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ORIGINAL ARTICLE

An ex vivo *loop system models the toxicity and efficacy of PEGylated and unmodified adenovirus serotype 5 in whole human blood*

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Polyethylene glycol coating (PEGylation) of adenovirus serotype 5 (Ad5) has been shown to effectively reduce immunogenicity and increase circulation time of intravenously administered virus in mouse models. Herein, we monitored clot formation, complement activation, cytokine release and blood cell association upon addition of uncoated or PEGylated Ad5 to human whole blood. We used a novel blood loop model where human blood from healthy donors was mixed with virus and incubated in heparin-coated PVC tubing while rotating at 37 °C for up to 8 h. Production of the complement components C3a and C5a and the cytokines IL-8, RANTES and MCP-1 was significantly lower with 20K-PEGylated Ad5 than with uncoated Ad5. PEGylation prevented clotting and reduced Ad5 binding to blood cells in blood with low ability to neutralize Ad5. The effect was particularly pronounced in monocytes, granulocytes, B-cells and T-cells, but could also be observed in erythrocytes and platelets. In conclusion, PEGylation of Ad5 can reduce the immune response mounted in human blood, although the protective effects are rather modest in contrast to published mouse data. Our findings underline the importance of developing reliable models and we propose the use of human whole blood models in pre-clinical screening of gene therapy vectors.

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Keywords: *human blood model; PEGylated adenovirus; immune response; cytokines; complement system; cell adhesion*

Introduction

Human adenovirus serotype 5 (Ad5) is among the most commonly used viruses for vector development and for oncolytic viral therapy of cancer.[1–3] Oncolytic Ad5 viruses have been used in numerous clinical phase I and II studies in the US and China.[4–9] There are two commercialized adenoviruses for treatment of cancer in China, one adenoviral vector expressing p53 and one E1B 55K-deleted replication-competent adenovirus.[10,11] Intratumoral injections have in some cases led to successful results.[7,9,12] However, when intravenous administration is used, the viral particles are quickly sequestered by blood cells. Ad5 is a common respiratory tract virus and consequently, a high proportion of the population has immunological memory in the form of anti-Ad5 antibodies.[13] In the US, the prevalence of neutralizing antibodies against Ad5 is between 33 and 60%.[14,15] Following the first dose of systemic adenoviral administration, 100% of patients test positive for anti-adenovirus antibodies and this is likely to be the case whichever serotype that is used.[16] The binding of antibodies together with activation of components of the innate immune system results in loss of viral infectivity after intravenous administration.[17]

In mice it has been shown that adenoviruses induce a number of inflammatory cytokines, including TNF, IL-1β, IL-6, IL-12, IP-10 and MCP-1.[18] Furthermore, adenoviruses activate the complement system through the classical and alternative pathways.[19] The complement system consists of about 20 plasma proteins that are important in the host defense against pathogens. The complement component C3 has a central role in the activation of all three pathways (classical, alternative and lectin) of the complement system. C4 is homologous to C3 and a part in the classical and lectin pathways, whereas C5 is involved in initiation of the late steps of complement activation. C3a, C4a and C5a are small fragments that result from cleavage of C3, C4 and C5 that can be used for quantification of complement activation. Kiang *et al.*[20] showed in a mouse study that a functional complement system is significant for enhancing several inflammatory responses against adenovirus. The interaction of adenovirus with the complement system can therefore have deleterious consequences both for the activity of the virus and the health of the host.

One way to prevent the destruction of adenovirus may be to coat them with a polymer such as polyethylene glycol (PEG). PEG is an uncharged, hydrophilic,

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non-immunogenic molecule with low toxicity.[21] PEG modification (PEGylation) is a well-established technique to protect therapeutic peptides and proteins that need prolonged circulation time in the bloodstream. The first successful attempt to modify the capsid surface of adenoviral gene transfer vectors by covalently attached PEG molecules were performed by O'Riordan *et al*.[22] and Croyle *et al*.[22] The binding of PEGylated adenovirus to human and rat erythrocytes and to human and mouse platelets is reduced *in vitro* compared with uncoated virus[23] and PEGylation of adenovirus leads to prolonged transgene expression in mice.[24,25] Furthermore, there is less uptake in mouse Kupffer cells when PEGylated viruses are used.[26] On the basis of previous *in vitro* experiments it is believed that high molecular weight PEG molecules achieve more effective detargeting of Ad5 than low molecular weight PEG molecules.[27,28] In mice it has recently been shown that viruses coated with 20K-PEG molecules undergo less uptake in the liver compared with viruses coated with PEGs of lower molecular weight.[28,29] Wisse *et al*.[30] propose that the physical size of the particle might be the determinant for infection. Adenoviruses with high molecular weight PEGs that are larger than the threshold of the liver sinusoidal fenestrae would be detargeted from the liver because of their size, not necessarily that the PEGylation is more efficient. In the presence of blood, adenoviral transduction is increased both *in vitro* and in mice as a result of blood factor-mediated infection. Recently it was shown that factor (F) IX and FX enhance transduction also of PEGylated adenoviruses.[29] This suggests that although PEGylation may decrease infectivity, the protection is sub-optimal.

Most data on PEGylation of adenovirus and immune responses are from mouse studies. In a recent *in vitro* study it was shown that human erythrocytes bind and inactivate Ad5 by the coxsackie adenovirus receptor (CAR) and the complement receptor 1 (CR1).[31] In contrast to human erythrocytes, mouse erythrocytes do not express CAR and CR1, which are important receptors for uptake of adenovirus in blood.[31] Therefore, it is important to use more appropriate models when testing adenoviruses for systemic delivery. *In vivo* models that have been used before are transgenic mice that express CAR or CR1 or immunodeficient NOD-SCID mice injected with human erythrocytes.[31–33] In these models, the circulation time increased and hepatocytic infection decreased because of erythrocyte binding and are in that way more similar to the human situation. Although these models provide a step forward, the transgenic mice have either CAR or CR1 but not both receptors. Furthermore, NOD-SCID mice have impaired T and B cell function and they lack the ability to stimulate complement activation, which is important for clearance of virus. Hence, there is still a crucial requirement for additional systems to model the interactions of Ad5 following systemic administration. In this paper, we developed and evaluated a novel *ex vivo* blood loop model where blood from healthy human donors was mixed with virus and added to heparin-coated PVC tubing and incubated at 37 °C while rotating for up to 8 h. The blood loop model was used to study cytokine release, complement activation and blood cell adherence after addition of uncoated Ad5 or Ad5 coated with either 2K- or 20K-PEG.

