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IgG Autoantibodies against Deposited C3 Inhibit Macrophage-Mediated Apoptotic Cell Engulfment in Systemic Autoimmunity

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Defective clearance of apoptotic cells has been shown in systemic lupus erythematosus (SLE) and is postulated to enhance autoimmune responses by increasing access to intracellular autoantigens. Until now, research has emphasized inherited rather than acquired impairment of apoptotic cell engulfment in the pathogenesis of SLE. In this study, we confirm previous results that efficient removal of apoptotic cells (efferocytosis) is bolstered in the presence of wild-type mouse serum, through the C3 deposition on the apoptotic cell surface. In contrast, sera from three mouse models of SLE, MerKD, MRLlpr, and New Zealand Black/WF1 did not support and in fact actively inhibited apoptotic cell uptake. IgG autoantibodies were responsible for the inhibition, through the blockade of C3 recognition by macrophages. Consistent with this, IgG removal reversed the inhibitory activity within autoimmune serum, and purified autoimmune IgG blocked both the detection of C3 on apoptotic cells and C3-dependent efferocytosis. Sera from SLE patients demonstrated elevated anti-C3b IgG that blocked detection of C3 on apoptotic cells, activity that was not found in healthy controls or patients with rheumatoid arthritis, nor in mice prior to the onset of autoimmunity. We propose that the suppression of apoptotic cell disposal by Abs against deposited C3 may contribute to increasing severity and/or exacerbations in SLE.  

The death and disposal of aged and damaged cells are essential for the maintenance of healthy tissues. In healthy individuals, apoptotic cells are cleared with remarkable efficiency. This is in part due to alterations of the dying cell surface, which are recognized as “eat me” signals by phagocytes, which ingest apoptotic cells through a process that has been termed efferocytosis (3). Numerous serum proteins, including C1q, IgM, C-reactive protein, and mannan-binding lectin, deposit on apoptotic cells, initiating and amplifying the deposition of C3 and its degradation products C3b and iC3b (5) and resulting in enhanced removal via recognition by complement receptors CR3 and CR4 (4, 6, 7).

The processes that govern dying cell clearance are defective in SLE. As a result, apoptotic cells accumulate in affected tissues (8–12). The persistence of apoptotic cells is thought to result in secondary necrosis and the release of proinflammatory and proimmunogenic intracellular constituents that contribute to the pathogenic autoantibody production. Therefore, insight into the pathways that govern apoptotic cell ingestion may be critical to uncovering the mechanisms of disease progression in SLE.

Reports correlating inefficient clearance with systemic autoimmunity have evoked inherited defects in either components of the apoptotic cell recognition mechanisms, such as C1q (13), or defects in the macrophages’ ability to phagocyte apoptotic cells (14–17). However, the contribution of acquired defects in efferocytosis (those that arise as a consequence rather than the cause of disease progression) in SLE has not been fully explored.

In the present work, we investigate the hypothesis that breaks in lymphocyte tolerance may precede and be the cause of apoptotic cell clearance defects in systemic autoimmunity. We show that IgG Abs that inhibit apoptotic cell uptake develop in three different strains of autoimmune mice. The inhibitory IgG Abs were directed against C3b components on the apoptotic cells, yet did not alter the total amounts or composition of the bound C3. Instead, they are suggested to block the interaction between C3b bound to the apoptotic cell surface and phagocyte C3 receptors. In mice, these Abs became apparent only as the animals developed their autoimmune state. Further analysis revealed higher titers of these Abs in patients with SLE compared with healthy control (HC) subjects or patients with rheumatoid arthritis (RA). Therefore, anti-C3 Abs that develop as a consequence of deficient lymphocyte tolerance may serve to initiate and/or exacerbate defects in apoptotic cell clearance and thereby accentuate the autoimmune and/or inflammatory processes in SLE.
Materials and Methods

**Mice**

C57BL/6, BALB/c, ICR, MRL, MRL\(^{+/+}\), and New Zealand Black (NZB)/WF1 mice were purchased from The Jackson Laboratory. Mer\(^{KD}\) mice were provided by Dr. Douglas Graham at the University of Colorado Denver Anschutz Medical Campus (Aurora, CO). C3\(^{−/−}\) mice were from Michael Carroll at Harvard University (Boston, MA). MRL mice were used as a control for MRL\(^{+/−}\)Nepo lupus autoimmune disease until later in life. All animal studies were performed in compliance with the United States Department of Health and Human Services Guide for the Care and Use of Laboratory Animals. The Institutional Animal Care and Use Committee at National Jewish Health approved all experimental procedures performed on the animals.

