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Antibody-neutralized reovirus is effective in oncolytic virotherapy

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M. Coffey is the president and chief executive officer at and has ownership interest in Oncolytics Biotech, Inc. A. Melcher is a consultant/advisory board member for Amgen and Bristol-Myers Squibb. No potential conflicts of interest were disclosed by the other authors.

Abstract: Immunotherapy is showing great promise for otherwise incurable cancers. Oncolytic viruses (OV), initially developed as direct cytotoxic agents, are now known to mediate their anti-tumour effects largely via activation of the immune system. With regard to OV however, the immune system represents a double-edged sword because, as well as anti-tumour responses, OV also stimulate anti-viral immune responses including the induction of anti-OV neutralizing antibodies. Current dogma suggests that the presence of either pre-existing anti-viral neutralizing antibodies in patients, or their development during viral therapy, is a barrier to systemic OV delivery rendering repeat systemic treatments ineffective. However, we have found that human monocytes loaded with pre-formed reovirus-antibody complexes, in which the reovirus is fully neutralized, deliver functional replicative reovirus to tumour cells resulting in their infection and lysis. This delivery mechanism is mediated, at least in part, by antibody receptors (in particular FcγRIII) which mediate uptake and internalization of the reovirus/antibody complexes by the monocytes. This finding has profound implications for oncolytic virotherapy and for the design of clinical OV treatment strategies in the future.

Introduction

The use of oncolytic virus (OV) therapy (a recognized form of immunotherapy) is progressing rapidly in the clinic, with confidence in the field increasing following FDA approval for the first agent in class, talimogene laherparepvec (T-Vec, a herpes Simplex virus encoding GM-CSF) to treat melanoma(1). However, OV are not currently used as widely as other types of immunotherapy such as checkpoint inhibitors, possibly owing to the current perception that systemic administration will be limited by neutralizing antibodies (NAb). NAb may be present at baseline, for those viruses prevalent within the human population e.g. herpes simplex virus type 1, and mammalian orthoreovirus type 3 (herein referred to as "reovirus"), or they may arise following initial doses of OV therapy. Such concerns

potentially limit systemic OV therapeutic strategies to a 'one shot cure' approach, whereby patients receive a single high dose of OV(2) or to direct OV injection into tumours. Indeed, FDA approval for T-Vec is for intra-tumoural (i.t.) delivery only and while this route ensures viral access to the tumour, it is technically challenging and limits treatment to readily accessible tumours. Systemic delivery has been shown to be safe, is broadly applicable in a clinical setting, and more suitable for targeting visceral or widespread metastatic disease. We and others have investigated an alternative approach that circumvents NAb-mediated neutralization by delivering virus within carrier cells(3, 4). This strategy is also clinically challenging but unexpectedly, developments from this work have indicated a potential positive role for NAb in OV therapy.

We showed that i.t. delivery of single agent reovirus was effective as an anti-tumour therapy in mice, while systemically administered reovirus was relatively ineffective(5). However, loading immune cells (T cells or dendritic cells) with reovirus *ex vivo* led to efficient systemic viral delivery to tumours, even in the presence of anti-reovirus NAb(3, 6). Following this, the results of a translational biological endpoint clinical trial (REO13) in patients with colorectal liver metastases, indicated that systemically delivered reovirus could access tumours and that functional virus was associated with immune cells in the blood but was not found in plasma(7). These data suggest that, while free reovirus is rapidly neutralized by NAb in the serum following intravenous (i.v.) delivery, replication-competent virus can be transported to tumours via carriage by blood cells. Consistent with this, pre-conditioning mice with GM-CSF – to mobilize the myeloid compartment to the systemic circulation prior to i.v. reovirus treatment – resulted in effective therapy, the virus associating predominantly with CD11b+ cells in the blood(8). Critically, GM-CSF pre-conditioning was only effective in reovirus-immunized mice with high serum anti-reoviral NAb, consistent with NAb contributing to therapeutic efficacy.

In the current study, a human *in vitro* assay is described, in which monocytes are loaded with fully neutralized reovirus in the form of reovirus/neutralizing antibody (reoNAb) complexes and co-cultured with tumour cell targets. Remarkably, whilst antibody-neutralized reovirus was unable to infect and kill tumour cells directly, it could be loaded onto human monocytes and delivered to melanoma cells in a functional/replicative form resulting in cell lysis. After loading, antibody-neutralized reovirus was internalized by monocytes and processed to release infectious viral particles. The internalization process involved surface Fc receptors (FcR), with FcγRIII expressed on non-classical monocytes playing a predominant role. These data have profound implications for the design of cancer therapy strategies and for mechanistically defining routes of viral dissemination and the initiation of adaptive immune responses which modulate infection.

Results

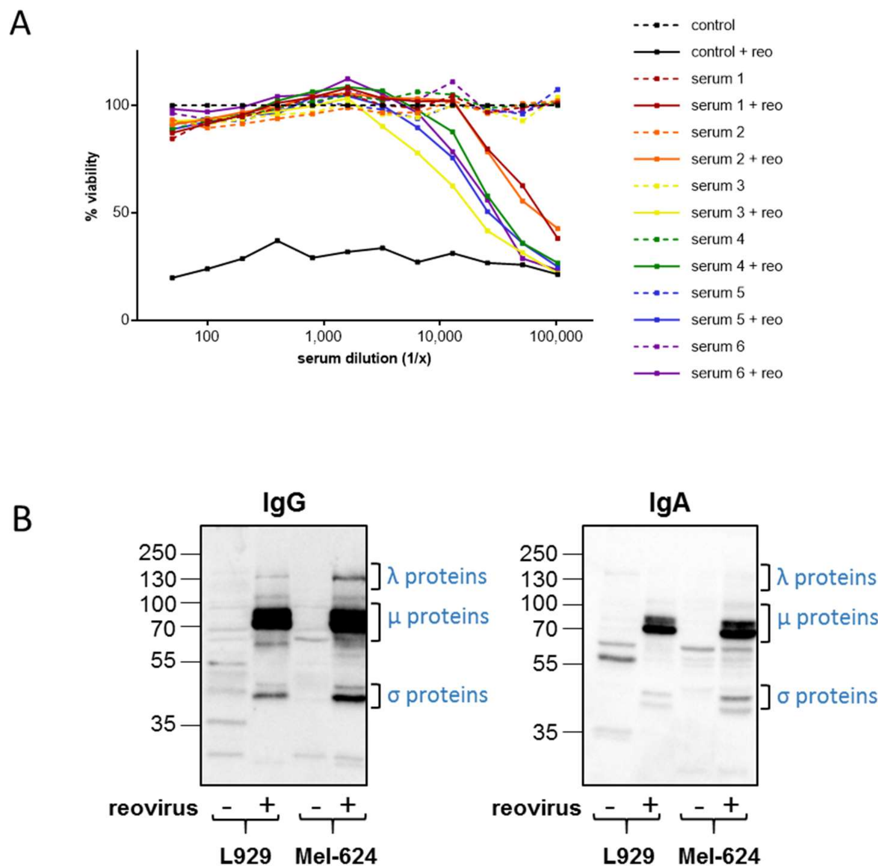
Reovirus is neutralized by IgG and IgA antibodies in patient-derived serum

Serum was obtained from patients on a biological end-point clinical trial (REO13-BRAIN) in which they received i.v. reovirus (1 x 10^{10} $TCID_{50}$) as monotherapy prior to surgical resection of brain tumours (primary or metastatic). Blood samples were taken at least 7 days post reovirus treatment to ensure a high titre of anti-reoviral NAb and the serum was isolated. A

standard neutralization assay indicated that serum from all patients was highly neutralizing towards reovirus (Fig. 1A), compared with serum from control donors (Fig. S1A). To demonstrate the presence of reovirus-specific antibodies in the patient-derived serum, western blots of lysates from reovirus-infected cells (L929 cells or Mel-624 cells) were performed using patient-derived serum as the primary detection antibody and anti-human IgG, IgA or IgM secondary antibodies. Both IgG and IgA antibodies in the serum recognized a range of reoviral proteins (Fig. 1B); IgM antibodies reactive to reovirus were not found. Depletion of IgG or IgA antibodies from the serum using specific anti-IgG or -IgA agarose beads showed that both isotypes contributed to reovirus neutralization, with IgG antibodies being predominant (Fig. 1C).

It has been suggested that complement plays a role in the neutralization of reovirus(9). We therefore investigated this via heat inactivation (HI) of patient-derived serum. Figure 1D shows that HI-serum demonstrated equivalent neutralizing capacity to untreated serum, suggesting that heat-labile factors such as complement are not fundamental to reovirus neutralization *in vitro*. Complement activity within patient-derived serum was verified (Fig. S1B).

These data show that patients receiving i.v. therapeutic doses of reovirus develop a high anti-reoviral antibody titre with IgG and IgA antibodies contributing to virus neutralization. By contrast, complement does not appear to play a major role in reovirus inactivation.



