Characterization of Endogenous Human FcγRIII by Mass Spectrometry Reveals Site, Allele and Sequence Specific Glycosylation

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In Brief
Characterization of endogenous FcγRIII glycosylation from healthy donors with different FcγRIIIB genotypes reveals site specific, and allele specific differences in glycosylation as well as noncanonical sequence specific differences in glycosylation. We propose these differences in glycosylation may influence the differential activity seen for neutrophils across genotypes.

Highlights
- Glycosylation of endogenous FcγRIII from neutrophils and matched plasma from more than 40 donors characterized at two sites involved in IgG binding.
- Glycosylation of soluble FcγRIII glycosylation at N45 can be used to assign FcγRIIIB alleles.
- FcγRIIIB allele specific differences in glycosylation at N162 may influence differential activity observed for primary cells.
Characterization of Endogenous Human FcγRIII by Mass Spectrometry Reveals Site, Allele and Sequence Specific Glycosylation*

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The importance of IgG glycosylation, Fc-gamma receptor (FcγR) single nucleotide polymorphisms and FcγR copy number variations in fine tuning the immune response has been well established. There is a growing appreciation of the importance of glycosylation of FcγRs in modulating the FcγR-IgG interaction based on the association between the glycosylation of recombinant FcγRs and the kinetics and affinity of the FcγR-IgG interaction. Although glycosylation of recombinant FcγRs has been recently characterized, limited knowledge exists on the glycosylation of endogenous human FcγRs. In order to improve the structural understanding of FcγRs expressed on human cells we characterized the site specific glycosylation of native human FcγRIII from neutrophils of 50 healthy donors and from matched plasma for 43 of these individuals. Through this analysis we have confirmed site specific glycosylation patterns previously reported for soluble FcγRIII from a single donor, identified FcγRIllb specific Asn45 glycosylation and an allelic effect on glycosylation at Asn162 of FcγRIllb. Identification of FcγRIllb specific glycosylation allows for assignment of FcγRIllb alleles and relative copy number of the two alleles where DNA/RNA is not available. Intriguingly, the types of structures found to be elevated at Asn162 in the NA2 allele have been shown to destabilize the Fc:FcγRIll interaction resulting in a faster dissociation rate. These differences in glycosylation may in part explain the differential activity reported for the two alleles which have similar in vitro affinity for IgG. Molecular & Cellular Proteomics 18: 534–545, 2019. DOI: 10.1074/mcp.RA118.001142.

Receptors for the Fc region of IgG (FcγRs) are critical in modulating the adaptive immune response. Interaction between the receptors and IgG in immune complexes or on opsonized cells promotes downstream effector function such as antibody dependent cellular phagocytosis (ADCP) and antibody dependent cellular cytotoxicity (ADCC). In humans, there are five activating FcγRs specifically FcγRI (CD64), FcγRIla (CD32A), FcγRIlc (CD32c), FcγRIllla (CD16A), and FcγRIlllb (CD16B) as well as the inhibitory FcγRIllb (CD16B) (1). FcγRIllla and FcγRIlllb are two closely related proteins with at least 95% homology in the amino acid sequence of the extracellular domains which are nearly indistinguishable when considering the common variants (Fig. 1).

FcγRIllla is expressed on NK cells, and subsets of monocytes, macrophages and dendritic cells. The cytoplasmic domain of FcγRIllla associates with the immunoreceptor tyrosine-based activation motif (ITAM) containing common FcRy chain which drives intracellular signaling events (2). The V158F polymorphism which is found in the extracellular domain of FcγRIllla results in increased affinity between the V158 variant and all IgG subclasses (3). Functionally, NK cells bearing FcγRIllla with the V158 variant exhibit enhanced response to immune complex stimulation (4). FcγRIlllb is a glycosphatidylinositol (GPI) linked protein expressed primarily on neutrophils and basophils (2).

FcγRIlllb is highly polymorphic with three common alleles differing at 5 sites in the protein (Fig. 1). Alleles named NA1, NA2 and SH or alternately HNA-1a, HNA-1b and HNA-1c have been described (2), (5) and additional variants have been detected (6). These variants do not influence the affinity of the FcγR-IgG interaction (3) but have been reported to influence neutrophil activity (7), (8), and (9). Both FcγRIllla and FcγRIlllb are heavily glycosylated. FcγRIllla contains five potential sites of glycosylation at N38, N45, N74, N162 and N169. The NA1 and NA2/SH alleles are distinguished by amino acid differences at four sites specifically R19S, N47S, D65N and V89I for the NA1 and NA2 alleles respectively. The SH allele is distinguished from the NA2 alleles by A61D substitution (5).

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site at N65. The NA1 allele on the other hand has only four potential sites of glycosylation because of allelic variation. The N45 and N65 sites are not glycosylated in the NA1 allele because of the presence of N47 and D65 respectively.

Glycosylation has long been established as a critical parameter influencing the Fc/H9253R-IgG interaction with core fucosylation (10) and sialylation (11) of the Fc domain of IgG being the best studied. Fc/H9253RIII glycosylation has additionally been reported to play a role in the Fc:Fc/H9253RIII interaction. Point mutations targeting each of the N-linked glycans from Fc/H9253RIII demonstrated that the glycans at N45 and N162 play a role in the formation of the Fc:Fc/H9253RIII complex. Removal of the N45 glycosylation site increased the affinity of the interaction between the Fc bearing nonfucosylated glycans and Fc/H9253RIIIa (12). A separate study using a similar approach demonstrated that the N-glycans at N162 were required for the higher affinity interaction seen for nonfucosylated glycans on the Fc (13).

The crystal structure of the complex between Fc/H9253RIIIa bearing high mannose type glycans (14) or neutral complex glycans (15) and the Fc domain for human IgG1 showed the interaction was stabilized by carbohydrate-protein and carbohydrate-carbohydrate interactions primarily involving Fc/H9253RIII glycans at N162. A recent study utilizing NMR to characterize the solution phase dynamics of glycoengineered Fc/H9253RI expressed in HEK cells identified unexpected contacts between the glycans at N45 and the polypeptide backbone (16).

