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Imlifidase-generated Single-cleaved IgG: Implications for Transplantation

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Background. Imlifidase is an immunoglobulin G (IgG)-specific protease conditionally approved in the EU for desensitization in highly sensitized crossmatch positive kidney transplant patients. Imlifidase efficiently cleaves both heavy chains of IgG in a 2-step process. However, low levels of the intermediate cleavage product, single-cleaved IgG (scIgG), may persist in the circulation. The study objective was to investigate Fc-mediated effector functions of scIgG and its potential impact on common clinical immunologic assays used to assess transplant eligibility. **Methods.** Imlifidase-generated scIgG, obtained by in vitro cleavage of HLA-sensitized patient serum or selected antibodies, was investigated in different complement- and FcγR-dependent assays and models, including clinical tests used to evaluate HLA-specific antibodies. **Results.** ScIgG had significantly reduced Fc-mediated effector function compared with intact IgG, although some degree of activity in complement- and FcγR-dependent models was still detectable. A preparation of concentrated scIgG generated from a highly HLA-sensitized individual gave rise to a positive signal in the anti-HLA IgG LABScreen, which uses anti-Fc detection, but was entirely negative in the C1qScreen. The same high-concentration HLA-binding scIgG preparation also generated positive complement-dependent cytotoxicity responses against 80%–100% of donor T and B cells, although follow-up titrations demonstrated a much lower intrinsic activity than for intact anti-HLA IgG. **Conclusions.** ScIgG has a significantly reduced capacity to mediate Fc-dependent effector functions. However, remaining HLA-reactive scIgG in plasma after imlifidase treatment can cause positive assay results equivalent to intact IgG in clinical assays. Therefore, complete IgG cleavage after imlifidase treatment is essential to allow correct decision-making in relation to transplant eligibility.

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INTRODUCTION

Highly sensitized patients with chronic kidney disease (CKD) harbor a broad spectrum of HLA-specific antibodies, which negatively impact the probability of finding a compatible organ. These patients are accumulating on kidney transplant waiting lists worldwide. Although recognition and prioritization within kidney allocations systems have led to improvement in rates of transplantation for highly sensitized patients, there remains a subset of very highly sensitized patients who are biologically incompatible with a high percentage of the donor pool and in whom a chance of a compatible organ offer is unlikely. In an adjusted negative binomal regression analysis, candidates with a calculated panel-reactive antibody (cPRA) of ≥99.9% had significantly lower transplant rates compared with nonhighly sensitized candidates, which has persisted despite substantial allocation priority.[1-3] For some selected patients, participation in kidney paired donation programs may be an option[4,5]; however, only a small fraction of patients will be transplanted through these programs, and a large percentage of these patients will continue to accrue extended waiting times.[6,7] In the absence of a compatible living donor, highly sensitized individuals may benefit from HLA-incompatible living or deceased donor transplantation given that preemptive desensitization protocols succeed in sufficiently reducing the level of circulating

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donor-specific antibodies.[4-7] The immunoglobulin G (IgG)-degrading enzyme of *Streptococcus pyogenes* (IdeS), with the international nonproprietary name imlifidase, is a 35-kDa cysteine protease that with high selectivity cleaves all 4 subclasses of human IgG just downstream of the hinge region (between G^{236} and G^{237}), thus generating a $F(ab')_2$ and a homodimeric Fc fragment.[8,9] In contradistinction to other desensitization methods, imlifidase provides an IgG antibody-free window based on its capacity to eliminate the entire pool of plasma IgG, including extravascular IgG.[10-13] As part of the clinical development program at Hansa Biopharma AB, to date, 46 HLA-sensitized patients with CKD have been transplanted after treatment with imlifidase.

Imlifidase cleaves the heavy chains of IgG in 2 separate reaction steps.[8,14] In the first reaction, one of the heavy chains of the intact IgG molecule is rapidly cleaved resulting in the intermediate product, single-cleaved IgG (scIgG). Due to a conformational change of scIgG, the second step of the reaction, where the complete separation of scIgG into $F(ab')_2$ and Fc-homodimer is achieved, requires longer time to completion compared with the rapid cleavage observed in the first step.

During the initial dose-finding study in sensitized patients with CKD,[13] a single intravenous imlifidase infusion of 0.25 mg/kg resulted in a near complete removal of circulating IgG and substantially reduced the levels of HLA-specific IgG within 24 h among a wide range of mean fluorescence intensity (MFI) levels evaluated with single-antigen beads (SAB). In comparison, the imlifidase 0.12 mg/kg dose in 1 patient with pretreatment level of anti-HLA antibody of 75 SABs with MFI > 20 000 resulted in the anti-HLA antibody signal remaining significant in the LABScreen assay 24 h posttreatment (34 beads with MFI > 5000). Preliminary investigations indicated that this remaining signal was attributed to residual scIgG.

As previously published data have mainly focused on clinical outcomes from phase 2 studies, little has been disseminated regarding the early preclinical and clinical experiences. The purpose of this study was to (a) further investigate the potential impact of scIgG in clinical assays used to assess transplant eligibility, (b) investigate scIgG with respect to potential residual Fc-mediated effector functions, and (c) provide guidance on clinical assay use and interpretation to facilitate clinical decision-making following desensitization with imlifidase as the drug advances from investigational to commercial use.

MATERIALS AND METHODS

Cells, Animals, and Human Serum

Human lymphoma cell lines Raji [ACC-319] and Daudi [ACC-78] were acquired from DSMZ. Human monocytic leukemia cell line THP-1 was donated from Dr Maria Allhorn, Lund University. Human erythrocytes were pelleted from heparinized blood collected from healthy donors. Cultured cells were maintained at 37°C in a humidified CO_2-incubator in R10 (RPMI 1640, 10% FBS and PEST) (Raji and Daudi) or R10 supplemented with 10 mmol/L HEPES and 0.05 mmol/L 2-mercaptoethanol (THP-1).

Female BALB/c mice from Taconic, Denmark, were housed at the research vivarium of Active Biotech AB in Lund. All animal experiments were performed in agreement with the ethical approval by Malmö/Lund animal ethics committee (permit M72-13).

Sera from HLA-sensitized patients with CKD, included in an earlier reported phase 2 dose-finding study[13] (EudraCT no. 2013-005417-13 and ClinicalTrials.gov Identifier NCT02224820) was used. The study protocol was approved by the regional ethics committee in Uppsala, Sweden (approval no. 2014/131), and all study subjects provided written informed consent.

Preparation of HLA-, Erythrocyte-, Thrombocyte-, and CD20-reactive scIgG and $F(ab')_2$

Human HLA-reactive patient serum, human control serum, rabbit antihuman erythrocyte (Rockland; 209-4139), rabbit antimouse erythrocyte antibody (Rockland; 210-4139), protein G-purified rabbit antimouse thrombocyte serum (Cedarlane, CLA31440), and rituximab (human monoclonal anti-CD20 IgG1, MabThera/Paranova Läkemedel AB, Sweden) were incubated with different concentrations of imlifidase at 37°C for 1 h, followed by heating (56°C, 30 min). Samples with desired amounts of scIgG and $F(ab')_2$ were identified by SDS-PAGE/Western blot and were tested for activity in different assays, with mock-treated intact IgG as positive control.

Single-antigen Bead Assays

Sera from the HLA-sensitized imlifidase-treated patients and fractions of intact IgG, scIgG, or $F(ab')_2$ generated from patient pretreatment serum were analyzed with the LABScreen and C1qScreen assays using a Luminex 200 instrument and HLA Fusion 2.0 software (One Lambda, ThermoFisher Scientific). All samples were heat-inactivated (56°C, 30 min) before the analysis.

CDC-PRA: Complement-dependent Killing by Panel-reactive Antibody

The CDC-PRA analysis was performed at the Department of Clinical Immunology and Transfusion Medicine, Lund, Sweden. Intact IgG, scIgG, and $F(ab')_2$ fractions, generated in vitro from HLA-sensitized patient serum, were tested against a mini panel of purified T and B cells from 5 representative donors, with cytotoxic fractions retested after serial dilution.

