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Complement Component C1q Regulates Macrophage Expression of Mer Tyrosine Kinase To Promote Clearance of Apoptotic Cells

Manuel D. Galvan,*† Deborah B. Foreman, † Erliang Zeng,*‡ John C. Tan,* and Suzanne S. Bohlson*†

Failure to efficiently clear apoptotic cells is linked to defects in development and the onset of autoimmunity. Complement component C1q is required for efficient engulfment of apoptotic cells in mice and humans; however, the molecular mechanisms leading to C1q-dependent engulfment are not fully understood. In this study, we used primary mouse macrophages to identify and characterize a novel molecular mechanism for macrophage-mediated C1q-dependent engulfment of apoptotic cells. We found that macrophage activation with C1q resulted in cycloheximide-sensitive enhanced engulfment, indicating a requirement for de novo protein synthesis. To investigate the cycloheximide-sensitive pathway, C1q-elicited macrophage transcripts were identified by microarray. C1q triggered the expression of Mer tyrosine kinase (Mer) and the Mer ligand growth arrest-specific 6: a receptor–ligand pair that mediates clearance of apoptotic cells. Full-length native C1q, and not the collagen-like tail or heat-denatured protein, stimulated Mer expression. This novel pathway is specific to C1q because mannose-binding lectin, a related collectin, failed to upregulate Mer expression and function. Soluble Mer-Fc fusion protein inhibited C1q-dependent engulfment of apoptotic cells, indicating a requirement for Mer. Moreover, Mer-deficient macrophages failed to respond to C1q with enhanced engulfment. Our results suggest that C1q elicits a macrophage phenotype specifically tailored for apoptotic cell clearance, and these data are consistent with the established requirement for C1q in prevention of autoimmunity.

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C1q is the recognition component of the classical complement pathway, and C1q is also required for efficient clearance of apoptotic cells (1). C1q deficiency is the strongest known susceptibility factor for lupus: C1q-deficient humans develop lupus with >90% penetrance (2). A leading theory for the development of lupus is that failure to efficiently clear apoptotic cells provides a reservoir of self-Ag, resulting in autoimmunity and immune complex deposition (3). Bridging molecules normally mediate clearance of apoptotic cells by binding to both the apoptotic cell and the phagocyte (4). For example, growth arrest-specific 6 (Gas6) is a well-described bridging molecule required for the efficient clearance of apoptotic cells. Gas6 binds to phosphatidylinerine, exposed on the apoptotic cell surface, and also binds to members of the Tyro3, Axl, Mer tyrosine kinase (Mer) (TAM) family of tyrosine kinases expressed by phagocytes (5). C1q is often depicted as a bridging molecule; however, the mechanisms leading to C1q-dependent engulfment of apoptotic cells and prevention of autoimmunity are not fully understood.

The ability of C1q to enhance phagocytosis was first described for engulfment of Ab- and complement-coated particles (6, 7). Subsequently, it became evident that the larger family of C1q-related proteins called defense collagens or collectins, including mannose-binding lectin (MBL) and surfactant protein-A (SP-A), shared the ability to enhance phagocytosis of multiple targets (8, 9). Korb et al. (10) made the important discovery that C1q bound specifically to apoptotic cells. This discovery led to numerous studies that demonstrated that C1q and the collectins enhanced engulfment of apoptotic cells (reviewed in Ref. 11).

It is generally accepted that C1q enhances engulfment by bridging apoptotic cells to phagocytes, and, therefore, C1q deficiency leads to a failure in apoptotic cell clearance, causing subsequent accumulation of apoptotic cell bodies. Importantly, MBL and other C1q-related collectins also enhance engulfment, but MBL deficiency does not lead to autoimmunity (12). In addition, the C1q/collectin-dependent phagocytosis pathway described to date is not specific for apoptotic cells because C1q/collectins enhance phagocytosis of multiple target particles, including Ab- and complement-opsonized particles as well as apoptotic cells (reviewed in Ref. 13).

These data suggest the existence of an alternative C1q-dependent engulfment mechanism with specificity for C1q and apoptotic cells. Because C1q alters monocyte/macrophage gene expression (14, 15), we hypothesized that C1q alters gene expression required...
for clearance of apoptotic cells. In support of our hypothesis, in this study, we identify and characterize a novel C1q-dependent apoptotic cell engulfment mechanism that requires Mer, a TAM family member that regulates engulfment of apoptotic cells and autoimmunity (16, 17).