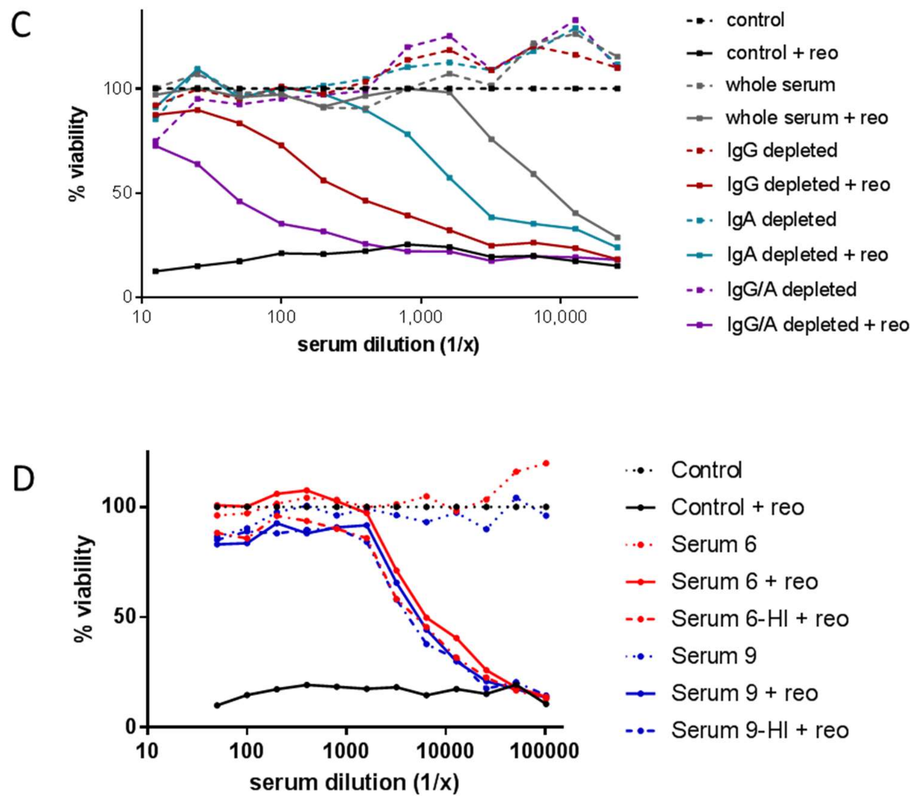


Fig. 1. Reovirus is neutralized by IgG and IgA antibodies in patient-derived serum
A) Reovirus neutralization assay. Each colour corresponds to one serum sample, the control (no serum) is shown in black. Solid lines show cultures containing reovirus and dotted lines those containing serum only. **B**) Western blot of mock or reovirus-infected lysates using patient-derived serum as primary antibody and anti-human IgG/IgA secondary antibodies. Blots are representative of three patient sera. **C**) IgG (red), IgA (blue) or both (purple) were depleted from serum and reovirus neutralization assays using depleted or whole sera (grey) were carried out, (control, black). **D**) Reovirus neutralization assay using whole or heat-inactivated (HI) serum. Two different patients' samples are shown in red or blue, whole serum (solid line) vs HI serum (dashed line); dotted lines show results in the presence of serum only (control, black).

Formation of reovirus/neutralizing antibody complexes

Our pre-clinical *in vivo* data led us to propose a model in which, following i.v. infusion, reovirus was rapidly bound by NAb to form reoNAb complexes which were delivered to tumours via monocytes(8). Therefore, the formation of the proposed reoNAb complexes was verified using electron microscopy (EM). Reovirus was allowed to adhere to EM grids which were then incubated with patient-derived serum or control serum from normal donors. Protein A gold labelling indicated the association of IgG with reovirus particles confirming the formation of reoNAb complexes (Fig. 2A). There were significantly more gold particles associated with the reovirus following incubation with patient-derived serum (76%) than with control serum (40%) (Fig. 2A and B). The presence of low level anti-reoviral NAb in control

serum is expected, as most people have had prior exposure to the virus(10-12); this data is also consistent with our previous clinical trial, in which NAb were present at baseline in patients, but significantly increased after i.v. reovirus administration(7).

Thus, the anti-reoviral antibodies present in patient-derived serum can bind reovirus producing reoNAb complexes, such as would be formed following systemic reovirus therapy, especially on repeat administration.

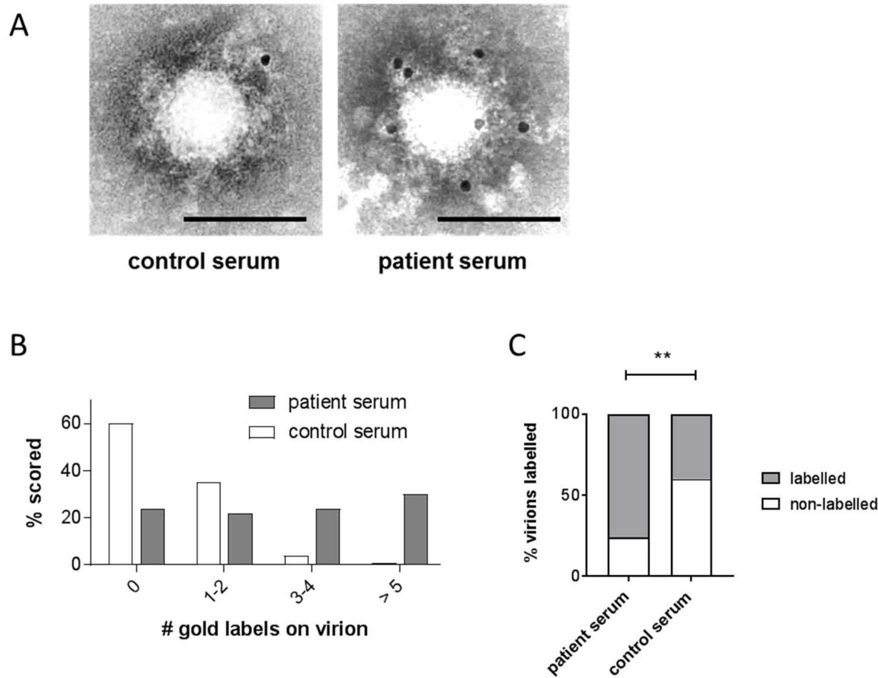


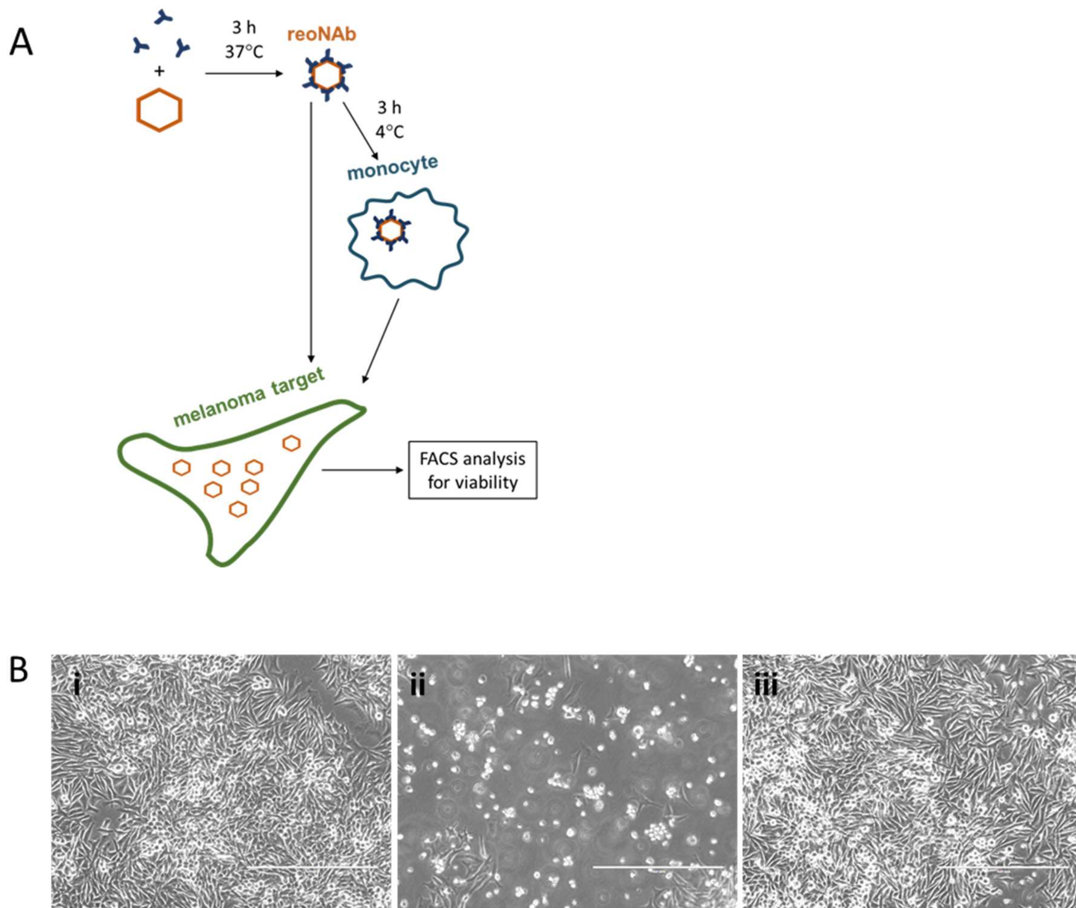
Fig. 2. Formation of reovirus/neutralizing antibody (reoNAb) complexes
Reovirus was bound to copper grids prior to incubation with control or patient serum and labelled with protein A-gold (10 nm). Preparations were fixed, negatively stained with PTA and visualised at 52,000 X magnification by TEM. **A**) Representative micrographs of labelled virions, demonstrating typical gold labelling patterns. Bar = 100 nm. **B&C**) Gold labels on individual virions were quantified, n > 340 virions for each condition. B) Percentage of virions having 0, 1-2, 3-4 or >5 gold labels associated per virion. C) Proportion of virions being gold-labelled when pre-incubated with patient-derived serum (76%) vs control (40%); $^{**}p < 0.01$ by $\chi 2$ test ($\chi 2 = 92.9$, cut-off 6.6 where df = 1).

Monocytes loaded with pre-formed reoNAb complexes mediate killing of tumour cells

In order to further develop the hypothesis that reoNAb can be delivered to tumours via monocytes in patients, the association of reovirus with human monocytes in the presence of neutralizing serum, was assessed. Whole blood from normal donors was mixed with patient-derived serum and reovirus was then added. In the presence of NAb, virus was loaded onto $CD14^+$ cells more efficiently than other immune cells (Fig. S2). Next, we designed a human *in vitro* assay (Fig. 3A) in which human monocytes were loaded with either live non-neutralized reovirus or pre-formed reoNAb complexes, and their ability to induce tumour cell death, was examined. ReoNAb complexes were generated by incubating reovirus with a pre-determined volume of neutralizing patient-derived serum at 37°C for 3 h. The complexes or

non-neutralized reovirus were loaded onto isolated human monocytes which were then co-cultured with melanoma target cells. Melanoma targets were also treated with reovirus or reoNAb complexes in the absence of monocytes. After 72 h, the cells were harvested and melanoma cell viability was determined by flow cytometry. Mel-624 cells treated directly with reoNAb did not show any loss of viability compared with controls; however, when tumour cells were cultured with monocytes carrying reoNAb complexes, significant cell death was observed (Fig. 3B and C). Monocytes loaded with non-neutralized reovirus induced higher levels of Mel-624 death than those loaded with reoNAb but the level of cell death induced by the latter was remarkable. Mel-624 cells cultured with monocytes alone showed no any loss of viability, nor reduction in growth rate (Fig 3B and C).

These results show that reovirus was fully neutralized within the reoNAb complexes and unable to infect melanoma targets directly, but following loading onto monocytes, the complexes induced tumour cell death.



