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Original Article

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Immunology

High-throughput characterization of the functional impact of IgG Fc glycan aberrancy in juvenile idiopathic arthritis

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Abstract

Juvenile idiopathic arthritis (JIA) encompasses all forms of chronic idiopathic arthritis that arise before age 16. Previous studies have found JIA to be associated with lower Fc galactosylation of circulating IgG, but the overall spectrum of glycan changes and the net impact on IgG function are unknown. Using ultra performance liquid chromatography (UPLC), we compared IgG glycosylation in 54 subjects with recent-onset untreated JIA with 98 healthy pediatric controls, paired to biophysical profiling of affinity for 20 IgG receptors using a high-throughput multiplexed microsphere assay. Patients with JIA exhibited an increase in hypogalactosylated and hyposialylated IgG glycans, but no change in fucosylation or bisection, together with alteration in the spectrum of IgG ligand binding. Supervised machine learning demonstrated a robust capacity to discriminate JIA subjects from controls using either glycosylation or binding data. The binding signature was driven predominantly by enhanced affinity for Fc receptor like protein 5 (FcRL5), a noncanonical Fc receptor expressed on B cells. Affinity for FcRL5 correlated inversely with galactosylation and sialylation, a relationship confirmed through enzymatic manipulation. These results demonstrate the capacity of combined structural and biophysical IgG phenotyping to define the overall functional impact of IgG glycan changes and implicate FcRL5 as a potential cellular sensor of IgG glycosylation.

Key words: FcRL5, IgG glycan, IgG receptor binding, juvenile idiopathic arthritis, machine learning

Introduction

Juvenile idiopathic arthritis (JIA), encompasses all forms of chronic idiopathic arthritis diagnosed before age 16 (Prakken et al. 2011). While the mechanisms leading to JIA remain incompletely defined, a role for antibodies is suggested by the presence of anti-keratin, anti-nuclear and anti-DEK antibodies in many patients and by

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characteristic decreases in galactosylation of circulating IgG (Moore et al. 1982; Parekh RP et al. 1988; Sumar et al. 1991; Bond et al. 1996; Hromadnikova et al. 2002; Mor-Vaknin et al. 2011; Ercan et al. 2012a). IgG Fc domain glycosylation is a key post-translational modification that modulates antibody binding to Fc receptors and consequently influences antibody effector function (Raju 2008; Anthony and Ravetch 2010). In humans, each IgG heavy chain is decorated in the Fc region with one of approximately 30 different sugar forms that vary in mannose, bisecting *N*-acetylglucosamine (GlcNAc), fucose, galactose and sialic acid content. The increase in agalactosylated (G0) IgG evident in JIA (Sumar et al. 1991) is observed also in adult rheumatoid arthritis (RA) (Parekh et al. 1985; Ercan et al. 2010a) systemic lupus erythematosus (Vuckovic et al. 2015), Lambert-Eaton syndrome (Selman et al. 2011), ANCA vasculitis (Holland et al. 2002, 2006), cancer (Kodar et al. 2012), infection (Moore et al. 2005) and aging (Kristic et al. 2014).

Whether hypogalactosylated/sialylated IgG contributes to the pathogenesis of inflammatory arthritis is uncertain. Elevation in G0 glycans correlates with arthritis severity as measured by clinical scores (Ercan et al. 2012b), and can predate overt joint swelling (Ercan et al. 2010b; Rombouts et al. 2015), suggesting a potential causative role. Further, aberrant hypogalactosylation tends to normalize during pregnancy-induced RA remission, recurring at the time of disease relapse (Rook et al. 1991; van de Geijn et al. 2009). The potential of G0 IgG species to contribute to disease is suggested by the observations that both agalactosylated IgG (Rademacher et al. 1994) and blockade of sialylation in B cells (Ohmi et al. 2016) enhance collagen-induced arthritis in mouse models. Recent studies in human RA cohorts have further strengthened these observations by showing that repressed sialyltransferase activity in antibody-producing cells is associated with the progression of active RA (Pfeifle et al. 2017). In sum, such studies favor the possibility that hypogalactosylated/hyposialylated IgG contribute to the pathogenesis of RA.

The mechanistic significance of these glycan changes has been controversial. While early studies found G0 IgG to exhibit enhanced binding to the complement-activating mannose-binding lectin (MBL) (Malhotra et al. 1995; Arnold et al. 2006), this pathway of complement activation appears to play little role in murine IgG-mediated arthritis (Nimmerjahn et al. 2007). Nevertheless, G0 IgG may also activate complement through the classical and especially the alternative pathway, thought to be more directly relevant for disease (Banda et al. 2008; 2012). Highly sialylated IgG is less effective at mediating complement-dependent cytotoxicity (Quast et al. 2015). Further, discovery of the immunosuppressive properties of sialylated IgG Fc (Kaneko et al. 2006) has suggested an additional mechanism, in which reduced IgG sialylation blunts immunosuppressive signaling mediated through a pathway involving DC-SIGN (Anthony et al. 2008, 2011). Finally, highly galactosylated (G2) IgG can block pro-inflammatory chemokine receptor signaling (Karsten et al. 2012). Thus, lack of sialic acid and/or galactose is likely to enhance the pro-inflammatory potential of IgG.