Complement-fixing Capacity of Single-cleaved HLA-reactive IgG

HLA-sensitized patient serum and nonsensitized healthy control serum were heat-inactivated (56°C, 30 min) and screened for IgG binding to Raji cells by flow cytometry using biotin-conjugated polyclonal donkey antihuman IgG (H+L) in combination with phycoerythrin-conjugated streptavidin.

To investigate the complement-fixating capacity of pretreatment patient and control IgG, scIgG, and $F(ab')_2$, Raji cells were incubated with the different IgG fractions using noncytotoxic human serum (10%) as complement source. After incubation, the cells were divided into different plates and stained for C1q and C4d before analysis by flow cytometry.

For detailed information, see **Supplemental Digital Content (SDC)**, http://links.lww.com/TP/C327.

Complement-dependent Cytotoxicity Activity of Single-cleaved Rituximab

To evaluate the potential cytotoxic capacity of scIgG, Daudi cells were incubated with different concentrations of rituximab preparations (Rtx, scRtx, or $F(ab')_2$ Rtx; 10 or 30 µg/mL depending on read-out), followed by addition of noncytotoxic human serum (8%–10%) as complement source. Plates were incubated at 37°C, and cells were analyzed with respect to cell viability (colorimetry) and cell death (7AAD), C1q and C4d (flow cytometry).

For detailed information, see **SDC,** http://links.lww.com/TP/C327.

Crossmatching

The complement-dependent cytotoxicity crossmatch (CDCXM) and flow cytometric crossmatch (FCXM) were performed according to standard procedures, as previously described.[15] The donor cells for the FCXM test were not treated with pronase.

Fcγ Receptor-mediated Interactions of Single-cleaved Antierythrocyte IgG (Binding, ADCC and ADCP)

For FcγR binding and ADCC experiments, THP-1 monocytes were preactivated with lipopolysaccharide (LPS) and IFN-γ, after which erythrocytes opsonized with antierythrocyte preparations (intact IgG, scIgG, and $F(ab')_2$) or PBS were added. After overnight incubation, the degree of erythrocyte retention to adherent THP-1 cells was evaluated. Supernatants were collected for analysis of hemoglobin content. For ADCP, erythrocytes were labeled with CellTrace Far Red dye, washed, and incubated with different concentrations of rabbit antihuman erythrocyte preparations. Opsonized erythrocytes were added to PMA- and LPS-activated THP-1 cells. After 5 h incubation at 37°C, nonphagocytized erythrocytes were lysed, and the percentage of THP-1 cells that had engulfed labeled erythrocytes was analyzed by flow cytometry. For detailed information, see **SDC,** http://links.lww.com/TP/C327.

Experimental Autoimmune Hemolytic Anemia

Eleven-week-old female BALB/c mice (Taconic) were weighed and injected intraperitoneally with 1 mg preparations of intact IgG, scIgG, or $F(ab')_2$ generated from rabbit antimouse erythrocyte antibody. Blood was collected into Microvette CB 300 µL, lithium heparin tubes (Sarstedt, 16.443). Hematocrit and erythrocyte number (by DAF assay; see **SDC,** http://links.lww.com/TP/C327) were assessed daily. For hematocrit, 30 µL blood was taken up in LightCycler Capillaries (Roche, 1909339) and centrifuged in capillary adapters (Roche, 1909312) for 10 min at 510 g, after which the ratio of packed erythrocytes to total blood volume was measured. Spleen weights were registered at termination (day 3).

Experimental Immune Thrombocytopenic Purpura

Nine-week-old female BALB/c mice were injected intraperitoneally with 0.25 mg preparations of intact IgG, scIgG, or $F(ab')_2$ generated from rabbit antimouse thrombocyte serum. Blood plasma was collected into Microvette CB 300 µL, K2 EDTA tubes (Sarstedt, 16.444) 1 d after

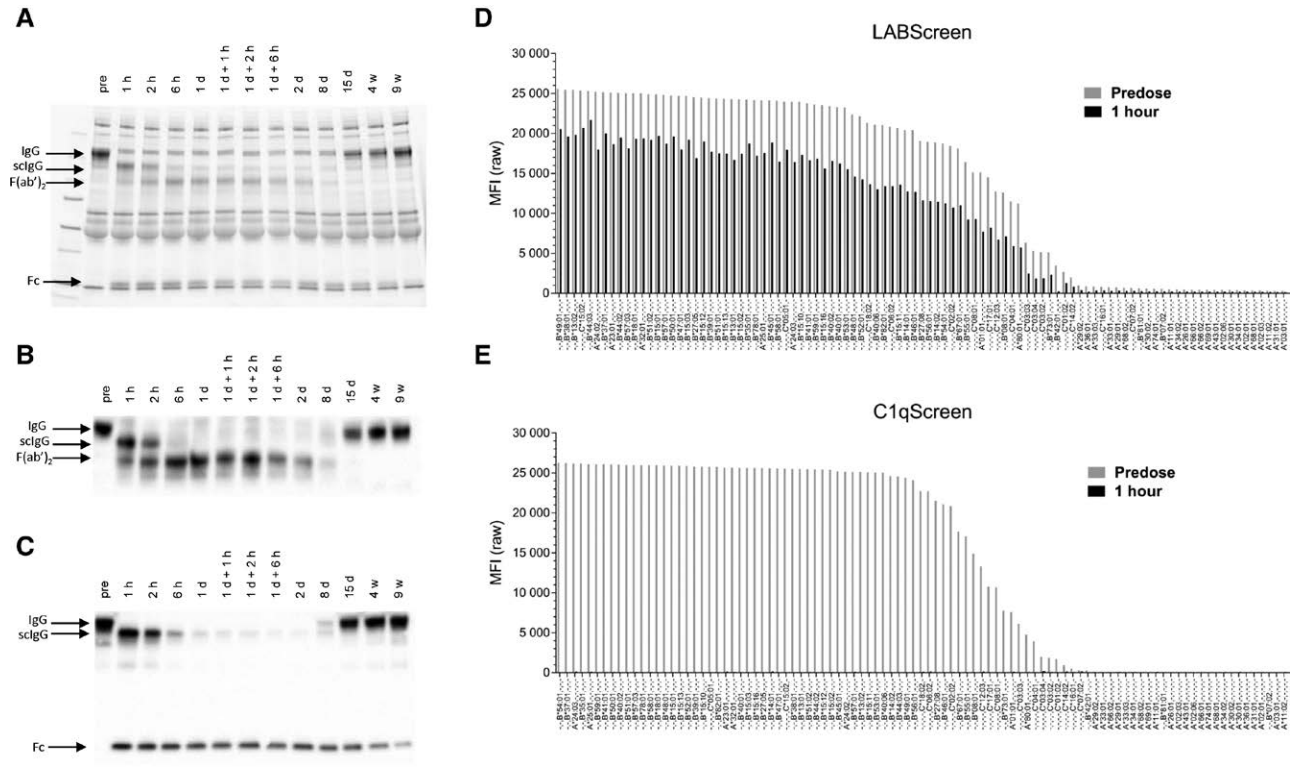


FIGURE 1. Analysis of serum samples obtained before and after treatment of an HLA-sensitized patient with 0.12 mg/kg imlifidase. In (A), SDS-PAGE analysis of IgG cleavage in consecutive serum samples obtained before (pre) and at various time-points after treatment, with an additional 0.12 mg/kg dose given after 30 h. In (B) and (C), anti-F(ab) and anti-Fc Western blots corresponding to the SDS-PAGE analysis in (A). In (D) and (E), LABScreen and C1qScreen SAB analyses of samples collected before (predose) and 1 h after imlifidase treatment, with each bar representing the signal obtained for a specific HLA class I-presenting bead. IgG, immunoglobulin G; MFI, mean fluorescence intensity; SAB, single-antigen beads; scIgG, single-cleaved IgG; SDS-PAGE, sodium dodecyl-sulfate polyacrylamide gel electrophoresis.

induction, and platelets were counted on a VetScan HM5 (Abaxis).

