Supplemental Figure Captions

Supplemental Figure S1. Expanded view of a precursor ion spectrum of a singly glycosylated peptide and its expanded view of product ion spectra. (a) The glycopeptide at $[1034+5H]^{5+}$ from a survey MS scan of MBP-GT tryptic digest at a concentration of 2 pmol/μL by direct infusion analysis; (b-f) MS/MS spectra of the five-charged, singly glycopeptide ion $[1033.23+5H]^{5+}$) LEDQNATGGDQNATGGDQNATGGDQNATVDHHHHHH. Several fragmented glycopeptides with various glycan compositions detected from CID of the precursor glycopeptide ion confirm the glycan composition consisting of one hexose, five *N*-acetylhexosamines and a species of mass 228 Da, consistent with a trideoxydiacetamidohexose.

Supplemental Figure S2. (a) Representative base peak chromatogram of PI (+204)-IDA analysis for AcrA-4x Lys C digests. Four major peaks were detected at elution times of 11.3, 38.5, 49.2 and 62.8 min, respectively corresponding to all four projected *N*-linked glycopeptides at asparagine residue 95, 101, 115 and 231 (see below) by the targeted glycopeptide detection. (b) MS spectra of NanoLC-PI Scan (+204) at elution time of 11.2 (top panel) and 12.7 min (bottom panel). The MS spectra for doubly and triply-charged ions eluted at 11.2 min show the mass of the glycopeptide matching to N95 peptide with expected seven unit of glycan consisting of one hexose, five *N*-acetylhexosamines and a species of mass 228 Da for a trideoxydiacetamidohexose. The MS spectra for doubly and triply-charged ions eluted at 12.7 min show the mass of the glycopeptide matching to N101 peptide with the expected seven unit of glycan consisting of one hexose, five *N*-acetylhexosamines and a species of mass 228 Da for a trideoxydiacetamidohexose. (c) MS spectra of NanoLC-PI Scan (+204) at elution time of 38.5 (top panel) and 62.8 min (bottom panel). The MS spectra for doubly and triply-charged ions eluted at 38.5 min show the mass of the glycopeptide matching to N115 peptide with expected

seven sugar units consisting of one hexose, five *N*-acetylhexosamines and a species of mass 228 Da for a trideoxydiacetamidohexose. The MS spectra for triply and quadruply-charged ions eluted at 62.8 min show the mass of the glycopeptide matching to N231 peptide with the seven sugar units consisting of one hexose, five *N*-acetylhexosamines and a species of mass 228 Da for a trideoxydiacetamidohexose. (d) Enhanced product ion mass spectra (*m/z* 100-2200) derived by collision-induced dissociation of the $(M + 2H)^{2+}$ glycopeptide precursor ions of *m/z* = 1086.6, which confirms the N95 *N*-linked glycopeptides with aforementioned seven sugar units of glycan composition consisting of one hexose, five *N*-acetylhexosamines and a trideoxydiacetamidohexose. (e) Enhanced product ion mass spectra (*m/z* 100-2200) derived by collision-induced dissociation of the $(M + 2H)^{2+}$ glycopeptide precursor ions of *m/z* = 1121.6, which confirms the N101-linked glycopeptides with aforementioned seven sugar units of glycan composition consisting of one hexose, five *N*-acetylhexosamines and a trideoxydiacetamidohexose. (f) Enhanced product ion mass spectra (*m/z* 100-2200) derived by collision-induced dissociation of the $(M + 2H)^{2+}$ glycopeptide precursor ions of *m/z* = 1448.5, which confirms the N115-linked glycopeptides with aforementioned seven sugar units of glycan composition consisting of one hexose, five *N*-acetylhexosamines and a trideoxydiacetamidohexose. (g) Enhanced product ion mass spectra (*m/z* 100-2200) derived by collision-induced dissociation of the $(M + 4H)^{4+}$ glycopeptide precursor ions of *m/z* = 1041.2, which confirms the N231-linked glycopeptides with aforementioned seven sugar units of glycan composition consisting of one hexose, five *N*-acetylhexosamines and a trideoxydiacetamidohexose.

Supplemental Figure S3. Binding of *E. coli*-derived human IgG1 Fc domains to FcγRI. (a) Glycosylated Fc^{DQNAT} was expressed in *E. coli* in a glycosylated (g) and aglycosylated (a) form.

The glycosylated form was purified from lysate (lane 1) by SBA affinity chromatography (lane 2) followed by Protein A/G affinity chromatography (lane 3) with coomassie staining of the resulting samples. (b) Purified glycosylated Fc^{DQNAT} (black triangles), as well as the aglycosylated wild-type Fc (white diamonds) and $Fc^{E382V/M428I}$ (gray squares), were each coated on ELISA plates and incubated with FcγRI. $Fc^{E382V/M428I}$ is an aglycosylated Fc variant that is known to bind FcγRI and serves as a positive control.

Supplemental Figure S4. Mean cell fluorescence (M) determined by flow cytometric analysis of: (a) plasmid-free wild-type cells; (b) wild-type cells carrying pACYC*pgl*; (c) wild-type cells carrying pACYC*pgl*mut; and (d) *waaL*-deficient cells carrying pACYC*pgl.* All cells were labeled with a fluorescently labeled version of SBA (SBA-Alexa Fluor 488).