as they form key elements of secondary structure within the hexon core and thus may be critical for hexon folding and virus stability. Consistent with these observations, our attempts to date to exchange larger regions or entire domains of the Ad5 hexon have failed to rescue virus (data not shown). In contrast, rAd5HVR48(1) and rAd5HVR48(1–7) vectors grew efficiently to high titres, although final yields of these chimaeric vectors were still three- to fivefold lower than yields of the parental rAd5 vectors. Transgene expression from rAd5HVR48(1) and rAd5HVR48(1–7) vectors expressing simian immunodeficiency virus (SIV) Gag proved comparable with that of rAd5 vectors, as measured by infection of A549 cells with varying amounts of virus followed by analysis of cell lysates by enzyme-linked immunosorbent assay (ELISA) (Fig. 1c). Moreover, specific infectivities of the chimaeric vectors were similar to those of rAd5 vectors, with virus particle to plaque-forming unit ratios <30. In addition, rAd5HVR48(1–7)-Gag vectors remained stable for at least 15 serial passages *in vitro* without detectable loss of the transgene (Fig. 1d) or changes to the chimaeric hexon sequence (data not shown).

We assessed the immunogenicity of rAd5, rAd5HVR48(1) and rAd5HVR48(1–7) vectors expressing SIV Gag in C57/BL6 mice either with or without anti-Ad5 immunity. Groups of mice were pre-immunized twice with 10^{10} virus particles of rAd5-Empty to generate high levels of anti-Ad5 immunity. These mice had Ad5-specific neutralizing antibody titres of 8,192–16,384, which represent the

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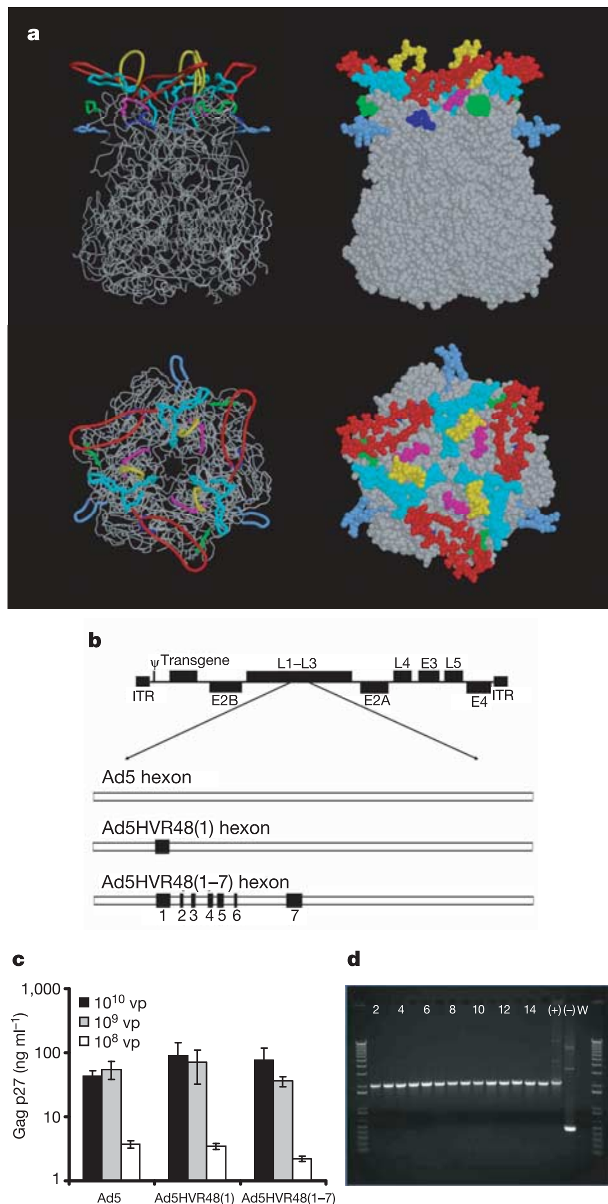