RESULTS

ScIgG Produces a Similar Signal as Intact IgG in the LABScreen Assay But Is Undetectable by C1qScreen

To investigate the potential influence of scIgG on SAB assays, consecutive serum samples from an HLA-sensitized patient treated with a suboptimal dose (0.12 mg/kg; C_{max} 2.1 μg/mL) of imlifidase were analyzed by SDS-PAGE and Western blot. All of the intact IgG in the pretreatment serum sample was cleaved to scIgG 1 h after the first dose and was further converted to $F(ab')_2$ and Fc fragments at 6 h postdose (Figure 1A–C). Pretreatment serum generated strong signals in both LABScreen and C1qScreen assays, with median MFI values >20 000. Despite the total elimination of intact IgG 1 h after imlifidase treatment (Figure 1B and C), the LABScreen signals were only slightly reduced in comparison with predose signals (Figure 1D). This indicated that HLA-binding scIgG is well-recognized by the Fc-specific detection antibody used in LABScreen. Importantly, even the very low levels of scIgG still present 2 d after treatment initiation and only very weakly detected by Fc-specific Western blot (Figure 1C; lane 2 d) gave a median MFI signal in the 2000–3000 range, with 4 individual beads being still above 6000 (data on file). In contrast, no signal was generated in the C1qScreen at any posttreatment time-point between 1 h (Figure 1E) and 2 d (data on file).

To confirm this observation, pretreatment serum was used to generate preparations with different proportions of

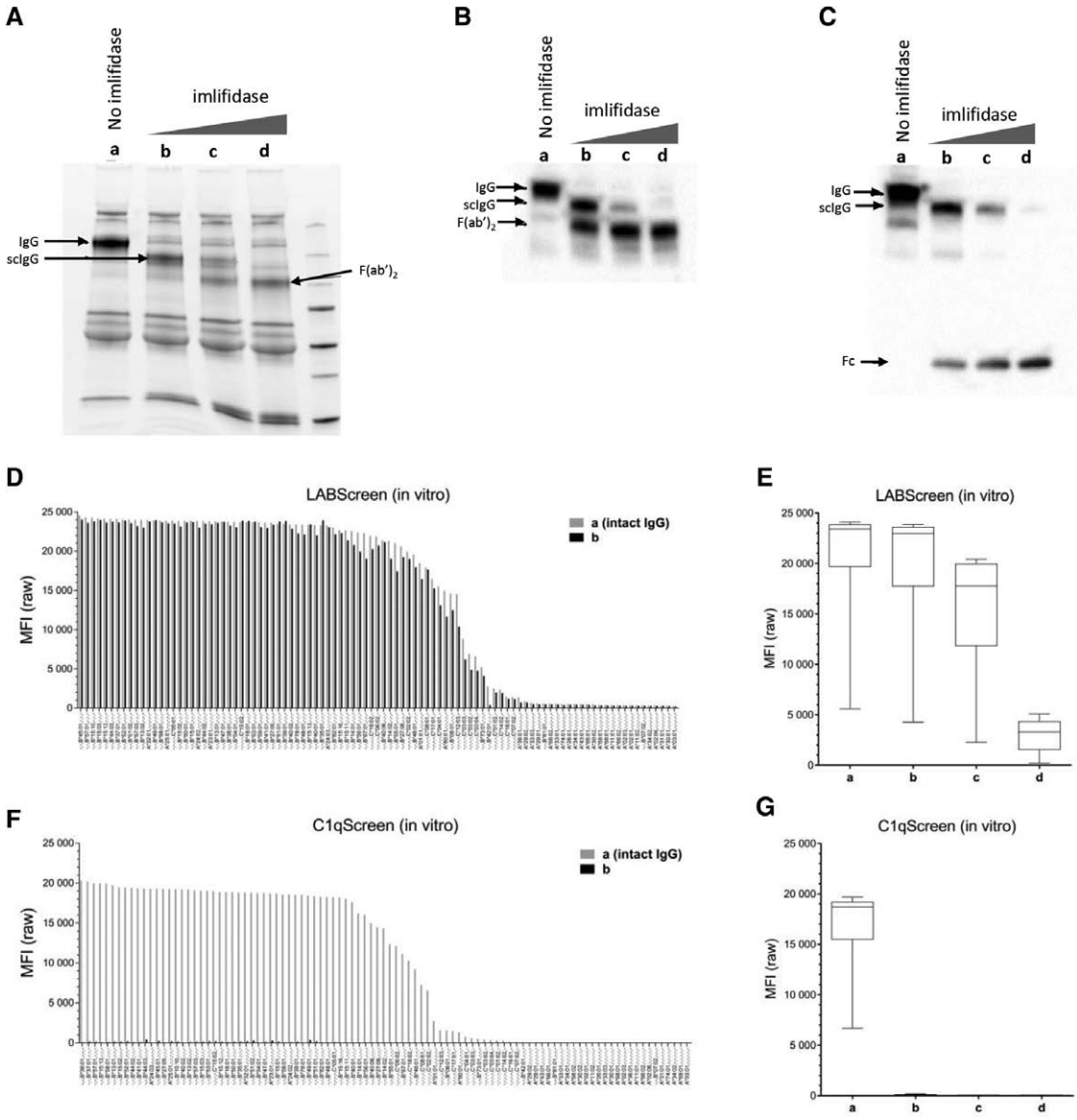


FIGURE 2. The impact of different imlifidase-generated anti-HLA fractions (intact IgG, scIgG, and $F(ab')_2$) on SAB analyses. Different IgG fractions were generated by in vitro treatment of serum from an HLA-sensitized patient with PBS (sample a) or increasing concentrations of imlifidase (samples b–d). In (A), SDS-PAGE analysis, in (B) and (C), anti-F(ab)- and anti-Fc-specific Western blots, respectively. In (D) and (F), the samples containing intact IgG (sample a) and mainly scIgG (sample b) were analyzed with LABScreen and C1qScreen, with each bar representing the signal obtained for a specific HLA class I-presenting bead. In (E) and (G), the LABScreen and C1qScreen data for all 4 samples are shown, with each box summarizing the signal obtained from all beads (median, interquartile range, and 10–90 percentile). IgG, immunoglobulin G; MFI, mean fluorescence intensity; SAB, single-antigen beads; scIgG, single-cleaved IgG; SDS-PAGE, sodium dodecyl-sulfate polyacrylamide gel electrophoresis.

intact IgG, scIgG, and F(ab′)$_2$ (referred to as samples a–d in accordance with Figure 2A–C). When analyzing the fraction in which all intact IgG had been cleaved to scIgG, but with only a small proportion of the scIgG having been further converted to F(ab′)$_2$ and Fc (sample b in Figure 2A–C) in the LABScreen assay, no reduction in signal was observed when compared with sample a (intact IgG) (Figure 2D and E). Although the MFI signal decreased somewhat for sample c, which contained mainly F(ab′)$_2$ fragments with only a small amount of residual scIgG, the reduction was significant only for sample d, which contained only trace amounts of scIgG (Figure 2E). Although sample d contained only trace amounts of scIgG (Figure 2A–C), the signals from many SAB were still considerably higher than background (median MFI [interquartile range]: 1977 [72-4202]; **Figure S1, SDC,** http://links.lww.com/TP/C327). In contrast, sample b, which contained mainly scIgG (Figure 2A), was entirely negative in the C1qScreen SAB assay (Figure 2F–G). This result was consistent with the C1qScreen analysis of the clinical trial serum sample collected 1 h after imlifidase treatment (Figure 1E).