Results

A human blood loop model was set up to more accurately model the immediate interactions with, and immune responses to, systemically delivered unmodified or PEGylated Ad5 vectors. Blood was collected and incubated with virus in heparin-coated PVC tubing at 37 °C while rotating. The loops with content and abbreviations are listed in Table 1.

Adenovirus-induced clot formation is linked to anti-adenovirus IgG titer and is reduced by PEGylation

First, an experiment with venous blood from six healthy individuals with five loops each containing Ad5, Ad5 + compstatin, 2K-PEGylated Ad5, 20K-PEGylated Ad5 or HEPES (negative control) was set up to study coagulation. This was achieved by counting the platelets at different time points during incubation. Formation of macroscopic blood clots was investigated by eye at the end of the experiment. The anti-adenovirus IgG titer was measured by ELISA as it was speculated that this would have an impact on the immune responses. Three donors who had anti-adenovirus IgG titer around 50 U ml^{-1} or below were defined as low IgG titer donors and three donors who had IgG titers higher than 100 U ml^{-1} were defined as high IgG titer donors.

In low IgG titer blood, clot formation was observed in the blood loops containing uncoated Ad5. Indeed, between 2 and 4 h after exposure the number of platelets decreased from above 80% to below 20% of the starting level (Figure 1a). Clotting and platelet decrease was not observed for Ad that had been modified with either 2K- or 20K-PEG. Interestingly the addition of the C3 inhibitor compstatin completely inhibited the Ad-induced clotting, indicating the involvement of the complement cascade. In high IgG titer blood, clotting was induced regardless of PEGylation or compstatin addition, with levels of platelets falling from 80% at 2 h to less than 10% at 4 h (Figure 1b).

Virus clearance ability varies between donors and can be divided into low and high ability to neutralize Ad5

The amount of infectious Ad5 particles remaining in blood after incubation was analyzed in a titration assay,

Table 1 Blood loops used in the experimental set up

Loop	*Content*	*Abbreviation*
1	Ad5 (1.2×10^{10} vp) in HEPES	Ad5
2	2K-PEGylated Ad5 (1.2×10^{10} vp) in HEPES	2K-PEG Ad5
3	20K-PEGylated Ad5 (1.2×10^{10} vp) in HEPES	20K-PEG Ad5
4	HEPES (50 mM, pH 8.0)	HEPES
5	2K-PEG in HEPES	2K-PEG
6	20K-PEG in HEPES	20K-PEG
7	Ad5 (1.2×10^{10} vp)+compstatin (20 μM)	Ad5+comp
8	2K-PEGylated Ad5 (1.2×10^{10} vp)+compstatin (20 μM)	2K-PEG Ad5+comp
9	20K-PEGylated Ad5 (1.2×10^{10} vp)+compstatin (20 μM)	20K-PEG Ad5+comp

The PEGylated and uncoated Ad5 are from the same batch and the HEPES, 2K- and 20K-PEG in the control loops (4–6) are from the same batches as in the experimental loops (1–3, 7–9).

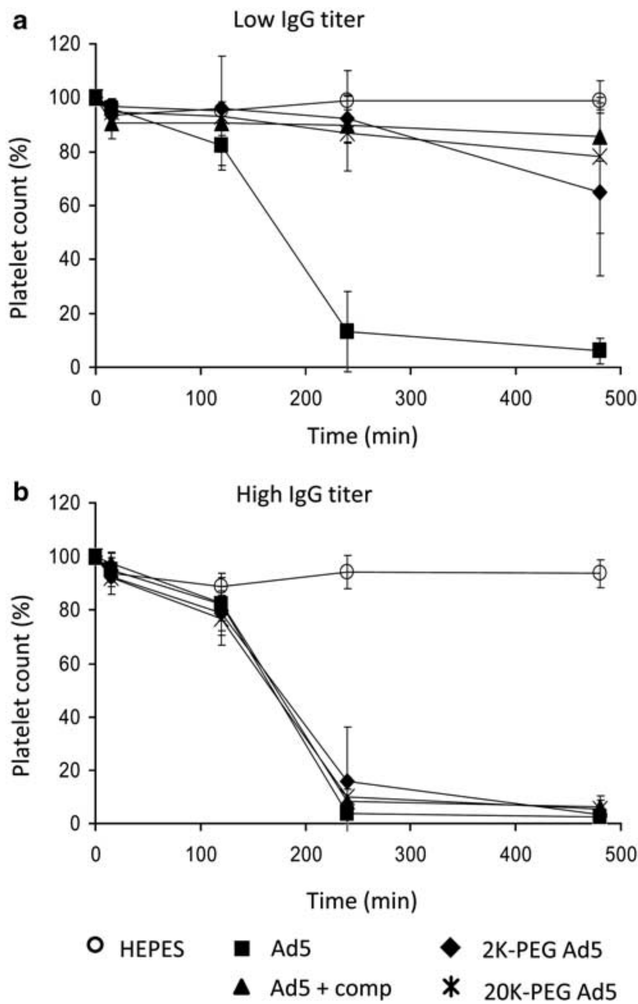

Figure 1 Platelet count in human blood exposed to Ad5 viruses. Virus was incubated in blood from three donors with low anti-Ad titers and three donors with high anti-Ad titers using the blood loop system. Blood samples were extracted at different time points when platelets were counted and the blood was inspected for clots. (**a**) In low IgG titer blood, clot formation only occurred in the loop with uncoated Ad5. (**b**) In high IgG titer blood, clot formation occurred in all virus-containing loops, regardless of PEGylation status or addition of compstatin.

as a measurement of the donors' ability to neutralize Ad5. This was done by collecting whole blood samples from the loop with Ad5 after 15 min and 6 h and directly adding them to 911 cells. As the viruses used in this study express EGFP, transduced 911 cells could be analyzed by flow cytometry the following day. The percentages of remaining virus activity at 15 min and 6 h were expressed in relation with Ad5 that had not been exposed to blood (set to 100%). Blood from 13 healthy volunteers was analyzed. Donors G, B, D and A had low ability to neutralize Ad5, as illustrated by the presence of infectious virus after 6 h of incubation in blood (Figure 2). In blood from donors L, O, N, M, S, R, F, E and I, the virus was cleared after 6 h but in most cases clearance was completed already after 15 min. Based on their ability to neutralize Ad5, the donors were divided into two groups, Low and High. The donors' anti-adenovirus IgG titers (as measured by ELISA, that is, both neutralizing and non-neutralizing anti-Ad5