**Human cells and serum**

Whole blood was collected from healthy donors for the isolation of mononuclear cells using CPT tubes (BD Biosciences) or sera in accordance with the guidelines of the Institutional Review Board at National Jewish Health (Denver, CO). Deidentified serum samples from 43 SLE patients, 18 patients with RA, and 19 nonautoimmune controls (HC) were obtained from a research protocol approved by the Institutional Review Board at University of Colorado, in which subjects had provided informed consent to allow their leftover samples to be used for additional research in association with linked clinical data. Deidentified serum samples from 10 additional SLE patients were obtained from the University of Colorado Clinical Rheumatology Laboratory and represent referrer samples after performance of clinical tests. Samples from lupus patients obtained from the Clinical Rheumatology Laboratory were identified based on ICD9 codes, but information was not recorded that could link the samples back to the patients; thus, the research involving these samples is not considered human subjects research and is exempt from the requirements of the United States Department of Health and Human Services under 45 CFR 46.101(b)(4). Deidentified serum samples from 16 additional HC subjects, also obtained from the University of Colorado Clinical Rheumatology Laboratory, were from anonymous blood bank donors used by the laboratory for quality control purposes, and thus, the research involving these samples is not considered human subjects research. SLE and HC sera were heat inactivated for all experiments involving macrophage uptake so that differences in how sera were handled or changes in sera complement levels for each subject would not affect the total amount of complement in each sample. SLE sera with the greatest ability to block C3 detection and HC sera that did not block C3 detection in initial experiments were selected for further studies involving protein G treatment.

**Abs and other reagents**

The FITC- or HRP-conjugated goat anti-mouse C3 and goat anti-human C3 Abs were purchased from MP Biomedicals and used at 1:25 for flow cytometry and 1:1,000 for Western blots along with anti-actin (Chemicon) and 1:1,000 for ELISA. Control (isotype) goat IgG was from Jackson ImmunoResearch Laboratories and used at the same concentration (minimum 200 cells, 2–3 replicate wells per condition) was scored by flow cytometry using either the FACScan or FACSCalibur systems running on CellQuest software (BD Biosciences), and plots were rendered using FlowJo software (Tree Star).

**Flow cytometry**

Apopotic Jurkat T cells were resuspended in DMEM alone or with 10% C57BL/6 mouse serum for 30 min at 37°C, washed with DMEM, and resuspended in DMEM alone or with 10% C57BL/6 mouse serum for phagocytosis or flow cytometry. For C3 blockade, apoptotic Jurkat T cells were resuspended in DMEM plus 10% freshly prepared C57BL/6 mouse serum with anti-mouse C3 or isotype IgG before coinoculation with macrophages. For mixed sera samples, 2.5% autoimmune serum was added to 5% control sera just before the initiation of the phagocytosis assay. For HMMD, uptake of apoptotic Jurkats was done with 10% fresh human sera alone (control) or mixed with 5% heat-inactivated SLE or HC sera in X-Vivo.

**Mouse serum fractionation**

DMEM with 6% sucrose and either Mer\(^{KD}\) or C57BL/6 sera was loaded onto a Superdex 200 gel filtration column, and 31 0.5–ml vol samples were collected. For each sample, the protein concentration was determined by absorbance at 280 nm, and phagocytosis assays were performed after adding C57BL/6 serum (5% final concentration) to duplicate wells for each fraction.