Intrigued by the possibility that differential glycosylation may tune the effector function of IgG and render IgG more inflammatory or less immunosuppressive in the context of JIA, we performed a comparative study of serum IgG purified from healthy and JIA-affected children. IgG glycosylation was defined by ultra-performance liquid chromatography (UPLC). Binding to IgG receptors including Fc receptors, C-type lectins and complement cascade-initiating proteins was characterized using a multiplex microsphere-based assay (Boesch et al. 2014). Machine learning methods were used to model and discover relationships between JIA status, IgG glycosylation and antibody interactions with Fc receptors. Both IgG glycan and Fc receptor binding data could robustly differentiate JIA-affected subjects from controls. JIA IgG demonstrated a reduction in both glycosylation and sialylation, while the Fc binding signature was driven principally by enhanced affinity of JIA IgG for Fc receptor-like protein 5 (FcRL5). These changes were co-linear, and experimental manipulation confirmed that affinity for FcRL5 was mediated through both galactose and sialic acid, identifying FcRL5 as a previously unrecognized cell surface sensor of IgG glycosylation status.

Results

IgG glycosylation in juvenile idiopathic arthritis

To model antibody glycan-function relationships, we analyzed samples from 98 healthy control children and 54 JIA patients. Antibody glycans were profiled by high-throughput UPLC (Stockmann et al. 2013) (Supplementary data, Figure 1). Antibody binding to a suite of Fc receptors and lectins (Supplementary data, Table 1) was characterized by a customized microsphere array assay (Brown et al. 2012; Boesch et al. 2014). We first sought to compare the IgG glycan profiles in JIA subjects with those observed in healthy controls. For IgG glycan profiles, 28 glycan peaks were automatically integrated and 14 derived aggregated glycan features were calculated based on peak compositions (Supplementary data, Table 2). Of the 28 individual glycan features, 12 showed significant differences between JIA subjects and healthy control children when adjusted for multiple comparisons (Figure 1). IgG from JIA subjects was less likely to bear galactose and sialic acid (NeuNAc) groups, demonstrated by increases in F6A2, F6A2B and F6A2[3]BG1 glycoforms. IgG from healthy controls was more fully galactosylated and sialylated, reflected in increases in F6A2[6]G1, F6A2G2, F6A2[6]G1S1, F6A2G2S1 and F6A2G2S2. Overall, patients with JIA demonstrated reduced IgG galactosylation and sialylation, without alteration in the presence of bisecting GlcNAc or core fucose (Figure 2).

We then sought to determine whether glycosylation differences could distinguish JIA patients from controls. A balanced classifier was trained to discriminate healthy controls from JIA subjects in the setting of 10-fold cross-validation using the elastic net generalized linear algorithm (Friedman et al. 2010). The resulting model successfully classified 85% of subjects (Figure 3A). Permutation tests conducted using the same approach but randomized subject labels demonstrated the robustness of the classifier, indicating that it reliably captured the quantitative contributions of different glycan compositions to meaningful subject group predictions (Figure 3B). In these models, features related to galactose and sialic acid content made strong positive contributions to the healthy phenotype (A2BG2, A2BG2S1, A2BG2S2 and galactosylated glycan) (Figure 3C). Collectively, the generalized classifier further supported the importance of agalactosylated IgG (G0) and lower sialic acid content in defining JIA.

We then tested whether glycan features could differentiate among International League of Associations for Rheumatology (ILAR) subtypes (Petty et al. 2004). Five subgroups of JIA were represented in the sample set: polyarticular rheumatic factor negative (Poly-RF−), polyarticular rheumatic factor positive (Poly-RF+) oligoarticular (Oligo), enthesitis-related arthritis (ERA), and juvenile psoriatic arthritis (JPsA). However, neither individual nor derived aggregated glycan prevalences exhibited statistically significant differences between subjects with different ILAR subtypes (all adjusted