Figure 1 | Construction of hexon HVR-chimaeric rAd5 vectors. a, Ad5 hexon trimer structure (Protein Data Bank 1P30)[13] with modelled and highlighted HVRs (HVR1, red; HVR2, green; HVR3, pink; HVR4, light blue; HVR5, yellow; HVR6, blue; HVR7, cyan). The remainder of the hexon trimer is shown in grey. The HVR1 loop, for which there are essentially no experimental constraints, was modelled arbitrarily, maintaining stereochemically correct geometry and avoiding self-collision. Ribbon diagrams (left panels) and space-filling models (right panels) are shown for both side views (upper panels) and top views (lower panels) of a hexon trimer. **b**, Schematic cloning strategy to construct rAd5HVR48(1) and rAd5HVR48(1–7) vectors by replacing either the first HVR or all seven HVRs, respectively, with the corresponding regions from Ad48. **c**, SIV Gag expression from rAd5-Gag, rAd5HVR48(1)-Gag and rAd5HVR48(1–7)-Gag vectors. Gag p27 ELISA analysis of cell lysates is shown after infection of A549 cells with 10^{10}, 10^{9}, or 10^{8} virus particles (vp) of each vector. Error bars are means ± s.e.m. **d**, Transgene stability of rAd5HVR48(1–7)-Gag vectors through 15 serial passages. PCR reactions were performed to amplify the transgene region using viral DNA extracted from crude lysates from passages 2–15 and then analysed by 0.8% agarose gel electrophoresis. PCR controls included the plasmid pAdApt-Gag (+), the plasmid pAdApt-Empty (−) and water (W).

upper bound of neutralizing antibody titres found in humans in sub-Saharan Africa[5,6,16]. Naive mice (Fig. 2a, c, e, g) and mice with anti-Ad5 immunity (Fig. 2b, d, f, h) ($n = 4$ per group) were immunized intramuscularly with a single injection of 10^9, 10^8, 10^7, or 10^6 virus particles of rAd5-Gag, rAd5HVR48(1)-Gag or rAd5HVR48(1–7)-Gag. Gag-specific $CD8^+$ T lymphocyte responses specific for the dominant D^b-restricted AL11 epitope (AAVKNWMTQTL)[7] were assessed by D^b/AL11 tetramer binding assays. All three vectors proved comparably immunogenic in naive mice, even at the dose-limiting level of 10^7 virus particles (Fig. 2e). These data show that modifying the hexon HVRs does not diminish the potent cellular immune responses elicited by rAd5 vectors.

In mice with anti-Ad5 immunity, the immunogenicity of rAd5-Gag was abrogated at all doses tested (Fig. 2b, d, f). The immunogenicity of rAd5HVR48(1)-Gag was similarly suppressed, indicating that exchanging only the first hexon HVR was insufficient to circumvent high levels of anti-Ad5 immunity. The immunogenicity of rAd5HVR48(1–7)-Gag, however, remained essentially unaffected by anti-Ad5 immunity, even at the dose-limiting level of 10^7 virus particles (Fig. 2f). In fact, responses elicited by rAd5HVR48(1–7)-Gag were significantly higher than those elicited by rAd5-Gag or rAd5HVR48(1)-Gag in mice with anti-Ad5 immunity ($P < 0.001$, comparing tetramer binding responses on day 21 by analysis of variance (ANOVA) with Bonferroni adjustments to account for multiple comparisons). These data demonstrate that simultaneously exchanging all seven hexon HVRs results in a vector that effectively evades anti-Ad5 immunity in mice. Functional interferon (IFN)-γ ELISPOT assays in response to a pool of overlapping Gag peptides, the $CD8^+$ T lymphocyte epitopes AL11 and KV9, and the $CD4^+$ T lymphocyte epitope DD13 confirmed these tetramer binding assays (Fig. 2i, j).