**Depleting and purifying IgG from sera**

Protein G-Sepharose (Zymed) or Sepharose 4B (Amersham) was washed with PBS, blocked with 1% BSA, washed again, and resuspended in a 50:50 bead:media (either DMEM + 1-glutamine or X-Vivo) slurry. A total of 50 \(\mu\)l sera and 300 \(\mu\)l beads was incubated for 2 h while rotating at 4°C and pelleted, and supernatant was collected. Beads were washed with media and mixed with the original supernatant to make 1 ml total volume. All samples were filtered through a 0.45-\(\mu\)m filter before phagocytosis or flow cytometry. Phagocytic assays were performed, depletion of IgG from sera was confirmed by Western blot. IgG was eluted with 0.1 M glycine HC1 (pH 2.8), neutralized with Tris (pH 9.0), and concentrated with an Amicon Ultra-4 (100,000 MWCO) filter.
Systemic autoimmunity has previously been associated with defects in apoptotic cell clearance that were attributed to either impaired phagocyte activity or lack of serum factors to support uptake (19). Accordingly, serum from the Mer<sup>KD</sup> murine model of systemic autoimmunity did not support the efficient uptake of human or murine apoptotic targets (Fig. 2A), regardless of the macrophages analyzed (primary or immortalized murine macrophages) (Fig. 2B). Moreover, the defect in effectorcellosis correlated with the chronological development of autoimmunity, as serum from preautoimmune, 6-wk-old Mer<sup>KD</sup> mice (20) promoted similar levels of apoptotic cell uptake as that from age-matched C57BL/6 controls (Fig. 2C). When sera from two other models of systemic autoimmunity were tested, neither MRL<sup>Lpr</sup> nor NZB/WF1 serum proved capable of supporting effectorcellosis to the levels seen in control sera (Fig. 2D, 2E). The inability for autoimmune serum to support engulfment may be explained by either the absence of an enhancing factor or the presence of an inhibitor. To distinguish between these possibilities, effectorcellosis was assessed in a mixture of nonautoimmune (C57BL/6) or preautoimmune (MRL) and autoimmune sera. The results demonstrated that sera from all three autoimmune strains inhibited apoptotic cell engulfment (Fig. 2D–F).

To determine whether autoimmune mouse sera blocked effercellosis by interfering with C3 opsonization, levels of C3 on serum-opposed apoptotic cells were determined by both FACS analysis and Western blotting. These studies showed that C3 was nearly undetectable when cells were exposed to autoimmune serum from either MRL<sup>Lpr</sup> (Fig. 3A) or Mer<sup>KD</sup> (Fig. 3C) mice. Furthermore, C3 levels were sharply reduced when autoimmune serum was mixed with pre- or nonautoimmune serum. By contrast, C3 protein levels (and the distribution of subfragments) were unchanged when analyzed by Western blot (Fig. 3B, 3D). Taken together, these results suggest that autoimmunity leads to development of an inhibitor of effectorcellosis in these mice that blocks the macrophage recognition of complement C3 on the surface of apoptotic cells.

**Results**

**Nonautoimmune mouse serum enhances murine macrophage-mediated apoptotic cell clearance in a C3-dependent fashion**

To examine the effect of autoimmune serum on the uptake of apoptotic cells by macrophages, we first examined the effect of nonautoimmune serum on this process. As shown in Fig. 1, ingestion of apoptotic cells by murine macrophages was markedly enhanced in the presence of mouse serum compared with standard tissue culture conditions using heat-inactivated FBS. The enhancement was equally effective with serum from three different mouse strains (Fig. 1B) and did not occur with viable cells (Fig. 1C). In keeping with studies using human serum reported earlier by Matsui et al. (4) and Mevorach et al. (6), the enhancement in this murine system was also shown to depend on complement activity and the presence of C3, that is, it was abrogated by heat inactivation of the serum, blockade with an anti-C3 Ab, or by use of serum from C3<sup>−/−</sup> mice (Fig. 1E, 1F). Pretreatment of apoptotic cells with fresh mouse serum resulted in deposition of C3 on the apoptotic cell surface, as demonstrated by FACS analysis (Fig. 1D, 1G). The latter approach indicated that C3b and iC3b could be detected on the apoptotic cells. By itself, the deposition of C3 on the apoptotic cells was insufficient to mediate uptake into macrophages because the subsequent removal of the serum prevented the ingestion of C3-opsonized apoptotic cells (Fig. 1H).