ScIgG Retains a Certain Degree of Activity in the CDC-PRA Test

As previously reported by Lorant et al,[13] pretreatment serum from the HLA-sensitized patient was positive against 90% of T cells and 100% of B cells in a CDC-PRA panel (n = 30). This patient was treated with an imlifidase dose of 0.12 mg/kg, which was subsequently judged suboptimal based on the insufficient C_{max} achieved in plasma (2.1 µg/mL to be compared with 5.8 µg/mL, which is the mean C_{max} achieved at the optimal dose level of 0.25 mg/kg). Although significantly reduced, 14% and 38% T- and B-cell positivity still remained 24 h after dosing.[13] At this time-point, intact IgG was no longer present, and scIgG was only very weakly detected by Western blot using anti-Fc detection (Figure 1C). To follow-up on these earlier findings and further investigate the clinical effect of scIgG, the samples generated from the pretreatment serum of the patient were tested against a mini panel of blood donor cells (n = 5). As shown in Figure 3, the serum fractions containing mainly F(ab′)$_2$ fragments with either small (sample c) or trace (sample d) amounts of scIgG (Figure 2A–C), were negative against all donor T and B cells in the mini panel. In contrast, mock-treated serum containing intact IgG (sample a) gave maximum reactivity in all donors (Figure 3). The serum preparation containing a substantial amount of scIgG but no intact IgG (sample b in Figure 2A–C) remained positive against 4/5 and 5/5 of T and B cells, respectively, however, with CDC reactivity scores being reduced in 2/5 (T cell) and 4/5 (B cell) donors. Follow-up analysis of serially diluted preparations of intact IgG (sample a) and scIgG (sample b) revealed a clear difference in their intrinsic capacity to trigger complement-dependent cytotoxicity, with 2048- and 256-fold dilutions being required to turn the intact IgG and the scIgG preparations completely inactive in all donor T- and the B-cell tests. It should here be emphasized that the extremely high level of scIgG in sample b is artificial and unlikely to be obtained at imlifidase C_{max} levels achieved at the selected clinical dose (0.25 mg/kg).

C1q Binding Is Impaired But Not Abrogated for scIgG

The CDC-PRA results indicated that the cytotoxic capacity of scIgG was clearly reduced though not eliminated. To further evaluate this, different preparations of rituximab (**Figure S2, SDC,** http://links.lww.com/TP/C327) were evaluated in a system with CD20-expressing Daudi cells using human serum as complement source.

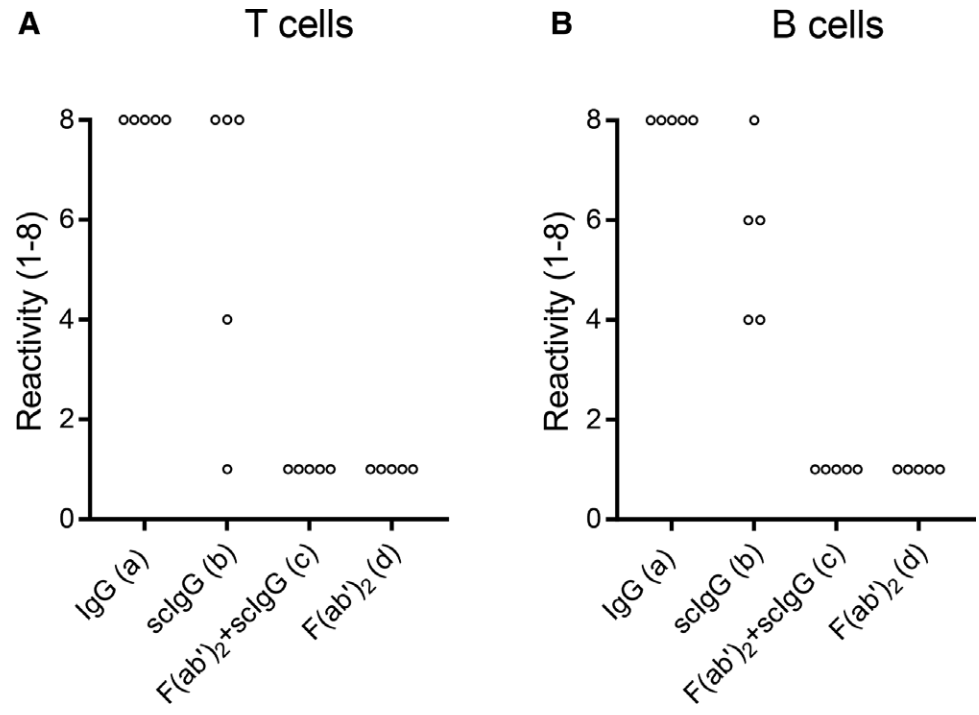


FIGURE 3. CDC-PRA analysis of HLA-reactive patient serum containing either intact IgG or different proportions of imlifidase-generated scIgG and F(ab′)$_2$ (samples a–d described in Figure 2A–C). The panel consisted of purified T cells (A) and B cells (B) from 5 donors. Data are presented as CDC reactivity to each donor cell pool, scored from 1 (negative) to 8 (maximum reactivity). CDC-PRA, complement-dependent cytotoxicity–panel-reactive antibody; IgG, immunoglobulin G; MFI, mean fluorescence intensity; scIgG, single-cleaved IgG.

In contrast to intact rituximab, single-cleaved and fully cleaved rituximab were both without detectable cytotoxic effect at 10 µg/mL (Figure 4A). When tested at 30 µg/mL, single-cleaved rituximab retained a certain capacity to bind C1q (Figure 4B), but even at this concentration, the binding was nonproductive and did not result in downstream complement activation (C4d deposition; Figure 4C) and cytotoxicity (7AAD; Figure 4D).

Because activation of complement by monoclonal antibodies depends on binding to a single antigen epitope, structural constraints related to epitope density, and geometry might influence the efficacy.[16,17] For this reason, serum from an HLA-sensitized patient with known CDC activity toward HLA-expressing Raji cells was used as a source of polyclonal IgG in an additional system (**Figure S3, SDC,** http://links.lww.com/TP/C327). HLA-sensitized patient serum showed strong reactivity with Raji cells (Figure 5A) and intact HLA-reactive IgG from the same patient was a strong inducer of C1q-binding (Figure 5B), in contrast to control IgG. Although HLA-reactive scIgG had clearly reduced capacity to induce C1q deposition, a certain degree of residual activity was still present when using the polyclonal antibody-based detection of C1q (Figure 5B). In contrast, a comparative assessment using the C1qScreen kit reagent indicated that only intact HLA antibodies were able to fix C1q (Figure 5C). Regardless of this ambiguity, single-chain cleavage of HLA-reactive IgG abrogated its capacity to induce downstream C4d deposition (Figure 5D).

ScIgG Retains a Certain Degree of FcγR-dependent Effector Function

To investigate whether complement-independent effector functions were maintained in scIgG, different FcγR-dependent in vitro models were used. FcγR- binding activity of intact IgG and scIgG was shown by the retention of opsonized erythrocytes on a confluent layer of

