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Anti-HA glycoforms drive B cell affinity selection and determine influenza vaccine efficacy

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SUMMARY

Protective vaccines elicit high affinity, neutralizing antibodies by selection of somatically hypermutated B-cell antigen receptors (BCR) on immune complexes (ICs). This implicates Fc-Fc receptor (FcR) interactions in affinity maturation, which, in turn, are determined by IgG subclass and Fc glycan composition within ICs. Trivalent influenza virus vaccination elicited regulation of anti-hemagglutinin (HA) IgG subclass and Fc glycans, with abundance of sialylated Fc glycans (sFc) predicting quality of vaccine response. We show that sFcs drive BCR affinity selection by binding the Type-II FcR CD23, thus upregulating the inhibitory FcγRIIB on activated B-cells. This elevates the threshold requirement for BCR signaling, resulting in B-cell selection for higher affinity BCR. Immunization with sFc HA ICs elicited protective, high affinity IgGs against the conserved stalk of the HA. These results reveal a novel, endogenous pathway for affinity maturation that can be exploited for eliciting high affinity, broadly neutralizing antibodies through immunization with sialylated immune complexes.

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AUTHOR CONTRIBUTIONS

TTW and SJS designed the clinical study. TTW, JM and JVR designed research and analyzed data. TTW, JM, GST, SB and CD conducted experiments. RA and PP provided intellectual input. FK contributed analytical reagents and intellectual input. TTW and JVR wrote the manuscript.

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INTRODUCTION

IC-FcR interactions mediate a wide array of cellular processes required for maturation of protective, vaccine-induced antibody responses including efficient transport of antigen to the germinal center, activation of T follicular helper cells and selection of high affinity B cells. Indeed, FcR signaling is responsible, in large part, for maintaining the balanced positive and negative signaling that culminates in appropriate immune responses (Pincetic et al., 2014). Two basic classes of FcRs have been identified: Type I FcRs are immunoglobulin superfamily members and include FcγRI, II, and III, while Type II FcRs are C-type lectin family members and include DC-SIGN and CD23 (Figure 1a). Perturbations in either signaling arm result in changes in antibody affinity and peripheral tolerance (Bolland and Ravetch, 2000). IC-FcR interactions can initiate activating, inhibitory or modulatory cell signaling depending on the pattern of FcRs engaged, which is determined by the structure of Fc domains within an IC. Fc structure, in turn, is regulated by IgG subclass and Fc glycan composition.

IgG antibodies exist as four subclasses in humans (IgG1-4) with IgG1 in highest abundance in serum followed by IgG2>IgG3>IgG4. This was demonstrated by the subclass distribution of baseline (pre-vaccination) anti-HA IgGs from this study's cohort of 10 healthy adult volunteers (Figure 1b, Figure S1). Each subclass is distinct in its ratio of binding to activating:inhibitory Type 1 FcgRs, with IgG1 and IgG3 having the highest activating receptor binding affinities (Figure 1c)(Bournazos et al., 2014; Morell et al., 1970).

The Fc glycan is an N-linked, complex, biantennary structure attached within the Cγ2 domain at Asn-297 of each IgG heavy chain and its presence is essential for all Fc-FcR binding interactions (Anthony and Ravetch, 2010). Composition of the core Fc glycan heptasaccharide can be modified by addition of specific saccharide units (fucose (F), N-acetylglucosamine (N), galactose (G) and sialic acid (S)) (Figure 1d); these modifications are dynamic and act to regulate the biological activity of IgG molecules by modulating Fc structure and, as a consequence, IC-FcR interactions. At baseline, a majority of IgG Fc glycoforms are of 'neutral' composition, defined by the presence of fucose and absence of sialic acid (Figure 1e, neutral glycans represented by +N and −S groups). sFc are present with an abundance of ~5–20% (Figure 1e, +S group) and afucosylated glycoforms are found with an abundance of ~5–15% (Figure 1e, -F group). This distribution was demonstrated by the baseline Fc glycoform composition on anti-HA IgG1 of this study's patient cohort (Figure 1e).

The most biologically significant modifications to Fc glycan composition are sialylation and fucosylation: the presence of sialic acid is inhibitory for Type I Fc receptor binding, while the absence of fucose enhances binding to the activating Type I FcγRIIIa. The presence of sialic acid alone is the determinant of Fc-Type II FcR binding (Figure 1f) (Anthony et al., 2008b; Sondermann et al., 2013). Sialylation has the effect of increasing the conformational flexibility of the Cγ2 domain, enabling the Fc to sample a more 'closed' conformation (Ahmed et al., 2014) thereby exposing binding sites for Type II FcRs with correspondingly reduced Type I FcR binding potential. Sialylation of the Fc glycan therefore represents a

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mechanism for regulating the effector activity of immunoglobulins through alternation of Fc conformations between open and closed states, thus regulating Fc binding to Type I or Type II FcRs, respectively (Sondermann et al., 2013). Studies on the bisecting GlcNAc modification show possible increased Type I FcγRIIIa binding affinity, however afucosylation is a far more potent determinant of strong FcγRIIIa binding (Hodoniczky et al., 2005; Shields et al., 2002; Shinkawa et al., 2003; Umana et al., 1999). Addition of galactose alone to one or both arms of the branched Fc glycan does not affect FcR binding, but is significant because galactosylation is a prerequisite for sialylation.

Shifting IgG Fc binding specificity from Type I to Type II FcRs can result in significant in vivo responses and precise regulation of sFc abundance is likely a fundamental homeostatic process. One known consequence of increasing Type II FcR signaling is anti-inflammatory activity, a classic example of which is the therapeutic anti-inflammatory activity of high dose intravenous immunoglobulin (IVIG) (Anthony et al., 2008a; Kaneko et al., 2006; Washburn et al., 2015). sFcs in IVIG, acting through binding of the Type II FcR DC-SIGN on innate effector cells, stimulate IL-33 production resulting in downstream anti-inflammatory processes (Anthony et al., 2011). Disrupted balance in Type I and Type II FcR signaling likely occurs in several inflammatory diseases such as rheumatoid arthritis and granulomatosis with polyangiitis in which decreased abundance of sFc are found on autoantibodies such as anti-citrullinated peptide (ACPA) and anti-proteinase 3 (PR3) antibodies, respectively. Sialylation of anti-ACPA and anti-PR3 Fcs is reduced during disease flares, while disease remission is correlated with elevated Fc glycan sialylation of those autoantibodies (de Man et al., 2014; Espy et al., 2011a; Espy et al., 2011b; Scherer et al., 2010; Tomana et al., 1988; van de Geijn et al., 2009; Wuhrer et al., 2015).

Just as sialic acid-modified Fc glycans play a critical role in the regulation of inflammatory processes, the presence or absence of a branching fucose moiety modulates the interaction of IgG Fcs with FcγRIIIa to enhance or inhibit IgG-mediated ADCC and monocyte/macrophage activation (Umana et al., 1999) (Shinkawa et al., 2003). Afucosylated Fc domains have increased affinity for the activating receptor FcγRIIIa that results from a stabilizing interaction between the N-glycan on FcγRIIIa with the afucosylated Fc glycan (Ferrara et al., 2011). Removal of fucose from monoclonal therapeutic antibodies such as rituximab and trastuzumab improved their clinical efficacy by increasing binding to FcγRIIIa, thereby enhancing ADCC activity (Dalle et al., 2011; Junttila et al., 2010). As with sialylated glycoforms, diseases associated with modulations in fucose levels on Fc glycans suggests strict regulation of Fc fucosylation; an example of this is fetal or neonatal alloimmune thrombocytopenia, in which IgG specific for human platelet antigens (HPA) have significantly diminished levels of fucosylated Fc glycans, with levels of afucosylated anti-HPA correlating with disease severity (Kapur et al., 2014).

