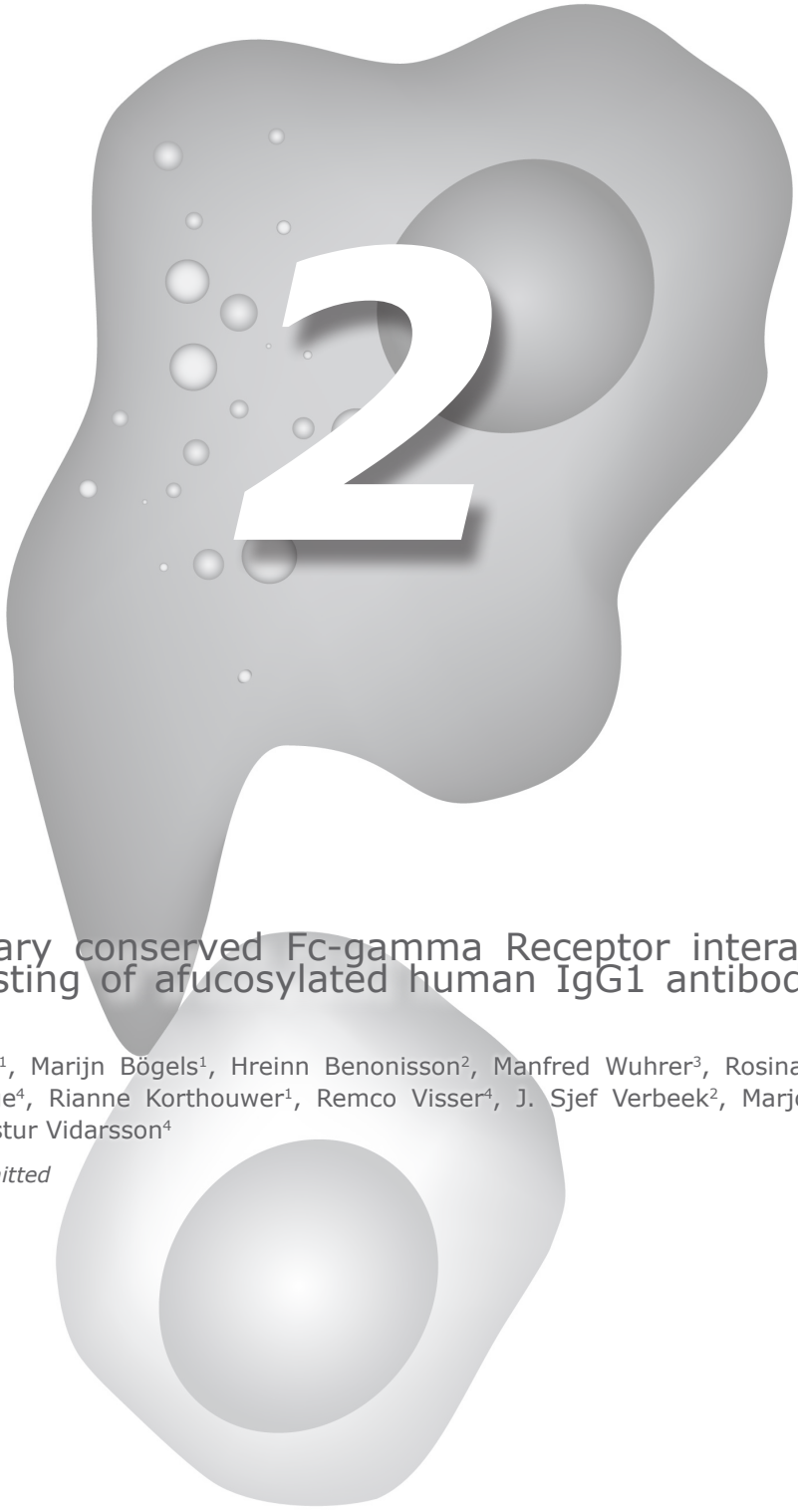

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Evolutionary conserved Fc-gamma Receptor interactions allows testing of afucosylated human IgG1 antibodies in mice

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Abstract

Cancer treatments are increasingly based on therapeutic antibodies to clear the tumour. One strategy is to maximize their effector functions, preferentially through natural and non-immunogenic modifications. The Fc domain of immunoglobulin G (IgG) antibodies contains an *N*-linked oligosaccharide in the second heavy chain constant domain (CH2) at position 297. Both the presence and composition of this glycan is crucial for IgG Fc receptor (Fcγ receptor) mediated antibody effector function. Human IgG1 antibodies lacking the core-fucose in this glycan have enhanced binding to human FcγRIII, resulting in enhanced antibody dependent cell cytotoxicity (ADCC) and phagocytosis through these receptors. *In vivo* mouse models are useful to predict effectiveness of human antibodies. However, it is not yet clear whether enhancing glycan modification of human IgG also translates into more effective treatment in these models. We generated humanized IgG1 TA99 antibodies targeting the B16-melanoma antigen gp75 with and without core-fucose at position 297 in the Fc-domain. C57Bl/6 mice that were injected intraperitoneally with B16F10-gp75 mouse melanoma developed significantly less metastasis outgrowth after treatment with afucosylated hIgG1 TA99 compared to mice treated with wildtype hIgG1 TA99. Human IgG1 without the core-fucose showed stronger interaction with the murine high affinity receptor FcγRIV, the mouse orthologous of human FcγRIIIa, indicating that this glycan change is functionally conserved between the species. In agreement with this, no significant differences were observed in melanoma outgrowth in FcγRIV$^{-/-}$ mice treated with human IgG1 TA99 with or without the core-fucose. These results confirm the potential of removing the core-fucose of therapeutic antibodies to increase efficacy. Moreover, we show that (afucosylated) human IgG1 antibodies act across species, supporting that mouse models are suitable to test afucosylated antibodies.

