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Berkeley, RA, Steele, LP, Mulder, AA et al. (8 more authors) (2018) Antibody-Neutralized Reovirus Is Effective in Oncolytic Virotherapy. Cancer Immunology Research, 6 (10). pp. 1161-1173. ISSN 2326-6066

https://doi.org/10.1158/2326-6066.cir-18-0309

Antibody-neutralized reovirus is effective in oncolytic virotherapy

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Running title
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Disclosure of Potential Conflict of Interest
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 promise for otherwise incurable cancers. Oncolytic viruses (OVs), developed as direct cytotoxic agents, mediate their anti-tumor effects via activation of the immune system. However, OVs also stimulate anti-viral immune responses including the induction of OV-neutralizing antibodies. Current dogma suggests that the presence of 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 tumor cells resulting in tumor cell 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 implications for oncolytic virotherapy and for the design of clinical OV treatment strategies.

Introduction

The use of oncolytic virus (OV) therapy (a recognized form of immunotherapy) is progressing 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, OVs are not used as widely as other types of immunotherapy such as checkpoint inhibitors, possibly owing to the perception that systemic administration will be limited by neutralizing antibodies (NAb). NAb may be present at baseline for viruses prevalent in the human population, e.g. herpes simplex virus type 1, and mammalian orthoreovirus type 3 (herein referred to as "reovirus"). NAb may also arise following initial doses of OV therapy. Such concerns potentially limit systemic OV

therapeutic strategies to a 'one shot' approach, whereby patients receive a single high dose of OV(2), or to direct OV injection into tumors. Indeed, FDA approval for T-VEC is for intra-tumoral (i.t.) delivery only. Although this route ensures viral access to the tumor, it is technically challenging and limits treatment to readily accessible tumors. Systemic delivery is safe, broadly applicable in a clinical setting, and more suitable for targeting visceral or widespread metastatic disease. We and others have previously investigated an 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 indicated a potential positive role for NAb in OV therapy.

We showed that i.t. delivery of single agent reovirus was more effective as an anti-tumor therapy in mice than systemically administered reovirus(5). However, immune cells (T cells or dendritic cells) loaded with reovirus *ex vivo* and administered systemically, delivered virus to tumors, even in the presence of anti-reovirus NAb(3, 6). The results of a translational biological endpoint clinical trial (REO13), in patients with colorectal liver metastases, indicated that free reovirus delivered systemically without cell carriage, could access tumors, and that functional virus was associated with immune cells in the blood but was not found in plasma(7). These data suggest that, although free reovirus is neutralized by NAb in the serum following intravenous (i.v.) delivery, replication-competent virus can be transported to tumors 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). 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 tumor cell targets. Antibody-neutralized reovirus was unable to infect and kill tumor cells directly, but when loaded onto human monocytes it was delivered to melanoma cells in a functional/replicative form which resulted 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), predominantly FcγRIII expressed on non-classical monocytes. These data indicate that circulating monocytes may be pivotal in preserving the therapeutic potency of OVs, despite pre-existing antiviral immunity.

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 using Mycoalert (Lonza) and found to be free of Mycoplasma infection (last test Aug 2017). Cell lines Mel-624, SkMel-28, PC-3 and SKOV-3 were obtained from the CRUK cell bank in 2003. Mel-624, SKMel-28 and SKOV-3 cells were re-authenticated in 2012 using STR profiling and comparison with the DSMZ database, in the absence of a reference profile within the DSMZ database, cell lines were shown to have an original STR profile which was distinct from all other cell lines within the database. PC-3 cells have not been re-authenticated. Vero, L929 and HCT116 cells were obtained from ATCC in 2008,

2012 and 2013 respectively and have not been re-authenticated. All cell lines were stored in liquid nitrogen. After thawing, cells were routinely passaged twice per week for no more than 20 weeks.

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 from patients enrolled in clinical trials: for reovirus, the REO13-brain trial (ISRCTN70443973); for CVA21, the STORM trial (NCT02043665). All patients gave written informed consent according to good clinical practice guidelines. Protocol, patient information sheet, and consent forms were approved by the United Kingdom Medicines and Healthcare products Regulatory Authority, regional ethics review committee, and institutional review board at St James's University Hospital. 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 reovirus-specific 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
OVs, NAb or OV/NAb 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, clone J10.4 (Santa-Cruz) for 30 min at 37°C. FcR on the monocytes were blocked by pre-treatment with 100 µg/ml $F(ab')_2$ fragment antibodies specific for FcγR (Ancell) or human recombinant anti-FcαR (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 tumors. Tumors were harvested on day 14, weighed and divided for analysis by plaque assay and qRT-PCR. For plaque assay, the tumor sample was homogenized and subjected to 3 cycles of freeze/thaw, then clarified by centrifugation and viral titer 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 $\Delta\Delta$CT method against GAPDH as comparator. Therapy studies: mice bearing 3 d established tumors were given one treatment cycle as described above. Tumors were measured three times per week, and mice were euthanized when tumors 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.

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 (1×10^{10} $TCID_{50}$) as monotherapy prior to surgical resection of brain tumors (primary or metastatic). Blood samples were taken at least 7 days post reovirus treatment to ensure a high titer 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 (Supplementary 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 secondary antibodies against human IgG, IgA or IgM. 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 investigated this via heat inactivation (HI) of patient-derived serum. Figure 1D shows that heat inactivation did not affect the neutralizing capacity of serum, suggesting that heat-labile factors such as complement do not neutralize reovirus *in vitro*. Complement activity within patient-derived serum was verified (Supplementary Fig. S1B).

Patients receiving i.v. therapeutic doses of reovirus develop a high reovirus specific antibody titer, with IgG and IgA antibodies but not complement contributing to virus neutralization.

