Generation of Ad5-HVR5*7*E451Q/F35++

A35 fiber (F35++),[1] including two amino acid changes (D207G and T245A), was generated using a strategy of gene assembling. The first DNA fragment was obtained by PCR using the oligonucleotides *Nde*IFibDIR: CCCGTGTATCCATATGACAC and Fiber35REV: CATAACACAAATTAGTTGTCGTCTTCTGTAATGTA with pWE15-A2 plasmid[1] and the second DNA fragment using the oligonucleotide 35FibDIR: ACGACAACTAATTTGTGTTATGTTTCAACGTGTT and FIBREV: GTTCCCTGCAGTGTATACCC using the same plasmid. Both fragments were amplified using *NdeI*FibDIR and FIBREV oligonucleotides by gene assembling generating a DNA fragment subsequently cloned into the strataclone shuttle plasmid pSCB-F35++ (Stratagene, UK). After sequencing all amplified nucleotides, the pSCB-F35++ and a shuttle vector containing the Ad5 fiber (pShuttle-Ad5Fiber) were digested by *Nde*I and *Mfe*I sites to exchange the fiber gene and generated the pShuttle-F35++ plasmid. Homologous recombination was performed using *Spe*I-opened pAd5CMVlacZ-HVR5*7*E451Q, previously described,[2] and the pShuttle-F35++ opened by *Xho*I to finally generate the plasmid pAd5CMVlacZ-HVR5*7*E451Q/F35++.

Cytokine and chemokine analysis

Increasing doses (1×10^{10}, 3×10^{10}, and 1×10^{11} vp/mouse) of Ad5, Ad5-HVR7(Ad26) or Ad5-HVR5*7*E451Q were administered to macrophage-depleted and control MF1 mice. Blood was extracted by venepuncture 1h and 6h post virus administration and serum was prepared by allowing whole blood to coagulate for 30 minutes at room temperature followed by centrifugation for 15 minutes at 4°C. Serum samples were analysed in a mouse cytokine 20-plex panel (Invitrogen,UK) following the manufacturer's instructions for the simultaneous quantification of the following cytokines: basic fibroblast growth factor (FGF-basic), granulocyte macrophage colony-stimulating factor (GM-CSF), interferon-gamma (IFN-γ), interleukin (IL) 1 alpha (IL-1α), IL-1β, IL-2, IL-4, IL-5, IL-6, IL-10, IL-12 (p40/p70), IL-13, IL-17, interferon-gamma–induced protein (IP-10), keratinocyte-derived cytokine (KC), monocyte chemo-attractant protein 1 (MCP-1), monokine induced by gamma interferon (MIG), macrophage inflammatory protein 1 alpha MIP-1α, tumor necrosis factor alpha (TNF-α) and vascular endothelial growth factor (VEGF). Data were analyzed using Bio-Plex manager software with 5PL curve fitting.

In vitro methods

CHO-BC1 and CHO-WTR were cultured in Dulbecco's modified Eagle's medium/F-12 (Invitrogen, Carlsbad, CA) supplemented with 10% fetal calf serum (PAA Laboratories, Teovil, UK), 1% penicillin, 100 μg/mL streptomycin, 500 μg/mL Geneticin (G-418), and 2mmol/L L-glutamine. For transduction studies, 4×10^4 cells/well were plated into 96 well plates and virus added at 1000 viral particles/cell for 3 hours at 37°C in the presence or absence of FX (10 μg/mL). Beta-galactosidase expression was analysed as previously described[2] and presented as relative light units per milligram of protein. Cell binding experiments were performed in 24-well plates with 2×10^5 cells/well in serum free media with 1000 viral particles/well in the presence or absence of FX as previously described.[2] Viral genome DNA was isolated using QIAamp DNA Mini Kit (Qiagen, Crawley, UK) and analysed by qPCR using 100 ng of DNA. Statistical analysis was generated using unpaired Student *t* test with statistical significance accepted at *P* below 0.05.

REFERENCES

1. Wang H, Liu Y, Li Z, et al. In vitro in in vivo properties of adenovirus vectors with increased affinity to CD46. *Journal of Virology*. 2008;82:10567–10579.
2. Alba R, Bradshaw AC, Parker AL, et al. Identification of coagulation factor (F)X binding sites on the adenovirus serotype 5 hexon: effect of mutagenesis on FX interactions and gene transfer. *Blood*. 2009;114:965–971.

6 hours

Virus	Dose	CL	FGF-basic	GM-CSF	IFN-g	IL-1a	Il-1b	IL-2	IL-4	IL-5	IL-6	IL-10
PBS		–	147 +/– 158	0	0	0	0	0	0	0	0	0
(n=4)		+	0	0	0	0	0	0	0	0	0	0
Ad5	1×10^{10}	–	0	0	0	0	5.27 +/– 4.6	0	0	0	0	0
(n=3)		+	117.0 +/– 104.7	0	0	53.29 +/– 92.3	42.6 +/– 61.6	21.7 +/– 37.6	42.7 +/– 74.1	0	0	79.9 +/– 70.7
Ad5	3×10^{10}	–	0	0	0	17.1 +/– 29.7	18.3 +/– 18.9	0	0	0	0	0
(n=3)		+	24.84 +/– 43.0	0	0	490.6 +/– 771.3	0	0	0	0	0	0
Ad5	1×10^{11}	–	136.6 +/– 20.9	9.59 +/– 11.09	773.2 +/– 837. 1	44.6 +/– 25.4	145.9 +/– 119.3	64.7 +/– 22.8	4.1 +/– 8.2	77.9 +/– 82.5	99.9 +/– 85.5	140.1 +/– 48.1
(n=8)		+	204.1 +/– 66.2	11.83 +/– 20.81	119.7 +/– 154	112.2 +/– 97.3	102.7 +/– 141.1	49.0 +/–64.2	54.4 +/– 91.6	13.0 +/– 26.0	124.8 +/– 175.3	115.1 +/– 151.1
HVR5*7*E451Q	1×10^{10}	–	0	0	0	1.6 +/– 2.7	29.8 +/– 26.3	3.6 +/– 6.2	1.77 +/– 3.0	0	0	0
(n=3)		+	69.7 +/– 63.1	0	0	0	56.1 +/– 4.1	1.3 +/– 2.2	7.6 +/– 8.4	6.9 +/– 12.0	0	48 +/– 83.1
HVR5*7*E451Q	3×10^{10}	–	0	0	0	12.8 +/– 22.2	24.8 +/– 15.6	4.1 +/– 7.2	5.0 +/– 8.6	18.6 +/– 32.3	0	22.7 +/– 39.3
(n=3)		+	68.9 +/– 60.1	0	0	0	5.0 +/– 8.7	0	0	0	0	0
HVR5*7*E451Q	1×10^{11}	–	77.4 +/– 16.4	5.5 +/– 9.9	816 +/– 951.9	48.9 +/– 26.6	119.8 +/– 121.6	80.0 +/– 11.6	0	124.1 +/– 70.6	191.2 +/– 149.6	189.6 +/– 114.5
(n=8)		+	170 +/– 61.1	2.2 +/– 4.5	194.7 +/– 161.3	55.4 +/– 84.1	48.0 +/– 48.3	50.0 +/– 37.1	0	8.3 +/– 6.4	12.7 +/– 22.5	75.1 +/– 45.7
HVR7(Ad26)	1×10^{10}	–	0	3.31 +/– 5.73	21.0 +/– 38.0	6.6 +/– 11.5	6.26+/– 7.8	0.6 +/– 1.1	9.2 +/– 16.0	0	0	0
(n=3)		+	0	0	0	6.8 +/– 11.7	0	0	0	0	0	0
HVR7(Ad26)	1×10^{11}	–	0	0	0	5.5 +/– 11.1	90.4 +/– 104.0	14.0 +/– 10.5	2.2 +/– 4.4	131.0 +/– 88.9	0	18.4 +/– 24.8
(n=4)		+	0	0	0	86.4 +/– 153.3	130.3 +/– 144.6	60.5 +/– 97.1	81.0 +/– 64.1	73.0 +/– 57.8	77.3 +/– 127.9	252.0 +/– 504.1

