Supplementary Appendix

This appendix has been provided by the authors to give readers additional information about their work.

Supplement to: Sadat MA, Moir S, Chun T-W, et al. Glycosylation, hypogammaglobulinemia, and resistance to viral infections. N Engl J Med 2014;370:1615-25. DOI: 10.1056/NEJMoa1302846

Supplemental material

Glycosylation, Hypogammaglobulinemia and Resistance to Viral Infections, by Sadat, MA et al.

S1- Patients, *CDGIIb* diagnosis

Briefly, an abnormal tetrasaccharide (Glu3Man1 or Glu4) was evident on a thin layer chromatography analysis of the urine, and pathological increases in abnormal high-mannose N-glycans (Glu3Man7GlcNAc2, Glu3Man8GlcNAc2 and Glu3Man9GlcNAc2) were detected by matrix–assisted laser detection desorption/ionization (MALDI) time-of-flight (TOF) on the plasma and skin fibroblasts lysates from the patients. These findings were biochemically diagnostic for MOGS-CDG (CDG-IIb), as had been determined in the only previously reported case of this disease[2]. Protein studies failed to detect MOGS expression on the patients' cell lysates by immunoblot analysis (Figure 1, suppl. material), and both patients are MOGS compound heterozygotes carrying a maternally inherited stop codon, c.370C>T, p.Q124X, and two paternally inherited missense changes, c.65C>A, p.A22E and c.329G>A, p.R110H. The maternally inherited premature stop codon (p.Q124X) activates nonsense mediated mRNA decay. The paternally inherited c.65C>A mutation, which in silico analysis predicts as generating an exonic splicing silencer (ESS; GGGAGG), reduces the efficiency of NM_006302.2 intron 1 and NM_001146158.1 intron 2 splicing resulting in protein translation into the intron, termination at a stop codon 19 amino acids later and nonsense mediated mRNA decay. The residual correctly spliced mRNA from the paternal allele encodes the R110H missense mutation; this mutant MOGS protein is rapidly degraded by the proteasome. These observations account for the lack of MOGS protein detectable by immunoblotting (Figure 1a).

S2- Studies of *CDG-IIb* and immunoglobulin

-MOGS expression

Lysates from EBV-transformed B cells from patients and healthy controls were sequentially immunoblotted with two polyclonal antibodies: anti-MOGS and anti α-GCS2 (glucosidase 2, the enzyme acting downstream of MOGS on the endoplasmic-reticulum N-glycan trimming process).

-Plasma IgG N-glycan analysis.

IgG obtained from plasma of healthy controls or patients was subjected to immunoglobulin affinity column purification and analyzed by MALDI-TOF as described previously[3].

-Unfolded protein response (UPR) and Endoplasmic reticulum-associated degradation (ERAD) in B cells

EBV-transformed B cell lines generated from normal donors or patients were stressed with different doses of tunicamycin (UPR inducer) and MG-132 (ERAD inducer) and evaluated for upregulation of the endoplasmic reticulum molecular chaperone BiP/GRP78 by immunoblotting.

-In vitro immunoglobulin production assays

Antibody-secreting cells (ASC) were generated from peripheral blood mononuclear cells (PBMCs) from the patients and a healthy control. Frequencies of ASC were measured by ELISPOT, as described [4]. Measurement of IgG recovered from the culture supernatant was performed by cytometric bead array (CBA) with a FACSArray instrument per the manufacturer's instructions (BD Biosciences).

-Ig half-life study

Adult RAG1 KO mice were injected subcutaneously with human plasma from patients or healthy human controls. Two sets of experiments were performed, in which mice received plasma normalized to contain 1 mg or 2.5 mg of IgG. Blood was collected from the tail vein at days 0, 2, 7, 14, 21, 30, 42 and 56 post-administration and human IgG levels were measured by ELISA.

