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Title: **Immunoglobulin G Fc glycans are not essential for antibody-mediated immune suppression to murine erythrocytes**

Short title: IgG Fc glycans are not critical for AMIS

Scientific category: Transfusion Medicine & Immunobiology

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The use of anti-D immunoglobulin to prevent hemolytic disease of the fetus and newborn (HDFN) is a success of antibody-mediated immune suppression (AMIS) in the clinic. A monoclonal antibody (mAb) to replace donor-derived anti-D would be beneficial to eliminate current issues with anti-D [1,2]. There have been several attempts to create replacement monoclonal anti-D therapies, however, no products have been as effective as polyclonal anti-D and some have led to enhanced alloimmunization [3]. This highlights the necessity of increased knowledge on anti-D mechanisms.

It has been suggested that IgG glycosylation on the Fc domain of AMIS antibodies may influence their ability to induce AMIS [3]. Removal of the IgG Fc glycans have been associated with impaired Fc receptor interactions, complement activation and antibody-dependent cellular cytoxicity [4,5]. In particular, variations in anti-D IgG Fc fucosylation have been shown to impact HDFN severity in patients with lower levels of fucosylation correlated with enhanced HDFN severity [6]. Glycosylation of several alloantibodies in pregnant women have been shown to have antigen-specific differences in glycosylation patterns compared to total IgG [7]. Differences in IgG glycosylation of monoclonal anti-D produced *in vitro* compared to polyclonal anti-D could potentially contribute to the decreased efficacy of monoclonal anti-D for prevention of RBC alloimmunization [3]. In this study, we completely removed the Fc glycan on four anti-red blood cell (RBC) antibodies and show that AMIS can occur independently of IgG Fc glycan-dependent RBC clearance in a mouse model of RBC alloimmunization.

This RBC alloimmunization model consists of immunizing C57BL/6 mice (6-8 weeks old, Charles River Laboratories, Kingston, NY, United States) with HOD (Hen egg lysozyme (HEL), Ovalbumin and human Duffy protein) transgenic RBCs [8]. All animal studies were approved by

the St. Michael's Hospital Animal Care Committee. We tested AMIS induction with a panel of four anti-RBC monoclonal antibodies: 1) MIMA 29 (mouse anti-Duffy (Fy^3); IgG2a) [9], 2) CBC-512 (mouse anti-Duffy (Fy^3); IgG1), 3) 4B7 (mouse anti-HEL; IgG1), and 4) 6D7 (mouse anti-HEL; IgG1). To evaluate the role of Fc glycans for IgG AMIS induction, Fc region deglycosylated antibodies were generated by treatment with the enzyme Peptide-N-Glycosidase F (PNGase F, cat# P0704S, New England Biolabs, Whitby, ON), as previously performed in [10].

Successful Fc glycan removal for each antibody was confirmed by UPLC (Figure 1A) as well as a band-shift assay and further UPLC data (Supplemental Figure S1A). The deglycosylated mAbs retained the ability to bind to HOD-RBCs similarly to the wild-type version (Supplemental Figure S1B).

The ability of anti-D to prevent HDFN has been long-thought to be due to IgG interactions with Fc receptors on phagocytic cells leading to rapid clearance of the erythrocytes [11]. IgG interactions with Fc receptors after removal of the conserved Fc N-linked glycan are impaired [4]. The two Duffy-specific mAbs (MIMA 29; IgG2a, CBC-512; IgG1) used in this study induce clearance of HOD-RBCs *in vivo* [12,13]. To study RBC clearance, HOD-RBCs were labeled with the fluorescent dye PKH26 to track the transfused cells *ex vivo* [14–16]. C57BL/6 mice were transfused with 10^8 PKH26-labeled HOD-RBCs (PKH26+ HOD-RBCs), and 24 hours later, mice were injected i.v. with 5 μg of wild-type or deglycosylated variants of MIMA 29 or CBC-512, or PBS. Peripheral blood was collected before (0) and 2 hr, 24 hr, 48 hr, and 72 hr after antibody injection to assess the *ex vivo* clearance kinetics of PKH26+ HOD-RBCs by flow cytometry (Figure 1B). Complete deglycosylation of the MIMA 29 antibody partially impacted its ability to clear HOD-RBCs (Figure 1B), whereas deglycosylation of the CBC-512 antibody

completely impaired its ability to clear the transfused HOD-RBCs (Figure 1B). This data suggests that CBC-512 clearance is fully dependent on Fc region glycans while MIMA 29 clearance is only partially dependent on Fc glycans.