That Fc glycan modulation may result in autocrine B cell signaling through IC-FcR interactions, potentially directing the antibody response to vaccination, is suggested by the observations that Fc glycan composition can change following vaccination (Selman et al., 2012) and that sFc can bind CD23, the Type II FcR expressed on activated B cells (Sondermann et al., 2013). The present study was designed to determine whether Fc structure within vaccine antigen-IgG ICs might be regulated as a mechanism of directing

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FcR-mediated processes involved in maturation of antibody responses. Our approach was to characterize modulations in the structural determinants of Fc domains (IgG subclass and Fc glycan composition) on IgGs elicited by administration of trivalent influenza virus vaccine (TIV) in healthy subjects. Next, we performed a series of directed experiments to determine what role, if any, the modulations played in determining vaccine responses. Our results on the natural regulation of Fc domain structure during the evolution of protective vaccine responses suggest immunization strategies involving administration of ICs containing sFc to elicit broadly protective antibodies against influenza viruses.

RESULTS

Characterization of Fc domain structure on TIV-elicited IgGs

Healthy adults were vaccinated with the 2012–2013 TIV. Sera were drawn at baseline (pre-vaccination), day 7, week 3, week 5 and week 7 following vaccination and antigen-specific IgGs were isolated for subsequent analysis of Fc glycan composition and IgG subclass (Figure S1a). At baseline, all subjects were positive for IgG against the H1 HA vaccine component, A/California/04/2009 (H1N1 virus), by H1-binding ELISA and the hemagglutination inhibition (HAI) test using homologous virus (Figure S2a,b). Baseline anti-H1 IgG was characterized for subclass distribution and Fc glycan composition (Figure 1b,e). Fc glycoforms on baseline anti-H1 IgG were predominantly neutral, with an inter-subject range of 66.3–88.3%, bisecting GlcNAc moieties were present with a range of 6.2–14.6%, sialylated glycoforms were present with a range of 4.6–18.5% and afucosylated glycans were present with a range of 4.27–15.07% (Figure S2c). Anti-H1 IgG at baseline contained significantly more sFc and less fucosylated (fucFc) than total IgG, while subclass distribution was not different between H1-specific and total IgG (Figure S2 d,e,f).

Following vaccination, modulations in the abundance of sFc glycoforms on H1-specific IgG were observed. sFc glycoforms were significantly elevated and at peak abundance by day 7 post vaccination; this was mirrored by an increase in fucFc by day 7. sFc glycoforms and fucFc on anti-H1 IgG were consistently elevated above pre-vaccination levels through week 7 except for a significant dip at week 3 post-vaccination (Figure 2a,b).

Because galactosylation is a prerequisite for sialylation of the Fc glycan, the presence of galactose could be a limiting factor in determining the abundance of sialic acid modification. Analysis showed that galactose (GalFc) levels were modulated to a small, but statistically significant degree following vaccination, but that the overall abundance of galactosylated glycans was over 90% at every timepoint (Figure 2c). This was several fold higher than sialylated glycans, suggesting that galactosylation was not limiting and that modulations in sialylation were independently regulated. There was no apparent regulation of bisected GlcNac glycoforms (bGlcNAcFc) (Figure 2d).

In addition to regulation of Fc glycoforms over time following vaccination, analysis of HA-specific IgG from a single timepoint, week 3, revealed that specific Fc glycoforms can be linked to Fab specificity, possibly due to differential glycosylaytion by distinct IgG-producing B cell subsets such as plasmablasts (PB) and memory B cells. Anti-HA (predominantly globular head-specific) and anti-HA stalk IgG differed significantly in Fc

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glycoform profile, with sialylated, fucosylated glycan levels highest on total anti-HA IgG and lowest on anti-HA stalk IgG (Figure 2e,f and Figure S2g). No differences were observed in levels of bisecting GlcNAc (Figure 2g).

Day 7 post vaccination was characterized by an increase in sFc on anti-H1 IgG, which would increase Type II FcR binding by ICs. In contrast, week 3 post vaccination was characterized by diminished sFc and elevated afucosylated Fc (afucFc), which would shift the signaling balance towards activating Type I FcRs (Figure 3a). This shift in Fc glycoform composition towards activating Type I FcR signaling at week 3 was mirrored by modulations in subclass distribution of anti-H1 IgGs, from a median IgG1 abundance of 35.2% at baseline, to a median level of 75.18% by week 3 (Figure 3b).

Fc glycoforms present following vaccination mirror glycosyltransferase expression in activated B cell subsets

Peak levels of sialylated and fucosylated Fc glycans at day 7 post-vaccination mirrored a peak in plasmablast (PB) abundance in the peripheral blood of our study subjects, in accordance with the well-described kinetics of plasmablast expansion that occurs following TIV administration (Figure S3a) (Wrammert et al., 2008). In addition, the amount of anti-H1 IgG produced during the early plasmablast response correlated with the change in sFc abundance on anti-HA IgG in the first 7 days following vaccination (p=.0065) (Figure S3b). These observations led us to hypothesize that sialylated and afucosylated Fc glycoforms may be produced, at least in part, by PB. Because the increase in relative abundance of afucFc and asialylated Fc glycoforms from day 7 to week 3 mirrored a small increase in peripheral memory B cells in our patient cohort (Figure S3c), as has been previously described (Pinna et al., 2009), we hypothesized that that those glycoforms may derive from memory B cells. Intracellular staining of PB and memory B cells for the relevant glycosyltransferases, ST6Gal1 and FUT8, revealed increased ST6Gal1 expression and non-significantly increased FUT8 expression in PB over memory B cells (Figure S3d,e). In addition, gene analysis of day 7 PB and memory B cells from six patients who received the 2009–10 TIV revealed elevated levels of *ST6Gal1* (p=.06) and *FUT8* (p=.03) in PB over memory B cells (Figure S3f,g), supporting the possible production of sialylated, fucosylated Fc glycans by PB and less sialylated, fucosylated Fc glycans by memory cells. Unlike *ST6Gal1* and *FUT8, B4GALT1*, coding for a galactosyltransferase involved in Fc glycan modification, was not significantly elevated in PB (Figure S3h).

Sialylated Fc abundance predicts influenza virus vaccine efficacy, defined by change in HAI+ titer post vaccination

To investigate the significance of the regulated changes in Fc glycan composition following TIV vaccination, we assessed association of specific Fc glycoform abundance with change in hemagglutination inhibition titer (HAI), a commonly used measure of TIV vaccine efficacy. In particular, since the degree of HA-specific plasmablast expansion on day 7 post vaccination has been observed to loosely correlate with vaccine response, we investigated any association between production of sialylated IgG on day 7 and vaccine efficacy as measured by HAI titer by week 3 (Nakaya et al., 2011; Wrammert et al., 2008). Indeed, the change in abundance of sialylated glycoforms on anti-HA IgG in the first week following

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vaccination was predictive of subsequent increase in HAI activity (Figure 4a). In addition, sialylated glycoform abundance predicted affinity of anti-HA IgG at week 3 post vaccination (Figure 4b). These data suggested that the abundance of sialylated Fc glycoforms produced following TIV vaccination could regulate the quality of the overall vaccine response.

Sialylated Fc glycoforms in ICs trigger upregulation of FcγRIIB on B cells, resulting in production of higher affinity IgGs

To determine the mechanisms by which sFc within immune complexes might modulate B cell activation, we studied the effects of sFc ICs on B cells in a variety of in vitro and in vivo assays. Pooled IgG from week 3 post-vaccination, either with native sFc levels (17.6% on anti-HA IgG) or neuraminidase treated (asialylated) (Figure S4) was complexed with California/04/2009 (Cal/09) H1 HA protein to form sialylated or asialylated IC (sIC or aIC). These ICs were incubated with human CD19+ PBMCs and analysis after 24 hour incubation revealed increased expression of FcγRIIB, the inhibitory Type I Fc receptor, on cells incubated with sIC but not aIC or HA protein alone (Figure 5a). Similarly, a recombinant anti-HA mAb, PY102 (Dinca et al., 1993), was expressed with sialylated Fc glycoforms (23.7% sFc), or in an asialylated form, and was mixed with A/PR8/1934 (PR8) H1 HA protein to form sIC or aIC. These mAb IC were then incubated with BJAB cells and, consistent with the primary B cell assay, incubation with sICs, but not aIC or HA protein alone induced upregulation of FcγRIIB expression (Figure 5b).