S3- Assays for Viral Infections

-HIV Infection assays

Patient and healthy control PBMCs were isolated and CD8-depleted CD4+ T-cell targets for HIV were prepared, as previously described [6]. Four HIV strains were tested: one using co-receptor CCR5 (BAL) and three using CXCR4 (IIIB -Applied Biotechnologies-, molecular clone ELI-6 [5]; and primary strain 93BR019 -BR, obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID-). For HIV entry assays, detection of HIV LTR using real-time PCR was used, as previously described [7]. For HIV replication assays, HIV p24 protein level was determined in the culture supernatants by ELISA. For the secondary infection experiments, target cells from two healthy donors were prepared as described above, incubated with normalized amounts of ELI-6 virus recovered from the two patients and one control, and viral entry was evaluated as described above. Primary fibroblasts from patients and healthy controls were transfected with an HIV envelope gp140 (gp120 plus the extracellular domain of gp41 of YU2)[4]-coding plasmid with and without a V5-tagged wild type (WT) MOGS expression vector using Turbofect ® according to manufacturer's protocol. For certain conditions, cells were treated with castanospermine (CS), a MOGS inhibitor, before and after transfections. Protein lysates from the transfected cells were digested with PNGaseF, an enzyme removing N-glycans form glycosylated proteins, resolved on PVDF membranes and probed with a rabbit anti human HIV polyclonal antibody (HIV-Ig; obtained through the AIDS Research and Reference Reagent Program) or a mouse anti V5 monoclonal antibody. For MOGS-mediated rescue of defective HIV replication, primary fibroblasts from Pt2 were co-transfected with full length HIV clone pYU2 and either WT MOGS expression or corresponding empty vector. Virus in culture supernatant at 72 h post-transfection was concentrated 30-fold by ultracentrifugation and used to infect TZM-bl indicator cells, as previously described (Gao LM et al., J Virol 2005, 79:10108-25). Briefly, serial dilutions of virus recovered from transfected fibroblasts were incubated with 10^4 TZM-bl cells per well of a 96-well plate in the absence of DEAE-dextran. Luciferase activity was quantified 48 h after infection and normalized by input p24. The infectious clone pYU2 and TZM-bl cells were obtained through the AIDS Research and Reference Reagent Program.

-Influenza A H1N1 infection assays

Monocyte-derived macrophages were generated from both patients and a healthy control using previously published methods 8. Three macrophage cultures were generated from each of the individuals. Macrophages were incubated with the 2009 H1N1 pandemic virus, washed and grown for 48h before HA assay and TCID50 were performed on supernatants using standard methods[9]. Viruses collected from macrophage cultures were measured and normalized and then used to inoculate Madin-Darby Canine Kidney (MDCK) cell cultures. Influenza-infected MDCK cell cultures were monitored for cytophatic effects (CPE) and HA assay positivity.

-Adenovirus type 5 (AdV5) infection assays

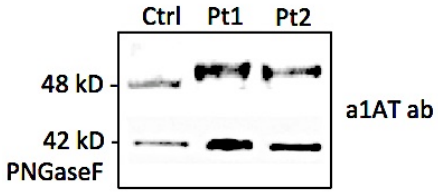
Primary fibroblasts from both patients and two healthy controls were infected with AdV5 starting at 2x106 particles per well, followed by two 1/6 serial dilutions. Cells were optically evaluated for CPE, then lysed and virus was titrated by qPCR as reported elsewhere[10]. The same infection experiment was reproduced in healthy control fibroblasts with normalized AdV5 virus produced either by the patients or healthy control cells.

-Poliovirus1/Mahoney (PV1) infection assays
Primary fibroblasts from both patients and two healthy controls were infected with 106 TCID50 of PV1. After 1 h adsorption, cells were washed, media was added and cells were incubated under CO2 for: 0, 1, 2, 4, 8, 24, and 48 h post-infection. Samples of supernatants were taken at each time point before freezing the plates. Viral stocks were prepared, and viral titers were determined by endpoint titration in HeLa cells. Uninfected controls were titrated in 4 replicates, and no CPE was observed from -2 to -4 dilution, suggesting titers were lower than 3.16×10^2 TCID50/ml or 2.5 log TCID50. CPE was read 72 h post inoculation, and titers were calculated using the Reed and Muench method.