To analyze the impact of deglycosylation on AMIS induction, sera from recipient mice were collected 7 days after injection of PKH26+ HOD-RBCs and the HEL-specific IgM and IgG response analyzed by ELISA [12,17]. Mice transfused with HOD-RBCs and injected with wild-type MIMA 29 (AMIS conditions) had minimal IgM and IgG anti-HEL responses (Fig 1C&D) while mice receiving deglycosylated MIMA 29 displayed less AMIS activity (Fig 1C&D). Therefore, the complete removal of the Fc glycan from MIMA 29 partially reduced AMIS activity and also partially impacted PKH26+ HOD-RBCs clearance *in vivo*. In contrast, the deglycosylated or wild-type version of CBC-512 both completely suppressed the IgM and IgG anti-HEL response to the transfused HOD-RBCs (Fig. 1C&D). Thus, complete removal of the Fc glycan from CBC-512 did not prevent the complete AMIS effect mediated by this antibody. The fact that deglycosylated CBC-512 was able to induce complete AMIS despite no ability to clear the HOD-RBCs provides direct evidence that AMIS can occur independently of IgG Fc glycan-dependent clearance of RBCs in the HOD model of red cell alloimmunization. This finding challenges the necessity for clearance of allogeneic RBCs for successful AMIS induction, which is considered important with anti-D [18,19].

In addition, two monoclonal anti-HEL specific antibodies (6D7; IgG1, 4B7; IgG1) that do not cause erythrocyte clearance but are known to mediate AMIS activity [12,17], were fully deglycosylated and despite complete Fc deglycosylation (Fig 1A) retained normal AMIS activity

(Supplemental Figure S1E&F). These data together show that removal of the Fc glycan did not critically impact AMIS induction for 3/4 tested anti-RBC antibodies. The one antibody that was partially impacted by Fc glycan removal (MIMA 29) was still able to mediate a partial AMIS effect despite the complete absence of any detectable glycan. It is possible that this deglycosylated IgG2a antibody may retain some residual Fc receptor binding activity, as studies using endoglycosidase S (which removes the majority of the IgG Fc glycan) results in an IgG2a with some Fc receptor I and IV binding activity [20,21]. Whether the complete removal of the glycan (as performed here) better abrogates Fc receptor binding is uncertain. Interestingly, the removal of the glycan from the MIMA 29 antibody also partially impacted its ability to clear RBCs and it remains possible that with this antibody RBC clearance may play a partial role in AMIS activity. Table 1 summarizes the characteristics of the panel of anti-RBC antibodies evaluated in this study and the impact of Fc glycan removal on AMIS induction.

This work demonstrates that the ability of monoclonal RBC-specific antibodies to prevent RBC alloimmunization is not critically dependent on the presence of IgG Fc glycans. However, the differential AMIS effect seen for one monoclonal antibody (MIMA 29) highlights the complexity of AMIS and provides support for the notion that several mechanisms may be involved in the ability for antibodies to mediate AMIS. This work is important for informing and guiding future developments in monoclonal efforts to replace polyclonal anti-D. This information also suggests that a shift in thinking for the criteria used to develop monoclonal anti-D therapies may be necessary, since past attempts have always emphasized the red cell clearance abilities of the monoclonal antibodies [3].

In summary, our data demonstrates that AMIS can be successfully achieved in the absence of IgG Fc glycan-dependent clearance of transfused allogeneic RBCs.

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Authorship Contributions

D.M. designed and performed experiments, analyzed data and wrote the manuscript; Y.C.L. performed experiments and analyzed data; L.B. analyzed data; N.P.L.L. and M.C. performed glycan analysis; X.Y. analyzed data; M.U contributed antibodies; A.H.L. designed research, analyzed data, provided grant funding, and wrote the manuscript. All authors commented on and approved the manuscript.