Materials and Methods

Reagents

All reagents were purchased from Fisher (Pittsburgh, PA) unless otherwise indicated. DMEM and RPMI 1640 were purchased from Life Technologies/Molecular Probes/Invitrogen (Carlsbad, CA). FBS was purchased from Hyclone Laboratories (Logan, UT) and heat inactivated for 30 min at 56°C. C1q was isolated from plasma-derived normal human serum by ion-exchange chromatography, followed by size-exclusion chromatography according to the method of Tenner et al. (18) and modified as described (19). Commercially available purified C1q was also purchased from Complement Technology (Tyler, TX). Purified C1q collagen-like tails were kindly provided by Dr. Andrea Tenner (University of California Irvine). MBL, goat anti-mouse Mer Ab, Mer-Fc, and goat anti-mouse Gas6 Ab were purchased from R&D Systems (Minneapolis, MN). Rabbit anti-mouse Mer Ab was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Whole goat IgG was purchased from Jackson ImmunoResearch Laboratories (West Grove, PA). PE-conjugated rat anti-mouse F4/80 was purchased from eBioscience (San Diego, CA). Protein G beads were purchased from GE Healthcare Life Sciences (Piscataway, NJ).

Mice

MeKDo 32 mice and wild-type (WT) C57BL/6 mice were maintained in the Animal Care and Use Committee of the University of Notre Dame Institute for Animal Care and Use Committee.

Cell culture

Bone marrow-derived macrophages (BMDM) were generated as previously described (20) and obtained from The Jackson Laboratory (Bar Harbor, ME) on a C57BL/6 background and referred to as mek−/−. C3-deficient mice on a mixed background were kindly provided by Dr. Mary-Ann McDowell (University of California, Irvine). C3-deficient mice were kindly provided by Dr. Martin Botto and Dr. Mark Walport (Imperial College, London, U.K.) and backcrossed onto C57BL16 for 11 generations. Macrophage-specific LRP-deficient mice were kindly provided by Dr. Dudley Strickland (University of Maryland School of Medicine) and described previously (21). All techniques were approved by the University of Notre Dame Institutional Animal Care and Use Committee.

Microarray analysis

Microarray and bioinformatics analyses were performed in collaboration with the University of Notre Dame Genomics and Bioinformatics Core Facility. Briefly, total RNA was extracted using the TRIzol (Sigma-Aldrich) method from BMDM adhered to HSA or C1q for 4 or 18 h. The resulting RNA was precipitated using sodium acetate and washed with ethanol to remove contaminants. Randomly selected RNA samples were analyzed with an Agilent 2100 Bioanalyzer (Agilent Technologies) to verify sample integrity prior to cDNA synthesis. Double-stranded cDNA was generated using the TransFlex Complete Whole Transcriptome Amplification Kit (Agilent Technologies) according to the manufacturer’s instructions, and the resulting product was precipitated with sodium acetate, washed with ethanol to remove residual primers and nucleotides, and stored at −20°C until labeled. Randomly selected cDNA were analyzed with an Agilent 2100 Bioanalyzer (Agilent Technologies) to verify sample integrity prior to labeling. cDNA was labeled with Cy3 using NimbleGen One-color DNA labeling kits according to manufacturer’s instructions (Roche NimbleGen, Madison, WI), isopropyl alcohol, and stored at −20°C. The labeled product was resuspended in hybridization buffer containing alignment oligonucleotides and unique sample tracking controls, loaded onto a custom murine gene expression microarray designed by NimbleGen (Roche NimbleGen), and hybridized overnight. After extensive washing, the array microchips were dried and then scanned using a NimbleGen MS 200 Microarray Scanner (Roche NimbleGen). Array data were extracted and analyzed using NimbleScan software v.2.5 (Roche NimbleGen). The mean fluorescence intensity was derived from a log2 transformation of the data and normalized using quantile normalization method. Heat maps were generated using Genesis software. The data were compared for statistical
significance using the Student t test and q value for control of false discovery rate.

