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Research Article

Fc-Fusion Drugs Have FcγR/C1q Binding and Signaling Properties That May Affect Their Immunogenicity

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Abstract. Fusing the human immunoglobulin G1 (IgG1) constant region (Fc-domain) to therapeutic proteins or peptides increases their circulating plasma half-life via neonatal Fc receptor (FcRn) binding and recycling. However, Fc-mediated interactions with other molecules including complement C1q and Fc gamma receptors (FcγRs) can have immunological consequences and the potential to modulate the immunogenicity of Fc-fusion therapeutics. In a comparative study, we carried out a comprehensive assessment of Fc-mediated interactions for five FDA-approved Fc-fusion therapeutics. C1q binding and complement activation were measured by ELISA, while FcγR binding and signaling were evaluated using BW5147:FcγR-ζ reporter cell lines. We demonstrate that FIX-Fc and FVIII-Fc bound C1q as well as activating and inhibitory FcγRs (I, IIA, IIB, IIIA). These coagulation factor Fc-fusions also signaled via FcγRIIIA, and to a lesser extent via FcγRI and FcγRIIB. TNFR-Fc and CTLA4-Fc bound FcγRI, while TNFR-Fc also bound FcγRIIIA, but these interactions did not result in FcγR signaling. Our comprehensive assessment demonstrates that (i) different Fc-fusion drugs have distinct C1q/FcγR binding and signaling properties, (ii) FcγR binding does not predict signaling, and (iii) the fusion partner (effector molecule) can influence Fc-mediated interactions.

KEYWORDS: Fc-fusion; Immunogenicity; Fc gamma receptors; Complement C1q.

INTRODUCTION

Fc-fusion is a protein engineering platform technology that is primarily used to increase the circulating half-life of protein and peptide therapeutics, where a biologically active effector molecule is genetically fused to a human immunoglobulin (IgG) heavy-chain fragment crystallizable (Fc) domain via the hinge region [1–5]. This bioengineering strategy results in an active protein drug with an extended therapeutic window, thus requiring less-frequent administrations. The enhanced pharmacokinetic profile is of particular importance to patients undergoing prophylactic replacement therapy, where frequent dosing regimens can negatively impact patient well-being in terms of ease of administration and compliance, especially in young children [6]. As a group, Fc-fusion proteins are one of the most successful therapeutics, whether measured in terms of the number of drugs approved, application to diverse disease areas, or the value of global sales [2]. Currently, there are 13 FDA-approved Fc-fusion drugs (including one biosimilar) used to treat a wide variety of clinical indications including cancer, transplant rejection, inflammatory diseases, and monogenetic disorders.

The increased terminal half-life of Fc-fusion proteins is achieved via binding to the membrane-bound neonatal Fc receptor, or FcRn [2]. FcRn is expressed broadly in different cell types including vascular endothelial cells, mucosal epithelial cells, immune cells, hepatocytes, and syncytiotrophoblasts [7, 8]. Following fluid-phase micropinocytosis, binding of Fc to the FcRn receptor occurs within acidified early endocytic vesicles and shuttles the antibody (or fusion protein) into a naturally occurring recycling pathway, avoiding lysosomal degradation and catabolism. This phenomenon is the mechanistic basis for the 3-week serum half-life of IgG1 [7].

A key biological property of the Fc region is binding and signaling via Fc gamma receptors (FcγRs). These receptors are expressed on the surface of many immune cell populations, particularly antigen-presenting cells (APCs) such as monocytes, macrophages, dendritic cells, and B cells [9]. FcγRs mediate APC interactions with IgG-opsonized

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particles resulting in activating or inhibitory immune signals. Furthermore, FcγR can also be expressed on the surface of granulocyte populations including neutrophils, basophils, eosinophils, and mast cells as well as on subpopulations of natural killer cell lineage lymphocytes. Fc-FcγR interactions can activate or inhibit diverse immune cell populations which leads to immune consequences. The Fc-FcγR interactions thus have the potential to affect the immunogenicity of Fc-fusion therapeutics. The influence of Fc-FcγR interactions on Fc-fusion immunogenicity may be most apparent in the potential to affect antigen uptake, processing, and presentation by dendritic cells. Another important biological interaction of the Fc region is engagement with the complement component C1q which triggers the classical activation pathway. Via C1q, the soluble complement system interacts with Fc regions through immune complexes leading to inflammation, phagocyte recruitment, clearance of opsonized pathogens and immune complexes via phagocytosis, and lysis of cells and pathogens [10]. Consequently, Fc-C1q interactions also have the potential to affect the immunogenicity of Fc-fusion therapeutics.

In this study, we tested the premise that therapeutic Fc-fusion proteins have the potential for Fc-mediated interactions with FcγR and C1q that may modulate immunogenicity [11]. We conducted a comparative study of FDA-approved Fc-fusion proteins for an assessment of Fc-FcγR and Fc-C1q interactions. We compiled a panel of five therapeutic Fc-fusions covering a variety of effector molecule fusion partners, clinical indications, cellular expression systems, and manufacturers. We also included three therapeutic monoclonal antibodies as human IgG1 controls. To our knowledge, these cross-product comparisons have not been made, as the preclinical characterization of each of these approved drugs was performed individually by the drug manufacturer. Our aim is to develop tools to evaluate Fc-mediated interactions which will allow for better assessment of Fc-fusion proteins during drug development.