-Vaccinia virus infection assays
Epstein-Barr virus (EBV)-immortalized B cells from the patients and a healthy blood donor were infected with a green fluorescent protein (GFP)-expressing recombinant vaccinia virus. After 24h, GFP-positive B cells were analyzed by flow cytometry. Forty-eight hours post-infection, cells were collected, washed and sonicated to release the intracellular vaccinia virus. Serial dilutions of the cell lysates were then tested for their capability to infect B cells from a healthy donor using the same infection protocol described above.

S4-Glycosylation patterns in plasma proteins

Plasma (1:40 dilution) from patients and healthy controls was resolved on 3-8% polyacrylamide Tris-acetate NuPage® gels (Novex), transferred to PVDF membranes and probed with a rabbit anti human α1-antitrypsin (a1AT) polyclonal antibody (Abcam), before and after digestion with PNGaseF (New England Biolabs), an N-glycan removing enzyme, per manufacturer's recommendations.



The patients' a1AT showed a higher molecular weight (MW) than the healthy control a1AT. Removal of N-glycans by PNGaseF digestion, eliminated the differences in MW.

S5- IgG binding to Fc receptors, Surface Plasmon Resonance Measurements

Binding to FcRn has been shown to protect IgG from degradation by the receptor-mediated transcytosis, thus avoiding the normal degradation pathway (Ward et al., 2003, Int Immunol 15:187-195; Ladinsky et al., 2012, Mol Biol Cell 23:2537-45). To investigate if FcRn contributes to the shortened half-life of the patient IgG compared with that of the healthy donors, the binding of IgG to FcRn was examined using surface plasmon resonance. Serum IgGs were purified from both patients as well as three healthy individuals using both protein A and protein G columns. In all cases, the separation resulted in co-elution of IgG1/2, but well resolved IgG3. The FcRn binding affinity for IgG1/2 varied between 30 - 100 nM among the healthy donors (Table 1 suppl. material). Similar binding affinity was observed for IgG3, consistent with the fact that IgG1/2 and IgG3 differ primarily in their lower hinge sequences outside of the FcRn binding site (Martin et al., 2001, Mol Cell 7:867-77). The IgG1/2 and IgG3 from the two patients displayed 20-60 nM binding affinities to FcRn. Thus, the glycan variation in IgGs from the patients do not affect significantly their binding to FcRn, consistent with the structural finding that FcRn binding site on Fc is distinct from the Fc glycosylation site.

We also examined the binding of patient IgGs to other known human Fc receptors including the high affinity FcγRI, medium affinity FcγRIIa, and a low affinity FcγRIIIb to investigate a potential involvement of these receptors to the shortened half-life of the patient IgG. Consistent with previously published affinities (Lu et al., 2011 286:40608-13), IgG1/2 from the healthy individuals bound to FcγRIa with 40-90 nM affinities (Table 1 suppl. material). The patient IgGs displayed similar binding affinities, between 50-80 nM, to the high affinity Fc receptor as the healthy donors. The bindings between IgG3 and FcγRIa displayed approximately 2-fold higher affinities than their IgG1/2 subtypes, measuring 25 and 40 nM, respectively, for the patient and healthy donors. The solution bindings between purified IgGs and immobilized FcγRIIIb were characteristic of low affinity bindings but consistent between the patients and healthy individuals (data not shown). Unlike FcγRIa and FcγRIIIb, significant differences were observed for IgG binding to FcγRIIa between the patients and healthy donors. The IgG1/2 from healthy donors bound to FcγRIIa with 20-140 nM affinities. In contrast, the IgGs from both patients showed approximately a 10-fold lower affinity to the receptor (Table 1 suppl. material). Three to five fold lower FcγRIIa binding affinities were also observed for the patient IgG3 compared to the healthy. These results show that IgGs from the patients displayed a weaker binding to FcγRIIa, suggesting this receptor may contribute to the half-life of serum IgG from the patients. However, FcγRIIa is an unlikely source of the experimentally observed shorter patient IgG half-life in mouse since the receptor is absent in mouse.

