Enzymatic inactivation of endogenous IgG by IdeS enhances therapeutic antibody efficacy

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Abbreviation list: Cmax, maximum concentration in serum; EndoS, endoglycosidase S; FcγR, Fc γ-receptor; IdeS, immunoglobulin G-degrading enzyme of *Streptococcus pyogenes*; IVIg, intravenous gamma globulin; PBMC, peripheral blood mononuclear cell; scIgG, single cleaved IgG.

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Abstract

Endogenous plasma IgG sets an immunological threshold that dictates the activity of tumor-directed therapeutic antibodies. Saturation of cellular antibody receptors by endogenous antibody limits antibody-dependent cell-mediated cytotoxicity (ADCC) and antibody dependent cellular phagocytosis (ADCP). Here we show how enzymatic cleavage of IgG using the bacterial enzyme IdeS can be utilized to empty both high and low affinity Fcγ-receptors and clear the entire endogenous antibody pool. Using *in vitro* models, tumor animal models as well as *ex vivo* analysis of sera collected during a previous clinical trial with IdeS, we show how clearing of competing plasma antibody levels with IdeS unblocks cellular antibody receptors. We show that therapeutic antibodies against breast cancer (trastuzumab), colon cancer (cetuximab) and lymphomas (rituximab and alemtuzumab) can be potentiated when endogenous IgG is removed. Overall, IdeS is shown to be a potent tool to reboot the human antibody repertoire and to generate a window to preferentially load therapeutic antibodies onto effector cells and thereby create an armada of dedicated tumor seeking immune cells.

Introduction

The mechanism of action of therapeutic antibodies against cancerous cells ranges from direct manipulation of endogenous signaling systems, direct target-induced cell death, to the recruitment of the immune system. However, the majority of therapeutic antibodies in clinical use induce complement-dependent cytotoxicity (CDC), antibody-dependent cytotoxicity (ADCC) and/or phagocytosis (ADCP) to kill target cells (1). CDC is triggered via the complement cascade which is initiated by complement factors binding to the Fc-part of an antigen-bound/aggregated

antibody and results in killing of target cells or pathogens. In contrast, ADCC and ADCP are triggered via interaction of the IgG Fc with cell-surface Fc γ-receptors (FcγRs) expressed by, for example, neutrophils, NK cells, monocytes, macrophages, dendritic cells and eosinophils (2,3).

While the interaction of a therapeutic antibody with host effector molecules is necessary for Fc-mediated immune recruitment, it is known that high serum concentration of endogenous plasma IgG has a negative impact on many *in vitro* assays. This suppressive effect has been attributed to the gamma globulin (IgG) fraction and its binding to FcγRs (4-6). The high plasma IgG concentrations means that all receptor classes are significantly occupied under physiological conditions even though the receptor affinities for non-complexed IgG span a wide range (K_a of 10^8-10^9 L/mol, $<10^7$ L/mol, and 10^6 L/mol for CD64/FcγRI, CD32/FcγRII, CD16/FcγRIII, respectively). Functionally, this means that the receptors are impeded by the endogenous IgG (7,8).

While all receptors are near saturation with plasma IgG, the high affinity receptor, FcγRI, which can functionally bind monomeric IgG is naturally most impeded. An alleviation of the suppressive effect of endogenous IgG is likely to have an enhancing effect on the cell killing activity on a wide range of immunological cells expressing FcγRI. To this end, aglycosylated antibodies which show abrogated binding to the low-affinity receptors but which have been engineered to engage FcγRI exhibited profound ADCC activity and points to the capacity of FcγRI as an important mediator of effector functions (9). A further example of potential therapeutic benefit of recruiting FcγRI+ cells is the use of bispecific antibodies capable of crosslinking FcγRI with target cells (10). It is not fully understood what the main function of the high affinity receptor is *in vivo,* however, animal experiments with FcγRI knock-outs have shown that at least the murine FcγRI is vital for therapeutic antibody efficacy (11).

In contrast to the high affinity receptor, there are three human FcγRs that mainly functionally respond to complexed IgG (CD16, CD32a and CD32b) (12). Their main functions to remove target structures by ADCC and ADCP are also well established *in vitro* and there is also accumulating evidence for their importance for clinical response to therapeutic antibodies (13-15). One strategy that has been employed to overcome the suppressive effects of competing IgG is to engineer the protein or glycosylation component of the therapeutic antibody Fc domain to generate enhanced receptor affinity (16-20). For example, the inhibitory effect of serum IgG on ADCC could be alleviated by defucosylation of anti-CD20 and anti-Lewis Y antibodies which enhances the binding to FcγRIIIA (21-23). However, this strategy is highly dependent on the target density and the concentration of the therapeutic antibody. Importantly, low target density renders the therapeutic antibody particularly prone to suppression by plasma IgG (6,24). Thus, strategies to directly manipulate the competing plasma IgG are being considered as a way to boost the efficacy and clinical outcome of therapeutic antibody therapy against cancer (25,26). It is envisioned that relief of suppression by plasma IgG would stimulate tumour killing activity by all immunological effector cells particularly by uncovering the most impeded and widely expressed receptor, FcγRI.

Adults have on average 10 g/L (6.7–14.5 g/L) intravascular IgG (27), however more than half of the total amount of IgG is present in the extravascular compartment (28). This high concentration and *in vivo* distribution results in a massive buffering capacity when trying to reduce circulating IgG levels by means of immunoadsorption or plasmaexchange. Thus, reducing IgG levels using these procedures takes several rounds of treatment allowing intra/extravascular equilibrium in IgG to establish between each round (29,30). These procedures can take several weeks and has to compete against the constant production of new IgG. Hence, accomplishing a

rapid and dramatic change in IgG levels in human subjects has not been possible until now. Recently, a clinical phase I study with IdeS demonstrated that upon injection of IdeS to human subjects the IgG levels dropped within minutes and IgG didn't start to reappear until several days later (31). IdeS cleaves IgG below the hinge region in a two-step reaction. It first cleaves one of the heavy chains generating a single cleaved IgG molecule (scIgG) which introduce a conformational change in the IgG. Secondly, the remaining heavy chain is also cleaved ultimately leading to the release of a $F(ab')_2$ fragment and a dimeric Fc fragment (31).

Here we show how IdeS can create a therapeutic window in which both high and low affinity FcγRs may be empty and can be armed with therapeutic antibodies thereby equipping them to be dedicated tumor seeking immune cells.

Material and Methods

Solid phase assays

Recombinant human CD64, CD16a, CD32a or CD32b/c (all R&D System) were coated in MaxiSorp plates at 2 µg/mL in PBS. Plates were blocked with 2% bovine serum albumin (BSA) in PBS and incubated with human IgG (IVIg, Octagam) at 1–2 µg/mL, either before or after IdeS treatment at indicated concentrations (37°C, 1 hour). PBS with 0.1% BSA was used for all dilutions and PBS with 0.05% Tween-20 (PBS-T) was used for all wash steps. Binding of IgG was detected using either a biotin-conjugated anti-Fab (109-066-097, Jackson ImmunoResearch) or anti-Fc (109-066-098, Jackson ImmunoResearch) antibody followed by horseradish peroxidase (HRP) conjugated streptavidin (SA-HRP, Pierce). Signals were developed using 3,3′,5,5′-tetramethylbenzidine substrate (TMB one component HRP, BioFX Laboratories),

stopped using 0.5 M H_2SO_4 and read at 450 nm. Blank was withdrawn from all samples and maximum binding using IVIg without IdeS was assigned 100%.

Human IgG concentration determination in mouse serum was measured using a Meso Scale Diagnostic (MSD) based assay developed at Hansa Medical AB. Briefly, a goat anti-human Fab fragment specific antibody was coated in MSD micro titre plates overnight. Plates were washed (PBS-T) and blocked in 2% BSA in PBS-T. A human IgG calibrator was applied as well as diluted serum samples and controls. Plates were washed and a biotinylated goat-anti-human Fc-specific antibody was applied together with streptavidin-sulfo (R32AD-1, MSD). Plates were washed, Read buffer T (R92TC-2, MSD) was added to plates and signals acquired using a MSD Sector Imager.