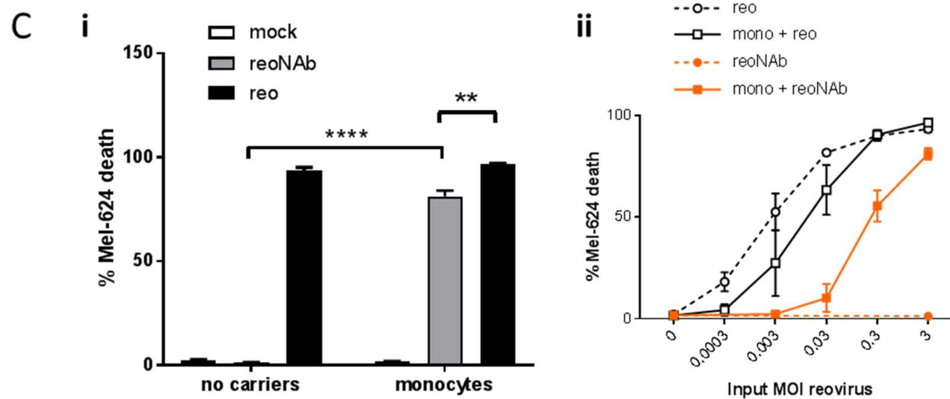


Fig. 3. Monocytes loaded with pre-formed reoNAb complexes mediate killing of tumour cells

A) Schematic of the hand-off assay. **B**) Representative microscopy images of Mel-624 cells treated with reoNAb complexes either directly (i) or following loading onto monocytes (ii) or co-cultured with monocytes alone (iii), scale bars = 400 µm. **C**) Mel-624 cells were cultured for 72 h with monocytes loaded ± reoNAb at MOI 3 (i) or at varying MOI (ii). Cells were harvested, stained with Live/Dead® stain and examined by flow cytometry. Monocytes were gated out and the percentage of viable tumour cells was determined. Graphs show mean +SD from 3 independent experiments; ****p = 0.00000094, Student's *t*-test.

Infectious reovirus mediates the killing of tumour cells by reoNAb-loaded monocytes

The observed tumour cell death could be mediated either by the monocytes themselves, following their activation by reoNAb complexes, or by release/transfer of infectious reovirus from the monocytes. Therefore, reoNAb complexes were generated using either live or UV-inactivated reovirus because both are able to activate monocytes (Fig. S3) but UV-inactivated reovirus is unable to infect and kill tumour cells directly(13). Monocytes loaded with UV-reoNAb complexes abrogated melanoma cell death following co-culture (Fig. 4A) suggesting that tumour cell death was due to reovirus infection and replication, rather than monocyte cytotoxicity. In support of this, reovirus titre within monocyte-reoNAb and tumour cell co-cultures increased over time (Fig. 4B), indicative of an on-going productive infection. Furthermore, blocking JAM-A (the known reovirus entry receptor) on the target melanoma cells, inhibited cell death (Fig. 4C), indicating that reovirus infection occurred via the normal entry route. However, separation of monocytes and tumour targets with a transwell, abrogated cell death (Fig. S4), suggesting that initial hand-over from the monocytes was contact dependent and that JAM-A was required for later viral spread between tumour cells. Reovirus replication occurred predominantly within tumour cells rather than monocytes, as reovirus titre did not increase over time in monocytes loaded with reoNAb complexes (Fig. 4D). This is in contrast to our previous observations in myeloid-derived human dendritic cells, where reovirus replication does take place albeit at a relatively low level(3).

These data indicate that fully antibody-neutralized reovirus can be loaded onto monocytes and delivered to tumour cells in a functional form, resulting in infection and oncolysis.

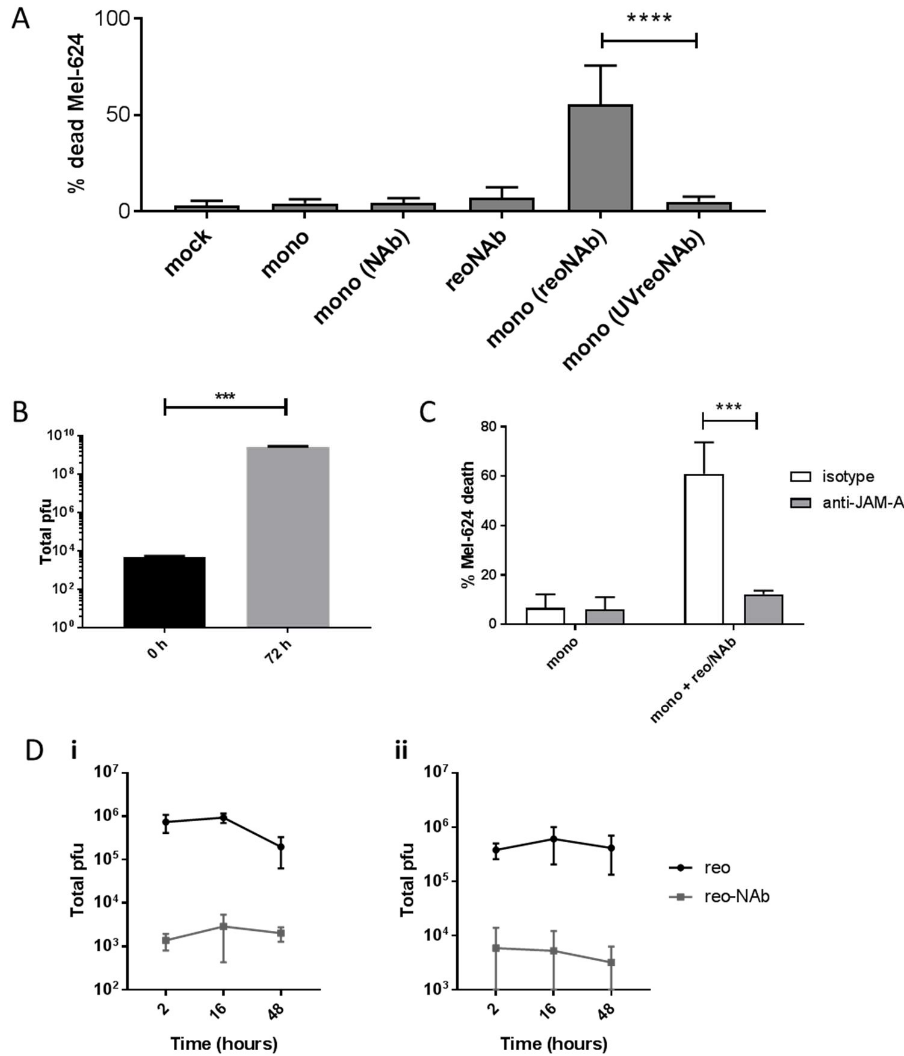


Fig. 4. Infectious reovirus mediates the killing of tumour cells by reoNAb-loaded monocytes

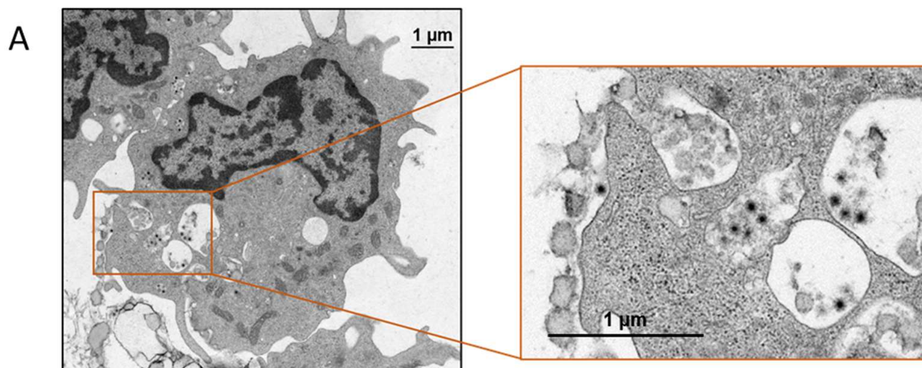
A) Monocytes were loaded ± NAb or reoNAb complexes formed using live or UV-inactivated reovirus (MOI 10) and co-cultured with Mel-624 cells. Cell death was analysed by flow cytometry. Graph shows mean +SD from 8 independent experiments; ****p = 0.000000245, Student's *t*-test. **B**) Monocytes were loaded with reovirus or reoNAb (MOI 10) and added to Mel-624 cultures. These were harvested at the times shown and viral titre determined by plaque assay. Graph shows mean +SD from four independent experiments; ****p = 0.00000129, Student's *t*-test. **C**) Monocytes were loaded as in (B). Mel-624 target cells were pre-incubated for 30 min with isotype control or anti-JAM-A antibodies at 10 µg/ml. Percentage Mel-624 cell death at 96 h was determined. Graph shows mean +SD from four independent donors; ***p = 0.00082, Student's *t*-test. **D**) Monocytes were loaded as in (B) and cultured for up to 48 h. Samples were harvested at the times indicated and reovirus titre in the cells (i) and culture supernatants (ii) was determined by plaque assay. Graphs show mean ±SD from 3 independent experiments.

ReoNAb complexes are internalized by monocytes prior to release of infectious virus

Previously, we showed that live reovirus could be internalized by dendritic cells for delivery to tumour cells(6); in the current study, the fate of reoNAb complexes following their loading onto monocytes, was investigated. EM demonstrated that reoNAb complexes were rapidly internalized by monocytes (Fig. 5A). Free reovirus was also internalized by monocytes but this appeared less efficient than reoNAb internalization, as virus particles remained on the monocyte surface following loading with non-complexed reovirus, but not with reoNAb complexes (Fig. 5B).

