

Novel methods to determine complement activation in human serum induced by the complex of Dezamizumab and serum amyloid P

Received for publication, March 8, 2021, and in revised form, August 23, 2021 Published, Papers in Press, August 28, 2021,

<https://doi.org/10.1016/j.jbc.2021.101136>

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Edited by Peter Cresswell

Lack of simple and robust methods to determine complement activation in human serum induced by antigen–antibody complexes is a major hurdle for monitoring therapeutic antibody drug quality and stability. Dezamizumab is a humanized IgG1 monoclonal antibody that binds to serum amyloid P component (SAP) for potential treatment of systemic amyloidosis. The mechanism of action of Dezamizumab includes the binding of SAP, complement activation through classical pathway, and phagocytosis; however, the steps in this process cannot be easily monitored. We developed two novel methods to determine Dezamizumab-SAP complex-induced complement activation. Complement component 3 (C3) depletion was detected by homogeneous time-resolved fluorescence (HTRF), and C3a desArg fragment, formed after the cleavage of C3 to yield C3a followed by removal of its C-terminal arginine residue, was determined using Meso Scale Discovery (MSD) technology. We found that the presence of both Dezamizumab and SAP was required for complement activation *via* both methods. The optimal molar ratio of Dezamizumab:SAP was 6:1 in order to obtain maximal complement activation. The relative potency from both methods showed a good correlation to Dezamizumab-SAP-dependent complement component 1q (C1q) binding activity in Dezamizumab thermal-stressed samples. Both SAP and C1q binding, as determined by surface plasmon resonance and the two complement activation potency methods described here, reflect the mechanism of action of Dezamizumab. We conclude that these methods can be used to monitor Dezamizumab quality for drug release and stability testing, and the novel potency methods reported here can be potentially used to evaluate complement activity induced by other antigen–antibody complexes.

During the past decades, therapeutic antibodies have become the most rapidly growing class of biological drugs. There are now over 60 antibody-based drugs approved for therapeutic use and are currently marketed as of 2016 (1). Among therapeutic antibodies, Immunoglobulin G (IgG) is the predominant isotype. The clinical efficacy of some therapeutic

antibodies relies on two types of interactions: target-specific binding by the antigen-binding fragment (Fab) to the antigen and immune-mediated effector functions *via* interaction of the crystallizable fragment (Fc) with receptors expressed on various cell types and complement component 1 (C1) circulating in serum. Such interactions include antibody-dependent cell-mediated cytotoxicity (ADCC), antibody-dependent cellular phagocytosis (ADCP), and complement-dependent cytotoxicity (CDC) (2).

Dezamizumab is a fully humanized IgG1 monoclonal antibody (mAb) for the potential treatment of systemic amyloidosis, a fatal disorder caused by pathological extracellular deposits of amyloid fibrils coated with the normal plasma protein, serum amyloid P component (SAP) (3). Dezamizumab is an anti-SAP antibody that triggers immunotherapeutic clearance of amyloid (4). In humanized murine models, binding of Dezamizumab to amyloid activates the classical complement pathway and attracts macrophages and other immune cells that phagocytose and remove amyloid complexes (5, 6). Since the mechanism of action of Dezamizumab includes the binding of SAP antigen and complement activation through the classical pathway, the binding and potency assays are chosen to reflect the critical quality attributes of Dezamizumab.

Three main pathways can activate the complement system: classical, lectin, and alternative (Fig. 1). Only the classical pathway (CP) is triggered by antigen–antibody complex formation. In the classical pathway, C1 circulates as an inactive serum complex, comprising six C1q molecules and two molecules each of the serine proteases C1r and C1s. After binding to the antigen, the Fc-portion of IgG or IgM interacts with the collagen-like tail of C1q. This interaction leads to a conformational change in C1 resulting in the sequential activation of the two proteases, C1r and C1s, which in turn allows C1s to cleave C4 and C2 forming inactive C4a and C2b and active C4b and C2a. C4b then binds to the cell surface and to C2a, to form the C2aC4b complex, which is C3 convertase (Fig. 1). The lectin pathway (LP) resembles the CP in that its activation also leads to formation of the C2aC4b, C3 convertase complex (Fig. 1).

Although the classical and lectin pathways are activated by antibody complex or lectin recognition respectively, the alternative pathway (AP) is constitutively active at low levels

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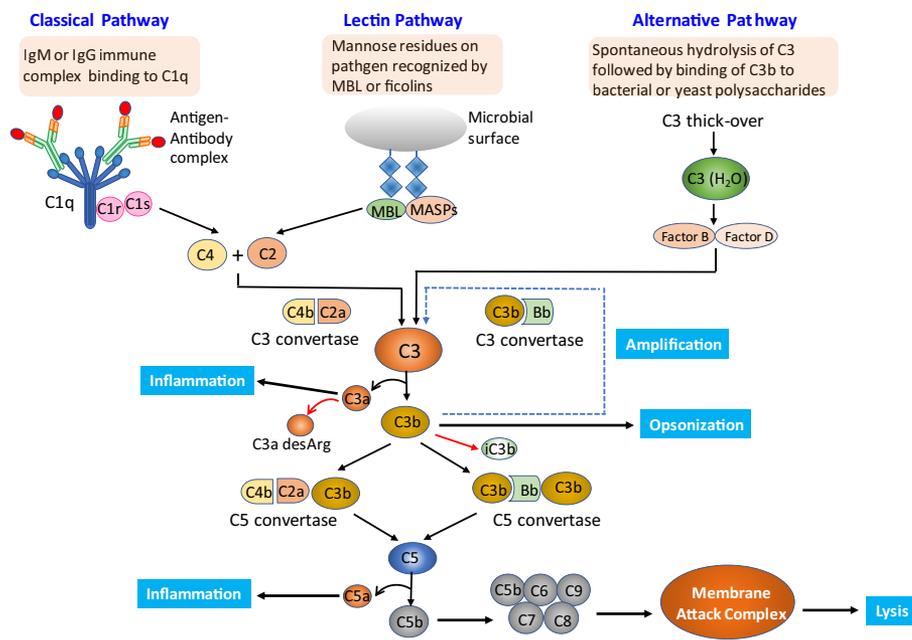


Figure 1. Schematic overview of the complement cascade illustrating the three activation pathways (classical, lectin, and alternative). The classical pathway is activated when C1q binds to antibody complexed with antigen. The interaction activates C1r and C1s, which cleave C4 and C2. The lectin pathway is activated when mannose-binding lectin (MBL) binds to conserved pathogenic carbohydrate motifs. The interaction activates the MBL-associated serine proteases (MASPs) and cleaves C4 and C2. The products from C4 and C2 cleavage form the classical and lectin pathway C3 convertase, C4bC2a, which cleaves C3 into C3b and C3a. C3b associates with C4bC2a to form the C5 convertase of the classical and lectin pathways, C4bC2aC3b. The alternative pathway (AP) is activated by spontaneous hydrolysis of C3. In the presence of Factors B and D, the AP C3 convertase (C3bBb) and AP C5 convertase (C3bBbC3b) are eventually formed. C5 convertase from all three pathways cleaves C5 to form C5a and C5b. C5b together with C6-C9 forms the membrane attack complex (MAC) that lyses targeted pathogens or damaged self-cells.

under resting conditions. This mechanism allows the system to stay primed for rapid and robust activation (Fig. 1).

The products of complement activation have three major effects: (1) anaphylatoxins (C3a and C5a), potent proinflammatory molecules that attract and activate leukocytes through interaction with the G-protein receptors. The biological activity of C3a and C5a is regulated by serum-carboxypeptidases (7, 8) that cleave the C-terminal arginine, yielding C3a desArg and C5a desArg. (2) opsonins (C3b, iC3b, and C3d), which decorate target cells and induce phagocytosis and (3) the terminal membrane attack complex (MAC, C5b-9) lyses targeted pathogens or damaged self-cells directly.

Although human complement system was discovered more than a hundred years, there is still lack of a simple and robust method to determine antigen-antibody complex induced complement activation *in vitro*. The challenges to develop such assays include: (1) selection of an appropriate component to determine complement activity from a complex mixture of more than 30 components and regulators. (2) selection of assay formats for easy assay performance. (3) identification of complement sources and appropriate handling and storage. (4) identification of optimal assay condition to maximize antigen-antibody complex induced complement activation and minimize nonantibody-dependent complement activation.

Here, we report two novel assay formats that we have used to characterize the activity of Dezamizumab-SAP complex induced complement activation in human serum. In the first method, C3a desArg formation was monitored using Meso Scale Discovery (MSD) technology. The MSD assay uses microplates with high binding carbon electrodes in the bottom

and electrochemiluminescent labels that are conjugated to detection antibodies. Sulfo-tag labels allow for ultrasensitive detection by the MSD instrument. In the second method, C3 depletion was detected by homogeneous time resolved fluorescence (HTRF), a frequently used assay technology that combines fluorescence resonance energy transfer technology (FRET) with time-resolved measurement (TR). In HTRF assays, a signal is generated through fluorescent resonance energy transfer between a donor and an acceptor molecule when in close proximity. Our results showed that relative potency from both methods had a good correlation to Dezamizumab-SAP dependent C1q binding activity for Dezamizumab thermal stressed samples. These methods can be also potentially used to evaluate complement activity induced by other antigen-antibody complexes.