SDS-PAGE and immunoblotting

IdeS-cleaved IgG generated for the solid phase assays was separated under non-reducing conditions on a 4–20% Mini-PROTEAN TGX Stain-Free gel (BioRad) in Tris-Glycine-SDS buffer. The gel was transferred to a 0.45 µm Nitrocellulose membrane and blocked in 5% non-fat milk (NFM) prior to incubation with biotin-conjugated anti-Fc in 1% NFM/PBS-T. The membrane was washed, incubated with SA-HRP and developed with LumiLight ECL solution (Amersham). Signals were acquired using a ChemiDoc MP system (BioRad).

Cells and cell lines

The breast carcinoma cell line SK-BR-3 (ACC736) and the colon adenocarcinoma cell line HT-29 (ACC299) were obtained from Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ) and maintained in McCoy's 5A with 10% fetal bovine serum (FBS) and penicillin/streptomycin (PEST). The B-cell lymphoma cell line Nu-DUL-1 (ACC579) and the Burkitt lymphoma cell line Daudi (ACC78), also from DSMZ, were maintained in RPMI1640 with 10% FBS and PEST (R10). The acute monocytic leukemia cell line THP-1, a kind gift from Dr. Maria Allhorn, Lund University, Sweden, was maintained in R10. No authentication was done by the authors for any of the cell lines. PBMCs were prepared by density gradient separation (FicollPaquePLUS) of blood obtained from healthy human volunteers.

Binding of IgG to cells

THP-1 cells were incubated with different concentrations of either polyclonal IgG (IVIg) or with a monoclonal IgG1, adalimumab (Humira, AbbVie) and stained using a biotin-conjugated anti-Fab or anti-Fc specific antibody followed by allophycocyanin-conjugated streptavidin (SA-APC). IgG binding was analyzed by flow cytometry. When IdeS was included in experiments, the antibodies were either pre-treated for 30 min at 37°C with different IdeS concentrations (0.03–30 µg/mL) or IdeS was added after an initial antibody/cell incubation of 30 min at 37°C. For kinetic experiments, samples were removed at indicated time points and fixed using 4% phosphate buffered paraformaldehyde (PFA), washed and stored (4–8°C) in PBS with 0.5% BSA until all samples were ready for staining.

Chase experiments were performed as follows. Plasma from heparinized peripheral blood was filtered (0.45 µm) and heat inactivated at 56°C for 30 min to inactivate remaining

complement activity. The plasma was split in two fractions, one treated with PBS and the other treated with 10 µg/mL IdeS for one hour at 37°C. A sample of PBMC was removed and fixed in PFA as a start point prior to plasma addition. The remaining PBMCs were split in three fractions and plasma (non-treated or IdeS-treated) was added back to the cells. After a 60 min equilibration in the incubator a time zero sample was removed from all samples and IdeS was added to one of the non-treated plasma samples (final IdeS conc. 10 µg/mL). Samples were removed after 30 min, 1, 2, 4 and 6 hours and immediately fixed in 4% PFA and stored cold until staining for flow cytometry analysis. Remaining IgG on cells was monitored using a biotin-conjugated Fab specific affibody (CaptureSelect IgG-CH1, Life Technologies) or Fc specific antibody followed by SA-APC. IgG binding was analyzed by flow cytometry. A plasma sample from each time point was removed and immediately mixed with an inhibitor (iodoacetic acid, Sigma) to block further IdeS activity. Samples were separated by SDS-PAGE on a 4–20% Mini-PROTEAN TGX Stain-Free gel in Tris-Glycine-SDS buffer and analyzed using a ChemiDoc MP system.

Antibody dependent cellular phagocytosis

Human capillary blood was collected in Li-Heparin tubes from healthy volunteer and centrifuged at 1000 g for 5 minutes. Pelleted RBCs were extensively washed and stained with Far Red DDAO-SE (Molecular Probe) at 2 µM. Labelled RBCs were opsonized with 20 µg/mL rabbit anti-human RBC antibody (anti-RBC) (209-4139, Rockland). THP-1 cells were seeded in a Deep Well 96-well plate prepared with the different test antibodies and opsonized RBCs were added. Non-opsonized RBC were used as controls. The plate was incubated at 37°C for 1.5–2 hours, the plate was centrifuged and the supernatant was removed. BD lysis buffer was added and the percentage of THP-1 cells positive for Far Red was evaluated using flow cytometry.

For ADCP using the therapeutic antibodies trastuzumab, cetuximab, rituximab and alemtuzumab, the following protocol was used: target cells (HT-29, SK-BR-3, Daudi and Nu-DUL-1) were calcein labelled (1 μg/mL) and incubated with therapeutic antibodies at different concentrations (5–100 μg/mL). Effector cells (THP-1) were Far Red labelled (1 μM) and incubated with R10, IVIg, IdeS-generated IVIg fragments (30 μg/mL IdeS, 37°C for 2 h and then heat-inactivated), papain-generated Fc-fragments (Calbiochem) or serum. Effector and target cells were mixed at 20:1 ratio and incubated at 37°C for 1–2 hours prior to fixation in 2% PFA and monitored using flow cytometry.

Human serum collected pre-dose and 24 hours post dosing of 0.25 mg/kg IdeS (Eudract No 2013-005417-13, approved by the Regional Ethics Committee in Uppsala, Sweden) were anonymized (approved by the Regional Ethics Committee in Uppsala) and heated at 56°C for 30 min in order to inactivate complement and remaining IdeS activity. Serum samples were diluted 10× in R10 prior to incubation with effector cells and subsequent mixing with rituximab opsonized target cells (Daudi).

Complement dependent cytotoxicity and proliferation

Daudi cells were harvested and 50,000 cells/mL were seeded in a 96-well plate. Wells without cells were filled with R10. Rituximab was diluted to 400 μg/mL in R10 and further 10× dilutions were prepared. Twenty-five μL of the rituximab dilutions were added and the plate was incubated at 37°C for 30 min. Baby rabbit complement (Cederlane) and heat-inactivated complement (56°C, 30 min) was diluted 5× in R10 and 25 μL was added to wells. The plate was incubated at 37°C in the CO_2-incubator for 45 min. Ten μL CCK-8 (Cell Counting Kit 8, Dojindo

Laboratories) was added to all wells and the plate was incubated for at least one hour at 37°C. The plate was read at 450 nm in an ELISA plate reader. Survival rates were calculated using the following formula: Survival rate (%) = $A_{sample} - A_{blank} / A_{neg.ctrl} - A_{blank} \times 100$. Negative control is cells without antibody treatment (maximum survival) and blank is wells without cells. Significance was calculated using GraphPad PRISM, One-way ANOVA, Sidak's multiple comparisons test, $P < 0.0001$ at 0.1 µg/mL rituximab.

For Daudi cell proliferation in the precense of test substances (IdeS, puromycin, rituximab and IVIg), the CCK-8 kit was used with the same set up as for CDC, but seeding 15,000 cells/well and incubating for 48 hours prior to addition of CCK-8. Test substances were used at 100 µg/mL and in 10× dilutions to 1 ng/mL. No complement was added to the proliferation experiments.

Xenograft model in SCID mice

Six week old female severe combined immunodeficient mice (CB17-SCID, Janvier Labs, France) were allowed to acclimatize for at least one week. Mice were inoculated subcutaneously (s.c.) at the right flank with 3×10^6 Daudi cells. Tumor volume was calculated every 2–3 days from day 10 using a caliper and the following formula: Volume (mm^3) = length × $width^2$ / 2. Tumor development was followed until 1500 mm^3 when mice were sacrificed and tumors were weighed. All animal experiments in this study were approved by an ethical committee at University of Lund (M68-14, Malmö/Lund animal ethics committee).

For IVIg dose titration, Daudi cells were inoculated as before and mice were treated with a single intraperitoneally (i.p.) dose of IVIg (0.01, 0.1 or 1 g/kg, Privigen) eight days after cell

inoculation followed by an i.p. administration of rituximab (Mabthera) at a dose of 20 mg/kg once every week starting 14 days after cell inoculation.

For IVIg serum concentration studies, naïve SCID mice were treated with a single i.p. dose of IVIg (1 g/kg) and serum levels of human IgG was followed up to 22 days after administration using the MSD assay outlined under solid phase assays.