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 bound by NAb to form reoNAb complexes which were delivered to tumors 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). More gold particles were associated with the reovirus following incubation with patient-derived serum (76%) than with control serum (40%) (Fig. 2A-C). Some anti-reoviral NAb in control serum is expected, as most people have had prior exposure to the virus(10-12). Our data are consistent with our previous clinical trial, in which NAb were present at baseline in patients, but increased 100-1000 fold after i.v. reovirus administration(7).

Thus, the reovirus specific antibodies present in patient-derived serum can bind reovirus *in vitro* producing reoNAb complexes. ReoNAb complexes would be formed *in vivo* following systemic reovirus therapy, and concentrations would increase upon repeat reovirus administration.

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

To determine whether monocytes might be capable of delivering reoNAb to tumors in patients, we assessed the association of reovirus with human monocytes in the presence of neutralizing serum. 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 (Supplementary 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. The ability of these monocytes to induce tumor 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 only with reoNAb complexes showed no loss of viability compared with controls; however, when tumor cells were cultured with monocytes carrying reoNAb complexes, significant cell death was observed (Fig. 3B and C). Monocytes loaded with non-neutralized reovirus induced more Mel-624 death than those loaded with reoNAb complexes (monocytes loaded with reovirus induced mean 96.5 ± 0.40% cell death, those loaded with reoNAb induced mean 81 ± 2.74% cell death). Mel-624 cells cultured with monocytes alone showed no loss of viability or reduction in growth rate (Fig 3B and C).

These results show that reovirus was fully neutralized within the reoNAb complexes but following loading onto monocytes, the complexes induced tumor cell death.

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

The observed tumor cell death could be mediated either by the monocytes themselves, following their activation by reoNAb complexes, or by release or transfer of infectious reovirus from the monocytes. Therefore, reoNAb complexes were generated using either live or UV-inactivated reovirus; both activate monocytes (Supplementary Fig. S3) but UV-inactivated reovirus is unable to infect and kill tumor cells directly(13). Monocytes loaded with UV-reoNAb complexes abrogated melanoma cell death following co-culture (Fig. 4A) suggesting that tumor cell death was due to reovirus infection and replication, rather than monocyte cytotoxicity. In support of this, reovirus titer within monocyte-reoNAb and tumor 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 tumor targets with a transwell abrogated cell death (Supplementary Fig. S4), suggesting that initial hand-over from the monocytes was contact dependent and that JAM-A was required for later viral spread between tumor cells. Reovirus replication occurred predominantly within tumor cells rather than monocytes, as reovirus titer 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, which do support reovirus replication(3).

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

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 tumor cells(6). Here we investigated the fate of reoNAb complexes following their loading onto monocytes. EM demonstrated that reoNAb complexes were internalized by monocytes (Fig. 5A). Free reovirus was also internalized by monocytes but this appeared less efficient than reoNAb internalization, as some non-complexed virus particles remained on the monocyte surface following loading with non-complexed reovirus, whereas no reoNAb complexes were visible on the surface (Fig. 5B).

Having previously demonstrated that Fc receptors (FcR) were involved in the delivery of reovirus to tumors 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 (Supplementary 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 tumor cells. By contrast, blocking FcγRIII significantly reduced reovirus loading onto monocytes (Supplementary Fig. S6) and also melanoma cell death following co-culture (Fig. 5C). Non-classical $CD16^+$ monocytes represent only a small fraction (approx. 10%) of the monocytic population. To analyze the involvement 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 free, non-complexed reovirus efficiently (Fig. 5D). Furthermore, RNA sequencing data showed that FcγR mRNA was up-regulated in monocytes loaded with reoNAb complexes, FcγRIII mRNA showing the greatest increase (Supplementary Fig. S7). FcαR may also mediate uptake of reoNAb by monocytes but the effect 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 tumor cell death was not abrogated by blocking these receptors.

The efficacy of reoNAb complexes is applicable beyond reovirus and melanoma cells

In order to show that the delivery of reoNAb complexes to tumor cells via monocytes was not melanoma-specific, tumor cells of other histological types were tested. ReoNAb complexes loaded onto monocytes were delivered to colorectal, prostate and ovarian tumor 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) tumors(8) or intra-cranial GL-261 (glioma) tumors(14). Thus, the therapeutic efficacy of anti-reoviral NAb is likely to be applicable over a range of tumor types.

Various OVs are candidates as therapeutic agents, therefore we asked whether antibodies against other OVs could contribute to therapy. Serum or pleural fluid was obtained from patients undergoing clinical trials with Coxsackievirus (CVA-21) or herpes simplex virus (HSV1716) and used to generate CVA/NAb and HSV/NAb complexes. Both of these OV/NAb complexes were ineffective when cultured directly with melanoma targets, indicating complete neutralization of the viruses. Following loading onto monocytes, CVA/NAb were comparable to reoNAb in mediating tumor cell death, whereas HSV/NAb complexes were ineffective (Fig. 6B). Thus anti-OV NAb may be useful in some but not all oncolytic virotherapies.

ReoNAb complexes deliver functional reovirus to tumors in vivo

Although we have demonstrated the importance of reovirus specific 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 tumor-bearing mice. Therefore, we used serum from mice that had been pre-immunized against reovirus – high anti-reoviral NAb (Supplementary Fig. S8) – to generate reoNAb complexes. These were injected i.v., with or without prior GM-CSF conditioning, into tumor-bearing mice. After three days the tumors were harvested and examined for functional reovirus by plaque assay. Functional reovirus was detectable within the tumors 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 antibody neutralization, functional reovirus can access tumors *in vivo*. These results are consistent with our previous data showing that i.v. administration of reovirus was not therapeutic in tumor-bearing mice unless the mice had been pre-conditioned with GM-CSF(8). Furthermore, administration of GM-CSF followed by reoNAb complexes delayed tumor growth and increased survival in tumor-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 systemic anti-reoviral NAb have an additional role in mediating therapy.

ReoNAb complexes formed using a reovirus-specific 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 dependent 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.