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Introduction

The use of monoclonal antibodies (mAbs) in cancer therapies is rapidly increasing. Nowadays development of new therapeutic mAbs is focused on inducing immune activation to further promote tumour cell death. These therapies include general checkpoint inhibitors such as α-CTLA4 (cytotoxic T-lymphocyte-associated protein-4) or α-PD-1/PD-1L (programmed cell death-1 Ligand), and tumour specific antibodies[1–3]. To enhance antibody effector functions, bi-specific antibodies and mini/nanobodies have been developed. Alternatively, new studies aim to modify antibodies to improve activation of the immune system via immunoglobulin (Ig) Fc receptors such as the IgG Fc receptor (Fcγ receptor). Preferentially, this has to take place without introducing any mutations, which may be immunogenic.

The Fc domain of human IgG has a highly conserved N-linked glycosylation site in the second heavy chain constant domain (CH2) at the asparagine at position 297 (Asn297)[4,5]. There are more than 30 different glycosylation forms found at this position. Modifying this glycan greatly affects the interaction and function of the IgG[6]. For example, removing the glycan results in the inability of IgG to interact with both complement and Fcγ receptors. Less dramatic modifications, such as cleaving the sialic acids from the glycan of intravenous IgG (IVIG) result in the loss of anti-inflammatory functions of IVIG in mice[7,8]. By contrast, removing the core-fucose results in a higher affinity binding of IgG to human FcγRIIIa[9–12]. Consequently, effector functions such as antibody dependent cellular cytotoxicity (ADCC) and phagocytosis (antibody dependent cell phagocytosis, ADCP) are increased [13]. Similarly, also the mouse FcγRIV, the orthologous of human FcγRIIIa, shows increased binding affinity for afucosylated mouse IgG2a compared to wildtype IgG2a[14–16].

Afucosylated antibodies exist in both human and mice, but the vast majority is fucosylated. Preventing core-fucosylation during production in mammalian cell lines is an attractive way to acquire therapeutic antibodies with enhanced effector function[17].

Novel mAb variants need to be tested in in vitro models, but also in in vivo models. Mice are one of the most popular experimental models to investigate cancer development and new potential therapies, partially because of their genetic homology with humans and our ability to easily (and relatively fast) manipulate their genome. For testing human antibodies in mouse models, it is vital that their effector functions and mechanism reflect the human situation as much as possible[18,19].

Human IgG1, the dominant subclass used for therapeutics in humans, does not interact as efficiently as mouse IgG2a with the murine high affinity receptors FcγRI and FcγRIV. Nonetheless, potent immune activation can still be observed in vitro[18]. Human IgG1 induced similar tumour cell lysis as mouse antibodies in vitro by mouse natural killer (NK) cells or polymorphonuclear cells (PMNs). Mouse macrophages were also able to kill tumour cells in the presence of any of the human IgG subclasses in an in vitro co-culture with the highest effect observed with IgG1. When macrophages were isolated from FcγRI and III deficient mice only mouse IgG2a, human IgG1 and human IgG3 were able to induce tumour cell death, suggesting they act through the remaining activating Fcγ receptor, FcγRIV[18].

In this study we investigated whether mice are a suitable model to test increased effector functions of IgG1 antibodies with lowered core-fucosylation. To this end, we humanized the murine TA99 antibody targeting gp75 by replacing the constant regions of the murine IgG2a heavy chain for the human IgG1. With a co-culture of human effector cells and B16F10-gp75 tumour cells in the presence of the wildtype or modified antibody, we identified the primary human effector cells and confirmed

its improved immune activation. We characterized binding affinities of human IgG1 TA99 as fucosylated and afucosylated variants on a biosensor array equipped with all mouse Fcγ receptors. Finally, we tested its capacity on tumour clearance of a B16F10-gp75 mouse melanoma in an intraperitoneal metastasis model.

Material and methods

Antibodies

We previously described the development of the anti-TNP antibodies that were used as isotype control[9,20]. Anti-gp75 antibodies (TA99) were generated in a similar fashion. In short, codon optimized VH and VL regions, previously cloned, were designed and ordered from Geneart (Life Technologies, Paisley, UK) with restriction overhangs compatible with our expression vector for human IgG1 and the human kappa light chain[9,20,21].

Antibodies were produced in the HEK-293F FreeStyle cell line expression system (Life Technologies) with co-transfection of vectors encoding p21, p27 and pSVLT genes as described in[22] to increase protein production. To generate antibodies with lower core-fucosylation 400μM 2-deoxy-2-fluoro-L-fucose (2F; Carbosynth, Compton, Berkshire, UK) was added during protein production[9].

Antibodies were purified on Protein A HiTrapHP columns (GE Healthcare Life Sciences, Little Chalfront, UK) using Akta-prime plus system (GE Healthcare Life Sciences) and dialyzed against PBS overnight. The Fc-fucosylation was determined by performing proteolytic digestion of IgG with trypsin (Promega, Madison, WI). Glycopeptides of this digestion were analysed using the nano LC-ESI-QTOF-MS as previously described[23,24].