For evaluation of IdeS effect in the xenograft model, Daudi cells were inoculated as before and mice were i.p. treated with IVIg (1 g/kg) day 0. One day after IVIg administration mice were intravenously (i.v.) treated with PBS or IdeS at 5 mg/kg for three consecutive days. Weekly i.p. treatment with rituximab at 20 mg/kg was initiated 14 days after cell inoculation.

For IVIg binding to splenocytes, naïve SCID mice were treated with a single i.p. dose of IVIg (0.1 g/kg) on day 0. One day after IVIg administration mice were i.v. treated with PBS or IdeS at 5 mg/kg for three consecutive days and spleens were removed. Splenocytes were prepare, fixed in 4% PFA and stained for human IgG using biotin-conjugated anti-Fc and anti-Fab antibodies and SA-APC. For double staining experiments, we used PE-conjugated rat antibodies NKp46 (BD, 560757), Ly6G/GR-1 (RM3004, ThermoScientific) and CD11b (BD, 553311). As Fc-block, rat polyclonal IgG was applied prior to the monoclonal antibodies. IgG binding was analyzed by flow cytometry.

Statistical analysis

GraphPad PRISM software was used for all statistical analysis and the method used is specified for each experimental type individually (method sections and figure legends).

Results

IdeS has the capacity to unblock cellular FcγRs

It has been shown that IdeS can cleave both free IgG, antigen-bound IgG as well as IgG constituting part of the IgG-type of B-cell receptor (32), however, whether IdeS can also cleave IgG when bound by FcγRs has not been established. In a set of experiments, we assessed the effect of IdeS on IgG-binding to different recombinant FcγRs, i.e. CD16a, CD32a and CD64 (Fig. 1). CD32b was also tested but we did not achieve signals above background using that receptor. Normal human IgG (IVIg) was treated with different concentrations of IdeS (0.03-30 μg/mL) prior to incubation with immobilized FcγRs. The results demonstrate that IgG cleaved by IdeS does not bind the low affinity FcγRs, CD16a or CD32a, even when low concentrations of IdeS were used and that IdeS completely prevented binding to the high affinity FcγRI receptor CD64 when used at higher concentrations above 3 μg/mL (Fig. 1a). As there was still signal from the high affinity receptor at IdeS concentrations below 3 μg/mL we investigated the composition of the IgG and IgG-fragments present during the binding. IgG was cleaved with different concentrations of IdeS (30 pg/mL to 30 μg/mL), the CD64 interaction assay was repeated and samples of the IdeS-treated IgG were also subjected to SDS-PAGE and immunoblot in order to address the digestion status of the IgG added to the plate (Fig. 1b). The results show that at high concentrations of IdeS (3 and 30 μg/mL) the IgG was completely cleaved into Fc and $F(ab')_2$ and there was no binding to CD64. However, at low concentrations (between 0.003 and 0.3 μg/mL) there was a fraction of scIgG present in the samples which interacted with CD64, although potentially with lower affinity. At very low concentrations of IdeS (30 and 300 pg/mL) un-cleaved IgG was present and responsible for the binding to CD64. Thus, it could be concluded that the high affinity receptor but not the low affinity receptors can bind scIgG.

In the next set of experiments IgG was pre-incubated in plates coated with the different FcγRs prior to addition of different concentrations of IdeS (0.03–30 μg/mL) and incubated for 60 min in order to investigate if the bound IgG was sensitive to IdeS activity. The results showed that the loss in signal was not complete even at the highest IdeS concentration (30 μg/mL)(Fig. 1c). The high affinity FcγRI (CD64) was only marginally affected by the addition of IdeS and the binding to the low affinity receptors (CD16a and CD32a) was reduced, but not abolished. IdeS treatment for 60 min using concentrations above 3 μg/mL completely cleaves free IgG, antigen bound IgG and IgG in the BCR (31,32), but this experiment indicates that cleaving of IgGs loaded onto FcγRs are protected from IdeS. However, when IgG bound to the high affinity receptor CD64 was treated with IdeS for up to 24 hours a close to complete emptying of the receptor could be demonstrated (Fig. 1d). Some wells were carefully washed with buffer at each time point to remove free IgG and allow a new equilibrium to settle. This resulted in almost 50% reduction of the signal in 24 hours. The delayed efficacy on IgG pre-loaded in FcγRs could be due to less efficient cleavage when the antibody is bound to the receptor or that bound IgG is protected from IdeS cleavage and only cleaved once released correlating to the on-off rates of the different FcγRs.

Next IdeS effect on IgG binding to FcγRs present on cells was investigated. We used the monocyte cell line THP-1 which express several FcγRs including the high affinity receptor (33). Cells were incubated with IgG to ensure maximal saturation of the FcγRs prior to addition of IdeS. The presence of IgG on cells was monitored using flow cytometry with Fab- and Fc-specific anti-human IgG antibodies. IdeS very efficiently prevented binding to FcγRs if IgG was pre-treated with IdeS (Fig. 2a, blue line). When the cells were pre-loaded with IgG prior to IdeS treatment emptying of FcγRs took at least two hours (Fig. 2a, red line). As a follow-up, we

prepared human peripheral blood mononuclear cells (PBMC) from healthy human volunteers and repeated the experiment using plasma as an IgG source. PBMCs incubated with plasma resulted in an increased signal compared to untreated PBMCs as IgG in plasma was loaded onto the cells FcγRs and the signal remained largely unchanged during the experiment (Fig. 2b, green line). When IdeS was added to the plasma-incubated PBMCs, there was a clear drop in MFI during the first two hours after IdeS addition (Fig. 2b, red line). Incubating PBMCs with plasma pre-treated with IdeS resulted in a small additional drop in MFI over time indicating that after standard density gradient PBMC purification there is still a small amount of IgG in the FcγRs, and this is gradually removed by IdeS (Fig. 2b, blue line). Two hours post IdeS treatment there was no difference between cells given pre-cleaved plasma or plasma followed by IdeS treatment and hence, IdeS treatment resulted in emptying of FcγRs also on primary cells. Plasma used in this experiment was separated by SDS-PAGE to visualize the composition of IgG and IgG-fragments at each time point (Supplementary Fig. S1). An inhibitor was added to the samples immideately to stop additional IdeS activity and the results clealy shows that IdeS efficacy on non-bound IgG is much faster than on FcγR-bound IgG.

One possible mechanism of IdeS-mediated receptor clearence is that IdeS cleavage occurs most readily once IgG is detached from the receptors thereby reducing the concentration of liganded receptor as the system shifts to maintain equilibrium, slowly shifting the equilibrium concentrations until the FcγRs are unliganded. To test this explanation for the observed slow decrease in cell-bound IgG, IdeS treated cells were compared to cells allowed to passively equilibrate against buffer only. THP-1 cells with bound IgG were washed repetedly to remove non-bound IgG prior to addition of either PBS or IdeS and aliquots were removed and analysed by flow-cytometry at different time points (Fig. 2c). The results showed that during the first hour

there was no major difference in IgG loss from the cells in PBS compared to the cells treated with IdeS, i.e. the on-off rate of IgG:FcγR was the main cause of the reduction in signal. After this initial phase the rate of IgG release was slowed down in the PBS samples as a new equlibrium was reached, however, in the IdeS treated samples the decrease in signal continued. We conclude that the most likely explanation is that IdeS does not cleave IgG present in FcγRs very efficiently, however, due to the clevage of IgG released from the cells the equilibrum of the IgG:FcγR interaction is constantly pushed toward the non-bound state and the net result is emptying of FcγRs.

The suppressive effect of polyclonal IgG on effector cell function is abrogated by IdeS

In the next set of experiments, the ability of IdeS to overcome the function-blocking effect of polyclonal IgG (human IVIg) was addressed *in vitro*. Initially an ADCP assay with human red blood cells (RBC) opsonized with an anti-RBC antibody and THP-1 as effector cells was used to investigate this effect. The effector cells very efficiently phagocytosed the opsonized and labelled (Far Red) RBCs with very little background uptake of non-opsonized cells (Fig. 3a). Opsonized RBCs were mixed with effector cells in the presence of different amounts of IVIg, IdeS-treated IVIg or IgG2/4 engineered to have minimal FcγR binding. The results showed that there was a clear dose-dependent inhibition of ADCP when using un-treated IgG and no inhibitory effect of IdeS-treated IgG (Fig 3b). The IgG2/4 with minimal FcγR binding did not inhibit ADCP. Surprisingly it could be demonstrated that very low concentrations (less than 0.1 g/L) of IgG inhibited effector functions.