Next, to determine how sialylated ICs might affect B cells in vivo, the human post-vaccination IgG/Cal/09 HA ICs (sIC and aIC) were used to prime mice, followed by HA protein boost 3 weeks later. Ten days post boost, peripheral B cells were analyzed for FcγRIIB expression; antigen-specific peripheral B cells from mice primed with sIC showed increased expression of FcγRIIB, whereas mice primed with aICs showed no such elevation (Figure 5c). Similarly, sIC or aIC made from mAb PY102 and PR8 HA protein were used to vaccinate mice; 3 days post vaccination, germinal center B cells from mice immunized with sIC, but not aIC or HA alone had increased FcγRIIB expression (Figure 5d). Because CD23 is the only Type II FcR expressed on B cells, we next determined whether upregulation of FcγRIIB was dependent on CD23 expression. CD23 deficient mice did not display upregulation of FcgRIIB on germinal center B cells, demonstrating that sICs acted through CD23 to trigger upregulation of B cell FcγRIIB (Figure 5d).

IgG elicited by sialylated ICs are higher affinity for antigen

Increased FcγRIIB expression is known to elevate thresholds for selection of B cells based on affinity of BCR, thus, we characterized the affinity of anti-HA IgG elicited by immunization with sICs (Bolland and Ravetch, 2000; Pearse et al., 1999). The affinity of IgGs elicited by human post-vaccination IgG/Cal/09 HA ICs was measured using an affinity ELISA assay that was modified from a nitrophenyl system for evaluation of anti-HA IgGs; this assay measures the ratio of high-affinity to all-affinity binding IgGs (Herzenberg et al., 1980; Kaisho et al., 1997). The affinity for Cal/09 HA1 was also evaluated using a method that measures the quantity of IgG remaining bound following treatment with 7M urea (Verma et al., 2012). IgGs elicited by the sIC priming protocol had significantly higher

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affinity for Cal/09 HA1 (primarily globular head), the highly conserved stalk domain of the H1 subtype HA protein, and for the complete Cal/09 HA glycoprotein (Figure 6a,b,c,d). Consistent with the lack of FcγRIIB upregulation using the sIC priming protocol in CD23 deficient mice (Figure 5d), elevated affinity of elicited IgGs was not achieved in CD23 deficient mice (Figure 6d). The affinity of IgGs elicited by mAb PY102/PR8 HA ICs for the PR8 H1 protein or the PR8 stalk domain was assessed using surface plasmon resonance (SPR) analysis (Verma et al., 2012). The polyclonal serum antibody dissociation off-rate for anti-HA IgG from mice primed with sIC was found to be approximately 10–20 fold lower than that of IgG from mice primed with aIC or CD23 deficient mice that received sIC (Figure 6e).

Higher affinity IgGs mediate broad protection against H1N1 influenza viruses

To determine any functional significance associated with increasing affinity of anti-HA IgGs, we tested pooled IgG from mice primed with polyclonal human post-vaccination IgG/Cal/09 HA IC or HA alone for protective activity (pools were derived from sera tested in Figure 6). IgG pools had equivalent HAI endpoint titers using virus expressing the homologous HA used for vaccination (A/Netherlands/602/2009) (Figure S5a) and equivalent binding activity for Cal/09 or Cal/09 stalk proteins (Figure S5b-g). For challenge experiments, virus was pre-incubated with purified IgG prior to intranasal infection; thus, weight loss was a function of dose of infectious virus remaining after incubation with IgG pools. On challenge, equivalent protection was observed against the H1N1 virus A/Netherlands/602/2009, which expresses the homologous HA used for vaccination (Figure 7a,b). In contrast, when we evaluated only anti-stalk IgGs for protective activity using a virus that expresses a chimeric hemagglutinin molecule (cH5/1) comprised of the highly conserved H1 stalk domain and an irrelevant head subtype domain (H5), we observed a difference in protective potency of the IgG pools. Only the higher affinity IgGs elicited by priming with sIC conferred anti-stalk-mediated protection (Figure 7c,d).

Next, equivalent challenge experiments were performed using IgG from mice immunized with mAb PY102/PR8 HA IC (sIC in wild-type mice, sIC in CD23-deficient mice or aIC) or PR8 HA alone. Purified IgG pools had equivalent binding titers for PR8 HA (Figure S5h). Mice were challenged with A/PR8/1934 virus, which expresses the homologous H1 HA used for vaccination and, as before, complete protection was achieved with all IgG pools (Figure 7e,f). In contrast, only IgG elicited by sIC protected mice from challenge with either A/FM/1/1947 H1N1 virus or A/Netherlands/602/2009 H1N1 virus (Figure 7g-j), demonstrating breadth of protection conferred by higher affinity IgG elicited by sIC.

DISCUSSION

That sFc produced during the early plasmablast response was found to correlate with subsequent production of HAI+ antibody suggested a possible requirement for immunomodulatory Type 2 FcR signaling in the ontogeny of protective TIV responses. Further experiments demonstrated that sFc within immune complexes triggered upregulation of B cell FcγRIIb, thus modulating the selection of B cells in favor of those expressing higher affinity BCR. Our studies suggest a model whereby TIV vaccination triggers

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plasmablast expansion and production of sFc IgG. Immune complexes formed with sFc IgG signal through the Type II FcR CD23 on activated B cells, triggering increased FcγRIIb expression. This results in an elevation of threshold for BCR affinity that is required for B cell survival and, ultimately, production of higher affinity, more potently protective IgG (Figure S6).

Several studies, including the present, have found that baseline titer of anti-HA IgG correlates negatively with the magnitude of TIV response, so that low baseline titer predicts greater vaccine response (Figure S7a) (Beyer et al., 1996; Sasaki et al., 2008; Tsang et al., 2014). Low baseline anti-HA titer also predicts greater plasmablast frequency (Tsang et al., 2014) and predicted increased production of sFc glycoforms by day 7 post vaccination (Figure S7b). Overall, low baseline anti-HA IgG predicts large plasmablast expansion and abundant production of sialylated glycoforms within the week following vaccination, resulting in a greater change in HAI+ IgG. Of note, greater production of sialylated Fc glycoforms by day 7 could also be predicted by baseline sialylated Fc abundance, with lower baseline sialylated glycoform abundance correlating with greater subsequent sFc production (Figure S7c).

The finding that protective anti-stalk IgGs can be elicited by sialylated ICs is significant as anti-stalk IgGs can mediate broad protection against antigenically distinct influenza viruses (Krammer et al., 2013; Pica et al., 2012; Wang et al., 2010). One practical application suggested by the observation that sialylated immune complexes can drive selection of higher affinity B cells, would be to use this as a strategy to selectively elicit higher affinity anti-stalk IgGs, thereby generating broader and more potent anti-HA responses. The finding also suggests an affinity requirement for protective anti-stalk IgGs that is not present for globular head-specific antibodies; higher affinity may be required of anti-stalk IgGs in order to restrict the conformation change in the HA that occurs at low pH, thus preventing fusion of the viral envelope with the host cell.

Because balanced FcR signaling is a requirement for generation of specific immune responses, strict regulation of Fc domain structure within ICs must occur (Bolland and Ravetch, 2000; Fukuyama et al., 2005). We show that this regulation occurs through synchronized modulations in determinants of Fc domain structure following exposure to antigen. The changes observed, over the weeks following vaccination, would regulate Type I and Type II FcR signaling within B cell follicles where antigen can be retained for months and even years following exposure (Nossal, 1992). PB that expand following TIV are a possible source of sFc IgG observed at day 7, while memory B cells may contribute to production of the less fucosylated, less sialylated Fc glycoforms observed at week 3. Production of less fucosylated, less sialylated glycoforms by memory B cells may be supported by the finding that these glycoforms are present with greater abundance on IgGs specific for the highly conserved stalk domain of the HA. A shift toward Fc domains with increased Type I FcR binding at week 3 was pronounced in both Fc glycoform and IgG subclass distribution; a possible function of the increased activating Type I FcR signaling may be to provide an adaptive mechanism for enhancing phagocyte activity during prolonged antigen exposure or infection.