Having previously demonstrated that Fc receptors (FcR) were involved in the delivery of reovirus to tumours via monocytes in mice(8), their role in the delivery of reoNAb by human monocytes was examined. Expression of FcγRIII (CD16), FcγRII (CD32), FcγRI (CD64) and FcαR (CD89) was confirmed by flow cytometry (Fig. S5) and the receptors were blocked prior to reoNAb loading. Blocking FcγRI or FcγRII had little effect on the amount of reovirus loaded onto the monocytes or the delivery of reoNAb to tumour cells. By contrast, blocking FcγRIII significantly reduced the level of reovirus loading onto monocytes (Fig. S6) and also melanoma cell death following co-culture (Fig. 5C). This was noteworthy, since non-classical $CD16^+$ monocytes represent only a small fraction (approx. 10%) of the monocytic population. To confirm the significance of FcγRIII, $CD16^+$ and $CD16^-$ monocytes were separated and their ability to deliver reoNAb to melanoma cells was compared. We confirmed that non-classical $CD16^+$ monocytes were more efficient in delivering reoNAb to induce melanoma cell death, whereas both classical and non-classical monocytes were able to deliver neat reovirus efficiently (Fig. 5D). Furthermore, RNA sequencing data showed that FcγR mRNA levels were up-regulated in monocytes loaded with reoNAb complexes, FcγRIII showing the greatest increase (Fig. S7). There appeared to be a potential role for the FcαR in mediating uptake of reoNAb by monocytes but this was not as marked as for FcγRIII (Fig. 5C).

These data show that FcR, particularly FcγRIII, are involved in the uptake of reoNAb complexes by monocytes, though they may not be the only mechanism of uptake since tumour cell death was not completely abrogated by blocking these receptors.



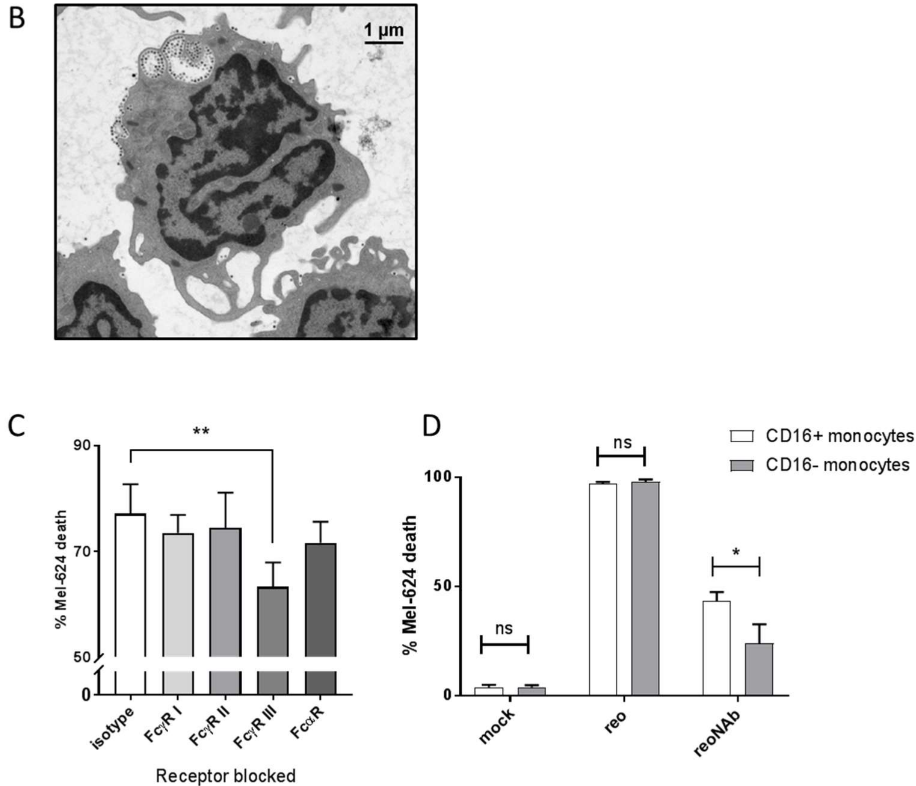


Fig. 5. ReoNAb complexes are internalized by monocytes prior to release of infectious virus
Negatively-selected monocytes were loaded with reoNAb (**A**) or reovirus (**B**) then washed, fixed and processed as described in M&M. TEM sections were viewed with an FEI Tecnai TWIN microscope at 120kV. **C**) Monocytes were pre-incubated with anti-FcγR antibodies or anti-FcαR human recombinant antibody, then loaded with reoNAb and added to Mel-624 targets; cell death was assessed by flow cytometry after 72 h. Mean +SD from four donors are shown; **p = 0.0089, Student's *t*-test. **D**) $CD16^+$ or $CD16^-$ monocytes were selected from PBMC, loaded with reovirus or reoNAb, washed and added to Mel-624 targets for 72 h. The proportion of dead Mel-624 cells was determined by flow cytometry. Graph shows mean +SD from four donors; *p = 0.036, Student's *t*-test.

The efficacy of reoNAb complexes is not restricted to melanoma cells and is applicable to other OV

In order to show that the delivery of reoNAb complexes to tumour cells via monocytes was not melanoma-specific, tumour cells of other histological types were tested. ReoNAb complexes loaded onto monocytes were delivered effectively to colorectal, prostate and ovarian tumour cells, resulting in significant cell death (Fig. 6A). In addition, we have previously shown that pre-conditioning with GM-CSF, followed by systemic reovirus treatment, enhances survival in reovirus-immunized mice bearing TC2 (prostate) tumours(8) or intra-cranial GL-261 (glioma) tumours(14). Thus the therapeutic efficacy of anti-reoviral NAb are likely to be broadly applicable over a range of tumour types.

Given the number of OV currently under investigation as therapeutic agents, it would be valuable to establish whether other anti-OV antibodies can contribute to therapy. Serum or pleural fluid was obtained from patients undergoing clinical trials with Coxsackievirus (CVA-21) and herpes simplex virus (HSV1716) and CVA/NAb and HSV/NAb complexes were generated. Both of these OV/NAb complexes were ineffective when cultured directly with melanoma targets, indicating complete neutralization of the viruses. However, following loading onto monocytes, CVA/NAb were comparable to reoNAb in mediating tumour cell death, while by contrast, HSV/NAb were ineffective (Fig. 6B). These findings show that the contribution of anti-OV antibodies to oncolytic virotherapy, while not being universally applicable, is not specific to reovirus.

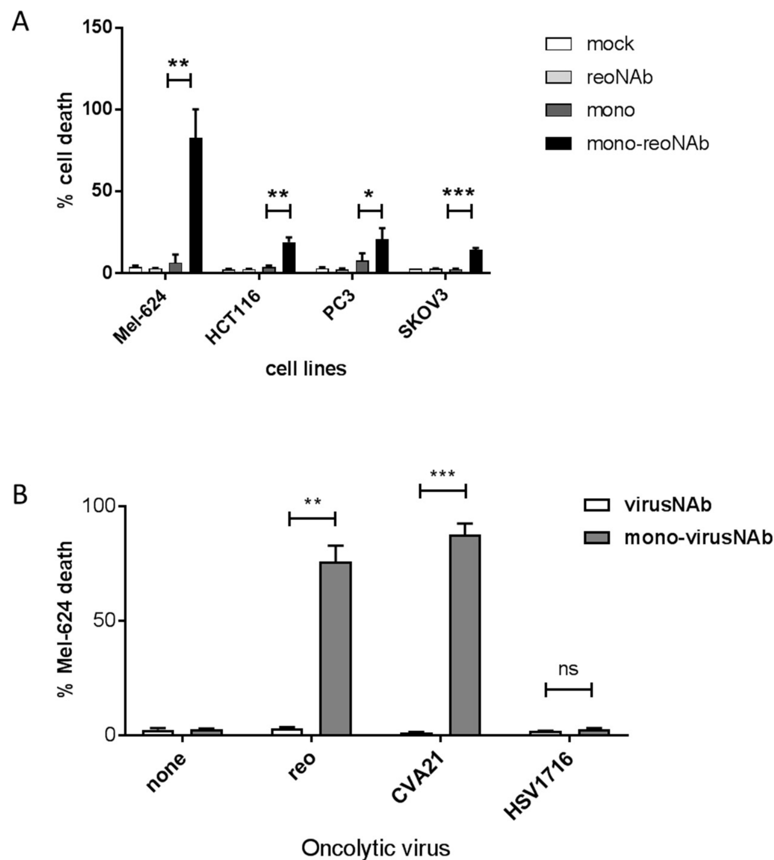


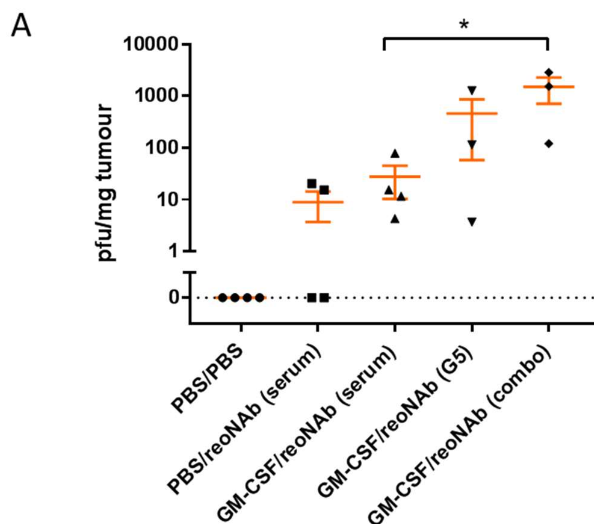
Fig. 6. The efficacy of reoNAb complexes is not restricted to melanoma cells and is applicable to other OV

A) Target cells were treated with medium, reoNAb complexes or reoNAb loaded monocytes, and cell death was assessed by flow cytometry after 72 h. Graph shows mean +SD from three independent experiments; Mel-624 **p = 0.0087, HCT116 **p = 0.0047, PC3 *p = 0.0373, SKOV3 ***p = 0.0007, Student's *t*-test. **B**) Virus-neutralizing antibody complexes (virusNAb) were formed using matched OV and patient-derived NAb. These were co-cultured with Mel-624 targets. Mel-624 cell death was assessed at 72 h by flow cytometry. Graph shows mean +SD from at least three independent experiments; reovirus **p = 0.0038, CVA21 ***p = 0.0008, ns = not significant, Student's *t*-test.