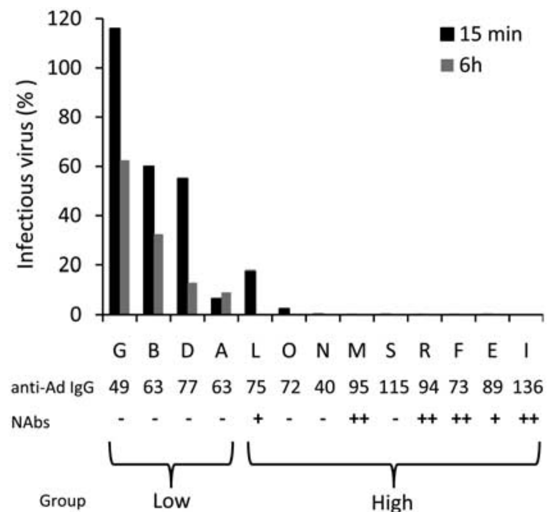

Figure 2 Ability of human blood to neutralize Ad5. Virus was added to freshly drawn human blood and incubated in the blood loop system. Aliquots were then extracted after 15 min and 6 h of incubation and the viral capability to infect 911 cells was evaluated. Infectivity was measured by detection of transgene (EGFP) expression by flow cytometry. The titer of Ad5 not exposed to blood was defined as 100% infectivity. Based on the infectivity assay, the donors were divided into two groups: Low (low ability to neutralize Ad5), that is, where infectious virus could still be detected after 6 h exposure to blood, and High (high ability to neutralize Ad5), that is, where infectious virus could not be detected after 6 h exposure to blood. The anti-adenovirus IgG titers (U ml^{-1}), determined by ELISA, are also given. The presence of NAbs was determined in untreated plasma samples using an EGFP reporter gene-based assay. The donors were scored as follows; high level of Nabs (++) with more than 50% inhibition at plasma dilution 1:500; low level of Nabs (+) with more than 50% inhibition at 1:10 to 1:50 dilution; and no detectable Nabs (−) with less than 50% inhibition at 1:10 dilution.

antibodies) are also listed in Figure 2. In group Low, the donors had rather low anti-Ad IgG titers. However, some donors in group High had also low amounts of anti-Ad IgG antibodies but were still able to neutralize Ad5 rapidly. Consequently, there was a tendency but no direct correlation between the total amount of anti-Ad IgG and the neutralizing capacity of the donor blood. The presence of neutralizing antibodies (Nabs) determined in plasma samples are presented in Figure 2. The donors were scored as follows; high level of NAbs (++) with more than 50% inhibition at plasma dilution 1:500; low level of NAbs (+) with more than 50% inhibition at 1:10 to 1:50 dilution; and no detectable NAbs (−) with less than 50% inhibition at 1:10 dilution. The four donors with low neutralizing ability (G, B, D and A) had also low levels of NAbs. However, three out of the nine donors with high neutralizing ability in whole blood did not have detectable levels of NAbs.

20K-PEGylation of Ad5 partially prevents complement activation and cytokine release

We next measured the activation of the complement factors C3a, C4a and C5a in plasma from the same 13 donors by flow cytometry using cytometric bead array. Human blood exposed to Ad5 showed a rapid release of C3a with values ranging from 24-373 ng ml^{-1} after 15 min. The C3a production in loops with

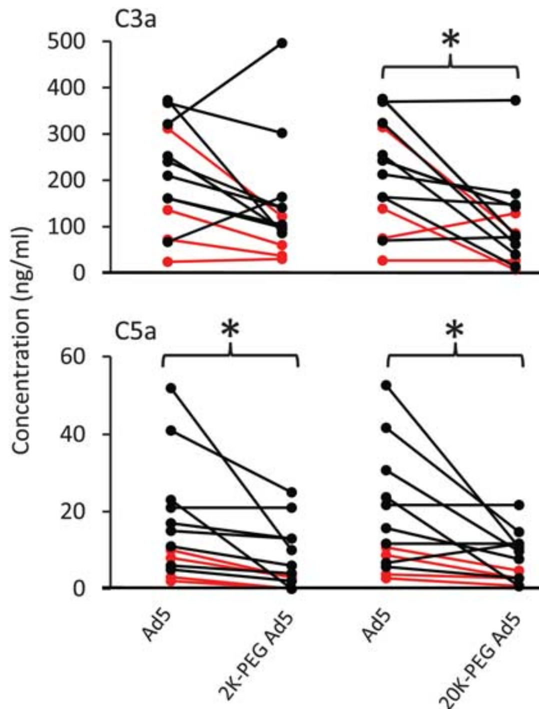

Figure 3 Complement activation in human blood exposed to Ad5 viruses. Blood and virus were incubated in the loop system. Plasma samples were analyzed for complement activation using cytometric bead array. Donors in group Low (low ability to neutralize Ad5) are represented by red dots and lines, whereas donors in group High (high ability to neutralize Ad5) are represented by black dots and lines. The upper panel shows C3a levels after 15 min of incubation. 20K-PEGylation resulted in a significantly (*) lower level of C3a release compared with uncoated Ad5 ($P=0.012$). The same tendency was observed for 2K-PEGylated Ad5 although no significant difference in C3a production was observed between 2K-PEGylated Ad5 compared to uncoated Ad5. In the lower panel, the C5a levels after 1 h of incubation is shown. Both 2K-PEGylation and 20K-PEGylation of Ad5 resulted in significantly (*) lower levels of C5a compared with uncoated Ad5 ($P=0.0005$ and $P=0.0068$, respectively).