The microarray data set discussed in this publication has been deposited in the National Center for Biotechnology Information’s Gene Expression Omnibus under Gene Expression Omnibus Series accession number GSE35280 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE35280).

Quantitative real-time PCR

Total RNA was isolated as described above. Quantitative real-time PCR (qRT-PCR) reactions were assembled with SABiosciences SYBR Green qPCR Master Mix (SABiosciences, Frederick, MD) according to the manufacturer’s instructions and amplified using Applied Biosystems Real Time PCR system (Applied Biosystems, Carlsbad, CA). Gene expression was quantified from standard curves prepared from a series of dilutions of control template cDNA with either GAPDH primers or gene-specific primers. All reactions were carried out in triplicate. Negative controls included reactions in which template cDNA or enzyme was omitted. The amplification specificity of the reactions was confirmed by melting-curve analysis. Results were normalized to GAPDH and reported as relative gene expression. The primers used were: protein S (forward: 5’-GCT TTC CAC TTG AGC CAA CAC CTT-3’; reverse: 5’-TGT GCT CTC AGC AGC TTA GGT TGA-3’), C3 (forward: 5’-GGG CTG TTA ATT GTG TGA TTC TG C3-3’; reverse: 5’-GAT GAG GAC GAA GGC TGT G-3’), Clqβ (forward: 5’-AGA AGC ATC ACA CAA GAC CAG CAG-3’; reverse: 5’-AGC TGC AAT CCA GAA ACC GAC ACC-3’), GAPDH (forward: 5’-CCA ATG TGG TGT CCG TCG TGC-3’; reverse: 5’-GGT GAA GTC GCA GGA GAC ACC-3’), milk fat globule-epidermal growth factor (MFG-E8) (forward: 5’-TAG CCC ACA GTG ATG ATG GT-3’; reverse: 5’-TCT GGG AGT GTG TGT TTG CC-3’), GAS-6 (forward: 5’-TCA AGA AGC AGT TGG TGC TC-3’; reverse: 5’-GTT GCC ATC ACT TAC CCT AGG GT-3’), and Mer (forward: 5’-CAC ATG GAC AAA GGA CTG ACG-3’; reverse: 5’-AGT AGC CAT CAA AAC CAG GG-3’).

Detection of Gas6

Gas6 protein levels in culture supernatants and lysates were assayed by ELISA according to the manufacturer’s instructions (AdipoBiotech, Beijing, China). BMDM were seeded at 1 x 10⁵ cells/ml in one-well Lab Tek Chamber slides (Nalge Nunc International) that had been precoated with 4 &middot; TAG CCC ACA GTG ATG ATG GT-3’; reverse: 5’-TCT GGG AGT GTG TGT TTG CC-3’), GAS-6 (forward: 5’-TCA AGA AGC AGT TGG TGC TC-3’; reverse: 5’-GTT GCC ATC ACT TAC CCT AGG GT-3’), and Mer (forward: 5’-CAC ATG GAC AAA GGA CTG ACG-3’; reverse: 5’-AGT AGC CAT CAA AAC CAG GG-3’).

Detection of Gas6

Gas6 protein levels in culture supernatants and lysates were assayed by ELISA according to the manufacturer’s instructions (AdipoBiotech, Beijing, China). BMDM were seeded at 1 x 10⁵ cells/ml in one-well Lab Tek Chamber slides (Nalge Nunc International) that had been precoated with 4 mM HSA, C1q, or MBL. At the indicated time point, BMDM lysates were generated with RIPA buffer supplemented with a mixture of protease and phosphatase inhibitors. Total protein concentration was determined by bicinchoninic acid according to the manufacturer’s instructions (Thermo Scientific), and equal protein concentrations were assayed within groups and between experiments.

Statistics

Statistics were performed using Prism version 5.02 (GraphPad). Statistical comparisons between groups were performed as indicated in the figure legends.