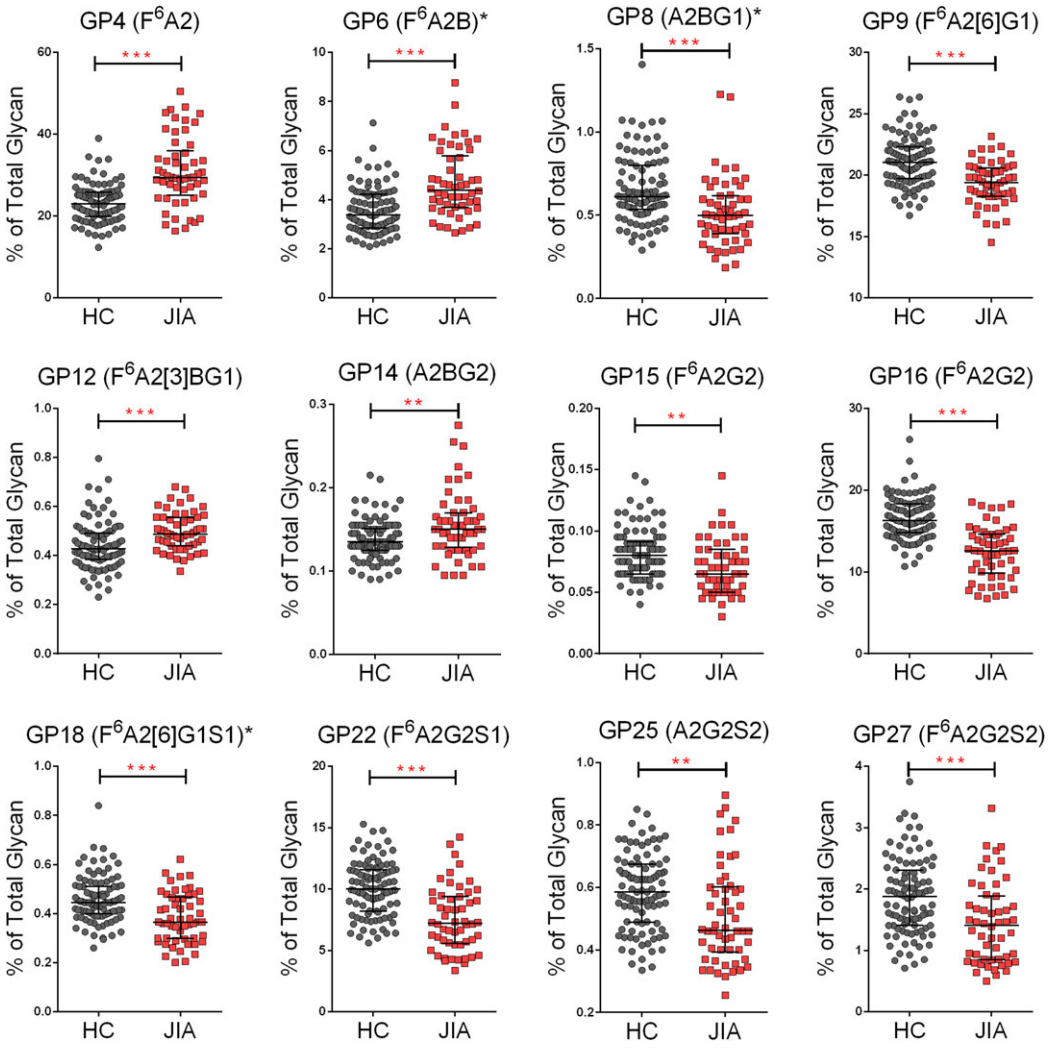


Fig. 1. IgG glycan compositions in healthy controls and JIA subjects. IgG glycoforms with variable prevalences in healthy control (HC) and JIA subjects. Significant differences were evaluated with a Student *t* test with a nonpaired, nonequal variance, two-sided hypothesis testing, and a confidence interval of 0.95. Calculated *P* values were adjusted by the Benjamini Hochberg False Discovery Rate (FDR) method (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$). This figure is available in black and white in print and in color at *Glycobiology* online.

P values exceeded 0.05). Although group sizes for these comparisons were small, no trends were observed to suggest that important differences would have emerged through analysis of a larger sample set (Supplementary data, Figure 3).

IgG function in juvenile idiopathic arthritis

To elucidate the functional consequences of differential IgG glycan profiles, the ability of polyclonal IgG to interact with functionally relevant antibody receptors and lectins was characterized using a multiplexed microsphere array. Lectins and Fc receptors were coupled to microspheres, and the interaction between polyclonal IgG and target proteins quantified by detection of bound IgG with a secondary antibody (Boesch et al. 2014).

A similar generalized classifier was used to classify JIA status using IgG binding data, resulting in an 80% classification accuracy (Figure 3D), exceeding the essentially random performance observed with permuted data (Figure 3E). Inspection of the features contributing significantly to the classification disclosed heavy reliance on one specific feature, binding to Fc-Receptor Like protein 5 (FcRL5) (Figure 3F). The coefficient weight of FcRL5 was 500% greater than any of the other features. Consistent with this strong contribution, the difference in binding to FcRL5 between subject groups was highly significant, with JIA subjects exhibiting roughly a 2.7-fold increase in recognition by this antibody receptor (Figure 4A).

Combining IgG glycan data and IgG binding data did not significantly improve the observed classification accuracy (Classification accuracy: 84.2%). IgG binding was comparable among ILAR subtypes of JIA (Supplementary data, Figure 2). When glycan, IgG binding, or the combination of both data types was used to train a classifier of ILAR subtypes, poor performance was observed. Collectively, these results indicate notable differences between healthy and JIA subjects, but a lack of differentiation among JIA subtypes as classified conventionally.

The binding of IgG to FcRL5 is associated with glycan composition

The significant differences observed between groups and the observed magnitudes and directions of glycan and IgG binding