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Further studies will be required to dissect the undoubtedly complex role of Fc glycoform modulations during evolution of humoral immune responses, including both sialylated and low fucose forms, as they are likely involved in regulation of multiple processes within the germinal center in addition to B cell activation and selection.

EXPERIMENTAL PROCEDURES

Clinical studies

The 2012–2013 TIV vaccination study was conducted at the Rockefeller University Hospital in New York City in accordance with a protocol approved by the Institutional Review Board of Rockefeller University (protocol #TWA-0804), in compliance with guidelines of the International Conference on Harmonization Good Clinical Practice guidelines, and was registered on www.clinicaltrials.gov (NCT01967238). Samples were drawn from 10 healthy adult volunteers (Figure S1). B cells analyzed for gene expression derived from six donors as part of a study approved by the Emory University institutional review board (IRB #00022916). Healthy volunteers received the 2009–2010 trivalent inactivated influenza vaccine. All samples were processed within 30 minutes of being drawn; sera and PBMCs were stored at −80°C and were thawed once prior to analysis.

Recombinant proteins and generation of immune complexes

Recombinant anti-HA mAb PY102 was expressed as a human IgG1 in 293T cells stably expressing human *ST6GAL1* and *B4GALT1* and purified using protein G chromatography as previously described (Bournazos et al., 2014). HA proteins were expressed in a baculovirus system as previously described (Pica et al., 2012). ICs were formed by incubation of molar ratio 30:1 (purified, polyclonal human IgG) or 3:1 (mAb PY102) IgG:HA trimer for 1h at 4°C. These IgG:HA ra tios were designed to generate 1:1 complexes with IgG and HA monomer; a 30:1 ratio was used for polyclonal IgG:HA based on a predicted anti-HA frequency of 1 in 10 IgGs post vaccination. IgG subclass and Fc glycan composition were determined by mass spectrometry; size of ICs was determined by size exclusion chromatography (See extended supplemental procedures for detailed protocols).

Fc glycan analysis

IgGs were isolated from serum by protein G purification. HA-specific IgGs were isolated on agarose resin (Pierce) coupled to HA protein. Total anti-H1 HA IgGs were captured on Cal/09-coupled resin while anti-H1 stalk proteins were captured using resin coupled to a chimeric hemagglutinin protein expressing the Cal/09 stalk and an H5 subtype globular head domain. IgG Fc-associated glycans were analyzed by mass spectrometry following tryptic digestion of purified IgG or on-bead IgG. Relative IgG subclass distribution was determined by mass spectrometric quantification of subclass-specific tryptic peptides. IgG4 was not in sufficient abundance to accurately quantify. See Supplemental Experimental Procedures for detailed mass spectrometric methods.

ELISAs

HA ELISAs were performed as described previously (Wang et al., 2010). Negative control (naïve mouse serum or binding values of human IgGs on the irrelevant protein bovine serum

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albumin) values were subtracted from readings given by test samples. Study subjects were determined to have positive IgG binding titers when binding was five times above background. For affinity ELISAs, sera diluted 1:200 were incubated on plates coated with 1ug/mL HA protein (low density) or 6ug/mL HA (high density). The affinity of HA-specific IgG was expressed as a ratio of binding to low density:high density HA-coated plates. This method was adapted from the well-established assay used for measurement of polyclonal anti-nitrophenyl-hapten affinity (Herzenberg et al., 1980). The 7M urea affinity ELISA was performed as previously described (Verma et al., 2012). HA1 protein (Sino Biological), chimeric cH5/1 protein (head domain derived from H5N1 strain A/Viet Nam/1203/04 and a stalk domain derived from H1N1 strain A/Puerto Rico/8/34) or Cal/09 or PR8 full-length HA proteins were used.

Hemagglutination inhibition assay

Surface plasmon resonance analysisSera were tested in a standard hemagglutination inhibition assay, as previously described (WHO, 2002).

Surface plasmon resonance analysis

The binding properties of serum antibodies were analyzed by SPR (Biacore T-200. GE Healthcare). Protein G-purified serum IgG from vaccinated mice was immobilized to the surface of a CM5 sensor chip (GE Healthcare) using amine coupling chemistry at a density of 1000 RU. Varying concentration (1 nM- 50 nM) of either PR8-HA or cH5/1 HA were injected sequentially over flowcells of the sensor chip. Samples were fit to heterogeneous ligand binding model and the off-rate constant (kd/s) was calculated.

In vitro studies

Human, $CD19^+$ PBMCs or BJAB cells were treated with IL-4 (200ng/mL) and CD40L (5ug/mL) to increase surface expression of CD23 prior to incubation with IC.

In vivo studies

All mice were maintained in a specific-pathogen–free facility at the Rockefeller University and all studies were approved by the Rockefeller University Institutional Animal Care and Use Committee.

Polyclonal IC immunization: For mouse immunizations using human serum-derived ICs, A/California/04/2009 HA protein (10ug) was delivered alone, or in complex with pooled, protein G-purified, week 3 post-vaccination IgG from vaccinated subjects. As control, asialylated IgG was prepared by treatment of IgG with α2-3,6,8 Neuraminidase (New England Biolabs) as described (Anthony et al., 2011). Mice previously administered ICs or HA in PBS were boosted, intraperitoneally, at 3 wk intervals, with 10ug HA in adjuvant (complete/incomplete Freund's or Alum).

For in vivo neutralization studies, anesthetized mice (female C57BL/6J; 6–8 wk old) were infected intranasally with 5 mLD50 of the A/Netherlands/602/09 (H1N1) or $cH5/1_{PR8}N1_{PR8}$ virus (the HA contains a head domain derived from H5N1 strain A/Viet Nam/1203/04 and a stalk domain derived from H1N1 strain A/Puerto Rico/8/34 - generated as described in (Hai

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et al., 2012)). IgGs from vaccinated mice were purified from pooled sera using protein G (GE Healthcare) and mixed with virus prior to intranasal infections. For the Netherlands/09 challenge, 10ug/ml IgG was mixed with virus, for the cH5/1 challenge, 200ug/ml IgG was used. Mouse body weight was recorded daily, and death was determined by a 20% body weight loss threshold.

Monoclonal IC immunizations: For PY102 mAb immunizations, 20ug PR8 HA and 50ug PY102 or 20ug PR8 HA alone was delivered intravenously to 6 wk old wild type or $CD23^{-/-}$ BALB/c mice. Boosts were administered as described above. For challenge studies, mice were anesthetized and infected as described above. A mixture of mouse-adapted virus (A/PR8/1934 (H1N1), A/FM/1/1947 (H1N1) or A/Netherlands/602/09 (H1N1) and purified polyclonal IgG (75 μg/mL) from vaccinated mice was pre-incubated at room temperature for 30 minutes. Six- to eight-week old female BALB/c mice were then infected with 5 mLD50 of virus. Mice were weighed daily to monitor morbidity and animals that exceeded 25% weight loss were euthanized.

Statistical analysis

All data were analyzed in Prism 6 (GraphPad). Results from multiple experiments are presented as mean ± SEM. Correlation analysis was used to determine the Pearson correlation coefficient, r. Linear regression was used to determine goodness of fit, R^2.

Two tail paired/unpaired student's t tests or ANOVA followed by Tukey posthoc analysis were performed to assess differences in the mean values of quantitative variables. Non parametric tests of significance were performed if normal distribution could not be assessed or if populations were not normally distributed.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGEMENTS

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Highlights

- Dynamic changes in Fc glycan composition following influenza vaccination.
- Sialylated Fc glycan abundance on anti-HA IgG predicts vaccine response.
- Engagement of CD23 by sialylated Fc glycans modulates B cell affinity maturation.
- IgGs elicited by sialylated Fc immune complexes protect broadly against H1 viruses.