Results

Clq-dependent engulfment of apoptotic cells requires de novo protein synthesis

Fraser et al. (14, 15) reported that Clq regulated the expression of proinflammatory cytokines from human monocytes, macrophages, and dendritic cells. Based on these data, we hypothesized that Clq-dependent gene expression may also regulate clearance of apoptotic cells. To investigate this possibility, we compared the effects of short-term and prolonged stimulation with Clq on the ability of mouse BMDM to phagocytose Ab-coated targets and apoptotic cells. In agreement with previously published observations, macrophages stimulated with Clq for 30 min showed enhanced phagocytosis of EAIgG (6). Clq enhanced both the percent of macrophages engulfing EAIgG and the number of EAIgG ingested per macrophage (data not shown). Clq-adhered macrophages showed a 2.6-fold increase in phagocytosis compared with control macrophages (Fig. 1A, 44 ± 3.6 versus 17 ± 2.3, respectively). Although Clq is known to enhance engulfment of numerous target particles, including Ab- and complement-coated particles, pathogens, and apoptotic cells (reviewed in Ref. 13), under these experimental conditions, Clq failed to enhance engulfment of nonopsonized apoptotic cells (Fig. 1B). These experiments were conducted in the absence of serum to eliminate the effect of factors (i.e., bridging molecules) that have been previously shown to influence engulfment of apoptotic cells.

Next, we investigated whether prolonged macrophage stimulation with Clq influenced macrophage phagocytosis. Macrophages were stimulated with Clq or control protein for 5 h before the addition of targets. As expected, similar to short-term stimulation with Clq, prolonged stimulation with Clq resulted in enhanced engulfment of EAIgG; there was a 1.6-fold Clq-dependent enhancement of phagocytosis of EAIgG over control (Fig. 1C, 58 ± 3.1 versus 37 ± 1.9, respectively). Interestingly, and in contrast to short-term stimulation with Clq, macrophages stimulated with Clq for 5 h ingested twice as many nonopsonized apoptotic cells compared to control macrophages (Fig. 1D, 22 ± 3.1 versus 12 ± 2.4, respectively). Together, these data demonstrate that prolonged stimulation with Clq enhances the ability of macrophages to engulf apoptotic cells.

Because prolonged macrophage activation with Clq resulted in enhanced engulfment of apoptotic cells (Fig. 1D), we hypothesized that de novo macrophage protein synthesis was required for the Clq-dependent effect. To assess the requirement for protein synthesis, macrophages were treated with cycloheximide, and Clq-
dependent engulfment of apoptotic cells was measured. Cycloheximide treatment completely ablated the C1q-mediated engulfment of apoptotic cells (Fig. 1F). Importantly, cycloheximide treatment did not alter C1q-dependent engulfment of EAIgG. Macrophages stimulated with C1q for 5 h ingested 3-fold more EAIgG compared with control macrophages in the presence and absence of cycloheximide (Fig. 1E). Together, these data demonstrate the existence of a novel C1q-dependent phagocytosis pathway for apoptotic cells that requires de novo protein synthesis. The data also demonstrate that this novel pathway is distinct from the previously described function of C1q in triggering a direct enhancement of phagocytic function (reviewed in Ref. 11) and suggest a role for C1q-dependent gene transcription in engulfment of apoptotic cells.

C1q stimulates expression of proteins required for engulfment of apoptotic cells

Because C1q-dependent enhanced engulfment of apoptotic cells required protein synthesis, we performed a microarray of C1q-elicted macrophage transcripts to identify genes involved in apoptotic cell engulfment. Cluster analysis of macrophage gene transcripts following 4- (Fig. 2A) or 18-h (Fig. 2B) adhesion to C1q demonstrated that C1q upregulated a number of genes encoding well-characterized proteins required for engulfment of apoptotic cells. The proteins included C1q, C3, MFG-E8, and Mer, as well as the Mer ligands, Gas6, and protein S (Fig. 2A, 2B).

qRT-PCR confirmed the microarray macrophage gene signature observed following adhesion to C1q. Consistent with the microarray data, we observed enhanced expression of mRNA encoding C1q, C3, MFG-E8, Mer and the Mer ligands, Gas6, and protein S (Fig. 2C–E). Mer is a member of the TAM family of receptor tyrosine kinases that also includes Tyro 3 and Axl. Mer is expressed on phagocytic cells, including macrophages and dendritic cells, and has a nonredundant role in clearance of apoptotic cells (17, 25). Importantly, Mer deficiency results in failure to clear apoptotic cells in vivo and onset of autoimmunity (16, 17, 26–29), similar to C1q deficiency (1). Because of the importance of Mer in apoptotic cell clearance, we confirmed C1q-dependent enhanced Mer mRNA and protein expression by qRT-PCR (Fig. 2E) and immunoprecipitation/Western blot, respectively (Fig. 2F). As expected, pretreatment of macrophages with cycloheximide prior to stimulation with C1q inhibited Mer expression (Fig. 2G). Additionally, 5-h macrophage stimulation with C1q resulted in twice as much Gas6 protein compared to control macrophages as measured by ELISA (Fig. 2H, 2900 ± 350 versus 1400 ± 130, respectively). Surprisingly, stimulation with C1q failed to enhance MFG-E8 protein levels above control as measured by ELISA (data not shown).