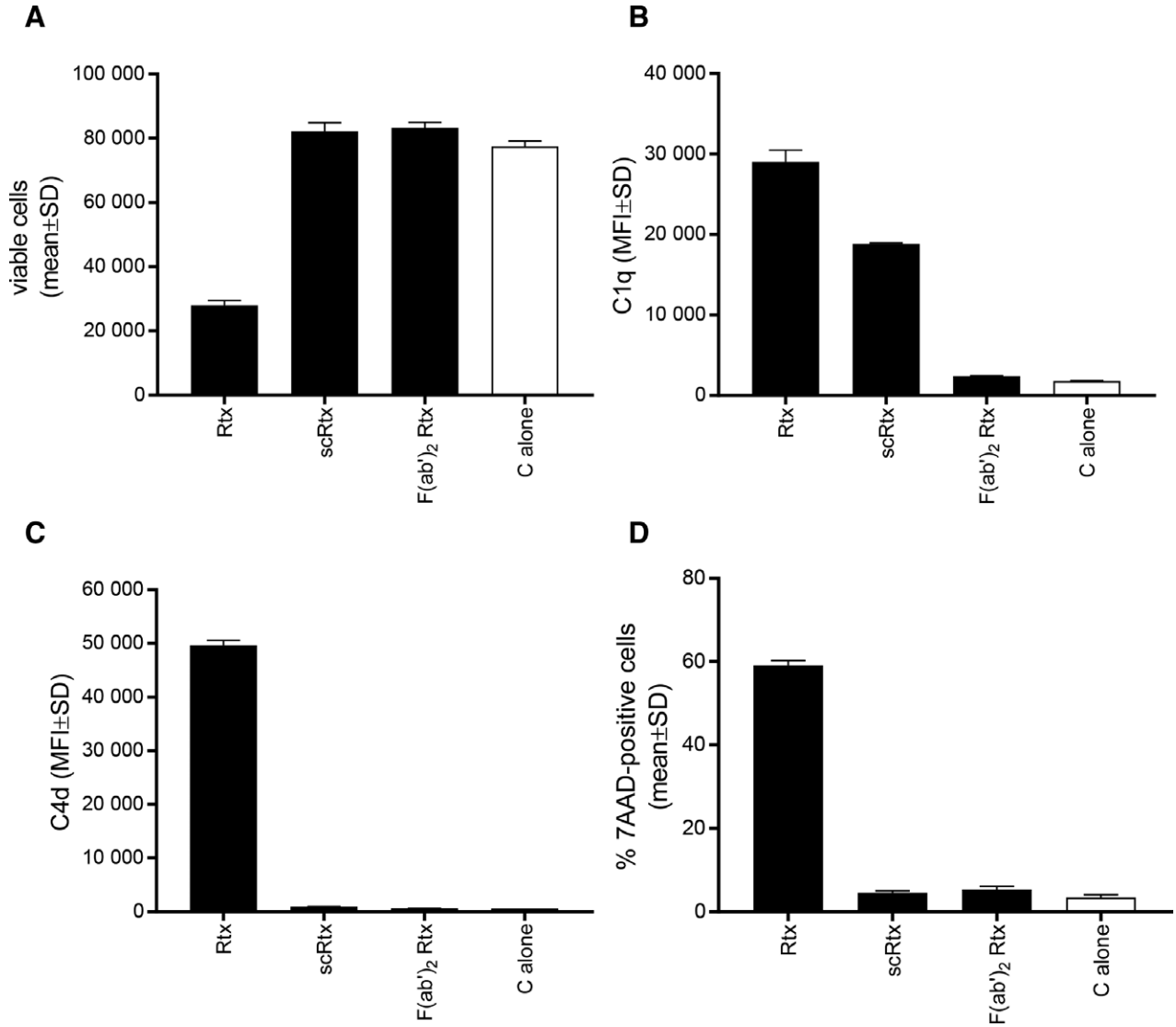


FIGURE 4. Complement activation and complement-dependent killing by intact rituximab (Rtx), scRtx, and F(ab′)$_2$ Rtx (**Figure S2, SDC,** http://links.lww.com/TP/C327) in CD20-expressing Daudi cells using human serum as complement source. In the presence of human complement, scRtx did not influence cell viability as determined by colorimetry at 10 µg/mL (A), in contrast to intact Rtx. At 30 µg/mL, scRtx retained a certain capacity to bind C1q (B), but the binding did not result in downstream C4d deposition (C) and did not result in detectable cytotoxicity (D; 7AAD). Complement (C) alone was entirely inactive. C alone, complement alone; F(ab′)$_2$ Rtx, fully cleaved rituximab; MFI, mean fluorescence intensity; scRtx, single-cleaved rituximab.

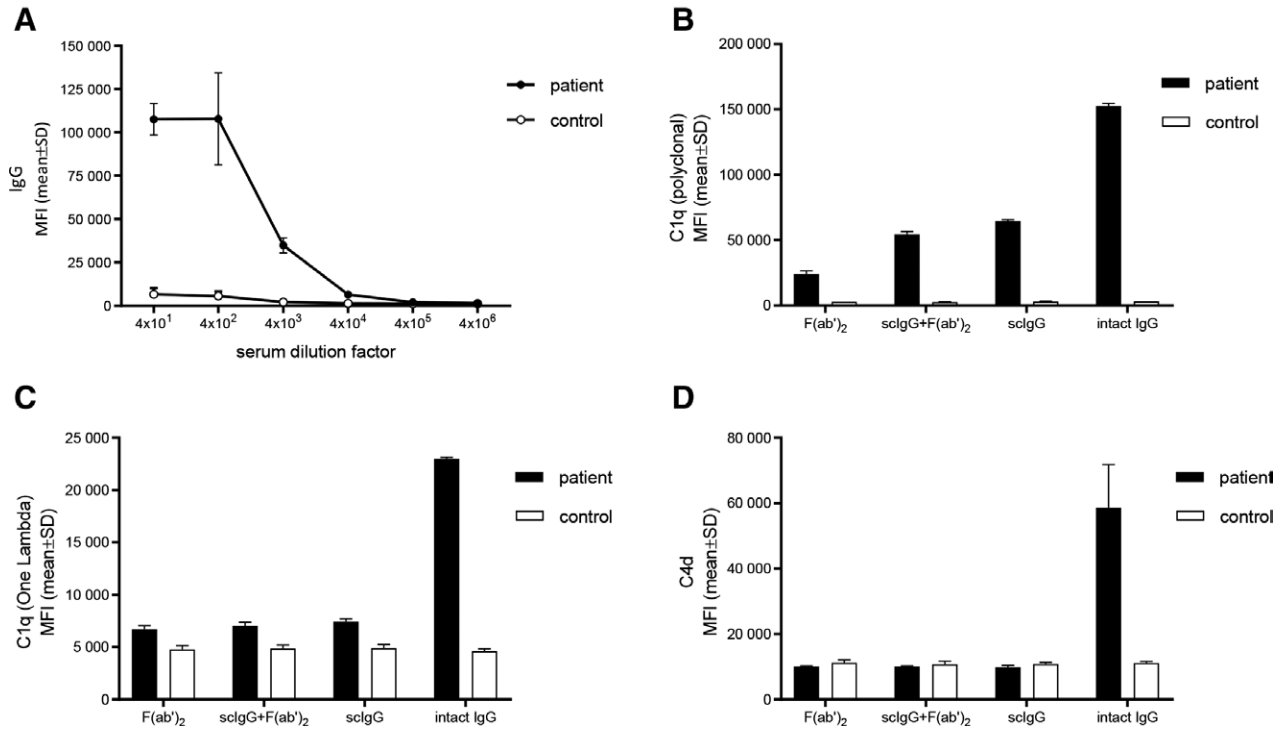


FIGURE 5. Complement activation by HLA-reactive IgG, scIgG, and F(ab′)$_2$ in Raji cells. In (A), IgG-binding activity of HLA-reactive (patient) and nonsensitized serum (control) at different dilutions. In (B) and (C), C1q binding to Raji cells induced by different imlifidase-generated IgG fractions (**Figure S3A, SDC,** http://links.lww.com/TP/C327), detected by polyclonal anti-C1q F(ab′)$_2$ antibody (B) and C1qScreen kit detection reagent (C). In (D), C4d deposition induced by the different imlifidase-generated IgG fractions. All analyses were performed on duplicate samples (n = 2). IgG, immunoglobulin G; MFI, mean fluorescence intensity; scIgG, single-cleaved IgG.