Discussion

Intravenous delivery of an oncolytic virus represents not only an optimal means of accessing disseminated neoplastic tissue, but also a practical way of stimulating a systemic response from the immune system. However, this route of infusion is often eschewed in favor of more local methods given the many hurdles to viral persistence present in the vasculature, for example the presence of neutralizing antibodies. As sero-prevalence for reovirus is common, in most individuals any i.v.-administered virus will encounter some 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 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 generation of virus-specific antibodies in circulation at high titer(15, 16), which is considered to preclude further therapeutic i.v. doses.

Our previous work, which focused on potentiating the delivery of reovirus to tumors by evasion of the anti-reoviral NAb response, 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 tumor 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. There was 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.

We generated ReoNAb complexes by incubating reovirus with a neutralizing volume of serum and confirmed their formation by EM. 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. The mechanism by which the reoNAb complexes are processed by monocytes and transferred to tumor 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. 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 some viral 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 examined the delivery of reoNAb complexes to other tumor cell lines and found that it is not restricted to melanoma, suggesting the applicability of our findings in influencing treatment design for cancer patients in general. Furthermore, we have demonstrated that the phenomenon of reoNAb delivery is not reovirus-specific because CVA/NAb complexes are delivered to tumor cells by monocytes in a similar manner, although HSV/NAb are not, suggesting that specific aspects of virus physiology may determine applicability. It is unclear which aspects govern the delivery of OVs via NAb complexes but given our observations with reovirus, CVA and HSV1716, one possibility is the presence or absence 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 tumor-bearing mice, functional reovirus can be retrieved from the tumors (Fig. 7), supporting our hypothesis that i.v. reovirus therapy in pre-immunized mice results in the formation of reoNAb complexes *in vivo* which are then delivered to tumors via monocytes(8). Although we know that following i.v. delivery, reovirus is 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 tumors. Although we have not ruled out this possibility, we have demonstrated that neutralized reovirus in the form of reoNAb can be delivered in a functional form *in vivo*. Furthermore, tumor-bearing mice treated with GM-CSF followed by pre-formed reoNAb have delayed tumor 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. We found that although a single neutralizing monoclonal antibody was as effective as anti-reoviral serum in mediating delivery of functional virus to tumors, a combination of monoclonal antibodies was significantly more effective. This suggests the possibility of pre-formed reoNAb complexes as a therapeutic in which the antibodies are selected to provide the most efficient viral delivery to tumors.

Although this reactivation and release of antibody-neutralized virus by human monocytes may appear counter-intuitive, there is some related evidence supporting our observations. Firstly, dendritic cells release macropinocytosed antigen in a native unprocessed form from late endocytic compartments to stimulate B cells(18) indicating that not all internalized antigen 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 that antibody-neutralized reovirus is internalized and processed by monocytes resulting in transfer of infectious virions that are able to infect and destroy tumor cells. Taken together with our previous data indicating the positive involvement of anti-viral NAb(8), we suggest that this provides a rationale for exploiting anti-viral NAb in OV therapy. Our results show that this approach is not specific to reovirus. Further research is needed to identify the factors that determine which OVs can be delivered in this manner.

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Writing, review and/or revision of the manuscript: EJI, RAB, LPS, RCH, AAMelcher, RGV
Material support: MCC

Acknowledgements

We thank Nik Matthews, Ritika Chauhan and James Campbell for assistance with transcriptomic profiling and analysis. This work was supported by a University of Leeds PhD scholarship award to Rob Berkeley. Publication costs were covered by Oncolytics Biotech.

Figure Legends

Fig. 1. Reovirus is neutralized by IgG and IgA antibodies in patient-derived serum
A) Reovirus neutralization assay. Each color 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. Graph is representative of two independent experiments. **B**) Western blot of mock or reovirus-infected lysates using patient-derived serum as primary antibody and as secondary, antibodies specific for human IgG/IgA. 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). Graph is representative of two independent experiments with different patient sera. **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). Graph is representative of two independent experiments with two different patient sera (four sera in all).

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, over 340 virions were scored 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 χ^2 test (χ^2 = 92.9, cut-off 6.6 where df = 1).

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

A) Schematic of the hand-off assay. **B**) 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. Images are representative of three independent experiments. **C**) Mel-624 cells were cultured for 72 h with reovirus or monocytes loaded ± reovirus or reoNAb at MOI 1 (MOI 3 wrt tumor cells) (i) or at varying MOI (ii). Cells were harvested, stained with Live/Dead® stain and examined by flow cytometry. Flow cytometry gates were set based on size and granularity which allowed monocytes to be excluded from the analysis and the percentage of viable tumor cells was determined. Graphs show mean +SD from three independent experiments; ****p = 0.00000094.

Fig. 4. Infectious reovirus mediates the killing of tumor 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 eight independent experiments; ****p = 0.000000245, Student's *t*-test. **B**) Monocytes were loaded with reoNAb (MOI 10) and added to Mel-624 cultures. These were harvested at the times shown and viral titer 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 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 titer in the cells (i) and culture supernatants (ii) was determined by plaque assay. Graphs show mean ±SD from three independent experiments.

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. Images are representative of at least 20 cells examined per condition. **C**) Monocytes were pre-incubated with antibodies specific for FcγR or human recombinant FcαR 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.

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

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.

Fig. 7. ReoNAb complexes deliver functional reovirus to tumors in vivo
A) Mice bearing 7 d established B16 tumors received 1 cycle of GM-CSF/reoNAb. ReoNAb were generated using either anti-reovirus mouse serum, a single reovirus-specific monoclonal antibody (G5), or a combination of reovirus specific monoclonal antibodies (combo). Tumors were harvested on day 14 and viral titer determined by plaque assay. Graph shows values for individual mice and mean ±SD pfu/mg of tumor; *p = 0.045, one way ANOVA. **B&C**) Mice bearing 3 d B16 tumors (8 per group) were treated as above. Mice were sacrificed when tumors reached 1 cm in diameter; GM-CSF/reoNAb vs control p = 0.022, log rank test.