20K-PEGylated Ad5 was lower ($P=0.0122$) compared with uncoated Ad5, when looking at individual donors (Figure 3). 2K-PEGylation of Ad5 did not result in significantly lower C3a levels compared with uncoated Ad5, although 11 out of 13 donors, including all donors in group Low (red dots and lines), had lower or same levels of C3a. Both 2K-PEGylated Ad5 and 20K-PEGylated Ad5 resulted in significantly less C5a production compared with uncoated Ad5 ($P=0.0005$ and $P=0.0068$, respectively) (Figure 3). The levels of C5a were generally lower in group Low (red dots and lines) than in group High (black dots and lines). The production of C3a and C5a was completely blocked by the C3 inhibitor compstatin. In loops containing compstatin, the C3a levels ranged from 0 to 14 ng/ml but in most cases the levels were undetectable. C5a levels ranged from 0 to 6 ng/ml when compstatin was used but also for this complement factor the levels were generally not measurable. No differences in C4a production were observed between uncoated and PEGylated viruses (data not shown).

Production of cytokines involved in inflammation was also measured in plasma by flow cytometry using cytometric bead array. The data were evaluated

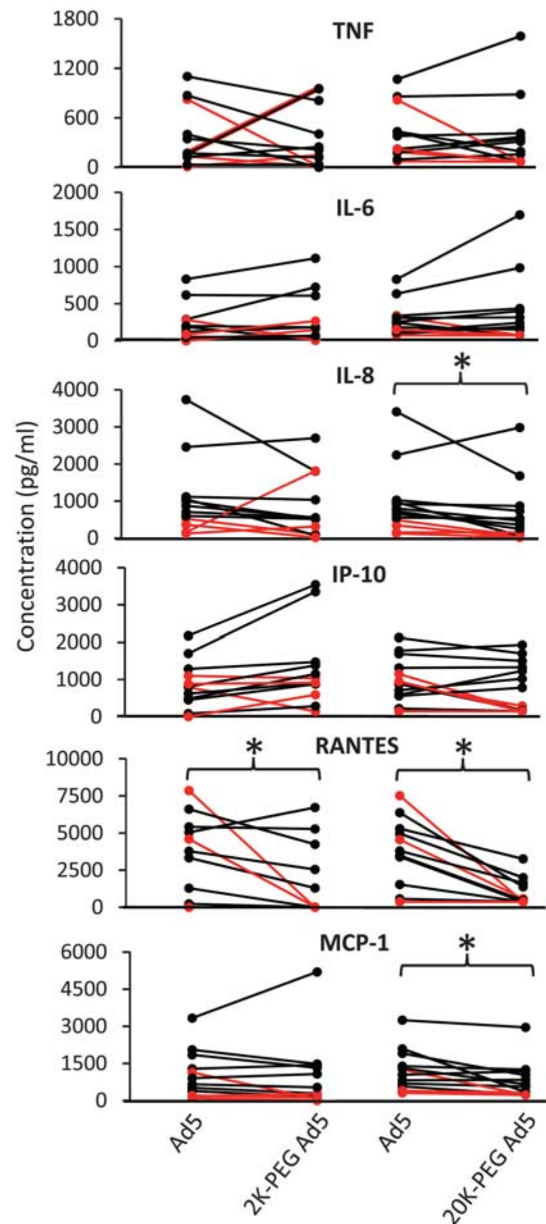

Figure 4 Cytokine levels in human blood exposed to Ad5 viruses. Blood and virus were incubated in the loop system for 6 h. Plasma samples were analyzed for cytokine release using cytometric bead array. Donors in group Low are represented by red dots and lines while donors in group High are represented by black dots and lines. 20K-PEGylation of Ad5 resulted in a significantly (*) lower release of half of the cytokines studied compared with uncoated Ad5. However, the production of cytokines was less for all donors in group Low (red dots and lines) when 20K-PEGylation of Ad5 was compared with uncoated virus.

as decrease in cytokine levels for individual donors (Figure 4). PEGylation reduced the levels of RANTES: 2K-PEGylated Ad5 versus Ad5 ($P=0.039$) and 20K-PEGylated Ad5 versus Ad5 ($P=0.0020$). Furthermore, 20K-PEGylation resulted in decreased levels of IL-8 ($P=0.017$) and MCP-1 ($P=0.0046$). The donors in the group with low ability to neutralize adenovirus (red dots and lines) appeared to have lower levels of all cytokines when 20K-PEGylated Ad5 was compared with uncoated Ad5. No differences in the release of IL-1β, IL-10,

IL-12p70 and MIG were observed between the loops with PEGylated Ad5, uncoated Ad5 or HEPES (data not shown). In summary, there was substantial variability in the inflammatory cytokine response to Ad5 among the donors. However, a trend towards lower cytokine release with 20K-PEGylated Ad5 especially in donors with a lower neutralizing capacity was apparent and for the cytokines RANTES, MCP-1 and IL-8 this difference proved to be significant.

PEGylation of Ad5 prevents binding to blood cells

We next wanted to investigate what proportion of viral particles that is associated with blood cells and what proportion remains in the plasma. After 15 min of incubation, blood was taken from the loops and viral DNA content in the plasma and cell fractions were evaluated by quantitative PCR. PEGylated Ad5 associated less with blood cells than uncoated Ad5, $P = 0.0017$ for 2K-PEGylated Ad5 versus Ad5 and $P = 0.0002$ for 20K-PEGylated Ad5 versus Ad5 (Figure 5a). In 9 out of 13 donors, including all donors in group Low (red dots and lines), more 20K-PEGylated Ad5 was associated with the plasma compared with the cell fraction (values below 50%). Addition of compstatin clearly reduced the cell-associated fraction of uncoated Ad5 ($P = 0.0007$) (Figure 5b). Compstatin together with 20K-PEGylated Ad5 led to further reduction in cell association ($P = 0.002$). Compstatin did not, on the other hand, reduce 2K-PEGylated Ad5 binding (data not shown). Therefore, the best combination to prevent virus from binding to cells appeared to be 20K-PEGylated Ad5 together with compstatin.

To study what cell types the virus is associated with, blood was incubated with either Ad5 or 20K-PEGylated Ad5 for 15 min in heparinized tubing. Blood cells were stained with antibodies and sorted by flow cytometry. Viral DNA from the cell fractions was purified and analyzed by quantitative PCR. Blood from four donors each from group Low and High was evaluated. The relative copy numbers per cell population and ml for Ad5 and 20K-PEGylated Ad5 are displayed in Figure 6. For the donors with low ability to neutralize Ad5 (A, B, D and G, left panel in Figure 6), 20K-PEGylation resulted in reduced binding to all cells analyzed compared with Ad5, with the exception of erythrocytes (CD235) and platelets (CD41) for donor D and B-cells (CD19) for donor G. In blood from donors with high ability to neutralize Ad5 (donors E, I, O and S), 20K-PEGylated Ad5 resulted in less binding to granulocytes (CD15) for all four donors and to T-cells (CD3) for three out of four donors. Equal or higher amounts of 20K-PEGylated Ad5 compared with uncoated Ad5 were bound to the other cell types with the exception of donor O, where all cells analyzed bound more uncoated Ad5 than 20K-PEGylated Ad5.