METHODS AND MATERIALS

Drug Panel and Controls

Therapeutic Fc-fusions (TNFR-Fc [etanercept]; CTLA4-Fc [abatacept; belatacept]) and monoclonal antibodies (anti-TNFα [adalimumab] and anti-CD20 [rituximab; obinutuzumab]) were acquired from the NIH Pharmacy through an Inter-Agency Agreement. Coagulation factor Fc-fusions (FIX-Fc [efmoroctocog alfa] and FVIII-Fc [eftrenonacog alfa]) were acquired from Biogen through a Material Transfer Agreement. Polyclonal human IgG was affinity-purified from pooled human serum (Sigma #H3667) using a GE HiTrap MabSelect SuRe column on a GE ÄKTAprime plus chromatography system. Forced heat-aggregation of samples and standards was achieved by incubation at 63 °C for 30 min, followed by immediate incubation on ice.

C1q ELISA

(i) Human C1q (Sigma C1740) was immobilized on F8 Maxisorp Nunc-immuno module strips (ThermoFisher 469949) at 10 μg/mL in PBS. Samples were diluted with borate buffer and detected with goat anti-human IgG-HRP (Abcam 97175) (1:5000; 100 ng/mL). TMB was used as the peroxidase substrate, and absorbance was read at 450 nm using a Biotek ELx808 plate reader. (ii) Fc-containing drugs were immobilized on F8 Maxisorp Nunc-immuno module strips (ThermoFisher 469949) at a concentration range from 1 μM to 61 pM in PBS. Human C1q (Sigma C1740; 1 μg/mL) was diluted with borate buffer and detected with goat anti-hC1q IgG (Quidel #A301; 1:1000) and rabbit anti-goat IgG-HRP (KPL# 14-13-06; 1:5000). TMB was used as the peroxidase substrate, and absorbance was read at 450 nm using a Biotek ELx808 plate reader.

Complement Activation Assay

Samples [therapeutic proteins: monomeric or heat-aggregated; heat-aggregated IgG (HAGG) (Quidel; cat# A114] (1 μM) were incubated in normal (Quidel; cat# A113) or C1q-depleted human plasma (Quidel; cat# A509) for 30 min at 37 °C. Following incubation, complement activation was quenched with 20 mM ice-cold EDTA. Complement C4d was detected in samples using the Microvue C4d Fragment EIA Kit following the manufacturer's instructions (Quidel; cat# A008).

FcγR Reporter Cell Assays

FcγR reporter cells [mouse BW5147 thymoma cells (ATCC TIB-47™) stably transfected to express an individual human FcγR on the cell surface with the cytosolic signaling domain replaced with the murine CD3ζ chain, so that FcγR engagement and signaling results in murine IL-2 secretion] (provided by Dr. Hartmut Hengel) [12] were used for FcγR binding and signaling assays. For binding assays, following a 90-min incubation with the BW5147:FcγR-ζ reporter cells on ice, cell-surface bound therapeutic proteins were measured by flow cytometry using an universal detection reagent [goat anti-human IgG Fc-PE; eBioscience cat# 12-4998-82]. For signaling assays, following an overnight incubation (~ 16–18 h) with the BW5147:FcγR-ζ reporter cells at 37 °C, cell culture supernatants were collected and secreted murine IL-2 was measured by ELISA as described in [12].

RESULTS

Differences in Fc Domain Amino Acid Sequence

Primary amino acid sequences for all therapeutic Fc-fusion proteins in the drug panel (Table I) were obtained from publicly available published sources [TNFR-Fc [13]; CTLA4-Fc [14]; FIX-Fc [15]; FVIII-Fc [16, 17]]. The Fc domain amino acid sequences for all therapeutic Fc-fusion proteins in the drug panel were aligned to the consensus human IgG1 Fc [IGHG1_HUMAN; accession number #P01857; EU numbering 216–447]. In all cases, the engineered Fc domain sequences deviate from the consensus amino acid sequence (Fig. 1). The engineered Fc sequence deviations can be broadly characterized as hinge region truncation (FVIII-Fc and FIX-Fc), disulfide bond removal (cysteine to serine substitution—CTLA4-Fc), or exchange of

Table I. Panel of Fc-containing therapeutic proteins

Class	No.	Description	Drug name	Proprietary name	Apparent molecular weight (kDa)	Mammalian expression system
Fc-fusion	1	TNFR-Fc	Etanercept	Enbrel®	150	CHO
	2	CTLA4-Fc 1	Abatacept	Orencia®	92	CHO
	3	CTLA4-Fc 2	Belatacept	Nulojix®	90	CHO
	4	FIX-Fc	Coagulation factor IX (recombinant), Fc-fusion protein	Alprolix®	98	HEK
	5	FVIII-Fc	Antihemophilic factor (recombinant), Fc-fusion protein	Eloctate®	220	HEK
mAb	6	Anti-TNFα	Adalimumab	Humira®	148	CHO
	7	Anti-CD20 1	Rituximab	Rituxan®	145	CHO
	8	Anti-CD20 2	Obinutuzumab	Gazyva®	150	CHO

CHO Chinese hamster ovary, *CTLA* cytotoxic T lymphocyte-associated antigen, *FIX* factor IX, *FVIII* factor VIII, *HEK* human embryonic kidney, *mAb* monoclonal antibody, *TNFα* tumor necrosis factor-alpha, *TNFR* tumor necrosis factor receptor

acidic (D → E—TNFR-Fc) and hydrophobic (L → M—TNFR-Fc) amino acid residues (Fig. 1b).

Fc-C1q Interactions Differ Between Therapeutic Fc-Fusion Proteins

To evaluate Fc-C1q interactions, we used two experimental setups. In the first setup, human C1q was immobilized on a plate surface and allowed to bind to the therapeutic protein (either in monomeric or forced aggregation forms). The coagulation factor Fc-fusion proteins (FIX-Fc and FVIII-Fc) bound immobilized C1q in their monomeric (soluble) as well as forced aggregation forms (Fig. 2a). Only force-aggregated forms of TNFR-Fc, anti-TNFα, and anti-CD20 1 bound immobilized C1q. However, CTLA4-Fc 1, CTLA4-Fc 2, and anti-CD20 2 did not bind C1q in either form. The observation that anti-CD20 1 (rituximab) bound C1q following forced aggregation while the glyco-engineered anti-CD20 2 (obinutuzumab) did not is consistent with some clinical findings (see "Discussion").