Results

MSD method used to detect C3a desArg formation from complement activation in human serum induced by Dezamizumab-SAP complex

The principle of the MSD method to detect C3a desArg formation from complement activation in human serum induced by the Dezamizumab-SAP complex is illustrated in Figure 2A. The cleavage of C3 in human serum by the increase activity of C3 convertase from the classical complement pathway induced by Dezamizumab-SAP complex will lead to the increase of C3a and C3b. C3a is short-lived in serum and is rapidly cleaved by carboxypeptidase B, a plasma protease that cleaves off the C-terminal arginine yielding an inactive but

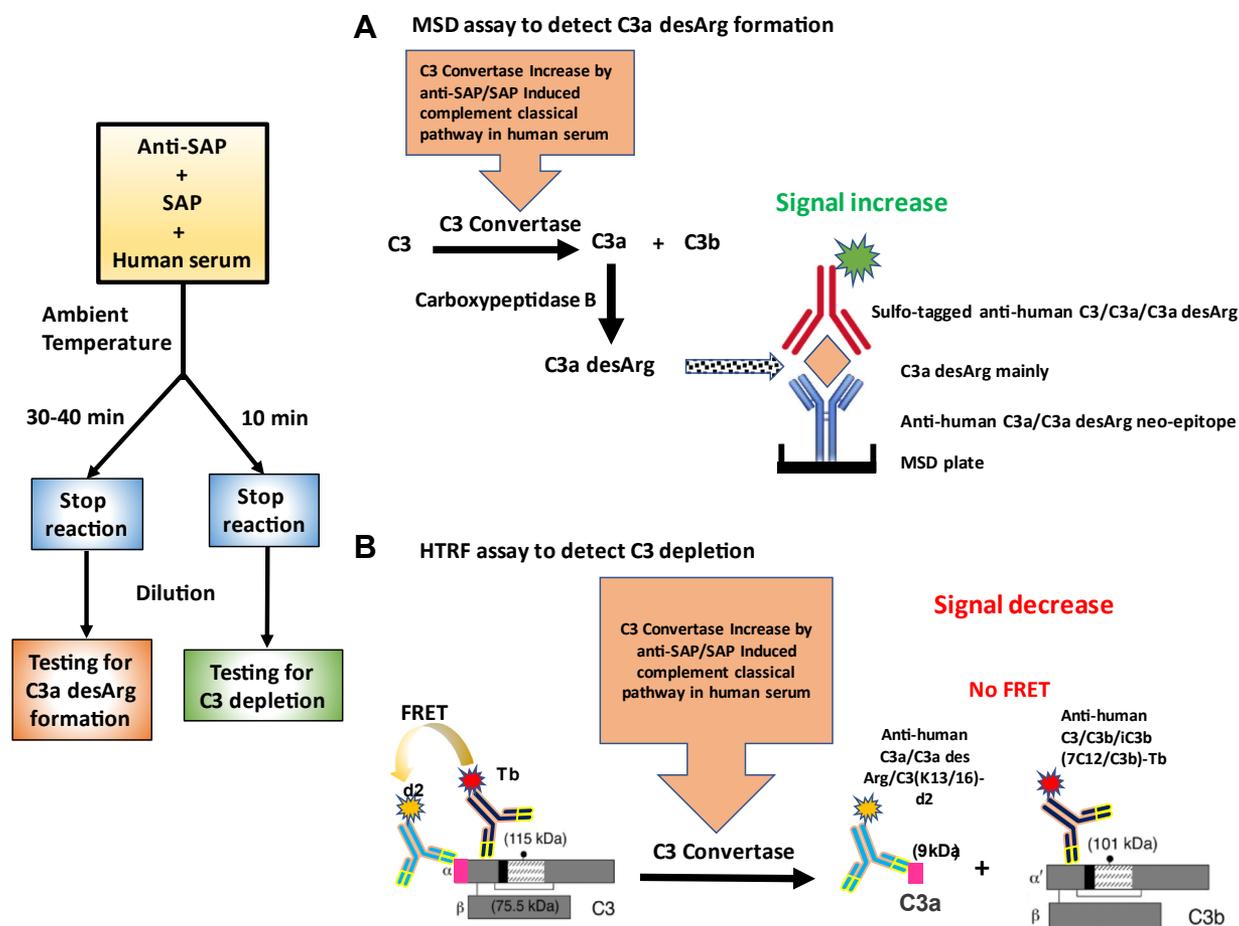


Figure 2. Methods for determination of complement activation induced by Dezamizumab-SAP complex in human serum. A, principle of MSD assay to determine C3a desArg formation. B, principle of HTRF assay to detect C3 depletion.

more stable C3a desArg (7). The MSD method utilizes the neo-epitope monoclonal antibody of human C3a/C3a-desArg coated microplate with carbon electrodes integrated into the bottom of each well. C3a desArg in the complement activation mixture bound to the coating antibody specifically. After plate washing to remove the unbound proteins, anti-human C3a/C3a desArg/C3 antibody conjugated with sulfo-tag for electrochemiluminescence was added and bound to the captured C3a desArg to form a complex. Tris-based read buffer containing tripropylamine (TPA) as a coreactant for light generation was added to detect the sulfo-tag complex formed. When the MSD plate was read, a voltage was applied to the plate electrodes causing the sulfo-tag near the bottom of the plate to emit light through a series of reduction and oxidation reactions. C3a desArg on the MSD plate can be quantified by the measurement of light density.

The high specificity of the coating antibody was the key to the success of the complement activation assay. After evaluation of several complement components and the antibodies against them, the monoclonal antibody 2991 from Hycult Biotech that recognizes the neo-epitope of human C3a/C3a-desArg showed high specificity against C3a desArg. When purified C3, C3a, C3a desArg, and iC3b were diluted and tested in the MSD method, the signal for C3a desArg was

>100-fold higher than C3a and >3500-fold higher than C3 and iC3b at 11 nM (Fig. 3A). These results indicated that the coating antibody is highly specific to C3a desArg. The other unbound complement components in human serum, such as C3, C3b, and C3a, can be removed by washing the plate to allow quantitation of C3a desArg by sulfo-tag conjugated anti-human C3/C3a/C3a desArg detecting antibody (Fig. 2).

The dilution factor needed to quantitate C3a desArg in human serum and complement activation induced by Dezamizumab-SAP complex were determined by performing threefold serial dilution of serum or complement activation mixture ranging from 5 to 10,935-fold. The highest signal, after back calculation for dilution factor, was observed with the dilution factors ranging from 3645-fold to 10,935-fold for serum only and 10,935-fold for complement activation mixture (Fig. 3B). This dilution range appeared appropriate to quantitate C3a desArg. Therefore, 10,000-fold dilution was routinely used for all serum samples before C3a desArg was detection by the MSD method.

The time and temperature dependence of Dezamizumab-SAP complex induced complement activation in human serum was determined at both 37 °C and ambient temperature (21–23 °C). Under control conditions (no Dezamizumab-SAP complex added) at 37 °C, C3a desArg increased significantly in

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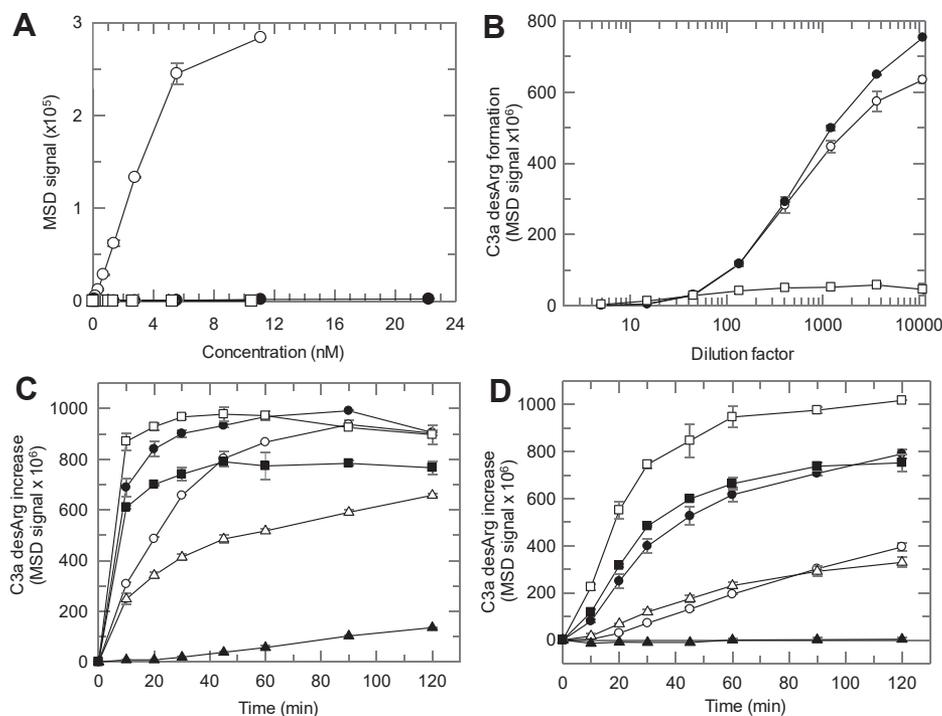


Figure 3. MSD method for determination of complement activation induced by Dezamizumab-SAP complex in human serum. A, specificity of the coating antibody. Purified C3 (■), C3a (●), C3a desArg (○) and iC3b (□) were diluted to various concentrations up to ~22.2 nM and captured in an MSD plate coated with neo-epitope of human C3a/C3a-desArg monoclonal antibody. In B–D, pooled human serum from Complement Technology was used and the final serum volume was 70% in the complement mixture. B, impact of dilution factor on quantitation of C3a desArg formation yielded from complement activation. Dezamizumab and SAP antigen was mixed at a 4:1 ratio (μg/ml) in the complement activation mixture. The final Dezamizumab concentration was 500 μg/ml (○), 250 μg/ml (●), and serum-only control (□). The reaction was terminated after 40 min at ambient temperature. The complement activation mixture and serum-only control were diluted with HBSP buffer at a range from 5 to 10,935-fold in threefold serial dilution before testing in the MSD assay. C, time course of complement activation at 37 °C. D, time course of complement activation at ambient temperature. In both C and D, Dezamizumab:SAP antigen ratio was 4:1 in μg/ml in the complement mixture. Complement activation was terminated with the stop solution at the indicated time. The complement activation mixtures were diluted 10,000-fold before testing in the MSD method. The MSD signal was back calculated by dilution factors and normalized by subtracting T = 0 signal. The final Dezamizumab concentration was 1000 μg/ml (○), 500 μg/ml (●), 250 μg/ml (□), 125 μg/ml (■), 62.5 μg/ml (Δ), and serum-only control (▲). Error bars for all panels represent the mean ± SD from n = 2 determination.

human serum within 2 h (Fig. 3C, ▲). Addition of the Dezamizumab–SAP complex induced complement activation, which increased rapidly in 10 to 20 min and reached equilibrium after 45 min. These results indicated that the complement pathway can have a low basal activation in human serum even without antigen–antibody complex formation. In contrast at ambient temperature, without addition of Dezamizumab–SAP complex, C3a desArg did not increase in human serum for up to 2 h indicating that the complement pathway has minimal spontaneous activation at ambient temperature. Meanwhile C3a desArg increase from complement activation induced by Dezamizumab–SAP complex was relatively linear up to 45 min (Fig. 3D). Therefore, complement activation at the ambient temperature appeared to be optimal and linear over 45 min in the MSD method and yielded the lowest background activity from human serum with high complement activity.