ReoNAb complexes deliver functional reovirus to tumours in vivo

Although we have demonstrated the importance of anti-reoviral antibodies in the therapeutic response to i.v. reovirus therapy following GM-CSF pre-conditioning in mice(8), we have not shown that pre-formed reoNAb complexes can mediate delivery of functional reovirus to tumour-bearing mice. Therefore, we used serum from mice that had been pre-immunized against reovirus – high anti-reoviral NAb (Fig. S8) – to generate reoNAb complexes. These were injected i.v., with or without prior GM-CSF conditioning, into tumour-bearing mice. After three days the tumours were harvested and examined for functional reovirus by plaque assay. Functional reovirus was detectable within the tumours of all of the mice that had received GM-CSF pre-conditioning, but in only two of four mice that did not receive GM-CSF (Fig. 7A). This indicates that in spite of complete antibody-neutralization, functional reovirus can access tumours *in vivo* and is consistent with our previous data showing that i.v. administration of reovirus was not therapeutic in tumour-bearing mice unless the mice had been pre-conditioned with GM-CSF(8). Furthermore, administration of GM-CSF followed by reoNAb complexes delayed tumour growth and increased survival in tumour-bearing, reovirus-naive mice (Fig 7B&C). This therapeutic effect was less than we had previously seen following GM-CSF/reovirus treatment in reovirus-immunized mice(8) and suggests that as well as contributing to reoviral delivery to tumours, systemic anti-reoviral Ab have an additional role in mediating therapy.

ReoNAb complexes formed using an anti-reoviral monoclonal antibody were delivered as efficiently as those generated using serum from reovirus-immunized mice (Fig 7A), further supporting our hypothesis that this is an antibody-mediated process rather than being dependant on other serum factors. Moreover, delivery was enhanced by using a combination of monoclonal antibodies rather than a single one. This has implications for therapy as it suggests the possibility of improving therapeutic outcome by manipulation of the antibodies coating the reovirus.



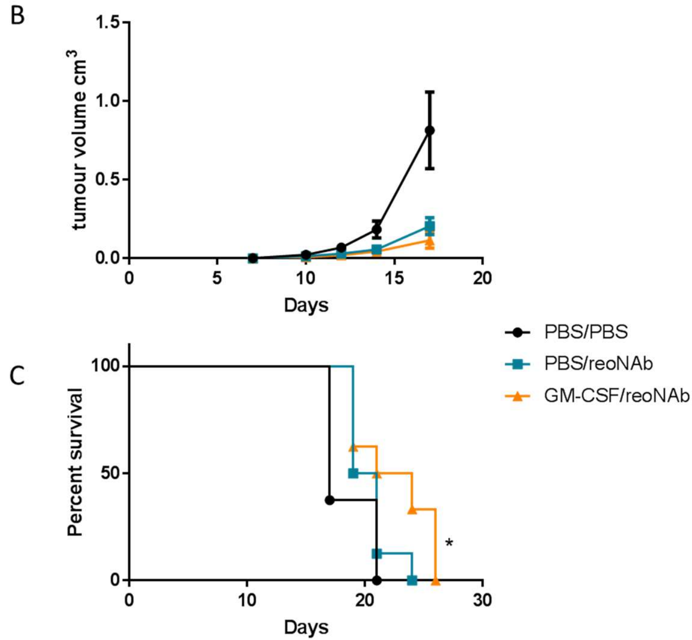


Fig. 7. ReoNAb complexes deliver functional reovirus to tumours in vivo
A) Mice bearing 7 d established B16 tumours received 1 cycle of GM-CSF/reoNAb. ReoNAb were generated using either anti-reovirus mouse serum, a single anti-reovirus monoclonal antibody (G5), or a combination of monoclonal antibodies (combo). Tumours were harvested on day 14 and viral titre determined by plaque assay. Graph shows mean ±SD pfu/mg of tumour; *$p = 0.045$, one way ANOVA. **B&C**) Mice bearing 3 d B16 tumours were treated as above. Mice were sacrificed when tumours reached 1 cm in diameter; GM-CSF/reoNAb vs control $p = 0.022$, log rank test.

Discussion

Intravenous delivery of an oncolytic virus represents not only the optimal means of accessing disseminated neoplastic tissue, but is also likely to be the most practical way of stimulating a systemic response from the immune system. However, this route of infusion is often eschewed in favour of more local methods given the many 'hurdles' to viral persistence present in the vasculature, one prominent perceived obstacle being neutralizing antibodies. As sero-prevalence for reovirus is common, in most individuals any i.v.-administered virus will encounter low-level NAb. A number of early-phase clinical trials have involved the administration of OV as a large i.v. bolus. Seen in the context of a pre-existing low-level immunity to the virus, these therapeutic infusions represent a re-exposure to abundant viral antigens and result in a large-scale anamnestic response. This is characterised by the rapid generation of antiviral antibodies in circulation at very high titre(15, 16), which is considered to preclude further therapeutic i.v. doses.

Our previous work, which focused on potentiating the delivery of reovirus to tumours by evasion of the anti-reoviral NAb response, unexpectedly uncovered a role for these antibodies in the therapeutic response(8). Here, we have further investigated the therapeutic potential of NAb, specifically in the form of reoNAb complexes where the virus is fully neutralized and

unable to infect susceptible tumour cells. The source of anti-reoviral NAb was serum from patients on the REO13-BRAIN clinical trial. All patients had high anti-reovirus NAb, comprising IgG and IgA isotypes, both contributing to reovirus neutralization (Fig. 1), with no evidence for involvement of the complement system. This contradicts a recent study in which an inhibitor of the complement C3 molecule precluded reovirus neutralization in plasma(9). The basis for this disparity is unclear. We employed a different strategy of disabling complement (HI vs inhibitor) and output method (MTT assay vs plaque assay), and used serum rather than anticoagulant-treated plasma; all of which could contribute to the difference in outcome.

ReoNAb complexes were generated by incubating reovirus with a neutralizing volume of serum and their formation was confirmed by EM (Fig. 2). Reovirus neutralization was confirmed by incubating the reoNAb complexes with susceptible melanoma target cells; no cell death was observed, indicating that the virus was fully neutralized and unable to infect the cells. However, following loading onto isolated human monocytes, the reovirus within the complexes could be transferred to melanoma targets to induce target cell death (Fig. 3). The mechanism by which the reoNAb complexes are processed by monocytes and transferred to tumour cells is currently the subject of further investigation in our laboratory but we have shown that it involves their internalization by the monocytes, this being partly dependent on FcγRIII (Fig. 5). This was surprising as non-classical monocytes expressing FcγRIII form the minor subset of peripheral blood monocytes but we have demonstrated that, within a mixed population, their contribution to reoNAb transport is proportionally larger than that of classical monocytes. Nevertheless, there appears to be some contribution by classical monocytes, which may depend on an alternative mechanism of uptake. In contrast to human myeloid-derived dendritic cells, which support low level replication, reovirus does not appear to replicate within freshly isolated human monocytes, indicating that viral amplification does not occur following internalization. The role of FcR in reoNAb transport suggests that NK cells and neutrophils, which express FcγRIII, may also play a role in reoNAb transport.

We have examined the delivery of reoNAb complexes to other tumour cell lines and found that it is not restricted to melanoma (Fig. 6A), suggesting the wide applicability of our findings in influencing treatment design for many cancer patients. Furthermore, we have demonstrated that the phenomenon of reoNAb delivery is not reovirus-specific because CVA/NAb complexes are delivered to tumour cells by monocytes in a similar manner, although HSV/NAb are not, suggesting that specific aspects of virus physiology may determine applicability (Fig. 6B). It is unclear which aspects govern the delivery of OV via NAb complexes but given our observations with reovirus, CVA and HSV1716, one possibility is the presence or otherwise of a viral envelope. However, a pre-existing immune response improves the therapeutic efficacy of Newcastle Disease virus (Jacob Ricca, abstract O15, SITC 2016) and Maraba virus(17), suggesting a possible role for OV/NAb delivery via monocytes for both of these enveloped viruses and therefore we postulate that delivery of OV/NAb complexes might be restricted to small RNA viruses rather than those with DNA genomes.

Finally, we have demonstrated that following i.v. delivery of reoNAb complexes to tumour-bearing mice, functional reovirus can be retrieved from the tumours (Fig. 7), supporting our hypothesis that i.v. reovirus therapy in pre-immunized mice results in the rapid formation of reoNAb complexes *in vivo* which are then delivered to tumours via monocytes(8). Although

we know that following i.v. delivery, reovirus is rapidly neutralized by NAb, this cannot be instantaneous and it is possible that transport of non-neutralized reovirus by monocytes was responsible for viral delivery to the tumours. Although we have not ruled out this possibility, we have demonstrated that fully neutralized reovirus in the form of reoNAb can be delivered in a functional form *in vivo*. Furthermore, tumour-bearing mice treated with GM-CSF followed by pre-formed reoNAb have delayed tumour growth and prolonged survival compared to controls, indicating that reoNAb have therapeutic potential. The therapeutic effect of reoNAb following GM-CSF pre-conditioning in naive mice, was less than we had previously shown using non-complexed reovirus in reovirus-immunized mice. Thus the enhanced therapeutic effect of a pre-existing anti-reoviral immune response(8) can only partly be mediated by reoNAb complexes formed after i.v. reovirus treatment and other immune mechanisms (e.g. ADCC or reovirus-specific CTL) must be involved. The data also suggest that reovirus therapy could be enhanced by manipulation of the antibodies bound to the virus. Whilst a single neutralizing monoclonal antibody was as effective as anti-reoviral serum in mediating delivery of functional virus to tumours, a combination of monoclonal antibodies was significantly more effective (Fig 7A). This suggests the possibility of pre-formed reoNAb complexes as a novel therapeutic in which the antibodies are selected to provide the most efficient viral delivery to tumours.

To our knowledge, this reactivation and release of antibody-neutralized virus by human monocytes has not previously been described and although it may appear counter-intuitive, there is some related evidence supporting our observations. Firstly, dendritic cells release macropinocytosed Ag in a native unprocessed form from late endocytic compartments to stimulate B cells(18) indicating that not all internalized Ag is necessarily degraded by myeloid cells. With regard to FcR involvement, Ab-neutralized adenovirus has been found to mediate gene transfer via an FcR dependent mechanism(19), though there was no viral release from the cells. The reports most closely related to our findings are of antibody-dependent enhancement (ADE) of infection. This occurs during infection with Flaviviruses including dengue virus, whereby patients previously exposed to another dengue virus serotype form non-neutralizing-Ab-virus complexes which are taken up by FcR expressing cells (including monocytes) resulting in enhanced virus infection(20, 21). ADE has also been reported for measles virus(22), another OV currently undergoing clinical trials. However, in contrast to our observations, ADE depends on the cross-reactivity of non-neutralizing antibodies whereas our research highlights a hitherto unidentified role for neutralizing antibodies in mediating viral dissemination.