Full-length native C1q, and not the C1q collagen-like tail, elicits phagocytosis and Mer expression independent of LRP and CD93

To investigate whether soluble C1q enhanced Mer expression, macrophages were incubated with increasing concentration of soluble C1q for 5 h. There was a dose-dependent increase in Mer expression with increasing concentrations of C1q up to physiologic concentrations (Fig. 3A). Moreover, physiologic C1q concentration (75 μg/ml) stimulated Mer expression to similar levels as 4 μg/ml immobilized C1q, the C1q concentration used for macrophage stimulation for the majority of the experiments reported in this study (data not shown). To demonstrate that the effects reported in this study were specific to C1q, C1q was heat inactivated at 56°C for 1 h. Heat-inactivated C1q failed to enhance Mer expression (Fig. 3B) and engulfment of Ab-coated targets and apoptotic cells (Fig. 3C, 3D). The collagen-like tails of C1q are sufficient for triggering C1q-dependent engulfment of EAIgG (7); therefore, the ability of C1q tails to upregulate Mer expression was tested. C1q tails failed to stimulate Mer expression when compared with full-length C1q (Fig. 3E), but enhanced engulfment of EAIgG (data not shown), suggesting that the whole C1q molecule is necessary for Mer expression. A number of putative receptors have been proposed to mediate C1q-dependent signaling...
on macrophages including CR1 (CD35), CD93, and LRP (CD91) (reviewed in Ref. 11). Because CR1 is not expressed by murine macrophages (30 and S.S. Bohlson and M.C. Greenlee-Wacker, unpublished observations), we focused our attention on CD93 and LRP and tested whether these receptors were required for C1q-dependent Mer expression using CD93- or LRP-deficient macrophages. C1q upregulated Mer expression in both CD93- and LRP-deficient macrophages at levels comparable to wild-type, demonstrating that these receptors are not required for C1q-dependent upregulation of Mer (Fig. 3F).

**MBL fails to enhance engulfment of apoptotic cells and expression of Mer and Gas6**

In addition to C1q, other members of the defense collagen family have been reported to stimulate macrophage phagocytosis. Using recombinant protein, Arora et al. (31) identified a 6-aa motif in the collagen-like tail of MBL that is conserved in C1q and required for enhanced FcR-mediated phagocytosis. Similar to C1q, MBL has been implicated in facilitating ingestion of apoptotic cells by acting as a bridging molecule (reviewed in Ref. 11). However, despite a function for MBL in enhanced phagocytosis of apoptotic cells, MBL deficiency is not associated with autoimmunity (12). Therefore, we hypothesized that C1q, and not MBL, would activate the novel engulfment pathway, resulting in Mer-dependent engulfment of apoptotic cells. As predicted, C1q, but not MBL, increased macrophage Mer expression and Gas6 protein levels, as measured by Western blot (Fig. 4A) and ELISA (Fig. 4B), respectively. Macrophages stimulated with C1q expressed significantly more Gas6 compared with control and MBL-activated macrophages (Fig. 4B). To investigate the effects of MBL on phagocytosis, macrophages were adhered to C1q or MBL for 5 h and then fed apoptotic cells. As shown previously, C1q enhanced engulfment of apoptotic cells compared with the control (Fig. 4C, 18 ± 0.8 versus 9.4 ± 1.0, respectively); however, MBL failed to significantly enhance engulfment above control levels (Fig. 4C, 12 ± 1.4 versus 9.4 ± 1.0, respectively).