Disclosure of Conflicts of Interest

The authors declare no relevant conflicts of interest.

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Figure Legends

Figure 1: **Analysis of wild-type and deglycosylated anti-RBC antibodies and their ability to induce RBC clearance and AMIS.** Wild-type and deglycosylated antibodies were first evaluated for the efficiency of glycan removal. Glycans were released from IgG heavy chains by in-gel PNGase F digestion, and then labeled with 2-aminoanthranilic acid and analyzed by UPLC. The symbols employed for different glycan structures is based on [22]. (**A**) Heavy chains of all samples had detectable glycan structures in the wild-type form of the heavy chains (blue lines), but no detectable structures after deglycosylation (black lines). The loss of the heavy chain signal after the deglycosylation reaction with PNGase F indicates that the Fc glycan removal was successful. (**B)** The ability of anti-RBC antibodies (MIMA 29 and CBC-512) to induce RBC clearance was analyzed by determining the percentage of surviving PKH26+ HOD-RBCs in mouse circulation. All mice except the naive treatment group received 10^8 PKH26-labeled HOD-RBCs i.v. by tail vein injection. After 24 hours, mice were injected with no antibody (HOD), or 5 μg of each antibody assessed: wild-type MIMA 29 (MIMA 29), deglycosylated MIMA 29 (deMIMA 29), wild-type CBC-512 (CBC-512) or deglycosylated CBC-512 (deCBC-512). The percentage of remaining PKH26+ HOD-RBCs in circulation was evaluated before (0), 2, 24, 48, and 72 hours after antibody injection. Mice were bled for serum 7 days after PKH26+ HOD-RBC transfusion and HEL-specific IgM (**C**) and IgG (**D**) antibody levels were evaluated by ELISA**.** Data represent individual values from mice from at least three separate experiments. Data were expressed as mean ± SEM and analyzed by one-way variance analysis (ANOVA), with Tukey's multiple comparison test. **p<0.01, ***p<0.001, ****p<0.0001

Table 1. Anti-RBC antibody characteristics and AMIS induction.