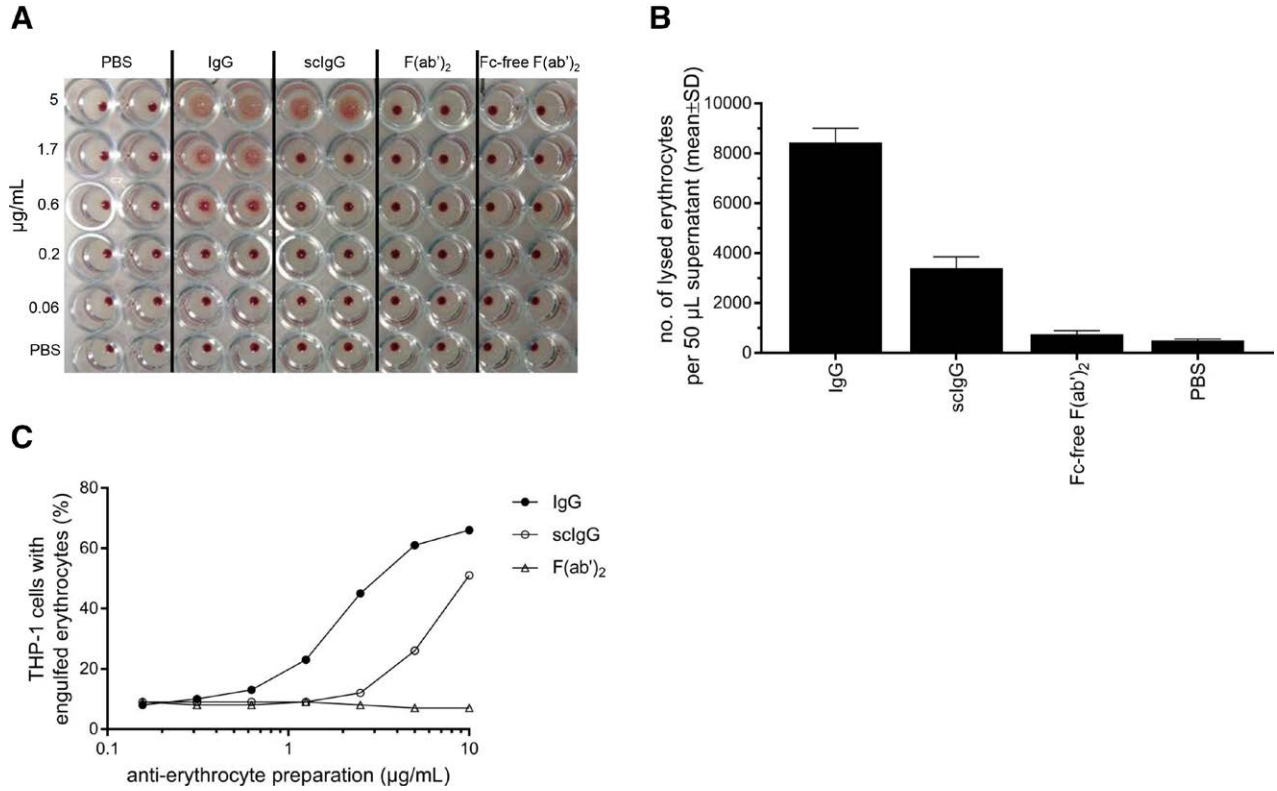


FIGURE 6. Complement-independent effector functions of imlifidase-generated antierythrocyte fractions. In (A), FcγR-dependent retention of erythrocytes to a confluent layer of THP-1 cells after opsonization with antierythrocyte IgG, scIgG, or F(ab′)$_2$ in the absence of complement (n = 2). In (B), ADCC activity of THP-1 cells after opsonization of erythrocytes with the different antierythrocyte IgG fractions (n = 2–3). In (C), ADCP activity of THP-1 cells after opsonization with the different antierythrocyte IgG fractions. For more information on the IgG fractions used, see **Figure S4, SDC,** http://links.lww.com/TP/C327. ADCC, antibody-dependent cellular cytotoxicity; ADCP, antibody-dependent cellular phagocytosis; IgG, immunoglobulin G; MFI, mean fluorescence intensity; scIgG, single-cleaved IgG.

THP-1 cells (Figure 6A), with intact IgG being effective already at 0.6 μg/mL, whereas 5 μg/mL scIgG was required for retention to occur. Regarding ADCC, antierythrocyte scIgG retained about 40% of the ADCC lysing activity at 5 μg/mL (Figure 6B). In line with this, 5 μg/mL antierythrocyte scIgG retained about 40% of the opsonizing activity of the intact IgG preparation leading to phagocytic uptake in the ADCP assay (Figure 6C).

The function of scIgG was also evaluated in 2 different antibody-mediated disease models in mice, experimental autoimmune hemolytic anemia (eAIHA), and experimental immune thrombocytopenic purpura (eITP), both considered largely FcγR-dependent.[18] At the used concentrations, scIgG had no effect on erythrocyte number and spleen weight in eAIHA (Figure 7A–C) but retained a limited capacity to reduce platelet count in eITP (Figure 7D).

The Flow Cytometric Crossmatch Test Does Not Discriminate Between Intact IgG and scIgG

Clinical serum samples from a second patient treated with a suboptimal dose of imlifidase (0.12 mg/kg) were analyzed by SDS-PAGE, visualizing the stepwise cleavage of IgG into $F(ab')_2$, via the scIgG intermediate. Comparison of clinical assay data with the SDS-PAGE pattern showed how the different assays are affected by remaining scIgG (Figure 8). Importantly, the data demonstrated a clear influence of remaining scIgG on assays using anti-Fc detection, including the FCXM. Although the nonamplified CDC turned negative 6 h posttreatment, the FCXM remained positive even after 24 h. The patient was given a second dose (0.12 mg/kg) of imlifidase in accordance with the trial protocol (NCT02224820). This resulted in FCXM conversion after another 6 h, which enabled successful transplantation of the patient.

DISCUSSION

The development of crossmatch testing before transplantation has significantly impacted graft survival by allowing the detection of preformed antibodies, which would result in hyperacute or antibody-mediated rejection.[19] These tests rely heavily on the determination of a donor-specific HLA antibody's ability to elicit cytotoxic responses.[20] In the setting of evaluating the success of a given desensitization regimen to enable an HLA-incompatible transplant, it