Consistent with previously published observations, MBL enhanced FcR-mediated phagocytosis at levels comparable to C1q when compared with the control (Fig. 4D), indicating that MBL was biologically active. Together, these data suggest that C1q, and not MBL, enhances engulfment of apoptotic cells via upregulation of Mer and the Mer ligand Gas6.
Mer tyrosine kinase is required for C1q-dependent engulfment of apoptotic cells

To determine whether Mer expression was required for the observed C1q-dependent engulfment of apoptotic cells, experiments were performed with macrophages from Mer-deficient mice (merk \textsuperscript{−/−}). Merk \textsuperscript{−/−} mice lack the tyrosine kinase signaling domain in the cytoplasmic tail (20) and fail to express Mer protein (32). Both wild-type and merk \textsuperscript{−/−} BMDM responded to C1q with enhanced FcR-mediated phagocytosis; there was a 3–5-fold enhanced FcR-mediated phagocytosis above control levels (Fig. 5A). However, whereas wild-type macrophages responded normally to C1q, merk \textsuperscript{−/−} macrophages failed to respond to C1q with enhanced engulfment of apoptotic cells (Fig. 5B). To confirm that the C1q-elicited response was conserved in other phagocytes, experiments were also carried out with thioglycollate-elicited peritoneal macrophages (PM\textsubscript{2}/2). Similarly, prolonged activation of wild-type PM\textsubscript{2}/2 with C1q resulted in enhanced Mer expression (Supplemental Fig. 2) and engulfment of apoptotic cells, and C1q-dependent engulfment was significantly reduced in merk \textsuperscript{−/−} PM\textsubscript{2}/2 (Supplemental Fig. 2). As expected, merk \textsuperscript{−/−} BMDM failed to show increased Mer expression upon stimulation with C1q compared with wild-type macrophages (Fig. 5E).

Because C1q upregulated macrophage expression of complement components including C3 (Fig. 2A–D), the role of C3 in the C1q-dependent engulfment of apoptotic cells was investigated using C3\textsuperscript{−/−} macrophages. As expected, C1q-elicited FcR-mediated phagocytosis was similar between wild-type and C3\textsuperscript{−/−} macrophages (Fig. 5C). As in previous experiments, wild-type and C3\textsuperscript{−/−} macrophages were allowed to adhere to C1q or control protein for 5 h and then fed apoptotic cells. In contrast to merk \textsuperscript{−/−} macrophages, C3\textsuperscript{−/−} macrophages responded to C1q with enhanced engulfment of apoptotic cells similar to wild-type controls (Fig. 5D). There was no significant difference in C1q-dependent engulfment of apoptotic cells between wild-type and C3\textsuperscript{−/−} macrophages (Fig. 5D).

C1q-dependent engulfment of apoptotic cells is inhibited by soluble Mer

The Mer-Fc fusion protein has been shown to inhibit Mer-dependent engulfment by competing for Mer ligands in solution (32). To determine if Mer ligands were required for C1q-dependent engulfment of apoptotic cells, macrophages were stimulated with C1q for 5 h and then cultured in the presence or absence of recombinant Mer-Fc fusion protein prior to coculture with apoptotic thymocytes. Apoptotic primary mouse thymocytes were used as targets to confirm that the observed C1q-dependent engulfment pathway was observed in a cell autologous system because previous experiments had used mouse phagocytes and a human cell line (Jurkat) as targets. C1q enhanced the engulfment of apoptotic murine thymocytes, similar to apoptotic Jurkats, as measured by flow cytometry (data not shown) and microscopy (Fig. 6A, 6B). As predicted, C1q enhanced engulfment of apoptotic thymocytes over control (Fig. 6B, 53 ± 8.6 versus 21 ± 4.5, respectively), and Mer-Fc inhibited the C1q-dependent engulfment of apoptotic thymocytes (Fig. 6A, 6B). To ascertain whether Mer-Fc was binding Gas6 in our culture system, culture media from macrophages stimulated with C1q was incubated with Mer-Fc followed by precipitation with protein G agarose. Western blotting and immunodetection with an Ab against Gas6 showed a single band corresponding to Gas6 of similar molecular weight to that found in murine serum (Fig. 6C).