1 hour

Virus	Dose	CL	FGF-basic	GM-CSF	IFN-g	IL-1a	Il-1b	IL-2	IL-4	IL-5	IL-6	IL-10
Ad5	1×10^{11}	–	0	0	27.7 +/– 41.6	0	35.9 +/– 58.6	6.9 +/– 8.8	32.5 +/– 72.7	26.8 +/– 59.9	50.3 +/– 36.34	167.0 +/– 334
(n=5)		+	78.9 +/– 91	0	0	0	17.2 +/– 34.4	1.7 +/–3.8	0	0	0	23.3 +/– 52.2
HVR5*7*E451Q	1×10^{11}	–	0	0	0	0	6.99 +/– 15.6	1.3 +/– 2.9	0	15.0 +/– 33.7	20.9 +/– 29.2	10.9 +/– 24.4
(n=5)		+	80.32 +/– 55.9	0	0	0	0.9 +/– 1.8	0	0	0	0	16.0 +/– 32.1

6 hours

Virus	Dose	CL	IL-12	IL-13	IL-17	IP-10	KC	MCP-1	MIG	MIP-1a	TNF-a	VEGF
PBS		**−**	24.6 +/− 42.7	0	0	0	0	0	5.3 +/− 8.2	0	0	0
(n=4)		**+**	19.8 +/− 34.4	0	0	0	0	0	33.9 +/− 14.3	0	0	0.003 +/−0.005
Ad5	**1×10^{10}**	**−**	67.1 +/− 31.3	0	0	200.8 +/− 144.0	184.3 +/− 319.3	87.5 +/− 64.8	51.1 +/− 63.3	0	0	0
(n=3)		**+**	35.8 +/− 79.9	0	0	39.6 +/− 68.6	0	48.8 +/− 81.8	0	0	36.3 +/−62.9	0
Ad5	**3×10^{10}**	**−**	106.6 +/− 66.4	0	0	190.9 +/− 99.1	0	159.3 +/− 261.7	37.3 +/− 19.6	21.5 +/− 37.3	0	0
(n=3)		**+**	34.8 +/− 36.0	0	0.2 +/− 0.3	0	0	0	64.9	0	0	0
Ad5	**1×10^{11}**	**−**	414.1 +/− 162.8	8.3 +/− 16.7	0.2 +/− 0.3	863.2 +/− 304.8	914.3 +/− 651.8	959.4 +/− 679.1	765.4 +/− 461.7	86.2 +/− 69.5	53.3 +/− 63.1	27.7 +/− 9.5
(n=8)		**+**	101.8 +/− 110.2	3.1 +/− 6.1	4.7 +/− 8.3	346.5 +/− 333.7	681.1 +/− 994.3	58.0 +/− 72.5	315.1 +/− 315.2	46 +/− 46	34.8 +/− 65.7	25.5 +/− 26.8
HVR5*7*E451Q	**1×10^{10}**	**−**	44.2 +/− 54.6	0	0	392.3 +/− 300.0	0	99.6 +/− 86.7	82.0 +/− 52.3	53.6 +/−66.1	0	0
(n=3)		**+**	54.6 +/− 13.8	0	0.1 +/− 0.3	21.1 +/− 23.1	223.9 +/− 195.2	18.8 +/− 3.6	0	0	0	0
HVR5*7*E451Q	**3×10^{10}**	**−**	111.4 +/− 40.4	0	0.3 +/− 0.6	364.1 +/− 86.5	0	261.7 +/− 11.2	23.1 +/− 12.6	0	0	1.28 +/− 2.2
(n=3)		**+**	27.1 +/− 43.2	0	0	0	0	1.57 +/− 2.7	0	0	0	0
HVR5*7*E451Q	**1×10^{11}**	**−**	815.9 +/− 268.2	18.9 +/− 36.9	0	1446.3 +/− 397.0	1404.7 +/− 607.7	1489.3 +/− 600.3	1420.1 +/− 663.2	107.4 +/− 81.5	79.4 +/− 52.7	24.3 +/− 3.3
(n=8)		**+**	316.3 +/− 143.7	16.2 +/− 32.4	0	701.0 +/− 313.8	1016.0 +/− 456.6	235.5 +/− 147.0	795.6 +/− 427.5	131.3 +/− 65	31.0 +/− 9.0	36.01 +/−20.2
HVR7(Ad26)	**1×10^{10}**	**−**	68.4 +/− 71.2	0	0	47.9 +/− 66.2	0	21.1 +/− 31.9	29.7 +/− 19.2	0	0	1.3 +/− 2.3
(n=3)		**+**	43.6 +/− 10.2	0	0	0	0	0	0	0	0	0
HVR7(Ad26)	**1×10^{11}**	**−**	440.9 +/− 236.2	0	0	810.8 +/− 132.8	332.9 +/− 361.8	1417.9 +/− 750.2	800.5 +/− 168.7	41.6 +/− 79.5	4.7 +/− 9.4	0.88 +/− 1.7
(n=4)		**+**	204.3 +/− 93.0	31.7 +/− 63.4	9.5 +/− 36.6	320.1 +/− 106.9	672.6 +/− 134.2	105.7+/− 60.0	324.1 +/− 380	275.7 +/− 350	53.8 +/− 43.9	7.6 +/− 15.3