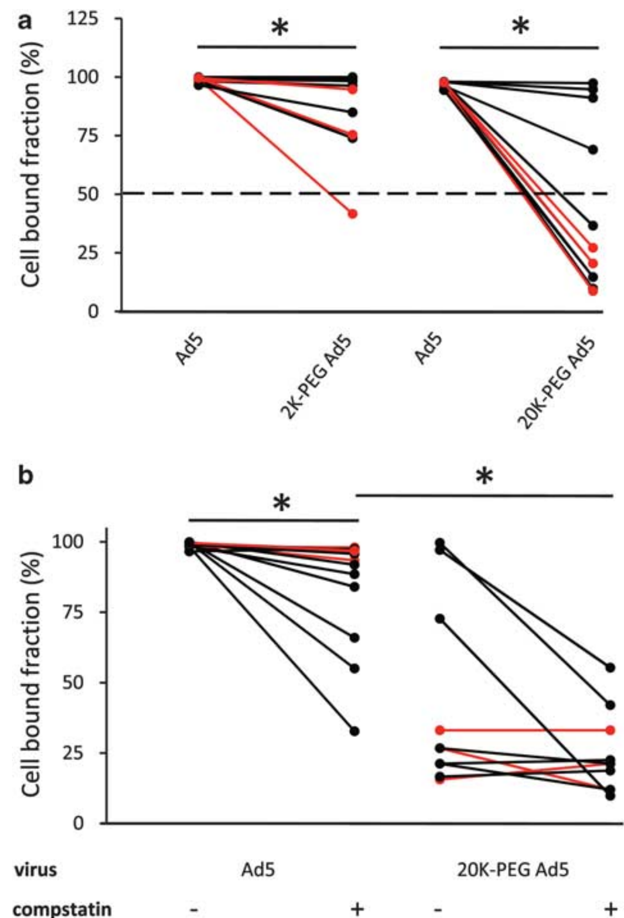

Figure 5 Association of Ad5 with human plasma and cell fractions. Whole blood was incubated with Ad5 viruses in the blood loop system for 15 min. Thereafter the plasma and cell fractions were separated by centrifugation and viral DNA was extracted from the two fractions. Ad5 DNA content in plasma and cell fractions was then measured by quantitative PCR. The percentage of cell bound fraction is displayed. 50% means equal amounts of virus in the plasma and cell fraction. (**a**) PEGylation of Ad5 with either 2K-PEG or 20K-PEG resulted in significantly (*) less cell binding ($P = 0.0017$ and $P = 0.0002$, respectively) compared with uncoated Ad5. (**b**) The effects of compstatin together with PEGylated and non-PEGylated virus were analyzed. Cell association was significantly (*) lower when Ad5 was administered together with compstatin ($P = 0.0007$). When 20K-PEGylated Ad5 was used together with compstatin the cell association was further decreased ($P = 0.002$).

Discussion

A high proportion of the human population has been infected with adenovirus serotype 5 (Ad5) and has consequently preformed antibodies against the virus. This causes a potential drawback because Ad5-based vectors are rapidly cleared upon systemic administration if the subject has anti-Ad5 antibodies. To overcome the antibody barrier, different strategies have been employed such as co-administration of immunosuppressive agents together with adenovirus or coating of virus with non-immunogenic molecules.[24] One popular approach has been to coat the virus with PEG to shield epitopes recognized by anti-Ad5 antibodies.

Many animal studies have been performed showing that PEGylation protects the virus from neutralizing antibodies.[24,26,34] Less is known about the protective effects of Ad5 PEGylation in human blood. However, Hofherr *et al.* have illustrated that 5K-PEGylated Ad5 reduces binding to human platelets and erythrocytes *in vitro*.[23] They also studied the formation of ultra large von Willebrand factor-platelet strings on endothelial cells using a flow chamber with defined flow and shear stress.[23,35] Although information can be obtained regarding cell association of Ad5 using purified platelets and