Figures

Figure 1

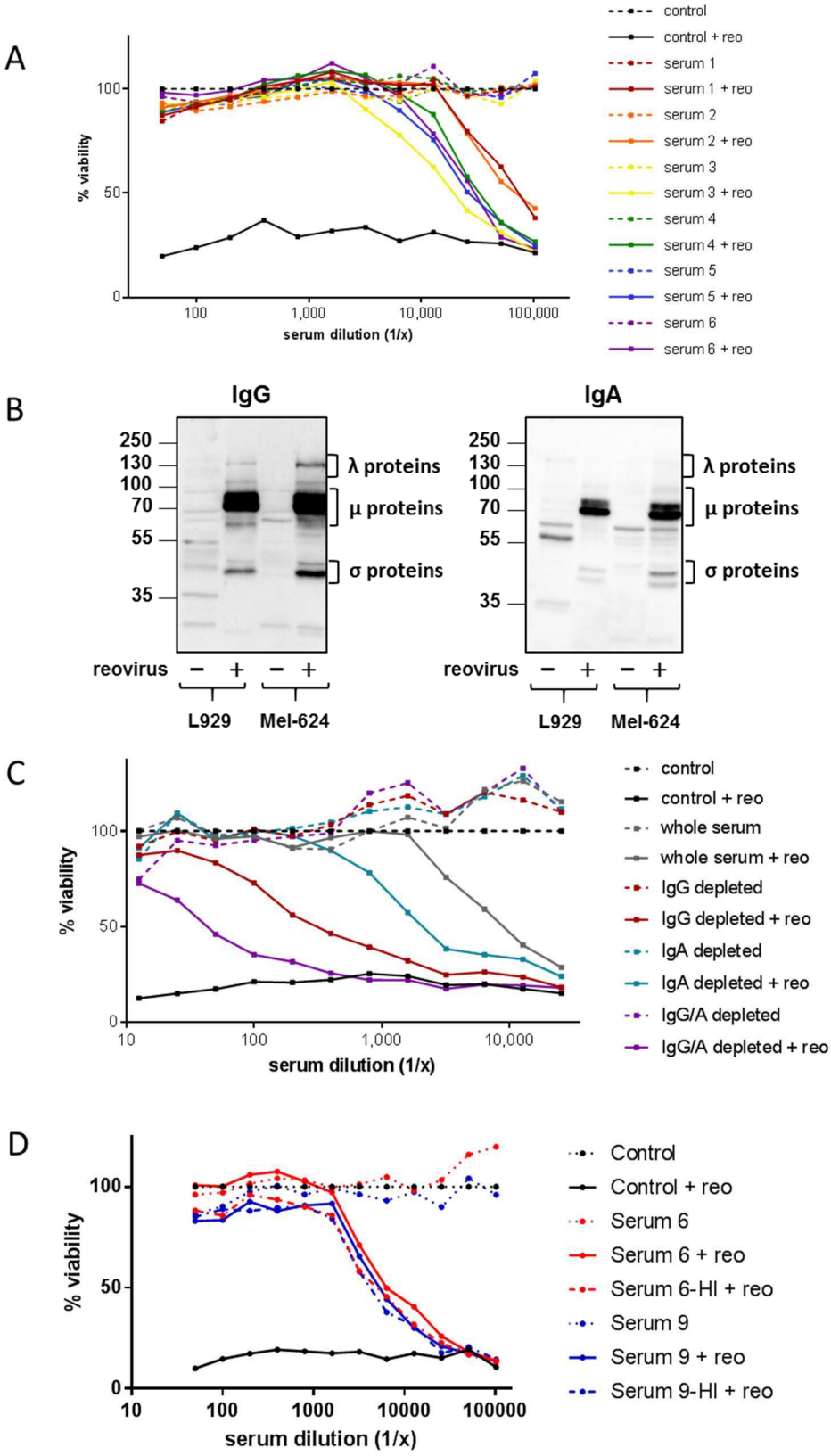
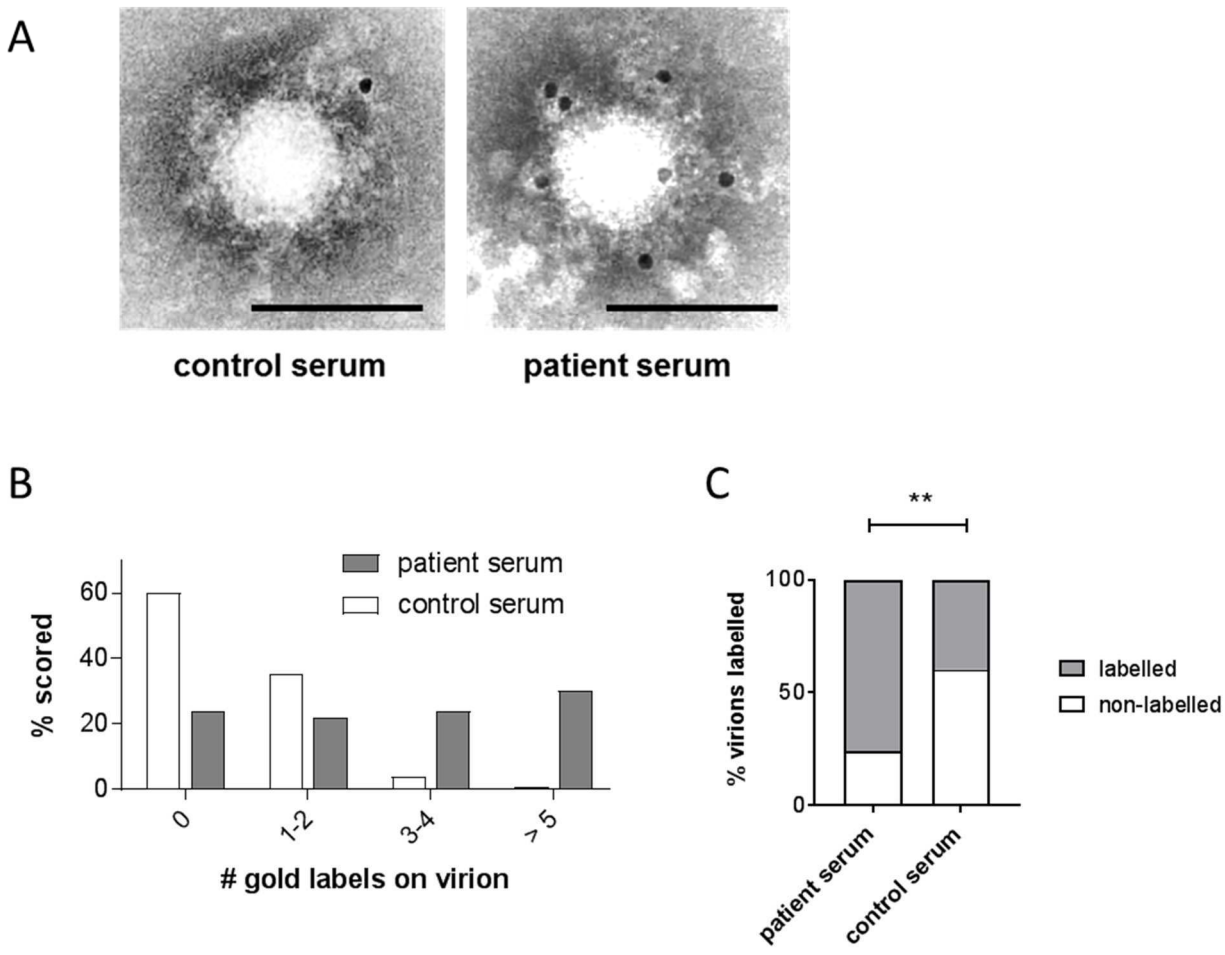