In the alternate experimental setup, the Fc-containing drugs were immobilized to the plate surface and then incubated with soluble human C1q. The C1q binding exhibited by the drugs (Fig. 2b) was consistent with the profile of binding to C1q following forced aggregation observed in the first experimental setup (Fig. 2a). TNFR-Fc, FIX-Fc, and FVIII-Fc bound C1q, while the CTLA4-Fc drugs did not (Fig. 2b). As for the therapeutic human IgG1 monoclonal antibodies, anti-CD20 1 bound C1q robustly, anti-CD20 2 bound C1q to a lesser extent while anti-TNFα did not show any binding to C1q (Fig. 2b). The differences in C1q binding observed between the human IgG1 Fc-containing therapeutics cannot be attributed to differential immobilization, as equivalent drug levels were verified using a Fc-specific universal detection reagent (data not shown).

To assess activation of the complement system's classical pathway by the drugs in the panel, complement C4d levels were measured in normal human plasma following incubation with each drug in both monomeric and forced aggregated forms. C1q binding was not sufficient for complement activation, as no Fc-fusion or monoclonal antibody tested activated the classical pathway in its monomeric form (Fig. 2c, left panel). This is the expected outcome for FDA-approved therapeutics which have been shown to be safe in clinical studies. However, it is notable that upon forced aggregation, FIX-Fc and anti-CD20 1 activated the classical pathway in a C1q-dependent manner (Fig. 2c, right panel). Aggregates can form during manufacturing and immune complexes may form if anti-drug antibodies (ADAs) are present. Formation of the latter in vivo could result in complement activation and could lead to clinical symptoms and adverse events such as hypersensitivity reactions due to systemic proinflammatory responses (see "Discussion").

Fc-FcγR Interactions Differ Between Therapeutic Fc-Fusion Proteins

A panel of transgenic reporter cell lines (BW5147:FcγR-ζ) [12] each engineered to express an individual human FcγR [see "Methods and Materials"] on the cell surface were used to evaluate Fc-FcγR interactions in the drug panel. The FcγR binding and signaling were measured for each drug and reporter cell line combination. Cell-surface bound drugs were measured using a Fc-specific universal detection reagent in a flow cytometry-based FcγR binding assay. All therapeutic proteins tested were observed to bind to FcγRI (the high affinity receptor), however, product-specific differences were observed for the FcγRIIA, FcγRIIB, and FcγRIIIA binding profiles (Fig. 3). The fold change in surface BW5147:FcγR-ζ mean fluorescence intensity (MFI) over the parental BW5147 (control cell line not expressing FcγR) for each drug-receptor pair are provided in Table II. FIX-Fc was observed to bind to both activating (FcγRI, FcγRIIA, FcγRIIIA) and inhibitory (FcγRIIB) FcγRs. TNFR-Fc bound FcγRI and FcγRIIIA. Although FVIII-Fc exhibited nonspecific binding to the BW5147 parental cell line (no FcγR expression), a 1.8–9.3 MFI fold change was observed when FcγRI, FcγRIIA, and