Dezamizumab and SAP concentrations affected complement activation significantly. As shown in Figure 3, C and D, with a Dezamizumab:SAP antigen concentrations of 250 μg/ml Dezamizumab to 62.5 μg/ml SAP (molar ratio 6.3:1) at 37 °C and ambient temperature resulted in maximal formation of C3a desArg. Higher and lower concentrations of Dezamizumab and SAP resulted in less and slower C3a desArg

formation. Surprisingly, significantly slower and less C3a DesArg formation was detected with 1000 μg/ml Dezamizumab and 250 μg/ml (Fig. 3, C and D) suggesting that very high Dezamizumab and SAP concentration inhibit complement activation. The impact of the ratio between Dezamizumab and SAP on complement activation in human serum was further evaluated and described in a later section.

HTRF method to detect C3 depletion from complement activation in human serum induced by Dezamizumab-SAP complex

A second method developed to measure complement activation used the HTRF method to detect C3 depletion from complement activation in human serum and is illustrated in Figure 2B. A pair of labeled antibodies binding to C3 at different regions was used. Antibody 7C12, which binds to an N-terminal proximal region of C3, was labeled with terbium and serves as a donor. A second antibody, K13/16, which binds the N-terminal C3a region of C3, was labeled with d2 and served as an acceptor. In the C3 HTRF method, binding signals are generated through fluorescent resonance energy transfer between the donor (Tb-labeled anti-C3) and the acceptor molecule (d2-anti-C3) when both bind to the same C3

molecule and are close enough to each other. The cleavage of C3 by C3 convertase activity will break the energy transfer and cause signal decrease.

To use this method, we first established the dilution factor needed to quantitate C3 change in human serum following activation by Dezamizumab-SAP complex. Dilutions were evaluated at 20, 40, 100, 250, 500, 1000, and 2000-fold dilution following complement activation. C3 signal from serum alone was highest with the dilution factors ranging from 20 to 100-fold (Fig. 4A). The complement activation after 10 min reaction at room temperature containing 250 $\mu\text{g}/\text{ml}$ of Dezamizumab and 62.5 $\mu\text{g}/\text{ml}$ showed a decrease of C3 signal with various dilution factors due to significant C3 cleavage. Low dilution factor (40- to 100-fold) was used for all serum samples before C3 detection by the HTRF method because C3 signal was highest from serum-only control and C3 depletion window was best between serum-only control and complement activation induced by Dezamizumab-SAP complex.

Based on the results of C3a desArg formation in the MSD method (Fig. 3, C and D), the time and temperature dependence of Dezamizumab-SAP complex induced complement activation in human serum was determined at the ambient temperature only because the complement activation at 37 °C was too fast to be quantitated accurately. In human serum alone, C3 decreased <12% in 50 min (Fig. 4B). In complement activation induced by Dezamizumab-SAP complex, C3 decreased 54% in 10 min and 84% in 50 min and reached equilibrium after 40 min (Fig. 4B).

We next investigated the time needed for the anti-human C3 antibody (K13/16)-d2 and the anti-C3 (7C12/C3b)-Tb antibody to bind to C3 in human serum. This was accomplished by reading the plate after the plate sat at ambient temperature for 4 h and ~24 h respectively. C3 depletion was similar when reading the plate after 4 h and ~24 h suggesting that 4 h incubation time was enough for the Tb and d2-conjugated antibodies to bind to C3 in human serum.

Effect of Dezamizumab to SAP ratio and concentration on complement activation

To investigate the optimal ratio of Dezamizumab to SAP on complement activation, the ratio of Dezamizumab to SAP was varied to achieve a molar ratio range from 1.3 to 84.4 between Dezamizumab and SAP. Three controls were evaluated including (1) serum only, (2) no Dezamizumab addition, but SAP was added at a final concentration ranging from 0.29 to 150 $\mu\text{g}/\text{ml}$ (eq. 0.0012–0.64 μM), and (3) no SAP addition, but Dezamizumab was added at a final concentration from 15.6 to 1000 $\mu\text{g}/\text{ml}$ (eq. 0.105–6.74 μM). SAP alone without addition of Dezamizumab at the indicated concentrations failed to activate complement as expected (Fig. 5B). Dezamizumab alone without addition of SAP activated complement in a bell shape at the indicated concentrations (Fig. 5C). The highest increase was 3.5-fold of C3a desArg formation above serum only and was observed at 0.84 μM (eq. 125 $\mu\text{g}/\text{ml}$) of Dezamizumab. Higher or lower Dezamizumab concentration than 125 $\mu\text{g}/\text{ml}$ without addition of SAP all yielded lower C3a desArg formation due to the limited availability of endogenous SAP in human serum (Fig. 5C). Therefore, the addition of both Dezamizumab and SAP is required to achieve optimal complement activation.

To determine the optimal ratio of Dezamizumab to SAP on C3a desArg formation, various ratios of Dezamizumab and SAP were tested. Maximal activation was achieved with a Dezamizumab:SAP molar ratio of 5.3:1 (Fig. 5A). At this ratio, a >12-fold increase of complement activation compared with serum alone was observed. Higher or lower Dezamizumab:SAP ratios resulted in lower C3a desArg formation (Fig. 5A). Dezamizumab:SAP molar ratios in the range of 3.6 to 9.9 were further evaluated. Complement activation showed a slight bell shape dose response curve with varying Dezamizumab:SAP ratios (Fig. 5D). Complement activation appeared maximal with Dezamizumab:SAP ratio between 4:1 and 7:1. Therefore, the optimal Dezamizumab:SAP ratio was selected to be 4:1 in $\mu\text{g}/\text{ml}$ (eq. 6.3:1 in molar ratio) for best

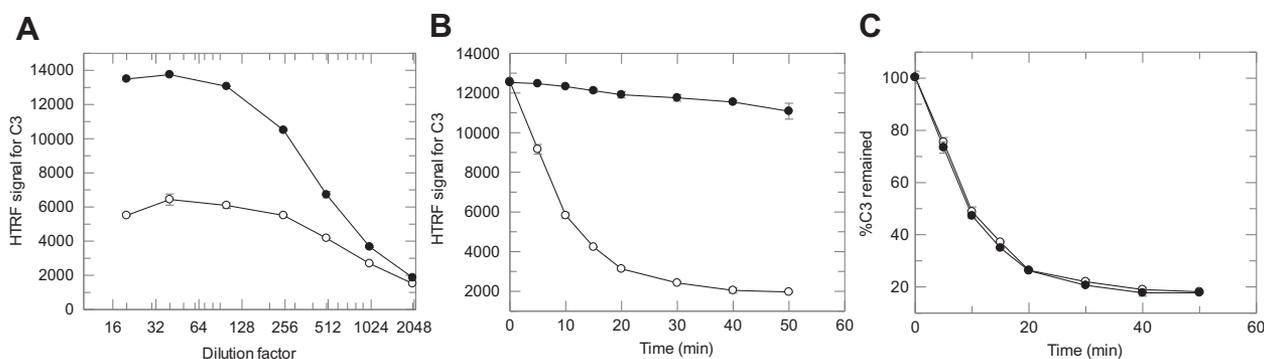


Figure 4. HTRF method for determining complement activation induced by Dezamizumab-SAP complex in human serum. Pooled serum from Complement Technology was used. The final serum volume was 40% and Dezamizumab:SAP antigen ratio was 4:1 in $\mu\text{g}/\text{ml}$ (molar ratio 6.3:1) in the complement activation mixture. **A**, impact of dilution factor on quantitation of C3 depletion yielded from complement activation. The complement activation mixture containing 250 $\mu\text{g}/\text{ml}$ of Dezamizumab (○) or serum-only control (●) and was terminated after 10 min at ambient temperature. The complement activation mixture and serum-only control were diluted with HTRF buffer ranging from 20 to 2000-fold. **B**, complement activation time course at ambient temperature. Complement activation mixture containing 250 $\mu\text{g}/\text{ml}$ of Dezamizumab (○) and serum-only control (●) was terminated at the indicated time with the stop solution. The complement activation mixtures were diluted 100-fold before testing in the HTRF method. **C**, impact of HTRF binding time before plate reading for C3 signal. The HTRF plate prepared as described in **B** containing 250 $\mu\text{g}/\text{ml}$ of Dezamizumab in the complement activation mixture was read at 4 h (○) and ~24 h (●) after incubation with Tb and d2-conjugated antibodies at ambient temperature. %C3 remained was calculated by HTRF signal from complement activation divided by the signal from serum-only control. Error bars for all panels represent the mean \pm SD from $n = 2$ determination.