Initial studies examining influence of FcγRI glycosylation on the FcγRIIa:Fc interaction relied on mutagenesis to selectively remove entire glycan chains. Subsequent studies utilized recombinant proteins produced in different cell types to examine the influence of the nature of the glycans present on the receptor on Fc:FcγRIIa binding. Several groups have published data on recombinant FcγR glycosylation from BHK (17), NS0 (18), CHO and HEK (19), (20). The FcγRIIa glycosylation pattern varied significantly among expression systems and was demonstrated to influence the kinetics but not the affinity of the interaction when comparing CHO and HEK expressed protein (19). A recent publication compared the effect of expression system on glycosylation pattern of recombinant FcγRI and FcγRIIa and reproduced the differential kinetics reported by Zeck et al. (20). The authors proposed that glycosylation differences, principally branching and sialylation, destabilized the interaction resulting in more rapid dissociation of the complex. These studies complement those utilizing point mutations and provide detail on the influence of FcγRI glycan structure on the Fc-FcγR interaction. The advances in LC-MS based characterization of glycopeptides in the past decade (21), provide a means for monitoring site specific glycosylation changes (22) of proteins from complex biological systems (23). Characterization of site-specific glycosylation patterns of endogenous human FcγRs can help to advance an understanding of the impact of FcγR glycosylation on immune cell activation.

Here we present the characterization of native FcγRIIib glycosylation from isolated human neutrophils as well as soluble FcγRIIa, which is a mixture of FcγRIIa and FcγRIIib, isolated from matched plasma. Through this analysis we identified FcγRIIib specific glycosylation at N45 and an allelic
influence on glycosylation of N162, which are consistent with and expand upon recent reports.

**Experimental Procedures**

**Healthy Donor Samples**—Matched plasma and neutrophils were obtained from healthy donors after informed consent through a combination of an internal blood donor program as well as a commercial source (Sanguine Bio; Sherman Oaks CA). The collection, handling and biomolecular analysis of healthy human neutrophils per experimental protocol 102013–001 was approved by the Western Institutional Review Board. Plasma was collected in EDTA tubes.

**Neutrophil Isolation**—Neutrophils were isolated from lysed whole blood by negative selection using the Neutrophil Enrichment Kit (StemCell Technologies, Vancouver, BC, Canada; Catalogue #19257) following manufacturer’s instructions. Freshly isolated neutrophils were pelleted and frozen at −80 °C until they were used for analysis. Paired neutrophils and plasma from a patient were from the same draw.

**Multiplexed Ligation-dependent Probe Amplification**—For 33 of these donors FcγRs polymorphisms and copy number were determined using Multiplexed Ligation-dependent Probe Amplification (MLPA). MLPA assays were performed using commercially available kits from MRC-Holland according to software manufacturer instructions. The NA1 allele is nonglycosylated at this site. The glycopeptide at N162 is common to FcγRIII and IgG. The glycopeptide at N45 is common to FcγRIII and the NA2 and SH alleles of FcγRIII whereas the NA1 allele is nonglycosylated at this site. The glycopeptide at N162 is common to FcγRIII and all alleles of FcγRIII (Table I).

**Proteolysis, Exoglycosidase Treatment and nLC-MS/MS Analysis**—The glycosylation pattern of FcγRIII N45 was characterized using a chymotrypsin (Sequencing Grade Promega, Madison, WI; V1061) which cleaves C-terminal to hydrophobic residues such as tyrosine, tryptophan and phenylalanine to generate the glycopeptides shown in Table I. A sequential digestion with endopeptidase GluC (Sequencing Grade Promega V1651) which cleaves C-terminal to glutamic acid followed by chymotrypsin was used to characterize the N162 glycopeptides.

We have focused on site specific glycosylation at N45 and N162 because removal of these two sites of glycosylation was demonstrated to influence the interaction between FcγRIII and IgG. The glycopeptide at N45 is common to FcγRIII and the NA2 and SH alleles of FcγRIII whereas the NA1 allele is nonglycosylated at this site. The glycopeptide at N162 is common to FcγRIII and all alleles of FcγRIII (Table I).

The peptides and glycopeptides were analyzed by nLC-MS/MS on a Dionex Ultimate 3000 nano RSLC (ThermoFisher Scientific) coupled to a QExactive mass spectrometer (ThermoFisher Scientific) equipped with an EasySpray nano-LC source (ThermoFisher Scientific). Peptides were separated on an EasySpray C18 column (0.75 × 250 mm 2 μm particle size ThermoFisher Scientific ES802). A data dependent acquisition was run initially to identify glycopeptides from each site. Glycopeptides were identified by searching GlycoWorkbench (26).

**Isolation of Neutrophil Fc Gamma Receptors**—Neutrophil FcγRIIib was isolated from 5 million neutrophils. Plasma FcγRIIib was isolated from 50 μL of plasma. Proteins were immunoprecipitated using biotinylated goat polyclonal antibodies against human FcγRIIib (R&D Systems, Minneapolis, MN; BAF1597). The proteins were isolated from neutrophils by first spinning down cells at 300 × g for 2 min and then washing 3 × 500 μL of ice cold PBS. Then 75 μL of IP Lysis Buffer (ThermoFisher Scientific, Waltham, MA; 87787) was added to each sample and cells were lysed by sonication and cell debris spun out at 10,000 × g for 5 min. PBS was added to the supernatant to bring the volume to 500 μL then the biotinylated antibody was added and allowed to incubate for 18 h at 4 °C. The antibody-FcγR complex was isolated using streptavidin magnetic beads (ThermoFisher Scientific 88816). The beads were washed two times 500 μL of IP Lysis buffer and two times with 500 μL of ice-cold PBS. The bound protein was eluted by incubating the beads in 50 μL of 6 M guanidine HCl. The eluted protein was reduced for 30 min at 65 °C with DTT at a concentration of 25 mM. Free cysteine residues were alkylated with iodoacetamide at a concentration of 75 mM. The isolated proteins were dialyzed across a 10kDa membrane against 4L of 25 mM ammonium bicarbonate for 18 h at 4 °C before proteolysis. Soluble FcγRs were isolated from 50 μL of plasma as described above omitting the cell lysis and centrifugation step.