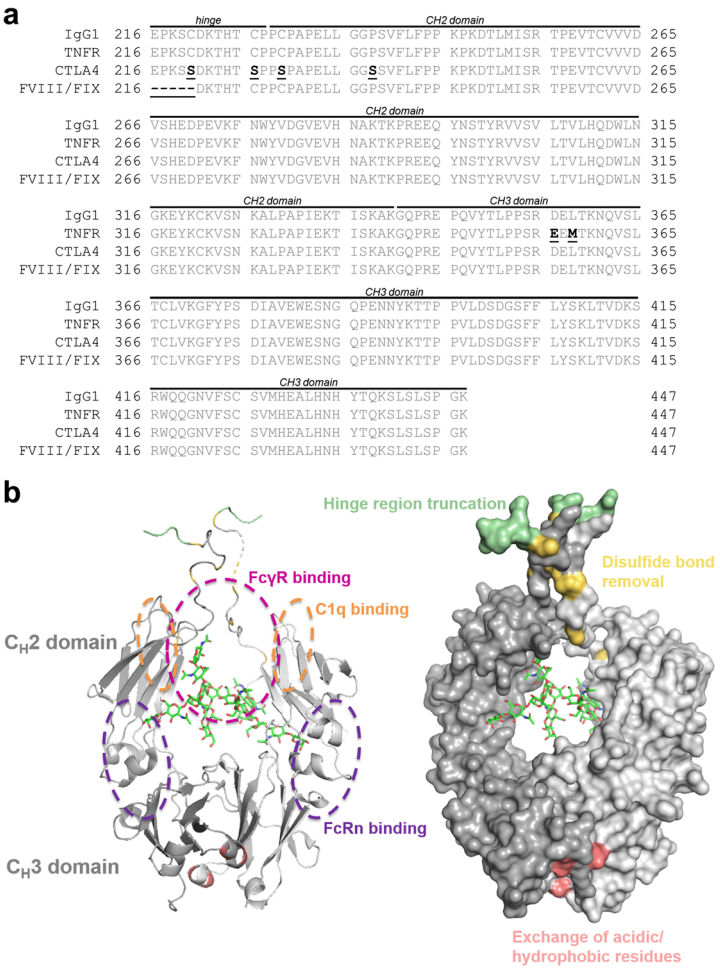

Fig. 1. Therapeutic Fc-fusion protein Fc domain amino acid sequence alignment. **a** Publicly available (nonproprietary) primary amino acid sequences for all therapeutic Fc-fusion proteins in our drug panel were compiled from published journal articles and patent applications [[TNFR-Fc [13]; CTLA4-Fc [14]; FIX-Fc [15]; FVIII-Fc [16, 17]]. The Fc domain amino acid sequences were aligned to the consensus human IgG1 Fc sequence [IGHG1_HUMAN; accession number #P01857; EU numbering 216–447]. Structural domains including the hinge (EU residues 216–227), constant heavy-chain region 2 (CH2; EU residues 228–340) and constant heavy-chain region 3 (EU residues 341–447) are illustrated. Product-specific deviations from the human IgG1 consensus sequence are highlighted in bold and underlined. **b** Left panel, structure of human IgG1 Fc domain is shown (PDB: 1HZH). Individual IgG1 heavy-chain monomers (EU residues 216–447) are colored light gray or dark gray. Regions of the Fc structure that interface with FcγR (magenta), C1q (orange), and FcRn (violet) are highlighted. Right panel, examples of amino acid residue deviations engineered in Fc-fusion panel are highlighted on the human IgG1 Fc domain structure [hinge region truncation (FVIII-Fc and FIX-Fc) [light green]; disulfide bond removal (cysteine to serine substitution—CTLA4-Fc) [light yellow]; or exchange of acidic (D → E—TNFR-Fc) and hydrophobic (L → M—TNFR-Fc) amino acid residues [light red]]. Cartoon and surface structures were generated in PyMol

FcγRIIIA expressing cells were used; indicative of specific binding to these receptors (Fig. 3, Table II). As a result of protein engineering, the CTLA4-Fc therapeutics only bound the high affinity receptor, FcγRI (see "Discussion"). FcγR binding was also evaluated for the control human IgG1 monoclonal antibodies in the panel. The anti-TNFα monoclonal antibody (adalimumab) was observed to bind to FcγRI and FcγRIIIA, while the anti-CD20 therapeutic monoclonal antibodies tested (rituximab, obinutuzumab) also bound FcγRI and FcγRIIIA, and to a lesser extent FcγRIIA (Fig. 3, Table II).

Differential FcγR binding profiles were observed for the Fc-fusion therapeutics and monoclonal antibody therapeutics included in the panel. However, FcγR cross-linking is required for these binding interactions to result in FcγR signaling [9]. We therefore tested FcγR-mediated signaling

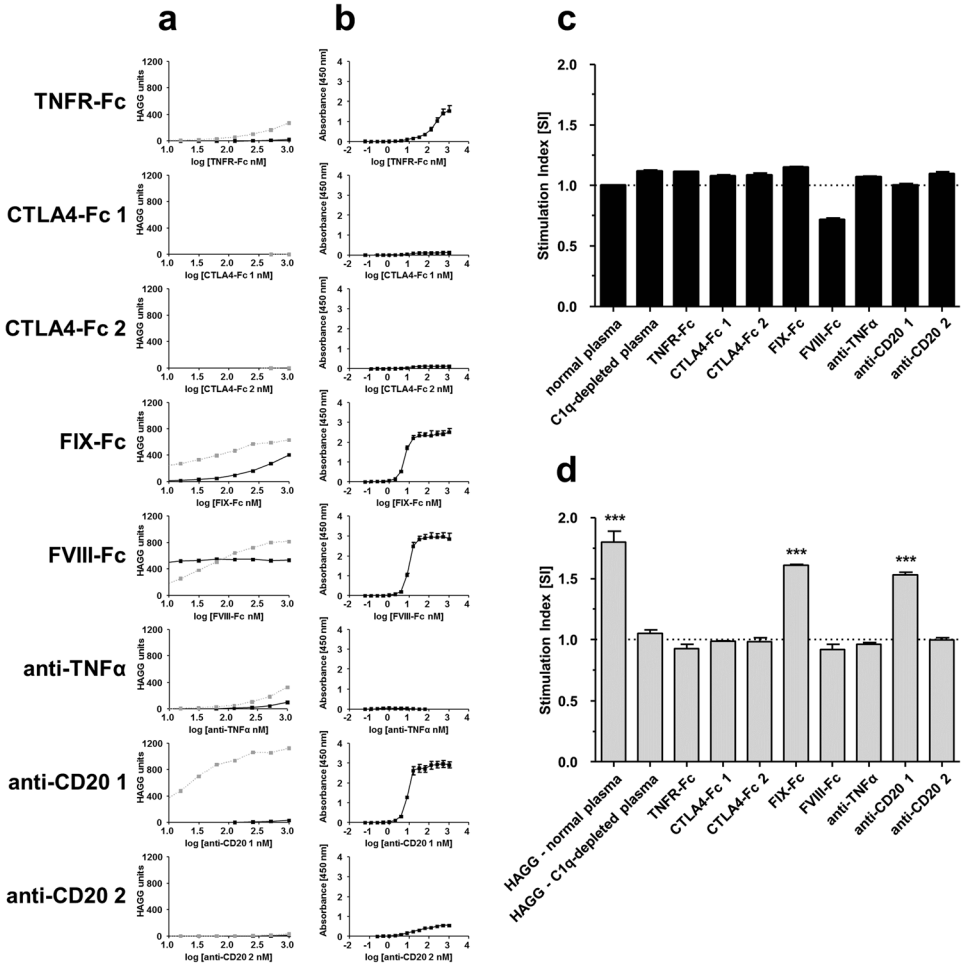

Fig. 2. Therapeutic Fc-fusion and monoclonal antibody C1q binding and classical complement activation. **a** Fc-C1q binding measured by ELISA with human C1q immobilized on the plate surface. Therapeutic proteins were tested in monomeric (black) or forced aggregation (gray) forms. Data from a representative experiment are presented. **b** Fc-C1q binding measured by ELISA with therapeutic proteins immobilized on the plate surface. Mean ± standard error (SEM) from 3 independent experiments is shown. **c** Activation of classical complement cascade was assessed by measuring C4d levels in human plasma following incubation with heat aggregated gamma globulin (HAGG) and monomeric (left) or force aggregated (right) therapeutic proteins. **d** Stimulation index is the ratio of C4d levels detected in normal plasma ± monomeric therapeutic (left) or the ratio of C4d levels detected in plasma containing monomeric therapeutic and plasma containing force aggregated therapeutic (right). Mean ± standard error (SEM) is presented. Student's *t* test compares the mean stimulation index to normal human plasma C4d level; ***P value < 0.001