Antibodies used for flow cytometry are anti-human CD16 (CD16, eBioscience), CD32 (6c4, eBioscience), CD64 (10.1, eBioscience), and HLA (L243, Biolegend, San Diego, California, USA), and anti-mouse CD3e (145-2c11, eBioscience), F4/80 (BM8, eBioscience), GR1 (RB6-8C5, eBioscience), NK1,1/CD161 (PK136, eBioscience), and NKp46/CD335 (29A1.4, eBioscience).

Isolation of primary effector cells

Human effector cells were isolated from peripheral blood that was obtained from healthy donors or buffycoats from Sanquin blood supply (Amsterdam, the Netherlands). All donors signed an informed consent. Blood was separated by Lymphoprep (Axis-Shield, Oslo, Norway) density gradient centrifugation. PMNs were obtained by lysing erythrocytes in ammonium chloride buffer (155 mM NH_4Cl, 10 mM $KHCO_3$ and 0.1 mM EDTA). Subsequently, PMNs were washed with PBS (B.Braun, Melsungen, Germany) and resuspended in complete medium. The mononuclear layer (Peripheral blood mononuclear cells, PBMC) obtained from the Lymphoprep separation was washed with PBS and resuspended in complete medium. NK cells and $CD14^+$ monocytes were isolated from the PBMC layer with cell separation beads (Miltenyi Biotech, Leiden, the Netherlands) according to manufacturer's protocol, washed and resuspended in complete medium. Peripheral blood lymphocytes (PBLs) were obtained as flow through from the $CD14^+$ monocyte isolation, washed and resuspended in complete medium.

Cell culture

The B16F10-gp75 mouse melanoma cell line was generated to stably express gp75 on the cell membrane as described in[25]. B16F10-gp75 tumour cells were cultured under humidified conditions (37°C, 5% CO_2) in medium RPMI 1640 (Gibco, Paisley,

United Kingdom) supplemented with 10% heat inactivated foetal calf serum (FCS, Lonza, Verviers, Belgium), glutamine (Glutamax, Lonza) and penicillin/streptavidin (Lonza), hereafter referred to as 'complete medium'.
Macrophages were obtained by culturing $CD14^+$ isolated blood monocytes with 50ng/ml macrophage-colony stimulating factor (M-CSF) (eBioscience, San Diego, CA) for 6 days in complete medium after which they were seeded for experiments in complete medium with 50ng/ml M-CSF for two additional days.

Flow cytometry
Fcγ receptor expression on human monocyte derived macrophages and composition of immune cells in peritoneal lavages were determined by flow cytometry. Samples were blocked with 5% serum diluted in PBS with 0.5% bovine serum albumin (BSA). Subsequently, cells were incubated with fluorescently labelled antibodies diluted in 0.5% PBS/BSA. Afterwards samples were washed with 0.5% PBS/BSA and erythrocytes were lysed with lysing solution diluted in MilliQ (10x concentrate, BD Biosciences). Data was acquired with BD LSRFortessa X-20 (BD Biosciences) and analysed with Flowjo X (Flowjo, LLC, Ashland, OR).

Antibody dependent cellular cytotoxicity (ADCC)
B16F10-gp75 tumour cells were seeded in a 96 wells plate (8.000 cells/well). Effector cells were isolated as described above. Antibodies and effector cells were diluted in complete medium. The effector to target (E:T) ratio for PMNs was 80:1, for PBMCs, PBLs and monocytes 10:1 and for NK cells 5:1. Antibodies were diluted to a final concentration of 1μg/ml or 5μg/ml when PMNs were used as effector cells. After 24hr of co-culture plates were carefully washed and a 3-hour cell titre blue assay (Promega, Leiden, the Netherlands) was performed according to manufactures protocol. Readout was performed on a Bio-rad Model 680 Microplate Reader (Bio-rad, Hercules, CA).

Antibody dependent phagocytosis (ADCP)
Antibody dependent phagocytosis was performed as described in[26]. In short, macrophages were seeded in the presence of M-CSF for 2 days prior to the experiment in 24 wells plates (200.000/well). At day 0 B16F10-gp75 were harvested, stained with cell proliferation dye eFluor 450 (eBioscience) according to manufacturer's protocol. Both antibodies (1μg/ml) and tumour cells (E:T=15:1) were diluted in complete medium. After 24hr of co-culture at 37ºC, cells were collected with Trypsin/EDTA and scratching. Subsequently samples were blocked with human serum, stained with anti-HLA-DR to stain macrophages, and fixed with 4% paraformaldehyde in PBS. Data was acquired and analysed with the BD LSRFortessa X-20 and Flowjo X.

B16 mouse melanoma metastasis model
8-week-old C57Bl/6 mice were obtained from Harlan. 8-10 week old C57Bl/6J $FcγRIV^{-/-}$ were generated and bred in the laboratory of Dr. J.S. Verbeek (Leiden University Medical Centre, Leiden, the Netherlands, their generation will be published elsewhere). Optimal tumour load was determined in both mouse strains prior to experiments, suboptimal conditions for TA99 hIgG1 in C57Bl/6 wildtype. B16F10-gp75 tumour cells were harvested at the start of the experiment, washed and diluted in PBS to a concentration of 50,000 cells/300μl. Antibodies were diluted in PBS at a concentration of 50 μg/300μl. Mice were intraperitoneally injected with first tumour cells right of the central line followed by antibodies left of the central line. 14 days

post injection mice were sacrificed with CO_2 and peritoneal metastasis were scored. Mice were kept under standard conditions with unrestricted access to food and water and wellbeing was observed on daily basis. Mouse experiments were performed according to the guidelines of the Dutch government assessed by the Medical Ethical Committee of the VU University Medical Centre Amsterdam and the Leiden University Medical Centre.