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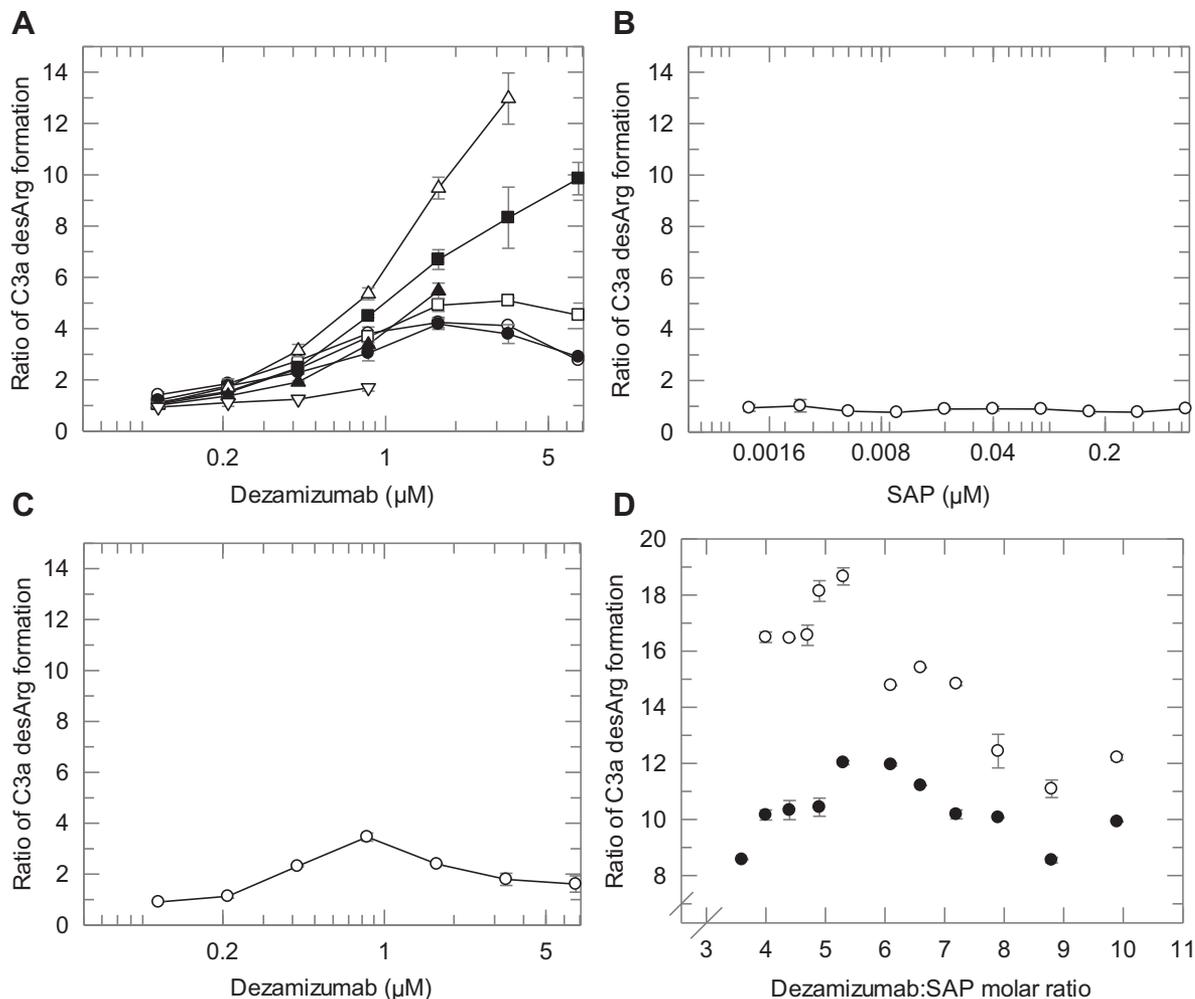


Figure 5. Effect of Dezamizumab:SAP ratio and concentration on complement activation. MSD method was used. Pooled human serum from Complement Technology was used and the final serum volume was 70%. After 40 min at the room temperature, the complement activation was terminated and diluted 10,000-fold to detect for C3a desArg formation. The Ratio of C3a desArg formation was calculated by sample signal divided by serum-only signal. *A*, two-fold serial dilutions were performed for Dezamizumab and SAP respectively. The diluted Dezamizumab and SAP were added to the assay plate followed by the addition of human serum with a final volume of 70%. After 40 min at ambient temperature, the complement activation was terminated with the stop buffer. The final Dezamizumab concentration in the complement activation ranged from 15.6 to 1000 µg/ml (eq. 0.105–6.74 µM) and SAP ranged from 0.29 to 150 µg/ml (eq. 0.0012–0.64 µM). C3a desArg formation was detected using the MSD method. The signal ratio of complement activation versus an average of serum-only background from eight determinations was plotted against the indicated Dezamizumab concentration at the indicated ratio between Dezamizumab and SAP in molar. Dezamizumab:SAP molar ratios were 84.4 (○), 42.2 (●), 21.1 (□), 10.6 (■), 5.3 (△), 2.6 (▲), and 1.3 (▽). *B*, SAP concentrations without the addition of Dezamizumab. SAP ranged from 0.29 to 150 µg/ml (eq. 0.0012–0.64 µM). *C*, Dezamizumab concentrations without the addition of SAP. Dezamizumab ranged from 15.6 to 1000 µg/ml (eq. 0.105–6.74 µM). *D*, Dezamizumab was mixed with SAP at the indicated molar ratio ranging from 3.6 to 9.9. The final Dezamizumab concentration was 500 µg/ml (○) or 250 µg/ml (●). Error bars for all panels represent the mean ± SD from *n* = 2 determination.

complement signal window between complement activation-induced Dezamizumab–SAP complex and serum-only control.

At the optimal Dezamizumab:SAP ratio, the highest Dezamizumab concentration that can be used in dose–response determination was 500 µg/ml (eq. 3.4 µM). A trend of decreasing complement activation was observed with 1000 µg/ml of Dezamizumab (eq. 6.7 µM) in Figure 5A indicating that high concentration of Dezamizumab may not be favorable for complement activation.

Minimal human serum component needed for complement activation

Since human serum was used as the complement source containing all components, the minimal serum component

needed to yield high complement activation was determined. Dezamizumab:SAP at a ratio of 4:1 in µg/ml (6.3:1 in molar) were mixed with different percent of human serum. C3a desArg formation increased >3.4-fold from complement activation induced by Dezamizumab-SAP comparing to serum alone with the serum component ranging from 40 to 70% in the presence of 250 µg/ml of Dezamizumab and 62.5 µg/ml of SAP (Fig. 6). When the serum component was reduced to 10 to 20% in the presence of 100 µg/ml of Dezamizumab and 25 µg/ml of SAP, a decrease of complement activation was observed, while complement activation remained high at 40% serum close to 40 to 70% (Fig. 6). Therefore, the minimal serum component in the assay mixture required for full complement activation is ≥40%.

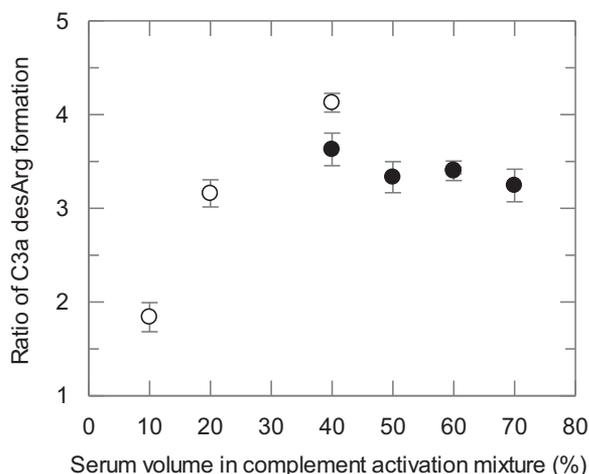


Figure 6. Minimal human serum component needed for complement activation. Dezamizumab: SAP antigen ratio was 4:1 in µg/ml (molar ratio 6.3:1) in the complement activation mixture. The final Dezamizumab concentration was 100 µg/ml (○) and 250 µg/ml (●). Pooled human serum from Innovative Research was used. The final serum volume in the complement activation mixture ranged from 10% to 70%. After 45 min at ambient temperature, complement activation was terminated and diluted 3645 and 10,935-fold respectively to detect C3a desArg formation in the MSD method. The ratio of C3a desArg formation was calculated by sample signal divided by corresponding serum-only signal. The ratio of C3a desArg formation from the 3645 and 10,935-fold dilution was plotted against percentage of serum volume in complement activation mixture. Error bars represent the mean ± SD from $n = 2$ determination.

Human serum and plasma as complement sources

The components of complement system such as inactive C1 circulate as a serum molecular complex. To identify the optimal complement sources yielding the lowest complement activation from nonclassical pathways and the maximal complement activation from the antigen-antibody complex induced classical pathway, we evaluated various complement sources including pooled human serum or plasma from different vendors and freshly prepared serum or plasma from individual donors.

Pooled human serum from Innovative Research yielded two to fivefold increase in C3a desArg at an optimal Dezamizumab-SAP complex (Fig. 6). Pooled human serum or plasma from several other vendors showed similar or less complement activation than the serum from Innovative Research (data not shown). In our hands, pooled human serum from Complement Technology showed good results of 16 to 20-fold increase in complement activation induced by Dezamizumab-SAP complex comparing to the serum-only control for C3a desArg formation in the MSD method (Figs. 7C and 8C). It is also a good source for C3 depletion in the HTRF method (Figs. 7D and 8D).