**Table I**

| Table I | Species-specific peptide sequences generated from proteolysis of FcγRIII |
|---------|-----------------|
| Sequence | Species | Variant |
| FIDA(61)ATDV(65)DSGEY | FcγRIIib NA1/FcγRIIla | FcγRII N65 |
| FIDA(61)ATVN(65)DSGEY | FcγRIIib NA2 | FcγRII N65 |
| FIDD(61)ATVN(65)DSGEY | FcγRIIib SH | FcγRII D61/N65 |
| FH(45)EN(47)LISSQAASSY | FcγRIIb NA2/SH/FcγRIIla | FcγRII S47/N45 glycopeptides |
| FH(45)EN(47)LISSQAASSY | FcγRIIb NA1 | FcγRII N47 |
| SDPQYELVH(89)GW | FcγRIIb NA2/SH/FcγRIIla | FcγRII I89 |
| SDPQYELVH(89)GW | FcγRIIb NA1 | FcγRII V89 |
| VGSKN(162)VSSE | FcγRII N162 | FcγRII N162 glycopeptides |
CHARACTERIZATION OF N162 GLYCOSYLATION

Species monitored for targeted MS/MS analysis of FcγRIII chymotryptic digest. Isolation width was set at 3 Da. The species monitored are described in the comments column. The species with glycan compositions described in the comments column. The species with glycan compositions were monitored for each species are shown in supplemental Table S1.

**TABLE II**

| Mass [m/z] | Start [min] | End [min] | (N)CE | Species |
|------------|-------------|-----------|-------|---------|
| 701.3      | 42          | 50        | 25    | FcγRIII N65D NA1/NA2 |
| 798.9      | 35          | 41        | 25    | FcγRIII N47 NA1     |
| 723.8      | 30          | 60        | 25    | FcγRIII D61 SH      |
| 683.3      | 47          | 53        | 25    | FcγRIII V89 NA1     |
| 690.5      | 50          | 56        | 25    | FcγRIII I89 NA2     |
| 1394.1     | 32          | 38        | 25    | FcγRIIIb S47 N45 M5 |
| 1475.1     | 32          | 38        | 25    | FcγRIIIb S47 N45 M6 |
| 1556.1     | 31          | 37        | 25    | FcγRIIIb S47 N45 M7 |
| 1094.8     | 36          | 42        | 25    | FcγRIII S47 N45 M4A1G1S1 |
| 1143.5     | 36          | 42        | 25    | FcγRIII S47 N45 FM4A1G1S1 |
| 1637.1     | 31          | 37        | 25    | FcγRIII S47 N45 M8  |
| 1718.2     | 30          | 37        | 25    | FcγRIII S47 N45 M9  |
| 1148.8     | 36          | 42        | 25    | FcγRIII S47 N45 M5A1G1S1 |
| 1040.0     | 36          | 42        | 25    | FcγRIII S47 N45 A1G1S1 |
| 1088.8     | 36          | 42        | 25    | FcγRIII S47 N45 FA1G1S1 |

**TABLE III**

| Mass [m/z] | Composition |
|------------|-------------|
| 1038.8     | N162 FA2G2S1F1 |
| 1111.4     | N162 FA3G3S1  |
| 1160.1     | N162 FA3G3S1F1 |
| 1209.2     | N162 FA3G3S2  |
| 868.0      | N162 FA1G1S1  |
| 989.7      | N162 FA2G2S1  |
| 892.7      | N162 FA2G2    |
| 1086.7     | N162 FA2G2S2  |
| 1135.4     | N162 FA2G2S2F1 |
| 1257.2     | N162 FA3G3S2F1 |
| 941.5      | N162 FA2G2F1  |
| 1306.2     | N162 FA3G3S3  |
| 453.7      | N162 Agycosyl |

Allelic variants based on peptide sequence information. The quadrupole isolation width was set to ±1.5 Da for the isolation of the parent ion of each of the species for the chymotryptic digest (Table II) and the chymotryptic − Glc Digest (Table III). The AGC was on the QExactive ion source set to 1,000,000 ions and a normalized collision energy of 27 was used for glycopeptide fragmentation. Targeted species were confirmed based on the full MS/MS and quantified based on the extracted ion abundance for the most abundant fragment. Glycopeptides fragmented by HCD give rise to a characteristic Y1 ion when detected with HR/AM provides a marker fragment unique to each glycopeptide backbone. Identification of the glycopeptides was dependent upon the presence of the Y1 ion in the MS/MS spectrum as well as the presence of at least two specific peptide fragments and the characteristic oxonium ions (supplemental Fig. S2, supplemental Fig. S3). The relative abundance of each glycopeptide was determined using the extracted ion abundance of the Y1 ion relative to the summed extracted ion abundance of this fragment from all glycopeptides at each site of glycosylation. The specific precursor and fragment ions monitored for each species are shown in supplemental information (supplemental Fig. S2).

**Data Analysis for Identification of Allelic Variants and Relative Quantitation of Site-specific Glycosylation**—Data analysis was performed using Xcalibur software Qual browser. Each glycopeptide species was quantified based on the MS/MS extracted ion current (XIC) for the ion corresponding to the Peptide + N-acetylgalactosamine fragment. The relative abundance was calculated by dividing the XIC area of each species by the summed XIC area for each site. FcγRIIIb alleles were identified based on the presence of marker peptides and specific glycosylation at N45; the specific markers used at each site for the assignment the FcγRIIIb alleles are shown below (Table IV). For assignment of alleles and comparison to copy numbers the abundance of N45 high mannose type glycopeptides was calculated relative to the sum of all high mannose glycopeptides plus the NA1 specific aglycosyl peptide. For presentation of N-glycopeptide distribution the aglycosyl NA1 peptide was not included in the calculation. In order to estimate site occupancy at N65 from the NA2 allele the area of the nonglycosylated peptide was normalized to the area of the D65 peptide from the NA1 allele for heterozygotes. Similarly, the area of the NA2 specific I89 peptide was normalized to the NA1 specific V89 peptide. The ratio of these normalized areas was calculated for donors having both alleles. Because the normalized area N65 to D65 but not the normalized area of I89 to V90 is affected by the presence of glycosylation at N65 a ratio close to 1 indicative of relative site occupancy.