These experiments support the notion that C3 is necessary, but not sufficient, for the effect of serum in supporting uptake of apoptotic cells, and that additional serum factors are required.

**Autoimmune serum inhibits apoptotic cell engulfment by blocking C3 detection**

Systemic autoimmunity has previously been associated with defects in apoptotic cell clearance that were attributed to either impaired phagocyte activity or lack of serum factors to support uptake (19). Accordingly, serum from the Mer<sup>KD</sup> murine model of systemic autoimmunity did not support the efficient uptake of human or murine apoptotic targets (Fig. 2A), regardless of the macrophages analyzed (primary or immortalized murine macrophages) (Fig. 2B). Moreover, the defect in effectorcellosis correlated with the chronological development of autoimmunity, as serum from preautoimmune, 6-wk-old Mer<sup>KD</sup> mice (20) promoted similar levels of apoptotic cell uptake as that from age-matched C57BL/6 controls (Fig. 2C). When sera from two other models of systemic autoimmunity were tested, neither MRL<sup>Lpr</sup> nor NZB/WF1 serum proved capable of supporting effectorcellosis to the levels seen in control sera (Fig. 2D, 2E). The inability for autoimmune serum to support engulfment may be explained by either the absence of an enhancing factor or the presence of an inhibitor. To distinguish between these possibilities, effectorcellosis was assessed in a mixture of nonautoimmune (C57BL/6) or preautoimmune (MRL) and autoimmune sera. The results demonstrated that sera from all three autoimmune strains inhibited apoptotic cell engulfment (Fig. 2D–F).

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**Identification of anti-C3 Ab as the inhibitor present in autoimmune serum**

Superdex 200 gel filtration of serum was used to identify candidate molecules that inhibited the uptake of apoptotic cells by nonautoimmune sera. Although several fractions from Mer sera displayed some inhibitory capacity, the strongest inhibitor found in autoimmune sera coeluted with IgG (Fig. 4A). The corresponding fractions from C57BL/6 serum did not inhibit engulfment (Fig. 4B). Protein G removal of IgG from either MRL<sup>Lpr</sup> or Mer<sup>KD</sup> sera removed the inhibitor of apoptotic cell clearance (Fig. 5A, 5D), and in the case of the MRL<sup>Lpr</sup> serum, restored the ability to detect C3 on the apoptotic cells by FACS analysis (Fig. 5B). Despite the observation that protein G treatment removed the inhibitory activity present in Mer<sup>KD</sup> serum, this treatment did not restore our ability to detect C3 on the surface of apoptotic cells by flow cytometry (Fig. 5E). Actual C3 deposition was unaltered by the presence of either MRL<sup>Lpr</sup> or Mer<sup>KD</sup> autoimmune serum (Fig. 5C, 5F). However, protein G-mediated removal of the inhibitory activity did not render either autoimmune sera capable of supporting higher levels of apoptotic cell uptake, like that which occurred in the presence of pre- or nonautoimmune serum (Fig. 5A, 5D). Protein G treatment may remove enhancing factors that are required to promote optimal clearance or autoimmune serum may inherently lack these factors.

Protein G-purified IgG from MRL<sup>Lpr</sup>, Mer<sup>KD</sup>, and their control serum counterparts was examined to determine whether IgG could directly block effectorcellosis and C3 detection. IgG from both autoimmune strains reduced phagocytosis by 90% (Fig. 6A, 6C) and extinguished C3 protein detection on the surface of apoptotic cells
IgG from control sera had no effect. Western blot analysis confirmed that IgG purified from all sera did not alter the levels of C3 protein deposited on apoptotic cells (Fig. 6E). IgG purified from MRLlpr or Mer KD serum was also shown to bind directly to C3 by immunoblotting (Fig. 6F).

