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Conserved FcγR- glycan discriminates between fucosylated and afucosylated IgG in humans and mice☆

Gillian Dekkers[a], Arthur E.H. Bentlage[a], Rosina Plomp[b], Remco Visser[a], Carolien A.M. Koeleman[b], Anna Beentjes[a], Juk Yee Mok[c], Wim J.E. van Esch[c], Manfred Wuhrer[b], Theo Rispens[d], Gestur Vidarsson[a,*]

[a] Department Experimental Immunohematology, Sanquin Research and Landsteiner Laboratory, Academic Medical Centre, University of Amsterdam, Amsterdam, The Netherlands
[b] Center for Proteomics and Metabolomics, Leiden University Medical Center, Leiden, The Netherlands
[c] Sanquin Reagents, Department R&D, Amsterdam, The Netherlands
[d] Department Immunopathology, Sanquin Research and Landsteiner Laboratory, Academic Medical Centre, University of Amsterdam, Amsterdam, The Netherlands

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ABSTRACT

The binding strength between IgG and FcγR is influenced by the composition of the N-linked glycan at position N297 in the Fc-domain of IgG. Particularly, afucosylation increases the binding affinity of human IgG1 to human FcγRIIIa up to ~20 fold, and additional galactosylation of the afucosylated IgG increases the affinity up to ~40 fold. The increase in affinity for afucosylated IgG has previously been shown to depend on direct carbohydrate-carbohydrate interactions between the IgG-Fc glycan with an N-linked glycan at position 162 unique to hFcγRIIIa and hFcγRIIIb. Here we report that the N162 glycosylation site is also found in the orthologous mouse FcγR, mFcγRIV. The N162-glycan in mFcγRIV was also responsible for enhancing the binding to mouse IgG with reduced fucose similar to hFcγRIIIa. However, unlike hFcγRIIIa, mFcγRIV did not bind more avidly to IgG with increased galactose and reduced fucose. Overall, these results suggest the N162-glycan in the human FcγRIII family and its orthologous mouse FcγRIV to be functionally conserved.

1. Introduction

Immunoglobulin G (IgG) is the main antibody class found in mammalian sera like humans and mice. IgG signals to the immune cells by first binding of its variable domains to foreign pathogens and then by binding of the constant domain (Fc) and subsequent crosslinking of Fc-gamma receptors (FcγR) on myeloid and NK cells. The capacity of IgG to perform this task is dependent on the affinity of each IgG subclass to the different subtypes of FcγR. Furthermore, this affinity is also influenced by the composition of the conserved glycan at position Asn297 in the Fc of IgG (Dekkers et al., 2017b; Jefferis, 2009; Shields et al., 2002; Shinkawa et al., 2003; Subedi and Barb, 2015; Yasuma et al., 2016). This bi-antennary glycan is composed of a core structure containing *N*-Acetylglucosamine (GlcNAc) and mannose groups and can be variably extended with fucose, galactose, sialic acid and an additional bisecting GlcNAc (bisection). Particularly the absence of core fucose (referred to as afucosylation from here on) on human IgG1 increases the binding affinity to human FcγRIIIa and FcγRIIIb (collectively referred to hereafter as hFcγRIII) by up to ~20 fold (Dekkers et al., 2017b; Shields et al., 2002). Addition of galactose to afucosylated IgG increases the affinity even further for hFcγRIIIa by another ~2 fold, resulting in up to ~40x better affinity of this glycoform for FcγRIIIa (Dekkers et al., 2017b; Houde et al., 2010). Intriguingly, galactose addition has no effect on binding of fucosylated IgG to hFcγR, and also limited effect on binding of afucosylated IgG to hFcγRIIIb (Dekkers et al., 2017b).

Of all human FcγR, FcγRIIIa and FcγRIIIb bear a glycan at position N162 in the binding site for Fc. For those FcγR lacking the N162 glycan, the IgG Fc glycan does not seem to make strong direct contact in the

Abbreviations: Asn, asparagine; Bisection, bisecting GlcNAc; Fc, fragment crystallizable; FcγR, Fc gamma receptor; GlcNAc, *N*-acetylglucosamine; HEK, human embryonic kidney; hFcγR, human FcγR; hIgG, human IgG; IgG, immunoglobulin G; mFcγR, —mouse FcγR; mIgG, mouse IgG; N162, asparagine at position 162; N162A, asparagine at position 162 substituted by alanine; N297A, asparagine at position 297 substituted by alanine; PBS, phosphate buffered saline; R_{max}, maximum response; SPR, surface plasmon resonance; V158, valine at position 158; WT, wild type

☆ The authors declare that they have no conflicts of interest.