Discussion

The ability of C1q and MBL to enhance phagocytosis has been well documented in the literature (reviewed in Ref. 13). The results presented in this paper are in accordance with previous published observations and show that brief macrophage stimulation with either C1q or MBL enhances phagocytosis of Ab-coated targets (Figs. 1A, 4D). However, unlike the previously described effects of C1q on macrophage phagocytosis, in this study, we show that prolonged macrophage stimulation with C1q elicited expression of Mer tyrosine kinase as well as Mer ligands (Figs. 2, 4A, 4B). Mer was essential for the C1q-dependent engulfment of apoptotic cells because treatment with soluble Mer-Fc fusion protein (Fig. 6) inhibited C1q-dependent engulfment of apoptotic cells, and Mer-deficient macrophages failed to respond to C1q with enhanced engulfment of apoptotic cells (Fig. 5B). This novel pathway is specific for C1q because MBL failed to elicit Mer and Gas6 expression (Fig. 4A, 4B). Together, these data demonstrate a novel C1q-specific mechanism for clearance of apoptotic cells.

The importance of C1q-dependent engulfment of apoptotic cells in prevention of autoimmunity has been appreciated for more than a decade (1, 10). However, previous studies to identify C1q-dependent molecular mechanisms were guided by the assumption that C1q provided a direct and immediate signal for enhanced phagocytosis (33, 34). The direct pathway was confirmed in this study by measuring C1q/MBL-dependent phagocytosis of EAIgG (Fig. 4D). However, this pathway is not specific for apoptotic cells because C1q also triggers engulfment of Ab- and complement-coated particles (6, 7). Moreover, this immediate signaling pathway is not specific for C1q because the collectins, including MBL and SP-A, trigger a similar enhancement (8, 9, 34). Together, these data fail to provide a satisfactory explanation for the observation that C1q deficiency (and not MBL or SP-A deficiency) results in a specific failure to clear apoptotic cells and resultant autoimmunity.
microarrays were captured at original magnification to apoptotic thymocytes that are not associated with macrophages. Photo-

FIGURE 6. C1q-mediated engulfment of apoptotic cells is inhibited by Mer-Fc. Macrophages were cultured in chamber slides precoated with 4 μg/ml C1q (right panels) or HSA (left panels) for 4 h at 37°C, 5% CO2. Mer-Fc was added to culture wells for 1 h, after which macrophages were fed apoptotic mouse thymocytes at a 1:10 macrophage/apoptotic cell ratio for an additional hour. (A) Representative photomicrographs of PE-conjugated anti-CD11b-labeled macrophages (red) fed CFSE-labeled apoptotic thymocytes (green) and adhered to HSA or C1q in the presence or absence of Mer-Fc. Inset in the upper right panel shows higher magnification (original magnification ×63) of cells. Arrows indicate apoptotic thymocytes that are associated with macrophages, and arrowheads indicate apoptotic thymocytes that are not associated with macrophages. Photo-
micrographs were captured at original magnification ×40. Scale bar, 50 μm. Inset scale bar, 10 μm. (B) Bars represent the mean ± SEM from three experiments. A minimum of 200 cells per experimental condition was quantitated. *p < 0.05, two-way ANOVA, Bonferroni multiple comparison tests. (C) Mer-Fc precipitated Gas6 from macrophage-conditioned media following 18-h adhesion to C1q.

The data presented in this study provide a new hypothesis: that C1q specifically elicits a macrophage phenotype optimized for efficient clearance of apoptotic cells. Although the data demonstrate that Mer is required for C1q-dependent engulfment of apoptotic cells in murine BMDM (Figs. 5B, 6), it should be noted that additional C1q-triggered BMDM transcripts were identified by microarray that are required for efficient engulfment of apoptotic cells. The significance of these transcripts was not explored in this study; however, C1q-dependent expression of other engulfment proteins may be important in clearance in specific microenvironments and/or in other phagocytic populations. In addition, the mechanism leading to C1q-dependent upregulation of Mer remains to be determined. Although LRP and CD93 have been implicated as receptors or coreceptors required for C1q-dependent signaling (33, 34), we found that neither protein was required for C1q-dependent expression of Mer (Fig. 3F). Two recent reports have demonstrated a role for nuclear receptor signaling in upregulation of Mer (35, 36). Nuclear receptors liver X receptor and peroxisome proliferator-activated receptor δ have been proposed to act as sensors for apoptotic cells. Binding of apoptotic cell-derived lipids to nuclear receptors stimulates nuclear receptor-dependent expression of engulfment machinery required for efficient clearance of apoptotic cells and prevention of autoimmunity. The mechanism(s) leading to C1q-dependent macrophage activation remain to be elucidated; however, based on these studies, nuclear receptors may be a viable avenue for investigation of C1q-dependent macrophage activation.