Surface plasmon resonance

Biotinylated mouse FcγRI, FcγRII and FcγRIV were purchased from Sino Biologicals (Bejing, China). Biotinylated mouse FcγRIII was not available. Therefore, a His-tag conjugated FcγRIII (Sino Biologicals) was used.

All Fcγ receptors were spotted using a Continuous Flow Microspotter (Wasatch Microfluidics, Salt Lake City, UT) onto a single SensEye G-streptavidin sensor array (Senss, Enschede, Netherlands) allowing for binding affinity measurements of each antibody to all Fcγ receptors simultaneously on the IBIS MX96 (IBIS technologies, Enschede, Netherlands) as described. Biotinylated Fcγ receptors were spotted in duplo in three-fold dilutions, ranging from 100 nM to 3 nM for FcγRII and 30 nM to 1 nM for FcγRI and FcγRIV in PBS 0.075% Tween-80 pH 7.4 (Amresco, Solon, OH). For the His-tagged FcγRIII, biotinylated anti-His IgG1 (GenScript, Piscataway, NJ) was spotted in duplo and three-fold dilution, ranging from 100 nM to 3 nM, onto the sensor and 30 nM FcγRIII (equally diluted in PBS 0.075% Tween-80, pH 7.4) was loaded onto the sensor before every antibody injection. Antibodies were then injected over the IBIS at 1.5 dilution series starting at 3.9 nM until 337.5 nM in PBS in 0.075% Tween-80. Regeneration after every sample was carried out with acid buffer (100 nM H3PO4, 0.075% Tween 80, pH 1.5). Calculation of the dissociation constant (K_D) was done by equilibrium fitting to Rmax=500. In the case of FcγRIII, anti-His association and dissociation curves were subtracted before calculation of IgG-binding affinity using SPRINT 1.9.4.4 software (IBIS technologies). Analysis and calculation of all binding data was carried out with Scrubber software version 2 (Biologic Software, Campbell, Australia).

Statistical analysis

GraphPad Prism 6 was used for data analysis. Data depicted are mean ±SEM. Data was analysed with a 1way ANOVA followed by Tukey's multiple comparison test.

Results

Lack of core-fucose in human IgG1 increases the activation of human effector cells

First, glycosylation profiles of humanized tumour targeting anti-gp75 antibodies (hIgG1 TA99) were investigated. Wildtype TA99 was highly fucosylated, but minimal fucose was present in afucosylated hIgG1 TA99 (Figure 1). B16F10-gp75 mouse melanoma cells were co-cultured with various human effector cells in the presence of humanized tumour targeting anti-gp75 antibodies (hIgG1 TA99) or nonspecific antibodies in antibody dependent tumour killing assays. Peripheral blood mononuclear cells (PBMC) were able to induce tumour cell killing in the presence of hIgG1 TA99 (Figure 2A). Moreover, in the presence of afucosylated hIgG1 TA99 significantly better killing of B16F10-gp75 cells was observed compared to incubation with the non-modified (wildtype) humanized IgG1 TA99 (Figure 2A). Isolated $CD14^+$ monocytes were hardly able to kill B16F10-gp75 cells in the presence of hIgG1 TA99 antibodies

(Figure 2C), whereas peripheral blood lymphocytes (PBL, including NK cells) were more potent in inducing ADCC. Moreover, B16F10-gp75 cells in the presence of afucosylated hIgG1 TA99 were more efficiently killed than with wildtype hIgG1 TA99 (Figure 2B). Within the PBL fraction NK cells were the primary effector cells (Figure 2D). NK cells targeting B16F10-gp75 cells showed in the presence of afucosylated hIgG1 TA99 significantly better ADCC than in the presence of wildtype hIgG1 TA99. Neutrophils, the most abundant effector cell in blood, did not show any capacity to kill tumour cells even with high E:T ratios and increased antibody concentrations (Figure 2E, and data not shown). $CD14^+$ monocyte derived M-CSF macrophages efficiently induced ADCP of B16F10-gp75 cells, particularly through afucosylated hIgG1 TA99 (Figure 2F).

Several studies have demonstrated that low core-fucosylation increases the interaction of human IgG1 to human FcγRIII (CD16)[9,27–30]. Human neutrophils, NK cells, and a small population of monocytes ($CD14^+$) express FcγRIII[31–34]. FcγRI (CD64) and FcγRII (CD32) expression was observed on monocytes and neutrophils. Moreover, also M-CSF cultured $CD14^+$ monocyte derived macrophages express all these Fcγ receptors (Figure 2G-I). These data support that human NK cells and macrophages are primary effector cells due to their expression of the transmembrane FcγRIIIa form, while neutrophils express the glycosylphosphatidyl-inositol (GPI)-linked FcγRIIIb glycoforms.