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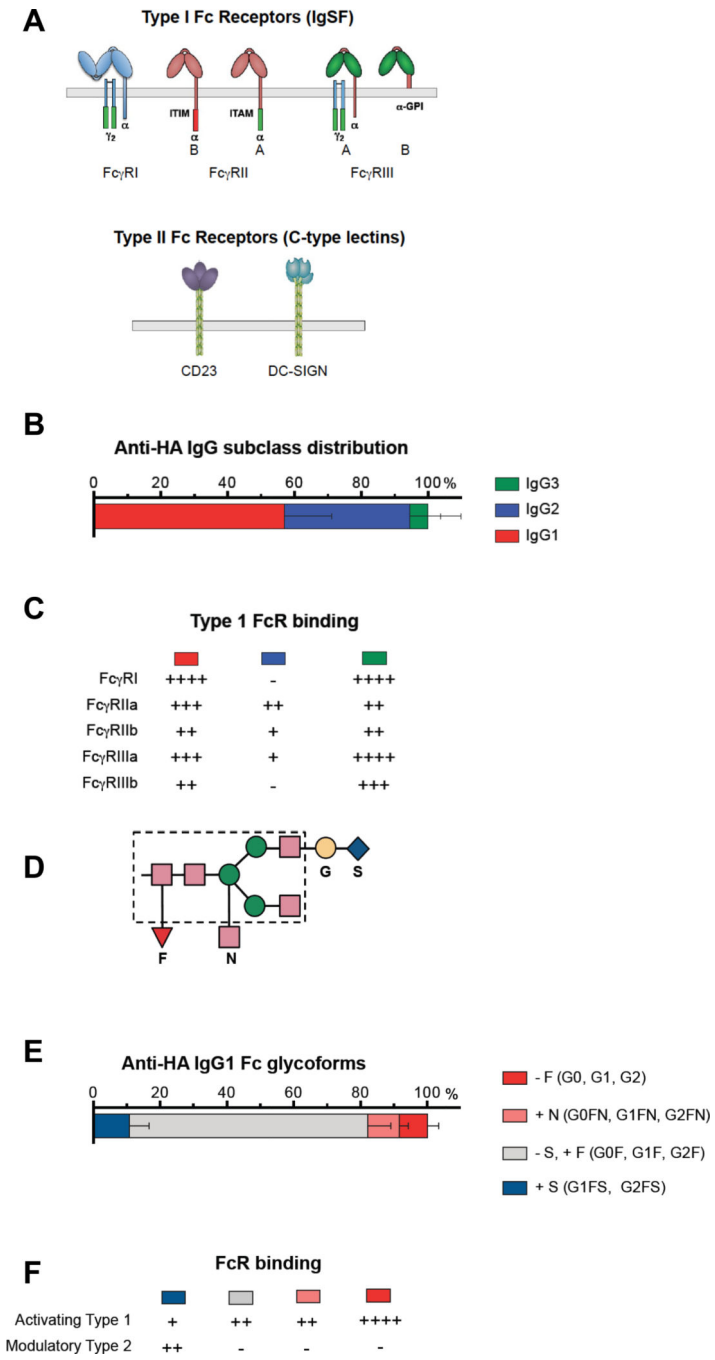

Figure 1. Type I and type II FcR binding characteristics of human anti-H1 IgG

(A) Overview of Type I and Type II FcR family (B) Subclass distribution of pre-vaccination anti-H1 HA (Cal/09) IgG from a cohort of 10 healthy adults. Mean IgG1: 56.18% (SD 14.16), IgG2: 37.64% (SD 15.14), IgG3: 5.37% (SD 3.82). IgG4 levels were below the limit of detection. (C) Type I FcR binding characteristics of IgG subclasses. (D) Schematic overview of the Fc-associated glycan structure. Composition of the core Fc glycan (boxed) can be modified by addition of fucose (F), N-acetylglucosamine (N), galactose (G) and sialic acid (S) residues. (E) Fc glycoform distribution on baseline anti-H1 HA IgG1 from our

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patient cohort and (F) binding characteristics for Type I and Type II FcRs. Fc glycovariants were categorized into: sialylated (blue; +S (G1FS, G2FS)), afucosylated (red; -F, (G0, G1, G2)) and 'neutral', defined by the presence of fucose and absence of sialic acid (with branching GlcNAc (+N, pink): G0FN,G1FN,G2FN, without branching GlcNAc (-S+F, gray) G0F,G1F,G2F). Error bars in (B) and (E) indicate standard deviation. See also Figure S1.

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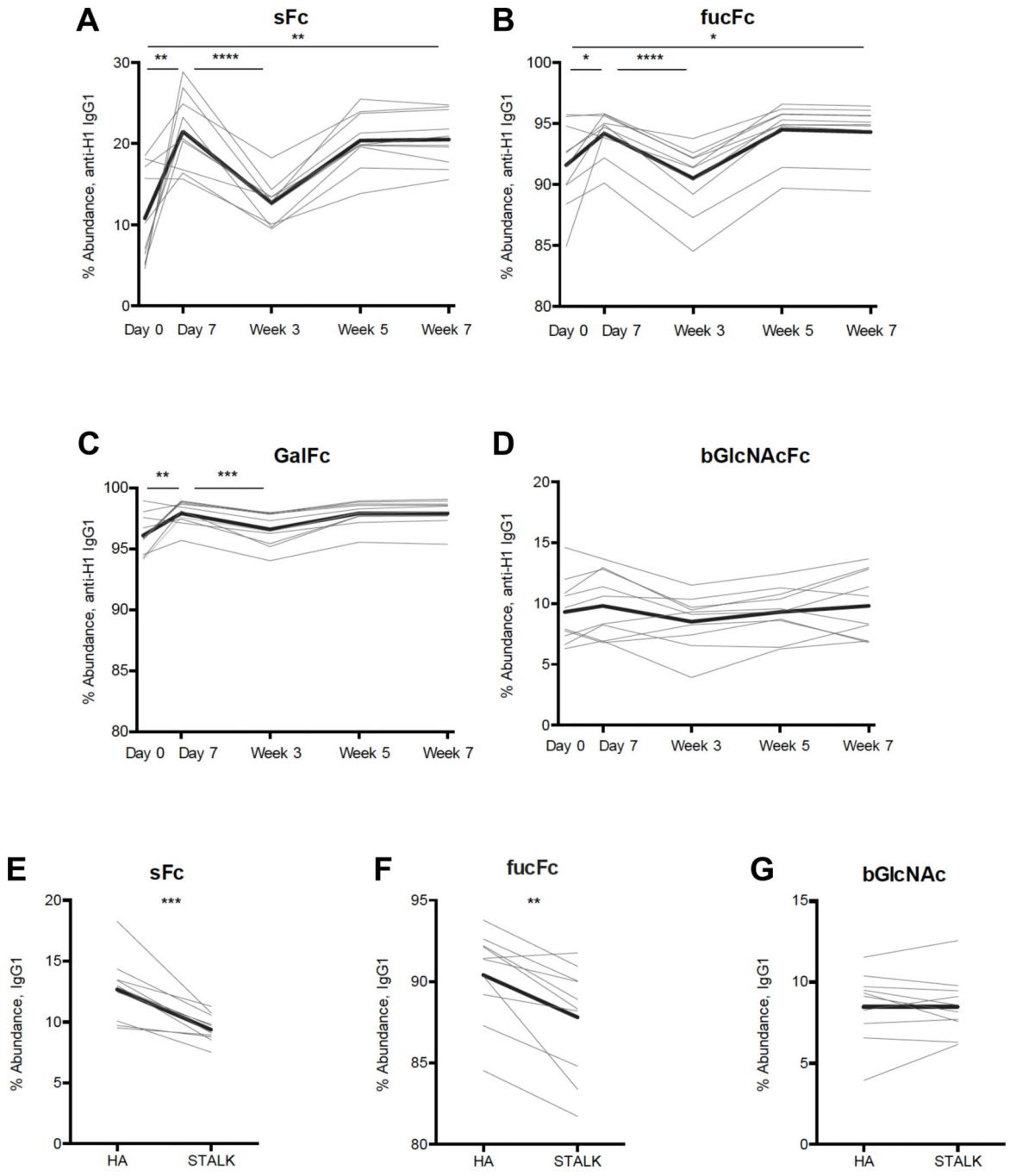