Based on the data presented it could be concluded that IdeS treatment has the capacity to

empty FcγRs and thereby abrogate the suppressive effect human polyclonal IgG has on FcγR mediated effector functions. This suggests a concept where IdeS-treatment could, by emptying FcγRs, potentiate the activity of cytotoxic antibodies. This concept was tested by using tumor cell lines as target cells for FcγR-mediated effector cell activity with commercially available therapeutic antibodies. Effector cells (THP-1) were pre-incubated with IVIg, IdeS-treated IVIg, or papain generated Fc-fragments, and alemtuzumab was used as a therapeutic antibody with labelled B-cell lymphoma cell line NuDUL-1 as target cells. Papain cleaves IgG above the hinge region and in contrast to IdeS-generated Fc-fragments these fragments still have the capacity to bind FcγRs. The results clearly showed that non-treated IgG and papain generated Fc-fragments completely blocked ADCP (Fig. 3c). However, IdeS-treated IgG had no blocking effect on the effector activity and rescued the therapeutic effect of alemtuzumab. The same suppressive pattern with non-treated polyclonal IgG blocking ADCP was seen with all tested antibodies, exemplified by cetuximab on the colon adenocarcinoma cell line HT29 and trastuzumab on the breast cancer cell line SK-BR-3 (Fig.3d and e).

IdeS potentiates therapeutic antibody treatment *in vivo*

The relevance of occupied FcγR was evaluated *in vivo* using rituximab as a therapeutic antibody and Daudi as target cells. SCID mice lack plasma IgG which is an advantage since IdeS does not effectively cleave murine IgG and instead mice were supplemented with human polyclonal IgG (IVIg). Flow cytometry analysis showed that rituximab binds well to target cells (Fig. 4a) and that rituximab has only a limited direct effect on the survival of Daudi *in vitro* (Fig. 4b). We further addressed the capacity of rituximab to fix complement and induce CDC in Daudi cells and rituximab was shown to significantly induce CDC at doses above 0.1 μg/mL (Fig. 4c). IdeS effect

on Daudi cell proliferation was also addressed in a 48 hour proliferation experiment in the presence of 0.1-100 µg/mL IdeS and there was no impact on Daudi cell proliferation in the presence of IdeS (Supplementary Fig. S2). An *in vivo* model was developed to be able to evaluate the effect of human polyclonal IgG (IVIg) on therapeutic antibody efficacy. Several cell lines expressing CD20 on the surface was monitored for tumor take in SCID mice and Daudi cells were selected. The effect of human IVIg on Daudi cell growth in SCID mice was evaluated in combination with rituximab. A single dose of 0.01 g/kg IVIg did not significantly change the growth suppressing effect of rituximab *in vivo*. However, both 0.1 and 1 g/kg IVIg significantly impaired the therapeutic effect of rituximab (Fig. 4d). A kinetic study of IVIg concentration in serum was conducted in order to monitor the level of human IgG after dosing (Supplementary Fig. S3). A single dose of 1 g/kg IVIg resulted in a human IgG concentration in mouse serum of 9 g/L measured 24 hours post dosing of IVIg. This concentration falls within the normal range of IgG in humans (6.7–14.5 g/L) and was thus considered to be a justified dose to use in the *in vivo* experiments. The concentration of human IgG was further followed weekly until day 22 and the concentration of human IgG in mouse serum remained well above 2 g/L throughout the experiment, a dose shown to be capable of blocking rituximab's therapeutic effect.

In the next set of experiments, IdeS ability to improve rituximab efficacy in the presence of IVIg was addressed. Mice were given human IVIg (1 g/kg) or PBS and inoculated with Daudi cells. Mice were further dosed with IdeS or PBS once daily for three days and then given rituximab or PBS once weekly starting on day 14 after tumor inoculation. There was no significant difference between the IVIg and control groups (Fig. 4e). Mice treated with rituximab showed a significant ($p < 0.0001$) therapeutic effect compared to control and IVIg alone. The growth inhibiting effect of rituximab was significantly ($p < 0.0001$) impaired by the presence of IVIg indicating that normal

IgG competes with the therapeutic antibody for effector cell binding. Interestingly, IdeS treatment after IVIg dosing significantly ($p < 0.0001$) rescued the therapeutic effect of rituximab (Fig 4.e). IVIg did not completely block the therapeutic effect of rituximab and this is most likely because not only FcγR-mediated activities, but also CDC and possibly direct effects, contributes to the therapeutic activity of rituximab in this model as suggested by the *in vitro* experiments in Fig. 4b and c and by others (34).

In order to address if human IgG was present on the potential effector cells in SCID mice, we injected mice with PBS, IVIg or IVIg followed by IdeS and prepared splenocytes. Cells were stained for the presence of human IgG and evaluated using flow cytometry. Mice given IVIg had a strong IgG-specific signal from the lymphocyte population whereas mice given IVIg followed by IdeS did not differ from the PBS control (Fig. 4f). Cells were also double stained for the presence of human IgG and either CD335, Ly6G (Gr-1) or CD11b and evaluated using flow cytometry. Human IgG was not found to be exclusively present on any selected cell populations, instead, it was present on approximately 30% of all cells and hence it is likely that the majority of cells carrying FcγRs have human IgG on their surface. The same pattern was seen from cells in the granulocyte gate (Fig. 4g) indicating that human IgG is present on the cell surface of these murine cells and that IdeS treatment *in vivo* empties the FcγRs.

The results presented here show that the presence of IgG very efficiently inhibits FcγR-dependent effector functions and that very low levels of IgG are needed to give a significant blocking effect. Furthermore, we have shown that IdeS has the capacity to empty FcγRs and thereby potentiate therapeutic antibody efficacy. We next addressed if a single dose of IdeS given to humans has the potential to potentiate the therapeutic activity of an antibody. A phase II study to evaluate the safety, tolerability, pharmacokinetics and efficacy of intravenous administration of ascending doses

of IdeS in chronic kidney disease patients has been conducted at Uppsala University Hospital, Sweden with Hansa Medical AB as sponsor (Eudract No 2013-005417-13). Pre-dose sera collected from subjects were screened by flow cytometry to address if they had natural plasma antibodies against Daudi and THP-1 cells. As expected many of the subjects were positive as they have anti-HLA antibodies against many potential donors (sensitized), however, two subjects were found to be negative. Sera collected pre-dose and at different time-points post dosing of 0.25 mg/kg body weight of IdeS were separated by SDS-PAGE to monitor IgG and the different IdeS-generated IgG fragments (scIgG, $F(ab')_2$ and Fc) and a representative gel is shown (Fig. 5a). Sera from two subjects were tested *ex vivo* for blocking capacity in a rituximab/Daudi experiment. The pre-dose sera from both individuals could significantly ($p < 0.0001$) inhibit rituximab mediated ADCP of target cells and the sera collected 24 hours post IdeS dosing did not block ADCP (Fig. 5b). These results suggest that a single dose of IdeS can reduce the IgG levels in humans to levels where the FcγRs are no longer occupied and hence IdeS treatment has the potential to potentiate therapeutic antibody treatment.

Discussion

IgG is one of the most abundant proteins in plasma with concentrations of approximately 10 g/L. These levels are known to curtail the effectiveness of anti-cancer therapies that exploit the effector functions of monoclonal antibodies. At a minimum, this elevates the doses required for efficient cell killing and, at worse, places an upper limit on cell killing by an antibody well under the theoretical efficacy. IdeS offers a solution to this problem by enzymatically unblocking FcγRs and thereby unleashing the full potential of the tumoricidal activity of therapeutic antibodies. The animal model presented here was designed to mimic the human IgG levels (9 g/L human IgG in

mouse serum reached the day after dosing). This had a significant impact on therapeutic antibody efficacy but importantly, the efficacy was restored by IdeS treatment.