**Experimental Design and Statistical Rationale**—The genotypes of the subjects included in this study were 29% (n = 14) NA1/NA1, 35% (n = 17) NA1/NA2 and 35% (n = 17) NA2/NA2 and 4% (n = 2) NA1/SH genotype. FcγR glycosylation from neutrophils and plasma was analyzed one time for each donor. Statistical comparisons between the alleles were performed using unpaired two-sided t test. Associations between glycoforms and copy number variants were assessed using one-way ordinary ANOVA, p < 0.05 was considered significant.

**RESULTS**

**Characterization of Neutrophil FcγRIIIb Alleles**—The FcγRIIIb alleles for 50 donors were determined from FcγRIIIb protein isolated from neutrophils via LC-MS/MS. The alleles were assigned based on the presence of allele specific peptide sequences including the glycopeptide containing N45 (Table IV). Using this approach several donors with noncanonical sequences were identified. Because of the variability observed in the protein the decision was made to genotype the FCGR3B gene in a subset of these donors using Multiplexed Ligand-dependent Probe Amplification (MLPA). This technique provides genetic information as well as information about copy number variants which have been reported to be common for FcγRIIIb (27). Our MLPA data shows that copy number variants are common for FcγRIIIb consistent with recent reports. Assignment of alleles was more than 95% concordant between the two methods with one of the noncanonical donors analyzed by both methods being the only divergent result out of the 22 donors (supplemental Table S1).

Assignment of FcγRIIIb allele based on the protein sequence and N45 glycosylation identified two types of noncanonical donors. Two donors appeared to be homozygous for
the NA1 allele based on the absence of the N65 peptide and N45/S47 glycopeptide. However, these donors had both the NA1 V89 and the NA2 I89 variant suggesting they have one canonical NA1 allele and one noncanonical NA1 (supplemental Fig. S9, S10, and S11). Two other donors appeared to be homozygous for the NA2 allele based on the absence of the NA1 specific aglycosyl N45/N47 and V89 peptides. However, these donors lacked the NA2 specific N65 peptide and instead had the NA1 D65 variant. Interestingly, the noncanonical NA2/NA2 donors also had lower levels of high mannose type glycans at N45 suggesting a possible influence of N65 on the glycosylation at N45 (see Fig. 4E, 4F).

Overview of FcγRIIlb Site Specific Glycosylation—Site specific glycosylation was characterized for N45 and N162 two of the sites common to both FcγRIIlb and FcγRIlla. These were selected because glycosylation of these two sites has been demonstrated to influence the interaction between FcγRIII and the Fc domain of IgG (13), (12). Consistent with recent reports (28) distinct site-specific glycosylation patterns were identified for these two sites with almost no overlap between species identified at the two (Fig. 2). The NA2 allele of FcγRIIlb contains two additional potential sites of glycosylation compared with the NA1 allele with N45 and N65 are specific to NA2 allele. N65 was recently reported to be glycosylated in native FcγRIII from human serum (28) we detected only the nonglycosylated N65 peptide in donors having at least one canonical NA2 allele. Comparing the ratio of marker peptides for the different alleles across the 20 heterozygous donors suggests the NA2 specific site at N65 is largely unoccupied with only 3 of the 17 heterozygous donors having a ratio <1 when analyzing FcγRIIlb from isolated neutrophils (Fig. 3). Although N45 was found to be fully occupied based on the absence of the nonglycosylated N45/S47 peptide.

N-glycans at N45 Are High Mannose and Sialylated Hybrid Type—Glycopeptides from FcγRII N45 were characterized using a chymotryptic digest which generated a single major glycopeptide backbone with the sequence FHN(45)ES(47)LISSQASSY. Ten unique FcγRIIlb N45 glycopeptide masses were identified which eluted at 15 distinct chromatographic peaks. The N-glycans identified at FcγRIll N45 are high mannose type and sialylated hybrid type glycans (Table V). The sialylated glycans were found to contain both α2–6 and α2–3 linked sialic acid with the former being more abundant (Table V). No nonglycosylated N45 peptide was detected in FcγRIII protein with the consensus NxS/T motif as is the case for FcγRIllla and the NA2 allele of FcγRIIlb suggesting this site is fully occupied in the NA2 allele and FcγRIlla. These results are largely consistent with recent reports which identified high mannose type glycans at this site from FcγRIll isolated from serum (28).

FcγRIIlb Contains High Mannose and Sialylated Hybrid N-glycans Whereas FcγRIllla Contains Only Sialylated Hybrid N-glycans—Comparing the glycosylation patterns between matched plasma and neutrophils from healthy donors suggests high mannose type glycans at N45 are specific to FcγRIIlb. FcγRIIl isolated from the plasma of donors homozygous for the NA1 allele show only hybrid sialylated structures (Fig. 4A). The lack of the consensus glycosylation motif in FcγRIIlb means that N45 glycans of plasma FcγRIIl from donors homozygous for the NA1 allele must be contributed

| FcγRIIlb NA1 | FcγRIIlb NA2 | FcγRIIlb SH | FcγRIlla |
|-------------|-------------|-------------|-----------|
| Aglycosylated | Glycosylated | Glycosylated | Glycosylated |
| FIDAA(61)ATVN(65)DSGEY | FIDAA(61)ATVN(65)DSGEY | FIDD(61)ATVN(65)DSGEY | FIDAA(61)ATVN(65)DSGEY |
| SDPVQLEVH | SDPVQLEVH | SDPVQLEVH | SDPVQLEVH |

Fig. 2. FcγRIII site specific glycan structures with average abundance across the 50 healthy donors. Crystal structure for FcγRIII (3SGK (14)) is also shown to illustrate the position of the glycosylation sites relative to the FcγRIII:Fc binding site.
entirely by FcγRIIIA. FcγRIIIA isolated from plasma of donors with at least one NA2 allele contains a mixture of high man-nose and hybrid structures (Fig. 4B). The lack of high man-nose type glycans in both plasma and neutrophils from NA1 homozygotes as well as the presence of these species in donors with at least one NA2 allele suggests high mannose type glycans at N45 can be used as a specific marker for the NA2 allele of FcγRIIIB.