In conclusion, we have demonstrated a highly novel phenomenon whereby fully antibody-neutralized reovirus is internalized and processed by monocytes resulting in transfer of infectious virions that are able to infect and destroy tumour cells. Taken together with our previous data indicating the positive involvement of anti-viral NAb(8), we suggest that this indicates a paradigm shift with respect to the current dogma regarding the ‘problems’ of anti-viral NAb for OV therapy. That this observation is not specific to reovirus but is likely widely applicable to other OV, increases the significance of our findings. Further research is needed to identify the factors that determine which OV can be delivered in this manner, as this will have a significant impact on the design of future clinical trials.

Materials and Methods

Cell lines
Cell lines were grown in DMEM or RPMI containing L-glutamine (Sigma) supplemented with 10% (v/v) heat-inactivated foetal calf serum (Life Technologies). Cell lines were monitored routinely and found to be free of Mycoplasma infection.

Viruses
Reovirus Type 3 Dearing strain (Reolysin™) supplied by Oncolytics Biotech (Calgary, Canada); Coxsackievirus type A21 (CVA21, CAVATAK™) supplied by Viralytics (Sydney, Australia); Herpes Simplex virus 1716 (HSV1716, Seprehvir™) supplied by Virttu Biologics (Glasgow, UK). Stock virus concentrations were determined by plaque assay on L929 (reovirus), SK-Mel-28 (CVA21), Vero (HSV1716) cells. UV-inactivation of reovirus was by 2 min UV-irradiation of 100 µl aliquots in a 96-well plate, using a Stratalinker UV 1800 (Stratagene); confirmed to be non-replicative by plaque assay.

Patient-derived serum/pleural fluid
Serum was obtained with ethical approval and consent from patients enrolled in clinical trials: for reovirus, the REO13-brain trial (ISRCTN70443973); for CVA21, the STORM trial (NCT02043665). Blood was collected into tubes containing a clotting activator. Samples were centrifuged at 2000 rpm for 10 min, the serum fraction harvested and stored at −70°C. Pleural fluid from patients treated with intrapleural HSV1716 (trial NCT01721018) was a gift from Joe Conner (Virttu Biologics). Where required, serum was heat-inactivated by incubation in a water-bath at 56°C for 30 min.

Complement activity assay
Untreated or heat-inactivated serum samples were diluted in Gelatin Veronal Buffered (GVB++) Saline (Sigma). Increasing volumes were added to vortexed sheep erythrocytes (Stratech) and GVB++ to a final volume of 1.5 ml according to the manufacturer's protocol (CompTech). Negative and positive controls were included to give background and 100% lysis values, respectively. Tubes were placed in a 37°C water bath for 60 min and cells were re-suspended every 10 min then placed on ice and centrifuged for 3 min at 800 g. Supernatants were transferred to a Maxisorp 96 well plate and absorbance at 540 nm was determined using a Multiskan EX plate reader (Thermo).
Percentage lysis = (OD test sample – OD blank)/(OD total lysis – OD blank) x 100.

Neutralization assay
Halving dilutions of serum or pleural fluid were added to 80% confluent monolayers of susceptible cells (see above) in a 96-well plate. Virus was added to achieve an MOI 0.05 (reovirus and CVA21) or MOI 1 (HSV1716). Cell survival was assayed at 72 h by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay.

Preparation of monocytes
PBMC were isolated from healthy donor leukapheresis cones by density-gradient centrifugation on lymphoprep (Axis Shield). $CD14^+$ monocytes were isolated from PBMC by positive selection with anti-CD14 Microbeads; for EM, monocytes were negatively selected from PBMC using the Human Pan Monocyte Isolation kit; $CD16^+$ monocytes were selected from PBMC using the $CD16^+$ Monocyte Isolation kit (all kits from Miltenyi).

Preparation of OV/NAb complexes

For *in vitro* assays, OV was incubated with a pre-determined neutralizing volume of patient-derived serum (reovirus, CVA21) or pleural fluid (HSV1716) for 2-3 h at 37°C. For *in vivo* experiments, the serum was obtained from mice pre-immunized i.p. with two doses of 2 x 10^7 pfu reovirus seven days apart; serum was obtained seven days after the second immunization. The anti-reoviral monoclonal antibodies used to generate the complexes were obtained from DSHB (Iowa) and were clones G5, 10F6, 8H6, 10G10, 10C1. Pre-determined neutralizing volumes were mixed with the virus and incubated for 2-3 h at 37°C.

Co-culture assay
OV, NAb or OVNAb complexes were added to isolated monocytes and incubated at 4°C for 2-3 h. Cells were washed 3x in PBS, re-suspended in RPMI and added to target cells either directly or separated by a 1 µm transwell (Greiner Bio-one) at a ratio of 3:1 (monocytes:targets). They were co-cultured at 37°C for 72 h, unless stated otherwise. Cell viability was analysed by flow cytometry using a LiveDead® stain (Thermo) according to the manufacturer's instructions. Where indicated, Jam-A on the target cells was blocked by pre-incubating with 10 µg/ml anti-Jam-A antibody, clone J10.4 (Santa-Cruz) for 30 min at 37°C. FcR on the monocytes were blocked by pre-treatment with 100 µg/ml anti-FcγR $F(ab')_2$ antibodies (Ancell) or anti-FcαR human recombinant antibody (Miltenyi) at 4°C for 45 minutes.

Depletion of antibody isotypes from serum
Serum was diluted 1:1 in PBS and incubated for 90 min at RT with agarose bead-conjugated antibodies specific for the human γ- or α-chain (Sigma). The samples were then centrifuged to remove beads (3,000 g, 15 s) and the supernatant harvested. Antibody depletion was confirmed by enzyme-linked immunosorbent assay (ELISA) using human IgG/IgA ELISA kits (Mabtech).

Western Blot
Lysates from reovirus-infected (MOI=1) Mel-624 or L929 cells (20 µg protein per lane) were separated by SDS-PAGE electrophoresis, transferred to nitrocellulose, blocked in 5% milk and probed using patient-derived serum (1:200 dilution) as primary antibody. Blots were washed 3x in PBST, and incubated with an HRP-conjugated goat secondary antibody against human IgG, IgA or IgM (all Thermo), diluted 1:5,000 in 5% milk/PBST. After a further three washes, blots were visualised with the chemiluminescent SuperSignal West Pico substrate (Thermo) on a Gel Doc XR system using Image Lab software (Bio-Rad).

Immunoprecipitation of reovirus
Reovirus was added to serum at a 1:5 (v/v) ratio and incubated at 37°C for 3 h. 1.5 ml Eppendorf tubes were blocked with 3% (w/v) bovine serum albumin for 1 h at 4°C, prior to the addition of reovirus-antibody samples. Pre-washed protein A resin beads (GenScript) in excess were mixed with samples and allowed to bind for 2 h at 4°C on a rotator. Samples were centrifuged (400 g, 2 min), washed 4x in 0.1% (v/v) Triton-X in PBS, then boiled (95°C, 5 min) in loading buffer to dissociate IgG from beads, and centrifuged (13,200 g, 2 min) to yield supernatant for analysis.

Electron Microscopy
Visualization of reoNAb complexes. Reovirus stock was dropped onto Veco 100-mesh copper grids (Electron Microscopy Sciences) and allowed to attach (RT, 5 minutes). Grids were washed 4x in PBS prior to incubation (90 min, RT) with patient-derived serum or

control serum, diluted 1:10 in PBS. After 4x washes in PBS, grids were incubated with protein A-conjugated 10 nm gold particles (1:300 in PBS + 1% v/v BSA) for 30 min at RT. After washing (4x PBS, 4x ddH_2O), grids were fixed for 1 h with 1.5% glutaraldehyde in 0.1 M sodium cacodylate. After 4x washes in ddH_2O, grids were negatively stained with 1% phosphotungstic acid for 30 s, then blotted and air-dried. Grids were visualised using an FEI Tecnai TWIN microscope at 120 kV (magnification 52,000 X).

Visualization of reoNAb-loaded monocytes.

Negatively selected monocytes were loaded, with either live reovirus or reoNAb at MOI 50, washed twice with ice-cold PBS and re-suspended in 2% (v/v) PFA + 0.2% (v/v) glutaraldehyde in 0.1 M PHEM buffer; they were then pelleted, re-suspended in storage buffer (0.5% w/v PFA in 0.1 M PHEM) and kept at 4°C prior to processing. Cells were post-fixed for 1 h at 4°C with 1% (w/v) osmium tetroxide in 0.1 M sodium cacodylate buffer, rinsed in buffer and re-suspended in 2% (w/v) agar. 0.5-1 mm^3 blocks were cut, dehydrated in ethanol followed by propylene oxide, then infiltrated with ascending ratios of LX-112 Epon resin/propylene oxide (1 h each) finishing in pure resin. Resin was polymerised at 70°C for 48 h, and 80 nm sections were cut using an Ultracut S microtome (Leica). TEM sections were viewed using an FEI Tecnai TWIN microscope at 120 kV.

RNA sequencing

Monocytes were loaded with live reovirus or reoNAb (MOI 10), re-suspended in complete RPMI and cultured for 24 h, then harvested, RNA extracted using the RNeasy mini kit (Qiagen) according to manufacturer's instructions and treated with DNase I. mRNA libraries were prepared using the NEBNext Ultra Directional RNA library prep kit (New England BioLabs) and sequenced using the HiSeq 2500 system (Illumina). Fastq files were analysed in R using the DEseq2 package (Bioconductor).

In vivo experiments

These were carried out at the University of Leeds or the Mayo Clinic, Rochester MN. All *in vivo* studies were approved by either the Leeds local ethics committee and UK Home Office or the Mayo IACUC. Six- to eight-week-old female C57Bl/6 mice were purchased from Charles River Laboratories (Margate, Kent) or Jackson Laboratories (Bar Harbor, Maine). Mice were challenged subcutaneously with 5×10^5 B16 melanoma cells. One treatment cycle of GM-CSF/reoNAb = 300 ng GM-CSF i.p. on 3 consecutive days followed by 2 x 10^7 pfu reoNAb complexes i.v on the following two days. Reovirus delivery: one cycle of treatment was given to mice bearing 7 d established tumours. Tumours were harvested on day 14, weighed and divided for analysis by plaque assay and qRT-PCR. For plaque assay, the tumour sample was homogenized and subjected to 3 cycles of freeze/thaw, then clarified by centrifugation and viral titre determined by plaque assay on L929 cells. For qRT-PCR, RNA was extracted using the RNeasy mini kit (Qiagen) according to manufacturer's instructions. cDNA synthesis was carried out using the SuperScript IV first-strand system (Thermo) according to manufacturer's instructions. Analysis was conducted using the ABI 7500 real-time system (Applied Biosystems) and reovirus S3 copy number was quantified using the ΔΔCT method against GAPDH as comparator. Therapy studies: mice bearing 3 d established tumours were given one treatment cycle as described above. Tumours were measured three times per week, and mice were euthanized when tumours reached 1 cm diameter.