To evaluate further the immunogenicity of the chimaeric rAd5HVR48(1–7) vector, we compared the immunogenicity of rAd5-Gag and rAd5HVR48(1–7)-Gag with the rare serotype vectors rAd35-Gag[4,7] and rAd48-Gag. In naive mice, rAd5-Gag and rAd5HVR48(1–7)-Gag proved significantly more immunogenic than rAd35-Gag and rAd48-Gag at the lower dose of 10^7 virus particles (Fig. 2k; $P < 0.001$), consistent with the lower immunogenicity of rare serotype recombinant adenovirus vectors as compared with rAd5 vectors in preclinical studies to date[3,7]. In mice with anti-Ad5 immunity, 10^7 virus particles of rAd5HVR48(1–7)-Gag proved significantly more immunogenic than rAd5, rAd35 and rAd48 vectors (Fig. 2l; $P < 0.001$), reflecting the intrinsic potency of this chimaeric vector combined with its capacity to circumvent anti-Ad5 immunity.

We next evaluated the immunogenicity of heterologous recombinant adenovirus prime–boost regimens in mice either with or without anti-Ad5 immunity. Groups of mice ($n = 4$ per group) were primed on day 0 with 10^9 virus particles of rAd35-Gag and then boosted on day 28 with 10^9 virus particles of rAd5HVR48(1–7)-Gag, rAd35-Gag, or rAd5-Gag. Naive mice primed with rAd35-Gag were boosted efficiently and comparably by the heterologous vectors rAd5HVR48(1–7)-Gag and rAd5-Gag (Fig. 3a, c). In contrast, re-administration of the homologous vector rAd35-Gag proved ineffective, presumably due to the generation of anti-Ad35 immunity by the priming immunization. In mice with pre-existing anti-Ad5 immunity (Fig. 3b, d), rAd5HVR48(1–7)-Gag proved significantly more potent than both rAd5-Gag and rAd35-Gag as a boosting vector ($P < 0.001$, comparing tetramer binding responses on day 49). These data suggest that rAd5HVR48(1–7) vectors function essentially as a novel serotype distinct from rAd5 vectors. Moreover, these studies show that rAd5HVR48(1–7) vectors remain highly immunogenic both as a prime and as a boost in the presence of high levels of anti-Ad5 immunity that ablate the immunogenicity of rAd5 vectors.

We measured vector-specific neutralizing antibody titres in mice immunized either once (Fig. 4a) or twice (Fig. 4b) with 10^{10} virus

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particles of rAd5-Gag, rAd5HVR48(1–7)-Gag, or rAd35-Gag. As expected, mice that received rAd5-Gag developed high titres of Ad5-specific neutralizing antibodies but substantially lower titres of Ad5HVR48(1–7)-specific neutralizing antibodies, confirming that the majority of Ad5-specific neutralizing antibodies are directed against the hexon HVRs. Conversely, mice that received rAd5HVR48(1–7)-Gag developed high titres of Ad5HVR48(1–7)-specific neutralizing antibodies but markedly lower titres of Ad5-specific neutralizing antibodies. These mice also developed high titres of Ad48-specific neutralizing antibodies, suggesting that the HVRs in this chimaeric vector are in fact presented in conformations comparable with their wild-type orientations in Ad48. Thus, exchanging the HVRs effectively swapped the predominant serologic determinants of the virus from Ad5 to Ad48. We also measured Gag-specific antibody responses in these mice by ELISA (Fig. 4c). High titres of Gag-specific antibodies were elicited by both rAd5-Gag and rAd5HVR48(1–7)-Gag, although there was a trend towards threefold lower antibody titres elicited by rAd5HVR48(1–7)-Gag. In contrast, rAd35-Gag failed to generate detectable Gag-specific antibodies in this system, consistent with our previous observations[7].