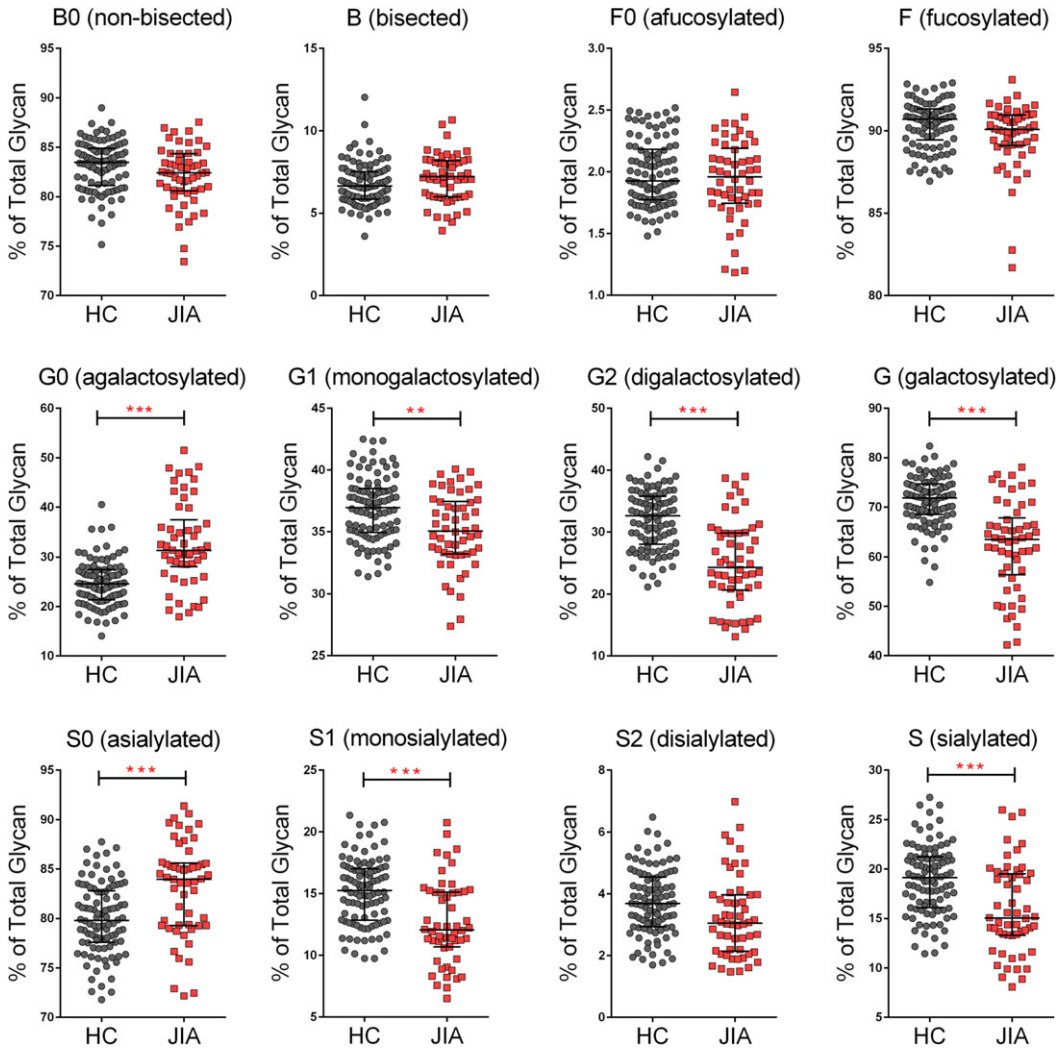


Fig. 2. Comparison of derived glycan compositions in healthy controls and JIA subjects. Derived glycan compositions were calculated and compared between healthy control (HC) and JIA subjects (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$). This figure is available in black and white in print and in color at *Glycobiology* online.

features in classification models suggested that the hypogalactosylated IgG could bind more effectively to FcRL5. Accordingly, we evaluated the correlation between FcRL5 binding data and IgG glycan composition. Interestingly, FcRL5 binding was significantly correlated with the prevalence of both agalactosylated and asialylated species (Figure 4B), and inversely correlated with the prevalence of various galactosylated (monogalactosylated, digalactosylated and galactosylated) and sialylated species (monosialylated, disialylated and sialylated) (Figure 4C). The level of galactosylated species demonstrated a marginally stronger correlation coefficient with FcRL5 binding signal (-0.54, $P = 1.34 \times 10^{-4}$) as compared to the sialylated species (-0.41, $P = 1.10 \times 10^{-4}$), and both observations were robust to the removal of apparent outliers identified by visual inspection. These observations are linked by the fact that the addition of sialic acid requires the presence of galactose. In contrast, no correlative relationships were observed between FcRL5 and glycan bisection or fucosylation (Supplementary data, Figure 3).

To directly test the role of glycosylation in FcRL5 binding, polyclonal IgG1 and IVIG were treated with neuraminidase to remove sialic acid alone; neuraminidase and galactosidase to remove both sialic acid and galactose; or control (IgG exposed to similar conditions but without enzymes) (Ackerman et al. 2013). Reduction in sialic acid, and reduction in both sialic acid and galactose, potentiated binding to recombinant FcRL5 in a dot-blot assay (Figure 5A and B). Similarly, binding to beads conjugated with FcRL5 increased for both desialylated and degalactosylated antibody pools (Supplementary data, Figure 4). Taken together, these results indicate that IgG binding to FcRL5 is enhanced by reduction in both sialic acid and galactose, potentially implicating this receptor in the functional impact of disease-associated IgG glycan changes associated with conditions such as JIA.

Discussion

JIA is one of the most common autoimmune diseases of children, with a prevalence estimated at 1 in 1000 children (Gare 1998; Manners and Bower 2002) and consequences that may include joint destruction and permanent disability (Bowyer et al. 2003). Previous studies had identified an increase in hypogalactosylated IgG in all JIA subtypes, but additional glycan changes and the resulting consequences for IgG effector functions remain unknown. Here, we show that sialylation is reduced in JIA in addition to galactosylation.