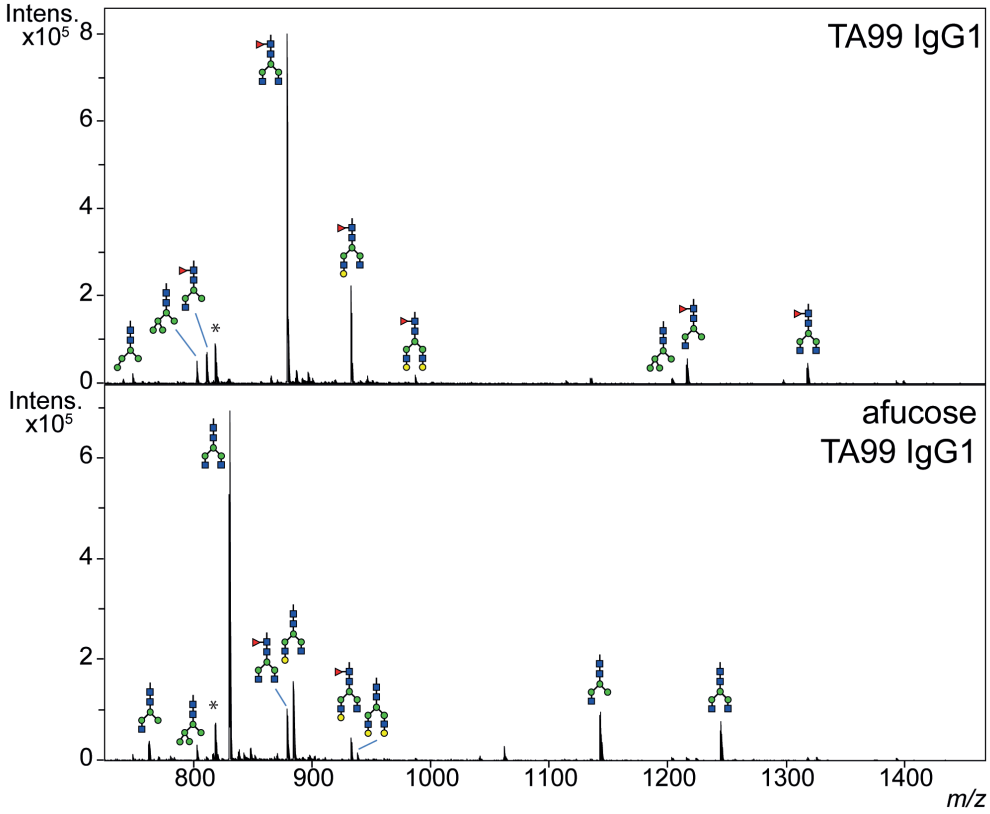

FIGURE 1. Afucosylated antibodies have minimal expression of fucose on glycans.
NanoLC-ESI-IT-MS spectra containing trypsin-generated glycopeptides of humanized wildtype IgG1 TA99 and afucosylated humanized IgG1 TA99. Green circle = mannose; yellow circle = galactose; blue square = N-acetylglucosamine; red triangle = fucose; * = unidentified peptide.

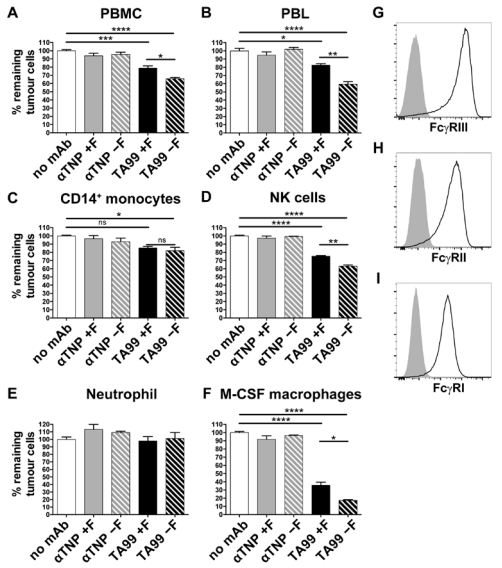

FIGURE 2. Lack of core fucose in the IgG1 Fc domain enhances antibody-mediated tumour killing by natural killer cells and human macrophages.
(A-D, F) ADCC or ADCP (E) with B16F10-gp75 cells in the presence of humanized anti-GP75 TA99 IgG1 (black bars) or control (anti-2,4,6-trinitrophenol, TNP, grey bars) antibodies, with or without core fucose (solid and striped bars resp.) by: (A) PBMC (D) PBL, (C) CD14+ monocytes, (D) NK cells or (F) PMN cells (predominantly neutrophils). (E) ADCP by M-CSF cultured CD14+ monocyte derived macrophages. Remaining tumour cells relative to the no antibody (white bars) co-culture were used as readout. (G-I) FcγRIII (CD16) (H) FcγRII (CD32) (I) FcγRI (CD64) expression on CD14+ M-CSF cultured macrophages was determined by flow cytometry. Plots and graphs represent data obtained in 3 to 5 independent experiments and healthy donors. All graphs represent mean ±SEM. *P<0.05; **P<0.01; ***P<0.001; ****P<0.0001.

Mice treated with afucosylated hIgG1 TA99 develop less peritoneal metastasis

To investigate if removal of the core-fucose affects tumour clearance in mice, a peritoneal metastasis B16F10-gp75 model was used. High tumour outgrowth was observed in mice that had been treated with nonspecific humanized anti-TNP antibodies (Figure 3A). No difference was observed in tumour outgrowth between the groups treated with wildtype or afucosylated anti-TNP. A significant reduction of tumour outgrowth was found in mice treated with TA99 compared to the nonspecific antibodies. Mice treated with afucosylated hIgG1 TA99 had almost no tumour outgrowth at all.