In most donors with at least one NA2 allele high mannose type species represented 80–90% of glycans at N45 from neutrophils but from only 40–60% in plasma derived FcγRIIIA based on relative abundance (Fig. 4C, 4D). This is not entirely unexpected as plasma derived FcγRIIIA is a mixture both FcγRIIIB and FcγRIIIA though most soluble FcγRIIIA has been reported to be FcγRIIIB shed from neutrophils (29). The differences between neutrophil and plasma FcγRIIIA glycosylation at N45 held true for more than 90% of donors tested. However, two donors out of 50 had low levels (<10%) of high mannose glycans in isolates from neutrophils and two additional donors showed higher levels of high mannose glycans in plasma than on isolated neutrophils (Fig. 4D). The two donors with low levels of high mannose also had noncanonical FcγRIIIB alleles with the NA1/FcγRIIIA D65 variant replacing the N65 variant found in the NA2 allele suggesting an interaction between the glycans at N45 and the side chain of N65/D65 (Fig. 4E, 4D).

The relative abundance of the individual glycopeptide varied substantially between donors (Table V). The identification of FcγRIIIB specific high mannose type glycans allows for the determination of FcγRIIIB genotype and relative copy number of the two alleles where DNA/RNA is not available. Plotting the area abundance of high man-nose type glycans in plasma relative to the nonglycosylated NA1 peptide for donors with different copy number variants shows a significant association between the levels of high mannose type glycans relative to the aglycosyl N45/N47 and the relative number of each allele as determined by MLPA (Fig. 5).

N162 Contains Complex Sialylated N-glycans With Variable Branching and Antennary Fucose—Glycopeptides at N162 were quantified from a combined chymotrypsin endoproteinase GluC digest as described previously (19). Under these conditions only a single predominant peptide backbone with
the sequence VGSKN(162)VSSE was formed (supplemental Fig. S1). Fourteen unique FcγRIIIb N162 glycopeptide masses were identified which eluted as 30 distinct chromatographic peaks. The N-glycans consist of complex type glycans which are predominantly sialylated and almost entirely core-fucosylated (Table VI). Sialylated N-glycans were found to contain both α2–6 and α2–3 linked sialic acid with the former being more abundant (Table VI). Antennary fucose was identified for...
a number of species including both Lewis and sialyl Lewis motifs based on the presence of characteristic nonreducing end fragments from the targeted MS/MS experiments (supplemental Fig. S4). Extraction of characteristic nonreducing end fragments from high resolution accurate mass (HR/AM) MS/MS allows for a clear differentiation between FA2G2F1S1 peak 1 and peak 3. From this analysis peak 1 is fucosylated on the nonsialylated antenna whereas peak 3 is fucosylated on the sialylated antenna. Fragmentation of the antennary fucosylated glycopeptide by low energy CID confirmed the assignments based on nonreducing fragments from HCD with HR/AM detection (supplemental Fig. S5). Interestingly there appears to be a preference for antennary sialylation of the sialylated antennae consistent with a sLex structure (supplemental Fig. S8). These structures are likely to represent sLe\(^x\) not sLe\(^x\) structures based on the presence of the preferred sLe\(^x\) substrate Ga\(\beta\)j\(\beta\)1–4GlcNAC found on N-glycans (30).

Highly branched structures and structures containing N-acetyllactosamine extensions were identified and characterized based on the detection of specific nonreducing end fragments. N-glycopeptides with up to three antennae and three sialic acids were identified as were species with N-acetyllactosamine extensions. One of the most abundant highly branched species was identified as FA3G3S1 (Table VI). Targeted MS/MS with extraction of diagnostic fragments from the nonreducing end reveals the presence of a fragment corresponding the di-lactosamine tetrasaccharide (diLacNAc) associated with the first chromatographic peak (supplemental Fig. S6). None of the 4 peaks generates a fragment suggesting the presence of sialylated di-LacNAc under HCD fragmentation. Low energy CID confirms the presence of di-LacNAc in the first peak (supplemental Fig. S7) as well as the absence of sialylated di-LacNAc even under lower energy fragmentation. There was substantial variation in the distribution of glycopeptide abundances at this site (Table VI). Similarly, to N45; N162 appears to be fully or highly occupied in these healthy donors.

The Fc\(\gamma\)RIIIb Allele Influences the Glycosylation at N162—Interestingly, a comparison between different Fc\(\gamma\)RIIIb polymorphic variants suggests glycosylation at N162 is influenced by the allele. Most of the glycans at N162 are biantennary however, a significant amount are triantennary or lactosamine extended species (Fig. 6A). A comparison of the N-glycopeptide relative abundances between alleles shows a trend toward increased levels of each of the branched species in the NA2 variant (Fig. 6A). When these similar glycoforms are combined there is a significant difference in the levels of these glycoforms across the different alleles (Fig. 6B). The association between the NA2 allele and higher levels of these larger

### TABLE V

| N45 glycopeptides | Proposed Structure | Range Neutrophils | Range Plasma |
|-------------------|--------------------|-------------------|--------------|
| M5                | ![structure](image) | 1.2%-16.2%        | 1.6%-20.5%   |
| M6                | ![structure](image) | 3.0%-23.7%        | 0.9%-23.2%   |
| M7                | ![structure](image) | 0.8%-29.8%        | 0.1%-27.4%   |
| M8                | ![structure](image) | 0.2%-33.3%        | 0.1%-24.4%   |
| M9                | ![structure](image) | 0.9%-9.8%         | 0.1%-4.6%    |
| FM4A1G1S1-1       | ![structure](image) | 0.5%-14.6%        | 1.0%-21.6%   |
| FM4A1G1S1-2       | ![structure](image) | 0.1%-15.1%        | 0.1%-13.1%   |
| M4A1G1S1-1        | ![structure](image) | 0.1%-15.1%        | 0.6%-37.9%   |
| M4A1G1S1-2        | ![structure](image) | 0.1%-14.3%        | 0.1%-17.3%   |
| M5A1G1S1-1        | ![structure](image) | 0.1%-6.3%         | 0.4%-18.3%   |
| M5A1G1S1-2        | ![structure](image) | 0.1%-8.0%         | 0.1%-6.7%    |
| A1G1S1-1          | ![structure](image) | 0.1%-9.4%         | 0.7%-13.6%   |
| A1G1S1-2          | ![structure](image) | 0.1%-9.3%         | 0.1%-7.1%    |
| FA1G1S1-1         | ![structure](image) | 0.5%-9.0%         | 0.7%-10.4%   |
| FA1G1S1-2         | ![structure](image) | 0.1%-8.1%         | 0.1%-4.1%    |
Endogenous Human FcγRIII Glycosylation

![Diagram of FcγRIII Glycosylation](image)

**Fig. 5.** High mannose glycan at N45 are specific to neutrophils and plasma abundance reflects allele distribution. The relative abundance of high mannose type glycopeptides compared with the nonglycosylated NA1 peptide is significantly associated with the relative number of the two alleles (p < 0.0001 one way ordinary ANOVA).