1 hour

Virus	Dose	CL	IL-12	IL-13	IL-17	IP-10	KC	MCP-1	MIG	MIP-1a	TNF-a	VEGF
Ad5	**1×10^{11}**	**−**	140.0 +/− 79.0	0	0	130.7 +/− 174.4	2818.9 +/− 746	472.9 +/− 447.7	245.2 +/− 190.7	243.9 +/− 545.4	0	6.7 +/− 7.1
(n=5)		**+**	72.9 +/− 44.5	0	0	0	43.5 +/− 97.4	9.2 +/− 12.8	21.6 +/− 23.5	53.0 +/− 81.3	0	1.1 +/− 2.6
HVR5*7*E451Q	**1×10^{11}**	**−**	118.8 +/− 68.2	0	0	61.1 +/− 101.0	4008.2 +/− 1326.8	432.7 +/− 240.8	171.1 +/− 38.6	61.2 +/− 93.1	11.9 +/− 26.7	7.0 +/− 7.8
(n=5)		**+**	61.7 +/− 49.4	0	0	0	0	3.7 +/− 7.4	23.7 +/− 18.3	0	0	4.9 +/− 5.7

Table S1. Chemokine/cytokine analysis for Ad5, Ad5-HVR7(Ad26) or Ad5-HVR5*7*E451Q

Inflammatory profiles at 1h or 6h after administration of 1×10^{10}, 3×10^{10}, or 1×10^{11} vp of Ad5, Ad5-HVR7(Ad26), or Ad5-HVR5*7*E451Q in the presence or absence of macrophage depletion (CL −/+, respectively). Cytokines and chemokines were quantified using mouse cytokine 20-Plex Panel (Invitrogen, UK). Analysis was carried out for the following cytokines: FGF-basic, GM-CSF, IFN-γ, IL-1α, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-10, IL-12 (p40/p70), IL-13, Il-17, IP-10, KC, MCP-1, MIG, MIP-1α, TNF-α, and VEGF. Values expressed as mean +/− standard deviation.

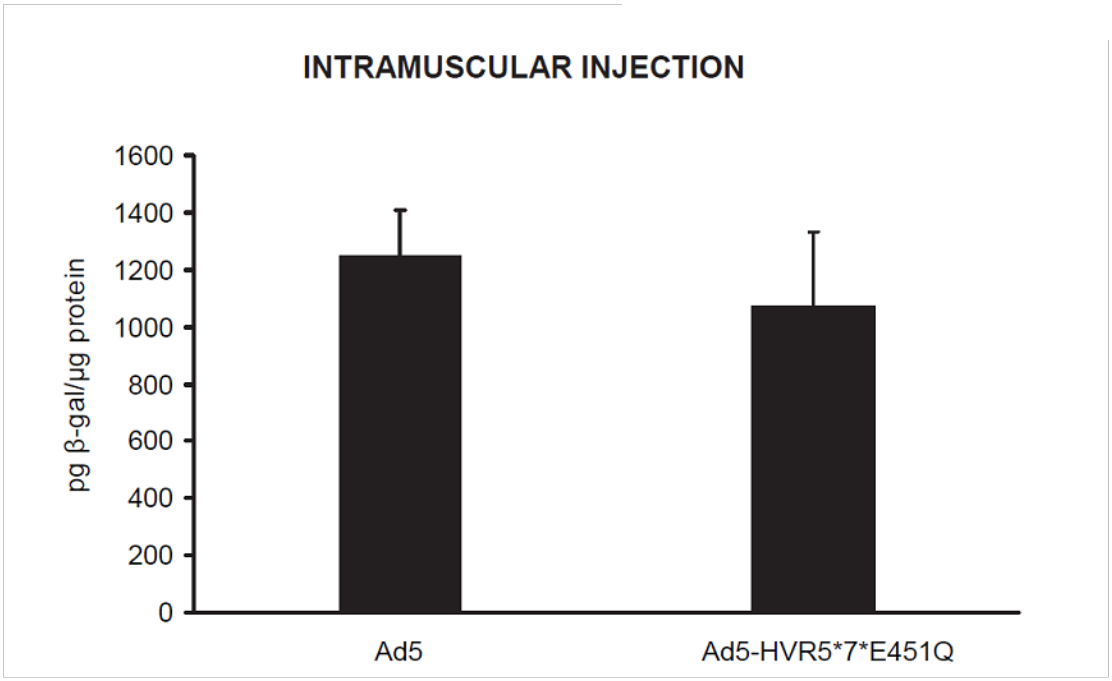

Figure S1. Intramuscular injection of Ad5 and Ad5-HVR5*7*E451Q. $1x10^{10}$ viral particles of Ad5 or Ad5-HVR5*7*E451Q were injected intramuscularly in tibialis anterior muscle of MF1 mice (n=5) and samples were harvested 48 h post-injection. Muscle was analysed using βgal ELISA kit (Roche, UK). We found no difference on transduction profiles between Ad5 and Ad5-HVR5*7*E451Q when these were administered intramuscularly. This result demonstrates Ad5-HVR5*7*E451Q is fully functional *in vivo* and the reduction observed in liver transduction is specific to the intravenous injection route.