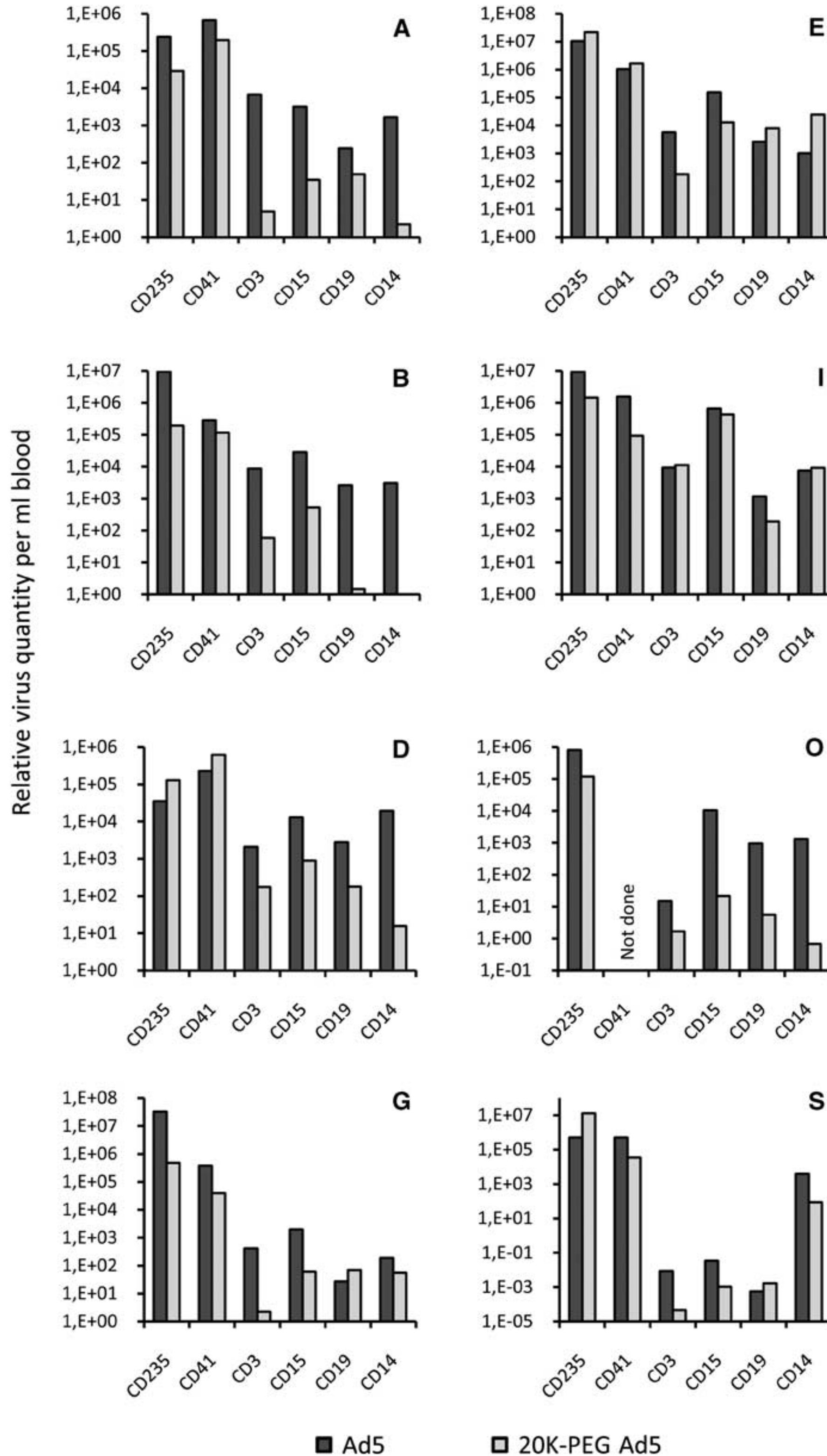

Figure 6 Association of Ad5 with various human blood cells. Ad5 and 20K-PEGylated Ad5 were added to blood and incubated for 15 min using the blood loops. Blood cells were stained with antibodies targeting erythrocytes (CD235ab), platelets (CD41), T-cells (CD3), granulocytes (CD15), B-cells (CD19) and monocytes (CD14) and then sorted by flow cytometry. Viral DNA content in the different cell fractions was measured by quantitative PCR. Relative virus quantity per cell population and ml is displayed. For donors A, B, D and G (left panel) with low ability to neutralize Ad5, 20K-PEGylation of Ad5 resulted in reduced binding to almost all cell types analyzed compared with Ad5. For donors E, I, O and S (right panel) with high ability to neutralize Ad5, 20K-PEGylation resulted in reduced binding to some cell types.

erythrocytes, other components present in whole blood such as antibodies and complement factors, which have an important role in binding, were not considered in those publications. Infectivity and immune responses that are partly dependent on antibodies and other plasma proteins cannot be studied in systems where one cell type at a time is present.

Complement activation caused by adenovirus differs between *in vitro* and *in vivo* systems. In a recent study, it was shown that antibodies are needed for activation of the classical pathway *in vitro* in contrast to mice where the major mechanism for complement activation was antibody-independent.[36] Instead of direct recognition of viral particles it was suggested that cell damage induced by adenovirus is the main complement activator in the *in vivo* situation. In the same study it was shown that PEGylation was able to reduce complement activation in mice.[36] The group of Amalfitano has shown that a functional C3 is needed for adenoviral-induced activation of endothelial cell markers (E-selectin, VCAM and ICAM) and release of several cytokines and chemokines.[37] Thrombocytopenia is a well-known side effect of adenoviral therapy and has been demonstrated to be dependent on C3 and Factor B in mice.[37] Complement receptors 1 and 2 are involved in the induction of neutralizing antibodies to adenovirus by activation of B cells.[38] The complement system is hence a major challenge for adenoviral therapy as it is involved in the regulation of both innate and adaptive responses against the virus.

In human blood, more than 90% of administered Ad5 is associated with blood cells whereas in mice, less than 1% of the virus is cell-associated.[17] This is probably a consequence of the fact that mouse erythrocytes lack CAR and CR1 whereas human erythrocytes express both receptors.[31] The infectivity of virus in human blood is dramatically decreased with time while mouse blood did not inhibit the infectivity of Ad5 in cell cultures.[17] Furthermore, it is known that complement activation differs between species.[39] Therefore, the immune response against, and the clearance of, uncoated and PEGylated Ad5 in human blood could be quite different to the immune response in mice, even if the mice have been immunized to have preformed anti-Ad5 antibodies or if immunodeficient mice are injected with human erythrocytes.

In the present paper, we wanted to study immune responses, infectivity and cell association in a system with all components of human blood. This was achieved by incubating whole blood from healthy donors together with virus in heparinized PVC tubing. Our data show rather small protective effects of adenovirus PEGylation with regard to clot formation, cytokine release, complement activation and cell adhesion in human blood. This is in contrast to what has been observed in mice where PEGylation had considerable capacity to reduce immune responses and to enhance transgene expression.[24–26,28] We observed that the donors' ability to neutralize Ad5 had a considerable impact on the immune response both for unmodified and PEGylated Ad5. Interestingly, the presence of NAbs in plasma did not directly correlate with the neutralizing ability in whole blood, indicating that other mechanisms may be involved as well or that non-neutralizing antibodies, as determined in plasma samples, may be neutralizing in whole blood. This suggests that a neutralizing assay with whole blood should be performed to more accurately determine the neutralizing ability of patients' blood. The cytokine and complement levels were generally higher in blood from donors with high ability to neutralize Ad5 when virus was added to the blood and PEGylation reduced blood cell adherence to a larger degree in blood with low ability to neutralize Ad5. Furthermore, clot formation occurred both against unmodified and PEGylated Ad5 when the IgG titer against adenovirus was high. Generally, 20K-PEGylation yielded better results than 2K-PEGylation. This is in agreement with previous observations, that high molecular weight PEG molecules have better ability to protect adenovirus in the circulation than low molecular weight PEG molecules.[28] It should be noted that complement activation and Ad5 binding to blood cells lead to rapid phagocytic clearance in the liver and spleen of mice.[23,40] Furthermore, splenectomy experiments have revealed that this organ is the major source for inflammatory cytokines.[23] Although our system accounts only for blood cell-released cytokines, it is an important step forward in the development of relevant human model systems.