We next assessed vector-specific neutralizing antibody titres in 265 serum samples from healthy individuals in South Africa (Fig. 4d). The median Ad5-specific neutralizing antibody titre in these samples was 1,024, whereas the median Ad5HVR48(1–7)-specific neutralizing antibody titre was significantly lower at 128 ($P < 0.0001$, Wilcoxon rank-sum test). These data suggest that 85–90% of Ad5-specific neutralizing antibodies in humans are directed against the hexon HVRs. The residual low-titre neutralizing antibodies against rAd5HVR48(1–7) presumably represent Ad5 fibre- and penton-specific neutralizing antibodies, because Ad48-specific neutralizing antibodies were particularly low in these samples. In previous adoptive transfer studies in mice, we demonstrated that Ad5 fibre- and penton-specific neutralizing antibodies did not efficiently suppress rAd5 vaccine immunogenicity[6], consistent with the observations in the present study that rAd5HVR48(1–7) vectors effectively circumvented anti-Ad5 immunity. Moreover, serum samples with Ad48- but not Ad5-specific neutralizing antibodies also exhibited neutralizing activity against rAd5HVR48(1–7) (data not shown), supporting the conclusion that dominant neutralizing antibodies are directed primarily against the hexon HVRs.

To confirm the murine immunogenicity studies, we performed a

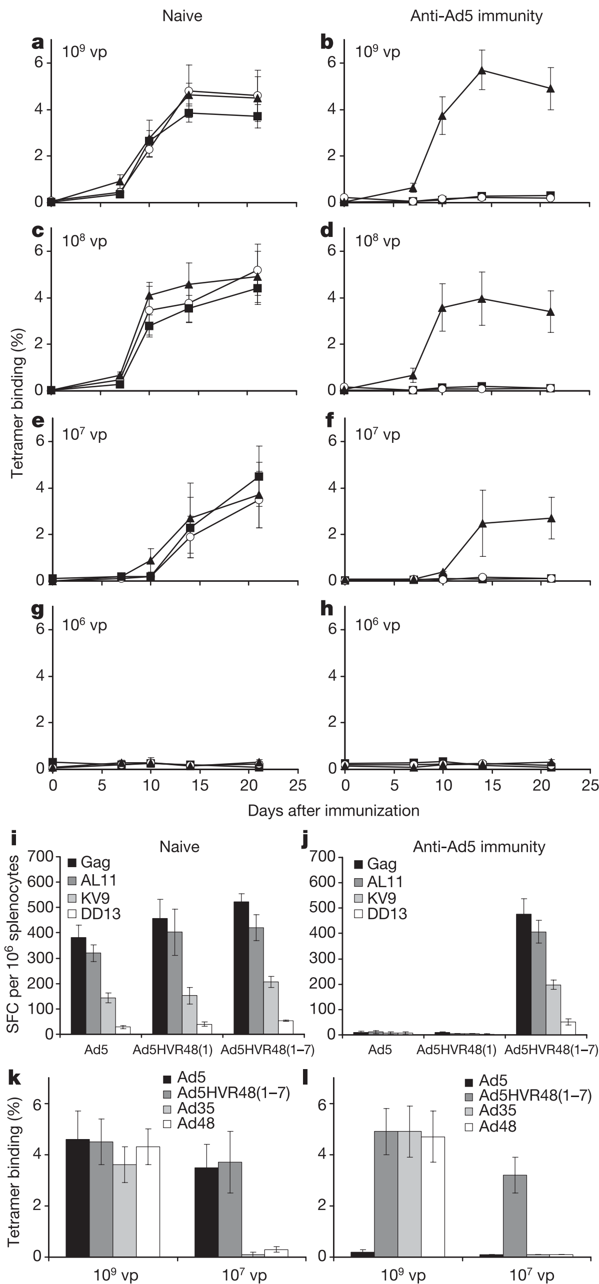

Figure 2 | Cellular immune responses elicited by hexon HVR-chimaeric rAd5 vectors. Naive C57/BL6 mice (**a**, **c**, **e**, **g**) or C57/BL6 mice with anti-Ad5 immunity (**b**, **d**, **f**, **h**) were immunized intramuscularly with 10^9 (**a**, **b**), 10^8 (**c**, **d**), 10^7 (**e**, **f**) or 10^6 (**g**, **h**) virus particles of rAd5-Gag (open circles), rAd5HVR48(1)-Gag (filled squares), or rAd5HVR48(1–7)-Gag (filled triangles). **a**–**h**, Gag epitope-specific $CD8^+$ T lymphocyte responses were assessed by D^b/AL11 tetramer binding assays at multiple time points after immunization. **i**, **j**, Gag-specific cellular immune responses to pooled Gag peptides as well as to AL11, KV9 and DD13 epitope peptides were assessed by IFN-γ ELISPOT assays on day 28 after immunization in mice that received 10^8 virus particles of each vector. Spot-forming cells (SFC) per million splenocytes are shown. **k**, **l**, Naive mice or mice with anti-Ad5 immunity were immunized intramuscularly with 10^9 or 10^7 virus particles of rAd5-Gag, rAd5HVR48(1–7)-Gag, rAd35-Gag, or rAd48-Gag. Gag epitope-specific $CD8^+$ T lymphocyte responses were assessed by D^b/AL11 tetramer binding assays on day 21 after immunization. Error bars are means ± s.e.m.