using the BW5147:FcγR-ζ reporter cell lines described in the "Methods and Materials" section. While the FcγR binding data illustrated that all tested therapeutics bound FcγRI (Fig. 3, Table II), only FIX-Fc and FVIII-Fc resulted in a signaling response (Fig. 4a). Moreover, the coagulation factor Fc-fusions (FIX-Fc and FVIII-Fc) generated signaling responses through both activating (FcγRI, FcγRIIIA) and inhibitory (FcγRIIB) FcγRs. As expected, obinutuzumab, the type II anti-CD20 monoclonal antibody glyco-engineered for enhanced ADCC via FcγRIIIA engagement [18] signaled via FcγRIIIA (Fig. 4a). All other drugs tested did not stimulate a signaling response from the BW5147:FcγR-ζ reporter cell lines under these conditions (Fig. 4a). Since FcγRIIIA is expressed on NK cells, these cells may be activated by these products in vivo.

The protein drugs, when preimmobilized to a plate surface, mimick protein aggregates or immune complexes. When we evaluated FcγR-mediated signaling under this condition, we observed substantively different FcγR signaling profiles (Fig. 4b). For instance, TNFR-Fc, which did not signal via FcγR when exposed to the reporter cells in solution (Fig. 4a) was found to signal through both activating and inhibitory FcγR when plate-immobilized (Fig. 4b). However, FcγR signaling was not universal, as the CTLA4-Fc therapeutics did not signal via FcγR when plate-immobilized. Upon plate-immobilization, we demonstrate signaling mediated by TNFR-Fc, FIX-Fc, and FVIII-Fc in cells expressing FcγRI, FcγRIIA, FcγRIIB, and FcγRIIIA; albeit to differing degrees (Fig. 4b). In several cases (TNFR-Fc: FcγRI, FcγRIIIA; FIX-Fc/FVIII-Fc: FcγRI, FcγRIIA, FcγRIIB, FcγRIIIA), the FcγR signaling profiles were dose-independent

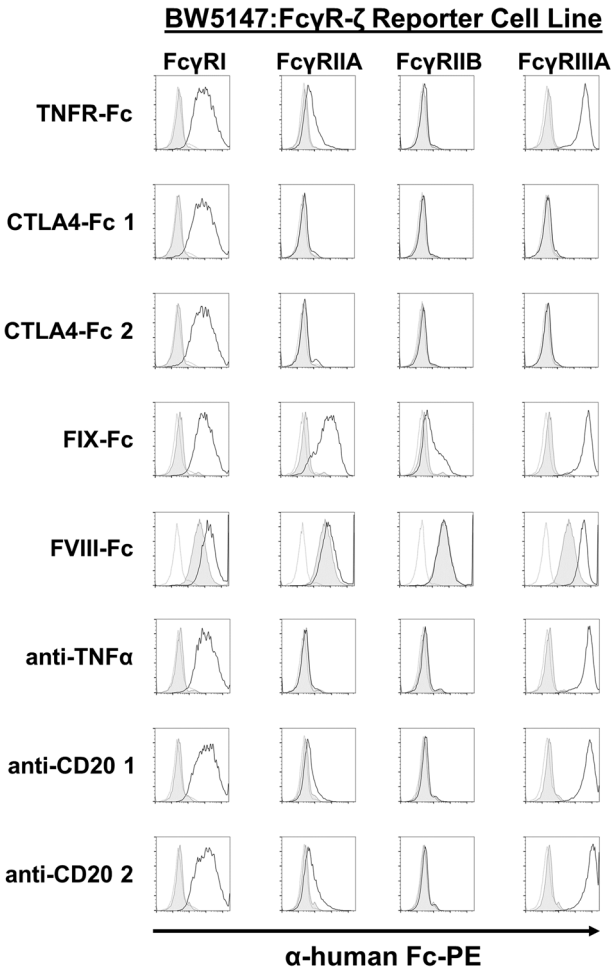

Fig. 3. Therapeutic Fc-fusion and monoclonal antibody FcγR binding. FcγR binding was measured using a flow cytometry-based binding assay. Therapeutic proteins were incubated on ice with FcγR-expressing reporter cell lines (each engineered to express an individual human Fc gamma receptor on the cell surface). Cell-surface bound drugs were detected using an universal (α-human Fc) reagent. Data from a representative experiment are presented. Each row represents an individual drug, while each column represents an individual BW:FcγR-ζ reporter cell line

and outside the linear dose–response range as the drug concentration range used (1 μM–15.625 nM) was kept constant for the FcγR signaling studies presented in Fig. 4a, b. Changes in the FcγR signaling profiles upon drug immobilization were not specific to therapeutic Fc-fusions as similar profile changes were detected for the human IgG1 monoclonal antibodies tested. Anti-TNFα, anti-CD20 1, and anti-CD20 2 stimulated signaling responses from the FcγRI and FcγRIIIA reporter cell lines upon drug preimmobilization to the plate surface (Fig. 4b). As stated earlier, protein therapeutics can form aggregates during manufacture and can form immune complexes if ADA are present. In vivo, this could lead to inflammation and adverse events.