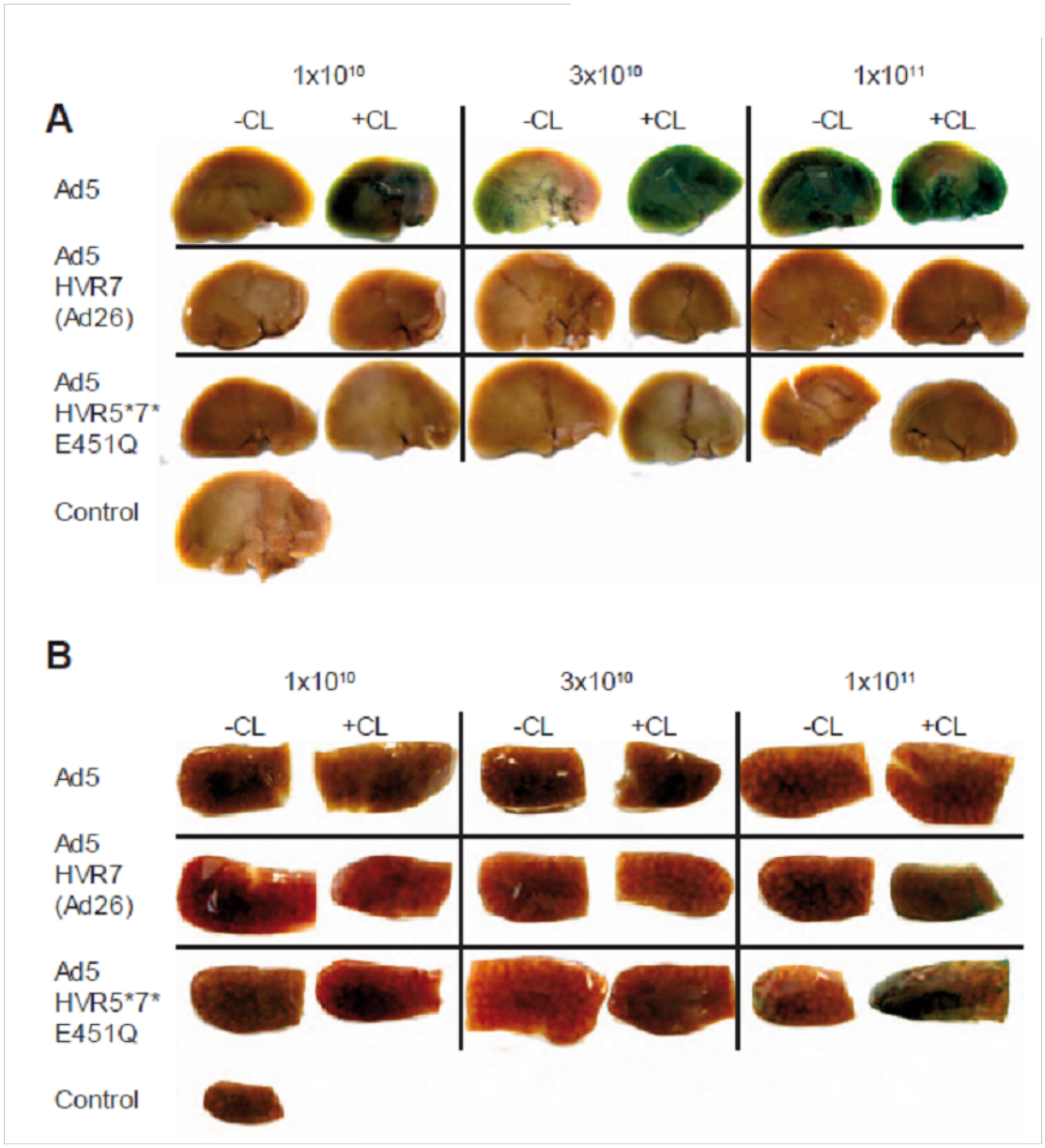

Figure S2. β-galactosidase staining of liver and spleen tissue at increasing viral doses. X-gal staining of liver lobe (A) and spleen (B) 48 h post-injection using $1x10^{10}$, $3x10^{10}$ and $1x10^{11}$ vp per mouse.