In the peritoneal lavage of untreated mice, the vast majority of the effector cells were F4/80 expressing cells (macrophages, F4/80^{+}GR1^{-} and monocytes, F4/80^{int}), followed by neutrophils (GR1^{+}) and NK cells (CD3^{-}NKp46^{+}NK1,1^{+}) (Figure 3B and C). When PBS was injected in the peritoneal cavity an influx of NK cells and neutrophils in the first 24 hours was observed (Figure 3C). Injection of tumour cells resulted in an increased percentage of monocytes, neutrophils and NK cells in comparison to PBS only. Moreover, monocytes were especially present in high amounts when antibodies were injected in addition to the B16F10-gp75, however no differences are observed between specific and nonspecific antibodies.

Afucosylated human IgG1 has increased affinity for mouse FcγRIV

By plasmon surface resonance array, monitoring the binding of all mouse Fcγ receptors we found that afucosylation of human IgG1 did not affect binding to FcγRI, FcγRII and FcγRIII (Figure 4A-C, table 1), whereas the binding to FcγRIV was increased three-fold (Figure 4D, table 1).

Interaction of afucosylated human IgG1 with FcγRIV is crucial for elevated tumour clearance

To explain the increased *in vivo* efficacy of afucosylated hIgG1 TA99 (Figure 3A) we first analysed Fcγ receptor expression of the different effector populations in the peritoneal cavity and blood. Peritoneal macrophages (F4/80^{+}) and monocytes

(F4/80^{int}) have low expression of FcγRIV whereas no expression was observed on neutrophils

(GR1^{+}) and NK cells (CD3^{-}NKp46^{+}NK1,1^{+}) (Figure 5A). Two populations of F4/80 expressing cells were identified in blood that had different expression profiles of Fcγ receptors, but both expressed higher FcγRI and FcγRIV compared to the other populations (Figure 5B). When FcγRIV$^{-/-}$ mice that had been injected with B16F10-gp75 cells, were treated with wildtype hIgG1 TA99 or afucosylated hIgG1 TA99, therapeutic ability of mAbs was abolished. (Figure 5C). This indicates that FcγRIV was the primary or even the sole receptor involved in the tumour clearance.

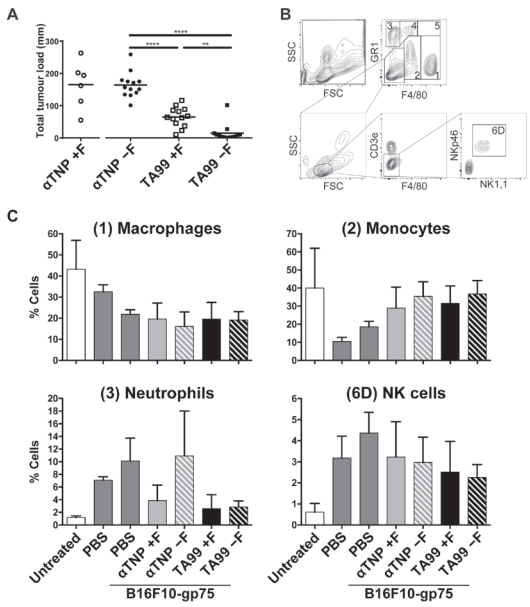

FIGURE 3. Treatment with afucosylated humanized IgG1 TA99 results in decreased tumour outgrowth in vivo.
C57Bl/6 mice were intraperitoneally injected with 50.000 B16F10-gp75 and 50μg nonspecific (○/●) or tumour specific antibodies (humanized TA99, □/■) with normal core-fucose (open symbols) or reduced core-fucose (closed symbols). Fourteen days post injection mice were sacrificed and metastasis outgrowth in the peritoneum was scored. N= 6 for αTNP +Fucose, n=13 for the other groups. (B) Populations identified in a peritoneal lavage. 5 populations are gated in a F4/80/GR1 plot, 1) F4/80+GR1-, 2) F4/80intGR1int, 3) F4/80-GR1+, 4) F4/80intGR1+, 5) F4/80+GR1+. The negative population was used to gate lymphocytes, CD3- cells and 6D) NK cells respectively. (C) Composition of the dominant populations in a peritoneal lavage of mice 24 hours after intraperitoneal injection with PBS, B16F10-gp75 with or without antibodies. N≥4. **P<0.01, ****P<0.0001.

Discussion

Antibodies are rapidly gaining ground as cancer therapeutic due to continuous and rapid discovery of new targets and ways to improve antibody effector functions. One promising modification strategy is changing glycosylation of mAbs, as this can enhance binding to Fcγ receptors. This study investigated whether mice represent a suitable model organism to investigate afucosylated mAbs. Currently various afucosylated antibodies are in clinical trials and a few are also approved for the treatment of haematological cancer types[35,36]. We found that in vitro only human NK cells and monocyte derived M-CSF macrophages are involved in ADCC or ADCP of mouse B16F10-gp75 melanoma cells in the presence of humanized hIgG1 TA99[37,38]. Furthermore, modifying the antibody by producing it without core-fucose on the Fc-glycan resulted in a significant better killing in comparison to the non-modified antibody. This is likely due to enhanced binding to FcγRIIIa that is expressed by both NK cells and macrophages. Neutrophils were unable to induce killing via hIgG1. It was previously demonstrated that granulocyte-macrophage colony stimulating factor (GM-CSF) stimulated human PMNs potently lysed A431 tumour cells via FcγRII in the presence of antibodies, whereas FcγRIIIb did not play a role[39]. However, the presence or absence of core-fucose did not affect neutrophil ability to kill A431 cells supporting that differences in mAb efficacy were not mediated via FcγRII.