Statistical Analysis

Data were analysed using GraphPad Prism software. Significance was evaluated using Student's t-test (multiple comparisons with Holm-Sidak correction), chi-squared test or one-way ANOVA (with Tukey correction) as appropriate, with $p < 0.05$ considered significant. Survival analysis was carried out using the log rank test.

Supplementary materials

Fig. S1. Patient-derived serum is highly neutralizing
Fig. S2. Patient-derived neutralizing antibodies favour routing of virus to CD14+ cells in whole blood
Fig. S3. UV-inactivated reovirus induces monocyte activation
Fig. S4. Hand-off of reoNAb by monocytes is contact-dependent
Fig. S5. Expression of Fc receptors on primary human monocytes
Fig. S6. FcγRIII receptor blockade impairs reo/NAb loading of monocytes
Fig. S7. Reo-NAb induce increased FcR RNA levels in monocytes
Fig. S8. Serum from mice immunized against reovirus is highly neutralizing

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Authors' Contributions

Conception and design: EJI, RAB, AAMelcher, RCH
Data acquisition and analysis: RAB, AAMulder, DvdW, EJI, LPS, TJK, JMT, RCH
Writing, review and/or revision of the manuscript: EJI, RAB, LPS, RCH, AAMelcher, RGV
Material support: MCC

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Supplementary Figures:

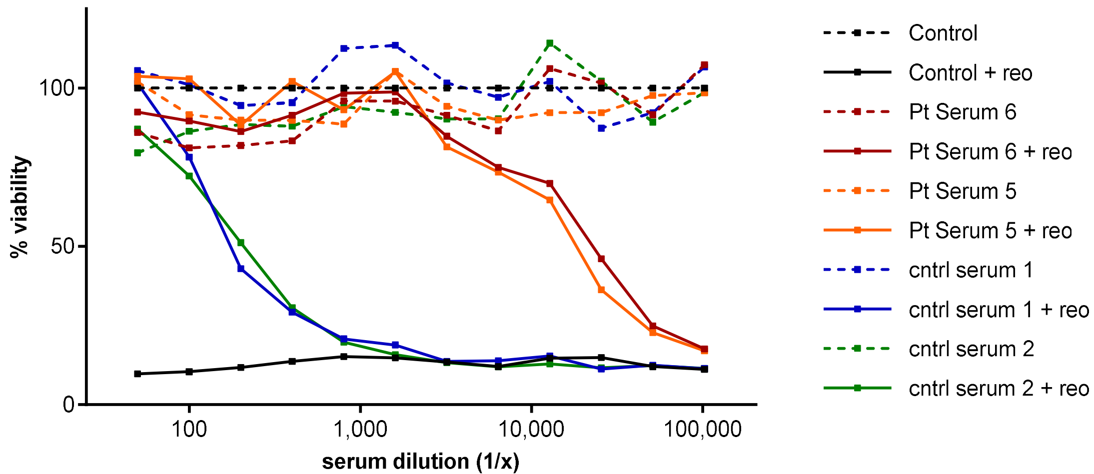


Fig. S1. Patient-derived serum is more highly neutralizing than normal donor serum
A standard MTT neutralization assay on L929 cells, using serum from two normal donors and two patients. The patient samples had a much higher neutralizing capacity than the normal donor samples, indicating a higher titre of anti-reovirus neutralizing antibodies.

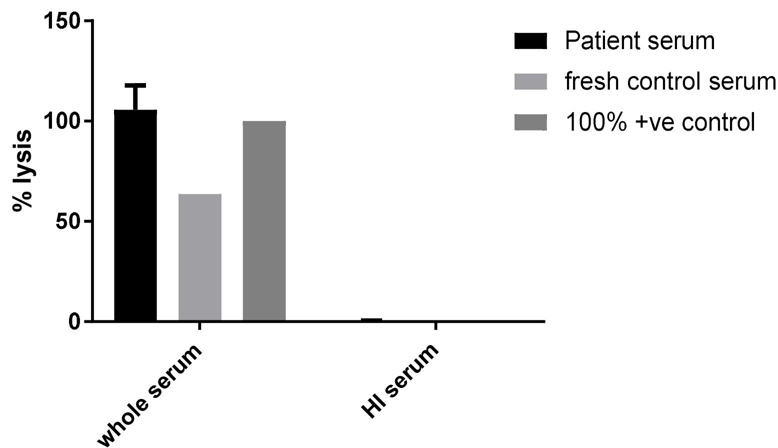


Fig. S2. Complement is active in the patient-derived serum samples
Patient-derived serum samples were left untreated or heat-inactivated and complement activity was determined from the level of lysis induced in antibody-coated sheep erythrocytes. The graph shows mean % lysis +SD from 4 patient samples and one fresh sample from a normal donor, compared to a 100% positive control. Complement was active in all the serum samples tested and the activity was completely removed by heat-inactivation.

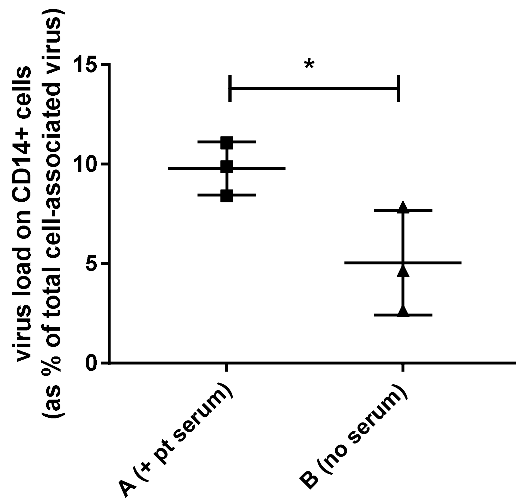


Fig. S3. Patient-derived neutralizing antibodies favour routing of virus to CD14$^+$ cells in whole blood

Whole blood from healthy donors was combined with patient-derived serum and incubated with reovirus for 30 min at 37°C. The CD14-positive and -negative populations were then selected and virus titre determined by plaque assay. Graph shows virus load on the CD14-positive population as a percentage of the total cell-associated virus. Mean ± SD from three individual donors are shown; *p = 0.049, Student's t-test.

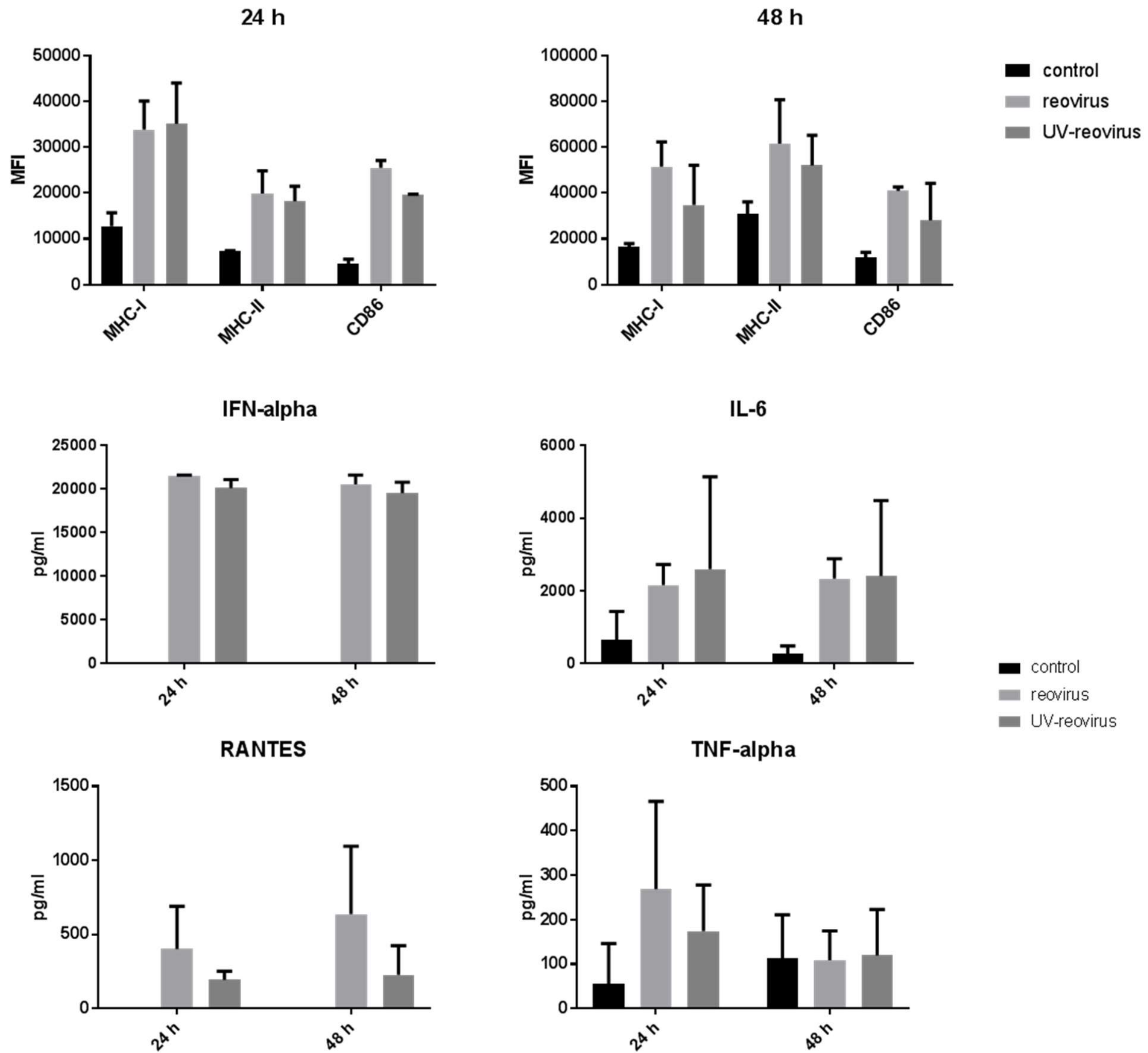


Fig. S4. UV-inactivated reovirus induces monocyte activation
Monocytes were treated ± reovirus at MOI 5. Cells and supernatants were harvested at 24 and 48 h and examined for expression of maturation markers as indicated (A) and cytokine secretion (B). Graphs show mean values +SD from three independent experiments. UV-inactivated reovirus induces similar changes in phenotypic and cytokine profiles as live reovirus.