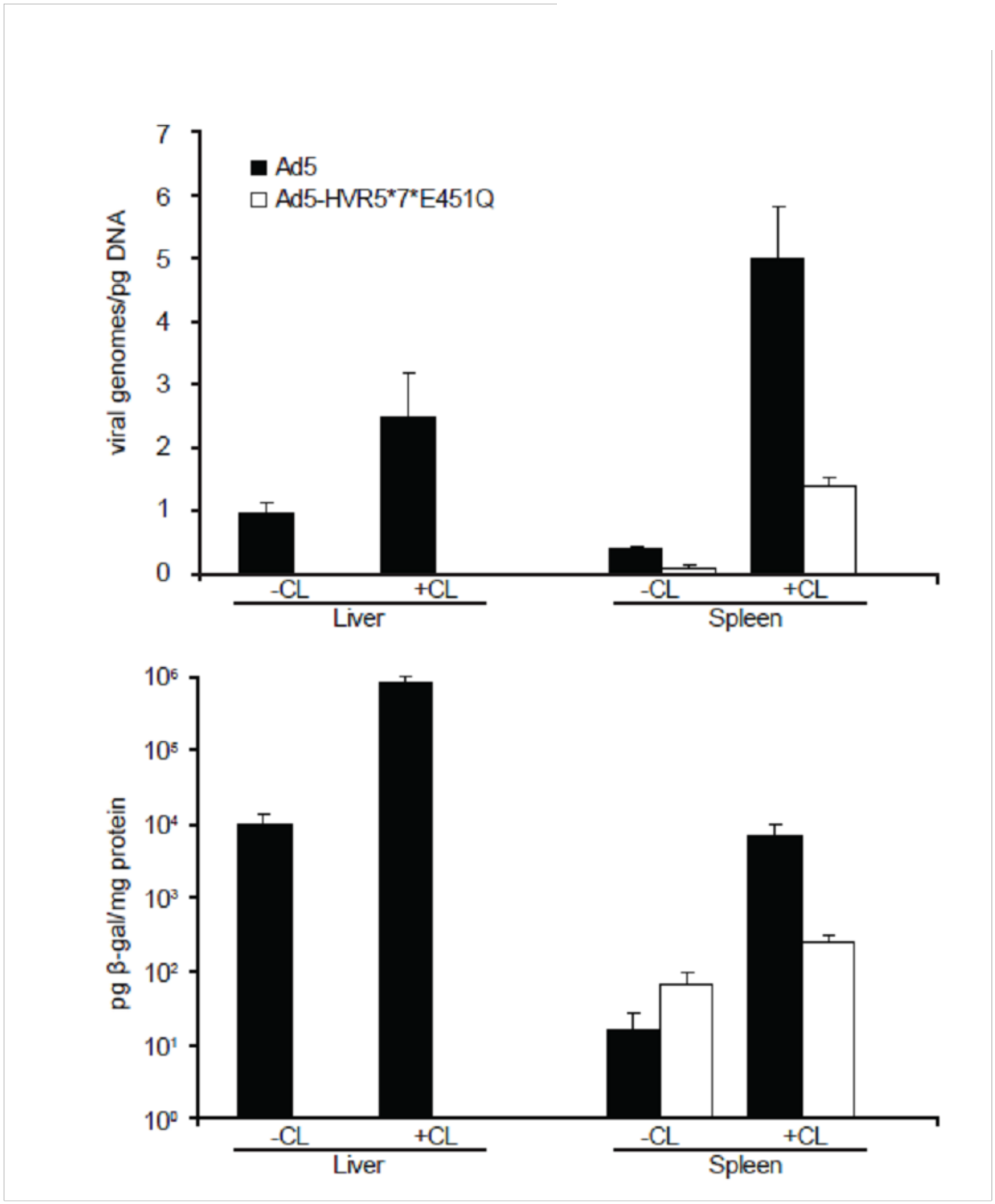

Figure S3. Viral genome content and transduction profiles in liver and spleen using Ad5 and Ad5-HVR5*7*E451Q adenoviruses in Brown Norway rats (n=5) after intravenous delivery of $4x10^{11}$ vp/kg. Ad5 and Ad5-HVR5*7*E451Q were injected intravenously in the presence or absence of clodronate liposomes (-CL, +CL). Liver and spleen tissue were harvested 48 hours post-injection and samples analysed for viral genomes by qPCR (upper panel) and transduction (lower panel) using β-galactosidase ELISA (Roche, UK). Collectively, results show FX-binding ablated Ad5 vectors preclude liver transduction and present lower levels of transduction in the spleen than Ad5 and as expected, Ad5-HVR5*7*E451Q resulted in higher levels of spleen transduction than those found in the liver.

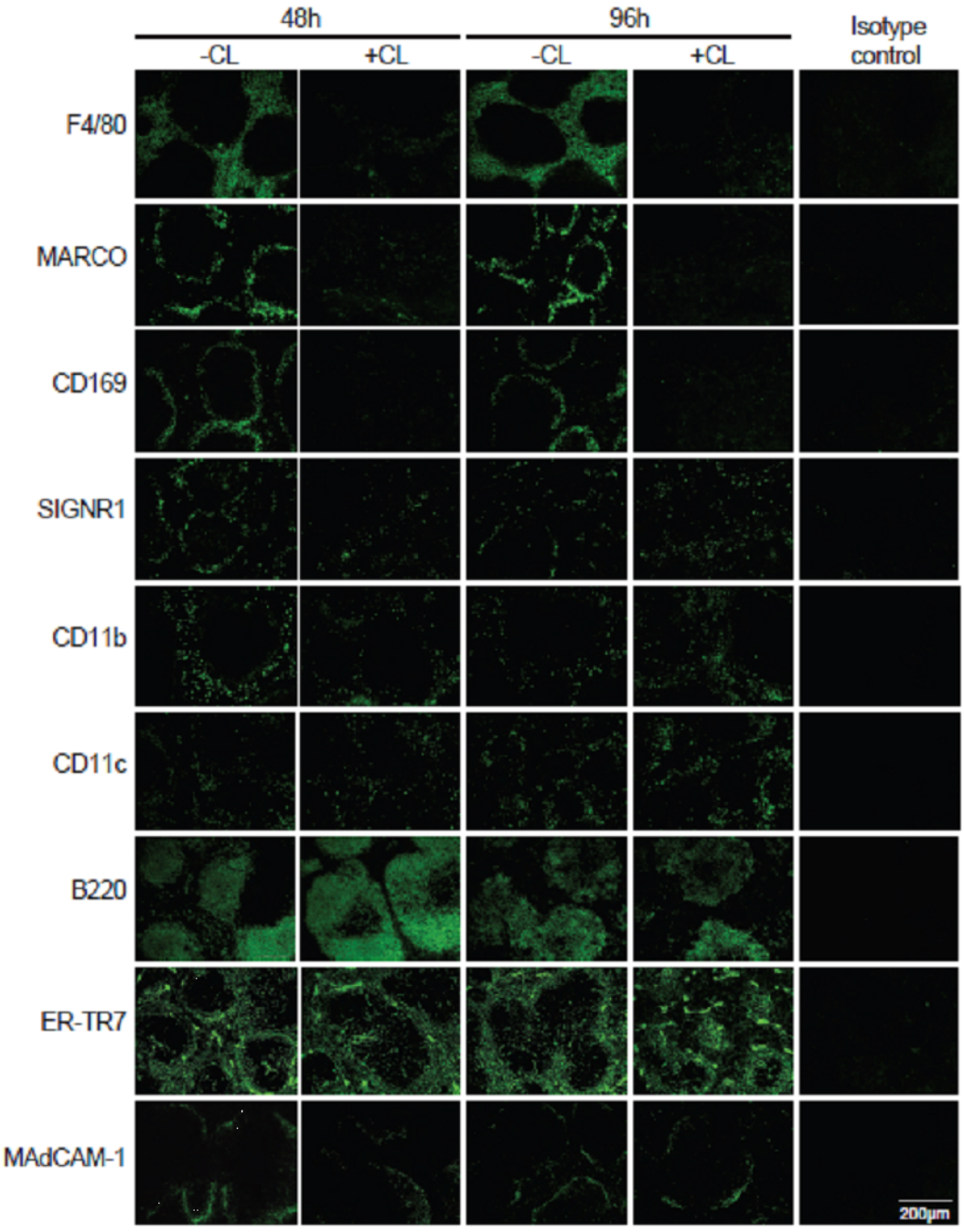

Figure S4. Selective depletion of spleen cell populations following treatment with clodronate liposomes. Spleens were removed from MF1 mice treated with PBS alone, or with clondronate-encapsulated liposomes (48h and 96h post-treatment). Frozen spleen sections (6µm) were stained for red pulp F4/80+, marginal zone MARCO+, SIGNR1+ and metallophillic marginal zone CD169+ macrophages, CD11b+ (macrophages and others), CD11c+ dendritic cell subsets and B220+ B cells. Sections were also stained for structural components of the white pulp; reticular fibroblasts (ER-TR7) and sinus lining endothelial cells (MAdCAM-1+). Isotype matched controls were included, and a representative image is shown in the panel on the far right. The 48h time-point indicates the cell types present at the time of virus injection (0h post-virus), and the 96h time-point indicates the cell types present at the time when the viral transgene expression was detected (48h post-virus) in the separate, virus-treated cohorts. CL = clodronate liposomes. Images are representative of multiple fields of view from 2-3 different animals.