In a series of experiments presented here we were able to show that although IdeS does not efficiently target IgG when bound to the FcγRs the net result of IdeS treatment is FcγR emptying as IdeS cleaves all IgG when not bound correlating to the on-off ratio of IgG:FcγR. These findings indicate that it is conceivable to make all FcγRs available for therapeutic antibody binding by means of IdeS pre-treatment. The C_{max} reached in humans after a single dose of 0.24 mg/kg was approximately 8 µg/mL (31) i.e. this concentration is within the range where we now show that IdeS is able to empty FcγRs. Many cancer cells produce small amounts of proteases with the capacity to cleave the hinge region of therapeutic antibodies and inactivate them when they bind to the cells (35,36) and thus, pre-loading the antibodies onto FcγRs might be one way of protecting them from the protease-rich environment in the tumor.

The idea to modify antibody binding to FcγRs and thereby increase therapeutic antibody efficacy has until now focused on modifications in the glycosylation pattern of these antibodies. For instance, an approach to increase therapeutic antibody efficacy, using the bacterial endoglycosidase EndoS, has been suggested (25). EndoS cleaves complex-type glycans on IgG thereby reducing its binding to FcγRs. IgG deglycosylated by EndoS was shown to bind less well to the low affinity receptor CD16a, but binding to other FcγRs including the high affinity receptor was not evaluated. Others have shown reduced binding of deglycosylated IgG to CD32a and CD32b (37). Yet another study addressed binding of immune-complexes (IC) to different FcγRs (38). They found that there was an IgG subclass difference in binding to the different receptors especially evident when analyzing binding to the low affinity receptors. However, they

also found that EndoS only had a marginal effect on IgG binding to CD64 irrespective of IgG-IC subclass.

That IVIg prepared from healthy humans can be used *in vivo* to inhibit many different antibody-mediated diseases is a well-known phenomenon and has been addressed by many research groups in animal models of different diseases like thrombocytopenia, acute lung injury and arthritis (39-43). Despite that the mechanism of action of IVIg is still a matter of debate, it is in clinical use for a wide variety of disorders. We are in favor of the interpretation that IVIg, by providing a dilution effect on the pathological antibodies regardless of disease, competes with FcγR and FcRn binding to change the half-life of the pathological antibodies. We target Daudi cells by rituximab and show that IVIg can significantly impact the efficacy of the therapeutic antibody and that removing the Fc-part of IVIg by means of IdeS treatment we improve efficacy of therapeutic antibodies. Others have used anti-platelet antibodies to target platelets and have shown that IVIg can significantly inhibit the removal of target cells. Campbell et al. even showed that a papain generated Fc-fragment is enough to achieve this effect and the $F(ab')_2$ part of IVIg was dispensable (43). Thus, these IVIg experiments all support our interpretation that endogenous IgG hampers efficient therapeutic antibody treatment and that IdeS treatment prior to therapeutic antibody administration is a general approach with potential in a wide range of diseases.

So far, IdeS has been used in two finalized clinical studies; one phase I study in healthy human subjects (31) and one phase II study (Eudract No 2013-005417-13) in kidney transplantation patients. Furthermore, IdeS is currently being investigated in two ongoing phase II studies, both in kidney transplantation patients (in press). Data on IdeS pharmacokinetics (PK) and pharmacodynamics (PD) profile have been collected as well as the safety profile of the

enzyme. IdeS shows a two-compartment distribution with an initial half-life of a few hours in the distribution phase and a much slower half-life in the elimination phase (unpublished observations by the authors). IdeS opens up a therapeutic window where endogenous IgG can be replaced by an antibody of choice, but due to the constant production of IgG in the body, this window is only open for 1–2 weeks. IdeS is of bacterial origin and consequently immunogenic. In healthy subjects IdeS induces an anti-IdeS response that peaks 2–3 weeks after treatment that then subsequently returns to normal levels (31). These data indicate that repeat treatment with IdeS preferably should follow a 6- to 12-month cycle. However, there is large individual variation in the magnitude of the response and if immune compromised cancer patients have the same anti-IdeS response remains to be investigated. IdeS in its present form is envisaged as a prime for antibody therapy with the emphasis that also tumor cells with low epitope density may be eradicated with a substantial benefit for the patients. Less immunogenic enzymes are under development and will likely increase the possibility of repeated dosing. Similarly, the clinical development of EndoS may enable the extension of the therapeutic window by the use of antigenically distinct proteins during therapy.

Future clinical trials addressing the therapeutic benefit of IdeS treatment in combination with different therapeutic antibodies has to bear in mind that IdeS cleaves all subclasses of human IgG including the therapeutic antibody and that IdeS activity can be measured in plasma for several days post dosing. This imposes a delay in the administration of the therapeutic antibody as cleavage of the therapeutic antibody would inactivate it, both through omitting Fc-mediated effector functions as well as manipulating the half-life of the antibody (loss of FcRn-recycling). One approach could involve the use a therapeutic IgG antibody engineered to be resistant to IdeS activity without loss of FcγR-binding (44). Another possibility is to make use of the natural

resistance of murine and rat IgG to IdeS (45). Therapeutic antibodies like catumaxomab (with Fc composed of mouse IgG2a and rat IgG2b) are likely not cleaved by IdeS and hence should be possible to utilize when there is still IdeS activity in the circulation. With the potential clinical development of further bacterial immune evasion factors, such as EndoS, there is scope to engineer therapeutic antibodies to be resistant to multiple factors and thereby creating a therapeutic that can be repeatedly administered during prolonged suppression of the endogenous antibody pool.

The usefulness of emptying FcγRs of bound IgG is not limited to cancer. All diseases which have a clinical benefit from IVIg treatment could be potentiated by IdeS pre-treatment. In autoimmune diseases, also auto-antibodies are present on FcγRs and removing these prior to IVIg therapy has the potential to improve IVIg efficacy and result in a clinical benefit for many patients.

Overall, we show how IdeS can be a potent tool to reboot the human antibody repertoire and to generate a window to preferentially load therapeutic antibodies onto FcγR bearing effector cells and thereby create an armada of dedicated tumor seeking immune cells.

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Figure legend

Figure 1. IdeS treatment inhibits antibody binding to recombinant FcγRs. (A) Human IgG (IVIg) was cleaved with IdeS (0.03–30 μg/mL, 37°C for 60 min) and then incubated with immobilized recombinant CD16a, CD32a and CD64 at RT for 60 min. Binding was monitored using an Fc-specific antibody and plotted as per cent binding compared to non-cleaved IVIg in each set-up. Error bars represent SD of duplicate wells. **(B)** IVIg was cleaved with IdeS (30 pg/mL to 30 μg/mL, 37°C for 60 min) and then incubated with immobilized recombinant CD64 at RT for 60 min. Binding was monitored as in A. Error bars represent SD of duplicate wells. The IdeS cleaved samples were also separated on SDS-PAGE and subjected to immunoblot using an Fc-specific antibody. The bands correlate to intact and single-cleaved IgG. **(C)** IVIg was incubated with immobilized recombinant CD16a, CD32a and CD64 at RT for 60 min followed by IdeS addition to the plates (0.03–30 μg/mL, 37°C for 60 min). Binding was monitored as in A. Error bars represent SD of duplicate wells. **(D)** IVIg was incubated with immobilized recombinant CD64 at RT for 60 min followed by addition of 30 μg/mL IdeS to the plate in reverse order i.e. starting with the 24 h sample and ending with the 30 min sample. At time zero the entire plate was washed and binding was monitored as in A. Error bars represent SD of duplicate wells. Some wells were manually washed each time point with 0.1% BSA in PBS to remove free IVIg.

Figure 2. IdeS treatment prevents antibody binding to cells and releases antibodies already bound to cells. (A) THP-1 cells were incubated with untreated IgG (no IdeS), IgG pre-cleaved with 30 μg/mL IdeS (IdeS first) or pre-incubated with IgG for 30 min and then mixed with 30 μg/mL IdeS (IgG first). The background from cells without IgG addition is also shown (no IgG).