* Corresponding author at: Department Experimental Immunohematology, Sanquin Research and Landsteiner Laboratory, Academic Medical Centre, University of Amsterdam, Plesmanlaan 125, 1066CX Amsterdam, The Netherlands.
E-mail address: G.Dekkers@sanquin.nl (G. Vidarsson).

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interaction, and only plays a minor contribution to affinity (Sondermann et al., 2000). However, a clear interaction has been observed between the IgG Fc-glycan and the N162-glycan of hFcγRIII (Ferrara et al., 2006). Interestingly, artificial removal of this hFcγRIII-N162-glycan increases affinity to fucosylated human IgG (dominant glycoform found in both human and mice), suggesting this glycan in human hFcγRIIIa to sterically interfere with binding. However, the hFcγRIII-N162-glycan also discriminates between fucosylated and afucosylated human IgG (Ferrara et al., 2011). When IgG without the Fc-core-fucose residue is compared to IgG with Fc-core-fucose closer glycan-glycan interactions are observed as well as enhanced glycan-protein interactions, which explains the higher affinity (Ferrara et al., 2011). If these interactions are also explaining the additional effects observed upon IgG-Fc galactosylation, is unknown.

For humans, the effect of the glycan composition of IgG1, in particularly fucosylation, on binding to the different FcγRs has already been thoroughly explored (Dekkers et al., 2017b; Subedi and Barb, 2016; Thomann et al., 2015). For mice this has been less extensively studied. The mouse repertoire of FcγRs consists of four FcγRs: mFcγRI, mFcγRIIb, mFcγRIII and mFcγRIV (Nimmerjahn et al., 2005) and their function and expression pattern differs slightly from humans, as reviewed by Bruhns and Jönsson (2015). The mouse orthologue of hFcγRIIIa is the mouse mFcγRIV (Nimmerjahn et al., 2005). Interestingly, it has been reported that afucosylated mouse IgG2a and IgG2b have an at least 10 fold increased affinity for mFcγRIV compared to normally fucosylated mouse IgG. Unlike the human counterpart, a slight increase in binding affinity to mFcγRIIb and mFcγRIII was observed for the afucosylated antibodies (Nimmerjahn and Ravetch, 2005). Galactosylation had no or minimal effect on binding of mouse IgG to mFcγR (Nimmerjahn et al., 2007). However, sialylated mIgG showed an almost 10 fold decrease in affinity for mFcγRIII and mIgG2a for mFcγRIV (Kaneko et al., 2006). The molecular mechanism by which glycosylation and sialylation of mouse IgG affect binding to mouse mFcγRIV is unknown.

Here, we investigated the effect of Asn297-glycosylation of human and mouse IgG on binding to FcγRs, with particular focus on the contribution of the N162-FcγR glycan.

2. Material and methods

2.1. Human and mouse IgG constructs

All restriction enzymes, ligases and accompanying buffers were from Thermo Fisher Scientific (Waltham, MA USA). Anti-human rhesus D (anti-D clone 19A10) IgG1 heavy and kappa light chain were both cloned into pEE14.4 expression vector (Lonza) as described previously (Dekkers et al., 2017a,b). Mouse anti-KEL1 (PUMA1 clone) expression vectors for mouse IgG1 and IgG2a heavy chain and kappa light chain were cloned as described previously (Dekkers et al., 2017a; Howie et al., 2016).

2.2. Human and mouse fusion Fc-FcγR constructs

In order to make the fusion Fc-FcγR constructs the extracellular domains of the human FcγRIIIa V158 (NCBI reference sequence: NP_001121065.1) and mouse FcγRIV (NP_653142.2) were reverse translated and codon optimized. Additionally, for both constructs a variant with N162 mutation was created. All four constructs were ordered from Geneart (Life Technologies, Paisley, UK) (FcγRIIIa variants) or Integrated DNA Technologies (Coralville, IA, USA) (FcγRIV variants) including 5′ HindIII restriction site, Kozak and 3′ EcoRI restriction site. The IgG2-Fc domain, composed of a human IgA1a hinge (PVPSTPPTPSPSTPPTPSPSCCH), human IgG2 Fc CH2 and CH3 domains including a mutation deleting the Fc-glycan (N297A), C-terminal biotinylation tag (BirATag) (GLNDIFEAQKIEW), 5′ EcoRI and 3′ EcoRV restriction sites was reverse translated, codon optimized at Geneart, and ordered from Integrated DNA Technologies (Dekkers et al., 2017b). FcγR and IgG-Fc constructs were ligated at EcoRI restriction site and cloned into pcDNA3.1 expression vector (Invitrogen, Carlsbad, California, USA) using flanking HindIII and EcoRV restriction sites.