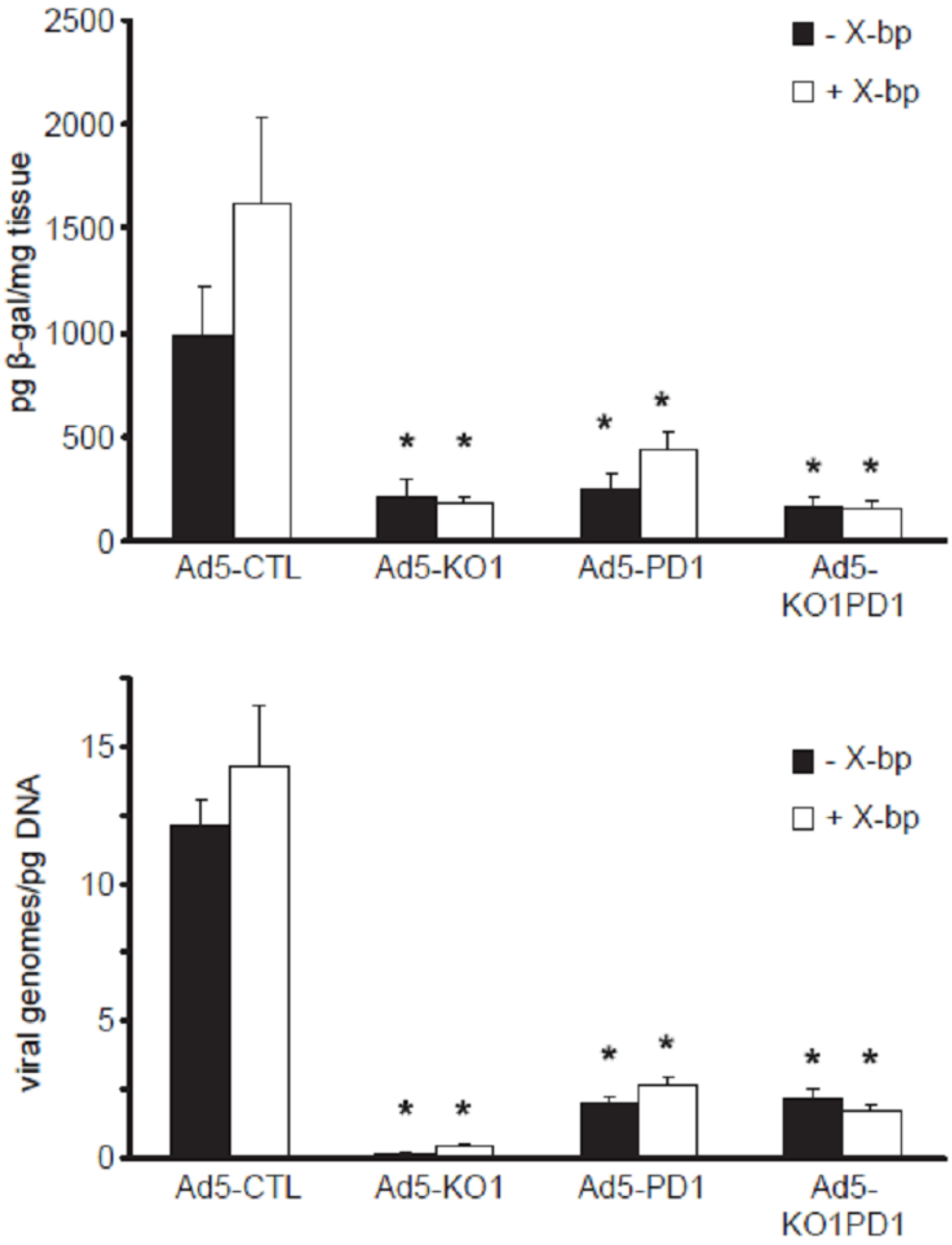

Figure S5. Transduction and viral genome content of CAR-binding ablated and integrin-binding ablated Ad5 vectors in the spleen in the presence of Xbp pre-treatment. 1x10^{11} vp of control Ad5-CTL and CAR-binding ablated (Ad5-KO1), integrin ablated (Ad5-PD1) or both CAR and integrin ablated Ad5 vectors (Ad5-KO1PD1) were injected intravenously into MF1 mice. Mice were pre-teated with clodronate liposomes 48 hous before injection of the virus and protein X binding protein (Xbp) was administered 30 minutes before the injection of the virus. Transduction data suggest CAR and integrin binding ablation significantly reduce spleen transduction although these modifications do not completely preclude the spleen transduction. (*P<0.05 versus Ad5CTL).