DISCUSSION

As a rule, therapeutic Fc-fusion proteins should not be considered as a monolithic class of drugs. Within the limited number of Fc-fusion therapeutics evaluated in this study, there was considerable diversity with respect to the (i) biology of the effector molecule (fusion partner); (ii) overall structure of the effector molecule (whether monomeric or homodimeric); (iii) the region of the molecule where dimerization occurs; (iv) the molecular mass; and (v) primary sequence of the Fc moiety. Exploitation of the Fc to enhance the half-life of therapeutic proteins can result in additional consequences; as the IgG1 Fc domain serves as an immune effector molecule through interactions with FcγRs and C1q, in addition to FcRn [11, 19, 20]. In this study, we assessed Fc-mediated interactions of marketed Fc-fusion proteins and provide evidence of variability in interactions with FcγR and C1q (summarized in Table III). Differences in Fc-mediated interactions may arise from product-related sources, including the nature of the bioactive fusion partner, the primary amino acid sequence used to encode the Fc domain, the use of a linker sequence, the protein expression system used to manufacture the drug, and posttranslational modifications such as glycosylation patterns. There is precedent for protein engineering via rational drug design to modulate human IgG1 functional characteristics in therapeutic Fc-fusion proteins. For example, replacement of three cysteine residues with serine in the Fc hinge region of the CTLA4-Fc-fusion protein (abatacept) improved protein yield and minimized the effects of antibody-dependent cellular cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC) [14]. Mechanistically, the CTLA4-Fc therapeutics would be expected to bind FcγRI, but not interact with FcγRIIA, FcγRIIB, FcγRIIIA, or C1q. Our data is consistent with this expectation; thus, CTLA4-Fc may be considered controls that validate the assays developed in this study to monitor Fc-FcγR and Fc-C1q interactions.

In this study, we observed diversity in the C1q binding and activation responses of therapeutic Fc-fusions. We found binding to complement C1q and subsequent activation were influenced by whether the Fc-fusion protein was in the monomeric or forced aggregation state. For example, FIX-Fc and FVIII-Fc bound C1q in the soluble and forced aggregated forms while TNFR-Fc only bound C1q following forced aggregation. However, the force-aggregated form of FIX-Fc activated the classical complement cascade while both aggregated FVIII-Fc and aggregated TNFR-Fc failed to do so. The Fc domain's primary amino acid sequence and engineered hinge region are identical for FVIII-Fc and FIX-Fc, suggesting that the nature of the fusion partner influences the Fc domain-mediated triggering of the complement cascade. However, it is plausible that the difference in classical complement activation observed between the forced-aggregated forms of FIX-Fc and FVIII-Fc may be related to differences in thermal stability or the degree of aggregation or both, and not Fc-mediated interactions.

The therapeutic antibody anti-CD20 1 (rituximab) bound C1q only following forced aggregation while the glyco-engineered anti-CD20 2 (obinutuzumab) did not. Obinutuzumab has been shown to induce reduced levels of complement-dependent cytotoxicity in vitro compared to rituximab [18]. While these anti-CD20 monoclonal antibodies differ in CD20 binding and lipid raft generation on target B cells thus impacting complement activation, variance in CDC induction may also be explained by differences in Fc glycosylation patterns influencing Fc-C1q binding. Importantly, our findings suggest processes (e.g., manufacturing,

Table II. Surface BW5147:FcγR-ζ binding by Fc-containing therapeutic proteins

Class	Drug	FcγRI		FcγRIIA		FcγRIIB		FcγRIIIA	
		MFI fold change[1]	Std. deviation	MFI fold change	Std. deviation	MFI fold change	Std. deviation	MFI fold change	Std. deviation
Fc-fusion	TNFR-Fc	35.5	1.4	2.2	0.5	1.1	0.0	94.2	38.3
	CTLA4-Fc 1	33.4	1.8	1.1	0.0	1.1	0.0	1.0	0.0
	CTLA4-Fc 2	31.2	2.1	1.1	0.1	1.1	0.0	1.0	0.0
	FIX-Fc	30.4	3.3	14.5	4.2	1.9	0.5	140.5	18.2
	FVIII-Fc	5.8	1.6	1.8	0.4	1.0	0.1	9.3	2.8
mAb	Anti-TNFα	43.0	1.5	1.0	0.0	1.2	0.1	181.8	26.3
	Anti-CD20 1	44.0	1.8	1.4	0.3	1.2	0.0	160.5	55.7
	Anti-CD20 2	40.8	0.9	1.9	0.5	1.2	0.0	293.8	6.6

CTLA cytotoxic T lymphocyte-associated antigen, *FcγR* Fc gamma receptor, *FIX* factor IX, *FVIII* factor VIII, *MFI* mean fluorescence intensity, *Std. deviation* standard deviation, *TNFα* tumor necrosis factor-alpha, *TNFR* tumor necrosis factor receptor
[1] Fold change calculated as MFI of BW5147:FcγR-ζ transfectants over MFI of BW5147 parental line (no FcγR expression). A MFI fold change threshold of ≥ 1.4 defines FcγR binding

storage, handling) that could induce aggregation or the formation of aggregates in vivo (due to immune complexes) may lead to complement activation, which in turn could lead to adverse events in patients. For example, commercial intravenous immunoglobulin (IVIg) preparations from the 1950s and 1960s associated with substantial aggregate formation were linked with clinical adverse reactions due to activation of the complement cascade [21, 22].

Using human cell lines or primary cells in the profiling of Fc-mediated interactions has the disadvantage that multiple FcγRs are coexpressed on the surface of immune cells [9, 23] making interpretation of results difficult. Consequently, in our comparative assessment of a panel of Fc-fusion proteins, individual evaluations of Fc-FcγR interactions were conducted for each drug-receptor pair using the engineered BW5147:FcγR-ζ reporter cell system [12]. It is important to note that comparisons of FcγR signaling among Fc-fusion proteins are only valid when comparing interactions within an individual BW5147:FcγR-ζ reporter cell line. Differences in FcγR signaling observed across reporter cell lines are not valid, as it was shown the BW5147:FcγR-ζ cell lines differ in their level of IL-2 production upon FcγR engagement [12, 24].