To compare the complement source between freshly prepared and pooled human serum or plasma, individual donors were recruited. Human serum or plasma was prepared as described in Experimental procedures. Complement activation induced by optimum Dezamizumab-SAP complex was significantly higher in serum than plasma indicating that serum is a better complement source than plasma. High variability in complement activation was observed between human serum samples from three individual donors.

Complement activation induced by optimal Dezamizumab-SAP complex from serum from different donors varied between 4 and 18-fold (Fig. 7B).

To mitigate the risk of high variability from complement among different donors, the pooled serum sources were used. Pooled human serum from Complement Technology showed similar complement activation to the best individual donors induced by Dezamizumab-SAP complex (Fig. 7, C and D).

Correlation between C1q binding and complement activation induced by Dezamizumab-SAP complex

The mechanism of action (MOA) for amyloid clearance by Dezamizumab includes: (1) Dezamizumab binding to SAP associated with amyloid deposits in the tissues; (2) activation of complement to attract and activate macrophages to mediate phagocytosis and clearance of the SAP deposits. To monitor the critical quality attributes of Dezamizumab, we developed the following MOA-reflective methods: (1) SAP binding to Dezamizumab by SPR method using Biacore; (2) C1q binding to Dezamizumab-SAP complex by SPR method; (3) relative potency of Dezamizumab to induce complement activation in human serum by the HTRF method to detect C3 depletion and (4) complement activation detected by the MSD method. To compare binding activity and potency, thermal-stressed Dezamizumab samples were generated by storage in a formulation buffer at pH 7.0 for 3 months at 2 to 8 °C, 25 °C, and 40 °C. These samples were tested in the binding and complement activation assays.

Dezamizumab binding to SAP was affected significantly by the storage temperature. Dezamizumab binding toward SAP was determined using a Biacore instrument and a protein A-immobilized chip as described in Experimental procedures. A calibration curve was generated from Dezamizumab reference standard. Dezamizumab binding concentrations to protein A and SAP antigen was extrapolated from the corresponding calibration curves. The capture of Dezamizumab samples on the protein A chip was constant irrespective of storage temperature (data not shown) indicating that Dezamizumab binding to protein A on the chip was not affected by storage conditions. Dezamizumab stored at 2 to 8 °C showed insignificant changes for SAP binding comparing to the reference standard (RS) for SAP binding, 94% binding activity comparing to the RS, indicating that Dezamizumab was stable in general when stored at 2 to 8 °C for 3 months. The Dezamizumab binding activity to SAP decreased from 94% for 2 to 8 °C to 84% for 25 °C and 51% for 40 °C of storage. Since the Fab region of an mAb is responsible for antigen binding, the loss of Dezamizumab binding activity to SAP at higher storage temperature was likely due to the changes of the Fab region in Dezamizumab (Table 1).

C1q binding activity to Dezamizumab-SAP complex also decreased dramatically for Dezamizumab stored at higher temperature. The C1q binding activity to Dezamizumab was determined using a C1q immobilized chip and a Biacore instrument as described in Experimental procedures. As anticipated, neither Dezamizumab nor SAP alone bound C1q (data

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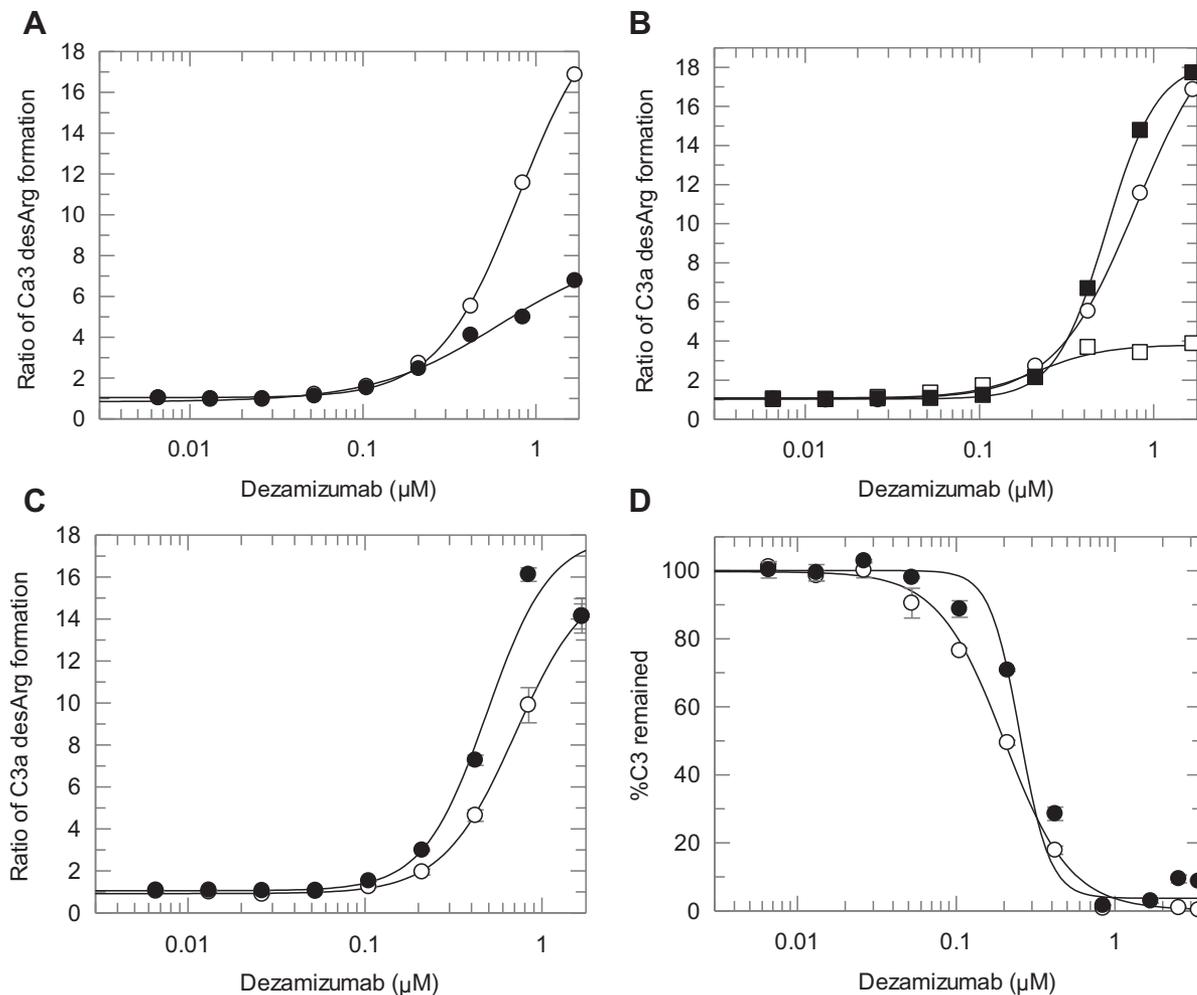


Figure 7. Evaluation of complement sources. Dose–response determination was performed with a final Dezamizumab concentration in complement activation mixture ranged from 0.007 to 3.36 μM and a fixed Dezamizumab:SAP molar ratio of 6.3:1. The final serum volume in the complement activation mixture was 40%. After 40 min at the room temperature, the complement activation was terminated and detected by the MSD (A–C) or HTRF method (D). A, human serum (○) and plasma (●) prepared from the fresh blood of a single donor. B, human serum prepared from the fresh blood of donor 1 (○), donor 2 (□), and donor 3 (■). In A and B, data were from a single determination due to the limited amount available from freshly prepared serum and plasma. C and D, human serum (○) prepared from the fresh blood of a single donor was compared with the pooled serum from Complement Technology (●) in the same experiment by the MSD method in C and HTRF method in D. In C and D, error bars represent the mean \pm SD from $n = 2$ determination.

not shown). Only the Dezamizumab–SAP complex bound C1q yielded high binding responses. Dezamizumab stored at 2 to 8 $^{\circ}\text{C}$ showed insignificant changes comparing to RS for C1q binding, 99% binding activity comparing to the RS, indicating that Dezamizumab was stable when stored at 2 to 8 $^{\circ}\text{C}$ for 3 months. C1q binding activity to Dezamizumab–SAP complex decreased from 99% for 2 to 8 $^{\circ}\text{C}$ to 75% for 25 $^{\circ}\text{C}$ and 16% for 40 $^{\circ}\text{C}$ of storage. Since C1q binds to the hinge region of a mAb, the further decrease of C1q binding in addition to the decrease of SAP binding indicating that there were also changes in the hinge region of Dezamizumab after thermal stress (Table 1).

A dose–response determination of Dezamizumab–SAP complex to measure complement activation was performed as described in Experimental procedures. %RP of Dezamizumab sample against the RS was calculated and compared by two approaches. Approach 1 was calculated by dividing the EC₅₀ of Dezamizumab RS by the EC₅₀ of a sample. Approach 2 was calculated by dividing the Y-range (upper asymptote

value minus lower asymptote value) of a sample by the Y-range of Dezamizumab RS. The potency of the Dezamizumab samples against the RS was determined by both MSD method for C3a desArg formation and HTRF method for C3 depletion in human serum. Like SAP and C1q binding results, Dezamizumab stored at 2 to 8 $^{\circ}\text{C}$ showed similar potency to the RS in complement activation regardless human serum sources (Fig. 8 and Table 1). These results were consistent with the SAP and C1q binding results indicating that Dezamizumab stored at 2 to 8 $^{\circ}\text{C}$ was stable for 3 months.