All incubations were performed at 37°C in the CO_2 incubator. The amount of IgG bound to THP-1 cells was monitored using a Fab-specific biotin-conjugated antibody followed by SA-APC and mean fluorescent intensity (MFI) was monitored in FL4. Error bars represent SD of duplicate wells. **(B)** Freshly prepared monocytes were incubation with plasma (no IdeS), plasma pre-cleaved with 10 µg/mL IdeS (IdeS first) or pre-incubated with plasma for 60 min followed by 10 µg/mL IdeS (plasma first). The background from cells prior to plasma addition is also shown (no plasma). The amount of IgG bound to PBMCs was monitored using a Fab-specific biotin-conjugated affibody followed by SA-APC and mean fluorescent intensity (MFI) was monitored in FL4. Error bars represent SD of duplicate wells. **(C)** THP-1 cells were pre-incubated with 100 µg/mL IgG at 37°C for 60 min and then washed 3× 50 volumes of buffer to remove free IgG. A zero sample was removed and PBS or 30 µg/mL IdeS was added to the cells and incubated at 37°C for the indicated time points. The amount of IgG bound to THP-1 cells was monitored using a Fab-specific biotin-conjugated antibody followed by SA-APC and mean fluorescent intensity (MFI) was monitored in FL4. Error bars represent SD of quadruplicate wells.

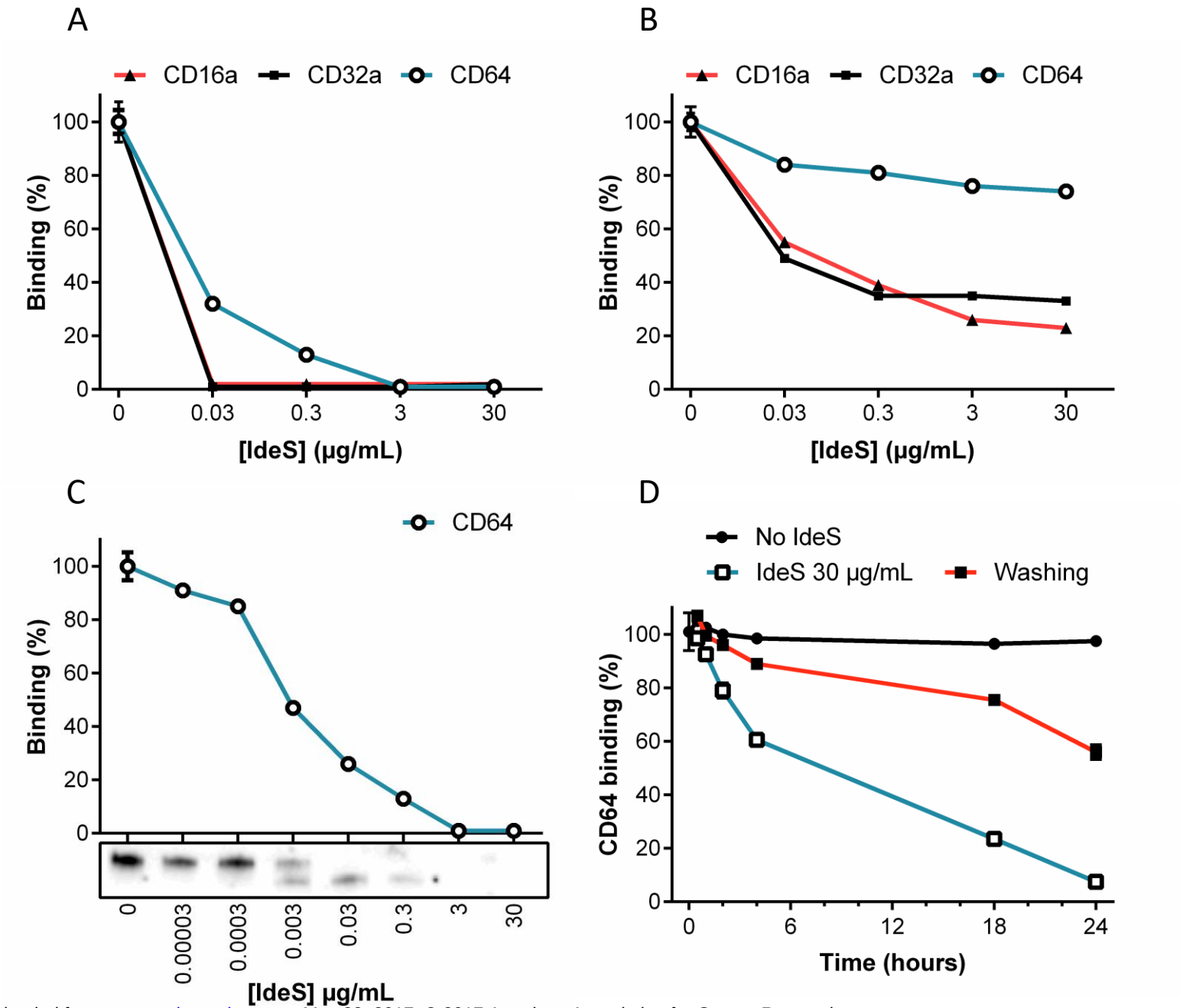
Figure 3. IdeS treatment enables efficient target specific antibody mediated phagocytosis by freeing antibody receptors from blocking non-target specific antibodies. (A) Visualization of engulfed red blood cells (RBC) after opsonization with anti-RBC IgG. **(B)** Effector cells were pre-incubated with different amounts of IgG being either capable (IVIg), IdeS-treated IVIg (IVIg/IdeS) or engineered to be un-capable (IgG2/4) to bind FcγRs. Far Red labelled target cells (RBC) were opsonized with anti-RBC IgG and mixed with effector cells (THP-1) at 20:1 E:T ratio. **(C)** Calcein labelled target cells (NuDUL-1) were opsonized with 5 µg/mL alemtuzumab and mixed with Far Red labelled effector cells (THP-1) at 20:1 E:T ratio in the presence or

absence of 100 µg/mL IVIg, IdeS-treated IVIg (IVIg/IdeS) or papain-generated Fc-fragments. **(D)** Calcein labelled target cells (HT29) were opsonized with 100 µg/mL cetuximab or BSA and mixed with Far Red labelled effector cells (THP-1) at 20:1 E:T ratio in the presence or absence of 400 µg/mL IVIg. (**E**) Calcein labelled target cells (SKBR3) were opsonized with 100 µg/mL trastuzumab or BSA and mixed with Far Red labelled effector cells (THP-1) at 20:1 E:T ratio in the presence or absence of 400 µg/mL IVIg. The percent of phagocytosed target cells was evaluated using flow cytometry.

Figure 4. IdeS-treatment potentiates the effect of anti-tumor antibody therapy *in vivo* in mice. (A) Verification of dose dependent binding of rituximab to Daudi cells by flow cytometry. **(B)** The direct anti-proliferative effect of increasing concentrations of rituximab and IVIg was evaluated *in vitro* on Daudi cells using a sensitive formazan based colorimetric assay (CCK-8). Error bars represent SD of triplicate samples. **(C)** Complement dependent cytotoxicity of Daudi cells was evaluated using increasing concentrations of rituximab in the presence of functional complement (CPL) or heat-inactivated complement (HI CPL) and survival was evaluated using the CCK-8 assay. Error bars represent SD of triplicate samples. **(D)** Tumor growth was evaluated in a SCID mouse model using s.c. injected Daudi cells (3×10^6/mouse). Mice were given 1, 0.1 or 0.01 g/kg IVIg (i.p.) on the day of inoculation. Palpable tumors were present from day 10 and rituximab (RTX) treatment was started on day 14 and given once weekly (n = 5/group). Error bars represent SEM. Statistical evaluation using GraphPad PRISM, one-way ANOVA with Dunnett's multiple comparisons test. **(E)** Mice (n = 10/group) were given IVIg (1 g/kg, i.p) or PBS (Ctrl) on the day of Daudi cell inoculation and IdeS or PBS was given at three consecutive days. Palpable tumors were present from day 10 and rituximab treatment was started on day 14.