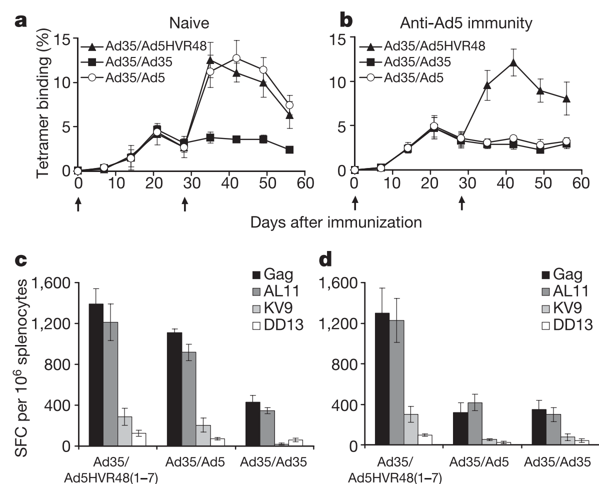

Figure 3 | Immunogenicity of heterologous recombinant adenovirus prime-boost regimens. Naive C57/BL6 mice (**a**, **c**) or C57/BL6 mice with anti-Ad5 immunity (**b**, **d**) were primed on day 0 with 10^9 virus particles of rAd35-Gag and boosted on day 28 with 10^9 virus particles of rAd5HVR48(1–7)-Gag, rAd35-Gag or rAd5-Gag. Arrows indicate immunizations. **a**, **b**, Gag epitope-specific $CD8^+$ T lymphocyte responses were assessed by D^b/AL11 tetramer binding assays at multiple time points after immunization. **c**, **d**, Gag-specific cellular immune responses to pooled Gag peptides as well as to AL11, KV9 and DD13 epitope peptides were assessed by IFN-γ ELISPOT assays on day 56 after the primary immunization. Error bars are means ± s.e.m.

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pilot study comparing the immunogenicity of rAd5-Gag and rAd5HVR48(1–7)-Gag in ten *Mamu-A*01*-negative rhesus monkeys (Fig. 5). Monkeys were pre-immunized twice with 10^{11} virus particles of rAd5-Empty to generate Ad5-specific neutralizing antibody titres of 16,384–32,768. Naive monkeys and monkeys with anti-Ad5 immunity were then immunized intramuscularly with a single injection of 10^{11} virus particles of rAd5-Gag or rAd5HVR48(1–7)-Gag. In naive monkeys ($n = 3$ per group), both vectors elicited potent and comparable Gag-specific IFN-γ ELISPOT responses (Fig. 5a, b). Multiparameter intracellular cytokine staining (ICS) assays[17] also suggested that these responses were comparable and consisted primarily of central memory $CD8^+$ T lymphocyte responses ($CD3^+CD8^+CD28^+CD95^+$) and effector memory $CD8^+$ T lymphocyte responses ($CD3^+CD8^+CD28^-CD95^+$), although lower frequencies of $CD4^+$ central memory T lymphocyte responses ($CD3^+CD4^+CD28^+CD95^+$) were also observed (Fig. 5c, d). In monkeys with anti-Ad5 immunity ($n = 2$ per group), low responses were still detected after rAd5-Gag immunization, presumably as a result of the high dose of the vaccine used in this study. These responses, however, were approximately fourfold lower than those observed in naive monkeys (Fig. 5a, c), consistent with the results of previous studies[8]. In contrast, responses elicited by rAd5HVR48(1–7)-Gag were not detectably suppressed by anti-Ad5 immunity (Fig. 5b, d). Although statistical analyses could not be performed due to the limited number of animals in this pilot study, these data nevertheless show that rAd5HVR48(1–7) vectors are highly immunogenic in nonhuman primates with anti-Ad5 immunity.