Table 1 suppl. material. Dissociation constants between IgG and Fc receptors

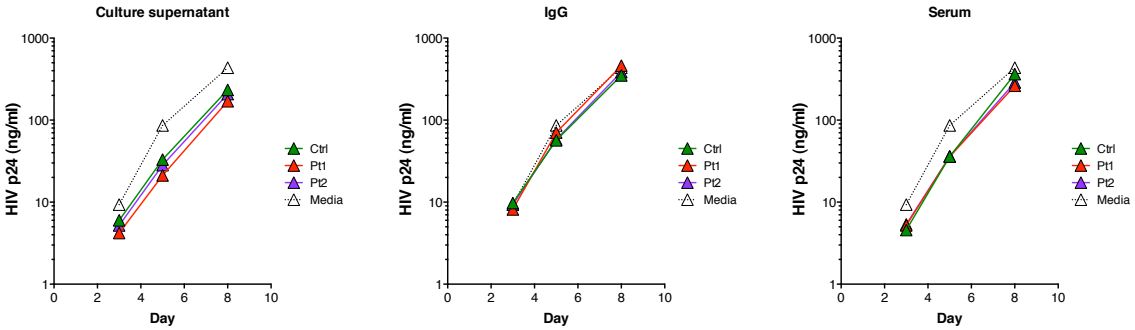
Peak	Donor	FcγRIa K_D (nM)	FcγRIIa K_D (nM)	FcRN K_D (nM)
$IgG_{1/2}$	P1	78.9 ± 69.9	1486.5 ± 1008.1	46.8 ± 30.9
	P2	53.9 ± 65.7	1639.5 ± 1153.4	23.4 ± 14.2
	HA	42.8 ± 35.8	18.7 ± 9.9	29.6 ± 23.8
	HB	N/C	N/C	29.1 ± 13.5
	HC	88.5 ± 16.8	136.1 ± 194.1	99.1 ± 71.9
IgG_3	P1	25.7 ± 8.9	540.2 ± 260.7	39.7 ± 34.6
	P2	24.4 ± 8.8	690.2 ± 447	N/C
	HA	41.1 ± 12.8	174.4 ± 311.7	40.8 ± 17.8
	HB	N/C	N/C	7.1 ± 0.4
	HD	N/C	N/C	60.7 ± 33