Figure 2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control serum
patient serum
B
patient serum
control serum
% scored
60
40
20
0
0
1-2
3-4
>5
gold labels on virion
C
**
100
50
0
% virions labelled
labelled
non-labelled
patient serum
control serum

Figure 3

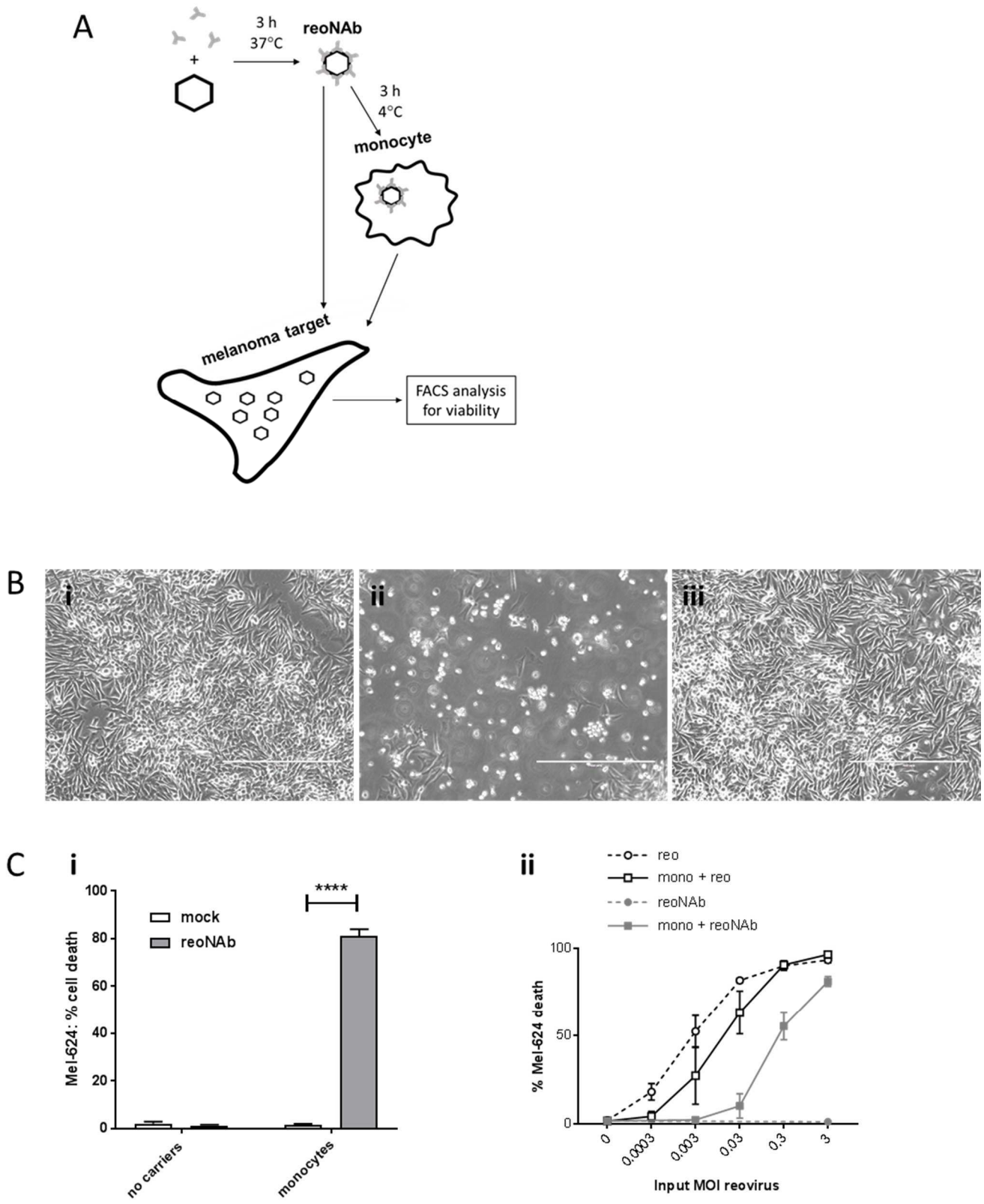

A
3 h
37°C
reoNAb
+
3 h
4°C
monocyte
melanoma target
FACS analysis
for viability
B
i
ii
iii
C
i
Mel-624: % cell death
100
80
60
40
20
0
mock
reoNAb
****
no carriers
monocytes
ii
reo
mono + reo
reoNAb
mono + reoNAb
% Mel-624 death
100
50
0
0
0.0003
0.003
0.03
0.3
3
Input MOI reovirus