These studies demonstrate that targeted HVR modification strategies can be used to construct chimaeric rAd5 vectors that effectively circumvent anti-Ad5 immunity in both mice and rhesus monkeys. Previous reports have shown that exchanging complete hexon genes between viruses from different adenovirus subgroups results in non-viable or poorly viable viruses[9], presumably due to viral structural constraints. In particular, highly immunogenic adenovirus subgroup C vectors (such as rAd5 vectors) containing hexons from rare serotype adenovirus subgroup D viruses (such as Ad48) have not previously been successfully constructed. We speculate that exchanging only the seven short HVRs on the surface of the Ad5 hexon protein effectively preserves the hexon core structure while removing key neutralizing determinants. The HVR-chimaeric rAd5 vectors also preserve many favourable features of rAd5 vectors, including vector stability, specific infectivity, high levels of transgene expression and potent immunogenicity. Production yields of the rAd5HVR48(1–7) vectors, however, remained several-fold lower than rAd5 vectors, suggesting that these vectors can be further optimized. It should also be possible to construct a series of HVR-modified rAd5 vectors with HVRs derived from other adenovirus serotypes as well as with synthetic sequences to facilitate heterologous recombinant adenovirus prime–boost vaccine regimens, although the extent of HVR sequence constraints in these chimaeric vectors remains to be determined.

The observations that rAd5HVR48(1–7) vectors exhibit primarily the serologic properties of Ad48 rather than Ad5 *in vitro* and effectively circumvent anti-Ad5 immunity *in vivo* demonstrate that dominant Ad5-specific neutralizing antibodies are focused primarily on epitopes within the hexon HVRs. We estimate that 10–15% of Ad5-specific neutralizing antibodies are directed against other Ad5 capsid proteins, including fibre and penton[18,19], based on virus neutralization studies using murine and human serum samples (Fig. 4). Fibre- and penton-specific neutralizing antibodies, however, are probably less relevant than hexon-specific neutralizing antibodies, as they are typically lower in titre and also seem inefficient at suppressing rAd5 vaccine immunogenicity *in vivo*[6], perhaps reflecting different mechanisms of virus neutralization[20]. Our conclusion that functionally relevant Ad5-specific neutralizing antibodies are focused primarily on the hexon HVRs is also consistent with studies that have shown that these solvent-exposed loops comprise the majority of adenovirus serotype-specific sequence variability[11,13]. We cannot, however, exclude the possibility that Ad5-specific neutralizing antibodies against other epitopes as well as Ad5-specific cellular immune responses may have important secondary roles in certain settings.

These studies have major implications for the development of recombinant viral vectors for vaccination and gene therapy. The identification and specific removal of dominant neutralizing antibody epitopes on the surface of viral capsid proteins offers a novel strategy to create viral vectors that evade pre-existing neutralizing antibody responses and that overcome the critical limitation of anti-vector immunity. In particular, HVR-chimaeric rAd5 vectors may prove useful as vaccine vectors for HIV-1 and other pathogens that