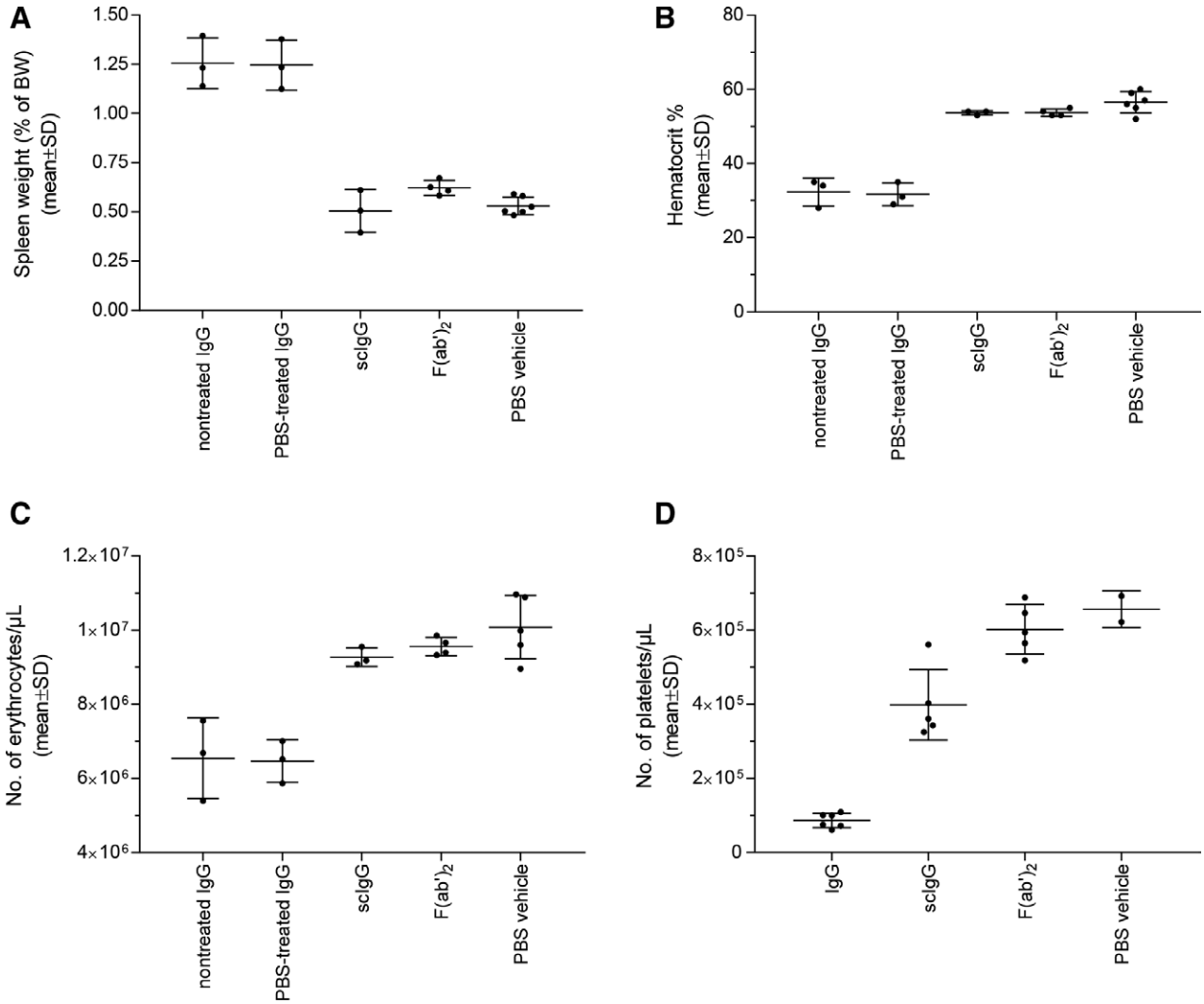
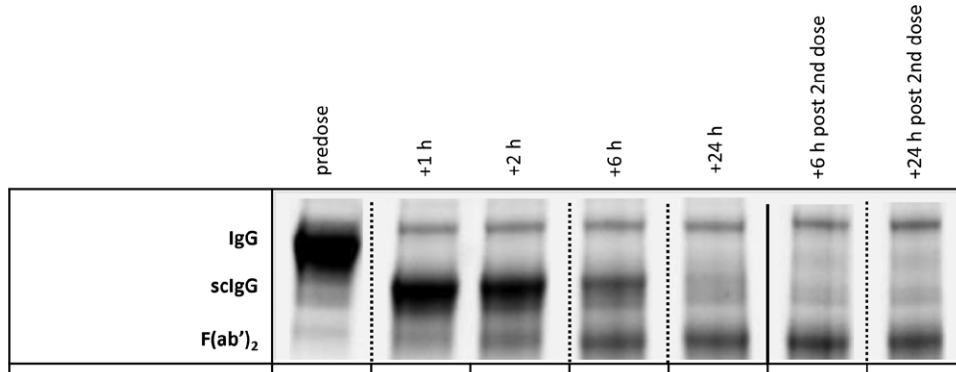


FIGURE 7. FcγR-dependent eAIHA (A–C) and eITP (D) in mice induced with the antierythrocyte/antiplatelet preparations shown in **Figure S5, SDC,** http://links.lww.com/TP/C327. In eAIHA, spleen weights (A), hematocrit (B) and erythrocyte number (C) were assessed at termination (day 3). In eITP, platelets were counted 1 d after disease induction (D). eAIHA, experimental autoimmune hemolytic anemia; eITP, experimental immune thrombocytopenic purpura; IgG, immunoglobulin G; scIgG, single-cleaved IgG.



	predose	+1 h	+2 h	+6 h	+24 h	+6 h post 2nd dose	+24 h post 2nd dose
SAB-HLA[a] (MFI)	5958	6312	4000	2700	1369	273	383
SAB-C1q[a] (MFI)	15 740	67			51		120
T-cell CDC[b] (pos/neg)	pos			neg	neg	neg	neg
B-cell CDC[b] (pos/neg)	pos			neg	neg	neg	neg
T-FCXM[c] (pos/neg; MCS)	pos 128			pos 91	pos 56	neg 22	neg 21
B-FCXM[d] (pos/neg; MCS)	pos 318			pos 196	pos 133	neg 22	neg 21

[a]mean fluorescence intensity (MFI) for DSA antigen HLA-B7
[b]nonamplified
[c]positive crossmatch defined as >40 mean channel shifts (MCS)
[d]positive crossmatch defined as >80 mean channel shifts (MCS)
/, not done

FIGURE 8. A summary of clinical data from a patient in a dose-finding study treated with a suboptimal dose of imlifidase (0.12 mg/kg) illustrates how remaining scIgG affects different clinical assays and in particular, assays using anti-Fc detection, ie, SAB-HLA and FCXM. Before imlifidase treatment, the patient had positive CDC and FCXM tests due to anti-B7 DSA. Although the nonamplified CDC turned negative 6 h posttreatment, the FCXM remained positive even after 24 h. The patient was given a second dose (0.12 mg/kg) of imlifidase in accordance with the trial protocol (NCT02224820). This resulted in FCXM conversion after another 6 h, which enabled successful transplantation of the patient. CDC, complement-dependent cytotoxicity; DSA, donor-specific antibody; FCXM, flow cytometric crossmatch; IgG, immunoglobulin G; SAB, single-antigen bead; scIgG, single-cleaved IgG.

is imperative to have strong understanding of the clinical assays involved in determining a crossmatch conversion from positive to negative. The mechanism of action of imlifidase includes the generation of a scIgG intermediate that may be indistinguishable from intact IgG when evaluated with assays using anti-Fc detection methods. This study concluded that when imlifidase is used in clinical practice, scIgG is unlikely to be pathologically important because relevant concentrations of the single-cleaved fragments are only present at very low concentrations after treatment with an optimal dose of imlifidase. Nonetheless, our results demonstrate that the presence of even low amounts of HLA-specific scIgG might have significant influence on assays that are commonly used for evaluating transplant eligibility. It should be emphasized that this influence is generally not attributed to the residual Fc-mediated effector function of scIgG but rather to limitations of the detection methods used, as discussed later and further illustrated in Figure 9 and Table 1.

Imlifidase-mediated cleavage of allo-antibody has previously been demonstrated to inhibit NK cell activation and ADCC.[21] In addition, a strong negative impact of single heavy chain cleavage on Fc-dependent effector functions has been suggested by others.[22,23] In our study, scIgG demonstrated a detectable capacity to initiate Fc-dependent processes. ScIgG binds C1q to some extent and might under certain conditions cause downstream complement activation eventually leading to complement-mediated cell killing. In addition, scIgG retains some degree of its ability to interact with FcγRs and has capacity to induce FcγR-dependent responses such as ADCC and ADCP in a complement-independent manner.

Amino acid residues in or adjacent to the imlifidase cleavage site in the lower hinge region (^{234}LLGGP^{238} in IgG1) have, together with residues in the CH2 domain, been shown to be involved in the interaction of IgG with C1q and FcγRs.[24-33] Thus, the retained ability of scIgG to carry out Fc-mediated effector functions might be considered unexpected. Our data show that the single-cleaved Fc chain portion in scIgG remains firmly attached to the intact Fc chain (data on file), presumably through a maintained CH3-CH3 interchain interaction.[34,35] It remains to be investigated whether the single-cleaved Fc chain might still contribute to C1q and FcγR binding or whether the remaining binding activity is maintained by the intact Fc chain alone.