Dezamizumab stored at 25 $^{\circ}\text{C}$ showed a different dose response in the MSD and HTRF methods. In the MSD method, C3a desArg formation reached the plateau lower than Dezamizumab RS, but EC₅₀ shift was insignificant comparing to the RS (Fig. 8, A and C, and Table 1). %RP using Approach 2 showed a good correlation (73% RP) to the C1q binding results for the Dezamizumab–SAP complex (75% activity) (Table 1). In the HTRF method, the C3 depletion range was similar between the Dezamizumab sample and RS, but EC₅₀ shifted to

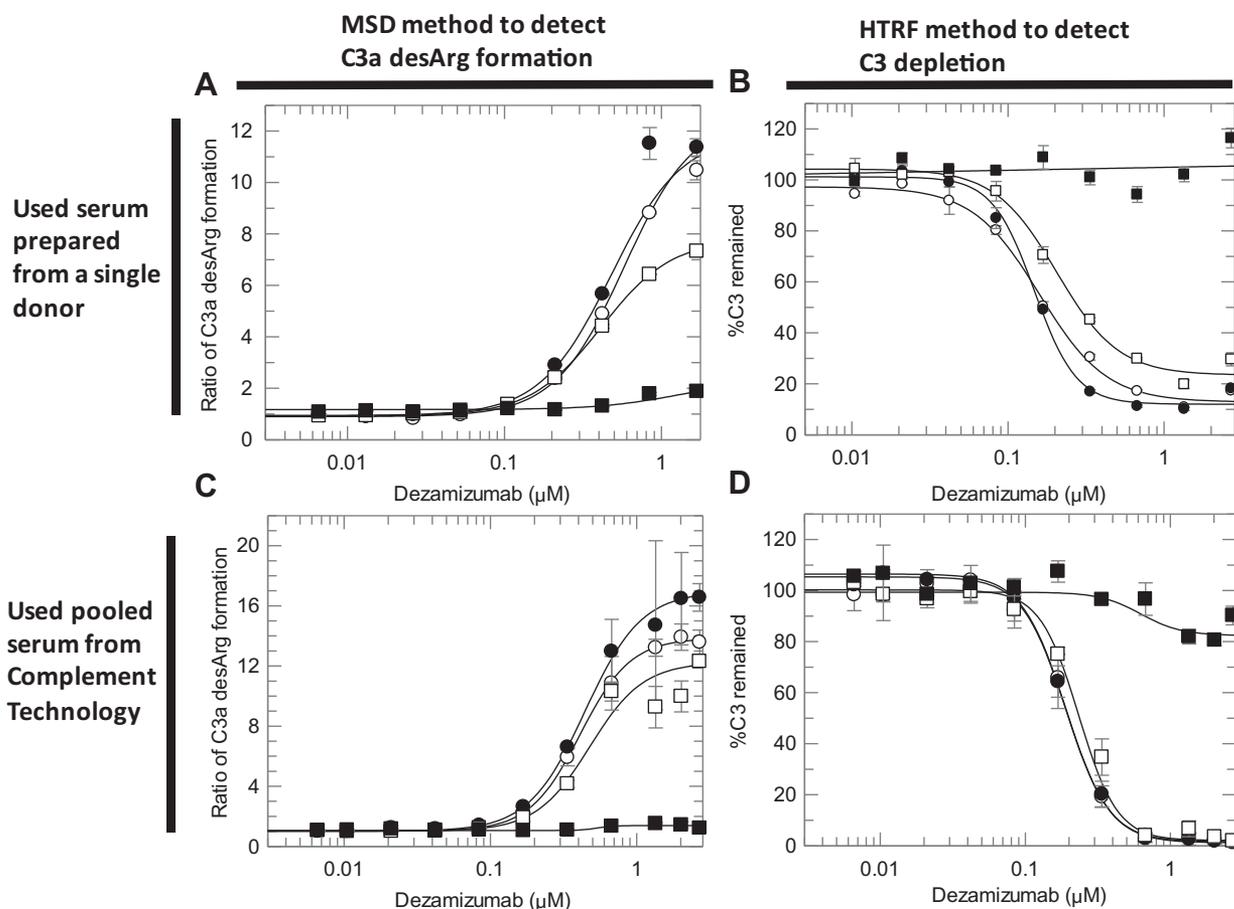


Figure 8. Relative potency determination between Dezamizumab reference standard and the thermal-stressed samples. Dose–response determination was performed with Dezamizumab reference standard (○), and Dezamizumab stored in a formulation buffer at pH 7.0 for 3 months at 2 to 8 °C (●), 25 °C (□), and 40 °C (■), or reference standard stored at –70 °C (○). Human serum in the complement activation mixture was either prepared from the blood of a single donor (A and B) or the pooled serum purchased from Complement Technology (C and D). C3a desArg formation by MSD method (A and C) and C3 depletion by HTRF method (B and D) were determined. The twofold serial dilution was performed with a fixed Dezamizumab:SAP molar ratio of 6.3:1. The final Dezamizumab concentration in the complement activation mixture ranged from 0.007 to 3.36 μM. The final serum volume in the complement activation mixture was 40%. The complement activation time was 40 min for A, C, and D, and 10 min for B at the ambient temperature. The complement mixture was diluted 10,000-fold for A and C in the MSD method, and 100-fold for B and 40-fold for D in the HTRF method. The ratio of C3a desArg formation in Dezamizumab samples to serum-only control was plotted against Dezamizumab concentration in the MSD method. Similarly, the %C3 remained in Dezamizumab samples versus the serum-only control was plotted against Dezamizumab concentration in the HTRF method. The average of n = 2 data with ± SD was fit using the full four-parameter EC50 model as described in [Experimental procedures](#). Error bars represent the mean ± SD.

higher value (Fig. 8, B and D). %RP using Approach 1 showed a good correlation (76% RP) to the C1q binding results (75% activity) (Table 1).

Comparing to serum-only control, Dezamizumab stored at 40 °C for 3 months showed very low C3a desArg formation (Fig. 8, A and C) and no significant C3 depletion (Fig. 8, B and D). These results were consistent with the result from C1q binding to the Dezamizumab–SAP complex (16% activity) (Table 1). Therefore, the loss of C1q binding to Dezamizumab–SAP complex also led to the loss of complement activation.

Discussion

Methods to determine complement activation in human serum directly induced by antigen–antibody complex through the classical pathway are not available due to many technical challenges. One of them is to obtain a good complement

source with minimal existed complement activation. A good complement source must contain all necessary complement components to yield high readout induced by antigen–antibody complex. However, alternative complement pathway activation can be easily induced in human serum by handling (9–11) yielding high complement background irrelevant to the classical pathway. In addition, the health status of a donor can also affect complement level leading to high variability.

Both serum and plasma are prepared from human blood and can be a complement source. Although both plasma and serum can be extracted from blood by centrifugation, anticoagulants are necessary to separate plasma, but are not used to obtain serum. Anticoagulant treatment (heparin) was used when we prepared plasma. Complement activation induced by Dezamizumab–SAP complex was significantly higher in serum than plasma suggesting that serum is a better complement source than plasma (Fig. 7A). Heparin is a natural polydisperse

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Table 1

Comparison of binding activities and relative potency of complement activation in human serum

Dezamizumab sample	%Dezamizumab binding to SAP ^a	%Dezamizumab-SAP binding to C1q ^a	%Relative potency against Dezamizumab reference standard ^b			
			MSD method		HTRF method	
			Approach 1 ^c	Approach 2 ^d	Approach 1 ^c	Approach 2 ^d
2–8 °C for 3 m	94	99	107 ± 20	109 ± 22	98 ± 10	104 ± 8
25 °C for 3 m	84	75	113 ± 39	73 ± 20	76 ± 5	109 ± 4
40 °C for 3 m	51	16	0	6 ± 5	0	0

^a Dezamizumab-binding activities for SAP and C1q were determined by Biacore instruments as described in [Experimental procedures](#). The assay variability was ±5% coefficient of variation (CV).

^b Average and standard deviation (SD) of % relative potency were calculated from the experiments using either in-house prepared human serum from a single donor or the pooled serum from Complement Technology. The average results in the HTRF methods were from the complement activation for 10 min at the ambient temperature and 40 and 100-dilution of the mixture before detection for C3.

^c Approach 1: %RP = $\frac{\text{EC50 of Dezamizumab reference standard}}{\text{EC50 of Dezamizumab sample}} \times 100\%$

^d Approach 2: %RP = $\frac{\text{Range of Dezamizumab sample}}{\text{Range of Dezamizumab reference standard}} \times 100\%$

sulfated copolymer of glucosamine and uronic acid. Fluid phase of heparin was reported to inhibit the interactions of C1, C4, and C2 during formation of the classic pathway C3 convertase (12–15). C1 inhibition by heparin-coated polyvinyl chloride tubing was also reported by increased levels of C1rs–C1-inhibitor complexes (16). Therefore, the lower complement activation induced by Dezamizumab–SAP complex in the prepared plasma could be due to using heparinized anticoagulant-treated tubes. Although we have not evaluated the impact of plasma prepared with other types of anticoagulant-treated tubes on complement activation, comparison between pooled human serum and plasma from several commercial sources showed that human plasma was inferior to serum in complement activation induced by Dezamizumab–SAP complex (data not shown). When generation of C3 activation products was compared at different temperatures *versus* incubation time among pools of serum, heparin-, citrate-, and EDTA-plasma, C3 activation products increased faster in serum than those in plasma at 37 °C (17). Similarly, the levels of complement biomarkers were also reported significant higher in serum than those in plasma (18). The classical pathway has a calcium-dependent step (C1q, C1r, C1s) and a magnesium-dependent reaction (the enzymatic action of C1s on C4 and C2) (19). Each SAP subunit also contains two calcium-binding sites essential for ligand binding (20). The effect of EDTA and citrate in preventing complement activation can be mediated *via* their binding of calcium and magnesium. Therefore, human serum is likely a better complement source than plasma for determination of antigen–antibody complex induced complement activation.