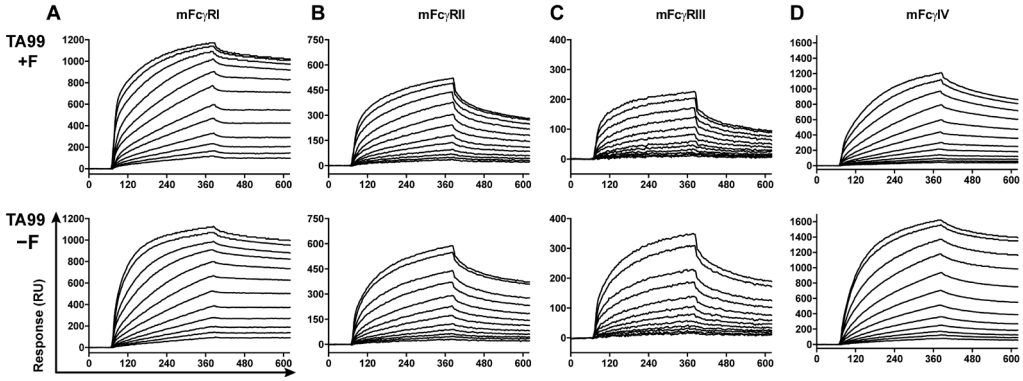

FIGURE 4. Lack of fucose in the Fc domain results in increased binding to mouse FcγRIV.
Binding affinity determined with a Surface plasmon resonance assay with calculated dissociation constants of the wildtype core-fucose (left side) or afucosylated (right side) humanized IgG1 TA99 with (A) FcγRI, (B) FcγRII, (C) FcγRIII and (D) FcγRIV. Dissociation constants are depicted in table 1.

TABLE 1. dissociation constants.

	FcγRI	FcγRIIb	FcγRIII	FcγRIV
TA99 hIgG1 +F	1,34E-09	9,30E-08	2,24E-07	8,97E-08
TA99 hIgG1 -F	1,67E-09	1,35E-07	1,90E-07	2,95E-08
Fold change	*1,25*	*1,45*	*1,18*	*3,04*

Immunoglobulins and their receptors, in particular their functional properties, have great homology between humans and mice. The murine FcγRIV is orthologous for human FcγRIIIa. Additionally, human IgG1 and mouse IgG2a have similar functions[14,16].

Overdijk *et al.* demonstrated that human IgG1 is able to activate mouse effector cells *in vitro*[18]. Our data support that monocytes and macrophages were the primary immune cells in the peritoneal cavity of mice. Even though other Fcγ receptor expressing cells such as NK cells and neutrophils were present and easily recruited, they were likely not involved in cytotoxicity. We observed significant better prevention of metastasis outgrowth in mice treated with afucosylated hIgG1 TA99 compared with wildtype hIgG1 TA99. The effect of core-fucosylation in the Fc domain of human IgG1 influences binding to both orthologous receptors, human FcγRIIIa and mouse FcγRIV. This was confirmed by analysing the binding capacity between highly fucosylated (wildtype) and afucosylated hIgG1 TA99, which showed only a strong influence on binding to FcγRIV. Moreover, mice lacking FcγRIV did not show any benefit from the TA99 treatment in our intraperitoneal metastasis model. This indicates that the interactions between hIgG1 and human FcγRIIIa or its orthologous mouse FcγRIV are conserved between these species, and that the mouse is a suitable model organism to further optimize antibodies for clinical use.

Both human and mouse macrophages express the Fcγ receptor involved in improved killing induced by afucosylated mAbs. In mice, we observed an influx of primarily neutrophils, monocytes, and NK cells after injection with B16F10-gp75 tumour cells into the peritoneal cavity. Of the cells present in the peritoneal cavity, only peritoneal macrophages and monocytes expressed FcγRIV. FcγRIV expressing on activated neutrophils after thioglycollate injection has been documented. We did, however, not observe FcγRIV on neutrophils, which likely reflects that neutrophils were less activated in our experiments[16].

Deleting neutrophils with GR1 antibodies in a subcutaneous mouse model resulted in a complete loss of tumour clearance with TA99 antibodies[40]. By contrast, in a

mouse intraperitoneal metastasis model, macrophages but not neutrophils were the likely effector cells, even though IgA antibodies were used, which can potently activate neutrophils[41–46]. Human NK cells have improved killing capacities with the afucosylated mAb. This is however, not the case in mice since mouse NK cells lack FcγRIV. Mouse NK cells only express the low affinity receptor FcγRIII, and therefore induce only a minimal effect in killing of tumour cells in mice[25,34,37,47–49]. This is also confirmed by the lack of suppression of tumour outgrowth in FcγRIV$^{-/-}$ mice in the presence of tumour targeting mAbs.