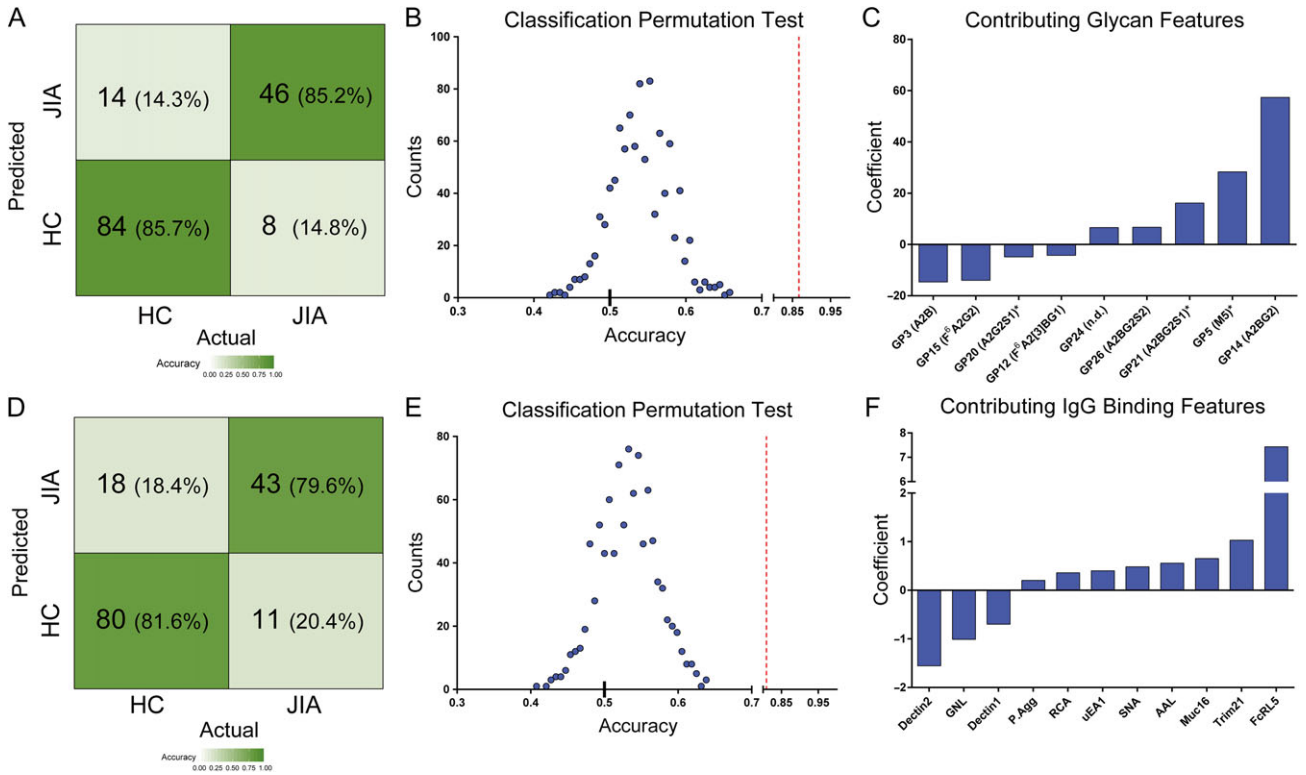


Fig. 3. Classification of healthy controls and JIA subjects. (**A**,**D**) Confusion matrices representing the cross-validated performance of an elastic net classifier trained to discriminate healthy control from JIA subjects using either glycan (**A**) or IgG phenotypic (**D**) data. (**B**,**E**) The accuracy of classification results in 1000-iterations of 10-fold cross-validation with permuted (blue) as compared to true subject labels (red dashed line) using either glycan (**B**) or IgG phenotypic (**E**) data. (**C**,**F**) The identity and relative contributions of major glycan (**C**) or IgG binding (**F**) features contributing to the classification. Features with coefficients smaller than 5% of the maximum coefficient were omitted. This figure is available in black and white in print and in color at *Glycobiology* online.

We could build robust classifiers that identify distinguishing features between healthy control and JIA-affected subjects. Application of machine learning tools allowed systematic evaluation of the ability of combinations of glycosylation and Fc receptor binding features to classify subjects based on JIA status, and provided specific coefficient weights for each contributing feature. Within the glycosylation data, hypogalactosylated/asialylated IgG glycoforms were the major contributors to successful subject classification. In models learned from Fc receptor binding data, one specific feature, IgG binding to FcRL5, contributed dominantly to the classifier, with a coefficient weight five times greater than any other.

Elevated agalactosylated IgG has been associated with several inflammatory diseases, but the consequences of this increase are poorly understood. In particular, it remains unclear whether elevated expression of hypogalactosylated/asialylated IgG in JIA is a cause or effect of disease. Evidence favoring the former includes the observation that agalactosylated antibodies exhibit greater arthritogenicity in various animal models (Rademacher et al. 1994; Banda et al. 2006, 2007, 2008). Longitudinal studies likewise support a potential role in pathogenesis, since aberrant glycosylation is observed months or years prior to the diagnosis of RA in adults (Ercan et al. 2010b; Rombouts et al. 2015), and RA remission in pregnancy and postpartum recurrence correlate with reduction and then recurrence of aberrant IgG Fc glycosylation, without accompanying changes in Fab glycans (van de Geijn et al. 2009; Bondt et al. 2016). While animal data have implicated IgG sialylation changes in IgG pathogenicity (Kaneko et al. 2006; Ohmi et al. 2016), mechanism(s) associated with this role remain controversial (Crispin et al. 2013; Campbell et al. 2014). In humans, RA- and (per this report) JIA-associated glycan variation encompasses both galactose and sialic acid, whereas in the murine system glycan changes appear relatively restricted to sialylation (Pfeifle et al. 2017). Further, in human pregnancy-induced RA remission, wherein changes to both IgG Fc galactosylation and sialylation levels are observed, disease severity is associated with galactosylation independently of sialylation (Bondt et al. 2013). Thus, whether IgG glyco-variation is a cause or effect of human disease, and whether variation in sialic acid or galactose content is more consequential, remains unresolved.

Aberrant IgG glycosylation can result from environmental exposures, including cytokines and hormones, as well as potentially from differential maturation states of B cells (Omtvedt et al. 2006; Wang et al. 2011; Ercan et al. 2017). How patients with JIA, RA and other inflammatory diseases develop aberrant IgG glycosylation remains to be determined. However, even if IgG glycan changes are driven by the inflamed milieu, it nevertheless is likely that these changes will in turn modulate the inflammatory process through their impact on IgG function. Our binding data implicate a previously unexpected pathway by which such an effect could be mediated: enhanced binding of hyposialylated and/or hypogalacyosylated IgG to FcRL5. FcRL5 is a B cell surface differentiation marker (Davis 2015; Sullivan et al. 2015). FcRL5 contains both immune-receptor tyrosine based inhibition motif (ITIM) and immune-receptor tyrosine based activation motif (ITAM) sequences, and has been shown to interact with the IgG Fc region in a subclass- and glycan-dependent manner (Wilson et al. 2012; Franco et al. 2013). FcRL5