Although anti-C3 IgG bound to apoptotic cells would be predicted to facilitate FcR-mediated macrophage uptake, neither nonautoimmune C57BL/6 nor autoimmune Mer KD sera promoted the uptake of viable Jurkats opsonized with mouse anti-human CD3 for FcR-mediated uptake by J774, suggesting that FcR-mediated mechanisms of uptake are not used in this system (Fig. 6G).

Finally, levels of anti-C3b were measured in Mer KD mice of both sexes and at varying ages and presumably stages of development of autoimmune disease. Anti-C3b levels were significantly higher in the sera of Mer KD mice that had autoantibodies to anti-dsDNA compared with Mer KD mice that lacked these autoantibodies (Fig. 6H). A similar trend was seen in Mer KD mice that had autoantibodies to anti-chromatin, but the results were not statistically significant.
significant due to the much lower number of mice that lacked anti-chromatin autoantibodies in this analysis (Fig. 6). There was no correlation of anti-C3b levels with age of the mice or titers of autoantibodies (data not shown). Taken as a whole, these data suggest that IgG autoantibodies are elicited in the course of autoimmune disease that can inhibit apoptotic cell engulfment by binding to C3 on the surface of apoptotic cells and blocking its interaction with macrophage complement receptors.

**C3 detection is blocked by Ig from the sera of SLE patients**

Human sera contains natural Abs to C3 (9) as well as induced autoantibodies (immunoconglutins) that are thought to be generated in response to acute or chronic inflammation involving complement activation (10), but these Abs have not been shown to directly interfere with macrophage uptake of C3-opsonized apoptotic cells. To test whether Abs to C3 contribute to the clearance defects in human SLE as in murine models, the capacity of sera from SLE patients and HC to block C3 accessibility and phagocytic uptake of serum-exposed apoptotic cells was evaluated. Sera from SLE patients contained significantly higher titers of anti-C3 Abs when compared with sera from nonautoimmune controls or patients with RA (Fig. 7A). Consistent with this, C3 detection on apoptotic cells incubated in fresh sera mixed with heat-inactivated HC sera (Fig. 7B). Protein G treatment of sera from the SLE patients with the highest level of inhibition of C3 detection restored the ability to detect C3 on apoptotic cells (Fig. 7C), and IgG purified from these sera blocked C3 detection (Fig. 7D). Deposition of C3 was not affected by SLE sera. Detection and deposition of C3 by sera from three HC were not changed by these treatments (Fig. 7C,7G). As previously shown by Mevorach et al. (6), human sera enhanced the uptake of apoptotic Jurkat cells by HMDM, and this was blocked by heat inactivation (Fig. 7D). However, the efferocytosis mediated by fresh sera was not blocked in these experiments by mixing either SLE or HC sera (Fig. 7E). Additional experiments were performed after blockade of macrophage Fcγ receptors to isolate complement-mediated uptake. In these experiments, protein G treatment of the majority of serum samples in both groups improved macrophage uptake of C3-opsonized cells, and IgG purified from these sera had no effect (Fig. 7F).

**Discussion**

The data presented in this work suggest that deficiencies in apoptotic cell engulfment need not precede the onset of systemic autoimmunity, but may instead arise as a result of C3-targeted
immune responses. This study describes a mechanism whereby C3-specific IgG Abs delay efferocytosis by blocking macrophage detection of activated C3 on apoptotic cells. Consistent with previous reports, C3 opsonization enhanced efferocytosis (4, 6) and autoimmune sera did not support apoptotic cell clearance (19). The current study expanded on these observations by demonstrating that autoimmune serum can contain Abs against opsonized C3 that inhibit apoptotic cell uptake. We propose a model in which acquired defects in efferocytosis may exacerbate intrinsic, inappropriate autoimmune responses.