Figure 4

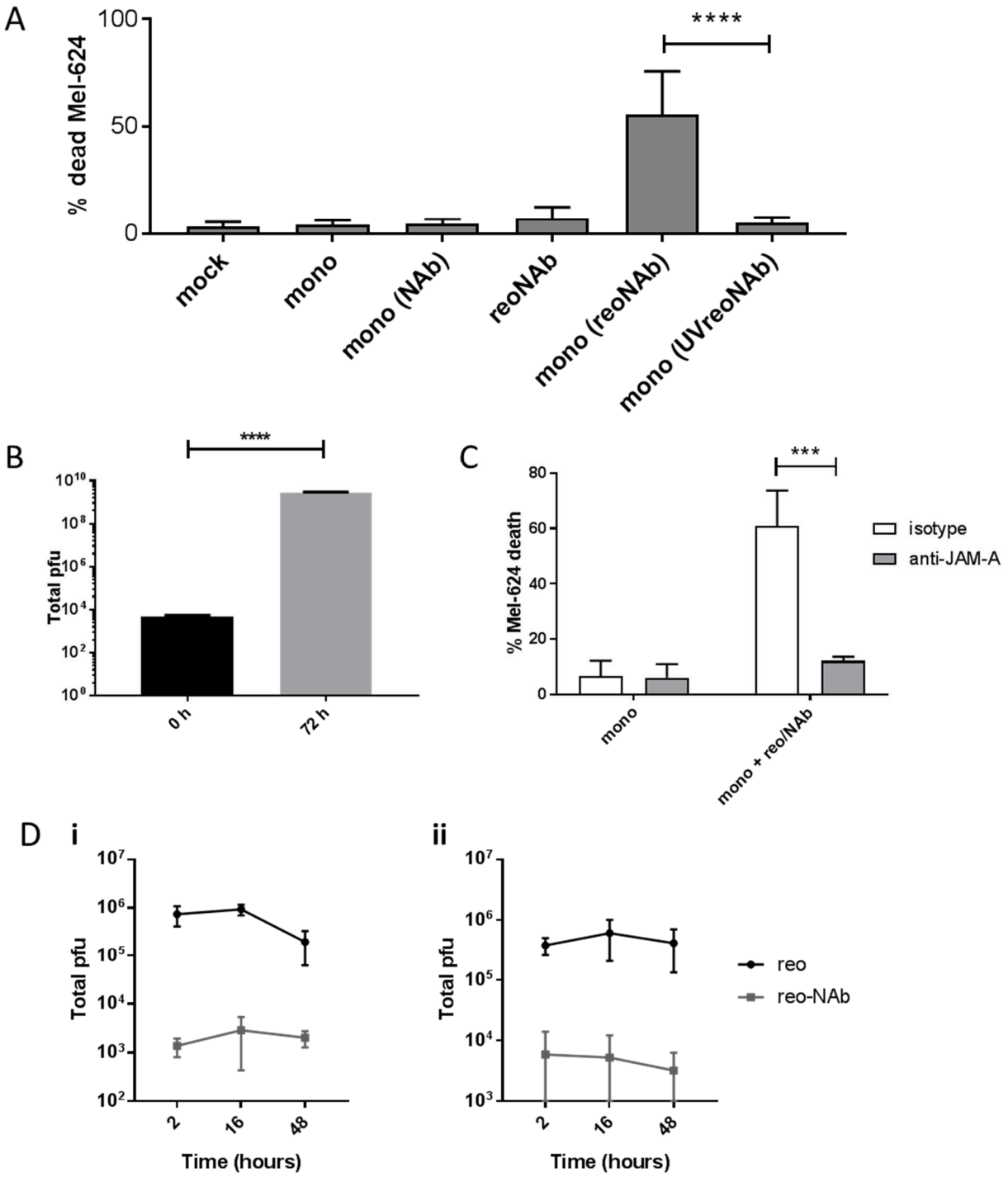

A
% dead Mel-624
100
50
0
****
mock
mono
mono (NAb)
reoNAb
mono (reoNAb)
mono (UVreoNAb)
B
****
Total pfu
0 h
72 h
C
***
% Mel-624 death
isotype
anti-JAM-A
mono
mono + reo/NAb
D
i
ii
Total pfu
Time (hours)
reo
reo-NAb