Error bars represent SEM. Statistical evaluation using GraphPad PRISM, one-way ANOVA with Dunnett's multiple comparisons test. (**F**) Mice were given PBS, IVIg or IVIg followed by IdeS and spleens were collected. Splenocytes were prepared and stained for the presence of surface bound human IgG using an Fc-specific detector antibody. Cells were analyzed using flow cytometry and gating on the lymphocyte population. Statistical evaluation using GraphPad PRISM, one-way ANOVA, unpaired t-test (Holm-Sidiak's multiple comparison test) (n = 4/group, each stained in duplicate wells). Error bars represent SD. **(G)** Same experiment as in (F), but gating on the granulocyte population.

Figure 5. Antibody dependent phagocytic capacity is increased in serum collected from human healthy subjects treated with IdeS. (A) SDS-PAGE separation of human serum under non-reduced conditions. The gel is from a representative subject (subject A) dosed with 0.25 mg/kg IdeS and shows serum collected pre-dose and at different time points post dosing of IdeS (14 min and up to 21 days post dosing). IgG, scIgG, $F(ab')_2$ and Fc are indicated by arrows. (**B**) Target cells (Daudi) were calcein labelled and opsonized with 10 µg/mL rituximab. Serum samples (subjects A and D) collected pre-dose and 24 hours post dosing of IdeS (0.25 mg/kg) were heated to inactivate remaining IdeS activity. Serum was then pre-incubated with Far Red labelled effector cells and mixed with Daudi cells at a 20:1 E:T ratio. The percent of phagocytosed cells was evaluated using flow cytometry. Maximum phagocytosis was determined using effector cells without serum treatment and was set to 100%. Error bars represent SD of quadruplicate samples. Statistical evaluation using GraphPad PRISM, one-way ANOVA, unpaired t-test (Holm-Sidiak's multiple comparison test).



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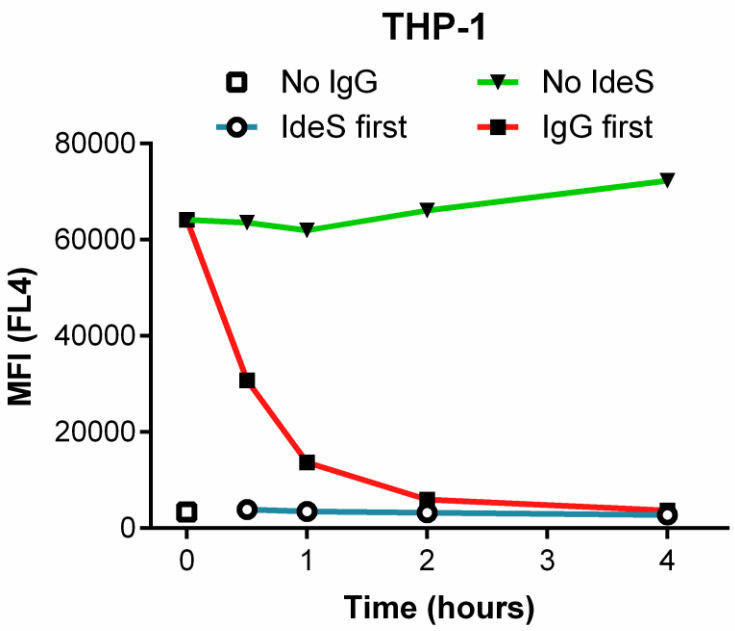

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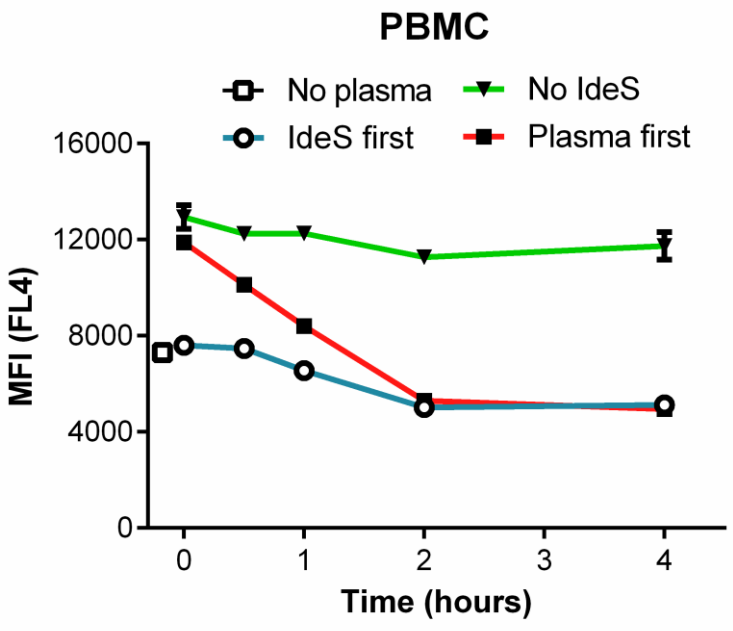

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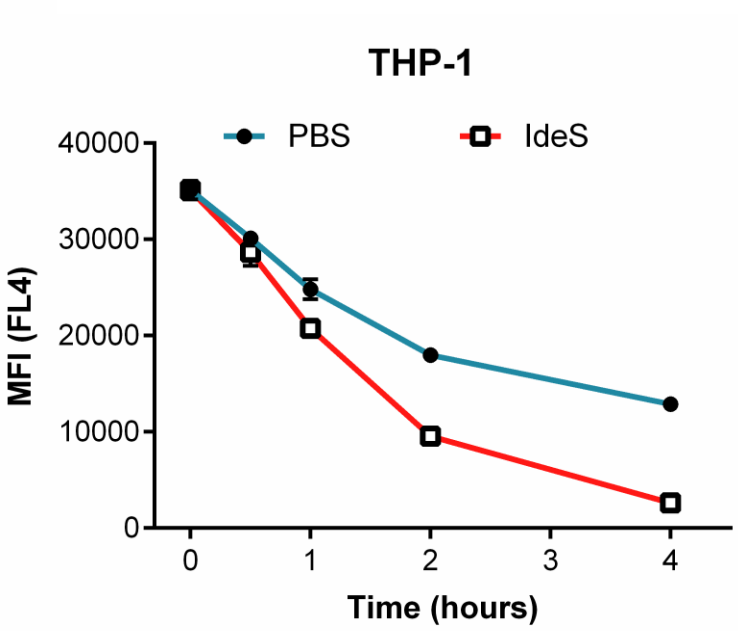