In the SAB assays, each individual antigen is presented on a separate solid phase bead, enabling identification of HLA antibodies with high specificity and sensitivity. However, these assays only remotely resemble the physiological situation in which a variety of potential antigens are presented on the semifluid surface of a cell. The C1qScreen assay adds information regarding C1q-binding but does not account for the complex and well-regulated

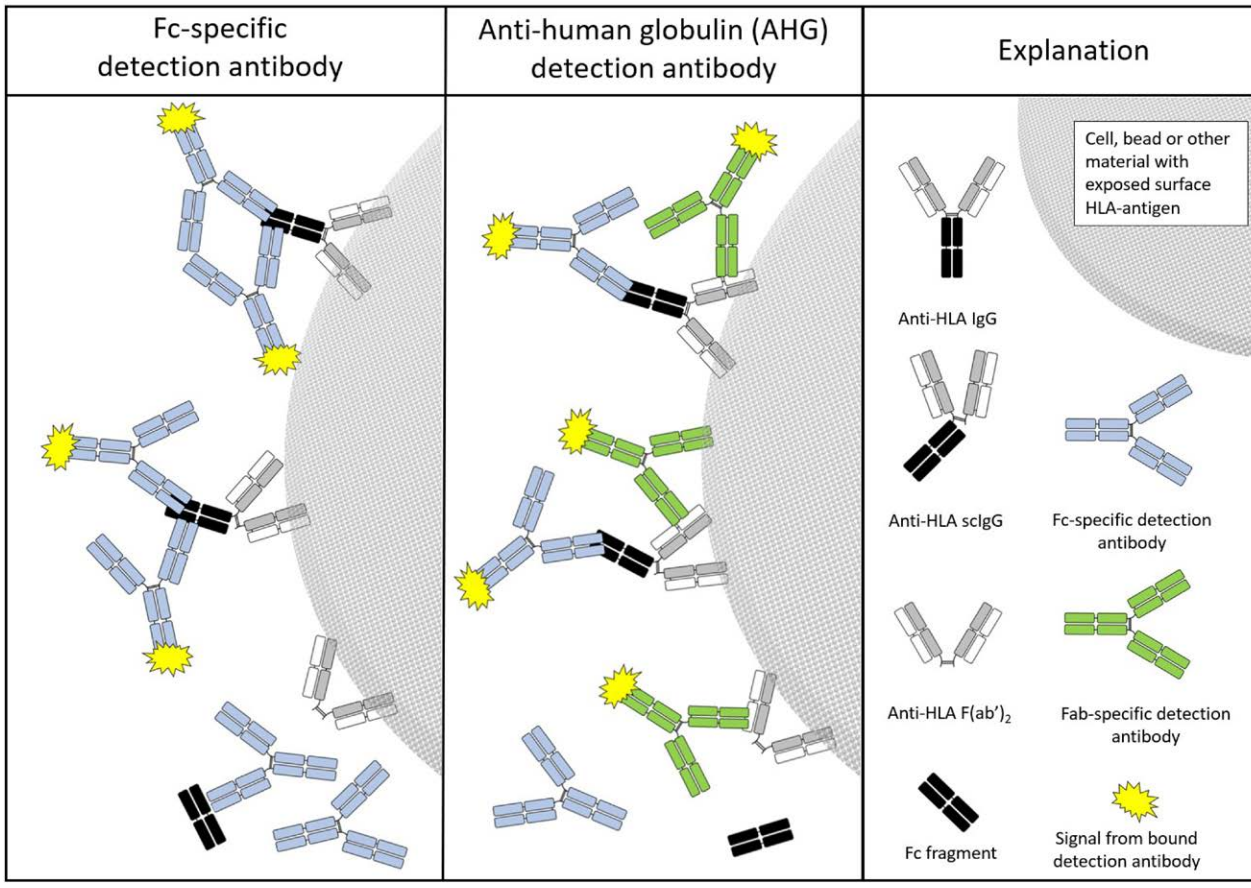


FIGURE 9. Schematic illustration of the detection of intact anti-HLA IgG, anti-HLA scIgG, and anti-HLA F(ab′)$_2$ fragments by an Fc-specific detection antibody (left panel) and by AHG (anti-IgG; middle panel). Explanation in right panel. AHG, anti-human globulin; IgG, immunoglobulin G; scIgG, single-cleaved IgG.

process of complement activation. Thus, a clinical decision to proceed to transplantation generally requires a negative crossmatch test involving donor cells.

The herein demonstrated impact of scIgG on the LABScreen assay has important implications for the most used crossmatch tests, that is, the antihuman globulin (AHG)-amplified CDCXM and the FCXM. These tests rely on anti-IgG (AHG) and anti-Fc antibodies (FCXM) in their respective detection steps. Consequently, the FCXM is unable to discriminate between intact IgG and scIgG (Figure 8), and the AHG-amplified CDCXM reacts to intact IgG and scIgG as well as F(ab′)$_2$. It should be emphasized that in the AHG-amplified CDCXM, the amplifying antibody is likely to be the main contributor to complement activation. This means that even in cases of complete imlifidase-mediated conversion of anti-HLA IgG into F(ab′)$_2$ and Fc fragments, there is a considerable risk that cell-bound anti-HLA F(ab′)$_2$ may cause a false positive result. Therefore, the AHG-amplified CDCXM test needs to be interpreted with caution in patients treated with imlifidase.

As suggested by this study, postimlifidase transplant eligibility is best evaluated in a system considering the complement-activating capacity of scIgG without giving scIgG (or F(ab′)$_2$) a disproportionate impact on assay outcome. Of the clinical systems currently in use, the nonamplified

TABLE 1.

Influence of imlifidase-generated HLA-reactive scIgG and F(ab′)$_2$ on different crossmatch tests

Assay for assessment of transplantation eligibility	Is the detection reagent compromised by the presence of HLA-reactive scIgG?	Is the detection reagent compromised by the presence of HLA-reactive F(ab′)$_2$?
CDCXM (nonamplified)[a]	No	No
AHG-amplified CDCXM	Yes	Yes[b]
FCXM	Yes	No
Anti-HLA SAB	Yes	No
C1q SAB	No[c]	No

[a]Includes no amplification step and thus provides a direct measure of the CDC activity of any IgG-derived component.
[b]AHG with F(ab′)$_2$ reactivity will detect HLA-binding F(ab′)$_2$ without intrinsic capacity for complement activation.
[c]With reservation that the residual complement-activating effect of scIgG in vivo might be underestimated.
AHG, anti-human globulin; CDCXM, complement-dependent cytotoxicity crossmatch; FCXM, flow cytometric crossmatch; IgG, immunoglobulin G; SAB, single-antigen bead; scIgG, single-cleaved IgG.

CDCXM is considered to best match these requirements. Although it is the least sensitive of the currently used crossmatch tests, CDCXM provides a physiologically relevant assessment of transplant eligibility by measuring lytic activity on live donor cells in which several different HLAs can be simultaneously expressed at relevant levels and in which potential clustering/capping of antigen is allowed.

Additional information on posttreatment donor-specific antibodies levels might be obtained by the highly sensitive SAB assays. However, our data demonstrate that the LABScreen assay is likely to underestimate the clinical efficacy of imlifidase, because even minute amounts of scIgG cause a signal of similar strength as intact IgG. On the other hand, the C1qScreen may overestimate the clinical efficacy of imlifidase, because scIgG is entirely undetected. In clinical practice, a combination of assays may be used for clinical decision-making and the pros and cons of each should be assessed.

Although AHG-amplified CDCXM is less compatible with transplant eligibility assessment after imlifidase treatment due to its recognition of both scIgG and $F(ab')_2$, results from other common methods that are based on anti-Fc detection, including FCXM and anti-HLA IgG SAB (eg, LABScreen), should also be interpreted with their limitations in mind. Although a positive posttreatment result in any of these assays is most often explained by low levels of residual scIgG, any signal above the predefined threshold for positivity will still require precautions, especially because the performance of FCXM tests is not standardized across different HLA laboratories and patient groups, including the use of pronase, which was not investigated in this study.

In conclusion, advances in new therapies often require a close evaluation of these medications on current assays, laboratory tests, and personalized medicine in decision-making. In the context of crossmatch conversion in desensitization therapies, imlifidase-generated scIgG impacts the clinical assays used to assess transplant eligibility, and interpretation of these tests should be made with careful consideration of the timing of the imlifidase dose in relation to the crossmatch testing. Knowledge of the pharmacokinetics/pharmacodynamics of imlifidase in evaluating the process of intact IgG through scIgG and ultimately to fully cleaved IgG as well as the clinical impact of scIgG, potentially maintaining residual Fc-mediated effector functions, should be considered to facilitate clinical decision-making following desensitization with imlifidase.

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