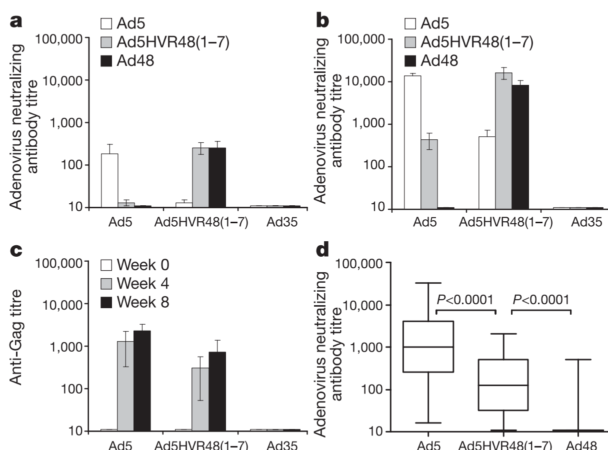

Figure 4 | Humoral immune responses to hexon HVR-chimaeric rAd5 vectors. a–c, Naive mice were primed at week 0 and boosted at week 4 with 10^{10} virus particles of rAd5-Gag, rAd5HVR48(1–7)-Gag, or rAd35-Gag. **a**, **b**, Ad5-specific (white bars), Ad5HVR48(1–7)-specific (grey bars) and Ad48-specific (black bars) neutralizing antibody titres were assessed by virus neutralization assays using serum samples obtained at week 4 (**a**) and week 8 (**b**). **c**, Gag-specific antibodies were assessed at weeks 0 (white bars), 4 (grey bars) and 8 (black bars) by ELISA. **d**, Ad5-specific, Ad5HVR48(1–7)-specific and Ad48-specific neutralizing antibody titres were assessed in 265 serum samples from South Africa and are presented as box-and-whisker plots (see Methods). Error bars are means ± s.e.m.

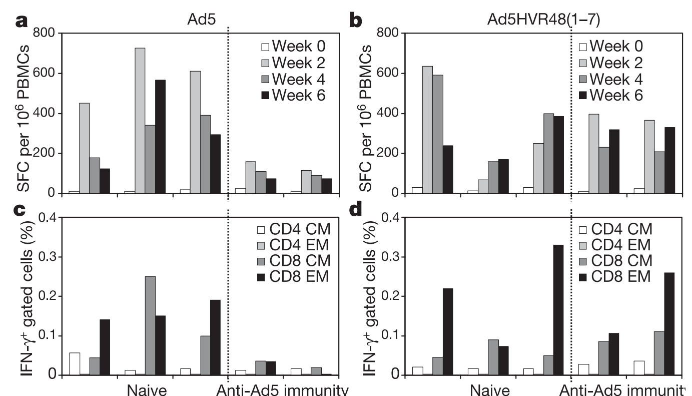

Figure 5 | Immunogenicity of hexon HVR-chimaeric rAd5 vectors in rhesus monkeys. Naive rhesus monkeys or rhesus monkeys with anti-Ad5 immunity were immunized intramuscularly with 10^{11} virus particles of rAd5-Gag (**a**, **c**) or rAd5HVR48(1–7)-Gag (**b**, **d**). **a**, **b**, Gag-specific cellular immune responses were assessed by IFN-γ ELISPOT assays at multiple time points after immunization. **c**, **d**, Gag-specific $CD4^+$ and $CD8^+$ central memory (CM; $CD28^+CD95^+$) and effector memory (EM; $CD28^-CD95^+$) T lymphocyte responses were evaluated by pooled peptide IFN-γ ICS assays at week 4 after immunization.

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are endemic in the developing world, where the vast majority of individuals have high levels of pre-existing anti-Ad5 immunity.

METHODS

Vector cloning and production. E1/E3-deleted, replication-incompetent rAd5 vectors containing chimaeric hexon genes were constructed using plasmid/cosmid recombination systems as described[4,21]. Partial Ad5 hexon genes containing the Ad5 HVRs exchanged with the corresponding regions from Ad48 were produced synthetically (GeneART) and cloned as *Apa*I-*Hpa*I fragments into a shuttle plasmid containing the complete Ad5 hexon gene. The Ad5 HVR regions were defined as amino acids 136–165 (HVR1), 188–194 (HVR2), 212–220 (HVR3), 248–258 (HVR4), 268–281 (HVR5), 305–310 (HVR6) and 418–451 (HVR7). *Asc*I-*Asc*I fragments containing the complete chimaeric hexon genes were then excised from the shuttle plasmids and used to replace the corresponding regions in the Ad5 cosmid pWE.Ad5.AflII-rITR.dE3. The resultant mutant Ad5 cosmids together with the adaptor plasmid pAdApt expressing codon-optimized SIVmac239 Gag under control of a CMV promoter were co-transfected into complementing PER.C6/55K cells, and homologous recombination yielded rAd5HVR48(1)-Gag and rAd5HVR48(1–7)-Gag vectors. These vectors were plaque-purified, expanded and purified by CsCl gradient centrifugation. Replication-incompetent rAd48 vectors were constructed using methods we have described for other serotypes[4] and exhibited comparable expression and yields.