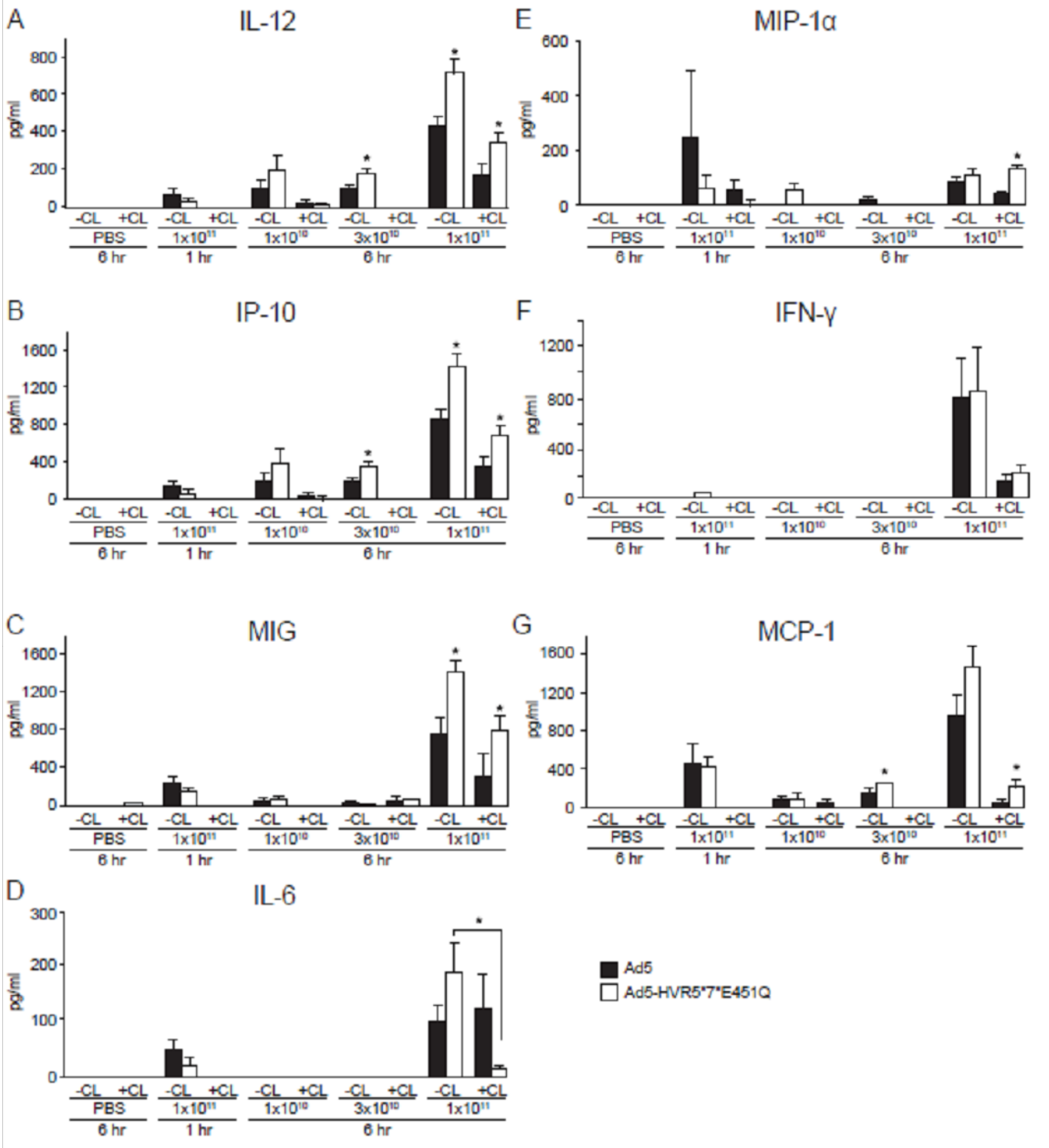

Figure S6. Analysis of chemokine/cytokine at increasing doses of control and FX-binding ablated Ad5 vectors. Inflammatory profiles of serum IL-12 (A), IP-10 (B), MIG (C), IL-6 (D), MIP-1α (E), IFN-γ (F) and MCP-1 (G) at 1 or 6 hours after administration of $1x10^{10}$, $3x10^{10}$ and $1x10^{11}$ vp of Ad5 and Ad5-HVR5*7*E451Q in the presence (-CL) or absence (-CL) of macrophage depletion. Cytokines and chemokines were quantified using a mouse cytokine 20-Plex Panel (Invitrogen, UK). (* $P<0.05$ versus Ad5).