2.3. IgG and Fc-FcγR production and isolation

All IgGs and Fc-Fusion FcγRs were produced by transient transfection of HEK-freestyle cells (Thermo Fisher Scientific), as previously described by Vink et al. (2014) and Dekkers et al. (2016). After 5 days the IgG-containing or Fc-Fusion-containing cell supernatant from these cells was harvested by spinning twice at maximum speed (> 4000 g) and subsequent filtration with 0.45 nm puradisc syringe filter (Whatmann, GE Healthcare, Little Chalfront, UK).

IgG and Fc-Fusion FcγR were isolated from cell supernatant with affinity chromatography columns HiTrap Protein A HP (GE Healthcare) for human IgG1, and all Fc-Fusion FcγR constructs or HiTrap Protein G HP (GE Healthcare) for mouse IgG1 and IgG2a on ÄKTA prime (GE Healthcare) according to standard procedures. Purified fractions were concentrated to concentration > 0.5 mg/mL using Protein Concentrators, 9K MWCO (Pierce, Thermo Fisher Scientific) and subsequently dialyzed against phosphate buffered saline (PBS) overnight using Slide-A-Lyzer™ Dialysis Cassettes, 10K MWCO (Thermo Fisher Scientific). Concentration of purified protein was determined using Nanodrop 2000c UV/VIS spectrophotometer (Thermo Fisher Scientific).

2.4. IgG glyco-engineering

Glyco-engineering of human IgG1 was optimized as described by Dekkers et al. (2016). In short: To decrease either fucosylation or galactosylation 0.4 mM (for hIgG1) or 0.2 mM (for mIgG) 2-deoxy-2-fluoro-L-fucose (2FF) (Carbosynth, Berkshire, UK) or 1 mM 2-deoxy-2-fluoro-D-galactose (2FG) (Carbosynth) respectively was added to the cell suspension. To increase galactose, 1% pEE6.4 + B4GALT1 encoding β-1,4-galactosyltransferase 1 (B4GALT1) enzyme was co-transfected with 99% IgG1-κ HC + LC vector and 5 mM D-galactose (Sigma Aldrich, Saint Louis, MO, USA) was added to the cell suspension 1 h before transfection.

2.5. Mass spectrometric analysis of IgG glycan composition

IgG Fc glycan compositions of produced human IgG1 and mouse IgG1 and IgG2a were determined by mass spectrometry as described previously by Dekkers et al. (2016) and de Haan et al. (2017). In short, trypsin-digested glycopeptide samples were analyzed by nanoLC-ESI-QTOF-MS. The separation was performed on an RSLCnano Ultimate 3000 system (Thermofisher, Breda, the Netherlands) with a gradient pump, loading pump and an autosampler. The resulting co-elution of the different glycoforms of the IgG1 Fc glycosylation site warrants fair comparison by ensuring identical ionization conditions for the various glycopeptide species. The LC was coupled to the MS detector *via* a CaptiveSpray source with a NanoBooster (Bruker Daltonics, Bremen, Germany). The Maxis Impact quadrupole-TOF-MS (micrOTOF-Q, Bruker Daltonics) was used as detector. MSConvert (Proteowizard 3.0) (Chambers et al., 2012) was used to convert the data files to mzXML format, and an in-house alignment tool (Plomp et al., 2015) was used to align the retention times of the data files.

A list of previously observed IgG glycans (Dekkers et al., 2016) was used for relative quantification, after manual examination of the current data did not reveal additional glycan structures. Peptide moieties of the glycopeptides differed based on the type of IgG: hIgG1 (EEQYNSTYR) (Dekkers et al., 2016), mIgG1 (EEQFNSTFR), and mIgG2a (EDYNSTLR) (de Haan et al., 2017). The highest intensity of selected peaks (within an m/z window of ± 0.04 and within a time window of ± 12 s surrounding the retention time) was extracted using the in-house developed 3D Max Xtractor software tool. If above a