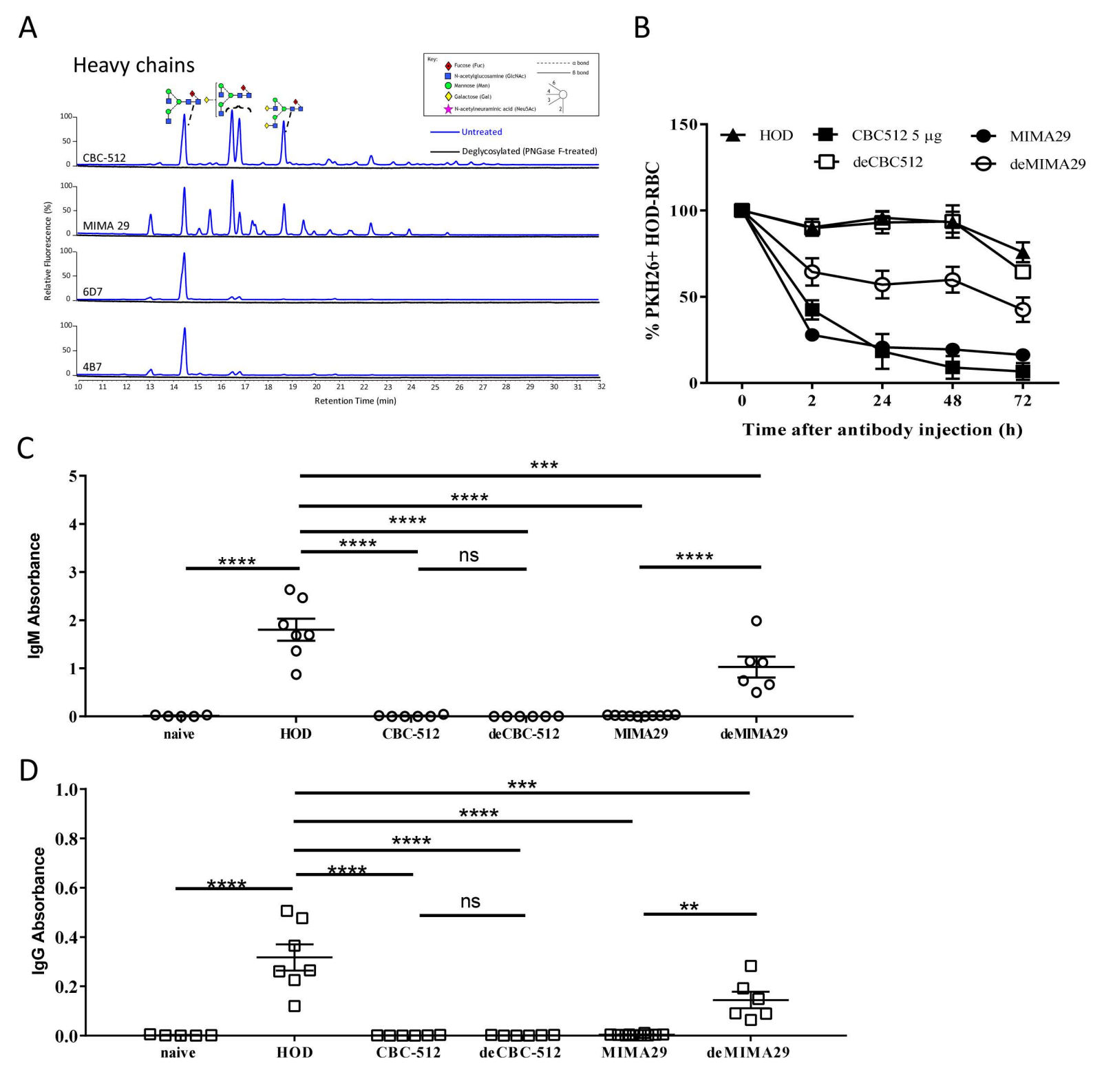
Antibody	Isotype	Reactivity	RBC Clearance [1]	Antigen Modulation[4]	AMIS
4B7	IgG1	HEL	-	no	Yes
de4B7	IgG1 (deglycosylated)	HEL	-[2]	no[2]	Yes
6D7	IgG1	HEL	unknown[3]	unknown	Yes
de6D7	IgG1 (deglycosylated)	HEL	unknown[3]	unknown	Yes
MIMA 29	IgG2a	Duffy	+++	yes	Yes
deMIMA 29	IgG2a (deglycosylated)	Duffy	++	unknown	Yes (Partial)
CBC-512	IgG1	Duffy	+++	unknown	Yes
deCBC-512	IgG1 (deglycosylated)	Duffy	-	unknown	Yes

1. RBC clearance determined by % cells remaining 2 hours after injection (+++, 50%; ++, <75%; -, >90%).

2. We presume that de4B7 does not induce RBC clearance or antigen modulation since the wildtype 4B7 does not.

3. Although we did not test RBC clearance by 6D7, all monoclonal and polyclonal anti-HEL antibodies tested thus far have not induced RBC clearance [12].

4. Antigen modulation (also known as antigen-loss) is a process by which an erythrocyte antigen becomes weakened or undetectable subsequent to antibody binding. The data for antigen modulation is based on previously published data by others [8,13].


A
Heavy chains
Key:
Fucose (Fuc)
N-acetylglucosamine (GlcNAc)
Mannose (Man)
Galactose (Gal)
N-acetylneuraminic acid (Neu5Ac)
α bond
β bond
Untreated
Deglycosylated (PNGase F-treated)
CBC-512
MIMA 29
6D7
4B7
Relative Fluorescence (%)
Retention Time (min)
B
HOD
CBC512 5 µg
MIMA29
deCBC512
deMIMA29
% PKH26+ HOD-RBC
Time after antibody injection (h)
C
IgM Absorbance
naive
HOD
CBC-512
deCBC-512
MIMA29
deMIMA29
ns
D
IgG Absorbance
naive
HOD
CBC-512
deCBC-512
MIMA29
deMIMA29
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