Another challenge in determining complement activation in human serum induced by antigen–antibody complex was to identify measurements correlated to the classic pathway directly. Although the complement system contains many components, C3 convertase (C4bC2a in [Fig. 1](#)) activation in classical pathway is a direct result of C1r and C1s activation triggered by antigen–antibody complex binding to C1q. Therefore, we decided to determine the cleavage of C3 by C3 convertase (C4bC2a in [Fig. 1](#)) using the HTRF method. We also targeted the determination of the C3 fragments yielded

from C3 cleavage. Since C3a and C3b are relatively short-lived in human serum, we tried to determine C3a desArg and iC3b that are the inactive and stable forms of C3a and C3b. The determination of iC3b by the MSD method was not successful due to poor specificity of the neo-antibody. However, we did identify a highly specific neo-antibody against C3a desArg allowing us to establish the MSD method to detect C3a desArg formation.

C3 cleavage of C3 convertase in human serum resulted from Dezamizumab–SAP complex induced complement activation was very fast even at ambient temperature. The reaction was linear over 10 min and reached equilibrium after 40 min ([Fig. 4B](#)). The relative potency of thermally stressed Dezamizumab samples were similar when the reaction time was 40 min ([Fig. 8D](#)) comparing to 10 min ([Fig. 8B](#)) except that slight increase of C3 depletion for Dezamizumab at 40 °C in 3 months was observed. In the MSD method, C3a desArg increase from Dezamizumab–SAP complex induced complement activation was relatively linear within 45 min at ambient temperature ([Fig. 3D](#)). Since C3a desArg was produced from a two-step cleavage from C3 by two proteases, C3 convertase and carboxypeptidase B, it is not surprising that the formation of C3a desArg was slower than C3 depletion. To ensure the methods are sensitive to determine the decrease of activity for degraded Dezamizumab, we had chosen the reaction time at the linear range (10 min for C3 depletion and 30–40 min for C3a desArg formation) at ambient temperature.

The third challenge in determining complement activation in human serum induced by Dezamizumab-SAP complex was to identify the optimal conditions for the antibody to antigen ratio allowing formation of oligomers that are required for C1q-binding and activation. In the classical complement pathway, antibodies can assemble at the cell surface to form hexameric structures that intern recruit C1q and induce complement activation (21). C1q structure resembles a tulip and six of these tulips assemble into a bundle (22). One subunit in the tulip is formed by a trimer of C1q A, B, and C chains that assemble into a collagen-like stalk connected to a globular headpiece (23). C1q-binding sites are found in the two Fc-CH2 domains of each IgG molecule (24). Only as oligomers

is IgG able to have sufficient binding avidity to activate complement (25–27).

Although SAP as the antigen is not bound to the cell surface, it is a pentameric glycoprotein consisting of five identical 25 kDa subunits arranged noncovalently in a flat donut-like configuration (20). SAP is secreted by the liver, and the plasma concentration of SAP is approximately 30 µg/ml (28). Importantly for complement activation, SAP exists as a pentamer *in vivo*. SAP as the pentamer has been shown to exist in serum or *in vitro* in the presence of physiological albumin concentrations (29). Without addition of SAP, complement activation with Dezamizumab alone was present but significantly lower than with the addition of SAP (Fig. 5, A and C). The results suggest that endogenous SAP in normal serum was suboptimal at forming an oligomer complex with Dezamizumab at concentrations higher than 125 µg/ml (eq. 0.84 µM).

The ratio between Dezamizumab and SAP affected complement activation significantly (Fig. 5, A and D). The purified SAP was assumed to be a decamer (two pentamer sandwiches) according to the vendor. High or low Dezamizumab:SAP ratio all led to low or nondetectable complement activation due to not being able to form enough oligomers to trigger the classical pathway. Complement activation appeared to be the highest for Dezamizumab:SAP molar ratio between 4 and 7 (Fig. 5D) suggesting that an average of six Dezamizumab binding to one SAP decamer to form a complex for C1q-binding avidity was best to activate complement. Also, one Dezamizumab appeared to bind to two 25 kDa subunits of SAP.

Interestingly, hook effects were observed at the highest Dezamizumab concentration. When Dezamizumab concentrations were >250 µg/ml (eq. 1.7 µM), unexpected slower formation and decrease of C3a desArg formation were observed (Fig. 3, C and D). Significant slower C3a desArg formation was detected in the presence 1000 µg/ml of Dezamizumab and 250 µg/ml of SAP (molar ratio of 6.3) (Fig. 3, C and D), suggesting that at these higher Dezamizumab concentrations, Dezamizumab was slower to form a favored complex to activate complement effectively.

The shape of dose–response curves for complement activation is interestingly different between the two methods for thermal-stressed Dezamizumab. As described previously, %RP was calculated by two approaches. Approach 1 is based on EC50 changes of Dezamizumab–SAP complex that gives half-maximal response. Approach 2 is based on the changes on Y-range (upper asymptote value minus lower asymptote value of response). The HTRF method showed a good correlation between % RP by Approach 1 based on EC50 change and the C1q-binding results (Table 1). The Y-range in %C3 depletion was comparable among Dezamizumab RS, 2 to 8 °C and 25 °C samples while the 25 °C sample showed an EC50 shift due to degradation (Fig. 8, B and D). Dezamizumab at 40 °C degraded severely and lost activity to induce complement activation. As a result, no significant C3 depletion was detected (Fig. 8, B and D). In the MSD method, C3a desArg was formed after two cleavage steps as described previously. The method showed a good correlation between %RP using Approach 2 based on

Y-range and the C1q-binding results (Table 1). Like the HTRF method, Dezamizumab at 40 °C degraded severely and C3a desArg formation was very low. However, %RP using Approach 1 based on EC50 change was not significantly different among Dezamizumab RS, 2 to 8 °C and 25 °C sample unexpectedly. Several factors could contribute to the unique mode of dose response in the MSD method: (1) Degraded Dezamizumab formed less Dezamizumab-SAP complex required to activate the classical complement pathway. The resulted lower complement activation led to less amount of C3a from C3 cleavage by C3 convertase. Since C3a is the substrate of carboxypeptidase B to form C3a desArg, the reaction velocity and equilibrium of C3a desArg formation were affected by C3a availability. (2) Less Dezamizumab–SAP complex formation due to Dezamizumab degradation resulted in higher concentration of unbound Dezamizumab and SAP that could inhibit complement activation at the higher concentration as discussed in the previous sections.

It is very exciting that the relative potency from MSD and HTRF methods showed a good correlation to Dezamizumab-SAP dependent C1q binding activity for Dezamizumab thermal stressed samples. This demonstrates that these methods reflect the MOA of Dezamizumab and are stability-indicating suitable to monitor Dezamizumab quality. The MSD method was more sensitive to the changes of C3a desArg formation in human serum and yielded higher signal ratio between complement activation induced by Dezamizumab–SAP complex and serum-only control. However, the MSD assay variability was significantly higher than the HTRF method (Table 1) likely due to more dilution, pipetting, and plate washing steps that were not needed in the HTRF method. Therefore, the HTRF method is a probably better choice in precision, time-saving, and ease of operation.

MSD and ELISA methods are similar in many ways including plate coating, blocking, washing, and incubation time for binding. Although the signal over background window for an MSD method may be better than an ELISA assay, the MSD method for detection of C3a desArg formation can be potentially converted into an ELISA method by replacing sulfo-tagged anti-human C3a/C3a desArg/C3 antibody (K13/16) (the detecting antibody in the MSD method) with a HRP-conjugated anti-human C3a/C3a desArg/C3 antibody (K13/16) if an MSD instrument is not available. In the HTRF method for C3 depletion, a pair of anti-C3 antibodies is commercially available and can be conjugated using the labeling kit or prepared directly by Cisbio. Any plate reader able to detect fluorescence resonance energy transfer can be used. The HTRF method is more user-friendly because of its easy operation and time-saving.

Experimental procedures

Materials and methods

Dezamizumab humanized monoclonal antibody reference standard and stressed samples were generated in-house. C1q and SAP from human serum were purchased from Complement Technology or Millipore Sigma respectively. Protein A

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was from Cytiva. Human serum was either prepared from individual donor or purchased as the pooled human serum from Innovative Research or Complement Technology. Anti-human Complement C3/C3b/iC3b (7C12/C3b)-Tb, anti-human C3a/C3a desArg/C3 (K13/16)-d2, and 96-well low volume white plate used in the HTRF method were purchased from Cisbio Assays. Monoclonal antibody 2991 recognizing the neo-epitope of human C3a/C3a-desArg used in the MSD method was from Hycult Biotech. Sulfo-tagged anti-human C3a/C3a desArg/C3 antibody was prepared by following the instruction of MSD and anti-human C3a/C3a desArg/C3 antibody (K13/16) was purchased from Biolegend. Gold Sulfo-tag NHS-ester, multi-array 96-well plate (L15XB), and MSD Read Buffer T (4×) were purchased from MSD. Blocker casein in PBS and 1.2 ml square well storage 96-well plate were purchased from Thermo Fisher Scientific. Phosphate Buffered Saline with Tween 20, pH 7.4 (PBST) was from Millipore Sigma. The solutions of 0.5 M EDTA at pH 8.0 and FUT-175 (Futhan) were purchased from Life Technologies and BD Biosciences respectively.

SAP binding assay by surface plasmon resonance (SPR) using Biacore

The SPR methods were carried out on the Biacore T100 or T200 instruments. Protein A was immobilized on two flow cells of a CM5 chip according to the instruction of amine coupling kit from Cytiva. Protein A was diluted to 0.4 mg/ml in 10 mM sodium acetate, pH 4.5, and injected over the activated surface.