In parallel with the PEGylated viruses, the C3 inhibitor compstatin was co-administered and evaluated in the same manner as the other samples. Compstatin is a complement inhibitor that prevents the cleavage of C3 into C3a and C3b.[41,42] As activation of C3 is essential in complement activation, compstatin could potentially decrease binding of virus to erythrocytes via CR1 and thereby decrease neutralization of adenovirus. We observed that C3a and C5a production was completely inhibited by compstatin in all donors regardless if uncoated or PEGylated Ad5 vectors were used. Compstatin was also able to reduce the cell-associated fraction of PEGylated Ad5 in blood with high ability to neutralize the virus. This may leave more viral particles free in the plasma fraction thereby increasing accumulation at tumor sites following intravenous delivery. It could therefore be used in combination with gene therapy to reduce the immune response against viral vectors. It is important to reduce the C3a and C5a levels to minimize adverse effects since these molecules are potent anaphylatoxins.[19,43] Opsonization of the virus by C3b will also be reduced if the C3 convertase is inhibited.

In conclusion, PEGylation of Ad5 did not convincingly reduce immune responses in the human blood loop model used here, as opposed to what has been seen in mouse models.[24,26,28] However, our data suggest that 20K-PEGylated Ad5, which showed the greatest reduction in immune responses and achieved the lowest level of cell association, in combination with compstatin, could represent a feasible avenue for further development of systemic adenoviral therapy. We want to emphasize that this study has again revealed the importance of using relevant models when studying immune responses against adenoviral vectors intended for use in humans. This blood loop system is an easy-to-handle and powerful preclinical model for studies of viral interactions with all components of whole blood, omitting the need for high-level anticoagulants that can alter the results by chelating the system of positive ions such as Ca^{2+} and Zn^{2+}. Furthermore, in contrast to the use of sealed small volume tubes, the loop system reported here also benefits from reproducing the continuous flow of

the blood stream and shear forces, which are important for platelet function. It should be pointed out that shear forces and flow rates are not equal in the whole body and no model exist that can provide such conditions. The data revealed that improved coating technologies probably in conjunction with genetic capsid modifications need to be developed to further decrease potentially deleterious interactions of Ad vectors with cellular and non-cellular blood components. The versatility of the loop model allows for rapid, reliable and reproducible analysis of diverse vector systems including extensively modified Ad vectors, different Ad species and serotypes, vectors based on other viruses and non-viral gene transfer vectors. It could preferably be used to measure virus cell association and immune responses of gene transfer vectors before administration to patients as a guide whether a specific therapy is appropriate for an individual patient.

Materials and methods

Cell lines

N52.E6 was cultured in Alpha Modification of Eagle medium supplemented with 10% fetal bovine serum (FBS). SKOV-3 was cultured in McCoy's 5A medium supplemented with 10% FBS and 10 mM HEPES. A549 and 911 cells were cultured in DMEM supplemented with 10% FBS and 1 mM sodium pyruvate. All cell culture reagents were purchased from Invitrogen (Carlsbad, CA, USA).

Virus production and PEGylation

Ad5 used for the studies presented herein is an E1-deleted first-generation vector harboring a human cytomegalovirus promoter-driven expression cassette for the enhanced green fluorescent protein (EGFP) that has been described earlier.[44] The vector was amplified in N52.E6 cells, purified by one CsCl step gradient, a subsequent continuous CsCl gradient, desalted by gel filtration using PD-10 columns (Amersham, Buckinghamshire, UK) and equilibrated in 50 mM HEPES (pH 8.0). Vectors were stored in HEPES with 10% glycerol. Vector titers were determined by a DNA-based slot blot procedure that has been described earlier.[45]

Ad vectors that were PEGylated with different amino-reactive PEG derivatives were from the same batch as vectors that were left un-PEGylated. In that way, batch-to-batch variability was avoided. 2K-SPA-PEG and 20K-SPA-PEG were purchased from IRIS Biotech Gmbh (Marktredwitz, Germany). PEGylation was performed in 50 mM HEPES buffer, pH 8.0. Amino-reactive PEGs were used in a 100-fold molar excess over particle surface amino groups (2K-SPA-PEG) or an 80-fold excess over particle surface amino groups (20K-SPA-PEG). The concentration of PEG in the reaction solutions did not exceed 2.5%. The reaction was performed at room temperature overnight. PEGylated vectors were stored in HEPES with 10% glycerol. The degree of capsid modification with PEG was 70% of the surface amino groups, determined by a standard fluorescamine assay according to Croyle *et al*.[22] PEGylation was quality-controlled by transduction of A549 cells with increasing amount of virus (20, 200 and 500 physical particle per cell). Quantification of EGFP expression by flow cytometry 24 h thereafter confirmed that the PEGylated vectors were detargeted. An infectivity assay with and without FX was also performed to verify detargeting of the PEGylated vectors (Supplementary Figure 1). Briefly, virus was incubated with physiological concentration (8 μg ml^{-1}) of FX for 1 h on ice. The virus/FX mixture was then added to adherent SKOV-3 cells and incubated for another hour on ice. Unbound viruses were removed by washing. After 24 h of incubation at 37 °C, the cells were harvested and EGFP expression was analyzed by flow cytometry (FACSCalibur Flow Cytometer, BD Biosciences, San Diego, CA, USA).

Human blood loop model

Permit to collect blood was approved by the regional ethical committee, 2008/264. Venous blood from 13 healthy individuals was collected in heparinized Falcon tubes and immediately mixed with 0.5 U ml^{-1} of heparin (LEO Pharma Nordic, Ballerup, Denmark). The blood was then transferred to heparin-coated PVC tubing, 35 cm long with an inner diameter of 4 mm (Corline, Uppsala, Sweden). Each loop was filled with 3 ml blood and virus and/or components as described in Table 1. The concentration of virus added was 1.2×10^{10} vp per three ml blood, which is equivalent to an injection of 2×10^{13} vp in an adult with 5 l of blood. The C3 inhibitor compstatin[46], used in some of the loops, was a kind gift from Professor John Lambris, Pennsylvania University, Philadelphia, PA, USA. Each tube was enclosed with a heparinized metal connector and incubated at 37 °C while rotating at 15 r.p.m. Samples were taken before the blood was added to the tubing and then after various time points of incubation. EDTA was added to a final concentration of 6.8 mM to block further reactions in samples used for cytokine, complement and platelet analyses. Plasma was obtained from untreated blood and blood rescued from the loops by centrifugation (3000 g, 20 min, 4 °C) and stored in aliquots at −80 °C until analysis.