Figure 2. Regulation of Fc glycan composition following TIV vaccination

Fc glycoforms on anti-H1 HA (Cal/09) IgG1 were analyzed. (A) Sialylated (sFc) and (B) fucosylated (fucFc) glycoforms were significantly elevated on day 7 post vaccination. (C) Galactosylation (GalFc) levels were modulated to a small degree following vaccination. (D) Bisected GlcNAc (bGlcNAcFc) modifications were not regulated. (E,F) Anti-HA (predominantly globular head-specific) and anti-HA stalk IgG1 differed significantly in Fc glycoform profile at week 3, with sialylated, fucosylated glycan levels highest on anti-HA IgG1 and lowest on stalk-specific IgG1. (G) No difference in level of bisecting GlcNAc was

observed. Bold line represents group mean in each panel. *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001 determined by two-tailed, paired students t tests. See also Figure S2.

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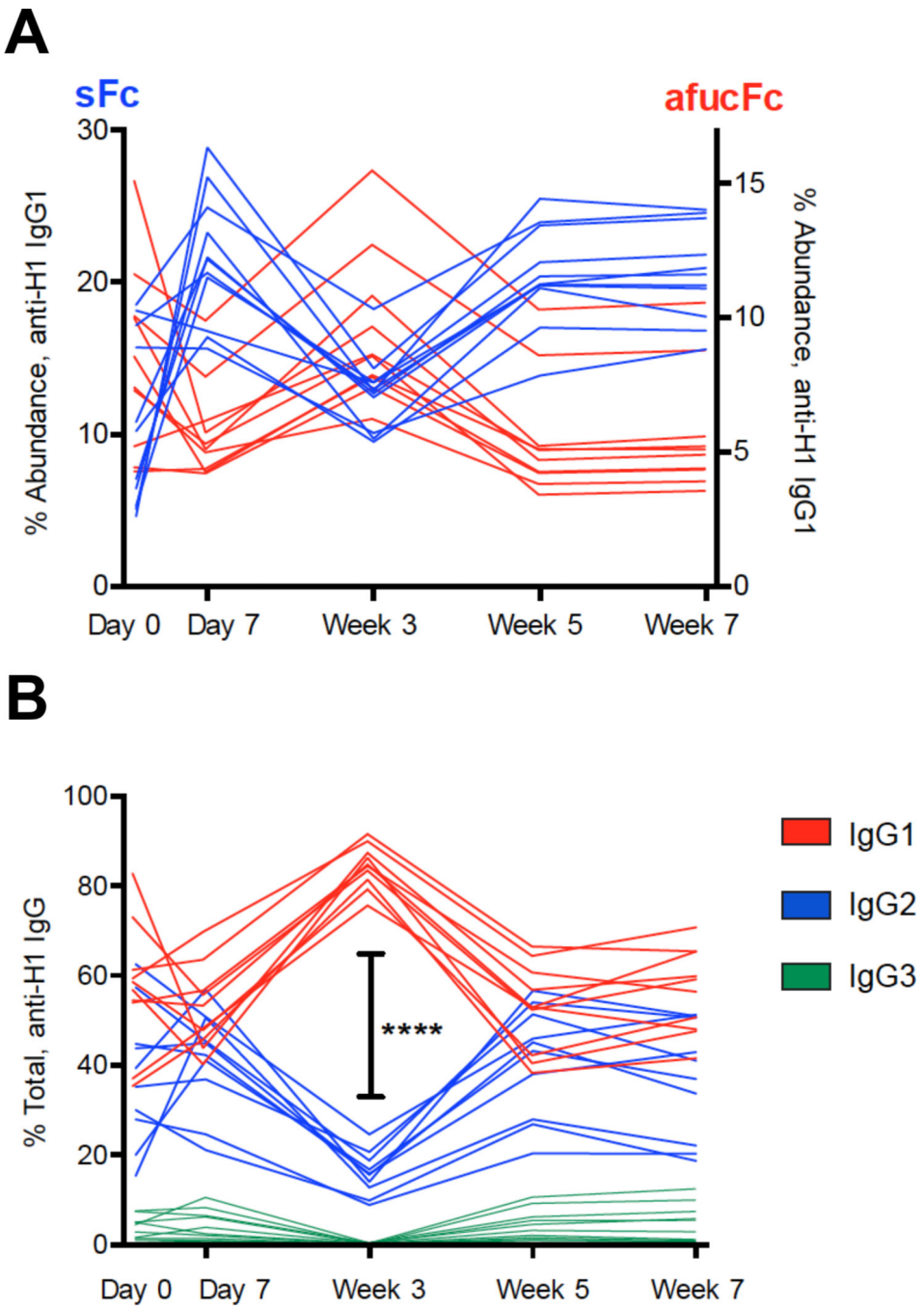

Figure 3. Modulations in Fc domain structure favor Type II FcR binding at day 7 and Type I FcR binding at week 3 post vaccination

(A) Fc glycoforms shift from Type II FcR binding (high in sFc) at day 7 to Type I FcR binding (high in afucFc content) by week 3. (B) The activating FcR binding glycoform profile at week 3 was mirrored by a peak in IgG1 subclass. IgG4 was not in sufficient abundance to accurately quantify. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$ determined by the Tukey post-hoc test. See also Figure S3.

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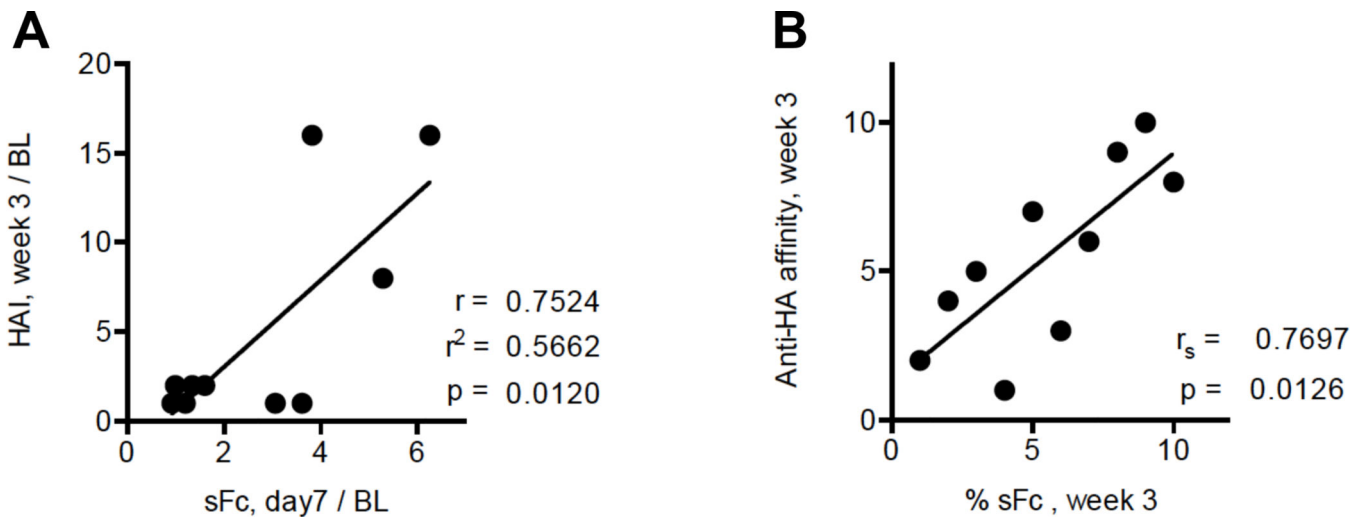

Figure 4. Sialylated Fc abundance predicts influenza virus vaccine efficacy

(A) Fold change in sFc on anti-HA IgG1 from baseline to day 7 post vaccination (sFc, day7/BL) predicted fold change in HAI titer from baseline to week 3 (HAI, week 3). (B) sFc abundance at week 3 post vaccination (% sFc, week 3) predicted affinity of anti-HA IgG (relative units) at week 3 (anti-HA affinity, week 3). Correlation analysis was used to determine the Pearson correlation coefficient (r) (following confirmation of normal distribution) and Spearman's rank correlation coefficient (r_s), linear regression was used to determine goodness of fit (r^2).