PBST was used as the running buffer and for preparation of Dezamizumab standard curve, control, and samples. The Dezamizumab samples (diluted to 10 µg/ml within the standard curve range generated from Dezamizumab reference standard, 0–20 µg/ml) were injected over the protein A-immobilized chip at the second flow cell with a flow rate of 10 µl/min for 1 min. SAP protein at 20 µg/ml was then injected to both flow cells for 2 min and bound to the captured Dezamizumab reference standard, control, or sample. The nonspecific binding of SAP antigen to protein A chip was subtracted from the specific binding of Dezamizumab to SAP. The surfaces were regenerated with 10 mM glycine, pH 1.5, and 3 M Guanidine HCl after each sample injection to remove the bound complex.

The active concentration of Dezamizumab sample bound to SAP was calculated from the corresponding Dezamizumab reference standard calibration curve. The specific binding activity was calculated by dividing the concentration of Dezamizumab bound to SAP by the total protein concentration of Dezamizumab.

C1q binding assay by SPR using Biacore

Two flow cells on a CM5 chip were used in this method. The second flow cell was immobilized with C1q by amine coupling and the first flow cell was treated similarly but without injection of any ligand and used as a blank control for reference subtraction. Human C1q was diluted to 50 µg/ml in

10 mM MES buffer, pH 6.5, and injected over the activated surface.

The C1q binding assay for Dezamizumab at the presence of SAP utilized a mix-and-inject instrument function where equal volume of Dezamizumab and SAP were mixed in the microplate and then immediately injected over the C1q surface. HBSP running buffer was used containing 10 mM HEPES, 150 mM NaCl, 0.005% P20, pH 7.4. Dezamizumab samples (diluted to 25 µg/ml within the standard curve range generated from Dezamizumab reference standard, 10–40 µg/ml) complexed with 15 µg/ml SAP antigen were injected over the C1q-immobilized chip at a flow rate of 10 µl/min for 1 min. The surfaces were regenerated with 700 mM Guanidine/6 mM EDTA after each sample injection to remove the bound complex.

The active concentration of Dezamizumab sample bound to C1q at the presence of SAP was calculated from the corresponding Dezamizumab reference standard calibration curve. The specific binding activity was calculated by dividing the concentration of Dezamizumab bound to C1q by the total protein concentration.

Preparation of human serum and plasma from fresh blood

Human blood from a donor was collected with a test tube made from Becton Dickinson (BD). The BD Vacutainer was used for serum preparation and anticoagulant-treated tubes (heparinized) was used to prepare plasma. For serum preparation, blood was to clot without disturbing at room temperature for 30 to 35 min. The clot was removed by centrifugation at 3000g for 10 min. For plasma preparation, human cells are removed by centrifugation at 1500g for 10 min immediately after blood collection. The serum or plasma was transferred and aliquoted into a clean Eppendorf tubes at 2 to 8 °C and stored at –70 °C.

Complement activation induced by Dezamizumab-SAP complex in human serum

Dilution or serial dilution to target the final indicated concentration for Dezamizumab and SAP was performed with HBSP buffer. The diluted Dezamizumab and SAP were added to a 96-well plate followed by the addition of undiluted human serum with gentle mixing by multichannel pipette. The complement activation was set at ambient temperature (21–23 °C) or indicated temperature. After indicated reaction time, the complement activation was terminated by addition of 5× or 10× stop buffer to reach the final concentration of 4 mM EDTA and 0.2 mM futhan (30) or 40 mM EDTA. The final concentrations of Dezamizumab and SAP and volume of serum indicated in figures and tables were all based on the solution after addition of the stop buffer. The complement activation mixtures were further diluted with HBSP buffer before testing for C3a desArg in the MSD method or HTRF buffer containing 100 mM NaCl, 25 mM EDTA, 0.1% BSA, and 0.01% PS20 before testing C3 in the HTRF method.

MSD method to detect C3a desArg formation

The multi-array 96-well MSD plate was coated with 100 µl of 2 µg/ml monoclonal antibody 2991 recognizing the neo-

epitope of human C3a/C3a-desArg with KPL coating buffer at 4 °C overnight. The coating solution was discarded, and the plate was blocked with 200 µl of blocker casein in PBS at room temperature for 1 h. After discarding the blocker solution, 100 µl of diluted complement activation solution as described in the section of “Complement activation induced by Dezamizumab-SAP complex in human serum” above was added to the plate. After incubation at room temperature for 2 h with shaking, the plate was washed three times with PBST buffer. Then 100 µl of 1 µg/ml sulfo-tagged anti-human C3a/C3a desArg/C3 antibody (K13/16) was added to the plate. After incubation at room temperature for 2 h with shaking, the plate was washed three times with PBST buffer. After addition of 150 µl of 2× MSD Read Buffer T diluted from 4×, the plate was read within 5 min in Meso Sector S 600 Multi-array microplate reader.

The non-complement-activation background (buffer only) signal was subtracted from all serum-only controls and serum samples.

HTRF method to detect C3 depletion

In a 96-well low volume white plate, 20 µl of diluted complement activation solution as described in the section of “Complement activation induced by Dezamizumab-SAP complex in human serum” above was added. Then 10 µl of 4× anti-human C3a/C3a desArg/C3 (K13/16)-d2 diluted with the HTRF buffer was added followed by the addition of 4× anti-human Complement C3/C3b/iC3b (7C12/C3b)-Tb. The final concentration of anti-human C3a/C3a desArg/C3 (K13/16)-d2 and anti-human Complement C3/C3b/iC3b (7C12/C3b)-Tb was 10 nM and 0.4 nM, respectively. The plate was sealed to protect from evaporation and light and allowed binding overnight (~24 h) at room temperature. The plate without sealer was read by PerkinElmer Envision 2104 Multi-label Microplate Reader with LANCE/DELTA D400/630 dual mirror, UV (TRF320) dug11 excitation filter, and LANCE (APC, Alexa) 665/7.5 and Europium 615/8.5 emission filters.

$$\text{HTRF signal} = \frac{\text{Counts from 665 nm}}{\text{Counts from 615 nm}} \times 10000$$

The HTRF buffer-only background signal with the labeled detecting antibodies was subtracted from all serum-only controls and serum samples.

Dose-response determination of Dezamizumab-SAP complex induced complement activation in human serum

The molar concentrations of Dezamizumab and SAP were calculated based on the molecular weight (MW) of 148 kDa and 235 kDa, respectively. The MW of SAP was based on the information of the vendor assuming SAP to be a decamer. The dose-response determination of Dezamizumab-SAP complex induced complement activation was performed with final Dezamizumab concentration in the complement activation ranged from 3.36 to 0.007 µM with Dezamizumab:SAP molar ratio of 6.3:1 or as indicated. The final serum volume in the

complement activation mixture was 40%. The diluted Dezamizumab and SAP were added to the assay plate respectively followed by the addition of undiluted human serum with gentle mixing using a multichannel pipette. The complement activation was set at ambient temperature (21–23 °C) for 10 min (or indicated time) for HTRF assay or 40 min for MSD assay. The complement activation was terminated with the stop buffer as described previously. The complement activation mixtures were diluted 10,000-fold for MSD assay or in a range of 40 to 100 folds for HTRF assay.

In both methods, serum-only without addition of Dezamizumab and SAP was used as the background.

The signal ratio of Dezamizumab induced complement activation *versus* serum-only background shown as the ratio of C3a desArg formation in the MSD method and C3 depletion in percentage in the HTRF method was plotted against the indicated Dezamizumab concentration. The dose-response data was fit with the four-parameter equation using GraFit software as shown below:

$$y = \frac{\text{Range}}{1 + \left(\frac{x}{\text{EC50}}\right)^s} + \text{Background}$$

In this equation, Range is the fitted uninhibited value minus the Background, and *s* is a slope factor. EC50 is the concentration of Dezamizumab that gives half-maximal response.

%Relative potency (%RP) of Dezamizumab sample against the reference standard was calculated and compared by two approaches:

Approach 1:

$$\%RP = \frac{\text{EC50 of Dezamizumab reference standard}}{\text{EC50 of Dezamizumab sample}} \times 100\%$$

Approach 2:

$$\%RP = \frac{\text{Range of Dezamizumab sample}}{\text{Range of Dezamizumab reference standard}} \times 100\%$$

Data availability

All data essential for the conclusions are contained within the manuscript. Data not shown are available upon request.

Acknowledgments—We are grateful to Dr Jay Hsu for sharing HTRF design to detect C3 cleavage and anti-human Complement C3/C3b/iC3b (7C12/C3b)-Tb and anti-human C3a/C3a desArg/C3 (K13/16)-d2 purchased from Cisbio. We would also like to thank Hanwen Yang for testing Dezamizumab thermal-stressed samples for SAP binding activity using Biacore.

Author contributions—J. M. and J. R. W. conceptualization; J. M. data curation; J. M. and Q. L. formal analysis; J. M. investigation; J. M. and Q. L. methodology; J. M. supervision; J. M. validation; J. M. visualization; J. M. writing—original draft; J. R. W. writing—review and editing.

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Conflict of interest—The authors declare that they have no conflicts of interest with the contents of this article.

Abbreviations—The abbreviations used are: ADCC, antibody-dependent cell-mediated cytotoxicity; ADCP, antibody-dependent cellular phagocytosis; CDC, complement-dependent cytotoxicity; CP, classical pathway; FRET, fluorescence resonance energy transfer technology; HTRF, homogeneous time-resolved fluorescence; IgG, immunoglobulin G; LP, lectin pathway; mAb, monoclonal antibody; MAC, membrane attack complex; MSD, meso scale discovery; RS, reference standard; SAP, serum amyloid P.

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