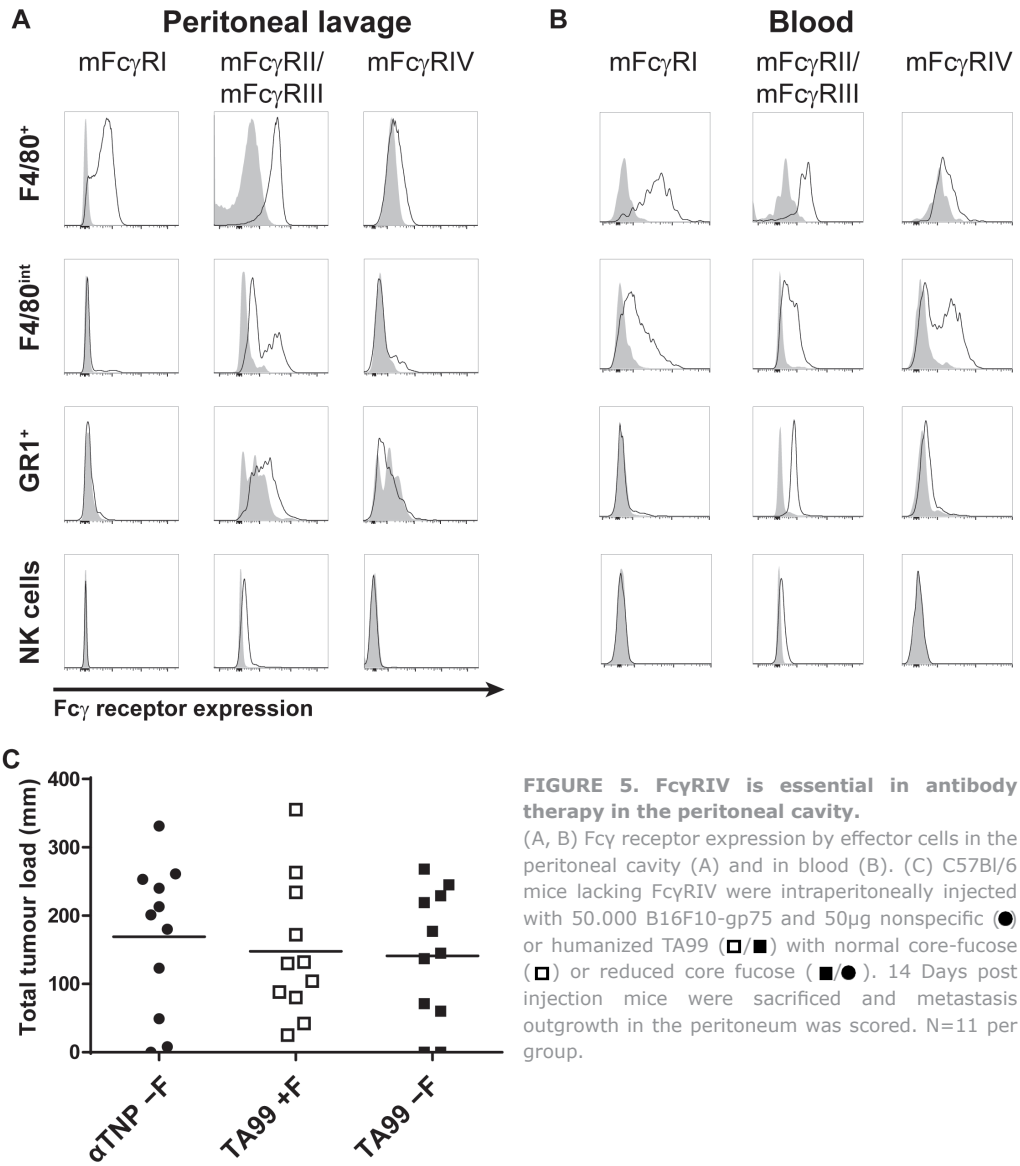

FIGURE 5. FcγRIV is essential in antibody therapy in the peritoneal cavity.
(A, B) Fcγ receptor expression by effector cells in the peritoneal cavity (A) and in blood (B). (C) C57Bl/6 mice lacking FcγRIV were intraperitoneally injected with 50.000 B16F10-gp75 and 50μg nonspecific (●) or humanized TA99 (□/■) with normal core-fucose (□) or reduced core fucose (■/●). 14 Days post injection mice were sacrificed and metastasis outgrowth in the peritoneum was scored. N=11 per group.

It was previously reported that the primary Fcγ receptor for mAb therapy differs between distinct locations of metastasis[15,50,51]. Otten *et al.* demonstrated that the high affinity receptors FcγRI and IV have redundant functions in a liver metastasis model. Knocking out or blocking one of the two was not sufficient to abolish antibody

treatment effects on metastasis outgrowth. Only when both receptors were absent mAb therapy was unsuccessful in preventing development of liver metastasis[51]. Similar to the liver, both the FcγRI and FcγRIV have been described in two independent papers as the primary receptor for antibody therapy in the lungs. Nimmerjahn and Ravetch demonstrated that FcγRIV was responsible for therapeutic effect in a B16F10 lung metastasis model[15]. On the contrary, Bevaart et al. identified FcγRI as main Fcγ receptor for mAb therapy in the B16F10 lung model[50]. The discrepancy in the outcomes in these latter 2 studies has not been clarified. Nonetheless, it is likely that tissue specific cell types are responsible for local effects of therapeutic mAbs, which might also be the case in the peritoneal cavity where, for example, in the omentum colonies of macrophages have been found that might be involved in this process[52].

In conclusion, our data demonstrate that removing the core-fucose of the Fc domain of human IgG1 induces increased tumour killing by human NK cells and macrophages via binding to FcγRIIIa. Importantly, these modified hIgG1 antibodies also have enhanced effector functions through the orthologous receptor of the human FcγRIIIa, FcγRIV, in mice. The potential of afucosylated human IgG1 antibodies can therefore be tested in therapeutic mouse models, which may simplify the road towards preclinical screening of candidate antibodies and the mechanisms of antibody-mediated tumour clearance.

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