N/C: These samples were not collected

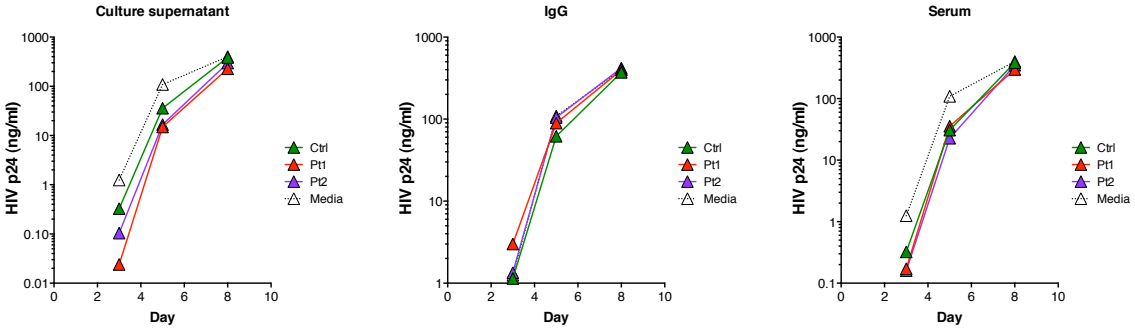
S6-Virus inhibition assay

Healthy control CD8-depleted $CD4^+$ T-cell targets were incubated with the following preparation prior to infection with HIV strains Bal or IIIB: 200 µl of fresh culture media or Day 3 culture supernatant from stimulated patient or healthy PBMCs (method that generates targets for HIV infection); 200 µl serum obtained from patient or healthy control; and 200 µl PBS containing10 µg of IgG isolated from the plasma of patient or healthy control. The infection was monitored over 8 days by measuring HIV p24 protein levels in the culture supernatants with periodic replenishment of fresh media. Of note, the Day 3 patient or control culture supernatant condition was maintained at 10% of total volume over the 8-day period (the supernatants were frozen at Day 3 and thawed on days of replenishment).

a)



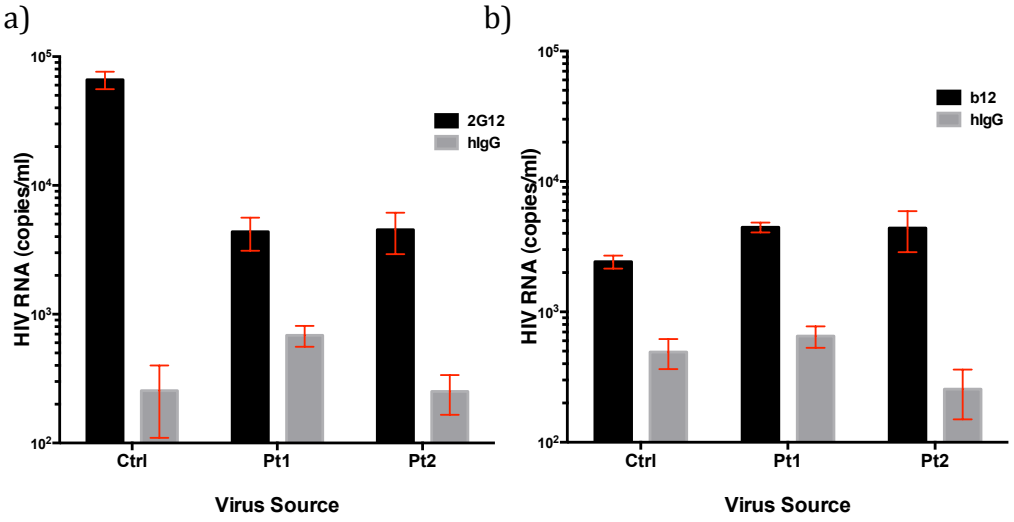
b)



The possibility that abnormal glycans secreted by *CDGIIb* patient cells contributed to the reduction in HIV viral kinetics shown in Figure 2 was addressed by pre-incubating CD8-depleted PBMCs from a healthy control with media or patient or a different healthy control-derived Day-3 culture supernatant (left panel), IgG (middle panel), or serum (right panel). This pre-incubation or continued incubation in the case of Day-3 culture supernatants had no effect on the replication kinetics of HIV strains a) IIIB or b) Bal. Of note, virus replication in target cells incubated with media was slightly higher than in cells incubated with culture supernatants or pre-incubated with sera. This is likely an indication that the latter two preparations contain HIV-inhibitory factors but that these are unrelated to the differences in protein glycosylation between the *CDGIIb* patients and the healthy control.

S7-Virus capture assay

Patient and healthy control CD8-depleted $CD4^+$ T-cell targets were infected with the HIV strain Ada8, a CCR5-dependent molecular clone derived from HIV-Ada (Theodore et al., AIDS Res Hum Retroviruses 1996, 12:191-4), whose envelope is known to bind the anti-HIV envelope mannose-dependent mAb 2G12 (Trkola et al., J Virol 1996, 70:1100-8; Haim et al., PLoS Pathog 2011, 7:e1002101). Binding of anti-HIV mAb 2G12 or control IgG to magnetic beads coated with protein A was performed according to manufacturer recommendations (Life Technologies). Each antibody-bead complex was then incubated for 90 min at 4C with patient or control cell culture supernatant from day 7 containing 1 ng of virus based on p24 protein level. Virus-antibody-bead complexes were washed extensively and beads were detached per manufacturer recommendation. The copy number of HIV RNA in the virus-antibody bead complexes was determined using Cobas Ampliprep/Cobas Taqman HIV-1 Test Version 2.0 (Roche Diagnostics).