Coagulation analysis

Blood was extracted from the loops and platelets were automatically counted at different time points using a Coulter AcT Diff Hematology Analyzer (Beckman Coulter, Miami, FL, USA). Macroscopic blood clots were observed by the naked eye.

Titration of infectious virus after blood exposure

Whole blood (55 μl) from the loop with uncoated Ad5 was taken after 15 min and 6 h, diluted 10 to 10 000 times in culture medium and added to confluent 911 cells in 12 well plates. After 2 h of incubation the plates were washed once with medium, after which fresh medium was added and the incubation continued for 24 h. The cells were harvested and EGFP expression was detected by FACSCalibur Flow Cytometer as a measurement of infectious viral particles. The titers obtained after 15 min and 6 h of incubation were related to the titer of uncoated Ad5 that had not been exposed to human blood, which was defined as 100% infectivity.

Anti-adenovirus IgG antibody titration

IgG antibody titers against adenovirus were measured in untreated plasma samples with an adenovirus IgG ELISA kit (Diagnostic Automation, Calabasas, CA,

USA), according to the provider's instructions. The cut-off control had a value of 10 U ml^{-1}, the weak positive control 35 U ml^{-1} and the strong positive control 150 U ml^{-1}. Importantly, this method reveals the combined level of the neutralizing and non-neutralizing anti-adenovirus antibodies present.

Neutralizing assay

Untreated plasma samples were heat-inactivated at 56 °C for 1 h. Uncoated Ad5 1.5×10^6 viral particles (vp), was added to a 96-well plate in a volume of 50 μl per well. Plasma dilutions (1:10 to 1:40 000) in a volume of 50 μl per well were then added and the plate was incubated at 37 °C for 1 h. Subsequently, 1.5×10^5 911 cells in a volume of 100 μl were added. After 24 h of incubation cells were harvested and analyzed by flow cytometry (LSRII Flow Cytometer, BD Biosciences).

Cytometric bead array analysis

The complement factors C3a, C4a and C5a were measured in plasma samples from time points 0, 15 and 60 min using Human Anaphylatoxin Kit (BD Biosciences). Plasma samples were diluted 1:600 in PBS before the assay, and the instructions from the manufacturer were followed. Samples were analyzed by FACSCalibur Flow Cytometer. The cytokines TNF, IL-6, IL-8, IP-10, RANTES, MCP-1, IL-1β, IL-10, IL-12p70 and MIG were measured at 0 and 6 h using Cytometric Bead Array Flex Set (BD Biosciences). Plasma samples were diluted 1:4 in Assay Diluent. The procedure was performed according to the manufacturer's instructions. Samples were analyzed by LSRII Flow Cytometer.

Quantitative real time PCR analysis

For DNA extraction, 10 μl of whole blood, plasma and cell fraction (separated by centrifugation at 3000 g, 20 min, 4 °C) were collected from the loops at 15 min. The viral DNA content in whole blood was used to verify equal virus load in the loops and comparable recovery after DNA purification. The volumes were adjusted to 200 μl with DMEM medium and viral DNA from each fraction was purified using High Pure Viral Nucleic Acid Kit (Roche, Mannheim, Germany), according to the manufacturer's instructions. Viral DNA content in the three fractions was analyzed by quantitative real time PCR using the iCycler IQ real-time detection system (Bio-Rad, Hercules, CA, USA). The specific PCR product was continuously measured during 40 cycles (95 °C for 15 s, 60°C for 60 s) using iQ SYBR Green supermix (Bio-Rad) and primers in the adenovirus E4 region: 5′-CATCAGGTTGATTCACATCGG-3′ (E4.Forward) and 5′-GAAGCGCTGTATGTTGTTCTG-3′ (E4.Reverse). All samples were amplified in duplicates. Copy numbers were related to a standard curve made by serial dilutions of a plasmid containing E4 open reading frame 1 (pCR2.1(Ad5E4orf1)). To compare copy numbers in plasma and cell fractions, the numbers were adjusted according to the packed cell volume (hematocrit), which is approximately 46% for men and 42% for women.[47]

Sorting of blood cells

In follow-up experiments, loop 1 (Ad5) and 3 (20K-PEG Ad5) with blood from donors A, B, D, E, I, O, G and S were incubated for 15 min at 37 °C while rotating. The anticoagulant hirudin (Pharmion Ltd, Windsor, UK), was added to a final concentration of 50 μg ml^{-1} before the blood was transferred to the tubing. Following incubation, blood (100–500 μl) was transferred to tubes and stained with antibodies: CD235ab^{PE}, CD41^{FITC}, CD3^{PE}, CD15^{APC}, CD14^{FITC}, CD19^{PE} and CD45$^{PerCP-Cy5.5}$ for 15 min at room temperature. The antibodies were purchased from Nordic Biosite (Taby, Sweden) and the amount of antibody used was according to the manufacturer's instructions. CD45 staining was used to separate leukocytes from erythrocytes and platelets. In leukocytes samples, erythrocytes were lysed with ammonium chloride solution (0.15 M NH_4Cl, 10 mM $NaHCO_3$, 1 mM EDTA dissolved in H_2O) for 10 min. Cells were washed once in PBS and then resuspended in PBS with 1% paraformaldehyde. Cells were sorted with FACSVantage SE DiVa (BD Biosciences). Viral DNA was then purified with High Pure Viral Nucleic Acid Kit (Roche) and analyzed by quantitative PCR as described above. Relative copy numbers of viral DNA per cell population and ml blood are presented.

Statistical analysis

GraphPad Prism (version 4.03) was used for statistical analysis. Wilcoxon matched pairs test was used to analyze complement, cytokine and quantitative PCR data. The differences in immune responses and viral cell bound fraction between uncoated and PEGylated Ad5 for individual donors were compared.

Conflict of interest

The authors declare no conflict of interest.

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Supplementary Information accompanies the paper on Gene Therapy website (http://www.nature.com/gt)