The exposure of phosphatidylserine on the surface of dying cells has been suggested to initiate activation of the complement system with subsequent cell surface C3 deposition that can then contribute to removal by CR3 and CR4 receptors (4, 6, 7, 12). However, as noted in the current studies, opsonization of the apoptotic cells with C3 still required additional serum factors for uptake. This raises the possibility that C3 receptors in this system serve primarily as essential tethering ligands that then need additional stimuli to optimally induce the actual ingestion (8). Because removal of the inhibitory anti-C3 IgG from autoimmune serum did not restore the ability of that serum to support efficient apoptotic cell engulfment, even when complement components (C1q or C3) were added back (data not shown), we concluded that C3 was necessary, but not sufficient for clearance, and that additional prophagocytic components (stimuli) were also lacking in the autoimmune serum.

Consistent with the hypothesis that serum from autoimmune animals inhibited efferocytosis by blocking the C3-dependent component of the uptake, depletion of IgG from MRL<sup>lpr</sup> mice with preautoimmune MRL serum (A) blocked detection of C3 on apoptotic cells by FACS analysis (n = 5), but not total C3 fragment deposition (B) determined by Western blotting of the apoptotic cell extracts (results shown here are representative of those obtained in three separate experiments). C and D, Similar results were obtained with autoimmune Mer<sup>KD</sup> serum mixed with nonautoimmune serum from C57BL/6 mice (n = 3). Unfilled histograms are isotype labeling; filled histograms are anti-C3 labeling. The geometric mean fluorescence for C3 (C3 Protein MFI) is expressed as a percentage of nonautoimmune serum control. *p < 0.05.

**FIGURE 3.** Sera from autoimmune mice inhibited C3 protein detection on the surface of apoptotic cells. C3 on apoptotic Jurkats incubated in media containing nonautoimmune serum (black), nonautoimmune serum mixed with autoimmune serum (dark gray), and autoimmune serum (light gray) was detected by FACS (A, C) or Western blot (B, D). Inclusion of serum from autoimmune MRL<sup>lpr</sup> mice with preautoimmune MRL serum (A) blocked detection of C3 on apoptotic cells by FACS analysis (n = 5), but not total C3 fragment deposition (B) determined by Western blotting of the apoptotic cell extracts (results shown here are representative of those obtained in three separate experiments). C and D, Similar results were obtained with autoimmune Mer<sup>KD</sup> serum mixed with nonautoimmune serum from C57BL/6 mice (n = 3). Unfilled histograms are isotype labeling; filled histograms are anti-C3 labeling. The geometric mean fluorescence for C3 (C3 Protein MFI) is expressed as a percentage of nonautoimmune serum control. *p < 0.05.
both C3 detection and apoptotic cell ingestion and also demonstrated significantly greater capacities to bind purified C3 protein and its degradation products, as shown by Western blot. Notably, both autoimmune strains produced IgG that specifically bound the \( \alpha \)-chain of the iC3b protein, suggesting that C3-specific Abs specifically block iC3b signaling through cognate receptors on macrophages.

Similar to the findings in autoimmune mice, IgG Abs that blocked the detection of C3 on apoptotic cells were elevated in human sera from SLE patients. However, there was not a statistical decrease in efferocytosis mediated by monocyte-derived macrophages when SLE sera were mixed with fresh human serum. There are a number of explanations for the minimal effect in this in vitro mixing assay. For one, there may just not be as much anti-C3 Ab present as in the murine systems. Second, the IgG against activated C3 may block FACS detection of C3 while still allowing the recognition of C3 by HMDM complement receptors. The anti-C3 IgG might itself promote FcR-mediated clearance by the human macrophages. Finally, the presence of autoantibodies in the SLE sera that interact with Ags on the apoptotic cells other than C3 may drive removal of apoptotic cells using nonefferocytic mechanisms (9). Conversion from C3-mediated to FcR-mediated uptake of the apoptotic cells would be a pathogenic switch from an immunosuppressive and anti-inflammatory process to one that was both proinflammatory and potentially immunogenic.