Animals and immunizations. Six-to-eight-week-old C57/BL6 mice (Charles River Laboratories) were injected intramuscularly with varying doses of replication-incompetent recombinant adenovirus vectors expressing SIV Gag in 100 μl sterile PBS divided equally in both quadriceps muscles. To induce active anti-vector immunity, mice were pre-immunized intramuscularly twice, separated by a 4-week interval, with 10^{10} virus particles of rAd5-Empty in 100 μl sterile PBS. Adult outbred rhesus monkeys that did not express the *Mamu-A*01* class I allele were injected intramuscularly with 10^{11} virus particles of recombinant adenovirus vectors expressing SIV Gag in 1 ml sterile PBS divided equally in both quadriceps muscles. To induce active anti-vector immunity, monkeys were pre-immunized intramuscularly twice, separated by an 8-week interval, with 10^{11} virus particles rAd5-Empty in 1 ml sterile PBS.

Tetramer binding assays. Tetrameric H-2D^b complexes folded around the immunodominant SIV Gag AL11 epitope (AAVKNWMTQTL) were prepared and used to stain epitope-specific CD8$^+$ T lymphocytes from C57/BL6 mice as described[7]. Samples were analysed by two-colour flow cytometry on a FACS Array (BD Pharmingen), and gated CD8$^+$ T lymphocytes were examined for staining with the D^b/AL11 tetramer. CD8$^+$ T lymphocytes from naive mice exhibited $<$0.1% tetramer staining.

ELISPOT assays. SIV Gag-specific cellular immune responses in splenocytes from vaccinated mice and peripheral blood mononuclear cells (PBMCs) from vaccinated monkeys were assessed by IFN-γ ELISPOT assays in response to overlapping 15-amino-acid peptides spanning the SIVmac239 Gag protein (NIH AIDS Research and Reference Reagent Program) as well as epitope peptides as described[6,7,22].

ICS assays. SIV Gag-specific CD4$^+$ and CD8$^+$ T lymphocyte responses in PBMCs from vaccinated monkeys were assessed by multiparameter ICS assays as described[17]. Cells were stained with monoclonal antibodies against CD4-FITC (L200), CD95-PE (DX2), CD28-PerCP-Cy5.5 (L293), IFN-γ-PE-Cy7 (B27), IL-2-APC (MQ1-17H12), CD3-Alexa700 (SP34) and CD8-APC-Cy7 (SK1) (BD Biosciences).

Virus neutralization assays. Ad5-specific, Ad5HVR48(1–7)-specific and Ad48-specific neutralizing antibody titres in murine and human serum samples were measured by luciferase-based virus neutralization assays as described[16]. Serum samples from healthy individuals randomly selected from a South African Medical Research Council measles vaccine study were used for the human adenovirus seroprevalence studies. Neutralization titres were defined as the maximum serum dilution that neutralized 90% of luciferase activity.

ELISAs. Gag-specific antibody responses in vaccinated mice were assessed by a direct ELISA using purified recombinant SIV Gag protein (Intracel) as described[7].

Statistical analyses. Statistical analyses were performed with GraphPad Prism version 4.01 (GraphPad Software). Immune response data are presented as means with standard errors. Comparisons of mean immune responses were performed by two-tailed ANOVA with Bonferroni adjustments to account for multiple comparisons. Neutralizing antibody data using human serum samples are presented as box-and-whisker plots depicting medians, 25–75% interquartile ranges and complete ranges. Comparisons of median neutralizing antibody titres were performed by two-tailed Wilcoxon rank-sum tests.

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