DISCUSSION

We characterized FcγRIIIb glycosylation from isolated neutrophils as well as matched plasma, which contains both FcγRIIIb and FcγRIIIa, for donors with different FcγRIIIb genotypes. The N45 glycosylation site of FcγRIIIb from neutrophils was found to contain primarily high mannose type glycans with lower levels of sialylated hybrid structures. This site in FcγRIIIb is specifically found on the NA2 allele and high mannose species were absent from soluble FcγRIII from donors homozygous for the NA1 allele suggesting the presence of these species in plasma is a marker for the NA2 allele. The allele specific nature of the high mannose type glycans we observed is consistent with previous reports which showed Concanavalin-A (Con-A) binding specifically to the NA2 allele of FcγRIIIb (31), (32). The NA2 specific glycosylation site N45 is likely responsible for the allele specific Con-A reactivity observed by these authors based on the lack of occupancy we observed at N65 the other NA2 specific site.

Previous work utilizing lectin binding identified cell type specific glycosylation of FcγRIIIa. In this study FcγRIIIa from NK cells was found to contain both high mannose and complex type glycans whereas monocyte FcγRIIIa was found to contain only complex type glycans (32). The authors utilized Con-A reactivity to characterize the glycosylation and many of the hybrid type structures found on soluble FcγRIIIa from donors homozygous for the NA1 allele would also bind the Con-A lectin (33). It may be that these hybrid structures are responsible for Con-A reactivity seen in NK cell derived FcγRIIIa. A recent report analyzing glycans released from FcγRIIIa isolated from NK cells identified high mannose type glycans though the specific site was not determined (34). This raises the possibility that FcγRIIIa from NK cells contains high mannose glycans at a site that was not analyzed in this study. It is also possible that in healthy donors very little sFcγRIII comes from NK cells which is consistent with their low frequency in peripheral blood.

The abundance of these high mannose type N45 glycopeptides in plasma relative to the NA1 specific aglycosyl peptide is proportional to the relative number of the two alleles as measured by MLPA which supports the finding that high mannose type glycans at N45 on soluble FcγRIIIa are a marker for the NA2 allele. This method of assigning genotype showed high concordance with MLPA using DNA from isolated neutrophils. The high concordance coupled with the low volume of plasma required for analysis suggest that this approach could be applied to characterize FcγRIIIb alleles from plasma in translational studies. In fact, we have utilized this approach to assign FcγRIIIb alleles in plasma samples from RA patients treated with anti-TNFα therapy. This has allowed us to evaluate the relationship between FcγRIIIb alleles and response to anti-TNFα therapy.

We observed an association between the amino acid at position 65 and the glycosylation at N45. The combination of N45/D65 was associated with predominantly sialylated hybrid structures at N45 whereas the canonical NA2 N45/N65 combination resulted in predominantly high mannose type glycans at N45. Interestingly, FcγRIIIa which has the N45/D65 combination contains sialylated hybrid type structures but not high mannose type structures at N45. The N65 residue was found to be largely unoccupied. Examining the crystal structure of glycosylated FcγRIIIa interactions between the glycans at N45 and the side chain of D65 are apparent (14). Solution NMR analysis of FcγRIIIa confirms that the glycans at N45 interact strongly with the protein backbone around N65 (16). This glycan protein interaction inhibits glycosylation of this site resulting in the observed lack of site occupancy of N65.

Comparing the glycosylation of FcγRIIIb from neutrophil and FcγRIII from matched plasma revealed differences between the cell surface and soluble glycoforms at both sites. Lower levels of high mannose type glycosylation at N45 and lower levels of antennary fucose at N162 were seen in plasma compared with isolated neutrophils. It is somewhat surprising that the glycosylation patterns between neutrophil FcγRIIIb and soluble FcγRIII are notably different given that most soluble FcγRIII has been reported to be neutrophil derived (29). The higher abundance of the sialylated species at N45 in plasma relative to neutrophils could result from preferential clearance of high mannose bearing FcγRIII. High mannose type glycans have been shown to interact with the mannose receptor resulting in reduced serum half-life for high mannose bearing glycoproteins (35). The lower levels of antennary fu-
cose observed in plasma compared with matched isolated neutrophils could arise from similar phenomenon. Neutrophil granule proteins bearing LewisX structures were cleared from serum by scavenger receptor C-type lectin (SCLR) (36). Site specific characterization of FcγRIII from isolated cell populations may help detangle the site specific glycosylation pattern for different cell types.

We have identified an association between the glycosylation pattern at this site and the FcγRIIIb allele. Comparing the levels of highly branched glycans at this sites reveals significantly higher levels of these bulky glycans are present in the NA2 allele. This effect is somewhat surprising as variant residues for the allele are found in the first Ig like domain while this glycosylation site is found in the second Ig domain. However, recent reports noting a distant interaction between the glycans at N45 and the residues in and around N162 may help explain this association (16). The N-glycans at N162 are found at the Fc:FcγRII binding interface and it