It has been shown that the paucity of Mer expression in Mer\(^{KD} \) mice leads to a primary defect in macrophages that prevents apoptotic cell ingestion (11) that contributes to the subsequent development of autoimmunity (20). However, sera from all three murine models of systemic autoimmune examined exhibited the inhibitory activity, independent of primary defects in macrophage-mediated efferocytosis. This suggests that autoimmunity in the Mer\(^{KD} \) mice might result from the in vivo clearance deficiency of the macrophages and be exacerbated by the development of the Ab inhibitor to efferocytosis. Our preliminary fractionation data suggested that there may also be other inhibitors present in Mer sera that we did not investigate. In the other autoimmune strains, as well as human patients, additional and/or alternative uptake defects may also contribute to the decreased apoptotic cell clearance that has been hypothesized as the source of autoantigens for the autoimmune response.

It is intriguing to note that autoantibodies termed immunoconglutinins, and later shown to react against fixed C3, have been noted since the 1930s (21), and in fact represented a subject of investigation for one of us in his PhD project (22). Elevated levels of immunoconglutinins, with varying Ig isotypes, have been reported in the sera from patients with chronic infection or autoimmune diseases, including SLE and RA, in which they increased during exacerbations (10). Whether in humans these exhibit variable effects in apoptotic cell clearance depending on isotype (i.e., ability to react with Fc receptors) or fine specificity against the C3 Ag is...
FIGURE 5. IgG depletion with protein G-Sepharose removed the inhibitory activity present in autoimmune serum. Analyses of MRL\textsuperscript{lpr} (A–C) and Mer\textsuperscript{KD} (D, E) sera treated with protein G-Sepharose beads to remove serum IgG. A and D, Engulfment assays using J774 macrophages and apoptotic Jurkat T cells. Single serum samples contained 5% serum each, whereas “Mixed Serum” samples contained 5% untreated pre- or nonautoimmune serum plus 2.5% autoimmune serum. Autoimmune serum was either untreated (black bars), control Sepharose bead treated (dark gray bars), or protein G-Sepharose bead treated (light gray bars). Removing IgG from MRL\textsuperscript{lpr} serum (A) and Mer\textsuperscript{KD} serum (C) using protein G-Sepharose did not restore the ability of these sera to promote uptake of apoptotic cells, but did prevent these autoimmune sera from inhibiting the uptake of apoptotic cells when mixed with nonautoimmune sera (n = 3). B and E, C3 was detected by FACS on apoptotic Jurkats treated with either pre- or nonautoimmune serum (black), autoimmune serum (dark gray), pre- or nonautoimmune serum mixed with autoimmune serum (medium gray), or pre- or nonautoimmune serum mixed with protein G-treated, IgG-depleted autoimmune serum (light gray). Protein G removal of IgG from MRL\textsuperscript{lpr} serum (B), but not Mer\textsuperscript{KD} serum (E), allowed C3 protein to be detected on the surface of apoptotic cells treated with mixed serum (n = 3). Western blot demonstrated that C3 protein and distribution of its fragments bound to the apoptotic cells were unchanged when exposed to MRL, MRL\textsuperscript{lpr}, or a mixture of both sera (C), or C57BL/6, Mer\textsuperscript{KD}, or a mixture of both sera (F) (results shown here are representative of those obtained in three separate experiments). PI is expressed as a percentage of nonautoimmune serum control. Unfilled histograms are isotype labeling; filled histograms are anti-C3 labeling. The geometric mean fluorescence for C3 (C3 Protein MFI) is expressed as a percentage of nonautoimmune serum control. *p < 0.05.
FIGURE 6. Apoptotic cell engulfment and C3 protein detection were inhibited by IgG purified from autoimmune sera. A and C, Engulfment of apoptotic Jurkats by J774 macrophages in pre- or nonautoimmune sera alone or mixed with IgG purified from either pre- or nonautoimmune or autoimmune sera. IgG purified from MRL<sup>lpr</sup> (A) and Mer<sup>KD</sup> sera (C) inhibited the uptake of apoptotic Jurkats seen in the presence of pre- or nonautoimmune sera (n = 4). B and D, C3 was detected by FACS on apoptotic Jurkat T cells incubated with pre- or nonautoimmune serum alone (black), or pre- or nonautoimmune serum mixed with IgG purified from pre- or nonautoimmune (dark gray) or autoimmune (light gray) mouse serum. The detection of C3 was inhibited by IgG purified from MRL<sup>lpr</sup> (B) and Mer<sup>KD</sup> (D) (n = 3). IgG purified from nonautoimmune sera had no effect on uptake or FACS detection of C3 (results shown here are representative of those obtained in three separate experiments). Phagocytic index (PI) is expressed as a percentage of nonautoimmune serum control. Unfilled histograms are isotype labeling; filled histograms are anti-C3 labeling. The geometric mean fluorescence for C3 (C3 Protein MFI) is expressed as a percentage of nonautoimmune serum control. E, C3 deposition was unaltered as detected by Western blot analysis of total C3 protein and distribution of its fragments bound to apoptotic cells exposed to pre- or nonautoimmune serum mixed with IgG purified from either autoimmune serum or pre- or nonautoimmune serum.
not clear, but the possible effect described in this work on blocking C3 recognition could be a contributing factor in the progression and/or exacerbations seen in systemic autoimmune diseases.