Figure 5

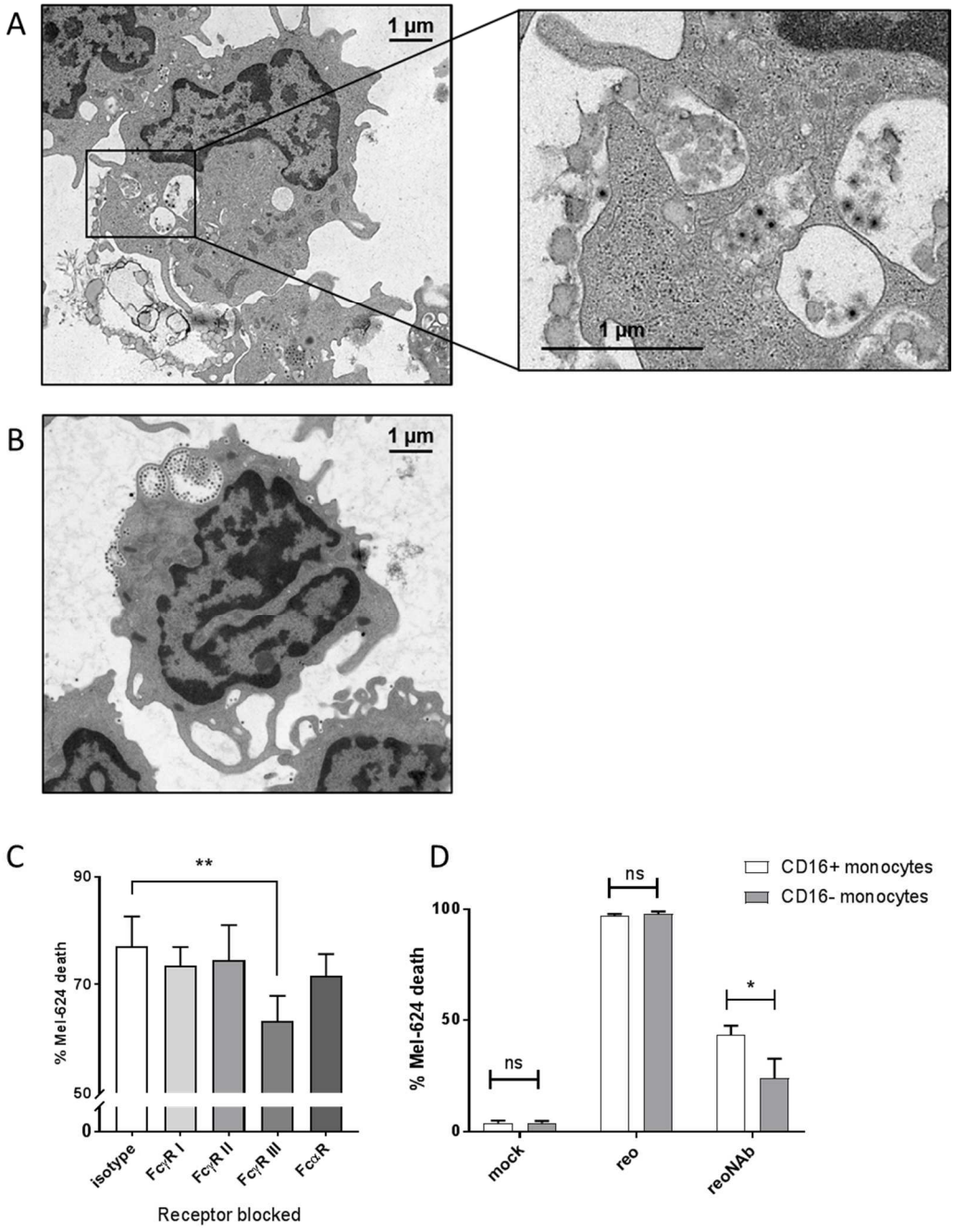

A
1 µm
1 µm
B
1 µm
C
**
90
70
50
0
% Mel-624 death
isotype
FcγR I
FcγR II
FcγR III
FcαR
Receptor blocked
D
CD16+ monocytes
CD16- monocytes
ns
ns
*
100
50
0
% Mel-624 death
mock
reo
reoNAb