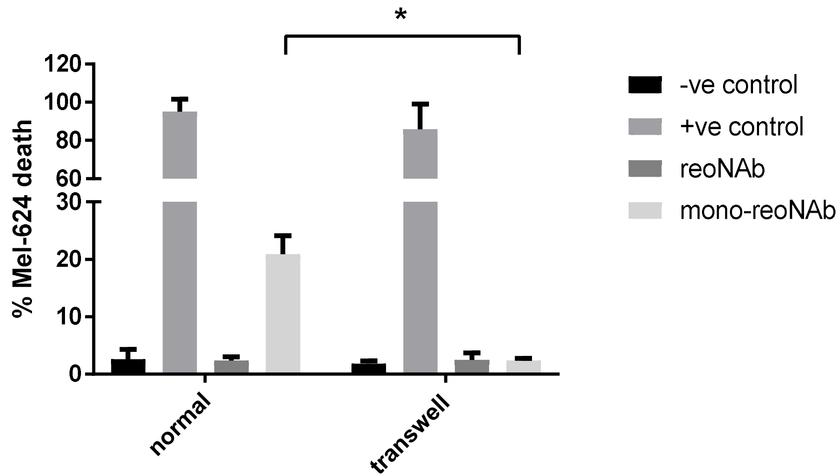


Fig. S5. Hand-off of reoNAb by monocytes is contact-dependent
Monocytes were loaded with reoNAb and co-cultured with Mel-624 cells either in the presence or absence of a transwell (monocytes added to transwell). Cells were harvested at 72 h and stained with Live/Dead®. Cell viability was analysed by FACS. Graph shows mean +SD from 3 independent experiments, *p = 0.0124.

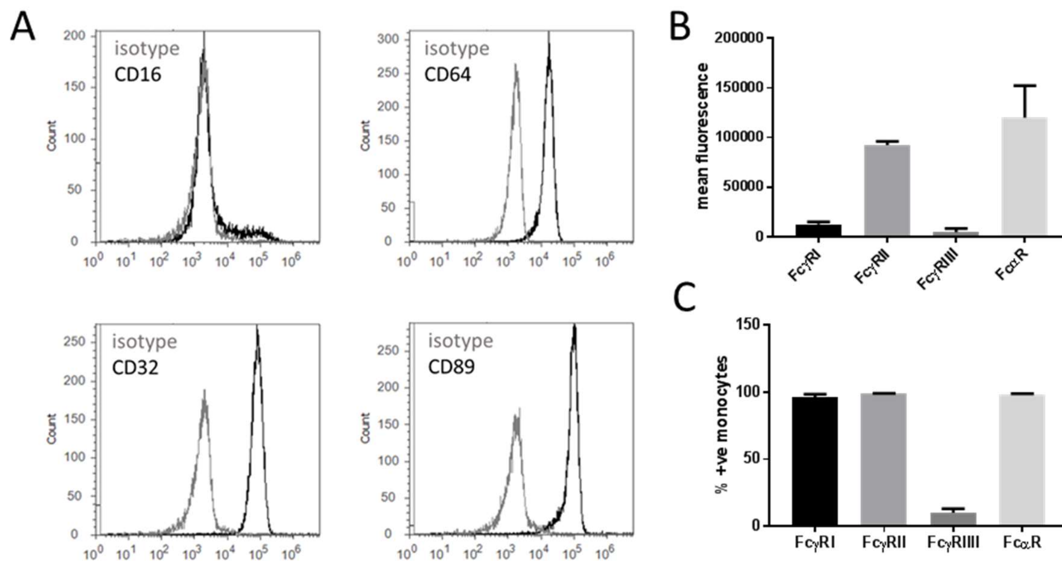


Fig. S6. Expression of Fc receptors on primary human monocytes
A) Following CD14 selection, monocytes were stained with fluorophore-conjugated antibodies against specific Fc receptors CD16, CD32, CD64 or CD89 (red), or isotype control (grey) and surface expression was examined by FACS. The percentage of cells staining positively for each marker is indicated. Plots are representative of 4 donors tested. B) Pooled median fluorescence values +SD for each FcR. C) Pooled % monocytes positive for FcγR expression +SD.

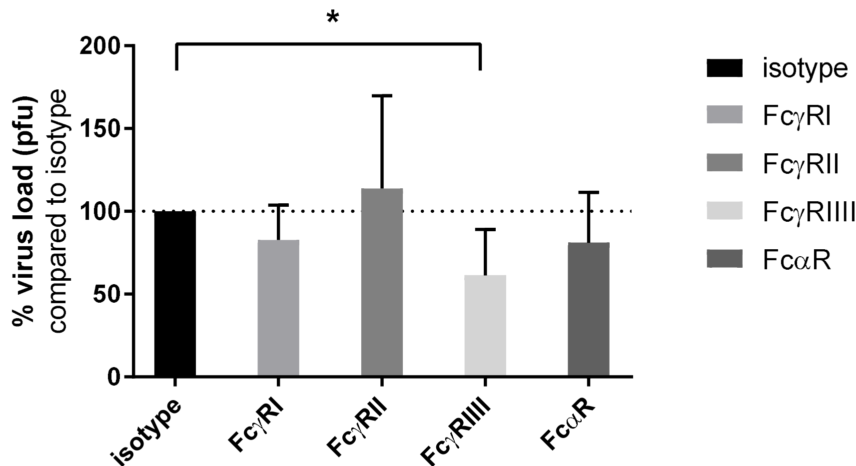
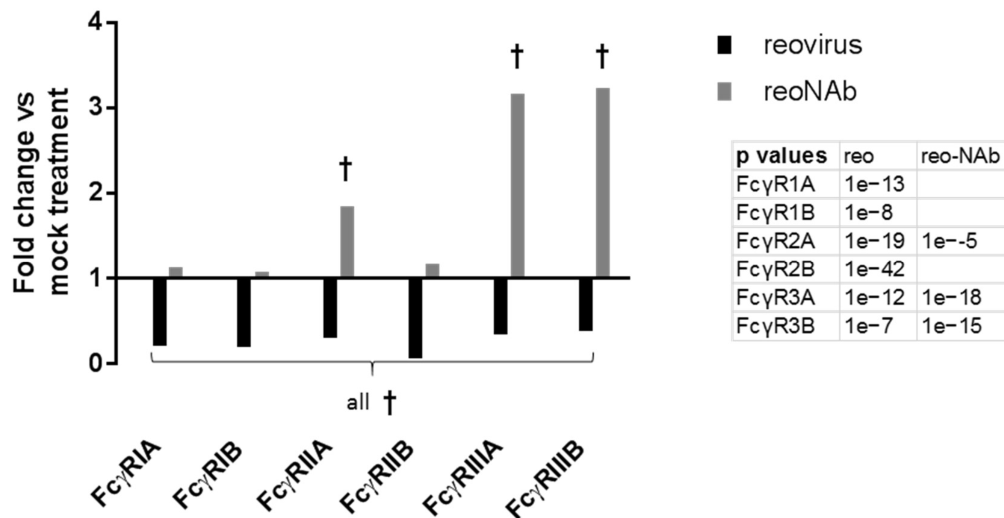


Fig. S7. FcγRIII receptor blockade impairs reo/NAb loading of monocytes
CD14-selected monocytes from healthy donors were treated with Fc receptor-blocking monoclonal antibodies, or isotype control (100 µg/ml) for 45 minutes at 4°C. Monocytes were then loaded with reo/NAb (MOI 10) and washed to remove unbound complexes. Virus load on the cells was determined by plaque assay. Data +SD from four donors are shown; *p = 0.031 by Student's t-test.



p values	reo	reo-NAb
FcγR1A	1e−13	
FcγR1B	1e−8	
FcγR2A	1e−19	1e−-5
FcγR2B	1e−42	
FcγR3A	1e−12	1e−18
FcγR3B	1e−7	1e−15

Fig. S8. Reo-NAb induce increased FcR RNA levels in monocytes
CD14-selected monocytes were treated with reovirus or reoNAb at MOI 10 (24 h). Cells were processed for RNA and differential gene analysis conducted by RNAseq. The fold change in expression of Fc receptor gene transcripts compared to mock-treated cells is shown. Values represent the mean of three monocyte donor samples. † indicates an adjusted *p* value < 0.0001, (see table) versus mock, by Wald test with Benjamini-Hochberg adjustment using the Enrichr platform.

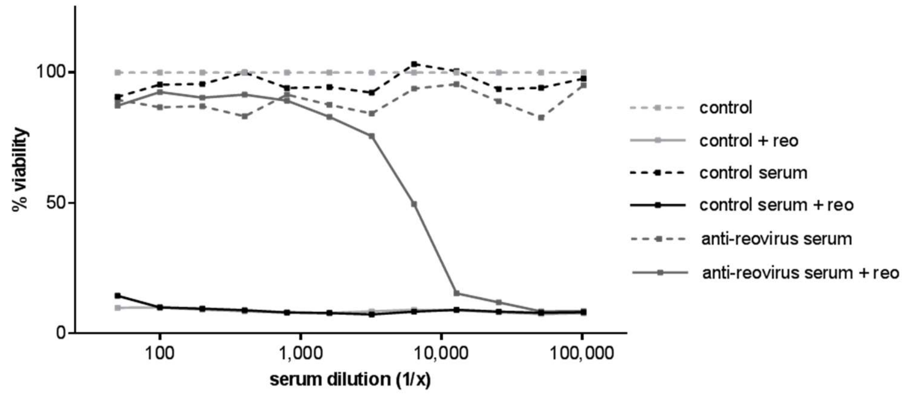


Fig. S9. Serum from mice immunized against reovirus is highly neutralizing
C57Bl/6 mice were immunized with two i.p. injections of 2 x 10^6 pfu reovirus one week apart. After a further week, the mice were sacrificed, blood was collected by cardiac puncture, pooled and the serum harvested. The serum was tested for its capacity to neutralize reovirus in a standard MTT neutralization assay and found to be highly neutralizing compared to serum from control, non-immunized mice.