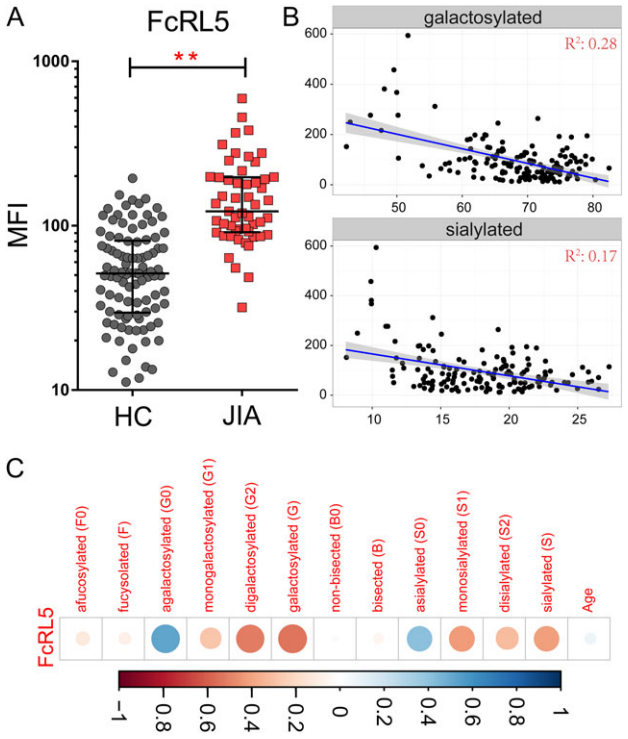


Fig. 4. IgG binding to FcRL5 distinguishes subjects and is associated with IgG glycan composition. (**A**) Median Fluorescent Intensity (MFI) of IgG binding to FcRL5 in healthy controls and JIA subjects (**$P < 0.01$). (**B**) Scatter plot between the MFI of IgG binding to FcRL5 (*y*-axis) and prevalence (% of total IgG glycan) of galactosylated/ sialylated IgG glycan content (*x*-axis). The linear fit of the scatter plot is presented as a blue line, with the confidence interval colored in gray. (**C**) Correlation coefficient heatmap presenting the magnitude and direction of correlation (Pearson) between derived antibody glycan compositions and binding to FcRL5. Blue denotes strong positive correlation, and red denotes strong negative correlation. This figure is available in black and white in print and in color at *Glycobiology* online.

ligation can suppress MAPK activation, calcium mobilization, and whole-cell pTyr in B cells by recruiting SHP-1 and SHP-2 phosphatase (Ehrhardt et al. 2003; Haga et al. 2007; Kochi et al. 2009; Jackson et al. 2010; Zhu et al. 2013). Mutation of the ITIM domain tyrosines in FcRL5 results in enhanced BCR-mediated calcium mobilization (Haga et al. 2007), and cross-linking of TLR9 and FcRL5 enhances B cell proliferation and isotype switching (Dement-Brown et al. 2012). Most previous work has focused on the ITIM, leaving the role of the ITAM as an activator of B cells unclear. Intriguingly, previous studies have found that whole-blood mRNA expression levels of *FcRL5* were an effective biomarker of the efficacy of B cell depletion using rituximab for RA treatment (Owczarczyk et al. 2011). Moreover, genetic studies have indicated associations between SNPs in the *FCRL* locus and chronic immune diseases in different populations, including RA (Kochi et al. 2009), autoimmune Addison's disease (Owen et al. 2007), multiple sclerosis (Martinez et al. 2007), autoimmune thyroid disease (Burton et al. 2007), and JIA (Ramirez-Bello et al. 2013). While this genetic predisposition is believed to be driven by the augmented expression of a related protein, FcRL3, with at least somewhat overlapping functionality (Kochi et al. 2005; Ehrhardt and Cooper 2011; Bajpai et al. 2012; Li et al. 2014), in combination with our findings these observations highlight the potential relevance of the FcRL family, including FcRL5, in autoimmune diseases such as JIA.

Given the presence of both ITIM and ITAM domains in FcRL5, reduced galactosylation and sialylation in JIA IgG could represent either a negative loop to constrain further B cell activation or a feed-forward loop that promotes B cell activity. The net effect of the interaction of aberrantly glycosylated IgG on FcRL5-expressing cells will help to identify whether this receptor could be an attractive therapeutic target, manipulated for example through depletion of FcRL5$^+$ cells or blockade of FcRL5 binding or signaling. Additional work is needed to determine the relevant signaling pathway(s) and the effect of FcRL5 ligation in specific contexts.

The current ILAR classification divides JIA into six principal subtypes (Petty et al. 2004). Similar IgG Fc glycosylation, assessed as the ratio of G0 to G1 glycans, has been reported among JIA subtypes (Ercan et al. 2012a), but a detailed comparison of IgG-specific glycans as well as antibody receptor binding profiling had not been conducted. In this study, wherein all forms except systemic JIA were represented, neither IgG glycans nor IgG binding profiles exhibited significant variation among JIA subtypes. We also attempted to build two multinomial classifiers to evaluate whether IgG glycan features and IgG binding features segregate with ILAR subcategories of JIA, but could identify no association. Our cohort was too small to suggest another typing schema based on glyco-variation. However, other lines of evidence have also suggested that the classification system is imperfect, including by HLA association data, blood mRNA profiling, and clinical phenotyping (Nigrovic et al. in press). Ongoing efforts are needed to more robustly classify biologically defined JIA subtypes.