Data presented in this paper demonstrate that subphysiologic concentrations of soluble C1q stimulate Mer expression (Fig. 3A). This is a significant finding because C1q is a soluble protein that circulates in blood, and therefore, this study suggests that under normal physiology, circulating C1q may program macrophages to respond efficiently to apoptotic cells. In addition, C1q is upregulated in response to injury (37), and therefore, increased C1q concentration following injury may further upregulate the Mer clearance system and promote the resolution of inflammation.

C1q deficiency is the strongest known genetic link to autoimmunity associated with lupus, and it is widely accepted that autoimmunity occurs, at least in part, due to a failure to clear apoptotic cells (1, 2, 10, 38). Therefore, the identification of a novel molecular mechanism leading to C1q-dependent engulfment of apoptotic cells has important implications in the development of therapeutics for autoimmune diseases. Our data demonstrate that C1q-dependent expression of Mer is required for C1q-dependent engulfment of apoptotic cells (Figs. 5B, 6). However, although a specific deficiency in Mer has been associated with lupuslike autoimmunity in mice, the association has not yet been reported in humans. Mer is a member of the TAM family of receptor tyrosine kinases that include Axl and Tyro3. TAM receptor signaling is thought to occur through hetero- and/or homodimerization. In addition, scavenger receptor-A (SR-A) signals via Mer during SR-A–mediated apoptotic cell engulfment (39). Rothlin and Lemke (5) and Lu and Lemke (40) demonstrated that deletion of all three TAM receptors resulted in profound autoimmunity in mice. More recent studies further indicate a critical role for Merkt, and not Axl or Tyro3, in prevention of autoimmunity in mice (28, 41). It is possible that C1q regulates expression of other TAM receptors and/or other engulfment proteins, such as SR-A, that may contribute to clearance of apoptotic cells and prevention of autoimmunity. Mer plays a nonredundant role in clearance of apoptotic cells in the retina, and Mer deficiency results in retinitis pigmentosa (RP) due to a failure in retinal pigment epithelial cell phagocytosis of photoreceptor outer segments (42, 43). It is interesting to note that it was recently reported that C1q levels are elevated in the retina of a mouse model of RP, and lack of C1q resulted in heightened susceptibility to RP, which is consistent with a role for C1q in apoptotic cell clearance (44). Therefore, it will be important to measure levels of Mer and other engulfment proteins in vivo under conditions in which deficiency in C1q results in pathology due to inefficient clearance of apoptotic cells.

The data presented in this study establish a previously unknown link between two well-characterized and important clearance systems: C1q and Mer. Identification of the signaling mechanisms downstream of C1q that result in upregulation of Mer and characterization of C1q-dependent expression of Mer and other engulfment proteins in vivo during normal physiology and autoimmune disease will provide additional insight into the contribution of this molecular pathway to pathogenesis.

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References


Supplemental Figure 1. MBL fails to upregulate Mer expression after 18 hours. Cell lysates from macrophages adhered for 5 (A) and 18 (B) hours to wells pre-coated with 4µg/ml HSA, heat inactivated (∆) C1q, C1q or MBL were analyzed for Mer expression by western blot.
Supplemental Figure 2. C1q elicits Mer expression in peritoneal macrophages (PMφ) and is required for engulfment of apoptotic cells. (A) PMφ were stained with isotype control or anti-F4/80 antibody and analyzed by flow cytometry. (B) Cell lysates from PMφ adhered for 5 or 18 hours to wells pre-coated with 4 μg/ml HSA or C1q were analyzed for Mer expression by western blot. Engulfment assays were performed as previously described using wildtype (WT) and mertk-/− PMφ. (C and D) C1q-adherent PMφ were fed EAIgG (C) or apoptotic Jurkat cells (D). Bars represent the mean fold change from four individual experiments ± SEM. * p < 0.05, *** p < 0.001, two-way ANOVA, Bonferroni multiple comparison tests.