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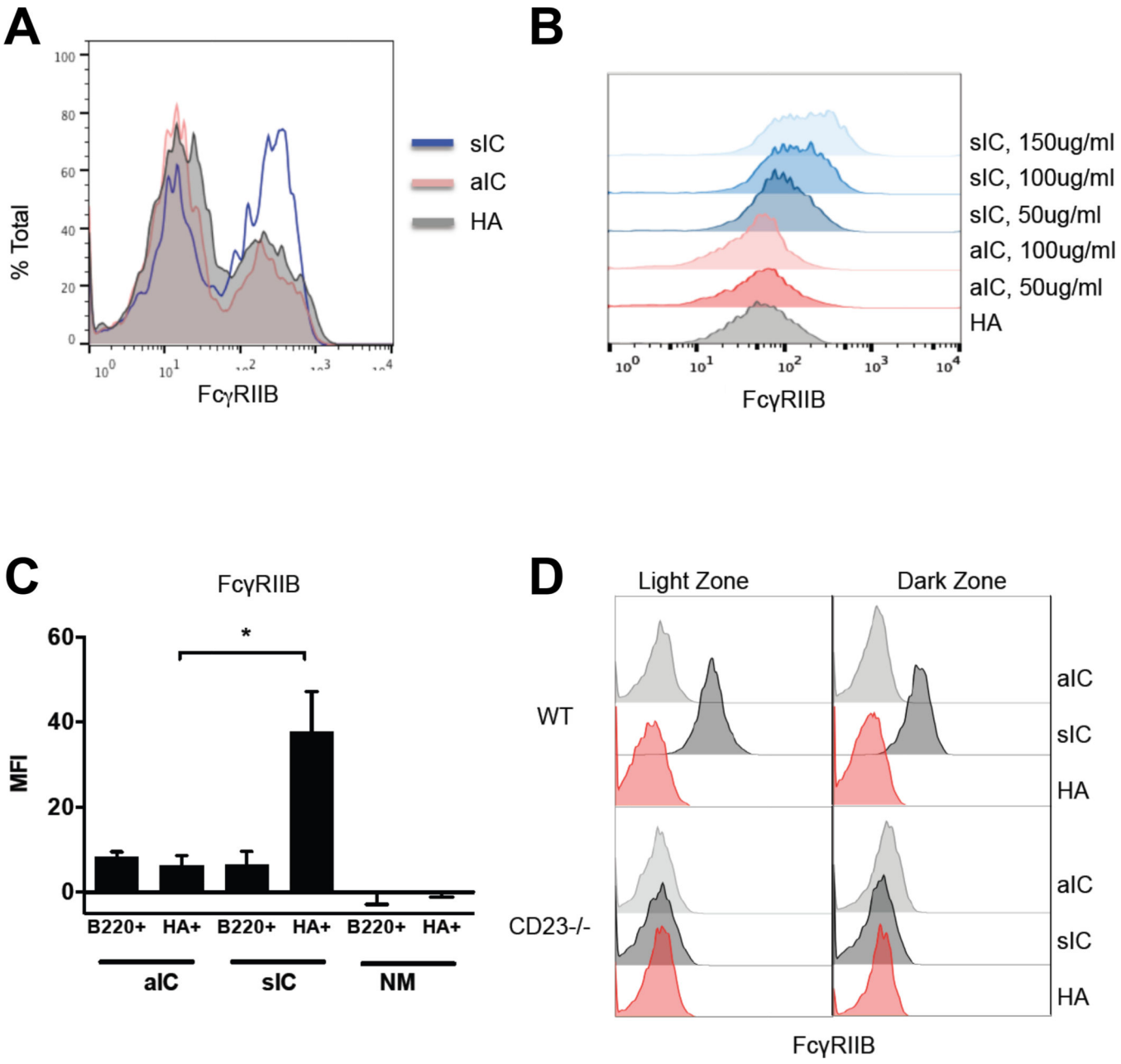

Figure 5. sFc in ICs trigger upregulation of FcγRIIb on B cells

(A) sIC or aIC were generated from pooled, post-vaccination human IgG and Cal/09 HA protein. Human, $CD19^{+}$ PBMCs were incubated with sIC, aIC or HA protein alone. FACS analysis following incubation revealed an increased number of cells expressing FcγRIIB with sIC but not with aIC or HA incubation. (B) sIC or aIC were generated from PY102 and PR8 HA protein. BJAB B cells were incubated with sIC, aIC or HA at the indicated IC concentrations. Incubation with sIC but not aIC or HA alone increased FcγRIIB expression on B cells. (C) Antigen-specific (HA-specific) peripheral B cells from mice primed with sIC showed increased FcγRIIB, whereas mice primed with aICs or naïve mice (NM) showed no elevation in peripheral B cell FcγRIIB expression. 5 mice per group. Data are represented as

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mean +/− SD D) Splenic B cells in the light and dark zones of the germinal center from mice primed with sIC, but not aIC or mock showed increased expression of FcγRIIB. Increased FcγRIIB expression was not present in CD23 knockout mice (CD23−/−) primed with sIC. All panels are representative of experiments performed in duplicate or triplicate. See also Figure S4.