| N162 glycopeptides                  | Graphical representation | Range Neutrophils  | Range Plasma  |
|-------------------------------------|--------------------------|--------------------|---------------|
| FA2G2S1-1                           | [Image]                  | 25.7%-70.5%       | 23.3%-57.6%   |
| FA2G2S1-2                           | [Image]                  | 8.4%-17.7%        | 5.8%-21.2%    |
| FA3G3S1F2                           | [Image]                  | 0.1%-5.0%         | 0.1%-1.7%     |
| FA3G3S1F2/FA2G2Luc152-1             | [Image]                  | 0.1%-9.2%         | 0.1%-7.9%     |
| FA3G3S1F2/FA2G2Luc152-2             | [Image]                  | 0.1%-8.1%         | 1.3%-9.1%     |
| FA2G2Luc1S1                         | [Image]                  | 0.1%-7.3%         | 0.1%-2.1%     |
| FA3G3S1                             | [Image]                  | 0.1%-10.2%        | 0.1%-5.1%     |
| FA2G2Luc1S1                         | [Image]                  | 0.1%-5.1%         | 0.1%-3.0%     |
| FA3G3S1                             | [Image]                  | 0.1%-4.9%         | 0.1%-6.9%     |
| FA2G2S2-1                           | [Image]                  | 0.1%-5.4%         | 0.5%-9.3%     |
| FA2G2S2-2                           | [Image]                  | 0.1%-12.3%        | 3.6%-8.4%     |
| FA2G2S2-3                           | [Image]                  | 0.1%-4.4%         | 0.4%-8.4%     |
| FA2G2SIF1-1 Leα                     | [Image]                  | 1.2%-10.4%        | 0.1%-5.7%     |
| FA2G2SIF1-2 Leα                     | [Image]                  | 0.1%-0.7%         | 0.1%-0.4%     |
| FA2G2SIF1 sleα                      | [Image]                  | 0.1%-5.9%         | 1.2%-12.6%    |
| FA2G2                             | [Image]                  | 0.1%-18.4%        | 0.9%-7.8%     |
| FA1G1S1-1                           | [Image]                  | 0.1%-4.5%         | 0.3%-7.5%     |
| FA1G1S1-2                           | [Image]                  | 0.1%-0.9%         | 0.1%-1.4%     |
| FA3G3SIF1/FA2G2Luc1SIF              | [Image]                  | 0.1%-0.5%         | 0.1%-0.5%     |
| FA3G3SIF1/FA2G2Luc1SIF              | [Image]                  | 0.1%-4.1%         | 0.1%-2.1%     |
| FA3G3SIF1/FA2G2Luc1SIF sleα         | [Image]                  | 0.1%-1.8%         | 0.1%-1.7%     |
| FA3G3SIF1/FA2G2Luc1SIF              | [Image]                  | 0.1%-1.7%         | 0.1%-0.8%     |
| FA2G2SIF2                           | [Image]                  | 0.1%-0.41%        | 0.1%-1.5%     |
| FA2G2SIF2                           | [Image]                  | 0.1%-1.2%         | 0.1%-4.8%     |
| FA3G3SIF1/FA2G2Luc1SIF              | [Image]                  | 0.1%-2.6%         | 0.1%-1.3%     |
| FA3G3SIF1/FA2G2Luc1SIFF             | [Image]                  | 0.1%-1.2%         | 0.1%-2.8%     |
| FA2G2F1                             | [Image]                  | 0.1%-8.4%         | 0.1%-0.4%     |
| A2G2S1                              | [Image]                  | 0.1%-0.6%         | 0.1%-3.3%     |
| FA3G3S3                             | [Image]                  | 0.0%-0.3%         | 0.1%-4.7%     |
has been proposed that differences in glycosylation at this site would have the most significant effect on the interaction (14), (19), (20).

Previous comparisons of the interaction kinetics between IgG and FcγRIIa produced in different cell lines suggests more highly branched species seen in the NA2 allele may have a destabilizing effect on the Fc:FcγR interaction. A faster dissociation rate for IgG binding to recombinant FcγRIIa expressed in different cell lines was associated with higher levels of highly branched and sialylated glycans (20). In examining the influence of FcγR glycosylation on IgG binding we observed that enzymatic desialylation of recombinant FcγRIIa suggested glycosylation can also influence affinity.

The allelic influence on the glycosylation profile is intriguing in light of functional studies examining the influence of FcγRIIb allele on FcγR mediated neutrophil activation. These studies have reported a greater response to several stimuli has been reported for neutrophils bearing the NA1 allele (7), (8), and (9). Unlike the well-studied polymorphic variants of FcγRIIIa and FcγRIIa no differences in affinity have been seen between IgG and the recombinant FcγRIIib variants (3). The expression system used to produce recombinant FcγRs has a strong effect of the glycosylation profile but it is unclear if the allelic differences in glycosylation we observed in endogenous FcγRIIib are reflected in the allelic variants of the recombinant protein. Our results coupled with the demonstrated influence of recombinant FcγRIIa glycosylation on the kinetics of the Fc:FcγRIIa interaction raise the possibility that the differences in neutrophil activity for these two alleles may be influenced by differences in glycosylation.

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10. Umaña, P., Jean-Mairet, J., Moudry, R., Amstutz, H., and Bailey, J. E. (1999) Engineered glycoforms of an antineuroblastoma IgG1 with optimized antibody-dependent cellular cytotoxic activity. Nat. Biotechnol. 17, 176–180.

11. Kaneko, Y., Nimmerjahn, F., and Ravetch, J. V. (2006) Anti-inflammatory activity of immunoglobulin G resulting from Fc sialylation. Science 313, 670–673.

12. Shibata-Koyama, M., Iida, S., Okazaki, A., Mori, K., Kitajima-Miyama, K., Saitou, S., Kakita, S., Kanda, Y., Shiota, K., Kato, K., and Satoh, M. (2009) The N-linked oligosaccharide at FcγRlla A sn-4s: an inhibitory element for high FcγRlla binding affinity to IgG glycoforms lacking core fucosylation. Glycobiology 19, 126–134.

13. Ferrara, C., Stuart, F., Sondermann, P., Brünker, P., and Umana, P. (2006) The carbohydrate at FcγRlla A sn-162. An element required for high affinity binding to non-fucosylated IgG glycoforms. J. Biol. Chem. 281, 5032–5036.

14. Ferrara, C., Grau, S., Jäger, C., Sondermann, P., Brünker, P., Waldhauer, I., Hennig, M., Ruf, A., Rufer, A. C., Stihle, M., Umaña, P., and Benz, J. (2011) Unique carbohydrate-carbohydrate interactions are required for high affinity binding between FcgammaRlla and antibodies lacking core fucose. Proc. Natl. Acad. Sci. USA 108, 12669–12674.

15. Mizushima, T., Yagi, H., Takezaki, M., Shibata-Koyama, M., Isoda, Y., Iida, S., Masuda, K., Satoh, M., and Kato, K. (2011) Structural basis for improved efficacy of therapeutic antibodies on defucosylation of their Fc glycans. Genes Cells 16, 1071–1080.