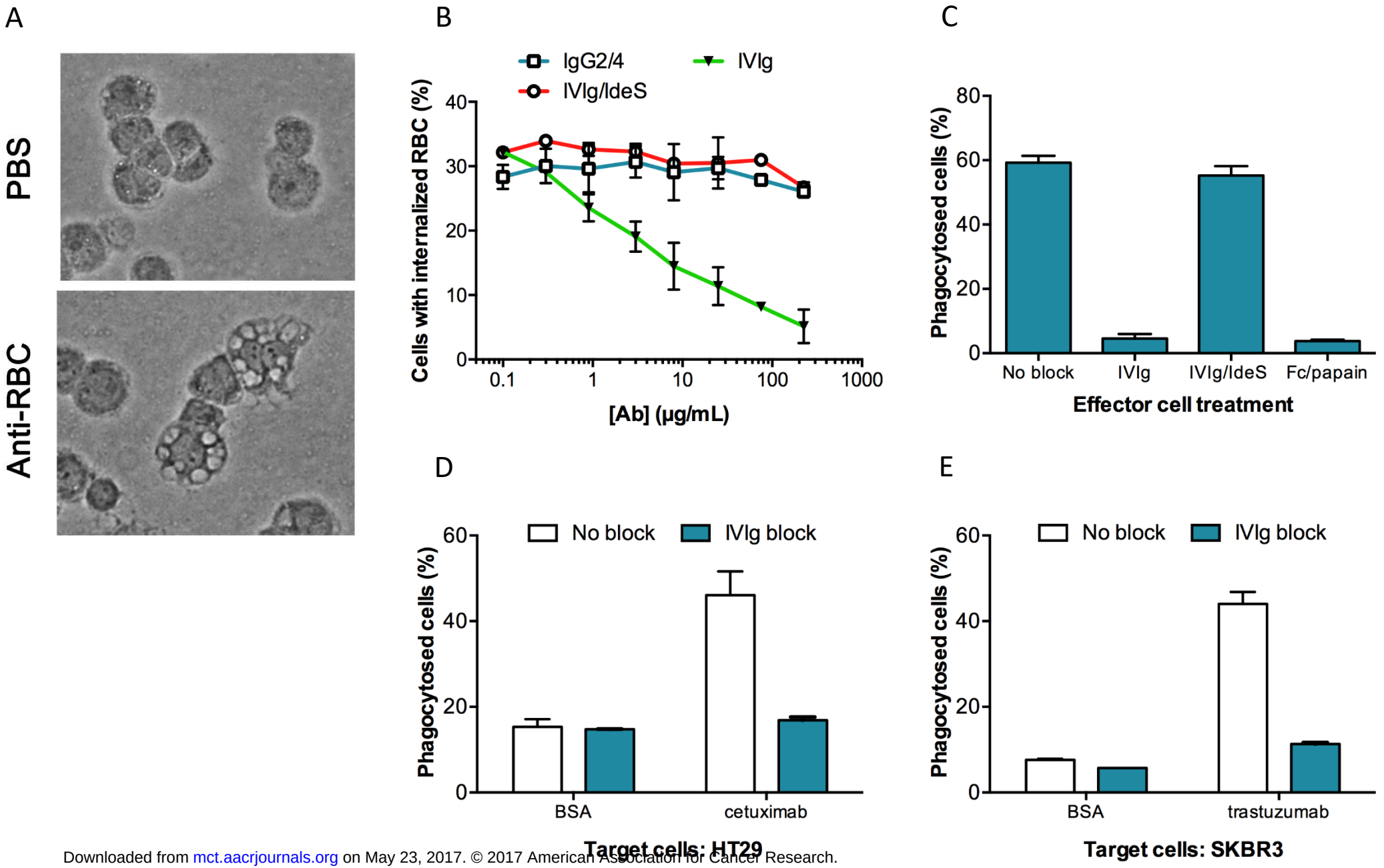

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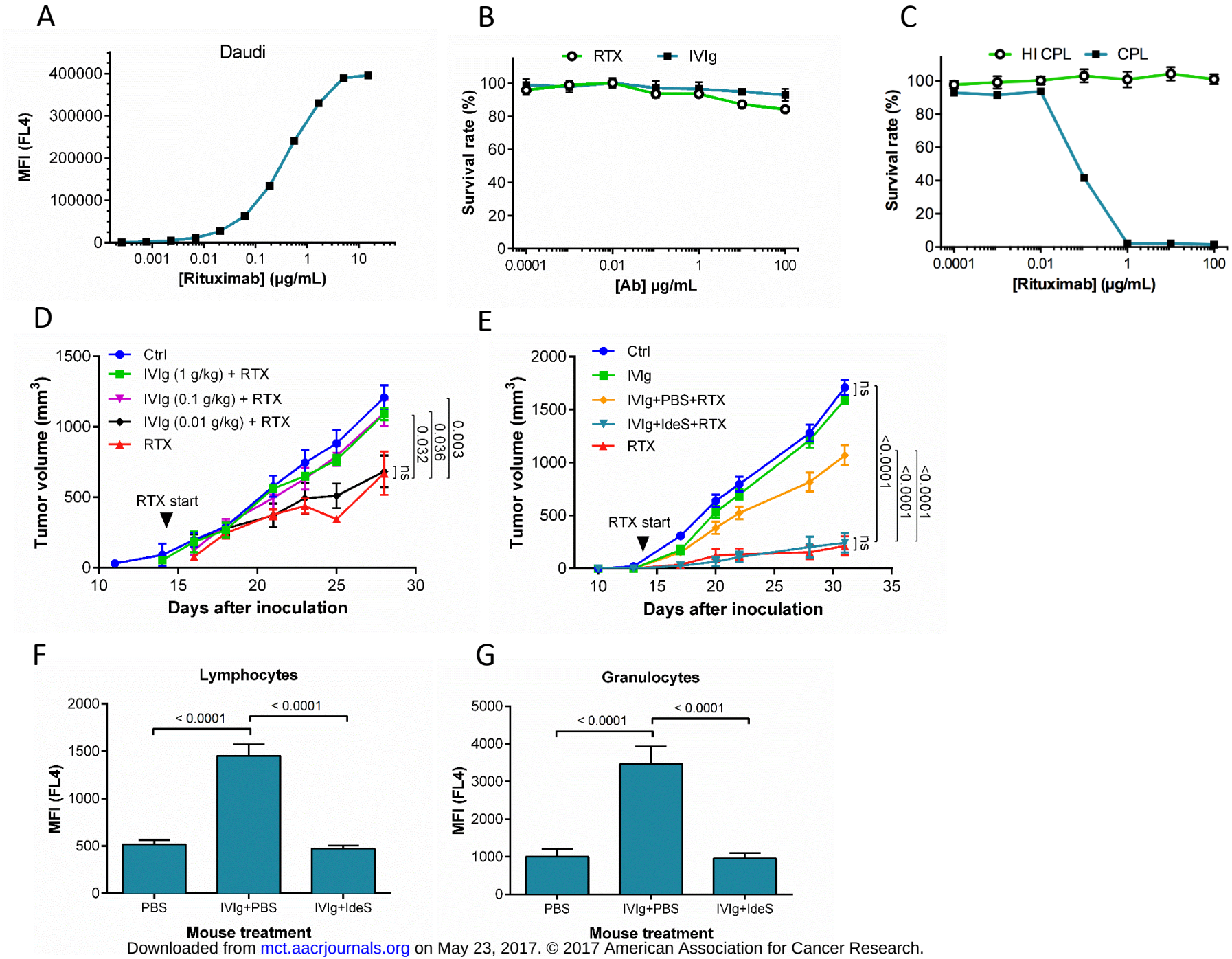

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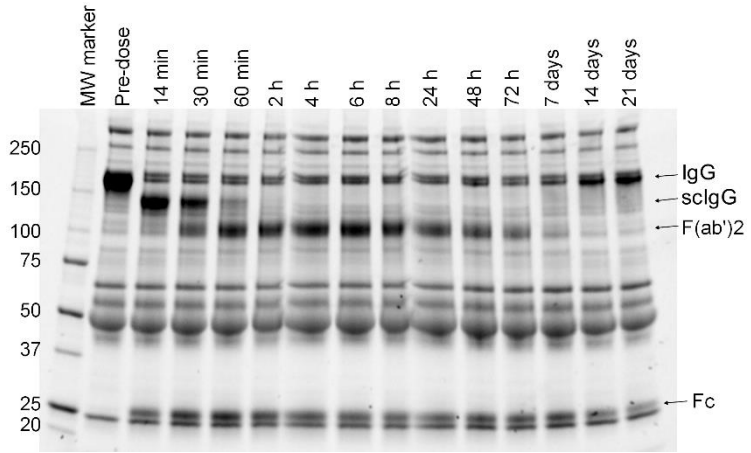

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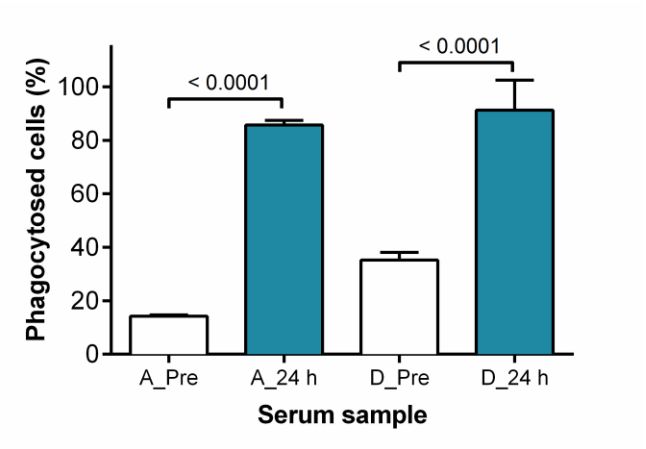

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Molecular Cancer Therapeutics

Enzymatic inactivation of endogenous IgG by IdeS enhances therapeutic antibody efficacy

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