Similar to C1q-Fc interactions, our assessment shows differences in the binding and signaling through FcγRs by therapeutic Fc-fusion proteins. Furthermore, FcγR binding did not accurately predict signaling responses. For example, FIX-Fc and FVIII-Fc induced FcγR signaling when free in solution and when plate-immobilized. Despite identical Fc structures, FIX-Fc exhibited more promiscuous FcγR binding properties when compared to FVIII-Fc. TNFR-Fc only induced FcγR signaling when plate-immobilized, thus mimicking aggregates or immune complexes. As expected, given the rational design strategy employed (see above and [14]), the CTLA4-Fc therapeutics did not signal through FcγRs. As predicted, obinutuzumab, glyco-engineered for enhanced FcγRIIIA engagement leading to greater clinical efficacy for the treatment of B cell lymphoma, bound and signaled via FcγRIIIA, while rituximab did not. Collectively, these results support the use of BW5147:FcγR-ζ reporter cells for the assessment of Fc:FcγR interactions.

Given the Fc domain's natural effector functions, Fc-mediated interactions have the potential to influence immunogenicity. For example, enhanced interaction with antigen-presenting cells via FcγR could impact a therapeutic protein's immunogenicity by influencing the activation state of a cell, the antigen processing and presentation pathways, and the secretion of pro-inflammatory cytokines, which collectively lead to enhanced antigen presentation to $CD4^+$T cells [25]. Alternatively, interaction with FcγRIIb may specifically inhibit anti-drug immune responses through the induction of plasma cell apoptosis [26, 27]. These Fc-mediated interactions could be considered either adverse or favorable as:

(1) Fc-mediated interactions leading to activation may increase the risk of anti-drug immune response development and therefore may compromise the safety and long-term use of the drug, or
(2) Fc-mediated interactions leading to inhibition may decrease the risk of anti-drug immune responses and may enhance the safety profile of the drug. There is also precedent for protein engineering strategies employing the human IgG4 subclass Fc domain. Compared to IgG1, the IgG4 Fc domain has lower binding affinity interactions with FcγR [25] and complement C1q [26]. As such, IgG4 Fc-fusion proteins and monoclonal antibodies have been developed to minimize Fc effector functions [27, 28]. For example, dulaglutide, approved by FDA in 2014 for the treatment of type 2 diabetes, is a glucagon-like peptide 1 (GLP-1) analog fused to a modified IgG4 Fc domain to minimize the Fc's effector function [29].

Previously, we have shown that human FIX-mouse Fc (hFIX-mFc) compared to FIX in mice induced a Th1-like response associated with elevated levels of plasma IFNγ [28]. In accordance with this finding, mice receiving FIX were more likely to have elevated plasma IgE compared to mice receiving hFIX-mFc. This could be beneficial if extrapolated to humans, because IgE anaphylaxis has been reported with

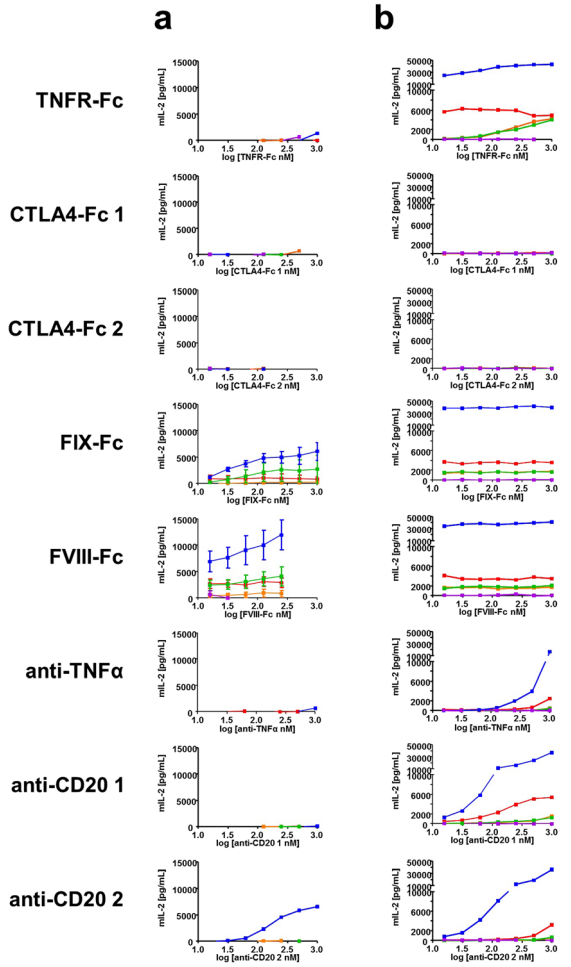

Fig. 4. Therapeutic Fc-fusion and monoclonal antibody FcγR signaling. FcγR signaling was measured by a reporter cell stimulation assay with **a** soluble drug or **b** plate-immobilized drug. Stimulated BW:FcγR-ζ reporter cell lines were incubated overnight at 37 °C/5% CO_2 with an individual drug. Murine IL-2 was detected in cell culture supernatants by ELISA. For each therapeutic protein, reporter cell lines are depicted using color code (red, FcγRI; orange, FcγRIIA; green, FcγRIIB; blue, FcγRIIIA; purple, parental [no FcγR expression]). **a** Mean ± standard error (SEM) from 2 to 3 independent experiments is shown, except for anti-CD20 1 and anti-CD20 2 where a single experiment is presented. **b** Data from a representative experiment are presented

FIX treatment [29] and possibly is less likely to occur with FIX-Fc. The finding in this study that FIX-Fc engages and activates FcγRIIIA suggests that it would activate this receptor in vivo and stimulate human NK cells to secrete IFNγ and thus protect against IgE responses.