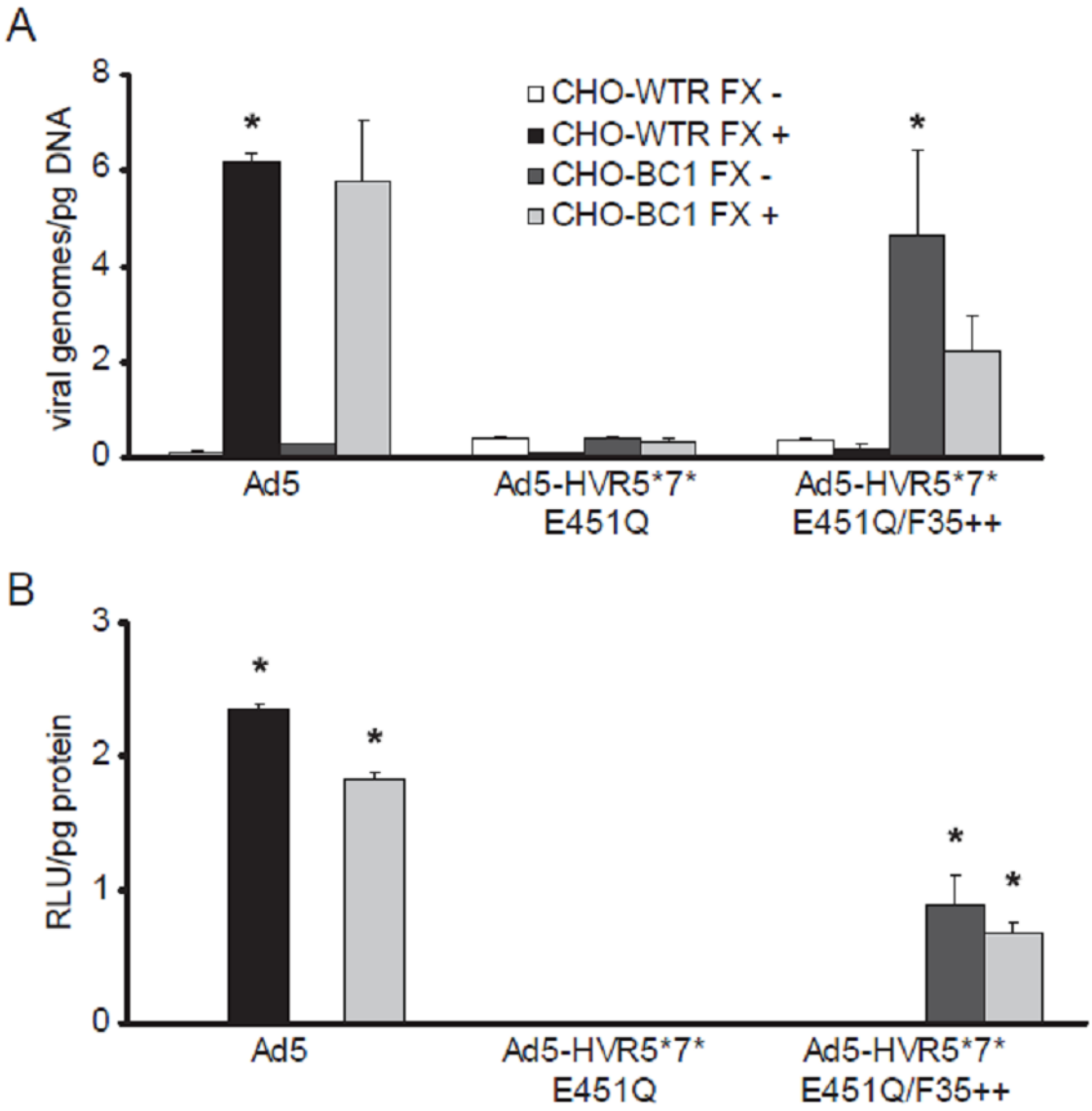

Figure S7. Cell binding experiments (A) and transduction profiles (B) in CHO-WTR and CD46 expressing CHO-BC1 cells. Cell binding and transduction experiments were performed using 1000 viral particles/cell of Ad5, Ad5-HVR5*7*E451Q or Ad5-HVR5*7*E451Q/F35++ in the presence or absence of FX (10 µg/mL). Ad5 bound and transduced both cell lines (CHO-WTR and CHO-BC1) but only in the presence of FX whilst Ad5-HVR5*7*E451Q failed to bind or transduce either cell line under any condition. Nevertheless, Ad5-HVR5*7*E451Q/F35++ efficiently bound and transduced CD46 receptor positive cells in a FX-independent manner. (* *P*<.05; FX vs non FX conditions; unpaired Student *t*-test).

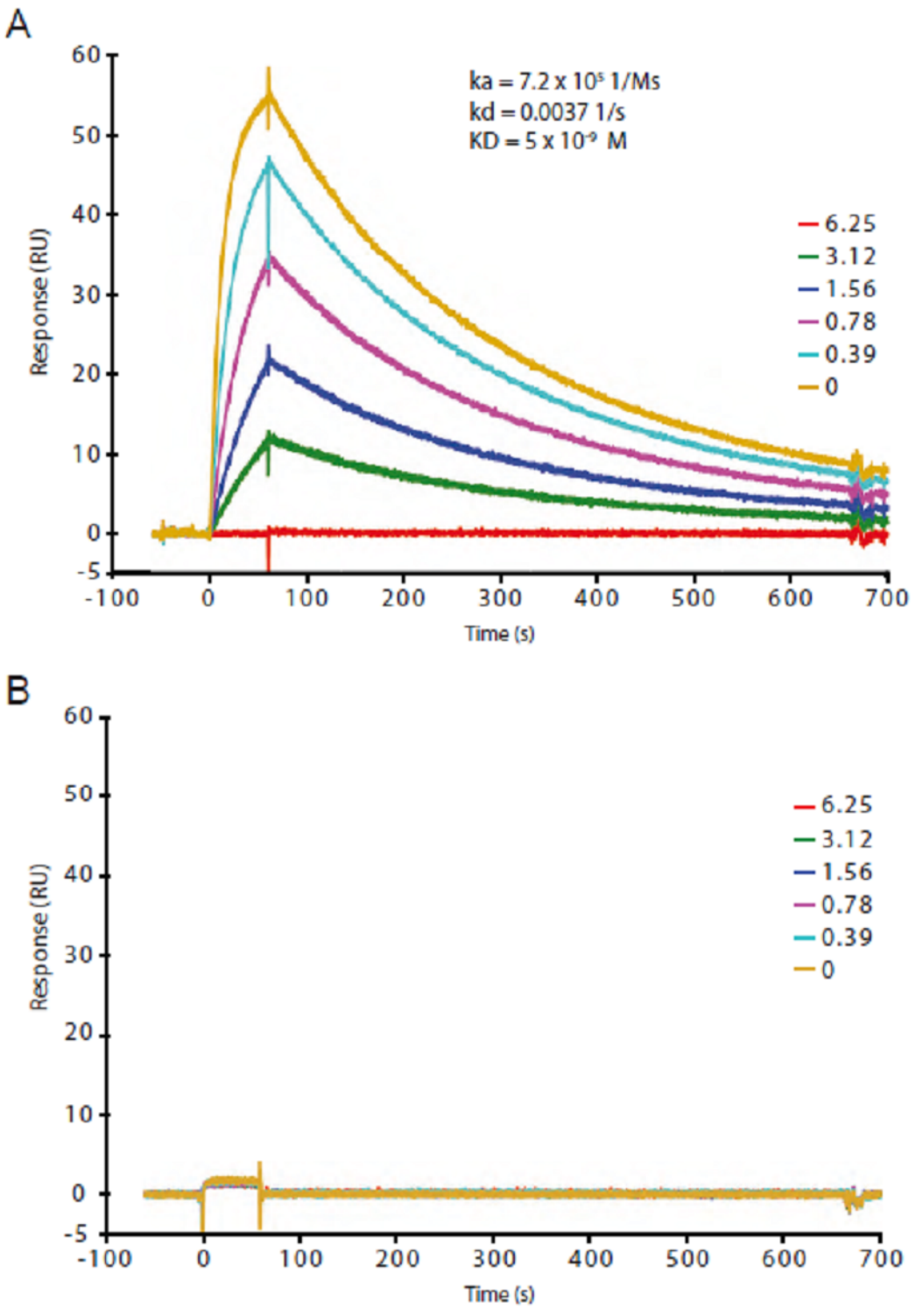

Figure S8. Representative sensorgrams of FX binding to immobilized hexon. Hexon was purified from Ad infected cells according to the method of Rux et al (1999). Ad 5 hexon (A) (592 RU) and Ad5-HVR5*7*E451Q hexon (B) (609) were covalently immobilized onto flowcells of CM5 biosensor chips by amine coupling according to the manufacturer's instructions. Factor X, 7 two-fold serial dilutions (6.25-0.39 µg/ml) analyzed in triplicate, were passed over the chip at 30 µl/min. Sensor-chips were regenerated between cycles by injection of 10 mM HEPES (pH 7.4), 150 mM NaCl, 3 mM EDTA, 0.05% Tween 20. Kinetic analysis was performed using Biacore T100 evaluation software and fitted using a 1:1 binding model. FX injections in triplicate over the two fold serial dilution range 6.25-0.39 µg/ml.