Figure 6

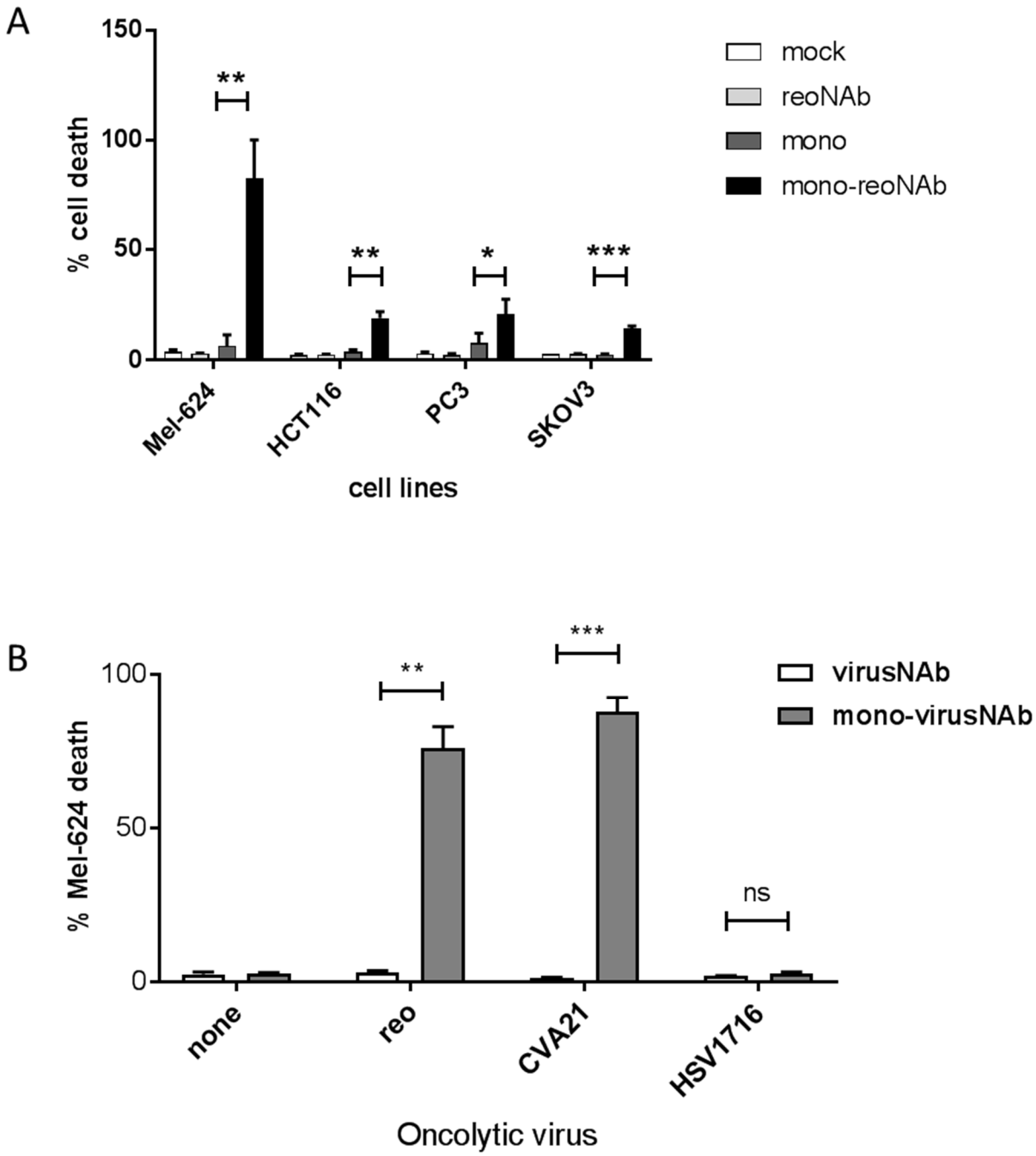
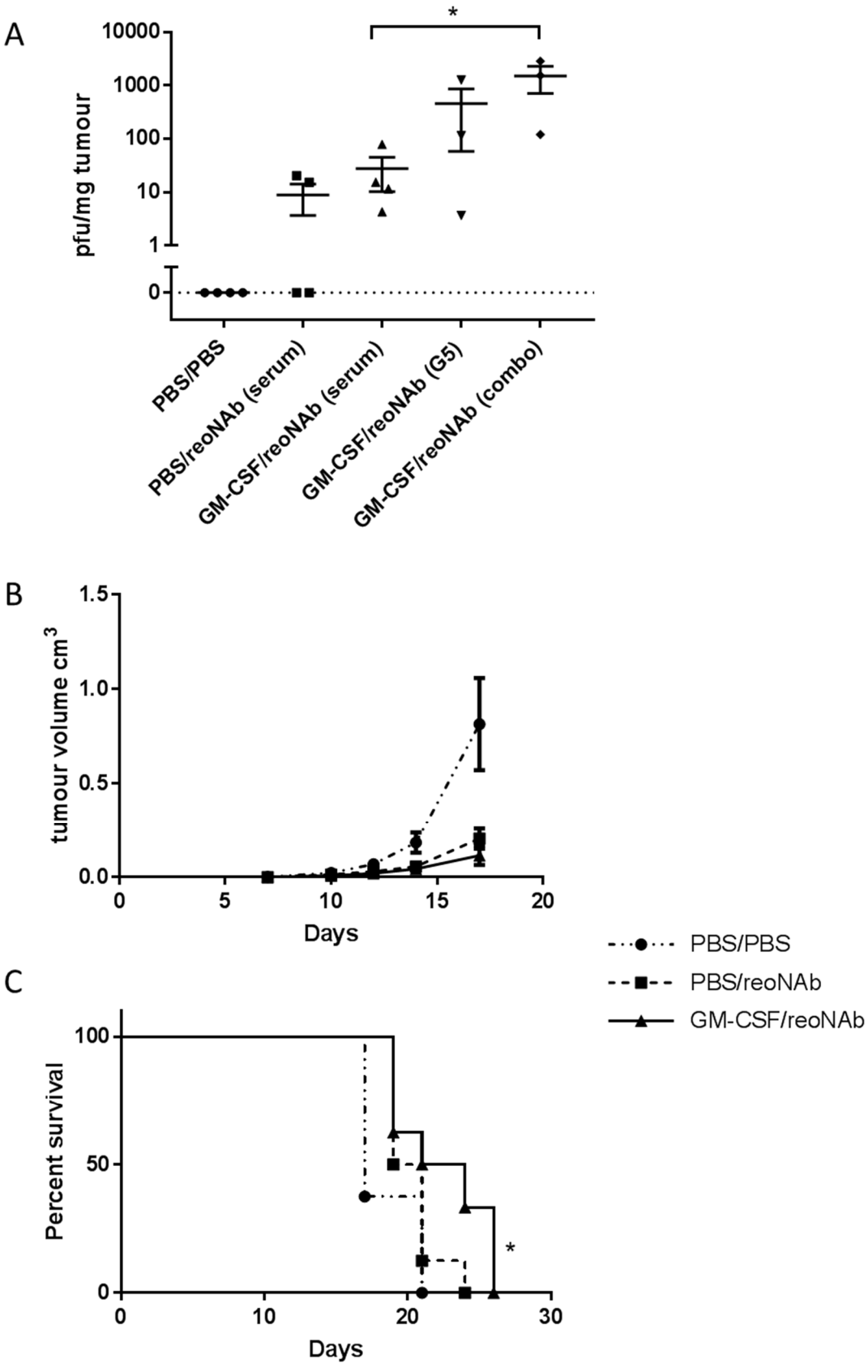

A
150
100
50
0
% cell death
**
**
*
***
mock
reoNAb
mono
mono-reoNAb
Mel-624
HCT116
PC3
SKOV3
cell lines
B
100
50
0
% Mel-624 death
**
***
ns
virusNAb
mono-virusNAb
none
reo
CVA21
HSV1716
Oncolytic virus

Figure 7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pfu/mg tumour
10000
1000
100
10
1
0
*
PBS/PBS
PBS/reoNAb (serum)
GM-CSF/reoNAb (serum)
GM-CSF/reoNAb (G5)
GM-CSF/reoNAb (combo)
B
tumour volume cm3
1.5
1.0
0.5
0.0
0
5
10
15
20
Days
PBS/PBS
PBS/reoNAb
GM-CSF/reoNAb
C
Percent survival
100
50
0
0
10
20
30
Days
*