An immunogenicity risk common to fusion proteins exists at the primary amino acid sequence level; as the potential exists for the introduction of neo-epitopes at the junction of the effector protein and the hinge region. However, compared to other protein therapeutics indicated for use in inflammatory diseases, TNFR-Fc (etanercept) and CTLA4-Fc (abatacept) elicit relatively low levels of anti-drug antibodies, in 2–20% and 0–13% of patients, respectively [30]. Notably, no previously treated hemophilia A or B patients developed neutralizing antibodies to FVIII-Fc or FIX-Fc in clinical trials [31–33]. Recently, FVIII-Fc was shown in vitro to elicit regulatory macrophage polarization [34], and the authors suggest that the immunomodulatory properties are Fc-mediated. Therefore, to further elucidate the underlying mechanisms involved in the immunogenicity of Fc-fusion proteins as well as potential immunomodulatory properties, understanding the milieu of product-specific Fc-mediated interactions is necessary.

In the case of monoclonal antibodies, there is evidence that sequence variations in the antigen binding (Fab) complementarity-determining regions (CDRs) can influence Fc-mediated interactions resulting in distinct functional characteristics between antibodies that share an identical Fc sequence [35, 36]. This effect may thus be expected to be even more pronounced in the case of Fc-fusion proteins where the effector molecule fusion partners vary greatly in their size and structure. Our results support this concept.

In this study, we observed Fc-mediated differences between the coagulation factor Fc-fusion products. FIX-Fc and FVIII-Fc were both engineered to generate a fusion molecule containing a monomeric coagulation factor and a dimeric Fc domain [37, 38] using identical Fc primary amino acid sequence and protein expression systems. The effect of the fusion partner on Fc-mediated interactions has been observed previously for TNFR-Fc, where Fc-FcRn interactions were altered upon papain cleavage [39]. Several outstanding questions remain: for example, (1) *does the size or nature of the fusion partner matter?* (2) *Can steric hindrance explain these functional differences?*

Approaches that enable the comprehensive assessment of therapeutic Fc-fusions through functional characterization can lead to a better understanding of product attributes. Dissimilar Fc-interaction profiles were observed for the different Fc-containing drugs that were evaluated here suggesting the need for comprehensive functional characterization studies during drug development. Such studies of Fc-containing candidates should include assessment of an active pharmaceutical ingredient (API) under optimal conditions, but also in suboptimal/altered states (e.g., forced degradation studies). Our studies demonstrate clear differences in immune consequences between interactions that occur in solution and when Fc-moieties are bound to a matrix. Thus, for those candidate drugs involved in binding to biological matrices as part of their mechanism of action, assessing Fc effector function following ligand binding would provide additional insights with respect to immunogenicity risk. Such a testing regimen could reveal potentially undesired Fc-mediated interactions. The US Pharmacopeia has recognized the importance of Fc function assays and has recently issued a compendial notice for a general chapter on Fc function assays. This proposed chapter, "is intended to provide knowledge regarding Fc-Fc gamma receptor complexes, Fc mediated effector function of therapeutic antibodies, and assessment of Fc functionality during antibody development and manufacturing" (https://www.uspnf.com/notices/fc-function-assays). Obviously, such information would also apply to Fc-fusion proteins and would be helpful to the industry and regulatory agencies. The results of these

Table III. Overview of Fc-mediated interactions

Class	Drug	Complement		FcγR			
		C1q binding	Activation upon aggregation	FcγRI bind/signal	FcγRIIA bind/signal	FcγRIIB bind/signal	FcγRIIIA bind/signal
Fc-fusion	TNFR-Fc	+	−	++/−	+/−	−/−	++/−
	CTLA4-Fc 1	−	−	++/−	−/−	−/−	−/−
	CTLA4-Fc 2	−	−	++/−	−/−	−/−	−/−
	FIX-Fc	+++	++	++/+	++/−	+/+	+++/+++
	FVIII-Fc	+++	–	+/+	+/−	−/+	+/+++
mAb	Anti-TNFα	−	−	++/−	−/−	−/−	+++/−
	Anti-CD20 1	+++	++	++/−	+/−	−/−	+++/−
	Anti-CD20 2	+	−	++/−	+/−	−/−	+++/+++

CTLA cytotoxic T lymphocyte-associated antigen, *FcγR* Fc gamma receptor, *FIX* factor 364 IX, *FVIII* factor VIII, *TNFα* tumor necrosis factor-alpha, *TNFR* tumor necrosis factor receptor

assays are not meant to be predictive but can be used to assign relative risk and more careful monitoring during clinical trials. Moreover, if adverse events do occur during clinical trials, the availability of in vitro assays can provide mechanistic explanations and targets for modulating the undesirable immune response(s). We have provided several examples of drugs that have been developed by engineering the Fc-domain of fusion proteins to inhibit (or enhance) specific Fc-FcR interactions. The assays described here would provide useful tools to evaluate the engineered Fc-fusion proteins designed to target or disengage from specific Fc receptors.

CONCLUSION

In a comparative study, we carried out a comprehensive assessment of Fc-mediated interactions for five FDA-approved Fc-fusion therapeutics. Our assessment demonstrates the following:

(i) Different Fc-fusion drugs have distinct C1q/FcγR binding and signaling properties;
(ii) FcγR binding does not predict signaling;
(iii) The fusion partner (effector molecule) can influence Fc-mediated interactions. These Fc-mediated interactions can have immunological consequences and the potential to modulate the immunogenicity of Fc-fusion therapeutics.

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COMPLIANCE WITH ETHICAL STANDARDS

Disclaimer My comments are an informal communication and represent my own best judgment. These comments do not bind or obligate FDA.

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