Our study has several limitations. First, study subjects ranged in age from 9 months to 16 years. While there was not a significant difference in the age between patients and controls ($P = 0.5$), healthy controls were not individually matched to JIA patients by age, which might confound study results as age can impact humoral immune responses (LeMaoult et al. 1997), and antibody glycosylation profiles are known to vary with age (de Haan et al. 2016). Second, our microsphere studies measure the effect of glycan changes on binding of free IgG. We cannot exclude the possibility that alternate binding properties might be encountered in antibodies engaged with antigen, or that are part of an immune complex. Finally, UPLC analysis of IgG glycans as employed here measures those liberated from the Fab region as well as from the Fc region. Fab glycans are generally fully developed, often to G2S2 forms, even in the context of inflammation (Holland et al. 2006). Thus Fab glycan contamination would be expected to introduce only conservative bias into our analysis. Moreover, the changes we have identified are consistent with those identified by Fc-specific glycopeptide analyses in RA (Scherer et al. 2009). IgG glycophenotyping findings proved co-linear with those from the microsphere assay, which is Fc specific, confirming experimentally that Fab glycans are unlikely to represent an important unmeasured confounder in our studies.

Conclusion

Taken together, these data demonstrate that JIA is associated with increases in agalactosylated and asialylated IgG, and that these glycan features can be employed to distinguish JIA patients from healthy controls. Further, we show that glycan changes result in altered IgG binding to FcRL5, confirming that glycan aberrancy in JIA translates into a net change in the effector potency of the antibody pool. Together with previous work linking FcRL5 to therapeutic responses in RA (Owczarczyk et al. 2011), our biophysical evidence of a direct impact on IgG glycans in FcRL5 binding identifies a potential new feedback loop in arthritis biology. Additional

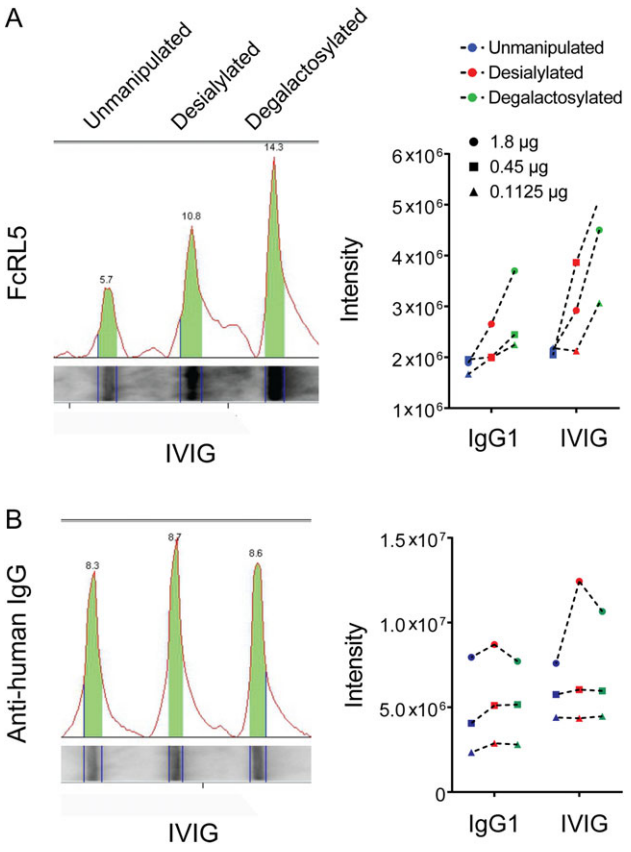


Fig. 5. IgG binding to FcRL5 is influenced by IgG glycan compositions. (**A**) Un-manipulated, neuraminidase-treated (desialylated) and neuraminidase- and galactosidase-treated (degalactosylated) IgG pools (IgG1 and IVIG) were slot blotted and the impact of differential glycosylation assessed by detection with FcRL5 (left, IVIG shown). Band intensities were quantified for both polyclonal IgG1 and IVIG at three different antibody concentrations (right). (**B**) As a test of equivalent antibody loading, the same membrane was stripped and re-blotted to detect (left, IVIG shown) and quantify (right) total IgG via detection with an anti-human IgG secondary. This figure is available in black and white in print and in color at *Glycobiology* online.

studies will be required to evaluate whether ligation of FcRL5 by aberrantly glycosylated IgG modulates B cell fate and function in order to play a determinative role in the pathogenesis of JIA and other autoimmune diseases.

Materials and methods

Subjects

Subjects with untreated recent-onset JIA (ICD10 M08.00) ($n = 54$) were recruited through the Boston Children's Hospital JIA Registry. Control samples ($n = 158$) were obtained through the same registry, from discarded samples after routine lead testing, and from a prospective consented study of healthy children (Ercan et al. 2012a). Subjects ranged in age from 9 months to 16 years. The study was reviewed and approved by the Institutional Review Boards of Boston Children's Hospital and Dartmouth College.

Multiplexed receptor and lectin microarray

In order to assess the ability of each IgG sample to interact with antibody binding proteins including human Fc receptors and lectins, we customized a multivariate microsphere assay from a previously described method (Brown et al. 2012; Boesch et al. 2014). In brief, Fc receptors and lectins (Supplementary data, Table 1) were covalently coupled to fluorescently coded magnetic microspheres as described. IgG was enriched from serum using Melon Gel purification kit (ThermoFisher Scientific, 45206) according to the manufacturer's instructions, diluted, and incubated with receptor/lectin conjugated microspheres (500 microspheres of each specificity) in a total 50 µL volume of incubation buffer (20 mM Tris pH7.5, 2 mM $CaCl_2$, 150 mM NaCl, and 0.05% (v/v) Tween-20), in black, clear bottom 384-well plates (Greiner Bio One, 781906) covered by micro-plate adhesive film (USA Scientific). Samples were mixed by ultrasonic shaking for 15 s, followed by the incubation on plate shaker (IKA, MTS 2/4) for 4 h at room temperature. Plates were then washed five times with incubation buffer using an automated plate washer (Biotek, 405 Select TS). To detect antibody bound to each receptor/lectin, a volume of 50 µL of anti-human IgG PE-conjugated antibody (Southern Biotech, 9040-09) was added to each well at a concentration of 0.65 µg/mL, followed by incubation at room temperature with shaking for 1 h. Finally, the plates were washed 5 times with and resuspended in Luminex sheath fluid buffer. Plates were sonicated for 15 s prior to data acquisition on a FlexMap 3D (Luminex, Bio-Plex Manager 5.0, Bio-Rad). The net median fluorescence intensity (MFI) was determined by subtracting the background signal (beads in the absence of antibody) from each test sample.