16. Subedi, G. P., Falconer, D. J., and Barb, A. W. (2017) Carbohydrate-Polypeptide Contacts in the Antibody Receptor CD16A Identified through Solution NMR Spectroscopy. Biochemistry 56, 3174–3177.

17. Takahashi, N., Cohen-Solal, J., Galinha, A., Fridman, W. H., Sauvé-Fridman, C., Kawasaki, N., and Kato, K. (2018) Site-specific N-glycosylation analysis of soluble Fcγ receptor IIIb in human serum. Sci. Reports 8, 2719.

18. Cosgrave, E. F., Struwe, W. B., Hayes, J. M., Harvey, D. J., Wormald, M. R., Lansing, M. C., and Rudd, P. M. (2013) N-Linked glycan structures of the human Fcgamma receptors produced in NS0 cells. J. Proteome Res. 12, 3721–3737.

19. Zeck, A., Pohlentz, G., Schlothauer, T., Peter-Kataliniæ, J., and Regula, J. T. (2011) Cell-type-specific and site-directed N-glycosylation pattern of FcγRlla. J. Proteome Res. 10, 3031–3039.

20. Hayes, J. M., Frostell, A., Karlsson, R., Müller, S., Martin, S. M., Pauers, M., Reuss, F., Cosgrove, E. F., Anneren, C., Davey, G. P., and Rudd, P. M. (2017) Identification of Fc gamma receptor glycoforms that produce differential binding kinetics for rituximab. Mol. Cell. Proteomics 16, 1770–1788.

21. Wuhrer, M., Catalina, M. I., Deelder, A. M., and Hokke, C. H. (2007) Glycoproteomics based on tandem mass spectrometry of glycopeptides. J. Chromatography B 849(1–2), p. 115–128.

22. Segu, Z. M., and Mchrehf, Y. (2010) Characterizing protein glycosylation sites through higher-energy C-trap dissociation. Rapid Commun. Mass Spectrometry 24, 1217–1225.

23. Thaysen-Andersen, M., and Packer, N. H. (2014) Advances in LC-MS/MS-based glycoproteomics: Getting closer to system-wide site-specific mapping of the N- and O-glycoproteome. Biochim. Biophys. Acta 1844, 1437–1452.

24. Breunis, W. B., van Mirre, E., Geissler, J., Laddach, N., Wolbink, G., van der Schoot, E., de Haas, M., de Boer, M., Roos, D., and Kuijpers, T. W. (2009) Copy number variation at the FCGR locus includes FCGR3A, FCGR2C and FCGR2B but not FCGR2A and FCGR2B. Human Mutation 30, E640–E650.

25. Nagelkerke, S., Tacke, C. E., Breunis, W. B., Geissler, J., Sins, J. W., Appelbo, B., van den Berg, T. K., de Boer, M., and Kuijpers, T. W. (2015) Nonallelic homologous recombination of the FCGR2/3 locus results in copy number variation and novel chimeric FCGR2 genes with aberrant functional expression. Genes Immunity 16, 422–429.

26. Ceroni, A., Maass, K., Geyer, H., Geyer, R., Dell, A., and Haslam, S. M. (2008) GlycoWorkbench: a tool for the computer-assisted annotation of mass spectra of glycans. J. Proteome Res. 7, 1650–1659.

27. Hollox, E. J., Detering, J. C., and Dehnugara, T. (2009) An integrated approach for measuring copy number variation at the FCGR3 (CD16) locus. Human Mutation 30, 477–484.

28. Yagi, H., Takakura, D., Roumenina, L. T., Fridman, W. H., Sauvé-Fridman, C., Kawasaki, N., and Kato, K. (2018) Site-specific N-glycosylation analysis of soluble Fcγ receptor IIIb in human serum. Sci. Reports 8, 2719.

29. Huizinga, T. W. J., de Haas, M., Kleijer, M., Nuijens, J. H., Roos, D., and von dem Borne, A. E. (1990) Soluble Fc gamma receptor III in human plasma originates from release by neutrophils. J. Clin. Invest. 86, 416–423.

30. Sakurama, H., Fushinobu, S., Hidaka, M., Yoshida, E., Honda, Y., Ashida, H., Kitaoaka, M., Kumagai, H., Yamamoto, K., and Katayama, T. (2012) 1,3–1,4–1-fucosylase that specifically introduces Lewis a/x antigens into type-1/2 chains. J. Biol. Chem. 287, 16709–16719.

31. Kimberley, R. P., Tappe, N. J., Merriam, L. T., Redeck, P. B., Edberg, J. C., Schwartzman, S., and Valinsky, J. E. (1989) Carbohydrates on human Fc gamma receptors. Interdependence of the classical IgG and nonclassical lectin-binding sites on human Fc gamma RIII expressed on neutrophils. J. Immunol. 142, 3923–3920.

32. Edberg, J. C., and Kimberley, R. P. (1997) Cell-type-specific glycoforms of Fc gamma RIIIb (CD16): differential ligand binding. J. Immunol. 159, 3849–3857.

33. Brewer, C. F., and Bhattacharyya, L. (1966) Specificity of concanavalin A binding to asparagine-linked glycopeptides. A nuclear magnetic relaxation dispersion study. J. Biol. Chem. 241, 7306–7310.

34. Patel, K. R., Roberts, J. T., Subedi, G. P., and Barb, A. W. (2018) Restricted processing of CD16a/Fcγ receptor IIIa N-glycans from primary human NK cells impacts structure and function. J. Biol. Chem. 293, 3477–3489.

35. Higel, F., Seidl, A., Sörgel, F., and Friess, W. (2016) N-glycosylation analysis of soluble Fcγ receptor IIIa binding affinity to IgG glycoforms lacking core fucose. Proc. Natl. Acad. Sci. USA 108, 12669–12674.

36. Graham, S. A., Antonopoulos, A., Hitchen, P. G., Haslam, S. M., Dell, A., Drickamer, K., and Taylor, M. E. (2011) Identification of neutrophil granule glycoproteins as LewisX-containing ligands cleared by the scavenger receptor C-type lectin. J. Biol. Chem. 286, 24336–24349.