The binding of HIV virions produced by the cells of *CDGIIb* patients or healthy control to glycan-dependent 2G12, and glycan-independent b12 anti-HIV envelope antibodies revealed that binding to b12 was similar for all three sources of virions, whereas binding to 2G12 was lower for the virions produced by the two *CDGIIb* patients when compared to virions produced by the healthy control cells. The binding experiments with 2G12 and b12 were performed separately on the same source of virus and each set included IgG as negative control. The data for 2G12 are representative of two independent assays and data shown are mean of triplicates ± SD. Moreover, these results were consistent with previously observed glycosylation dependency for 2G12 recognition and strongly suggested that the HIV envelope glycosylation pattern on virions produced by patient cells was similar to the reported effect of the MOGS inhibitor N-butyldeoxynojirimycin (Doores KJ et al., 2010 J Virol, 84:10510-21).

S8- Influenza virus susceptibility

Glycosylation of influenza A virus surface proteins hemaglutinin (HA) and neuraminidase (NA) play an important role in receptor binding, infectivity, virus release, and virulence (Vigerust et al., Trends Microbiol 2007, 15:211-8). Post-translational, host-cell dependent glycosylation of the HA molecule in particular is crucial to proper folding of the molecule and modulating interaction with sialylated receptors needed for infection. All strains of influenza, including the 2009 H1N1 pandemic strain, have varying degrees of glycosylation of the HA that is specific to that strain or subtype, adding to the uniqueness of each strain or subtype (Kiel W et al., Virology 1984, 133:77-91). Small changes in the unique glycosylation pattern found on a particular strain, such as the 2009 H1N1 pandemic virus could interfere with the virus's ability to bind to receptors on host cells leading to a defective virus unable to cause infection. As a first step on the evaluation of influenza viruses' susceptibility, titers to seasonal H1N1, H3N2, and the 2009 pandemic H1N1 were determined in both patients' serum. Neither of the two siblings had measureable antibodies to the viral strains tested by hemagglutination inhibition assay. In order to evaluate if the N-glycosylation defect would affect the ability of influenza viral HA to agglutinate the patient's RBCs, a standard hemagglutination assay was also performed. The pandemic H1N1 virus showed no differences in its ability to agglutinate the patient's RBCs when compared to control RBCs.

Based on our results, in the case of these two individuals the host cells are likely not able to adequately glycosylate the surface proteins of the virus leading to this specific situation of a virus that can not function properly.

S9- Immunization records

	Pt1	Pt2
DTaP	2m/4m/7m/19m/5y3m/*10y9m	2m/4m/6m/16m/5y2m/*6y10m
Hepatitis B	Birth/2m/8m/*10y9m	Birth/1m/6m/*6y10m
HiB	2m/4m/15m/3y11m/*10y9m	2m/4m/6m/12m/*6y10m
IPV	2m/4m/19m/5y3m	2m/4m/14m/5y2m
MMR	15m/5y3m	12m/5y2m
Varicella	19m/5y3m	12m/5y2m
PCV	-	2m/4m/6m/12m
PPV	*10y9m	*6y10m

DTaP: diphtheria, tetanus, acellular pertussis vaccine; Hepatitis B: hepatitis B vaccine; HiB: *haemophilus influenza B* vaccine; IPV: inactivated polio vaccine; MMR: measles, mumps, rubella vaccine; Varicella: varicella vaccine; PCV: pneumococcus conjugated vaccine; PPV: pneumococcus polysaccharide vaccine; m: months; y: years. * Vaccines given to evaluate functional antibody responses.