High-throughput glycan analysis

UPLC analysis was performed as described previously (Stockmann et al. 2013). IgG purification, glycan release, labeling and clean-up were performed on the Hamilton STARlet robotic liquid handler (Hamilton, Reno, NV). IgG was purified from serum samples using Protein G plates (ThermoFisher Scientific, 45204). IgG *N*-glycans were released by digestion with recombinant *N*-glycosidase F (ProZyme, CA), and then labeled with 2-aminobenzamide (Royle et al. 2008), cleaned-up using solid phase extraction and dried. Purified glycans were then separated by HILIC-UPLC on a Waters H-Class instrument (Waters, Milford, MA) with a Waters BEH glycan column (150 × 2.1 mm, 1.7 µm BEH particles) using separation settings as described previously (Stockmann et al. 2013). Data was processed automatically: chromatograms were integrated into 28 peaks (Pucic et al. 2011), as described in Supplementary data, Table 2, and the prevalence of each glycoform or peak was reported as percentage of total glycan or total integrated peak area. Derived glycans (e.g., total agalactosylated glycans) were calculated as described in Supplementary data, Table 3.

Western blot analysis

Soluble FcRL5 with a C-terminal His_6 tag was expressed by HEK293 cells and purified using the standard Ni-NTA chromatography. Soluble FcRL5 was then biotinylated on primary amines (EZ-link Sulfo-NHS-Biotin, Thermo Fisher) according to the manufacturer's instructions. Human serum IgG1 and intravenous Immunoglobulin (IVIG) were treated by neuraminidase, or both neuraminidase and galactosidase respectively, as previously described (Ackerman et al. 2013). For blotting, 18 µg of each sample in 100 µL PBS was loaded onto a nitrocellulose blotting membrane (GE Healthcare) through the dot-blot microfiltration apparatus (Bio-Rad). The membrane was then blocked in blocking buffer (PBS with 3% BSA) on a shaker at room temperature for 1 h. After that, the membrane was washed three times with blocking buffer and

incubated with 100 μg/mL biotinylated-FcRL5 at room temperature for 2 h. The membrane was then washed again, prior to incubation with avidin-HRP conjugate at 2.5 μg/mL (Thermo Fisher) for 1 h. Blots were washed three times and developed by the ECL western blotting substrate (Thermo Fisher) and photographed by the Bio-rad imager. The same membrane was then stripped with 0.1 M glycine-HCL pH 3.0 for 1 h and re-blotted with goat anti-human IgG HRP conjugate (Thermo Fisher) and developed and imaged to quantify total IgG. Band intensities were determined by the Image Lab5.0. The size and range of the band was identified by the "Lanes and Bands" tools from the software. Each band's intensity was then reported and plotted in the GraphPrism.

Statistical analysis

Age is known to impact IgG glycan compositions (Parekh et al. 1988; Kristic et al. 2014). To reduce the impact of age as a factor in our study, the 98 healthy controls that were best age matched to the 54 JIA subjects ($P = 0.5$) were selected for statistical analysis. Student's t test analysis was performed by the Stats R software package, version 3.1.2. Both the glycan and the microarray measurements were compared under nonpaired, nonequal variance and two-sided hypothesis testing with a confidence interval of 0.95. Calculated P values were adjusted by the Benjamini Hochberg False Discovery Rate (FDR) method (Benjamini and Hochberg 1995).

Binomial classification models were built by the Glmnet R package (Friedman et al. 2010). To facilitate comparison of model coefficients, antibody microarray binding data was scaled and centered prior to modeling. Antibody glycan data was measured as the percentage of total glycan, thus no normalization was performed before modeling. Binomial classifiers were trained using either glycan or microarray measurements with the elastic net mixing parameter at 0.4, and balanced observation weights. Coefficient weights of each IgG measurements contributing to the model were evaluated at a specific tuning parameter value (lambda min), which yielded the minimum cross-validated mis-classification error. The contribution of different features to model predictions was generally stable over variable values of lambda (from lambda min to 1 standard deviation of lambda min). Predictive robustness was compared to model performance when group labels were randomly permuted and models trained with 250 iterations of 10-fold cross-validation. Models with prediction accuracy greater than two standard deviations away from the permutation mean (2 SD + Mean) were accepted.

Abbreviations

ERA, enthesitis-related arthritis; ILAR, International League of Associations for Rheumatology; JIA, Juvenile idiopathic arthritis; JPsA, juvenile psoriatic arthritis; MBL, mannose-binding lectin; MFI, median fluorescence intensity; RA, rheumatoid arthritis; UPLC, ultra-performance liquid chromatography.

Supplementary data

Supplementary data are available at *Glycobiology* online.

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Conflict of interest statement

The authors declare no competing financial interests.

Authors' contribution

M.E.A. and P.A.N. conceived, planned and secured funding for the project. I.A.H. and P.A.N. provided study samples. H.D.C., H.S., B.A., C.A.M., A.E. performed experiments and analyzed data. H.D.C. created the figures. H.D.C., M.E.A. and P.A.N. wrote the manuscript. All authors critically reviewed, edited and approved the final version.

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