**FIGURE 7.** SLE sera contained IgG against C3b that blocked opsonized C3 detection. A, ELISA showed that the mean anti-C3b titers were higher in sera from SLE patients (n = 53) compared with sera from HC (n = 35) or patients with RA (n = 18). B, FACS detection of C3 on apoptotic Jurkats preincubated with fresh human sera (Control) was reduced by mixing heat-inactivated SLE (n = 16), but not heat-inactivated HC sera (n = 16). C, Removal of IgG with protein G treatment restored the FACS detection of C3 from selected SLE sera (n = 3), whereas the IgG purified from these SLE sera blocked C3 detection (n = 3). Removal of IgG and purified IgG from HC sera had no effect on the detection of C3 (n = 3). D, HMDM uptake of apoptotic Jurkats was enhanced by fresh, but not heat-inactivated (ΔH) human sera (n = 16). E, Mixing sera from SLE patients (n = 14) or HC (n = 16) with fresh human sera did not inhibit uptake of apoptotic Jurkats compared with fresh sera alone (Control). F, Uptake of apoptotic cells with fresh human sera mixed with either SLE (n = 6) or HC (n = 7) was increased by protein G treatment of these sera, whereas the purified IgG had no effect. G, Western blots of lysates revealed no change in total C3 bound to apoptotic cells incubated in fresh sera and mixed with SLE (n = 6) or HC (n = 7). Dots represent each individual sera, horizontal lines are the mean for the group, and whiskers are the SEM. The geometric mean of C3 staining (MFI) on apoptotic Jurkats preincubated in 15% human sera alone was used as the control. Phagocytic index of apoptotic Jurkats by HMDM in 10% fresh human sera alone was used as the control. *p < 0.05.

**Disclosures**

J.M.T. is a stockholder in and consultant for Taligen Therapeutics, Inc. The other authors have no financial conflicts of interest.

nonautoimmune controls. F, IgG from autoimmune MRL<sup>lpr</sup> and Me<sup>KD</sup> sera bound purified C3 and its degradation products by immunoblotting (blot shown here is representative of results obtained in multiple experiments). G, J774 macrophage phagocytosis of apoptotic Jurkats (Efferocytosis), but not anti-C3d–coated viable Jurkats (Fc-mediated), was enhanced by C57BL/6 sera (n = 3). H and I, Sera from 23 Me<sup>KD</sup> mice were tested by ELISA for anti-C3b and markers of autoimmunity (anti-dsDNA or anti-chromatin). Each dot represents an individual animal, with the line representing the mean for the group and the whiskers representing the SEM. Elevated levels of anti-C3b were detected in the sera of Me<sup>KD</sup> mice with anti-dsDNA (n = 17) (H) or anti-chromatin (n = 20) (I) autoantibodies compared with the Me<sup>KD</sup> mice lacking these autoantibodies (n = 6 and 3, respectively). *p < 0.05.
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