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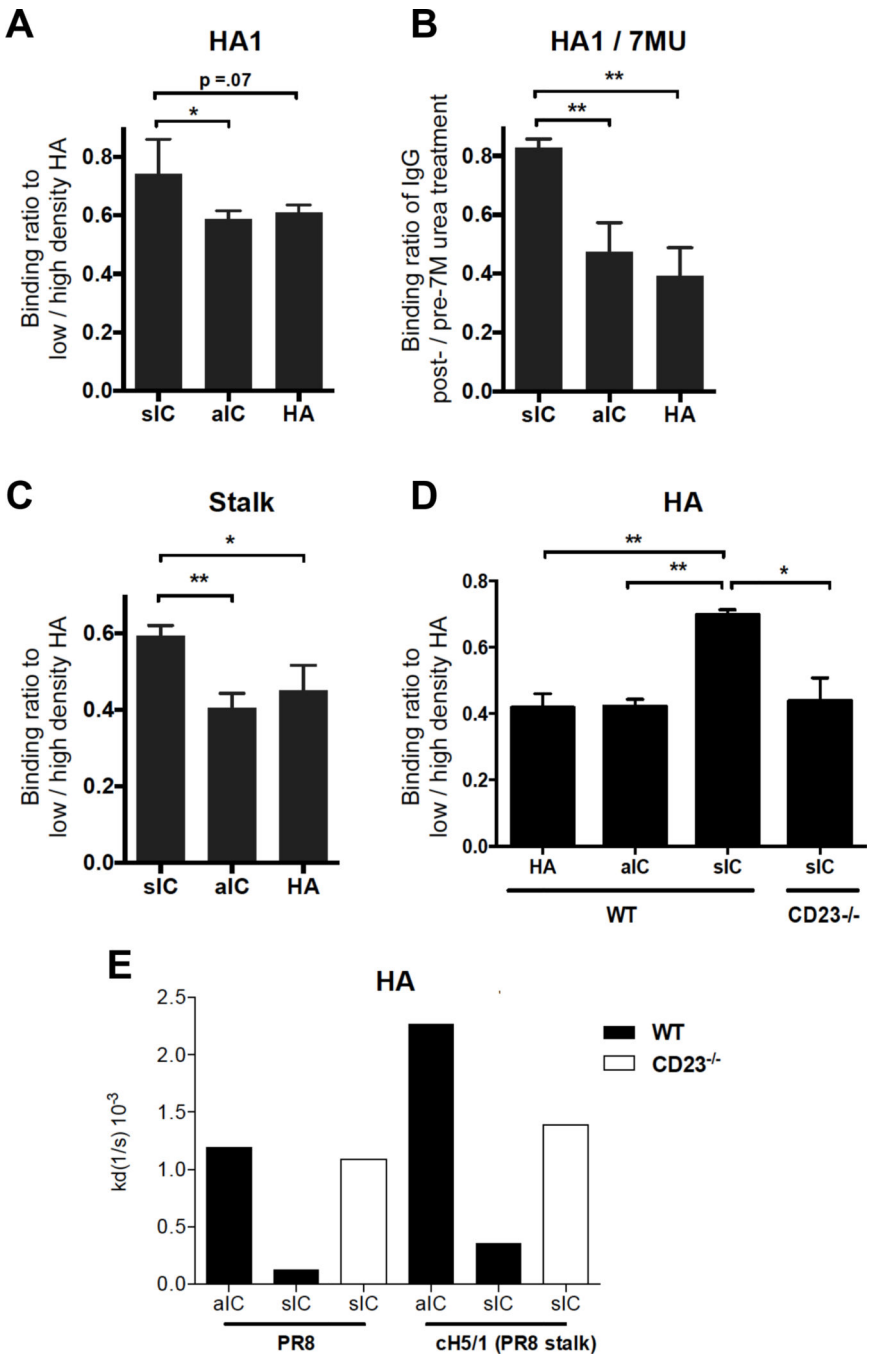

Figure 6. sFc in ICs elicit higher affinity IgG

(A,B) IgGs elicited by sIC had significantly higher affinity for the globular head, (C) the stalk domain of the HA protein, (D) and the wild-type Cal/09 H1 subtype HA protein when compared with the affinities of IgG elicited by aIC or HA alone. Only wild-type mice and not CD23 deficient mice generated higher affinity, anti-HA IgGs in response to an immunization protocol with sIC priming. Affinity measurement in (B) was determined by 7M urea ELISA, expressed as IgG bound to HA following 7M urea treatment/IgG bound without 7M urea treatment. Data are represented as mean +/− SEM. (E) SPR analysis of off-

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rate constant of polyclonal IgG elicited by mAb PY102-HA ICs in wild-type or CD23 −/− mice. sIC priming protocol in wild-type mice elicited approximately 10–20 fold higher affinity IgGs over aIC priming or sIC priming in CD23 −/− mice. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$ determined by two-tailed student's t test. See also Figure S4.

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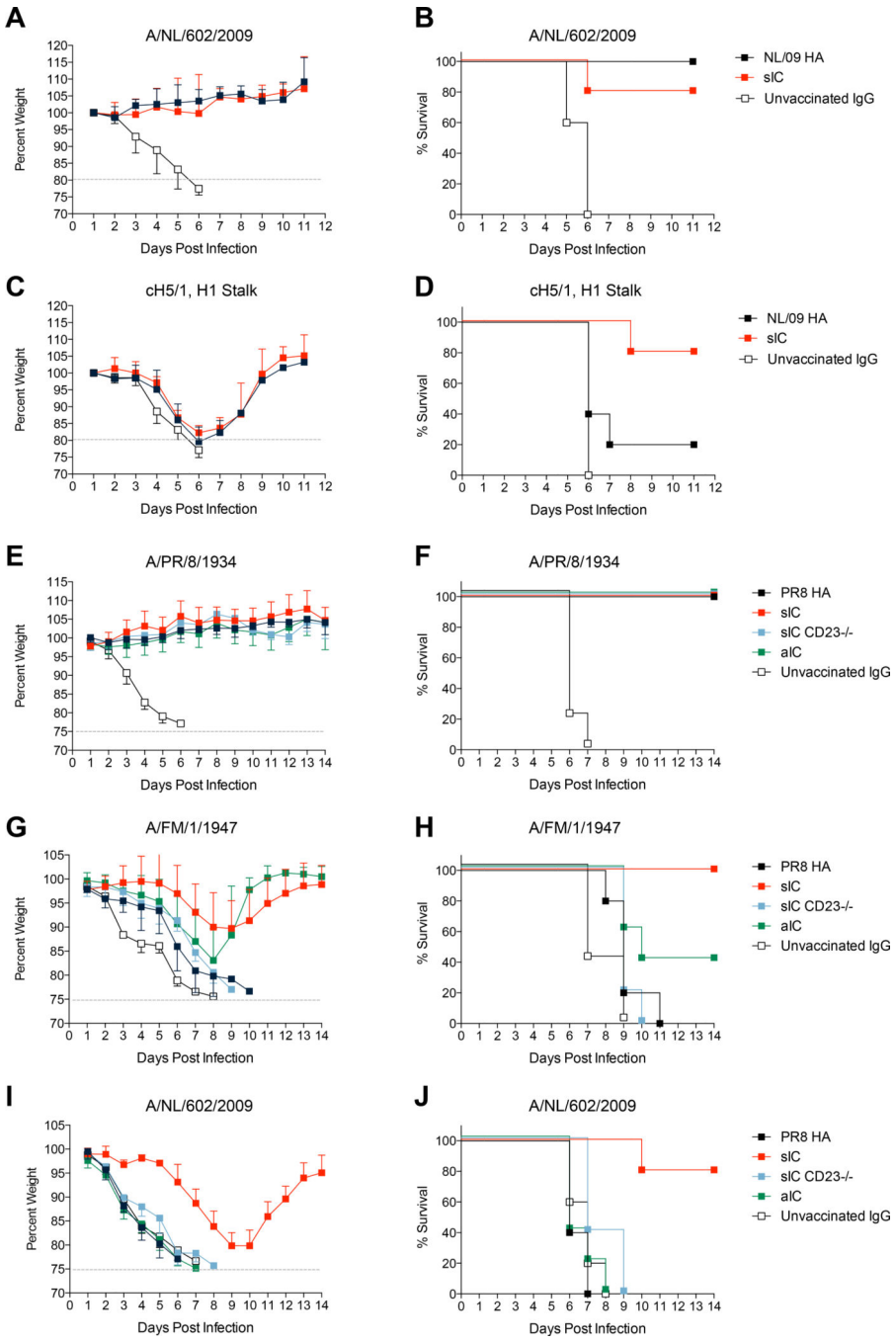

Figure 7. Immunization with sIC elicits IgGs with greater breadth of protective potency against distinct H1 influenza viruses

(A,B) Purified IgGs were pooled from mice primed with sIC (Cal/09 HA) or Cal/09 HA alone followed by two boost immunizations of Cal/09 HA. IgG from either pool conferred equivalent protection against A/Netherlands/602/2009 (H1N1). (C,D) In contrast, only IgGs elicited by priming with sICs conferred anti-stalk-mediated protection against the chimeric cH5/1 virus that expresses an HA with an H1 stalk domain and an H5 subtype globular head. (E,F) Purified IgGs were pooled from mice primed with monoclonal PY102-PR8 HA ICs or PR8 HA alone. ICs used were: sIC in wild-type mice, sIC in CD23−/− mice or aIC in wild-

type mice. IgG from all pools conferred equivalent protection against A/PR8/1934 virus (H1N1). (G,H) In contrast, only IgG elicited by sIC protected mice from challenge with A/FM/1/1947 (H1N1) virus or (I,J) A/Netherlands/602/2009 virus. The number of animals used was 5–10 per group in each experiment. See also Figure S5.

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